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POSTER ABSTRACT GUIDE

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POSTER SESSIONS

Posters 101 – 580 are in-person poster presentations.

Presentation times listed below are in CEST time zone.

Posters will be continuously displayed during Exhibit Hall hours Wednesday – Friday.

IMPORTANT: Poster presenters are responsible for removing their posters at the take down time listed below in their assigned session. Any posters that are not removed at the end of the session will be discarded.

Individual poster presentation date, time and details can be found in this Poster Abstract Guide and Mobile App.

WEDNESDAY, 10 JULY: POSTER SET-UP & TAKE DOWN HOURS

Set-up: 2:30 PM – 5:30 PM • Take down: 7:45 PM – 8:00 PM

POSTER SESSION I: ODD numbered poster presentations

5:45 PM – 6:45 PM

POSTER SESSION I: EVEN numbered poster presentations

6:45 PM – 7:45 PM

THURSDAY, 11 JULY: POSTER SET-UP & TAKE DOWN HOURS

Set-up: 11:00 AM – 1:30 PM • Take down: 5:45 PM – 6:00 PM

POSTER SESSION II: ODD numbered poster presentations

3:45 PM – 4:45 PM

POSTER SESSION II: EVEN numbered poster presentations

4:45 PM – 5:45 PM

FRIDAY, 12 JULY: POSTER SET-UP & TAKE DOWN HOURS

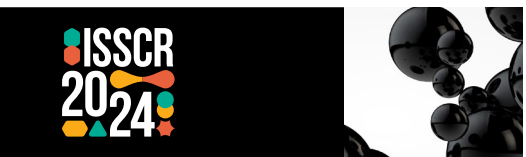
Set-up: 11:00 AM – 1:30 PM • Take down: 5:45 PM – 6:00 PM

POSTER SESSION III: ODD numbered poster presentations

3:45 PM – 4:45 PM

POSTER SESSION III: EVEN numbered poster presentations

4:45 PM – 5:45 PM



POSTER ABSTRACTS

WEDNESDAY, 10 JULY, 2024

TRACK: CLINICAL APPLICATIONS (CA)

Session I: Odd

5:45 PM – 6:45 PM

TOPIC: CARDIAC

101

IMMUNE-SHIELDED AND SAFE IPSC-DERIVED CARDIOMYOCYTES FOR HEART REPAIR

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Heart failure (HF) is a global health burden, aggravated by the marginal regenerative capacity of cardiomyocytes. A single myocardial infarction can cause irreversible loss of a billion cardiomyocytes, leading to HF with reduced ejection fraction (HFrEF). Despite maximal medical treatment, HF worsens over time, and eventually leaves ventricular assist devices or rare heart transplantation as last treatment options for end stage HF patients. Regenerative cell therapy holds the potential for a novel treatment of HFrEF with curative character, and active investigations are ongoing to re-muscularize the failing heart with stem cell-derived cardiomyocytes. To offer a durable and safe therapeutic effect, we generate genetically engineered off-the-shelf allogeneic hiPSC-derived cardiomyocytes (iCM) that are shielded from the patient's immune system ("cloaking") and carry a drug-inducible kill-switch. Using a fully characterized GMP hiPSC line as starting material, we have established a 10-day GMP-compatible 3D differentiation protocol that enables robust production of iCM in small scale GMP-compliant bioreactors with very high purity (>97% cTNT+). We utilized in silico modeling to identify influencers of cell growth during our bioreactor process and hence could stabilize high final cell yields that exceed the input material. To shield our iCM from allogeneic immune rejection, we genetically engineered cloaked hiPSC. Our differentiation protocol applied to these cloaked hiPSC enabled production of iCM with comparable purity and yield to wild type cells. We further established in vitro co-culture assays of iCM with human primary T cells or NK cells to demonstrate the efficiency of our immune-shielding strategy. Cloaked iCM showed inhibitory activity against T cell and NK cell killing. In conjunction, these results pave the way for pre-clinical development of our hiPSC-derived cardiomyocytes as cell therapeutics by enabling transition to large-scale production in bioreactors and demonstrating the potential of gene-edited cloaked iCM in counteracting allorejection.

Keywords: regenerative cell therapy, iPSC-derived cardiomyocytes, gene engineering

5:45 PM – 6:45 PM

103

A NOVEL INTEGRATED STRATEGY FOR EXPANSION AND METABOLIC MATURATION OF HUMAN IPSC-DERIVED CARDIOMYOCYTES IN BIOREACTORS: BIOPROCESS DEVELOPMENT AND SCALE-UP

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Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) hold significant potential for cardiovascular disease cell therapies, enhancing cardiac regeneration post-myocardial infarction. However, generate a relevant number (> 1 billion) of cardiomyocytes (CM) is labour-intensive. Also, immature state of hiPSC-CM restricts the mimicry of adult CM, limiting utility in drug screening and disease modelling. In vitro expansion and maturation of hiPSC-CM faces limitations, and the ability to produce relevant numbers would advance drug screening, disease modelling and cell therapy applications. We developed a bioreactor-based protocol for production and maturation of hiPSC-CM, aiming for increased scalability and reproducibility, maintaining quality attributes. Sustained WnT signaling pathway activation combined with oxygen control enhanced hiPSC-CM expansion, retaining cells' identity, purity and functionality. A maturation step integrated after expansion applying a metabolic-based approach generated CM with a more mature phenotype, metabolism and functionality. hiPSC-CM cultured as 3D aggregates in stirred-tank bioreactors (STB) were expanded by CHIR99021 addition through continuous perfusion. Maintaining a mild-hypoxia (10% O2) in the STB reduced ROS production by 30 % and increased Ki67+ proliferative cells by 70%, compared to normoxia (21% O2). After 11 days, the mild-hypoxia STB yielded 400 million of hiPSC-CM per 200mL run, showing spontaneous beating and a higher percentage of cells expressing cTnT and α-actinin cardiac markers (98%), compared to static culture (90%). Importantly, we successfully scaled-up the bioprocess to a 2L single-use STB and efficiently produced clinically relevant numbers of hiPSC-CM with improved quality: approximately 4 billion hiPSC-CM with > 95% cTnT+ and α-actinin+ cells. hiPSC-CM expanded in the 2L STB showed ability to further mature after integration of a metabolic maturation step, evidenced by increased expression of adult CM markers and improved functionality. This work presents a robust bioprocess to (1) produce billions of iPSC-CM in STB, attaining relevant cell numbers for cell therapies and (2) induce metabolic and functional maturation of hiPSC-CM, leveraging future applications in drug screening and disease modelling.

Funding Source: FCT PhD fellowship SFRH/BD/145767/2019, EU-funded project BRAV3 (H2020, ID:874827), iNOVA4Health (UIDB/04462/2020 and UIDP/04462/2020) and the Associate Laboratory LS4FUTURE (LA/P/0087/2020).

Keywords: iPSC-derived cardiomyocytes, bioprocess scale-up, cardiomyocyte metabolic maturation

5:45 PM – 6:45 PM

105

NOVEL STRATEGIES TARETING INFLAMMATION AND ER STRESS TO ENHANCE CARDIAC REGENERATIVE THERAPY

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Cardiovascular disease (CVD) is the leading cause of death in the US. A significant loss of cardiomyocytes from myocardial infarction can result in progressive deterioration of cardiac function and heart failure (HF). Therefore, there is a compelling need to seek new therapeutic options for patients in end-stage HF. Induced-pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) represents a new promising approach for the treatment of HF. However, one of the main challenges of cardiac stem cell therapy is the survival and retention of transplanted cells in the hostile milieu. We and others have shown that a high rate of transplanted stem-cell loss, ~90% within the first few days, has been observed due to ischemic environment, inflammation, and apoptosis. The cytochrome P450 products, epoxyeicosatrienoic acids (EETs), are anti-inflammatory metabolites of arachidonic acid with cardioprotective effects, however, EETs are rapidly metabolized by soluble epoxide hydrolase (sEH) enzyme. Our team has developed novel inhibitors of sEH (sEHIs) to prevent the catalysis of EETs, thereby enhancing their cardioprotective activity. Indeed, we were the first to demonstrate the beneficial effects of sEHIs in clinically relevant models of CVD to enhance cardiac stem cell therapy. To test the hypothesis that the survival of hiPSC-CMs can be improved by reducing inflammation and Endoplasmic Reticulum (ER) stress-induced apoptosis, we performed CRISPR/Cas9 mediated gene silencing of sEH in hiPSC-CMs. Multidisciplinary techniques were used to assess survival and ER-stress in gene-edited hiPSC-CMs. Our analyses showed a significant decrease in the activation and phosphorylation of apoptosis proteins, Annexin V, ER stress, and reactive oxygen species production in gene-edited hiPSC-CMs. In vivo bioluminescence imaging demonstrated that treatment with sEHI resulted in a significant increase in the survival and retention of transplanted hiPSC-CMs compared to cell treatment alone. Functional analysis showed a significant improvement in the fractional shortening by echo in the sEHI treated compared to the non-treated mice. Our findings suggest that suppression of inflammation, ER Stress and resolution of pre-existing fibrosis using sEH inhibition represents a potential and promising adjuvant to cardiac cell therapy.

Funding Source: Geneen Award (PT); NIH F31HL168956 (DD); NIH R01 HL085727, HL085844, HL137228, VA I01 BX000576, I01 CX001490 and 23SFRNCCS1052478 (NC); NIH R56 HL167932, AHA SFRN 23SFRNPCS1060482, TRDRP T32KT4729, and UCD Int Med Chair's Award (PS).

Keywords: cardiac, regenerative, inflammation

5:45 PM – 6:45 PM

107

CD34 AS A NOVEL MARKER FOR THE ISOLATION OF HPSC-DERIVED SINOATRIAL NODE PACEMAKER CARDIOMYOCYTES FOR BIOLOGICAL PACEMAKER APPROACHES AND DISEASE MODELLING

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The regulation of the heartbeat relies on pacemaker cells situated in the sinoatrial node (SAN). Dysfunction in these crucial cells can result in a dangerously slow heart rhythm, necessitating treatment with an electronic pacemaker (EPM). Although EPMs are the standard of care, treatments are associated with a complication rate ranging 10-16% and come with several caveats including surgical battery replacements and lack of response to the autonomic nervous system. Therefore, the development of a biological pacemaker that replaces the damaged tissue with new functional pacemaker cells presents a promising alternative therapy. Our lab has previously generated sinoatrial node-like pacemaker cells (SANPLC) from human pluripotent stem cells (hPSCs) and demonstrated their ability to function as a biological pacemaker in rat hearts. To translate these results to cell therapy applications, a method for the identification and purification of SAN cells from differentiations is required. Currently, due to the lack of surface markers, a NKX2-5:GFP transgenic reporter is required for the negative selection of NKX2-5- SANLPC. To address this issue, we present the first comparison between single-nuclei/cell RNA sequencing of human fetal SAN cells and hPSC-derived SAN cells. Through this comparison, we have identified several novel SAN markers including MYH11, BMP4, and most importantly the cell surface antigen CD34. We show that hPSC-derived SAN cells closely resemble fetal SAN cells on the transcriptional level. Furthermore, we demonstrate that CD34 is specifically expressed by SAN pacemaker cells but not by other cardiomyocyte subtypes. We establish that isolation of CD34+ cells through fluorescent or magnetic activated cell sorting from cardiac hPSC differentiations results in the enrichment of SAN-like cells exhibiting the molecular profile of SAN pacemaker cells. These CD34+ cardiomyocytes also display typical SAN action potentials and can function as a biological pacemaker. In summary, this work advances the development of cell-based therapies for cardiac pacing. Furthermore, this newly discovered SAN pacemaker cell surface marker will be highly valuable for future endeavors in hPSC-based modeling of SAN diseases, drug discovery, and targeted delivery of therapeutics to SAN cells.

Funding Source: Canadian Institutes of Health Research, Canadian Foundation for Innovation, Canadian Stem Cell Network

Keywords: biological pacemaker, sinoatrial node, surface marker

5:45 PM – 6:45 PM

109

IDENTIFICATION AND VALIDATION OF NOVEL ENGINEERING STRATEGIES IN IPSCS TO RESIST ALLOGENEIC IMMUNE CELL REJECTION

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Human induced pluripotent stem cell (hiPSC)-derived therapies are a promising approach for treating a diverse array of diseases characterized by the necessity for tissue repair and replacement. However, allogeneic hiPSCs face a significant obstacle limiting their clinical application—namely, their inherent immunogenicity can lead to rejection of the administered cells. Gene-editing technologies allow for the generation of engineered, allogeneic iPSC-derived therapies that resist immune rejection. To develop “off the shelf” medicines, we have established end-to-end capabilities for the identification of novel targets, editing, differentiation and functional assessment, both in vitro and in vivo, of the engineered cells. As shown in this study, we validated our extensive platform capabilities by combining target deletion as well as over-expression approaches to generate hiPSC-derived cells capable of evading both, T and NK cell rejection. Integration of the transgenes in our Sustained Transgene Expression Loci (STEL) ensured robust and sustained target expression in both PSCs and their derivatives including cardiomyocytes. We then evaluated the ability of the engineered hiPSCs and their differentiated cardiomyocyte derivates to evade NK as well as T cell killing by multiple allogeneic donors in co-cultures

in vitro. We then confirmed the ability of the engineered cardiomyocytes to avoid allogeneic immune rejection in vivo via co-injection of the cardiomyocytes and the immune cells in NSG mice. In contrast to non-engineered cells, we observed that engineered cardiomyocytes remained viable after encountering either allogeneic T or NK cells in vitro and in vivo. Importantly, the diversity among donors allowed us an assessment of the robustness and consistency of the immune evasion strategy across a range of genetic backgrounds, helping us to understand the broader applicability and effectiveness of this approach. In conclusion, these data highlight our advanced platform capabilities across engineering, differentiation and robust functional in vitro and in vivo assessment. We further demonstrate the identification of a novel immune evasion strategy for hiPSCs, overcoming a major hurdle towards the clinical application of these therapies, by leveraging our platform capabilities.

Keywords: allo rejection, immune evasion, cardiomyocytes

5:45 PM – 6:45 PM

111

PROTEIN-FREE, LOW COST MEDIUM FOR EFFICIENT HPSC-CARDIOMYOGENIC DIFFERENTIATION AND UPSCALING TO 2000 ML STIRRED-TANK BIOREACTORS

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Harnessing the full potential of human pluripotent stem cell (hPSC)-derived cardiomyocytes (CMs) for cell therapy, tissue engineering, and drug discovery, will require the cost-effective, large-scale production of cell products. Chemically defined, low-cost media of constant quality are important in such processes. The broadly utilized “Chemically defined medium, 3 components” (CDM3) for cardiac differentiation presents a first step towards this, however, it depends on utilizing the costly and batch-quality dependent recombinant human albumin. To overcome these drawbacks we have developed entirely protein-free, chemically defined media formulations (PFMF) that efficiently support cardiomyocyte yield and purity. The applicability of these novel media formulations is demonstrated across numerous culture platforms, including conventional Erlenmeyer flasks (20 mL volume) followed by process upscaling to 150 mL stirred tank bioreactors (STBRs) and ultimately 2000 mL STBR scale. The observed CMs yield of 1.5-2×106 viable cells/mL in PFMF enables the production of about 1.5 billion (1.5×109) viable cells in a single 2000 ml STBR run, presenting an important achievement regarding clinical translation. Comprehensive downstream analysis of the cell product includes RNA sequencing, electrophysiological analysis by patch clamping, and functional assessment of contractile properties by generating bioartificial cardiac tissues, fully confirming the expected quality and function of the generated CMs. All components of the newly developed media are commonly utilized in the biomedical industry and are known to be biocompatible, in line with the required clinical applicability of hPSC-derived cardiomyocytes; moreover, media cost reduction of at least 30% compared to CDM3 medium is achieved, further promoting commercial aspects of cell therapies for heart regeneration.

Keywords: hPSC-bioprocessing, cardiomyocytes, up-scaling

5:45 PM – 6:45 PM

113

MESODERM POTENTIATION VIA SINGLE FACTOR TRANSFECTION PROMOTES MATURATION OF INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES

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Following the discovery of induced pluripotent stem cells, various strategies were developed to promote guided mesoderm specification and cardiac differentiation. While a high differentiation yield was obtained, derived progeny exhibited an immature phenotype akin to neonatal cardiomyocytes. Various strategies have been investigated to enhance the maturity of induced pluripotent stem cell derived cardiomyocytes. We here introduce a novel single-factor transfection approach to enhance the maturity of cardiac cells derived from pluripotent stem cells. Transfection with the mesoderm transcription factor, Brachyury prior to standard cardiac differentiation promoted upregulation of cardiac specific transcription factors. In addition, transfected cells exhibited improved structural and functional maturity. Namely, sarcomere length and the proportion of sarcomere with I-bands were increased. Transfected cells exhibited augmented action potential kinetics with a

reduced relaxation constant and a faster upstroke velocity. In parallel, use of Brachyury promoted augmented calcium handling characterized by a larger area under the curve of calcium trainsets and improved calcium transient shortening with pacing. Thus, we here introduce a single-factor maturation protocol that provides more mature cardiomyocytes under standard differentiation conditions. Such findings provide a novel and simple protocol for enhancing pluripotent stem cell-derived cardiomyocyte maturity and suggest that early differentiation cues may influence later maturity.

Keywords: induced pluripotent stem cells, cardiac maturation, cardiac differentiation

5:45 PM – 6:45 PM

115

TRANSIENT KNOCKDOWN OF HCN4 IN EMBRYONIC STEM CELL-DERIVED CARDIAC PROGENITOR CELLS REDUCES PACEMAKING CELLS AND INCREASES VENTRICULAR CARDIOMYOCYTES LINEAGE COMMITMENT

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Following myocardial infarction (MI), millions of cardiomyocytes (CM) are replaced by non-contractile scar tissue due to a lack of regenerative potential in adult human heart. Human embryonic stem cell-derived cardiac progenitor cells (hESC-CPC) are a promising avenue for cell-based therapy in MI. However, the occurrence of transient ventricular arrhythmias after cell transplantation, known as engraftment arrhythmias (EA) is a safety concern. One cause of EA is the presence of immature cells with pacemaker activity upon transplantation. The expression of specific genes, such as the hyperpolarization-activated cyclic nucleotide-gated potassium channel HCN4, contribute to the automaticity of these cells. In this study, we hypothesise that the transient knock down (KD) of HCN4 in hESC-CPC can favour a reduction of non-ventricular, pacemaker-like CMs, towards a majorly of ventricular like cells (vCM) during differentiation, reducing cell automaticity and EA. To test our hypothesis, hESCs were transfected with a transposon-based system constitutively expressing RFP, which includes a shRNA sequence targeting HCN4 controlled by a doxycycline induced promoter (TetON). Firstly, the hESC line was transfected, and efficiency was evaluated by measuring the RFP signal. Secondly, HCN4 KD was performed by inducing the shRNA expression with doxycycline for 2 days during differentiation of hESC towards CPC, and KD was confirmed in terms of gene and protein expression levels. Thirdly, we purified the CPC population by negative selection for the stem cell marker TRA-1-60. Action potential was measured in single cells after 15 days of differentiation. We show that HCN4 transient KD resulted in a reduction of both atrial and pacemaker-like cells, consequently increasing the vCM population. Ongoing experiments analysis of individual cell gene expression profile by scRNA-seq and their molecular profile after transplantation and differentiation in an in vivo rodent kidney capsule model will give additional insights on cells identity after HCN4 transient KD in hESC-CPC and derived CM. These preliminary data look promising to explore the transient KD of various targets associated with pacemaker activity, either individually or in combination, opening possibilities to reduce EA after cell implantation.

Funding Source: Skövde University and AstraZeneca support this project.

Keywords: cardiac progenitor cells, arrhythmia, myocardial infarction

5:45 PM – 6:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

119

IN VIVO CONJUNCTIVAL REGENERATION USING AN ORGANOID-BASED THERAPY: A PRE-CLINICAL STUDY

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Ocular surface disease pose a significant clinical challenge, with limited treatment options available. A cell therapy using conjunctival organoid–derived cell sheets offers a promising approach for conjunctival regeneration. We evaluate the safety and efficacy of conjunctival organoid–derived cell sheets in repairing conjunctival defects in a pre-clinical rabbit model. Human conjunctival organoids were established from small conjunctival biopsies (~1 mm2, N=6), recapitulating key features of the conjunctival epithelium, including an abundance of goblet cells. The organoids were then dissociated and seeded as single cells on a fibrin scaffold. A conjunctival defect was induced in immunosuppressed rabbits (N=34) by conjunctival excision (5.0 mm), followed by transplantation of the cell sheets (6.0 mm) onto the denuded area. Examinations were performed on postoperative days (POD) 7, 14, and 21 to assess conjunctival defects and wound healing. Immunohistological analysis was conducted on POD 7 and 21 to evaluate the regenerative capacity of the transplanted cells in more detail. Clinical examination showed no signs of conjunctival defects or adverse effects on POD 7, 14, and 21. Histological examination revealed complete conjunctival regeneration with normal morphology and organization on POD 7 and 21. A regular distribution of mucin-producing goblet cells was observed in the fornix (18 cells/mm) and bulbar (9 cells/mm) conjunctiva. Mild infiltration of inflammatory cells in the episcleral and conjunctiva was observed on POD 7 and POD 21. Expression of human-specific CK14 on POD 7 (100%) and 14 (75%) indicated that transplanted cells specifically repopulated the defect, not the neighboring rabbit conjunctiva.

Conjunctival organoid–derived cell sheets demonstrate promise for repairing conjunctival defects, offering a potential therapeutic strategy for ocular surface disorders

Keywords: organoid, conjunctiva, regeneration

5:45 PM – 6:45 PM

121

QUALITY CONTROL OF HUMAN PSC-DERIVED RPE CELLS FOR REGENERATIVE THERAPY

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Human retinal pigment epithelial (RPE) cells form a specialized monolayer of polarized pigmented epithelial cells located between the blood vessels of the eye and the neural retina. The key function of RPE cells is the maintenance and protection of light-sensitive photoreceptors. Degeneration of RPE cells accompanies age-related eye diseases and can lead to the loss of vision. The rescue of patient´s vision can be achieved by replacement of the RPE cells. Therefore, we aim to differentiate human embryonal stem cells and human induced pluripotent stem cells into fully-functional RPE cells that would be suitable for the regenerative therapy To ensure the RPE cells are not released for the treatment of the patient until the quality has been considered satisfactory, we designed a panel of quality control tests that characterize a) functionality: phagocytosis, specific cytokine secretion, b) morphology, c) identity and viability: marker detection by immunocytochemistry and flowcytometry, d) immunogenicity: lymphocyte-graft cell immune reaction, HLA expression of RPE cells. These tests are prepared for the translation into advanced therapy medicinal product manufacture.

Funding Source: Created in collaboration with MED MUNI through the CZECRIN project (LM2023049), supported by the national budget through MEYS. Supported by MED MUNI fund, MUNI/A/1598/2023, and by Ministry of Health of the Czechia, NU22-08-00629.

Keywords: retinal pigment epithelial cells, pluripotent stem cells, quality control

5:45 PM – 6:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

123

SINGLE-CELL EPIGENOMICS REVEALS MECHANISMS OF MOUSE DENTAL GERM DEVELOPMENT

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The process of dental development is orchestrated by a dynamic interplay between cellular differentiation and the chromatin state, which serves as a pivotal determinant of the gene regulatory network. Despite its fundamental importance, the epigenetic intricacies that govern odontogenesis remain largely uncharted. Leveraging the power of single-cell technologies, our research endeavours to illuminate the epigenetic underpinnings that facilitate cellular diversity during the formation of dental structures. Building upon our prior work, which established a single-cell transcriptome atlas of mouse dental germs, we identified distinct populations of CD24+ and Plac8+ odontogenic cells, respectively. In this ongoing investigation, we employ single-cell assay for transposase-accessible chromatin by sequencing (scATAC-seq) on both incisors and molars from E10.5-E16.5 mouse dental germs. This approach enables us to unravel the unique chromatin accessibility patterns that are specific to cell types within the developing dental germs. Integrating this novel data with our preceding scRNA-seq findings, we perform comprehensive analyses to pinpoint genomic regions that exhibit significant shifts in accessibility, correlating with cell-type and region-specific differentiation events in tooth development. Our integrative analysis also aids in the prediction of candidate regulatory elements that are cell-type-specific. Through a systematic comparison of chromatin accessibility between dental pulp and follicle regions, we uncover the surprising diversity among dental progenitor cells. The diversity sheds light on the pivotal signalling pathways that underpin odontogenic lineage identities. Moreover, by fusing single-cell chromatin accessibility with gene expression data, we construct enhancer networks that reveal hub enhancers, which play a crucial role within these networks. Our findings contribute a novel perspective to the understanding of how chromatin states influence the complex patterns

of cell type diversity and cell fate determination during dental development. The insights gained through this research hold the potential to inform stem cell biology and regenerative medicine, offering a blueprint for future explorations into the epigenetic regulation of organogenesis.

Keywords: dental germ development, single-cell ATAC-seq, epigenetics of odontogenesis

5:45 PM – 6:45 PM

125

REPRODUCIBLE AND SCALABLE DIFFERENTIATION OF OVARIAN SUPPORT CELLS FROM A CLINICAL-GRADE HIPSC LINE TO BE APPLIED AS A NOVEL IN VITRO MATURATION STRATEGY FOR INFERTILITY TREATMENT

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In vitro maturation (IVM) is an assisted reproductive technology in which immature oocytes are retrieved with abbreviated stimulation and matured outside the body, offering a safer alternative to generate mature eggs for infertility treatment. Despite the clear benefits to the patient, when compared with conventional protocols, IVM yields in general less usable mature oocytes, limiting its utilization as standard of care. We recently demonstrated that ovarian support cells (OSCs) derived from hiPSCs in co-culture with immature human oocytes mimic the ovarian environment allowing for higher rates of oocyte maturation and euploid embryo formation, offering a novel solution to overcome the limited yield in IVM. Translating novel cell-based IVM products to clinics however represents certain challenges beyond efficacy, namely manufacturing at scale, integration of commercial-grade raw materials, and ensuring purity, reproducibility, and safety. As we translate this technology towards clinical manufacturing and application, we optimized our manufacturing protocol to deliver increased scale and substituted raw materials with appropriate animal origin-free alternatives, which included a systematic methodology to select an ideal substrate. These modifications improved the efficiency, purity, and consistency of the differentiated OSC population, ultimately improving consistency of functional outcomes. Most importantly, we sourced a compliant female clinical/commercial-grade hiPSC line as starting material, and operated in controlled environments to create a new cell line by applying an identical engineering strategy to originally create the research-use-only (RUO) hiPSC line. OSCs differentiated from the new hiPSC line delivered similar quality attributes and clinical outcomes previously demonstrated with our RUO line, with an increase in consistency and reproducibility across batches. This technology is currently being applied in a first-in-human OSC-IVM observational study with reported pregnancies. Overall, we have demonstrated that our strategic approach to product development meets scalable manufacturing needs and ultimately supported a product of improved efficacy, quality, and safety.

Keywords: in vitro maturation, manufacturing, infertility

5:45 PM – 6:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

127

SCF AND 740 Y-P, A PI3K ACTIVATOR, DIFFERENTLY MODULATE SIGNALING PATHWAYS AND COOPERATE TO ACHIEVE REPRODUCIBLE EX VIVO HUMAN HAEMATOPOIETIC STEM CELL EXPANSION SUITABLE FOR GENETIC ENGINEERING

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Haematopoietic stem cell (HSC) is a clinically relevant blood-generating stem cell where HSC transplantation (HSCT) remains only a curative option for multiple blood or non-blood disorders. Therapeutic applications of HSC are, however, limited partially due to a lack of methods that allow HSC to maintain or expand stably ex vivo. Stable ex vivo HSC expansion would address multiple current challenges in the field of HSC biology and clinical hematology, including low robustness on HSC research, a considerable rate of engraftment failure in umbilical cord blood (UCB) HSCT, and limited success of genetic modifications in HSCs. Our group previously developed stable ex vivo mouse HSC expansion protocol, using a polymer-based medium with thrombopoietin (TPO) and stem cell factor (SCF). A cytokine-free ex vivo human HSC expansion protocol that replaces SCF with 740 Y-P, a PI3K activator, has been reported with successful expansion of single UCB. Nevertheless, it remains unclear how stable the cytokine-free protocol is. Here, we aimed to develop more stable protocol for human HSC expansion. Screening more than 100 molecules targeting SCF/TPO signaling pathways, we identified that adding SCF and 740 Y-P in the cytokine-free medium (SCF+740 protocol) significantly improved the expansion of immunophenotypic hematopoietic stem/progenitor cell (iHSPC: Lin-CD34+) and HSC (Lin-CD34+EPCR+) derived from human CD34+ UCB during 28-day culture. We confirmed reproducible HSPC expansion in SCF+740 protocol during 28-day culture (n=7 independent donors; mean fold change of viable cells: 105.2 ranging 36.3 – 402.3; mean purity of iHSPC on day 28: 56.0% ranging 47.8-69.2%). In a xenograft model, human CD34+ UCBs expanded in SCF+740 protocol

for 14 days showed higher human multilineage chimerism in NSG mice 16 weeks post-transplantation (mean chimerism: 22.9% [expanded] vs 0.96% [unmanipulated]). Analyses on protein phosphorylation revealed different effects of SCF and 740 Y-P on signaling pathways in expanded human iHSPCs. Importantly, our protocol supported successful homology directed repair-based targeted knock-in in HLF-positive HSCs and lentiviral transduction in HSCs engraftable in NSG mice. Taken together, these findings underscore the SCF+740 protocol for advancing HSC research and therapeutic applications.

Funding Source: National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, The Uehara Memorial Foundation Stanford Cancer Institute

Keywords: ex vivo haematopoietic stem cell expansion, genetic engineering, haematopoietic stem cell transplantation

5:45 PM – 6:45 PM

129

RECONSTITUTION OF ACTIN NETWORK DYNAMICS: A PROMISING APPROACH TOWRADS EFFICIENT IN VITRO PRODUCTION OF RED BLOOD CELLS

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An erythroid progenitor cell line capable of efficient in vitro production of mature red blood cells (RBCs) represents a promising alternative for traditional red blood cell concentrates. Since an erythroblast cell line (imBMEP), previously established in our laboratory, showed limitations in efficient maturation, a knockout of the enucleation inhibitor miR-30a-5p was performed. Although this modification led to a significant promotion of terminal erythropoiesis, insufficient enucleation remains a major challenge. Through a comparative transcriptome analysis of imBMEPs and hematopoietic stem cells (HSCs), we aimed to systematically investigate possible causes for the inhibited final maturation. Differential gene expression analysis revealed high variance between imBMEP cells and HSC controls. Using Ingenuity Pathway Analysis (IPA), these differences in gene expression were assigned to specific pathways and functions. A notable number of significantly altered pathways were associated with the cytoskeleton and the actin network. In particular, actin polymerization appeared to be inhibited at several levels within the regulatory networks. While Western Blot data of actin, α-tubulin and vimentin are showing no quantitative differences in protein expression between imBMEPs and HSCs, the immunofluorescence analysis of the cells showed distinct structural changes. The actin network in particular appears to be altered, with actin forming unstructured accumulations within the imBMEP cells instead of a homogeneous network as it was seen in HSCs. The observed structural changes indicate that primarily actin polymerization and reorganization seem to be affected. With regard to terminal erythropoiesis, this could be a reason for the limited enucleation of the cell line, as the actin network plays a decisive role in this process. It is now necessary to identify key candidates that are responsible for the observed differences and whose modification leads to a restoration of the actin network dynamics. By restoring an effective enucleation process, imBMEP cells could provide a potentially unlimited cell source for the efficient in vitro production of modifiable RBCs for transfusion to patients who are currently difficult to supply with compatible donor derived erythrocytes.

Keywords: erythropoiesis, enucleation, transcriptome analysis

5:45 PM – 6:45 PM

131

FACTORS INFLUENCING SUCCESSFUL GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS FROM PERIPHERAL BLOOD MONONUCLEAR CELLS

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Human induced pluripotent stem cells (hiPSCs) have demonstrated significant potential in their ability to differentiate into diverse tissues. We investigated the clinical and experimental characteristics associated with the successful generation of hiPSCs from peripheral blood mononuclear cells (PBMCs). In this study, hiPSCs were generated from PBMCs obtained from patients who underwent pancreatectomy. The Sendai viral vector system was utilized to reprogram PBMCs with Oct4, Sox2, Kl4 and c-Myc transcription factors. The pluripotency of iPSCs was confirmed through alkaline phosphatase staining and immunocytochemistry for pluripotency markers. Among the 17 PBMCs samples, hiPSCs were generated successfully in 9 (52.9%) samples. There were no differences in age, sex, or comorbidities such as diabetes, hypertension, and malignancy. Among 11 individuals without diabetes, iPSCs were generated in 7 (63.6%), whereas it was only 33.3% (n=2) among the 6 individuals with diabetes. Those with successful hiPSCs generation exhibited higher body mass index (BMI) and lower hemoglobin (Hb) levels compared to those without hiPSCs generation. Interestingly, the PBMC count obtained from complete blood count tests conducted before surgery tended to be higher in those with successful hiPSCs generation compared to those without. Blood volume, hemolysis, and PBMC purity after isolation did not differ between the two groups. The time required to generate colonies was shorter in those with successful hiPSCs generation compared to those without, but not significant. Notably, a distinct boundary of the colony was the significant factor for the successful generation of hiPSCs (odds ratio of 4.9). In this study, hiPSCs successfully derived from PBMCs exhibited pluripotency markers, with generation rates correlating positively with higher BMI and lower Hb

levels, and a tendency towards higher PBMC counts pre-sampling. Colony morphology also emerged as a significant factor influencing successful hiPSCs generation, highlighting the potential clinical relevance of these findings for regenerative medicine applications.

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MIST) (No. NRF-2021R1C1C1013016).

Keywords: human induced pluripotent stem cells, peripheral blood mononuclear cells, reprogramming

5:45 PM – 6:45 PM

133

GENERATION OF FUNCTIONAL IPSC-DERIVED CAR-T CELLS FOR CANCER IMMUNOTHERAPY VIA CHEMICAL-INDUCED EPIGENETIC REPROGRAMMING

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Cancer immunotherapy employing patient-derived T cells modified with chimeric antigen receptors (CAR) has proven remarkably effective against lymphoid malignancies, but such autologous cell therapies entail labor-intensive cell processing. Using human induced Pluripotent Stem Cells (iPSCs) to produce CAR T cells holds considerable promise for realizing allogeneic off-the-shelf cancer immunotherapies. However, generating fully mature iPSC-T with robust functionality remains challenging. Previously, we established a stroma-free culture system for iPSC differentiation into T cells (Jing et al, Cell Stem Cell, 2022). Building upon this platform, we conducted small molecule screens to identify epigenetic regulators that influence lymphoid development, and focused on the H3K9-directed histone lysine methyltransferase G9a/GLP as a suppressor of T cell fate. Analyses by ATAC-seq and RNA-seq revealed that G9a/GLP inhibition governs chromatin accessibility and gene expression patterns associated with lymphoid differentiation. As a consequence, G9a/GLP controls the cell fate choice between myeloid and lymphoid lineages in HSPCs. We also demonstrated that inhibiting G9a/GLP promotes lymphopoiesis in zebrafish, illustrating the evolutionary conservation of G9a/GLP's role in T cell development. Most importantly, chemically-induced epigenetic reprogramming via G9a/GLP inhibition facilitates the generation of highly functional iPSC-T cells with a molecular signature closely resembling that of mature alpha-beta T cells from peripheral blood. When epigenetically reprogrammed iPSC-T cells were engineered to express an anti-CD19 CAR, they exhibited enhanced effector responses and tumor cell-killing capability in vitro. Furthermore, CAR iPSC-T cells derived with G9a/GLP repression displayed robust antitumor activity in a xenograft lymphoma mouse model, resulting in improved survival in treated animals. Notably, we show that mice treated with epigenetically modulated iPSC-CAR-T were resistant to tumor cell rechallenge, demonstrating that iPSC-CAR-T cells derived via G9a/GLP inhibition can persist and elicit sustained remission. These findings facilitate the efficient production of clinically relevant iPSC-derived T cells for adoptive cell therapies.

Funding Source: This study was supported by grants from NIH NHLBI U01HL134812, NIH NIDDK 2U01DK104218, Emerson Collective Cancer Research Fund to GQD, and NIH NHLBI 5U24HL134763 sub-award 1701192 and NIH NHLBI K99HL170075 to RJ.

Keywords: iPSC-derived T cells, CAR T therapy, epigenetic modulation

5:45 PM – 6:45 PM

135

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF AN ALLOGENEIC IPSC-DERIVED NK THERAPEUTIC PRODUCT MANUFACTURED AT SCALE

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Natural Killer (NK) cell therapies do not require HLA-matching, do not cause graft-vs-host disease (GvHD) and have demonstrated promising data in treating solid tumours, thereby offering a highly attractive allogeneic therapeutic strategy. Whilst several sources of NK cells are available, the differentiation of human induced pluripotent stem cells (iPSC) offers many advantages over primary NK cells, such as unlimited expansion of the cell bank and reduction in costs. The clinical and industrial utility of developed iPSC-derived NK (iNK) therapies relies on efficient, robust and cost-effective differentiation and manufacturing methods. Plasticell uses a proprietary, high-throughput combinatorial screening technology - CombiCult® - to develop efficient methods for differentiation and expansion of hematopoietic cells. CombiCult® was used to test over 4000 cell culture protocols in parallel to discover highly efficient, cost-effective, feeder-, serum- and xeno- free, GMP-ready protocols for differentiation of iPSC into mature and functional NK cells. Based on the yield and purity of NK populations, six protocols were chosen for in-depth characterization of NK phenotype. Ratios

of inhibitory, activating and co-activating receptors expressed on the surface of iNK cells were analyzed by FACS and compared to the repertoire of receptors expressed on the surface of NK cells derived from human peripheral blood (PB). The cytotoxic activity of iNK cells was tested on multiple cancer cell lines established from hematopoietic and solid tumours. The longevity of iNK cells in vitro was tested in long term cell cultures revealing persistent expression of surface receptors and potent anti-tumor activity. Several stationary and agitated cell culture approaches were used to test the scalability of differentiation protocols. Differentiation of iPSC in agitated culture conditions such as shaker flasks or STR bioreactors resulted in generation of fully functional mature iNK cells with increased yield, efficiency (number of iNK cells per iPSC) and purity of iNK population. The transcriptomes of iNK cells obtained by different protocols and alternative manufacturing methods were compared to those of donor PB NK cells to confirm the suitability of iNK cells manufactured at scale to enter pre-clinical and clinical studies.

Funding Source: Innovate UK Biomedical Catalyst 2021 Round 2: Feasibility & Primer Awards

Keywords: allogeneic iPSC derived NK cell therapy, combinatorial stem cell screening technology, scale up manufacturing of iPSC derived NK cells

5:45 PM – 6:45 PM

137

VASCULAR DEVELOPMENT FROM HUMAN PLURIPOTENT STEM CELL-DERIVED PROGENITORS

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The ability to revascularize ischemic tissue through the transplantation and engraftment of vascular progenitors (VPs) offers unprecedented opportunities to develop novel cell-based therapies to treat a range of diseases resulting from vascular dysfunction and ischemia. While significant effort has been directed at identifying transplantable VPs over the past decade, little progress has been made in this endeavour as most published papers to date report only limited survival and engraftment of the transplanted cells. This failure could be due to the fact that VPs simply cannot engraft host tissue without support of additional cell types and/or factors or that the wrong VPs have been transplanted. To overcome this challenge, many in the field have used engineering strategies to generate three-dimensional vascular tissues for transplantation. While these tissues can engraft, the vasculature that does develop is largely restricted to the graft site, potentially limiting its usefulness for revascularizing extended areas of ischemic tissue. In this study we have developed a strategy to generate a novel human pluripotent stem cell (hPSC)-derived VP population that displays robust engraftment potential. Following transplantation of single cell suspensions of these VPs into either the mammary fat pad or hindlimb skeletal muscle of NSG mice, they differentiate and form human capillary-like vessels that are dispersed throughout the target tissue. Many of the human vessels contain mouse erythrocytes, indicating they are perfused and functioning as part of the vasculature of the host animal. Kinetic studies showed functional vasculature can develop within 14 days of transplantation and persist for up to 3 months in the skeletal muscle indicating that the hPSC-derived vessels are stable and can function for long periods of time in the recipient animal. Together, these findings show it is possible to revascularize tissue with hPSC-derived VPs and in doing so, pave the way for developing cell-based therapies to treat ischemic disease.

Funding Source: BlueRock Therapeutics

Keywords: revascularization, ischemia, PSC-endothelium

5:45 PM – 6:45 PM

139

ENGINEERING STAGE-SPECIFIC DEVELOPMENTAL CUES TO GENERATE NON-ALLOGENIC, IPSC-DERIVED CHIMERIC ANTIGEN RECEPTOR T CELL FOR THERAPY

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Chimeric antigen receptor (CAR) T cell therapy has demonstrated remarkable results in otherwise treatment-refractory, hematologic malignancies. Currently, the process of engineering individualized, autologous cellular therapies pose complex manufacturing challenges, compounded by inherent biological variations in T cells and premature exhaustion, resulting in a less efficacious CAR T product and inconsistent clinical responses. Thus, there is growing interest in generating functionally enhanced CAR T cells from a universal and self-renewing allogeneic source of induced pluripotent stem cells (iPSCs). However, there are critical barriers to generating CAR T cells from iPSCs, such as spontaneous differentiation into unwanted, innate cells (i.e. Natural Killer cells) due to improper antigen-independent signaling of CARs in immature T cells. In addition, additional genetic modifications necessary to remove the risk of alloreactivity by removing expression of endogenous T cell receptors (TCRs) prevent conventional T cell development. To address these barriers, our work combines the first-in-class, 3-D Artificial Thymic Organoid (ATO) culture system and a novel CRISPR/Cas9-gene editing strategy to achieve precise, stage-specific expression of CARs during T cell differentiation from iPSCs. This approach restricts CAR expression to mature T cells, preventing fate diversion during early development. Furthermore, by introducing the CAR target antigen in the ATO microenvironment, developing T cells undergo positive selection in the absence of endogenous TCRs. This

approach produces iPSC-derived, TCR-null CD8+ CAR T cells that present a mature, naïve phenotype with homogeneous, high expression of CD62L, CCR7, CD27, and CD45RA, with robust antigen-specific cytotoxicity both in vitro and in vivo, over multiple tumor rechallenges. We present a proof-of-concept, platform that can be adapted to other tumor targets, CAR designs, and additional genetic modifications that could further enhance T cell function and persistence in vivo. Development of iPSC-engineered T cell therapy would ensure immediate access to treatment that delivers a more durable and consistent response.

Funding Source: Ruth L Kirschstein Individual Predoctoral NRSA (1F30CA278297-01), UCLA BSCRC Pre-doctoral Training Fellowship, CIRM (DISC0-14422), UCLA-Caltech Medical Scientist Training Program (T32GM008042)

Keywords: CAR T, iPSC, ATO

5:45 PM – 6:45 PM

141

AUTOMATED IPS CELL PRODUCTION USING CHEMICALLY DEFINED SCAFFOLD FOR IMMUNOTHERAPY

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Cellular immunotherapy is a promising tool in the treatment of cancer that uses immune cells such as T - and NK cells. Widespread use of this therapy requires a stable supply of immune cells which can be derived from human iPSCs, preferably produced in a closed system. There have been several attempts to investigate the production of immune cells. However, these processes need to address the issues of variability, reproducibility, and robustness to make them suitable for manufacturing. These issues are often related to the complex nature of the scaffold, many of which are animal derived proteins with high variability and cannot be easily adapted to automated systems. We have developed a polymer-based scaffold that is synthesized chemically making it suitable for automated and closed systems. This Chemically-Defined Scaffold (CDS) is a hydrophobic polymer, insoluble in water, room temperature stable and can be precoated on different culture ware in a sterile manner. We have previously shown that combining the CDS with an automated system for maintenance and passaging of iPSCs significantly reduced variability introduced by raw materials, operator, and handling processes; thus, reducing the overall noise in the biological experiments. We aim to introduce this reproducibility in the entire manufacturing process from reprogramming to differentiation into immune cells, a part of which is reflected in this study. We developed a modified protocol that allowed reprogramming of PBMCs using Sendai virus vector and formation of iPSC colonies without the manual picking process. This “picking-less” protocol allowed increased number of iPSC colonies while simultaneously reducing the presence of non-iPSCs when compared to conventional protein scaffold. This process was then tested in a closed automated system where whole blood was used to derive iPSCs. Here, a similar result was observed when higher number of iPSC colonies were observed with CDS when compared to a conventional protein scaffold. This CDS surface could potentially be used for differentiation of iPSCs into immune cells. Our results show that the chemically defined scaffold is a platform that can automate complex biological processes like reprogramming and has the potential to accelerate the automation of immune cell production.

Keywords: automation, animal origin free, reprogramming

5:45 PM – 6:45 PM

143

A UNIVERSAL DIFFERENTIATION PLATFORM FOR THE GENERATION OF IPSC-DERIVED MULTIPOTENT CD34+ HEMATOPOIETIC PROGENITOR CELLS

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Cancer is currently the second leading cause of death and due to increased life expectancy and western lifestyle, its incidences and deaths will further raise. Hence, there is an urgent need for therapeutic treatments. Gene and cell therapy represent the most recently developed measures in cancer therapy that have shown promising clinical results. Adoptive transfer of autologous immune cells is an example for successful cell therapy, however, it has major drawbacks as the process is very expensive and laborious. To overcome those drawbacks, new strategies have evolved to utilize induced pluripotent stem cells (iPSCs) for Off-the-Shelf cell therapy as they can be easily genetically-modified, massively expanded and differentiated into immune cell types relevant for cell therapy. To address the need for iPSC-derived immune cells, we have established a universal differentiation platform utilizing our GMP-compliant iPS cell lines. In contrast to current protocols, we are following a fully defined 2D approach. Adding cytokines in a timely-defined and sequential manner leads to the formation of mesoderm, followed by hemogenic endothelium and finally to CD34+ hematopoietic progenitor cells (HPCs), via the endothelial-to-hematopoietic transition. Those HPCs represent an important intermediate in the process of immune cell differentiation that can be banked and supplied Off-the-Shelf for a shortened differentiation process. Multipotency of those HPCs could be validated by their capability to differentiate into cells of the myeloid (monocytes) and lymphoid lineage (NK cells). To further test for multipotency we are currently establishing a protocol for the generation of iPSC-derived T cells. By systematically analyzing optimal T cell differentiation conditions, in particular medium and

cytokine composition, we could prove their capacity to differentiate into T cells, albeit at a low frequency. However, using T cell-derived iPSCs with the same protocol, we successfully generated T cells, indicating that the protocol is working but needs optimization for the generation of iPSC-derived T cells. Taken together, our data shows that we can successfully generate iPSC-derived HPCs using our universal differentiation platform that can be further differentiated into immune cells that might be used in cell therapy.

Keywords: iPS cells, T cells, immunotherapy

5:45 PM – 6:45 PM

145

PRECLINICAL EVALUATION OF HPFH3 EDITING FOR ENHANCED FETAL HEMOGLOBIN REACTIVATION IN SICKLE CELL DISEASE AND BETA-THALASSEMIA

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The Hereditary Persistence of Fetal Hemoglobin (HPFH) represents a naturally occurring mutation known to reactivate fetal hemoglobin (HbF) at adult stage, holding significant therapeutic promise and physiologically more acceptable. However, the therapeutic potential of a deletion form of HPFH remains relatively unexplored in preclinical settings. In this study, we engineered the HPFH3 genotype (Indian), which is 50kb deletion, into the erythroid progenitor cell line, HUDEP-2 using CRISPR/Cas9 approach. HPLC analysis of heterozygous clonal deletion cells revealed reactivation of HbF up to 25%. Moving beyond cell lines, the therapeutic abilities of HPFH3 were assessed using patient-derived hematopoietic stem/progenitor cells (HSPCs). The generation of HPFH3 genotype demonstrated therapeutically relevant levels of HbF reactivation in both Sickle Cell Disease (SCD) (31.53%) and β-thalassemia (BT) (27.66%) as observed in HPLC analysis. Notably, this genetic editing not only induced substantial HbF reactivation but also led to the reversal of pathological phenotypes including reduced sickling and ROS levels in SCD HSPCs derived reticulocytes and globin chain balance and reduced ROS in BT HSPCs derived reticulocytes invitro. Additionally, genome editing of HSPCs did not affect the multi-lineage potential of HSPCs. Taking a critical step towards translational medicine, HPFH3-edited HSPCs were xenotransplant into immunocompromised NBSGW mice. Remarkably, this in vivo approach exhibited the retention of genome integrity, with no discernible adverse effects on HSPC regeneration or differentiation into various blood cell lineages, including erythroid, myeloid, T, and B cells. These preclinical findings collectively emphasize the robust therapeutic potential of HPFH3 editing, positioning it as a universal strategy for enhancing HbF levels. This study not only sheds light on the molecular intricacies of HbF regulation but also offers a promising avenue for the development of a broadly applicable therapeutic approach for both SCD and BT.

Keywords: fetal hemoglobin reactivation, beta-hemoglobinopathies, genome editing

5:45 PM – 6:45 PM

TOPIC: LIVER

147

GENERATION OF AN IPSC-DERIVED HEPATIC ORGANOID FOR LIVER CELL THERAPY

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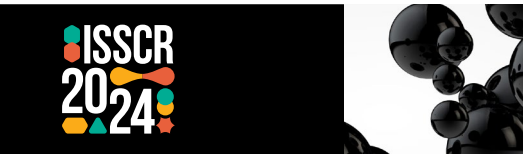
The dramatic increase in the global incidence of liver diseases represents an unmet clinical need and social burden. Cell therapy is a promising effective alternative treatment for many genetic liver diseases, such as metabolic disorders, where little improvements in organ function can lead to a reversion of the disease. Currently, primary cryopreserved hepatocytes are the gold standard for liver cell therapy but they are characterized by low cell renewal, loss of phenotypic identity and functions once isolated, and poor engraftment in the host. In order to overcome these issues, we are developing transplantable liver organoids mimicking the complex structure and function of the liver starting from induced pluripotent stem cells (iPSC). One of the major drawbacks of cell-based regenerative medicine is the need to generate patient-specific cells in order to avoid their rejection, a time consuming and costly process that is very challenging for inborn liver diseases carrying the disease-causing mutations. Here, we have generated a heterologous hypoimmunogenic iPSC cell line (hypo-iPSC) that could be exploitable for all patients without any risk of immune-rejection, by deleting through the CRISPR/Cas9 system the B2M and CIITA genes, essential for the correct surface expression of HLA-I and HLA-II proteins. Hypo-iPSC were successfully committed towards hepatic endoderm-like cells (HNF4α+ and AFP+), endothelial like cells (vWF+ and CD144+) and stellate-like cells (PDGFR-β+, VIM+ and PCDH7+). Hepatic endoderm-like cells were combined in a 3D structure with endothelial-like cells and stellate-like cells whose paracrine signals are likely to increase hepatic maturation and functions. Remarkably, we successfully generated hypoimmunogenic 3D hepatic spheroids characterized by a shell of hepatic stellate-like cells and a core of endothelial-like cells embedded in mature hepatocyte-like cells (ASPGR1+, ALB+) able to perform mature hepatocyte functions (albumin secretion and uptake and release of the indocyanine green dye). Our results pave the way for the generation of an hypoimmunogenic liver tissue with the intrinsic ability of long-term survival in a fully allogeneic recipient without immunosuppression, representing a promising cost-effective and off-the shelf cell therapy for liver diseases.



Funding Source: This study is supported by: 1- Fondazione Aiuti per la Ricerca sulle Malattie Rare (ARMR), Italy. 2- Fondazione Telethon, Italy (grant No. GGP20073)

Keywords: hypoimmunogenic liver organoid, regenerative medicine, iPSC derivatives

5:45 PM – 6:45 PM

149

THE THERAPEUTIC POTENTIAL OF ADSCS IN AMELIORATING LIVER FIBROSIS THROUGH STELLATE CELL SENESCENCE: INSIGHTS FROM CCL4 INJURY AND ECHINOCOCCUS MULTILOCULAR INFECTION MODELS

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To investigate the impact of adipose stem cell (ADSCs) transplantation on hepatic stellate cell (HSC) senescence in liver fibrosis induced by CCl4 chemical liver injury and multilocular Echinococcus infection, ADSCs were isolated from mouse inguinal adipose tissue and co-cultured with activated hepatic stellate cells for 24 hours. The upregulation of the activation marker α-SMA was assessed using Western blot analysis. Pathological examination was conducted to evaluate the effects of ADSCs transplantation on two liver fibrosis models. The influence of ADSCs on hepatic stellate cell senescence both in vivo and in vitro was evaluated through β-galactosidase staining. Aging-related indicators following co-culture of JS-1 cells were analyzed using Western Blot, and immunohistochemistry was employed to assess P16 and P21 expression in the fibrosis model post ADSCs transplantation. Additionally, immunofluorescence was used to determine the co-localization of P21 and α-SMA. The findings revealed that hepatic stellate cells were activated in the fibrosis model, with a significant increase in α-SMA expression. ADSC transplantation led to a reduction in fibrosis area, as well as a notable decrease in ALT and AST levels. Moreover, the expression of α-SMA was significantly reduced, and an increase in SA-β-Gal stained positive cells was observed alongside a rise in P21-positive cells. Notably, P21 was co-expressed with α-SMA-positive cells, indicating that ADSCs mitigate fibrosis progression by promoting the senescence of activated stellate cells post transplantation.

Funding Source: This work was supported by National Natural Science Foundation of China (82060371 for RL, 32060223 for XB), Key Program of Xinjiang Natural Science Foundation (2022D01D59 for RL)

Keywords: adipose stem cell, cell senescence, liver fibrosis

5:45 PM – 6:45 PM

151

GENERATION AND CHARACTERISATION OF UNIVERSAL LOW-IMMUNOGENIC HUMAN PRIMARY CHOLANGIOCYTE ORGANOIDS FOR TREATMENT OF BILE DUCT DISORDERS

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Other than complex surgery or transplantation, there are no current curative therapies for bile duct diseases/cholangiopathies affecting the intra- or extrahepatic biliary tree. We have previously shown that human bile duct epithelial cells can be cultured as 3D organoids to generate mature primary cholangiocyte organoids (PCOs) for the treatment of cholangiopathies. Since the generation of autologous PCOs is likely to remain logistically and economically prohibitive for the foreseeable future, immune rejection of allogeneic PCOs remains a key outstanding barrier to their clinical translation. We thus aimed to develop 'universal' low-immunogenic cholangiocyte organoids for regenerative medicine applications. After systemic testing of numerous conditions, human leukocyte antigen (HLA) I and II double knock out (DKO)-edited PCOs (ePCOs) were generated using CRISPR-Cas9 by dissociating PCOs into single cells, electroporation with the guide-Cas9 complex and sorting for the specific double negative cells. Assessment comparing to parental wild-type (WT) cells was carried out by flow cytometry, functional readouts, co-culture with human peripheral blood mononuclear cells (PBMC) in vitro, and by engraftment under kidney capsule of immunodeficient mice subsequently humanised. Mutational load and CRISPR-driven off-target genetic mutations of parental vs ePCOs was quantified using whole genome sequencing and Nanoseq techniques. The HLA I and II DKO ePCOs generated maintain a mature PCO phenotype demonstrated by flow cytometry and functional analyses. Immune characterization in vitro by co-culture with PBMC experiments and in vivo with humanised mice, show that ePCOs have reduced PBMC cell activation, reduced local immune infiltration and increased graft survival. Additionally, off-target analysis and mutation burden of parental vs ePCOs do not show CRISPR-driven off-target sites nor excess mutation in ePCOs. Human PCOs lacking HLA I and HLA II can be successfully generated using a CRISPR-Cas9 approach. ePCOs retain the phenotypic characteristics of mature PCOs and show reduced immunogenicity when co-cultured with PBMC and using humanised mouse models compared to WT/parental cells. Genomic data show no CRISPR-driven off-target mutational burden in ePCOs.

Keywords: immunogenicity of cell therapies, human primary organoids, humanised mouse model

5:45 PM – 6:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

153

THE RATE OF GRAFT SURVIVAL AFTER CELL ASSISTED LIPOTRANSFER IN OUR INSTITUTION

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Traditional lipoinjection has some problems, such as unpredictability and a low rate of graft survival due to partial necrosis. Cell-assisted lipotransfer (CAL) has emerged as a promising technique in reconstruction by soft tissue augmentation, leveraging adipose-derived stem cells to enhance graft viability and contour restoration. This presentation aims to broaden understanding and utilization of CAL across diverse applications in relatively small scale reconstruction. Here we present a retrospective study which shows the utility of CAL in various deformities beyond breast reconstruction. Four patients (all four were female aged 37.5 ± 16.7) underwent CAL for reconstruction. Cases included breast reconstructions for unilateral congenital hypoplasia and residual concavity deformation after unilateral mastectomy and reconstruction using autologous tissue (2 cases), reconstruction of unilateral congenital lower leg concavity due to constriction ring syndrome (1 case), and post-tumor resection facial concavity (1 case). One CAL was performed per patient. Standard preoperative assessments and surgical techniques were employed. Adipose tissue harvest, stem cell isolation, and CAL procedure were performed as per our protocol (Japanese regulation No. PC3220186/ Cell Processing Center FC3220129). Postoperative monitoring assessed outcomes and complications. The median postoperative observation period was 107.5 days (range 258-27 days). The median rate of graft survival was 81.3% (range 95-70%). In all patients, no complications other than temporary postoperative pain occurred. Our result showed the versatility and efficacy of CAL beyond breast reconstruction, showcasing its utility in addressing various deformities. CAL can offer reliable outcomes with minimal complications, natural-looking results and enhanced patient satisfaction.

Keywords: cell-assisted lipotransfer, CAL, rate of graft survival, adipose-derived stem cells

5:45 PM – 6:45 PM

155

INNOVATING ORAL CANCER TREATMENT: HUMAN DENTAL PULP STEM CELLS IN TARGETED SUICIDE GENE THERAPY

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Oral squamous cell carcinoma is one of the most prevalent forms of head and neck cancer. Current therapies include radiotherapy, chemotherapy, and surgical resection, causing systemic side effects and patient discomfort. Hence, there is a need for more specific and targeted therapies. In this project, we introduce a dental pulp stem cell (DPSC)-mediated suicide gene therapy based on the herpes-simplex thymidine kinase 1 (HSV1-TK)/ganciclovir (GCV) system. The HSV1-TK gene is incorporated into the genome of DSPC (HSV1-TK+ DPSC), enabling the conversion of the non-toxic prodrug GCV into its cytotoxic form. We hypothesize that the HSV1-TK+ DPSC migrate towards tumor tissue, facilitating the transfer of cytotoxic GCV to neighboring cells via gap junctional intercellular communication, inducing apoptosis. Four different DPSC donors with similar HSV1-TK expression were used, indicating connexin-43 presence in 2D and 3D DPSC/OSCC co-cultures, suggesting gap junction formation. Microinjections of the non-permeable dye lucifer yellow demonstrated functional gap junctions in both proximal and distal cells. Therapeutic efficacy was tested in DPSC-OSCC 2D and 3D in vitro co-culture models and an in vivo xenograft model. In vitro analyses showed successful HSV1-TK+ DPSC-mediated OSCC cell killing in different ratios (1:1, 1:5, 1:10) through Incucyte confluence analysis, alamarBlue viability assays, and cleaved caspase-3 expression. In vivo, GCV-treated OSCC xenografts showed a significant reduction in tumor growth compared to PBS controls. These findings highlight successful DPSC-mediated OSCC cell killing through gap junctional intercellular communication in 2D and 3D co-cultures, positioning DPSC as promising carriers for targeted OSCC treatment. Moreover, the validation of the HSV1-TK/GCV system in vivo shows its translational relevance. Further research is required to gain in-depth knowledge about the influence of other biological systems (e.g. the immune system) on this therapy.

Funding Source: FWO vlaanderen Limburgs kankerfonds

Keywords: oral squamous cell carcinoma, human dental pulp stem cells, suicide gene therapy

5:45 PM – 6:45 PM

157

EXPLORING THE USE OF 3D BIOPRINTING AS A METHOD FOR HOMOGENOUS CELL SEEDING ON DECELLULARISED AMNION AND INTESTINE-DERIVED EXTRACELLULAR MATRICES

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3D bioprinting is a promising technique used for creating cardiac regenerative medicine relevant constructs that have the capability to grow, remodel and regenerate in vivo. Due to its ability to control the positioning and distribution of cells, it could be used as a method to seed homogenous cells on natural scaffolds. In this work, we explored bioprinting as a cell seeding approach on in-house and commercial scaffolds. Firstly, the in-house scaffold was prepared by decellularizing the human amniotic membrane (AM) and histological characterization assessment (H&E, EVG and DAPI staining) was conducted to study the effectiveness of decellularization of AM scaffold (i.e. deECM AM). Secondly, no-grid 3D bioprinted constructs composed of pig thymus mesenchymal stem cells encapsulated by alginate hydrogel with various cell seeding densities at both static and dynamic culture conditions were bioprinted on in-house deECM AM and commercial decellularized extracellular matrix ProxiCor (deECM ProxiCor) scaffolds. A cell viability assessment was conducted and a comparison between their cell viabilities/total number of cells was studied. Confocal microscope was used to create Z-stacked images showing the distribution of cells on the bioprinted constructs. H&E, EVG and DAPI staining showed successful decellularization and maintenance of extracellular matrix structure of deECM AM scaffold compared to native AM. A significantly higher number of live cells was present in no-grid 3D bioprinted constructs deposited on deECM AM scaffold compared to deECM ProxiCor scaffold. Furthermore, a significantly higher number of live cells were observed on no-grid 3D bioprinted constructs on both natural scaffolds during dynamic culture condition compared with static culture condition. Finally, Z-stacked images affirmed the precise positioning of homogenously distributed cells on no-grid 3D bioprinted constructs deposited on both tested natural scaffolds at static and dynamic conditions. In conclusion, 3D bioprinting can precisely position and homogeneously distribute cells on different types of natural scaffolds at both static and dynamic culture conditions. This approach holds promise to produce good quality of in-house stem-cell patches that could, potentially, be used in cardiac tissue engineering applications.

Keywords: bioprinting, mesenchymal stem cells, decellularization

5:45 PM – 6:45 PM

159

ENHANCED THERAPEUTIC EFFICACY IN INFLAMMATORY BOWEL DISEASE THROUGH DESENSITIZATION OF UTERINE MYOMETRIUM-DERIVED MESENCHYMAL STEM CELLS TO INFLAMMATORY STIMULI-INDUCED IMMUNOGENICITY

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This study aimed to compare the efficacy of uterine myometrium-derived mesenchymal stem cells (UtMSCs) to bone marrow-derived MSCs (BMMSCs) in treating inflammatory bowel disease (IBD) while investigating the underlying mechanisms. Human BMMSCs and UtMSCs were intravenously administered to mice with dextran sulfate sodium-induced colitis to assess their impact on physical activity, intestinal repair, microbiome composition, and immunity. Bioinformatics analyses were employed to delineate therapeutic roles, and the potential mechanism was validated using allogeneic T cells. Treatment with UtMSCs, in contrast to BMMSCs, significantly reversed alterations in the gut microbiome, diminished interferon-gamma (IFN-γ)- and interleukin-17A (IL-17A)-secreting T helper (Th) cells in mesenteric lymph nodes, and ameliorated disease severity. Transcriptomic data revealed substantial downregulation of Major Histocompatibility Complex (MHC) II-related pathways, pivotal for Th activation, in UtMSCs compared to BMMSCs. Notably, IFN-γ treatment enhanced BMMSC immunogenicity to activate T cells, mirroring local immune responses in IBD patients unresponsive to anti-tumor necrosis factor-alpha (TNF-α). However, IFN-γ had an opposing effect on UtMSCs, desensitizing their ability to activate allogeneic T cells and impeding the differentiation of inflammatory T cells. This effect was attributed to low responsiveness of UtMSCs to IFN-GR-mediated upregulation of MHC II molecules. UtMSC treatment not only hinders the antigen-presenting role in eliciting Th activation under inflammatory condition such as exposure to IFN-γ but also establishes UtMSCs as a superior source for IBD therapy, particularly in individuals resistant to immunosuppressants. This study contributes valuable insights into the potential of UtMSCs as a promising therapeutic avenue for IBD management.

Keywords: uterine myometrium-mesenchymal stem cells, inflammatory bowel disease, major histocompatibility complex II

5:45 PM – 6:45 PM

161

ELUCIDATION OF CELL BEHAVIOR IN THE 3D PRIMARY CULTURE OF UMBILICAL CORD-DERIVED HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells (MSCs) are multipotent stem cells which can be isolated from various tissue sources, with great potential for cell therapy. One obstacle for MSC therapy is the heterogeneity in MSC property due to the donor profile, tissue source, isolation method and culture condition. To supply MSCs of sufficient number and quality, the development of a manufacturing process is required which is based on the understanding of the effect of the process on MSC property. Many studies have considered the development of the expansion culture process of the raw material MSCs. However, few studies have considered the development of the isolation and primary culture process, and the effects on raw material MSC property remains unknown. In this study, we investigated two methods for the isolation of MSCs from umbilical cord, and found that whilst one isolation method resulted in the conventional 2D primary culture, where MSCs proliferated as a monolayer, the other method resulted in a novel 3D primary culture phenomenon, where MSCs initially proliferated as a monolayer, but subsequently formed spheroids. The 2D and 3D primary cultures are expected to provide distinct mechanical cues for the isolated MSCs, which may have implications for MSC property. Thus, we aimed to elucidate the mechanisms underlying the two primary cultures. Phase-contrast timelapse of the 2D and 3D primary cultures were analyzed, revealing that both cultures initially showed slow migration, but a switch to faster migration was seen in the 3D primary culture before spheroid formation. In addition, passage of single cells to the day 7 culture surface of the 3D primary culture, but not to the fresh culture surface, was sufficient to cause spheroid formation, revealing that the switch in migration was due to a temporal change in the culture substrate, which ultimately gave spheroid formation. Overall, we identified and elucidated the behavioral mechanism of a novel 3D primary culture method, which may provide an improvement to the conventional 2D primary culture method.

Funding Source: Project Focused on Establishment of QbD-based control strategy and advanced core ecosystem in cell manufacturing from the Japan Agency for Medical Research and Development (AMED) under Grant Number JP20be0704001.

Keywords: mesenchymal stem cell (MSC), primary culture, isolation

5:45 PM – 6:45 PM

163

BONE MARROW-DERIVED MESENCHYMAL STROMAL CELL THERAPY AMELIORATED DONOR LUNG INJURY AND IMPROVED TRANSPLANTATION OUTCOMES IN A PORCINE MODEL

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Lung transplantation (LTx) remains the only treatment option for end-stage pulmonary diseases, yet faces challenges related to donor lung scarcity and primary graft dysfunction (PGD), limiting long-term patient survival. Aspiration injury in donor lungs is one of the main causes why donor organ offers are declined as it increases the PGD risk. Mesenchymal stromal cell therapy holds promise in regenerative medicine and immunomodulation but has not been sufficiently explored in the field of lung transplantation. We used early passage bone marrow-derived mesenchymal stromal cells (MSCs) during ex vivo lung perfusion (EVLP) and post-LTx to mitigate lung injury and PGD incidence in our extensive porcine lung transplantation model. We aimed to regenerate marginal donor lungs to make them available for transplantation and to address gaps in PGD prevention strategies. We herein create the opportunity to better understand MSC's regenerative potential, by proposing a novel MSC therapy integration with EVLP and post-transplantation care. Our experimental results demonstrate that repeated administration of early passage MSCs during ex vivo lung perfusion and post-transplantation significantly improved lung function and histopathological outcomes in pigs with aspiration-induced lung injury. Compared to single-dose or placebo groups, animals receiving repeated MSC doses showed reduced lung injury scores, lower signs of inflammation, supported tissue regeneration, and had significantly lower incidence of PGD, 3 days post-transplantation. Our findings represent a leap in lung transplantation research by showcasing the effectiveness of repeated early passage MSC administration in improving outcomes for recipients. This

novel approach offers promising therapeutic potential to mitigate lung injuries and PGD, thus possibly increasing the available donor lung pool, enhancing LTx outcomes and potentially advancing the success rates of lung transplantation procedures.

Funding Source: This project is supported by the Swedish Research Council, CAMP, Sweden´s Innovation Agency, the Knut and Alice Wallenberg Foundation, and ALF funding from the Swedish government and county councils.

Keywords: MSC therapy, lung transplantation, lung injuries

5:45 PM – 6:45 PM

165

B2M-KO IMSCS BETTER SUPPRESS T CELL PROLIFERATION BY UPREGULATING IDO1 IN RESPONSE TO PROINFLAMMATORY SIGNALS

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Mesenchymal stem cells (MSCs) are multipotent, relatively non-immunogenic, and can differentiate according to environmental cues, making them an ideal candidate for numerous allogeneic cell therapies. Moreover, MSCs have consistently demonstrated excellent safety profiles in clinical trials, but poor therapeutic responses have impeded their translational success. Induced pluripotent stem cell (iPSC)-derived MSCs (iMSCs) engineered with stealthing features are designed to address the shortcomings of traditional MSC therapeutics, including source heterogeneity, limited expansion potential, and suboptimal pharmacokinetics. Here, we report on the development and characterization of β2 microglobulin-knockout iMSCs (B2M-KO iMSCs) derived from mRNA-reprogrammed iPSCs that were gene edited to eliminate B2M expression. B2M is a critical protein of the human leukocyte antigen class I complex (HLA-I), which has been implicated in T cell-mediated immune clearance. Accordingly, unlike traditional MSCs that express some degree of HLA-I and minimal HLA-II, B2M-KO iMSCs expressed neither HLA-I nor HLA-II. Interestingly, relative to tissue-derived MSCs and wild-type iMSCs (WT iMSCs), B2M-KO iMSCs better suppressed T cell proliferation in an activated PBMC co-culture assay ($p = 0.042$). To investigate the mechanism of increased immunosuppression, B2M-KO iMSCs and WT iMSCs were primed with IFNγ for 48 hours and then assessed for expression of the immunoregulatory enzyme, indoleamine 2,3-dioxygenase 1 (IDO1) via western blot and immunofluorescence staining. B2M-KO iMSCs showed greater IDO1 production in response to IFNγ relative to WT iMSCs. At 54 hours post IFNγ-exposure, B2M-KO iMSCs also showed sustained low levels of IDO1 production, whereas WT iMSCs did not show any residual IDO1 expression. Addition of the small molecule IDO1 inhibitor, Epacadostat, significantly reduced suppression of T cell proliferation for all MSC groups ($p = 0.032$), revealing that IDO1 is required for suppression of T cell proliferation by MSCs. These results suggest that B2M-KO iMSCs may prove useful for the treatment of T cell mediated inflammatory conditions.

Keywords: mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), allogeneic cell therapies

5:45 PM – 6:45 PM

167

THE APPLICATION OF HUMAN WHARTONS JELLY STEM CELLS WITH NANOSCAFFOLD FOR TREATMENT OF WOUNDS IN THE ELDERLY

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Elderly patients develop pressure ulcers that are very difficult to treat and current treatments have several limitations. We evaluated the wound healing potential of an aloe-vera-polycaprolactone (AV/PCL) nanoscaffold impregnated with human Wharton's jelly stem cells (hWJSCs + AV/PCL) or its conditioned medium (hWJSC-CM + AV/PCL) using in vitro and in vivo model. For in vitro: hWJSCs and human skin fibroblasts were derived and characterized using our previously published protocols. The influence of hWJSC-CM on elderly human skin fibroblasts (eHSFs, n=6) were compared with that of young HSFs (yHSFs, n=6) and untreated eHSF controls. For in vivo: Following ethical committee review, a total of 56 mice (42 old and 14 young) were divided into 4 groups of 14 mice per group. The 28 wounds (14 mice) of each group were treated with the wound dressing patches as follows (i) Young mice-UCM + AV/PCL; (ii) Elderly mice-hWJSC + AV/PCL; (iii) Elderly mice-hWJSC-CM + AV/PCL; (iv) Elderly mice-UCM + AV/PCL. Wound healing was monitored for day 3, 7, 14 and 21. Scratch wounds of hWJSC-CM-treated eHSFs completely closed by day 2 compared to untreated eHSF controls. Collagen and elastin levels were significantly increased while senescence-related genes were significantly down-regulated in hWJSC-CM-treated eHSFs compared to untreated eHSFs. Angiogenesis assays produced significantly greater tubule numbers and ring formation in the presence of CM from hWJSC-CM-treated eHSFs. Wound closure rates were significantly greater for hWJSC + AV/PCL (D7: 59.00 ± 4.08%; D14: 94.83 ± 1.29%; D21: 99.52 ± 0.30%) and hWJSC-CM + AV/PCL (D7: 65.67 ± 3.63%; D14: 94.65 ± 1.27%; D21: 99.20 ± 0.47%) compared to UCM+AV/PCL (D7: 44.14 ± 5.89%; D14: 65.72 ± 2.94%; D21: 82.12 ± 6.47%) ($p< 0.05$). Epidermal and dermal thickness, CD31 and gene expression of markers associated with wound healing (MMP2, MMP9, TFPI-2, COL1A1, COL3A1, TGF-β, ANGPTL1, ANGPTL2, ANGPTL3, FGF2, HFG, VEGF, CXCL10 and EREG) were significantly greater in elderly mice treated with hWJSC+AV/PCL and hWJSC-CM+AV/PCL. Our data show that hWJSCs in combination with the stem cell niches in nanoscaffolds provide an attractive wound dressing patch for treatment of chronic wounds in the elderly. The safety and efficacy of this wound dressing patch will be evaluated in a human clinical trial.

Keywords: human Wharton's jelly stem cells, nanoscaffolds, elderly and young skin, preclinical elderly mouse model, wound dressing patch

5:45 PM – 6:45 PM

169

CARDIOPOIETIC CELL THERAPY IMPROVES QUALITY-OF-LIFE IN ADVANCED HEART FAILURE: OUTCOMES FROM A PHASE III CLINICAL TRIAL

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Patients with heart failure experience a poor quality-of-life. Hence, they appreciate interventions which may help alleviate debilitating symptoms and daily suffering. Cell therapy for heart failure has proven feasible and safe. However, in cardiac regenerative clinical trials, limited emphasis has been placed on the patients' perception of their state of health. Here, in the setting of a multinational, randomized, double-blinded Phase III study, measures of health-related quality-of-life were added to traditional mortality and hospitalization outcomes. All participants suffered from refractory chronic heart failure characterized by reduced left ventricular ejection fraction due to ischemic heart disease or previous myocardial infarction, and had an episode of worsening symptoms in the past 12 months despite guidelines-directed medical therapy. The Minnesota Living with Heart Failure Questionnaire (MLHFQ), a Food and Drug Administration qualified instrument for evaluating therapeutic effectiveness, was applied through the 1-year follow-up. Patients either received the sham procedure (n = 151) or were cell-treated (n = 120) through endomyocardial delivery of 600 million cells. Cells were pre-conditioned using the cardiopoiesis optimizing protocol aimed at promoting the cardiorestorative capacity of autologous adult mesenchymal cells. Sham and cell-treated cohorts reported comparable improvement in MLHFQ scores (mean treatment difference with baseline adjustment -3.2 points, p = 0.107). Superiority of cell treatment over sham in the betterment of the MLHFQ score was demonstrated for patients with pre-existing advanced left ventricular enlargement (baseline-adjusted mean treatment difference -6.4 points, p = 0.009). In this highly responsive subpopulation, benefit on the MLHFQ score paralleled reduction in the number of deaths and hospitalization post-cell therapy (adjusted Mann-Whitney odds 1.43, 95% confidence interval 1.01-2.01; p = 0.039). These results suggest that cell therapy has the potential to improve both quantity and quality of life in patients with advanced ischemic heart failure. Further studies are required to validate the reach of regenerative therapy in achieving whole person care.

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Keywords: clinical trial, quality-of-life, heart failure

5:45 PM – 6:45 PM

171

REVERSIBLE IMMORTALIZATION PRESERVES THE CHARACTERISTICS OF MESENCHYMAL STEM CELLS AND FACILITATES THE LARGE-SCALE PRODUCTION OF IL10-ENRICHED EXOSOMES TARGETED TOWARD CHONDROCYTES

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Osteoarthritis (OA) is the most common painful disease with chronic articular cartilage degeneration. MSCs offer potential for OA therapy, as their exosomes harbor bioactive molecules such as non-coding RNAs and proteins. However, primary MSCs and their exosomes exhibit intrinsic limitations, including restricted exosome production due to replicative senescence, inconsistent therapeutic efficacy in OA treatment, and challenges in targeting owing to the dense, avascular extracellular matrix of cartilage. Here, we demonstrate the reversible immortalization of human umbilical MSCs using lentivirally delivered excisable hTERT and SV40T transgenes, which extends cell proliferation while retaining stem cell characteristics. We engineered IL10-enriched MSC exosomes through two distinct approaches: utilizing the XPack MSCV-XP-MCS-EF1α-Puro Cloning Lentivector, employing an optimized N-terminal peptide sequence to direct IL10 to the inner surface of the exosome membrane; and employing CRISPR/dCas9 activation (CRISPRa) to enhance the transcription of endogenous IL10, resulting in its enrichment within exosomes. By fusing a chondrocyte-affinity peptide (CAP) with the lysosome-associated membrane glycoprotein 2b protein on the surface of exosomes, we generated CAP-exosomes capable of specifically targeting chondrocytes in vitro. Finally, we combined the aforementioned strategies to produce ample bioengineered exosomes, which were applied to an in vitro osteoarthritis model to mitigate inflammation. Based on the traits of MSC-derived exosomes, these works increase the therapeutic efficacy and establish a novel platform for delivering therapeutic molecules in OA therapy.

Keywords: reversible immortalization, mesenchymal stem cell, exosome

5:45 PM – 6:45 PM

TOPIC: MUSCULOSKELETAL

173

MACHINE LEARNING AND STATISTICAL CHARACTERIZATION OF BMAC PROTEOME FROM THE ILIAC CREST AND HUMERAL HEAD

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Bone marrow aspirate concentrate (BMAC) is a widely used orthobiologic due to its high concentrations of anti-inflammatory proteins and anabolic growth factors. It is typically derived from the iliac crest for its high yields of marrow and progenitor cells, as well as anecdotal evidence for better patient outcomes. BMAC holds promise for various orthopedic applications including enhancing tissue repair and regeneration. However, the feasibility of obtaining BMAC from alternative sites, such as the humeral head, remains largely unexplored. This study aims to assess whether the BMAC derived from the humeral head is biochemically comparable to that of the iliac crest. Specifically, we investigate whether the proteomic profiles of BMAC samples from these two sites exhibit significant differences. To address this, we conducted a comprehensive statistical and machine learning analysis comparing the proteomes of BMAC derived from the iliac crest and humeral head. Our statistical analysis of antibody microarray data revealed significant differences in the expression levels of 30 out of 110 proteins sampled between BMAC specimens from the iliac crest and humeral head. Subsequent classification using two SVM machine learning models identified 33 unique proteins and 7 overlapping proteins as key in distinguishing the origin of BMAC samples. Notably, 4 proteins consistently emerged across all three analyses, warranting further investigation into their roles within BMAC. These findings shed light on the potential suitability of using BMAC derived from the humeral head for orthopedic procedures, such as full-thickness rotator cuff repairs. By demonstrating the biochemical comparability of BMAC from different sites, our study opens avenues for clinicians to consider local BMAC extraction and use as an augment to corrective surgeries. Moreover, the identified proteins offer valuable insights into the mechanisms underlying BMAC's therapeutic effects, facilitating future research aimed at optimizing its clinical applications. Ultimately, this work contributes to advancing personalized orthobiologic therapies and improving patient outcomes in orthopedic medicine.

Funding Source: Pennsylvania Infrastructure Alliance grant and the LUCE foundation for supporting research student salaries Sachdev Orthopaedics for providing support for materials.

Keywords: biochemical comparability of bone marrow aspirate concentrate (BMAC) from Iliac crest and humeral head, machine learning and statistical characterization of bone marrow aspirate concentrate proteome from Iliac crest and humeral head, identification of key site based proteins

5:45 PM – 6:45 PM

175

INDUCTION AND EXPANSION OF HUMAN PRRX1+ LIMB-BUD-LIKE MESENCHYMAL CELLS FROM PLURIPOTENT STEM CELLS

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Current protocols for the differentiation of human pluripotent stem cells (hPSCs) into chondrocytes do not allow for the expansion of intermediate progenitors so as to prospectively assess their chondrogenic potential. Here we report a protocol that leverages PRRX1–tdTomato reporter hPSCs for the selective induction of expandable and ontogenetically defined PRRX1+ limb-bud-like mesenchymal cells under defined xeno-free conditions, and the prospective assessment of the cells' chondrogenic potential via the cell-surface markers CD90, CD140B and CD82. The cells, which proliferated stably and exhibited the potential to undergo chondrogenic differentiation, formed hyaline cartilaginous-like tissue commensurate to their PRRX1-expression levels. Moreover, we show that limb-bud-like mesenchymal cells derived from patient-derived induced hPSCs can be used to identify therapeutic candidates for type II collagenopathy and we developed a method to generate uniformly sized hyaline cartilaginous-like particles by plating the cells on culture dishes coated with spots of a zwitterionic polymer. PRRX1+ limb-bud-like mesenchymal cells could facilitate the mass production of chondrocytes and cartilaginous tissues for applications in drug screening and tissue engineering.

Keywords: limb bud, chondrocyte, cartilage

5:45 PM – 6:45 PM

177

SCALABLE GENERATION OF MUSCLE SPHEROIDS FOR REGENERATIVE MEDICINE

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Skeletal muscle disease is associated with severe limitations of quality of life and devastating, potentially life- threatening functional consequences. Regenerative therapies using tissue engineered muscle are hampered by lack of quantitatively expandable muscle progenitors. Human induced pluripotent stem cells may provide an unlimited source of human muscle progenitors to exploit in regenerative strategies and tissue engineering. The aim of this study was to enable generation of

human muscle spheroids by recapitulating embryonic muscle development in a scalable bioreactor process. To direct pluripotent stem cells into muscle, pluripotent cells were expanded in a bioreactor (volumes between 50-300 ml) to obtain homogenous populations of aggregates with a mean diameter of 140±40 µm (n=4 bioreactor runs). Pluripotency was confirmed by detection of NANOG/OCT4/Tra1-60 using flow cytometry. Characteristic stages of muscle embryonic development were then induced by modulation of Wnt/BMP and Notch signaling. Transcriptome analyses and immunostainings confirmed progression from a neuromesodermal progenitor (SOX2+/TBXT+) to paraxial mesoderm and formation of somite progenitors. Of note, migratory progenitor cells that delaminate from the dermomyotomal somite compartment in the embryo were identified by LBX1 and MET expression. Migratory progenitors contribute to limb muscle and diaphragm which are particularly affected in many muscle diseases. By further maturation in serum-free conditions spontaneously contracting muscle spheroids with an average yield of 1x10E6 cells/ml were obtained from the bioreactor process. The development of human skeletal muscle in muscle spheroids provides a scalable method to derive regenerative progenitor cells as well as muscle building blocks to generate quantitative amounts of skeletal muscle for regenerative medicine.

Keywords: bioreactor, somitogenesis, spheroids

5:45 PM – 6:45 PM

179

CLINICAL-GRADE STEM CELL-DERIVED MINIMAL INJECTABLE UNIT CHONDROSPHERE INJECTION ALLEVIATE CARTILAGE DEGRADATION IN OSTEOARTHRITIS

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The defected cartilage tissue is almost impossible to self-recover. Current treatment options only tend to reduce pain or delay the progression of cartilage degeneration that can lead to osteoarthritis (OA). Recently, induced pluripotent stem cells (iPSCs) are used as a cell source for the development of stem cell-based therapeutics. The development of a successful cell therapeutic, however, requires an efficient and reproducible manufacturing process that generates a safe and efficient product. In this study, the development of an novel iPSC-derived OA therapeutics "MIUChon" is reported, which are small 'Minimal Injectable Unit' chondrospheroids that can be delivered by intra-articular injections. The development of a successful cell therapeutic requires an efficient and reproducible manufacturing process that generates a safe and efficient product. We also attempted to develop a clinical-grade manufacturing process that meets all these requirements. The protocol that we have generated efficiently reproduced safe and efficacious MIUChons. The in vivo and in vitro results demonstrate MIUChon treatment effectively reduced cartilage degeneration and destruction. Overall, the development of MIUChon offers a new strategy for cartilage treatment in OA via intra-articular injections.

Keywords: induced pluripotent stem cells, cartilage, osteoarthritis

5:45 PM – 6:45 PM

181

MYOGENIC PROGENITORS AND FIBROBLAST SEEDED DECELLULARISED SCAFFOLDS GENERATES GRAFTS THAT INTEGRATE IN A PORCINE MODEL OF ESOPHAGEAL DEFECT DEVELOPING CONTRACTILE MUSCLE

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Tissue engineering aims to address the critical organ shortage for transplantation but also has great potential in situations where transplantation is not possible, for example in the esophagus. Esophageal tissue deficits due to cancer or congenital defects currently undergo complex replacement procedures by refashioning of the stomach, colon or small bowel but are associated with significant, long-term complications and poor quality of life. Despite the effort of the scientific community to generate engineered esophagi, this technology is far from clinical translation. We previously repopulated decellularized esophageal scaffolds with pericyte-like bipotent Mesoangioblasts (MABs), which give rise to smooth and skeletal muscle, and fibroblasts (FBs) by microinjection, followed by bioreactor maturation. To assess the function, safety, and integration of this construct, we developed autologous grafts that were transplanted in a pre-clinical esophageal defect mini-pig model (n=8). Single nucleus transcriptomic analysis performed on MABs and FBs (7:3 ratio) pre-injection and post- bioreactor shows an increased proangiogenic phenotype in the graft (e.g. ⬆VEGFA, ⬆PTGS2, ⬇THBS1, ⬇TIMP1), potentially enhancing integration upon orthotopic transplantation into an avascular environment. Grafts were safe and functional, allowing for enteral autonomy and appropriate growth of animals. Spontaneous re-epithelialization of grafts was seen within 3 weeks, and development of contractile muscle shown by the end of the follow-up period (6 months). Spatial transcriptomic analysis at two timepoints post-implantation showed increased proportions of smooth and skeletal muscle and a trend towards resolution of fibrosis at 6 vs. 3.5 months, and a closer recapitulation of tissue architecture compared to native controls. For the first time a bioengineered muscular graft shows contractility with full functionality for esophageal peristalsis. Furthermore, the transcriptomics characterization of our model represents a proof-of-concept of the application of spatial and single-nucleus RNAseq technologies in the evaluation of bioengineered graft integration and of the regenerative mechanisms involved. These results suggest that our construct may offer an alternative solution to esophageal replacement.

Keywords: esophagus, tissue engineering, regenerative medicine

5:45 PM – 6:45 PM

TOPIC: NEURAL

183

FUNCTIONAL IMPROVEMENT UPON TRANSPLANTATION OF FORWARD PROGRAMMED EXCITATORY AND INHIBITORY HUMAN NEURONS INTO PHOTOTHROMBOTIC CORTICAL STROKE LESIONS

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Cerebral infarction is a major cause of disability. Cell therapy using neural precursor cells (NPCs) has yielded beneficial results in preclinical models. However, in vivo differentiation of transplanted NPCs is difficult to control, thus precluding a defined cell composition of the graft. To address this problem, we here explored the transplantation of postmitotic neurons. Specifically, we used induced expression of the transcription factors NGN2 or ASCL1/DLX2 in human pluripotent stem cells to generate defined fractions of 'forward programmed' induced excitatory and inhibitory neurons (iNs), respectively. Different in vitro studies were conducted to simulate and optimize the harsh transition of the donor cells from standard cell culture conditions to the in vivo post-stroke environment. In that context, we found that neurons treated with CEPT (chroman 1, emricasan, polyamine, trans-ISRIB) show higher viability under hypoxic conditions. Furthermore, in vitro time course multielectrode array (MEA) analyses were used to pinpoint the transition of depolarizing to hyperpolarizing GABA signaling (GABA shift) and to assess the functional connectivity between the NGN2- and ASCL1/DLX2-induced iNs. For the in vivo studies, mice were subjected to photothrombotic middle cerebral artery occlusion. Two days later, 105 cells containing 75% NGN2- and 25% ASCL1/DLX2-iNs were stereotaxically transplanted into the infarct border zone. In contrast to control conditions such as fibroblast grafts and buffer injections, iN transplants led to significant behavioral improvement. Immunofluorescent analyses revealed survival of the engrafted iNs for at least 12 weeks. Donor neurons incorporated into the host brain tissue, displaying axon outgrowth into ipsi- and contralateral forebrain regions. Moreover, engrafted iNs were decorated with vGLUT1 and SHANK2-positive punctae, indicating synapse formation in vivo. Taken together, our data suggest an improved regimen for transplantation of post-mitotic human neurons into the lesioned adult brain and an entry point for studying the mechanisms underlying behavioral improvement upon iN transplantation in stroke.

Funding Source: Overseas Research Fellowship, Japan Society for the Promotion of Science Uehara Memorial Foundation research fellowship European Union Horizon 2020 research and innovation program, grant agreement no. 874758 (NSC-Reconstruct)

Keywords: cerebral infarction, NGN2, ASCL1/DLX2

5:45 PM – 6:45 PM

187

NEW CLINICAL-GRADE IPS CELL LINES AND GMP-COMPATIBLE DIFFERENTIATION PLATFORM FOR THE GENERATION OF RETINAL PIGMENT EPITHELIUM

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Cell therapeutic applications based on induced pluripotent stem cells (iPSCs) as a universal starting point appear highly promising and challenging at the same time. Here, the regulatory framework of good manufacturing practice (GMP) imposes a necessary yet demanding set of requirements in terms of quality and consistency on the manufacture of iPSCs and their differentiated progeny. Given the scarcity of accessible GMP iPSC lines, we sought to establish a corresponding workflow and develop improved manipulation procedures with a GMP mindset. Hence, we generated a first set of compliant iPS cell banks via an approved manufacturing workflow based on episomal reprogramming. Donors were from the EU and US and fulfilled corresponding eligibility criteria. The newly generated iPSC lines met a comprehensive set of release specifications and were further characterized at the molecular and functional levels. Accordingly, the cells did not carry lesions in critical cancer-associated genes and displayed a low global mutation load reflecting their neonatal origin, cord blood. Using these same iPSC lines, we have systematically optimized gene editing procedures and developed new and GMP-compatible differentiation procedures for cell replacement therapy: Here, we present a new protocol for the generation of retinal pigment epithelium (RPE) in treating age-related macular degeneration. This new differentiation method features a particularly high degree of simplicity, efficiency, and overall GMP friendliness in that it merely relies on a series of easy-to-perform media changes under adherent culture conditions. Mechanistically, the platform combines small molecule-based neuroectodermal conversion with powerful RPE specification based on the activation of TGFβ signaling. The workflow spans a total of 6 weeks and yields pure RPE cells without need for enrichment or cell selection strategies. Importantly, our optimized conditions for RPE cryopreservation will enable the generation of banks of RPE cells in view of cost-effective routine administration of off-the-shelf patient doses.

Keywords: GMP, iPS cells, RPE

5:45 PM – 6:45 PM

189

DEVELOPMENT OF CLINICAL-GRADE HUMAN INDUCED PLURIPOTENT STEM CELLS FOR AUTOLOGOUS CELL REPLACEMENT THERAPY IN PARKINSON'S DISEASE

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Parkinson's disease (PD) is a prevalent neurodegenerative disorder primarily characterized by the selective loss of midbrain dopamine (mDA) neurons in the substantia nigra. Consequently, cell replacement therapy has emerged as a promising treatment avenue for PD patients. To establish human induced pluripotent stem cell (hiPSC)-based personalized cell therapy, we have developed a platform of core techniques, including novel methodologies of reprogramming and in vitro differentiation along with a chemical approach to eliminate undifferentiated cells from the final cell product. This platform was successfully employed in treating the first sporadic PD patient. In the current study, our focus was to address whether we can generate clinical grade hiPSCs from multiple PD patients and to test their mDA cell products' safety and efficacy for the feasibility of personalized cell therapy for numerous PD patients. Utilizing our second-generation reprogramming method, which combines Yamanaka 4 factors with metabolism-regulating microRNAs, we successfully established clinical-grade hiPSCs from fibroblasts of multiple PD patients. Importantly, our analyses revealed that these patient-derived hiPSCs possess genomic integrity and demonstrate unbiased pluripotent differentiation potential, devoid of cancer-causing mutations. Furthermore, we generated high-quality mDA cells from these hiPSCs and confirmed their authenticity as mDA progenitors, free from residual undifferentiated hiPSCs or genetic aberrations in cancer-related genes. These promising outcomes have led to the recent FDA approval of a phase I/IIa clinical trial for autologous cell replacement therapy for PD. We will discuss our findings regarding the safety and efficacy of our approaches as well as regulatory criteria for an autologous cell therapy approach for PD.

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Keywords: human induced pluripotent stem cells, autologous cell replacement therapy, Parkinson's disease

5:45 PM – 6:45 PM

191

REPROGRAMMING HUMAN GLIA INTO DOPAMINERGIC NEURONS FOR PARKINSONS DISEASE THERAPY

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Parkinson's Disease is a neurodegenerative disorder that results in the loss of dopaminergic (DA) neurons in the substantia nigra. Stem cell-based cell replacement therapy to replace the lost DA neurons has now reached clinical trials. An alternative approach involves direct reprogramming of resident brain glial cells into new dopamine-producing neurons. Previous studies in rodents have demonstrated the feasibility of reprogramming resident glia into functional neurons, but it remains an outstanding question if human glia can be converted into subtype-specific neurons within the adult brain. Our in vitro studies demonstrate the successful reprogramming of human stem cell-derived glia progenitor cells (GPCs) into DA neurons using Ascl1, Lmx1a, and Nurr1 (ALN)-factors. To study if human glia can be converted using these factors in vivo, we introduce a novel humanized pre-clinical reprogramming model by transplanting human GPCs into the dopamine-depleted rat striatum. The transplanted GPCs expand within the rat brain while maintaining their glia progenitor identity as well as generating astrocytes. By delivering lentiviral vectors encoding ALN-factors into the rat striatum post-human GPC transplantation, we investigate the conversion of human glia into cells expressing key markers, including tyrosine hydroxylase, indicative of dopaminergic neurons. Our forthcoming studies aim to comprehensively characterize the reprogrammed cells.

Funding Source: Lundbeck Foundation Postdoc Fellowship (R347-2020-2522).

Keywords: direct glia-to-neuron reprogramming, dopaminergic neurons, in vivo conversion

5:45 PM – 6:45 PM

193

GENERATING AND CHARACTERISING MHC CLASS I KNOCKOUT HUMAN EMBRYONIC STEM CELL DERIVED DOPAMINERGIC NEURONS FOR USE IN PARKINSON´S DISEASE

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In Parkinson's disease (PD), the core issue is the loss of midbrain dopaminergic (DA) neurons, driving key motor symptoms. Although effective dopaminergic treatments exist, prolonged use leads to side effects. This emphasizes the need for reparative approaches, like dopamine cell replacement. Human embryonic stem cell (hESC)-derived dopaminergic neural progenitor cells (NPCs) show promise, with ongoing early-stage clinical trials, including our STEM-PD trial. However, the potential immune response to these cells poses a risk of graft rejection, requiring immunosuppression, which introduces challenges. To address this, CRISPR editing was used on the RC17 hESC line to create a β2M knockout (KO) hESC line—a de facto MHC class I KO line. Subsequently, these cells were differentiated into NPCs by day 16 and matured into mesencephalic dopamine (mesDA) neurons by day 45+ in vitro. The MHC-I KO line exhibited delayed maturation, particularly early in culture. Immunofluorescence analysis and calcium imaging at day 45 showed decreased firing of KO-derived neurons, with morphological changes diminishing at later time points, suggesting slower maturation without MHC class I. Immunogenicity testing in vitro indicated that KO NPCs did not induce T cell activation. Notably, the WT line also showed no T cell activation and had immunosuppressive properties. MHC-I KO is associated with natural killer (NK) cell activation through "missing self." Preliminary experiments testing NK activation capacity revealed both WT and KO-NPCs inducing a response, though whether this was more pronounced in the KO line remains unclear. Further investigation is underway to clarify this aspect. As the PD field progresses towards second-generation products, this research addresses a vital knowledge gap on genetically modifying cells for immune response mitigation during DA neuron transplantation. While MHC-I KO ESCs successfully generate functional NPCs and neurons in vitro, indicating potential for transplantation with a reduced risk of graft rejection, caution is warranted, as such manipulations may have unexpected consequences on cell maturation and functionality.

Keywords: stem cells, neurons, treatment

5:45 PM – 6:45 PM

195

TRANSPLANTATION OF DIRECTLY REPROGRAMMED STRIATAL PRECURSOR CELLS INTO A RAT MODEL OF HUNTINGTONS DISEASE

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Huntington's disease (HD) is a neurodegenerative disorder characterized by progressive motor, cognitive, and psychiatric impairments as a result of the loss of the loss of medium spiny neurons (MSNs). Currently there is no cure for HD. Cell replacement therapy is a potential option for the treatment of HD. Our lab developed a direct reprogramming protocol that generates human striatal precursors from dermal fibroblasts. The protocol eliminates the risks of tumorigenesis and oncogenic mutations upon transplantation and therefore provides a clinically viable cell source for transplantation therapy. This study investigated the potential for striatal precursor cells generated by direct reprogramming to survive and generate functionally integrated MSNs following transplantation into a rat model of HD. Human striatal precursors were derived from dermal fibroblasts by direct reprogramming using SOX2 and PAX6. Striatal precursors were transplanted into the rat striatum 3 weeks following quinolinic (QA) lesioning (n = 15). Control animals were injected with sterile saline (n = 15). Motor function was assessed up to 14 weeks post transplantation using the cylinder test. The rats were killed 14 weeks following transplantation and the survival and maturation of transplanted hiLGEPs determined by immunohistochemistry. Striatal precursors expressed the human-specific marker STEM121 and co-expressed the neuronal marker MAP2 (76.8% ±9.6) and the MSN specific marker DARPP32 (80.7% ±5.4) 14 weeks following transplantation. At 14 weeks post-transplant, transplanted rats demonstrated restoration of motor function with no difference in ipsilateral forelimb use compared to baseline (p = 0.8). In contrast, saline treated rats retained a significant increase in ipsilateral forepaw use compared to baseline (p = 0.02). This study demonstrates the therapeutic potential of directly reprogrammed striatal precursor cells for transplantation therapy for HD.

Keywords: Huntington's disease, cell replacement therapy, direct cell reprogramming

5:45 PM – 6:45 PM

197

CHARTING PATIENT PERSPECTIVES FROM TRIAL PARTICIPATION: A QUALITATIVE STUDY OF PATIENT EXPECTATIONS ABOUT FUTURE PARKINSON'S TREATMENTS

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Advancements in biomedical sciences have always been accompanied by optimism about future applications and possibilities. Such optimism develops in a complex interplay across scientific laboratories, popular culture and the lives of patients and relatives. As clinical trials to evaluate stem cell-based interventions are rolled out, understanding participants' experiences of taking part in a trial are crucial, as are their expectations about what future treatment possibilities the biomedical sciences might bring. This poster presents initial qualitative data from a series of workshops where people living with Parkinson's disease reflected on their experiences as participants in a placebo-controlled, randomized, double-blind phase II UK-based clinical trial in two stages. The trial was undertaken in 2012 – 2017 and involved infusions of the drug Glial Cell Line Derived Neurotrophic Factor (GDNF) proposed to be capable of stimulating the regeneration of dopaminergic neurons. While the trial did not involve transplantation of stem cells, the focus on administering a regenerative product to ameliorate dopaminergic neuron degeneration in Parkinson's disease, provides a unique learning opportunity for future stem cell-based clinical trials. The objectives of the workshops were therefore to investigate: (1) how might the participants' trial experiences affect their relationship with their bodies? (2) how do the trial participants envision the future treatments for Parkinson's disease? and (3) what values do the participants ascribe to advancements in biomedicine—and their own role in such advancements? Preliminary findings and empirical tendencies of this work-in-progress will be presented to discuss how to assist future trial participants and translation of stem cell therapies.

Funding Source: This work was supported by The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), Novo Nordisk Foundation grant number NNF21CC0073729.

Keywords: patient perspectives, trial participation, Parkinson's disease

5:45 PM – 6:45 PM

199

REGION SPECIFIC NEURAL PROGENITOR CELLS WITH SPINAL CORD IDENTITY ENHANCE FUNCTIONAL RECOVERY IN CERVICAL SPINAL CORD INJURY

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While neural progenitor cell (NPC) transplantation holds promise for spinal cord injury (SCI) treatment, optimal therapeutic effects likely hinge on matching the regional identity of NPCs to the host tissue. This study investigates whether NPCs with a spinal cord identity exhibit superior functional recovery compared to unpatterned forebrain NPCs in a rodent model of cervical SCI. We generated spinal cord progenitor cells (spNPCs) from human pluripotent stem cells (hPSCs) using a combination of posteriorizing and caudalizing morphogens (FGF8b + Wnt3a, Retinoic Acid). Compared to forebrain NPCs (fbNPCs), spNPCs displayed reduced expression of forebrain markers (FOXG1+, OTX2+) and increased expression of spinal cord markers (HOX+). Importantly, both NPC lines exhibited similar tripotent differentiation potential in vitro. Following transplantation into injured cervical spinal cords, spNPCs not only demonstrated superior survival and integration but also exhibited an unprecedented ability to differentiate into functional neural lineages that established robust synaptic connections with endogenous cells. Notably, Most strikingly, animals receiving spNPCs displayed significant enhancements in electrical signal transmission across the injury site, alongside remarkable improvements in motor function tests, without exacerbating neuropathic pain—a testament to the precise and effective regeneration of the injured spinal cord. These findings highlight the therapeutic potential of region-specific spNPCs for cervical SCI. Matching the regional identity of transplanted NPCs to the host tissue promotes effective cell integration, enhances synaptic connectivity, and leads to superior functional recovery. This approach holds considerable promise for advancing stem cell-based therapies for SCI.

Keywords: neural progenitor, pluripotent stem cell, spinal identity

5:45 PM – 6:45 PM

201

HUMAN CORTICAL NEURONS RAPIDLY GENERATED BY DIRECT ES CELL PROGRAMMING INTEGRATE INTO STROKE-INJURED RAT CORTEX

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Stroke is one of the major causes of long-term disability and death in adult humans worldwide. A shortage of blood flow to a specific brain area causes neuronal death, leading to motor, sensory, and cognitive impairments. Replacement of dead neurons by transplantation to reconstruct damaged neuronal networks might become a new strategy for functional recovery in patients. This study aimed to determine whether a rapid and efficient protocol for direct programming of human embryonic stem cells to neurons (ES-iNs) can be applied for cell replacement in ischemic stroke. After 7 days of programming, cells were transplanted into the somatosensory cortex adjacent to an ischemic lesion in rats subjected to cortical stroke. The grafted neurons, their axonal myelination, and afferent and efferent synaptic inputs were studied using immunohistochemistry and immunoelectron microscopy (iEM). After intracerebral transplantation, the ES-iNs survived and did not give rise to teratomas. Three months after grafting, individual ES-iNs expressed markers of immature or mature neurons as well as markers for projection neurons of upper or deep cortical layers. The grafted neurons sent widespread axonal projections to the ipsilateral and through the corpus callosum, also to the contralateral to lesion cortex. Importantly, iEM data showed that ES-iNs received synaptic inputs from host cortical neurons, and host-derived oligodendrocytes myelinated their axons. In conclusion, our study showed for the first time that cortical neurons can be produced efficiently and rapidly by direct programming of human ES cells with the capacity to integrate into the stroke-injured brain.

Keywords: induced pluripotent stem cells, stroke, regeneration

5:45 PM – 6:45 PM

203

DEVELOPMENT OF TRANSPLANTABLE CEREBRAL ORGANOIDS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS FOR THE TREATMENT OF STROKE PATIENTS

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Stroke is one of the world's biggest problems for elderly people. Although drugs or interventional approaches are clinically available, many people suffer motor disability because of stroke. Our aim is to improve motor dysfunction caused by cerebral infarction (stroke) by reconstructing neural circuits through cell transplantation. To achieve this goal, we are trying to produce a human iPSC-derived cell product for cell transplantation. Cerebral organoids can be induced reproducibly and efficiently from a clinical-grade human iPSC line using the modified-SFEBq (serum-free embryoid body quick) culture protocol. We have completed the cell culture process by adding additional manufacturing steps, namely the exclusion of non-purpose cells by morphology, sphere-forming, and freezing steps. We have also confirmed that neural fibers from transplanted cells extend along the cerebrospinal tract when the dissociated organoid cells are transplanted into immunodeficient mice. We are conducting a proof-of-concept experiment in rat and monkey stroke models.

Keywords: cerebral organoids, induced pluripotent stem cells, cell therapy for stroke

5:45 PM – 6:45 PM

205

NEAR-INSTANT DETECTION OF GLIOMA STEM-LIKE CELLS IN LIVE HUMAN GLIOBLASTOMA TISSUE

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Glioblastoma is classified as a grade IV glioma, a devastating brain tumor with poor outcome. Emerging research suggests that failure to target glioma stem-like and progenitor cells (GSCs) could explain the poor survival of glioblastoma patients. Glioblastoma tumors often show an infiltrative growth pattern with protrusions into the surrounding brain tissue. In the apex of the tumor, tumor cells co-exist with normal cells, immune cells, GSCs, etc., which makes GSC detection and removal particularly difficult. Here we demonstrate the use of an oligothiophene named p-HTMI or GlioStem (GS), for selective real-time detection of GSCs ex vivo in live human glioblastoma-tissue parallel to surgery. More than 110 brain tumors including >60 glioblastoma patient biopsy samples were stained and analyzed for the presence of GS+ cells. A subset (n=21) of glioblastoma patient samples was stained with a panel of cancer stem and progenitor cell markers and GS-positive cell populations sorted. Bulk RNA-Seq for 7 glioblastoma patient samples with two paired populations of sorted cells, GS+ and GS-, revealed that the transcriptomic signatures of GS+ samples from different patients clustered together, whereas the GS- populations did not cluster close to one another or the GS+ populations pointing to a certain level of homogeneity regarding the GS+ populations, independent of intra-patient or patient-to-patient heterogeneity. Notably GS+ samples exhibited significantly higher expression of 37 genes associated with stem and progenitor cells compared to the GS- samples, including markers being associated with the pre-oligodendrocyte precursor cell (pre-OPC) and pro-neural subtypes but also cancer cells. Single cell annotation proved that GlioStem detected GSCs of different identities (e.g., pro-neural-, OPC-, radial glia-like cells) at different stages of maturity, again with a mix of cancer cell-signatures. We propose that GlioStem is a novel pan-GSC-marker in fresh glioblastoma tumor tissue with potential for immediate use in clinical settings.

Keywords: cancer cell progenitor, clinical trial, neurosurgery

5:45 PM – 6:45 PM

207

DEVELOPMENT OF A CEREBRAL INFARCTION MODEL OF CYNOMOLGUS MONKEYS (MACACA FASCICULARIS) FOR PRECLINICAL STUDIES OF CELL TRANSPLANTATION THERAPY

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Ischemic stroke represents a significant challenge to global nursing care. While thrombolytic therapy has emerged as an acute intervention, its efficacy declines beyond the first hours after stroke onset, leaving a therapeutic gap that is difficult to fill with subsequent drug regimens and rehabilitation. Consequently, novel approaches such as cell transplantation therapy have attracted attention in recent years. Despite promising results in preclinical rodent models, the translation of these findings to human clinical trials has often proved unsuccessful. To bridge this gap, studies in non-human primates, which have closer anatomical and functional parallels to humans, are essential. However, stroke models in macaque monkeys remain scarce. In this study, we established a stroke model in cynomolgus monkeys (Macaca

fascicularis) by surgical occlusion of the middle cerebral artery. Post-operatively, all monkeys exhibited severe paralysis with Non-Human Primate Stroke Scale (NHPSS) scores ranging from 13 to 15. While symptoms gradually improved, residual mild paralysis and fine motor deficits persisted at 3 months. The reproducibility of this model is high, and the larger brain size of cynomolgus monkeys compared to smaller primates such as marmosets enhances its utility for investigating drug dosage and administration site for cell transplantation therapy in preclinical settings.

Funding Source: This work was supported by the Network Program for Realization of Regenerative Medicine from the Japan Agency for Medical Research and Development (AMED) and JSPS KAKENHI Grant Number 22K09230.

Keywords: Ischemic stroke, cerebral infarction model, cynomolgus monkeys

5:45 PM – 6:45 PM

209

HUMAN STEM CELL DERIVED ENTERIC NERVOUS SYSTEM PROGENITOR TRANSPLANTATION RESTORES FUNCTIONAL RESPONSES IN HIRSCHSPRUNG DISEASE PATIENT-DERIVED TISSUE

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Hirschsprung disease (HSCR) is a severe congenital disorder that results from failure of enteric nervous system (ENS) progenitors to fully colonise the gastrointestinal tract during embryonic development. This leads to aganglionosis in the distal bowel, resulting in disrupted motor activity and impaired peristalsis. Currently, the only viable treatment option is surgical resection of the aganglionic bowel. However, patients frequently suffer debilitating, lifelong symptoms and often require multiple further surgical procedures. Hence, alternative treatment options are crucial. An attractive strategy involves the transplantation of ENS progenitors generated from human pluripotent stem cells (hPSCs). We have generated hPSC-derived ENS progenitors using an accelerated protocol. Here we describe their characterisation through a combination of single cell RNA-sequencing, protein expression analysis and calcium imaging. Our protocol consistently gives rise to high yields of cell populations exhibiting transcriptional and functional hallmarks of early ENS progenitors. Crucially, these hPSC-derived ENS progenitors have the capacity to integrate, migrate and form neurons within surgically removed explanted human HSCR colon samples. The transplanted HSCR tissue displays increased basal contractile activity and increased responses to electrical stimulation compared to control tissue. Together, our findings demonstrate the potential of hPSC-derived ENS progenitors to repopulate and restore functional responses in human HSCR patient colonic tissue.

Funding Source: This work was supported by the Medical Research Council, European Union Horizon 2020 Framework Programme and NC3Rs

Keywords: enteric/vagal neural crest, Hirschsprung disease, enteric nervous system

5:45 PM – 6:45 PM

211

TRANSPLANTED HUMAN IPSC-DERIVED NEOCORTICAL PRECURSORS FORM VASCULARIZED TISSUE IN VIVO

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Recent progress in cortical stem cell therapy has demonstrated its potential to repair the brain. However, current stem cell transplant models have yet to demonstrate that the circuitry of transplant-derived neurons can encode useful function to the host after neurodegeneration or trauma. This may be due to missing cell types, their proportions, and the abnormal graft tissue cytoarchitecture. In addition, there is a need for vasculature to increase viability and function of transplanted cells. Here, as a platform for further improvements in neocortical cell grafts, we devised a reproducible aspiration lesion and transplant paradigm. In this paradigm, dissociated mouse fetal donor cells differentiate into upper and deeper layer neurons, glial cells are present, and grafts are vascularized. The grafted neurons project outside of the graft to appropriate brain areas. The graft becomes fully vascularized by 2 weeks post-transplant and perfused with blood. We also find that, with this paradigm, we can organize cells into layers. Finally, when donor neurons are transplanted into the visual cortex, they have spontaneous action potentials, mature similarly to visual pyramidal neurons, and respond to sensory input. This model can serve as a cell replacement

strategy using human induced stem cell (hiPSC) differentiated into the aforementioned cell types. We have preliminary data demonstrating that hiPSC-derived neural precursors can survive, differentiate into upper and deeper layer neurons, and glial cells are present in the graft. When donor neural cells are transplanted with human vascular cells, they form vessels and are perfused with fluorescently labeled red blood cells. Additionally, we find that specific immunocompromised mouse strains are more conducive to the survival of hiPSC-derived cells in this particular paradigm. Overall, we have developed a preclinical model that should allow us to build complex hiPSC-derived neocortical tissue, containing many of the precursor cell types and cytoarchitecture necessary for a properly developing cortex. Importantly, this in vivo model could also be used for investigating development and diseases.

Keywords: neocortical transplant, pluripotent stem cells, regeneration

5:45 PM – 6:45 PM

TOPIC: NO TISSUE SPECIFICITY

213

THE CHALLENGES OF TRACKING THE PROGRESS OF HPSC-BASED CELL THERAPIES

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More than a quarter of a century after the first human embryonic stem cell lines were derived and 14 years after the discovery of human induced pluripotent stem cell lines, human pluriptotent stem cell (hPSC)-derived cells are finding their way into clinical translation. Since 2019, the human pluripotent stem cell registry (hPSCreg®) has maintained a clinical study database to track the progress of hPSC-based cell therapies. As of February 2024, the clinical study database holds 136 clinical studies spanning 14 clinical indications (defined by ICD-10 chapters). Drawing from the experience of hPSCreg as a registry for hPSC lines, the first step in establishing the clinical studies registry was to populate the database with manually curated information. The studies are searched manually from clinical trials which involve hPSC-based cell products for interventional treatment and have been registered at national clinical trial registries, such as clinicaltrials.gov. However, finding data about relevant clinical studies for the hPSCreg resource requires extensive manual curation. Incremental changes in clinical trial reporting and journal publication requirements attempt to improve transparency of clinical trial results, for example, by the registration of clinical trials to national regulatory bodies, making data sharing statements in the clinical trial entry obligatory and placing timelines on reporting results. In the present work, we show an update of the clinical database content and outline plans for its ongoing development.

Funding Source: European Commission Horizon Europe Programme under grant agreement no. 101074135.

Keywords: human pluripotent stem cell-derived cell products, clinical trial database, hPSC-based cell therapy

5:45 PM – 6:45 PM

215

TESTING MAD7 NUCLEASE IN A GMP WORKFLOW TO PRODUCE HYPOIMMUNE IPSC FOR CLINICAL APPLICATION

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Induced pluripotent stem cell (iPSC)-based therapies have the potential to offer broad clinical applicability and cost-effective scalable manufacturing to meet global patient demand. However, there are several challenges on the path to clinical manufacturing of allogeneic iPSC lines, including complicated licensing terms for nucleases that limits freedom to operate, as well as manufacturing difficulties for generating multiple edits without compromising iPSC quality, editing efficiency, or functionality. To successfully develop iPSC-based therapies and meet patient demand, universal iPSC lines need to become commercially available. To address these challenges and enable hypoimmune iPSC for use in iPSC derived cellular therapeutics, CCRM has developed a GMP compatible workflow using the IP friendly MAD7 nuclease to perform multiple gene edits of knock-out and knock-in for immune cloaking. The GMP workflow was designed and optimized to allow single cell sorting of edited iPSC with the proof of clonality that can be used in a regulatory package for IND submission. This optimized strategy was tested with MAD7 nuclease using sequential and multiplex editing approaches to knock out HLA-I/II complexes, and MAD7 showed comparable editing efficiency to Cas9. Further, assays were developed to test the functional efficacy of the knockouts in iPSC for enhanced product characterization. Both MAD7 and Cas9 mediated HLA-I/II complex knockout iPSC lines were characterized using the developed HLAI/II functional tests as well as standard iPSC characterization. These gene edited iPSC lines are commercially available for researchers and therapeutic developers to test in their respective differentiation processes with unedited parental lines as a control. Access to these iPSC lines enables groups to evaluate how standard hypoimmune edits may affect various applications, such as their specific downstream differentiation process, in vivo model assays or downstream drug product. These iPSC lines offer a solution to address crucial challenges in clinical manufacturing while commercial availability to different research groups will broaden the application of hypoimmune iPSC derived cell therapies. The long-term goal of this work is to produce off-the-shelf GMP hypoimmune iPSC for therapeutic developers.

Keywords: hypoimmune, MAD7, gene editing

5:45 PM – 6:45 PM

217

EFFICIENT REPROGRAMMING OF BLOOD CELLS TO PLURIPOTENCY WITH A SYNTHETIC, SELF-REPLICATING RNA THAT ENCODES A NOVEL REPROGRAMMING FACTOR COMBINATION

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We set out to design and develop a robust blood cell reprogramming method to make clinical grade iPSCs for our cell therapy programs. RNA was the preferred nucleic acid for reprogramming since it minimizes the risk for accidental genome mutation. We selected blood cells to reprogram since they have fewer mutations relative to other somatic cell types. Using blood as our starting material also increased access to donor candidates compared to other somatic cell types that are more difficult to obtain. The challenge with method is that RNA reprogramming has not traditionally worked well with blood cells, likely due to technical limitations with repeated introduction of RNA. To bypass these limitations, we developed a synthetic, self-replicating single RNA that encodes reprogramming factors capable of reprogramming blood cells after only one electroporation. Our novel platform provides an efficient strategy for robustly reprogramming blood cells into iPSCs, the raw material needed for our cell therapies.

Keywords: reprogramming, clinical, iPSC

5:45 PM – 6:45 PM

219

LARGE-SCALE EXPANSION OF 3D PLURIPOTENT STEM CELL SPHEROIDS IN A NEW XENO-FREE SUSPENSION CULTURE MEDIUM

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Pluripotent stem cell (PSC)-derived allogeneic cell therapies continue to be limited by the need for scalable and efficient PSC expansion. Three-dimensional (3D) suspension culture has the potential to enable large-scale production of high-quality PSCs, which are grown as aggregates or spheroids. However, the adoption of suspension culture to assist in workflows is limited by the lack of commercial options for PSC suspension culture media. To address this, we have developed the new GMP manufactured Gibco™ Cell Therapy Systems (CTS) StemScale™ PSC Suspension Medium. CTS StemScale is a xeno-free formulation and promotes the self-aggregation of single cells into 3D spheroids. Both induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are capable of forming spheroids, with cell-line dependent growth in the range of 5X – 10X expansion per passage. These spheroids maintain pluripotency and genomic stability over multiple passages and are capable of trilineage differentiation to the three germ layers. Furthermore, expanding PSCs as spheroids enables easy scale-up in various suspension culture vessels, from small scale (< 100 mL) to large-scale (>1L) culture systems, including bioreactors. Notably, we were able to expand 450 million cells to 5 billion cells over a 5 day period by using this PSC culture medium to grow spheroids inside a bioreactor. Due to the large number of cells necessary to seed large-scale vessels, we also successfully cryopreserved these harvested cells at high densities in the range of 50 – 100 million cells/mL. Cells thawed from these vials showed high viability and were able to form spheroids which expanded at normal rates over multiple passages. Ultimately, cells grown in CTS StemScale have the flexibility to differentiate as 3D spheroids, dissociate into single cells and be utilized in downstream applications, or be cryopreserved as single cells for future use.

Keywords: spheroid, bioreactor, scale-up

5:45 PM – 6:45 PM

221

ADVANCED MATRIX-FREE HUMAN PLURIPOTENT STEM CELL MANUFACTURING BY SEED TRAIN APPROACH AND INTERMEDIATE CRYOPRESERVATION

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Human pluripotent stem cell (hPSCs) derivatives have great potential for advanced drug screening, in vitro disease modelling and regenerative therapies. However, the envisioned routine application of these cells will require robust and economically viable production processes, compatible with industry and regulatory standards. Instrumented stirred tank bioreactors (STBR) are routinely applied for mammalian cell lines cultivation in the biopharmaceutical industry. This platform has also been adapted to the matrix-free suspension culture of hPSCs and recently enabled advanced high density bioprocessing of hPSCs by metabolic control and in silico modelling by our group. To further close the gap between the research state and industry-compliance, we here

demonstrate a seed train approach to ensure straightforward process upscaling. Chemical, STBR-controlled dissociation of the matrix-free cell-only hPSC aggregates was established; this crucially supported efficient single cell recovery and process control at each upscaling step. In contrast to prior suspension culture processes, which relied on conventional monolayer culture (2D) for process inoculation, our advanced strategy enabled the uninterrupted maintenance of exponential hPSC cell growth for 8 passages (4 days per passage; 32 days in total) in serial suspension culture. Furthermore, we show the applicability for intermediate high density cryopreservation of suspension-derived hPSCs followed by the direct re-inoculation of 3D suspension culture in STBRs, thereby entirely excluding the requirement of 2D cultivation. Gene expression profiling reveals novel insights into molecular mechanisms associated with continuous hPSCs 3D culture compared to conventional 2D controls; importantly, hPSCs' karyotype stability and differentiation potential was fully maintained after long-term suspension culture. By completely omitting the need for conventional 2D matrix-dependant cultivation of hPSC, the novel culture strategy fosters process automation and facilitates the development of GMP-compliant closed system manufacturing, paving the way for hPS cells expansion and differention at clinically relevant conditions and quantities.

Keywords: stirred tank bioreactor, hPSC bioprocessing, Serial passaging

5:45 PM – 6:45 PM

223

BREAKING THE LIMITS OF 2D: REVOLUTIONIZING STEM CELL CULTIVATION IN STIRRED-TANK BIOREACTORS FOR CELL THERAPY SCALE-UP

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Human induced pluripotent stem cells (hiPSCs) are a powerful tool for innovative approaches, such as drug discovery, in vitro disease modelling, or regenerative therapies. However, such procedures require high cell numbers to be sufficiently applicable, a criterion that is hard to satisfy with traditional 2D culture methods. Stirred-tank bioreactors on the other hand offer a 3D culture environment suitable to provide, control, and maintain optimal growth conditions for the cell of choice. In this study, stirred-tank bioreactors were utilized to systematically optimize process parameters of a hiPSC culture in a step-by-step process. This approach led to a more than 10× increase in cell density (almost 35 × 10^6 cells/mL) compared to uncontrolled conditions while stem cell features and viability were retained.

Keywords: high-density stem cell bioprocessing, process optimization, industrial and therapeutic applications

5:45 PM – 6:45 PM

227

INTEGRATIVE VARIANT CALLING USING RNA SEQUENCING FOR COMPREHENSIVE RISK ASSESSMENT OF ATMPS

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Risk assessment for Advanced Therapeutic Medicinal Products (ATMPs), which include gene and cell therapies, is a longstanding challenge in medical innovation. We developed a bioinformatics pipeline to analyze potentially hazardous genetic changes from RNA-sequencing data for this purpose. Utilizing a combined database of disease-associated variants, comprised of ClinVar, COSMIC, and intoGen annotations, along with AlphaMissense-predicted pathogenicities, we evaluated two variant calling software tools, GATK and Monopogen. Our analysis of single-cell and bulk RNA-seq data from human pluripotent stem cells revealed that combining GATK and Monopogen for single-cell RNA-seq provides the most comprehensive identification of variants. Importantly, this included mutations in risk-associated genes such as TP53, TET1, and OCT4 which. In bulk RNA-seq, however, we identified approximately 20% less genetic variances. This platform will be leveraged for the risk assessment of ATMPs.

Keywords: ATMPs, variant calling, risk assessment

5:45 PM – 6:45 PM

229

OVER 1,000-FOLD EXPANSION OF HIGH-QUALITY, GENETICALLY STABLE IPSCS IN A BIOREACTOR FOR CLINICAL APPLICATIONS

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Human pluripotent stem cells (hPSCs) are attractive tools for drug screening and disease modelling, as well as promising candidates for cell therapy applications. A key requirement for cell therapies is high-quality and genetically stable starting material to produce hPSC-derived cells. However, long expansion times along with increased passage numbers to reach the high number of cells required results in many cycles of mitosis and potential accumulation of genetic abnormalities. Here we present Cellartis® DEF-CS™ 500 Xeno-Free Culture Medium (DEF-CS XF) a chemically defined, feeder-free medium, without human- or animal-derived components for scaled-up hiPSC

culture. In initial 2D culture, the medium supported expansion and culture of eight different hiPSC lines, where the cells remained over 98% positive for TRA-1-60 and SSEA-4 and lacked expression of differentiation markers for up to 20 passages. In addition, no karyotype abnormalities were reported for any of the tested cell lines. 3D culture was initiated by aggregate formation in the medium, which led to the formation of homogenous spheroids with robust proliferation rates in a perfused bioreactor system. By optimizing perfusion rates and dissolved oxygen levels, hiPSC could be expanded 1,100-fold within 3 passages over 11 days to a final concentration of 5 million cells per millilitre. Throughout the expansion, cells maintained their pluripotency, with over 80% of the population positive for Oct-4 and 90% for SSEA4, whilst lacking differentiation markers. Finally, expanded hiPSCs were differentiated into beta cells using a directed differentiation protocol, achieving a cell concentration of at least 10^6 cells/mL throughout the differentiation process. Beta cells produced from 3D-expanded hiPSCs were at least 51% positive for insulin expression, demonstrating the utility of this medium to produce large quantities of therapeutically relevant cells. In summary, our xeno-free culture system allows for efficient, robust, and scalable production of hiPSCs, thus facilitating the use of hiPSCs for research and large-scale 3D suspension for clinical applications.

Keywords: scale-up, 3D expansion, robust proliferation

5:45 PM – 6:45 PM

231

SCALING UP PLURIPOTENT CELLS: EXPANSION OF IPSCS IN A HOLLOW FIBER BIOREACTOR

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The number of applications for expanded induced pluripotent stem cells (iPSCs) have increased notably in recent years. iPSCs can provide the raw material required for differentiated cell types, a tailored substrate for drug development and testing, or produce the therapeutic agent themselves in the case of iPSCs derived exosomes. As the range of applications for iPSCs grows, so does the need for reliable methods for large-scale expansion of this versatile cell type. The Quantum Flex hollow fiber bioreactor (HFB) provides such an option for the scale-up and manufacture of iPSCs for a range of applications. In this work, human iPSCs from a single donor were expanded in the small (2000 square cm) HFB of Quantum Flex. HFBs were coated with either 1 mg recombinant laminin (LN; 17µg/mL; 0.5µg/square cm) or 2 mg recombinant vitronectin (VN; 34µg/mL; 1.0µg/square cm) prior to cell seeding using a method designed specifically for the small bioreactor system to maximize the evenness of cell distribution in that system. Regardless of coating, iPSCs were seeded at 7500 iPSCs/square cm (1.5E+07 total cells/HFB) and expanded for ~4 days following methods that were kept as aligned as possible with typical manual culture methods to facilitate ease of process transfer. In that time, iPSCs expanded on LN coated HFBs yielded a mean of 5.0E+08 iPSCs (n=4; SD=7.1E+07) and iPSCs expanded on VN coated HFBs yielded a mean of 2.5E+08 iPSCs (n=3; SD=9.4E06). Of those yields, an average of 12% (LN) or 10% (VN) were lost to the system washout that occurs prior to the introduction of 60 mL Accutase that was used to harvest the cells. Mean iPSC viability upon harvest was 96.8% (LN) or 97.9% (VN). Cells recovered from the system were assayed via flow cytometry for 4 common iPSC markers (SOX2, OCT4, TRA-1-60, SSEA4) and all cells from the system harvest displayed all 4 markers at rates >90%. Interestingly, when cells recovered from the pre-harvest washout were also interrogated for the same markers, these cells displayed those markers at frequencies that were 10-19% (LN) or 4-8% (VN) lower than that seen in the cells recovered from the harvest only. This may indicate that the preharvest washout can help prune less desirable (potentially more differentiated) iPSCs prior to harvest and leave a less differentiated population behind for the final harvest.

Keywords: iPSC, bioreactor, manufacture

5:45 PM – 6:45 PM

233

SAFETY AND EFFICACY EVALUATION OF INTRA-ARTICULAR KNEE TRANSPLANTATION OF THREE CONSECUTIVE DOSES OF HUMAN UMBILICAL CORD BLOOD PLATELET-RICH PLASMA IN KNEE OSTEOARTHRITIS PATIENTS: PHASE I/IIA

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Knee osteoarthritis (OA) is a chronic degenerative disorder that affects about 370 million people around the world. Although autologous Platelet-Rich Plasma (PRP) has shown beneficial therapeutic effects on Knee osteoarthritis, its efficacy depends on some patient-related factors such as age, gender, or patient' comorbidities. Due to these limitations, application of allogenic PRP has been proposed as an alternative approach. This study aims to investigate the safety and efficacy of human Umbilical Cord Blood Platelet-Rich Plasma (hUCB-PRP) utilization in Knee osteoarthritis. Based on the study's eligibility criteria, six patients with knee OA enrolled in a pilot phase-I open-labeled clinical trial assessing the safety and efficacy of triple intra-articular injection of allogenic hUCB-PRP at intervals of 0, one, and two months and followed for 12 months after the first injection. The safety and efficacy were assessed based on the Common Terminology Criteria for Adverse Events version-5 and Visual Analog Scale (VAS) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC), 36-item Short Form (SF-36) and the cartilage volume of the knee joint. Six participants with an average age of 63±3.74 years old were enrolled in this study. After cell-infusion, no Serious Adverse Events (SAEs) were identified. Pain was the most prevalent complaint of participants after the injection which was mostly resolved by 24 hours. The average

score of WOMAC was 55.33 (±22.6), 35.50 (±16.7), and 33.33 (±14.7) respectively at the initial, first, and second visits. A repeated measure ANOVA test was performed and all subscales of WOMAC including pain, stiffness, and physical activity were significantly improved (P-values< 0.05) between the initial visit and follow-ups. The VAS score was not significantly changed during the intervention. The SF-36 significantly improved after the interventions (P-value< 0.05). The cartilage volume is going to be assessed as the clinical improvement outcome at 12-month follow-up. This study showed the safety and efficacy of hUCB-PRP during a phase-I clinical trial during a 6-month follow-up as an alternative treatment for autologous PRP for knee osteoarthritis.

Funding Source: Royan Stem Cell Technology Company.

Keywords: knee osteoarthritis, Umbilical Cord Blood Platelet-Rich Plasma, clinical trial

5:45 PM – 6:45 PM

235

A SINGLE-STEP PROCESS FOR ENGINEERING HYPOIMMUNOGENIC PLURIPOTENT STEM CELLS WITH THE PIN-POINT PLATFORM

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Pluripotent stem cells (PSCs) hold great promise for the manufacturing of numerous advanced cell therapies. Off-the-shelf allogeneic products derived from PSCs engineered to be compatible with large cohorts of patients have the potential to dramatically broaden access to these therapies, however their sensitivity to DNA damage presents challenges for efficiently performing the complex genome editing operations necessary to realise much of their potential. Base editors represent a potential solution to these challenges due to their reduced genotoxicity compared to nuclease-based technologies. We have developed the Pin-point™ platform, which enables the modular assembly of base editors composed of DNA binding Cas and DNA modifying deaminase components associated via an aptamer encoded in the sequence-targeting guide RNA (gRNA). Owing to the aptamer-dependent recruitment of the deaminase component to target DNA sequences, the Pin-point platform uniquely allows multi-purposing of a single Cas nickase component for simultaneous multiplexed base editing and targeted transgene knock-in. Transient delivery of mRNAs encoding a Pin-point base editor composed of Rat APOBEC1 and SpCas9 nickase in combination with synthetic aptamer-encoding gRNAs achieved durable target protein knockout, and substantially improved cell viability, editing efficiency, and genome integrity following multiplexed base editing compared to CRISPR-Cas9 with no adverse impacts on pluripotency. To demonstrate the utility of the Pin-point platform for the engineering of allogeneic PSCs we generated a panel of clonal hypoimmunogenic iPSC lines with a range of genotypes using an automated clone tracking and picking workflow. Hypoimmunogenic iPSC lines generated via both multiplexed base editing and simultaneous base editing with targeted transgene integration retained pluripotency and exhibited the expected human leukocyte antigen (HLA) phenotypes when differentiated to therapeutic cell products. The Pin-point platform therefore represents a safe and efficient solution to simultaneously perform multiple genome engineering operations via a novel single step process compatible with downstream automation, offering the opportunity to dramatically streamline the development of allogeneic iPSC-derived cell therapies.

Keywords: base editing, multiplexed knockout, targeted knock-in

5:45 PM – 6:45 PM

TOPIC: PANCREAS

239

INTEGRATION OF STEM CELL-DERIVED PANCREATIC AGGREGATES INTO FN-SILK NETWORK FOR IN VITRO MATURATION

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Diabetes type 1 is a life-threatening disease that accompanies a life-long insulin dependency and limits the quality of life. A potent way to treat diabetes is pancreatic islet transplantation. However, pancreatic islet transplantation has significant challenges, e.g., donor shortage and massive loss of islets. These problems could be solved by using insulin-producing pancreatic aggregates differentiated from human pluripotent stem cells, combined with a scaffold to protect the pancreatic aggregates during the transplantation. FN-silk is a recombinantly produced silk protein, functionalized with a fibronectin motif to promote cell adhesion, that has the unique ability to form a 3D network that supports cell growth in an environment mimicking the extracellular matrix. Incorporation of pancreatic aggregates derived from pluripotent stem cells FN-silk networks may support a novel cell therapy to treat type 1 Diabetes. We analysed the viability of pancreatic aggregates incorporated in FN-silk networks during a cultivation period of 3 weeks, and compared the functionality of incorporated versus free pancreatic aggregates by measuring the expression of c-peptide (insulin) and glucagon expression. Moreover, the transcriptome was analyzed on a single-cell level to compare gene expression profiles and heterogeneity. We observed high viability of pancreatic aggregates incorporated in FN-silk networks over 3 weeks of cultivation, and enhanced pancreatic islet function with increased insulin and glucagon expression. In particular, the maturation of beta cells seems to be improved in FN-silk networks. Overall, the single-cell transcriptome analysis revealed deep insight into the different cell types within the pancreatic aggregates, and the changes in gene expression profile when cultivating pancreatic aggregates in a 3D environment. FN-silk is an excellent biomaterial to incorporate pancreatic aggregates and could potentially be used for transplantation to diabetic patients.

Funding Source: The project has received funding from the European Union´s Horizon 2020 framework programme for research and innovation under the Marie Sklodowska-Curie grant agreement No 813453.

Keywords: biomaterial, diabetes, human pluripotent stem cells

5:45 PM – 6:45 PM

241

ENRICHMENT OF STEM-CELL ISLETS USING DENSITY GRADIENT SEPARATION METHOD

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Pluripotent stem cell-derived pancreatic islets (SC-islets) hold great promise for β cell replacement therapy in patients with type 1 diabetes. Despite significant progress in the differentiation protocols, current SC-islet preparations contain non-target (non-islet) cells, which raises the safety risk and increases the transplant volume. Here, we describe a clinically-compliant, full 3D differentiation protocol that includes a purification step relying on the principle of isopycnic centrifugation (density gradient separation) for the generation of the final cell product enriched in SC-islet cell clusters. Before gradient separation, the cell clusters contained on average 52.5 ± 5.4% SC-β cells (C-peptide positive), 8.4 ± 2.7% SC-α cells (glucagon positive), and 4.0 ± 1.7% bihormonal cells. Importantly, these cells were organized in islet cell-rich and endocrine cell-poor clusters, as assessed by dithizone (DTZ) staining. During density gradient separation, islet cell-rich clusters appeared at a density of 1.053–1.088 g/ml, which is similar to primary human islets. Pooled enriched SC-islet fractions consisted of 93.9 ± 0.8% DTZ-positive tissue, while the tissue volume for transplantation was reduced by 22 ±13% after purification. Enriched SC-islets displayed improved functionality both in vitro and in vivo. In vivo, stimulated human C-peptide was increased by 25.6 fold 6 months post transplantation in enriched SC-islet transplants, while non-purified SC-islet transplants showed a 9.5 fold increase as compared to day 14 post transplantation. We propose density gradient separation as a GMP-compliant purification method that is fast, easily scalable, and cost-effective to enrich SC-islets in the final cell product. In contrast to antibody-based single-cell sorting approaches, this method does not destroy the islet cytoarchitecture, which is associated with alteration of islet function and cell loss. This method raises the safety of the final cell product and provides a smaller transplant volume without adverse effects on the biological activity of the cells.

Funding Source: RegMedXB consortium DON Foundation The Dutch Diabetes Research Foundation Bontius Foundation Novo Nordisk Foundation Center for Stem Cell Medicine reNEW (NNF21CC0073729).

Keywords: diabetes, PSC-derived cell therapy, density gradient separation method

TRACK: DISEASE MODELING AND DRUG DISCOVERY (DMDD)

5:45 PM – 6:45 PM

TOPIC: CARDIAC

243

EVALUATION OF THE ROLE OF NANOTOPOGRAPHY CUES IN INDUCED-PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTE DIFFERENTIATION

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Nanotopography (physical) cues have been reported to regulate biological processes such as proliferation, tumor metastasis, and stem cell differentiation via multiple cascade mechanisms. The nanodots chip, as defined with name of nanoscale dots of topography, has been used on several cell types including cancer cells, normal cells, and stem cells for the understanding of topology-dependent behaviors. Studies of stem cell-topography interactions have been focused on maintenance of pluripotency, yet, little is known about the effects on stem cell differentiation specifically in cardiomyocytes (CMs) differentiation. In this study, we aimed to investigate the roles of a set of nanodots chip (with gradually increment dot size) on iPSC-derived CM differentiation. Firstly, homogenous nanodots were fabricated and characterized. iPSC lines were seeded on sets of nanodots chips following by CMs differentiation. The CMs differentiated cells were characterized using immunofluorescence (IF) staining and gene expression profiling. The gene expression profile was analyzed using bioinformatics approaches to identify gene ontology (GO) and targeted drug candidates. Cell assays were carried out to validate the effects of the drugs on CM differentiation efficiency. In short, we identified two statistical significance gene expression trends via CM differentiation on nanodots. According to the trends, four drug candidates were identified. Cell assays revealed that addition of the drugs candidates enhanced CM differentiation efficiency. This finding suggested that nanodots chip could be served as a screening platform to identify potential enhancers for CM differentiation.

Keywords: nanotopography, iPSC-derived cardiomyocyte differentiation, drug screening

5:45 PM – 6:45 PM

245

HYPERTROPHIC CARDIOMYOPATHY AND PROTAC THERAPY IN CARDIOIDS

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Hypertrophic cardiomyopathy (HCM) is characterized by abnormal thickening of the heart muscle, leading to impaired cardiac function. Despite multifactorial influences, the specific factors triggering hypertrophic responses in cardiomyocytes remain elusive, with a lack of pharmaceuticals tailored for HCM treatment. In our study, we utilized three-dimensional cardioids derived from induced pluripotent stem cells (iPSCs) as an ex vivo model to explore potential hypertrophic factors, including endothelin-1 (ET-1), isoproterenol (ISO), and angiotensin-2 (Ang II). Following a 7-day exposure, only ET-1 induced significant morphological changes in cardioids, notably reducing cavity size and increasing cell size and wall thickness of cardioids, particularly pronounced in those carrying the TnnT2 I79N variant, manifesting severe hypertrophy. Immunoblotting confirmed elevated expression of hypertrophic markers, such as atrial natriuretic peptide (ANP), exclusively in ET-1 treated cardioids. Bulk RNA sequencing unveiled that ET-1-induced HCM is associated with modifications in sarcomere components, the disruption of microtubules, along with activation of the MAPK pathway. Moreover, we evaluated the efficacy of a PROTAC targeting JNK1 in attenuating ET-1-induced hypertrophy. A significant over 50% reduction in ANP expression has been shown after PROTAC treatment within 2 days, highlighting its promising therapeutic potential in relieving HCM through degradation of JNK1. Overall, our study explores various factors that may induce HCM, confirms ET-1 as a direct hypertrophic factor in cardiomyocytes, and the innovative use of PROTAC to reduce HCM.

Keywords: cardioids, ET-1-induced HCM, PROTAC

5:45 PM – 6:45 PM

247

DEVELOPING NEXT-GENERATION IN VITRO MODELS OF DUCHENNE MUSCULAR DYSTROPHY BY GENETIC- AND TISSUE-ENGINEERING HUMAN IPS CELLS AND THEIR MYOGENIC DERIVATIVES

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Muscular dystrophies, a heterogeneous group of genetic disorders characterised by skeletal muscle wasting and weakness, often exhibit concurrent cardiac involvement, such as Duchenne muscular dystrophy (DMD). While less prominent in infants, this cardiac pathology worsens with age and significantly contributes to mortality due to dysfunctional dystrophin protein. However, progress in cardiac research is restricted by limited access to heart tissue from living patients and inadequate DMD animal models. To date, while in vitro platforms for modelling DMD have been developed across both 2D and 3D formats, none have been designed to recapitulate the phenotypic presentation of both striated muscle tissues jointly. Therefore, we propose a patient specific strategy utilizing human iPSC derived cardiac and skeletal muscle cells within 3D engineered biomimetic tissues. By elucidating disease mechanisms and establishing DMD specific readouts in this platform, we aim to accelerate therapeutic development. To fulfil these purposes, isogenic DMD and healthy control hiPSC lines, engineered with a reporter cassette for real-time monitoring of dystrophin expression and localisation, were differentiated to cardiac and skeletal myogenic lineages and combined with biomaterials into engineered heart or skeletal muscle tissues (EHTs and ESMTs, respectively). Molecular analyses of maturation markers (cTNI, MHY6/7) revealed enhanced tissue maturation in EHTs compared to conventional monolayer cultures. Notably, DMD EHTs exhibited a reduction in the cardiomyocyte population (cTNT & cTNI positive), accompanied by an increase in the fibrogenic cell population, reminiscent of the fibrosis observed in vivo. Analysis of excitation-contraction coupling demonstrated contraction abnormalities in DMD EHTs also resembling disease associated defects described in vivo. These EHTs and ESMTs were used to assess toxicity and efficacy of mutation specific DMD therapeutics: pilot findings indicate increased time-resolved dystrophin expression in 3D tissues engineered with the aforementioned reporter cassette. This study will now leverage a platform integrating EHTs and ESMTs to comprehensively investigate DMD

disease biomarkers and further assess the efficacy of novel therapeutic approaches in both tissue systems.

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Keywords: Duchenne muscular dystrophy, 3D cultures-engineered muscle tissues, hiPSC-derived myogenic cells

5:45 PM – 6:45 PM

249

MULTIDOSE AUTOLOGOUS UMBILICAL CORD BLOOD-DERIVED MONONUCLEAR CELL THERAPY ENHANCES CARDIAC FUNCTION AND CARDIOMYOCYTE PROLIFERATION IN A PORCINE SINGLE VENTRICLE MODEL

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Single ventricle congenital heart diseases (SV-CHDs) requiring surgical palliation mandate novel therapeutic approaches to optimize long-term outcomes. Multiple data sets have shown some promising beneficial effects of stem cell-based therapies in functional cardiac repair. In particular, umbilical cord blood mononuclear cells (UCB-MNCs) are a source of multipotent stem cells with regenerative capacity and have been evaluated for augmenting cardiac remuscularization and enhancing cardiomyocyte proliferation. However, whether the proliferation of cardiomyocytes potentiated by UCB-MNCs guarantees the restoration of cardiac function is not known. Here, we assessed a novel approach for heart function restoration using multi-dose UCB-MNC therapy. Cesarean delivery of the piglets allowed for UCB collection. Deploying established safety protocol, UCB was processed to the final product—UCB-MNCs, administered as test therapy. A right ventricular pressure overload porcine model was created by pulmonary artery binding (PAB). Piglets were grouped into; Placebo (PAB +DMSO), Single Dose (PAB + 3 million MNC cells/kg), and Multidose (PAB + 3 million/kg MNCs and additional UCB-MNCs cell injection 3 weeks after the first dose). All cell injections were administered intramyocardially across all groups and each group followed for 12 weeks. Multidose intramyocardial administration of UCB-MNCs promoted a significant increase in Ki-67-positive cells and a rise in cardiomyocyte proliferative activity, as demonstrated by a high incorporation of BrdU in cardiomyocyte nuclei compared to single dose and placebo-treated group. An increase in RV-free wall thickness and decreased RV chamber size was observed in the cell-treated groups compared to placebo. Fick cardiac output measurements revealed an improvement in cardiac index in the multidose-treated group compared to other groups. We found more CD31-positive microvessel density across the multidose-treated group compared to the single and placebo group. This result depicts that multidose administration of UCB-MNCs may favorably affect dysfunctional RV performance by simultaneously enhancing cardiac proliferation and function. This portends significant advancement in managing single ventricle-CHD utilizing stem cell regenerative therapy.

Funding Source: Todd and Wanek Program for Hypoplastic Left Heart Syndrome

Keywords: single-ventricle congenital heart disease, umbilical cord blood mononuclear cell therapy, cardiac function restoration

5:45 PM – 6:45 PM

251

AUTOMATED ANALYSIS OF ELECTROPHYSIOLOGICAL EFFECTS IN AUXOTONICALLY CONTRACTING HIPSC-CM ENGINEERED HEART TISSUE

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Cardiomyocytes derived from human induced pluripotent stem cell are an interesting model to study compound effects on cardiac electrophysiology in contracting heart tissue. Assay format and protocol details are not well established. We differentiated hiPSC-CM from a control hiPSC line and produced adeno-associated virus serotype 6 containing the genetically encoded voltage sensor ArcLight driven by cardiomyocyte-specific troponin T promoter. We generated auxotonically contracting strip-format engineered heart tissue (EHT) between flexible polydimethylsiloxan (PDMS) posts and transduced hiPSC-CM during EHT casting with AAV6 ArcLight. We developed an advanced test system to allow for simultaneous analysis of auxotonic contraction by video-optical recording and ArcLight fluorescence intensity detection by photomultiplier. AAV6 ArcLight-transduced hiPSC-CM show characteristic decline in fluorescence intensity during depolarization/contraction in 2D and EHT format. ArcLight signals were detectable for several weeks. The test system allowed for automated, sterile, and manipulation-free recording. Motion artefacts in EHT analysis were controlled by whole tissue recording. For simultaneous electrophysiology and force analysis in EHT reveal characteristic time delay between start of depolarization and contraction. Indicator compounds inhibiting repolarizing hERG channels revealed characteristic changes in voltage and force recording. We have established a prototype for simultaneous automated and sterile analysis of voltage and force in auxotonically contracting EHTs and validated the test system with indicator compounds.

Funding Source: This project was funded by BMBF - PRAEDIKARD

Keywords: engineered heart tissues, genetically encoded voltage sensors – GEVIs, drug screening

5:45 PM – 6:45 PM

253

SCALE OUT AND UP OF HIPSC-DERIVED CARDIOMYOCYTE PRODUCTION USING DIFFERENT KINDS OF BIOREACTORS

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Cardiovascular diseases, the world's leading cause of death, necessitate reliable in vitro models for testing the efficacy and safety of numerous drug candidates. The availability of primary human heart cells is limited in both quality and quantity, and they lack standardization. On the other hand, animal experiments often fail to provide reliable human predictions. However, cardiomyocytes derived from induced pluripotent stem cells (hiPSC-CMs) hold immense potential to fulfill these requirements. In vitro models based on hiPSC-CMs offer new avenues to replicate both physiological and pathophysiological conditions. Already validated in safety pharmacology, hiPSC-CMs are a promising model for drug development and a potential replacement for animal testing. We have devised a cost-effective, reliable method for the concurrent production of hiPSC-CMs from various hiPSC lines in impeller-free, small-scale bioreactors. This protocol has been successfully applied to a range of cell lines, including healthy and diseased hiPSC lines from the EBiSC (European Bank for induced pluripotent Stem Cells). The process was later scaled up to an automated, monitored bioreactor system, resulting in a standardized, reproducible cardiac differentiation procedure. The capability to produce one billion hiPSC-CMs, and even larger quantities within a single batch, is now achievable within 8 days of differentiation. Over 90% expression of cardiac Troponin T in these cells signifies their high purity and quality. Consequently, no further downstream processing for purification is required, but an extended 2D culture of the hiPSC-CM is necessary to ensure assay readiness. We are now capable of concurrently producing hiPSC-CM from various hiPSC lines e.g. for variation studies on a disease line and its corrected isogenic counterpart, as well as amplifying output to generate billions of hiPSC-CM from a single line. This progress is particularly beneficial for applications such as tissue engineering and large-scale drug screening that require substantial cell quantities.

Funding Source: This work has received support from the EU / EFPIA / Innovative Medicines Initiative 2 Joint Undertaking (EBiSC2 grant n°821362, see www.ihi.europa.eu).

Keywords: human induced pluripotent stem cells, cardiomyocytes, bioreactor

5:45 PM – 6:45 PM

255

PCBP1 REGULATES ALTERNATIVE SPLICING OF AARS2 IN CONGENITAL CARDIOMYOPATHY

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Alanyl-transfer RNA synthetase 2 (AARS2) is a nuclear encoded mitochondrial tRNA synthetase that is responsible for charging of tRNA-Ala with alanine during mitochondrial translation. Homozygous or compound heterozygous mutations in the Aars2 gene, including those affecting its splicing, are linked to infantile cardiomyopathy in humans. However, how Aars2 regulates heart development, and the underlying molecular mechanism of heart disease remains unknown. Here, we found that poly(rC) binding protein 1 (PCBP1) interacts with the Aars2 transcript to mediate its alternative splicing and is critical for the expression and function of Aars2. Cardiomyocyte-specific deletion of Pcbp1 in mice results in defects in heart development that are reminiscent of human congenital cardiac defects. Loss of Pcbp1 leads to an aberrant alternative splicing and a premature termination of Aars2 in cardiomyocytes. Indeed, Aars2 mutant mice recapitulate heart developmental defects observed in Pcbp1 mutant mice. Mechanistically, we found dysregulated gene and protein expression of the oxidative phosphorylation pathway in both Pcbp1 and Aars2 mutant hearts; these data provide further evidence that the infantile hypertrophic cardiomyopathy associated with the disorder oxidative phosphorylation defect type 8 (COXPD8) is mediated by Aars2. Our study therefore identifies Pcbp1 and Aars2 as critical regulators of heart development and creates novel animal models to reveal important molecular insights into infantile mitochondrial cardiomyopathy.

Funding Source: This research is supported by National Institutes of Health (R01HL149401 and R01HL138757) and Additional Ventures.

Keywords: heart development, mitochondrial cardiomyopathy, RNA splicing

5:45 PM – 6:45 PM

257

METFORMIN IN STEM CELL MODELS OF MITOCHONDRIAL DISEASE

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Mitochondrial diseases are the heterogeneous group of diseases characterized by dysfunction in oxidative phosphorylation. The most typical manifestations in patients include neuronal disorders, diabetes and cardiomyopathy. There are no curative treatments for mitochondrial diseases. Metformin is the most widely prescribed oral drug for the treatment of type 2 diabetes with blood sugar lowering

effect in patients. Furthermore, multiple studies have suggested that in addition to diabetes, metformin also has beneficial effects on several other diseases including neuronal and cardiovascular diseases. Thus, metformin has been observed beneficial for several of the symptoms most common in mitochondrial patients. However, the use of metformin in mitochondrial patients is controversial due to the increased risk of lactic acidosis, which is also one symptom of mitochondrial disease. The mechanism of action of metformin is still unclear, and needs to be investigated further, especially in mitochondrial diseases patients. In our project we have studied the effects of metformin in induced pluripotent stem cells (iPSC) and iPSC derived cardiomyocytes from patients with primary mitochondrial disease.

Keywords: cardiomyocytes, mitochondrial disease, metformin

5:45 PM – 6:45 PM

259

THE USE OF STEMRNA IPSC DERIVED HUMAN VENTRICULAR CARDIAC TISSUE (IHCT) FOR CLINICAL APPLICATIONS

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The generation of synthetic human tissue necessitates mimicry of native tissue cell composition, architecture, and molecular fidelity. Here we detail the establishment of developmentally staged iPSC-derived human ventricular cardiac tissue (iHCT) from various clinically relevant StemRNA iPSCs. StemRNA iPSCs are generated from ethically sourced starting material, are integration-free as created by mRNA reprogramming, are eligible for further GMP manufacturing, and therefore provide an ideal starting point for cardiac organoid creation for future clinical applications. StemRNA iHCTs comprised of all cell-types of native human cardiac ventricles with their concordant molecular, metabolic, structural, and physiologic characteristics. iHCTs exhibit vascularization, necessary for physiologic nutrient and oxygen distribution within the organoid proper, and for effective distribution of drugs and test compounds. By virtue of its self-organized nature and its composition of all relevant cardiac cell types, StemRNA iHCTs allows for more precise modeling and interrogation of drug effects on the heart compared to 2D monoculture systems typically containing only one relevant cardiac cell type. We assessed StemRNA iHCT response to a selection of reference cardiovascular drugs, for which drug effects and function are well established. In these studies, we successfully recapitulated the expected cardiac responses for 95% of compounds tested. Thus, cardiac tissue generated from StemRNA iPSC provides a clinically relevant method for reliable testing and detection of potential beneficial or cardiotoxic effects of new pharmaceutical compounds on the heart and its functionality. We have further established disease models for indications including myocardial infaction (MI), heart failure with preserved ejection fraction (HFpEF), diabetic cardiomyopathy and cardioinflammatory disease for later indication-specific efficacy assessment. Taken together, StemRNA iHCTs can facilitate accurate and precise drug testing to increase screening efficiency and better streamline drug development workflows.

Funding Source: REPROCELL, Inc. and Genome Biologics

Keywords: cardiac organoids, drug discovery, GMP

5:45 PM – 6:45 PM

261

CHARACTERISATION OF PROLIFERATION AND HYPERTROPHIC RESPONSE OF HYPOPLASTIC LEFT HEART SYNDROME PATIENT-DERIVED HIPSC-CMS BY HIGH-CONTENT AND GENE EXPRESSION ANALYSIS

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Hypoplastic Left Heart Syndrome (HLHS) stands as a rare yet impactful condition, characterised by underdevelopment of the left side of the heart, resulting in an inability to adequately support systemic circulation. While HLHS is primarily oligogenic in nature, its precise aetiology remains elusive, and surgical intervention stands as the sole treatment option. Our research intends to understand the convergence of the genetic heterogeneity of HLHS in cellular processes driving heart development; how these cellular processes are altered, and how such alterations contribute to the disease phenotype. By leveraging human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), we circumvent the limitations of animal models, which have failed to reproduce the disease phenotype. In the present study, we aim to elucidate how patient-derived hiPSC-CMs differ from those derived from healthy donors in terms of proliferation and stress response by subjecting hiPSC-CMs to either proliferation-inducing compounds or pro-hypertrophic stimuli. Briefly, hiPSC-CMs, derived from two healthy donors (control CMs) and two HLHS patients (HLHS CMs), were exposed to either endothelin-1 (ET-1) at 100nM to induce pathological hypertrophy, or proliferation-inducing compounds, CHIR99021 at 5μM and SB203580 at 10μM for 24 hours. We analysed the effects of ET-1 using quantitative PCR for hypertrophic gene expression, high-content analysis (HCA) for pro-B-type natriuretic peptide (proBNP) expression, and bromodeoxyuridine (BrdU) incorporation for cell proliferation. Our results revealed a heightened sensitivity of HLHS CMs to pro-hypertrophic stimuli, as evidenced by significantly increased proBNP expression compared to control. Moreover, while both groups responded similarly to proliferative stimuli, HLHS CMs exhibited a trend towards a higher basal proliferation rate, indicating intrinsic differences. Further studies are underway to determine if these differences extend to other cellular processes and within 3D models. Our findings contribute to the understanding of HLHS disease mechanisms and lay the groundwork for future research including the development of more effective treatments.

Funding Source: Research Council of Finland (projects 321564, 353109) Sigrid Jusélius Foundation The Finnish Foundation for Cardiovascular Research

Keywords: Hypoplastic Left Heart Syndrome, disease modeling, hiPSC cardiomyocytes

5:45 PM – 6:45 PM

263

SELF-ASSEMBLING HUMAN HEART ORGANOIDS AS A PLATFORM FOR STUDYING CARDIOMYOCYTE REGENERATION

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Heart disease is a leading cause of morbidity worldwide. A hallmark of heart disease is the loss and insufficient regeneration of cardiomyocytes. Recently, human self-forming cardiac organoids (HCOs) have been developed from induced pluripotent stem cells (hiPSCs) that partially mimic the tissue composition and complexity of the heart, making HCOs a powerful model system to study regeneration of the maturing human heart. Human cardiac organoids were generated using hiPSCs modified with a cardiomyocyte-specific fluorescent ubiquitination-based cell cycle indicator (FUCCI) that visualizes different phases of the cell cycle in living cardiomyocytes (TNNT2-FUCCI). HCOs were differentiated for 7 days and maintained in culture for at least 20 days. HCOs contained the three major cell lineages of the heart, including cardiomyocytes (67.0%), endothelial cells (10.4%), mesenchymal cells (14.1%) and epicardial-like cells (8.5%). We demonstrated a decrease in cell cycle activity in TNNT2-FUCCI HCOs by real-time recordings at multiple time points during HCO differentiation and maturation. Multicellular cardiac organoids derived from TNNT2-FUCCI hiPSC are a powerful tool for studying cardiomyocyte proliferation in a complex 3D microenvironment. In the future, this platform can be used for high-throughput live approaches using any type of screening library.

Keywords: cardiomyocyte renewal, organoid, cell cycle

5:45 PM – 6:45 PM

265

THE ROLE OF TELOMERE BIOLOGY IN CARDIAC DIFFERENTIATION AND FUNCTION

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Telomere attrition is a key driver of aging and short telomeres are tightly associated with aging-related disorders including cardiovascular disease. The telomere-synthesizing enzyme telomerase (TERT) is downregulated after birth, leading to progressive telomere shortening throughout life. We previously showed that therapeutic reactivation of TERT, via its non-canonical role in mitochondria, is cardioprotective in mouse models (myocardial infarction (MI) and doxorubicin cardiotoxicity) and in human induced pluripotent stem cell (hiPSCs) derived cardiomyocytes (CMs). Thus, we hypothesized that CMs with short telomeres could be more susceptible to stress conditions and exhibit poor regenerative capacity. Hence, we generated an in vitro platform of human CMs with either long or short telomeres to delineate the role of TERT and telomere length status for CM health. To do so, we utilized human induced pluripotent stem cells (hiPSCs) in a dish and further combined it with differentiation techniques to derive human CMs in vitro. We employed the state-of-the-art inducible CRISPRi technology in hiPSCs to modulate the TERT gene, which enabled us to shorten the telomere lengths at will as validated by TRAP assay and telomere qFISH respectively. Despite an increasing deficit in the CM differentiation potential of hiPSCs with short telomeres, we successfully generated CMs with long and short telomeres. We performed detailed analysis of cardiomyocyte differentiation efficiency using flow cytometry and single cell RNA sequencing to establish how short telomere length status diminishes cardiac differentiation efficiency for the hiPSCs. Metabolic activity, viability and contractility parameters of the resulting CMs were measured using mito stress test, caspase activity and multi electrode array respectively. CMs with shorter telomeres exhibited drastically impaired mitochondrial function, strikingly higher levels of basal caspase activity and significantly reduced contraction profile. Collectively, our data suggests that intact telomeres are essential for development and maintenance of healthy CMs. The CRISPRi TERT hiPSC system represents an excellent platform to investigate telomere driven disorders, potentially beyond cardiomyocytes.

Keywords: telomere disease modelling, cardiac development, telomerase

5:45 PM – 6:45 PM

267

A HIPSC-DERIVED CARDIOVASCULAR MODEL FOR THE ANALYSIS OF OXIDATIVE STRESS IN HYPERTENSION

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Hypertension is an important risk factor for various cardiovascular diseases, such as stroke, acute myocardial infarction and chronic kidney disease. It is a complex, multifactorial disease whose molecular mechanisms are not well understood. Recently, several studies have focused on the role of oxidative stress in the development of hypertension. Reactive oxygen species (ROS) are essential for cellular physiology, but in an unbalanced situation, an exacerbated production of ROS can damage cellular components and trigger pathological processes. Among the different ROS sources present in the cardiovascular system, NADPH oxidases (NOXs) are particularly important because they are involved in many features of cardiovascular dysfunctions. In the context of hypertension, clarification is needed regarding redox signalling regulation and the role of Noxs in cardiovascular pathologies. In this scenario, human induced pluripotent stem cells (hiPSC), which model various diseases in vitro, can be a powerful tool for understanding molecular mechanisms in response to oxidative stress in hypertension–induced cardiovascular dysfunction. Here we used hiPSC-derived cardiomyocytes, endothelial and vascular smooth muscle cells from both normotensive and hypertensive subjects to dissect the redox mechanisms through which Nox regulates cardiovascular function. Human iPSC lines were derived from 6 hypertensive (3 responsive and 3 not responsive to medication) and 3 normotensives subjects, and differentiated into cardiomyocytes, endothelial and vascular smooth muscle cells (VSMCs), Cell-type identities were confirmed through immunofluorescence staining for α-actinin, CD31, CD144, α-SMA, CNN, and TAGLN. We analyzed the pattern of Ca2+ influx in hiPSC-derived VSMCs. VSMCs from the hypertensive group exhibited increased Ca2+ influx in the presence of Angiotensin II and Endothelin-1 stimuli compared to those of the normotensive group. We are currently completing this analysis with gene and protein expression, as well as ROS measurement. Our initial results indicate that these cells can maintain the hypertension-related phenotype in vitro after nuclear reprogramming during hiPSC generation.

Funding Source: Fundação de Amparo a Pesquisa do Estado de São Paulo - FAPESP, CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico

Keywords: hiPSC, hypertension, ROS

5:45 PM – 6:45 PM

269

FUNCTIONAL ANALSYSIS OF ANTHRACYCLINE INDUCED CARDIOTOXICITY IN VITRO

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Anthracyclines, such as doxorubicin, are commonly used in chemotherapy for the treatment of several cancers but can be cardiotoxic, leading to severe cardiovascular complications. In this study, we aimed to investigate the mechanisms of doxorubicin using an in vitro model of doxorubicin-induced cardiotoxicity in human pluripotent stem cell-derived cardiac cells. The spatiotemporal mechanisms of doxorubicin-induced cardiotoxicity were investigated using high-throughput live imaging. With this technology, we assessed doxorubicin kinetics, focusing on cellular viability, uptake, and excretion profiles, as well as cell-type-specific toxicity. Subsequently, molecular profiling via single-nuclei RNA sequencing was performed at multiple time points following doxorubicin exposure. These investigations highlight the potential of in vitro models to study doxorubicin-induced cardiotoxicity and will provide a foundation of mechanistic data that may be used to develop therapies for the prevention of doxorubicin-induced cardiotoxicity.

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Keywords: cardiotoxicity, anthracycline, disease modelling

5:45 PM – 6:45 PM

271

ENGINEERED HEART TISSUE MODEL OF PEDIATRIC RESTRICTIVE CARDIOMYOPATHY WITH TNNI3 MUTATION EXHIBITS IMPAIRED RELAXATION

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Restrictive cardiomyopathy (RCM) is a rare cardiomyopathy characterized by ventricular diastolic dysfunction. Genetic mutations in the sarcomere proteins have been implicated in RCM, among which TNNI3 mutations are the most common and result in poor prognosis. TNNI3 is a protein involved in myocardial relaxation and is believed to play an important role in the diastolic dysfunction of RCM. However, no studies have used human induced pluripotent stem cells (iPSC) derived from patients with RCM harboring TNNI3 mutations. The present study investigated whether iPSC-derived cardiomyocytes (CMs) and engineered heart tissue (EHT) from an RCM patient with a TNNI3 mutation exhibited diastolic dysfunction. Whether correction of the TNNI3 mutation or overexpression of normal TNNI3 would ameliorate the phenotype was also assessed. An iPSC line from a pediatric patient with severe RCM harboring the TNNI3 R170W mutation (R170W-iPSC) was generated. The properties of R170W-iPSC-CMs and -EHTs were evaluated and compared with an isogenic iPSC line in which the mutation was corrected by genome editing technology, and a TNNI3 overexpression R170W-iPSC (TNNI3-iPSC) line in which normal TNNI3 was consistently expressed. Evaluation of calcium (Ca2+) dynamics revealed that R170W-iPSC-CMs exhibited a decrease in maximum fluorescence intensity and a prolongation of time to maximum fluorescence intensity, which are characteristics of failing heart tissue. The Ca2+ tau, a time for signal decreased from 100% to 20%, which represents Ca2+ reuptake efficacy during relaxation, was also significantly prolonged in R170W-iPSC-CMs. These results suggest that R170W-iPSC-CMs reflect diastolic dysfunction in RCM. Additionally, significant improvement in Ca2+ dynamics was observed in isogenic- and TNNI3-CMs. Subsequently, EHTs were generated to investigate contractile dynamics. Force of the EHTs was calculated from the movement of pillars using video microscopy. R170W-EHTs showed a prolonged time to relaxation and an increase in force during relaxation, suggesting relaxation impairment. These contractile kinetics were improved in isogenic- and TNNI3-EHTs. In conclusion, R170W-iPSC-CMs and -EHTs were useful as in vitro pathological models, capturing the phenotype of diastolic dysfunction characteristic of RCM.

Funding Source: Japan Agency for Medical Research and Development (22bm0804008h0006, 22bm0804035h0001 and 23ek0109684h0001)

Keywords: restrictive cardiomyopathy, induced pluripotent stem cells, engineered heart tissue

5:45 PM – 6:45 PM

273

EVALUATING CARDIOTOXICITY THROUGH A HUMAN LIFE CYCLE MIMICRY MODEL BASED ON PLURIPOTENT STEM CELLS

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During the human life cycle, the risk of exposure to potentially harmful substances such as drugs and chemicals is gradually increasing. Therefore, there is a need to develop in vitro models for toxicity assessment that can accurately predict the adverse effects of these xenobiotics. Human pluripotent stem cells (hPSCs) have emerged as a cell source for such models with their ability to reflect human physiology. In particular, hPSC-derived cardiomyocytes (hPSC-CMs) have been developed as a model to detect electrophysiological cardiotoxicity of various chemicals. In this study, we investigated the possibility of hPSCs as an in vitro model to simulate the whole human life cycle for toxicology tests with xenobiotics. We assessed the toxicity from hPSCs to 3D cultured hPSC-CMs by bisphenol A (BPA) treatment, one of the most commonly known toxic substances. As a result, the BPA showed cytotoxicity at all stages. This result demonstrates that human pluripotent stem cells are a proper cell resource that can be used for cytotoxicity evaluation. In conclusion, hPSC-based cytotoxicity assessment could be an evaluation system for xenobiotics-induced cytotoxicity across the entire human lifespan.

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Keywords: human pluripotent stem cell, human pluripotent stem cell-derived cardiomyocytes, cytotoxicity

5:45 PM – 6:45 PM

275

CARDIOVASCULAR DISEASES IN 3D: HUMAN ORGANOIDS AS MULTIPURPOSE TOOL TO RECAPITULATE CARDIAC PATHOPHYSIOLOGY IN VITRO

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The prevalence of cardiovascular diseases (CVDs) is expected to increase in the coming decades. Therefore, there is an urgent need for sustainable and complex in vitro models to sufficiently recapitulate the physiology of the human heart. This is especially important regarding drug discovery and development, leading towards translatable in vitro testing of safe and potent drug candidates. Here, three-dimensional (3D) cultivation has emerged as a broad and versatile field utilizing multiple cell types within a 3D environment. We established a multicellular human cardiac organoid (hCO) platform whereby hiPSC-cardiomyocytes (CMs) are combined with fibroblasts, endothelial cells and adipose tissue derived stem cells and organize into a functional hCO structure that contracts spontaneously or induced by electrostimulation. In disease modelling approaches, we generated HCM hCOs based on patient-derived hiPSC-CMs. Initially, we treated these hCOs with Mavacamten which is the first-in-class small inhibitor of the cardiac myosin ATPase approved for treatment of HCM patients with hyper contractility. Chronic and acute treatment of the hCOs affected the contractile performance, leading e.g. to a reduction in contraction amplitude. Apart from genetic heart diseases, these hCOs can be used to model CVDs such as a myocardial infarction (MI). Here, a post-MI resembling hCO is induced by cultivation under low oxygen concentration and norepinephrine stimulation. Combined, this leads to altered fibrosis associated gene expression and changes in calcium handling gene expression that is further reflected in impaired contractile function after reoxygenation. Moreover, we stimulated healthy hCOs with phenylepinephrine-isoprenaline and endothelin-1 (ET1) to induce a hypertrophic phenotype. Both altered the contractile function, leading to an increased beating frequency. ET1 treated hCOs further showed an upregulation of ANP and BNP expression indicating their potential to study cardiac hypertrophy. In summary, our preliminary data highlight the versatility of the multicellular hCOs to serve as a functional drug screening platform and to study cardiovascular pathophysiology.

Keywords: cardiac organoids, disease modelling, cardiac hypertrophy

5:45 PM – 6:45 PM

277

HUMAN MULTI-TISSUE ORGANOIDS AS AN ADVANCED TERATOGENICITY MODEL

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Infamous history such as the thalidomide disaster combined with an increasing number of women taking pharmaceuticals during pregnancy presents a pressing need for a reliable and ethically sound teratogenicity assay. Human pluripotent stem cell (hPSC)-based organoids allow developmental recapitulation in a scalable manner. We recently described a hPSC-derived multi-tissue organoid model termed “heart-forming organoids” (HFOs), properly recapitulating patterns of early heart, vasculature and foregut development, including anterior foregut endoderm (AFE; early lung anlagen) and posterior foregut endoderm (PFE; liver anlagen). HFOs pose an exciting potential to assay teratogenicity. In addition, the developmental mimicry allows the identification of specific teratogenic cell type susceptibly, and the affect this has on developmental patterning. This enables a mechanistic understanding of teratogenic pharmaceuticals currently on the market in a scalable manner, and, based on this validation, promotes the safe development of new drugs. The organoids were exposed to FDA-categorized teratogens Valproic acid (V) and Thalidomide (T). Flow cytometry (FC) and immunofluorescence staining (IF) revealed the expected anti-angiogenic properties of T and V as well as the cardio-toxic effect of T, suggesting HFOs capability to recapitulate the in vivo teratogenic phenotype of these compounds. Moreover, FC revealed a decrease in the endodermal (EPCAM+/CD117+) population of V and T HFOs. Notably, following whole-mount IF, V HFOs showed a relative increase of AFE (FOXA2+/SOX2+) over PFE (FOXA2+/HNF4α+) and a concomitant increase in cardiac progenitors (NKX2.5+/FOXA2+) suggesting a coupled delay in development and an anterior bias. In contrast, T treatment resulted in disruption of the endodermal patterning, juxtaposing AFE and PFE. These results highlight HFOs potential to shed light on teratogenic mechanisms in a quantitative and morphological context, an ability previously difficult with in vitro models. Furthermore, this provides a proof–of-concept to establish a higher throughput assay capable of not only identifying teratogenicity but also understanding the cellular mechanisms that cause them.

Keywords: teratogenicity, organoid, development

5:45 PM – 6:45 PM

279

ESTABLISHMENT OF DSG2 MUTATION ARRHYTHMOGENIC RIGHT VENTRICULAR DYSPLASIA (ARVD) HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIAC DISEASE MODEL

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Arrhythmogenic right ventricular dysplasia (ARVD) is a rare familial heart muscle disease in which the cardiac muscle in the right ventricle is damaged, leading to fibro-fatty myocardial scarring, ventricular arrhythmias, and sudden cardiac death. Mutations in desmosomal proteins, including desmoglein-2 (DSG2), can cause ARVD. In this study, we used human-induced pluripotent stem cells (hiPSCs) from an ARVD patient with a heterozygous DSG2 mutation (c.2358delA) to establish hiPSC-derived cardiac tissues (hiPSC-CMTs) as a disease model. Human amniotic fluid-derived stem cells (AFSCs) and embryonic stem cells (ESCs) were used as healthy controls to differentiate cardiomyocytes (CMs). Flow cytometry data confirmed a purity of over 75% in CMs, as evidenced by the expression of cardiac markers, including Troponin T (TNNT2) and Signal-regulatory protein α (SIRPα) on day 20. CMs were mixed with fibroblasts and endothelial cells in a collagen/fibrinogen extracellular matrix (ECM) and then added to a microfabricated tissue gauge (μTUG) to create hiPSC-CMTs. Maturation media (TDI), consisting of thyroid hormone, dexamethasone, and IGF-1, were used to promote the maturation of CMTs. CMs treated with TDI displayed a more mature phenotype with an elongated spindle shape, larger contraction, a faster electrical pacing frequency, and a proper adrenergic response. DSG2-mutated CMTs exhibited a longer sarcomere length, a decline in CASQ2, and changes in desmosome (PKP2/DSG2/JUP) genes, as well as slower calcium kinetics. They were highly sensitive to isoproterenol testing and showed electrical alternants compared to the AFSC and ESC CMTs. In conclusion, this study suggests that hiPSC-CMT models recapitulate and provide insight into ARVC pathophysiology, and they can be used to identify potential treatment methods for future therapeutic development.

Funding Source: NSTC 112-2636-M-006 -010

Keywords: arrhythmogenic right ventricular dysplasia, microfabricated tissue gauge, hiPSC-derived cardiac tissues (hiPSC-CMTs)

5:45 PM – 6:45 PM

281

REDUCING MICROTUBULE DETYROSINATION IMPROVES CONTRACTILITY AND PROLIFERATION AND PREVENTS HYPERTROPHY IN HUMAN-INDUCED PLURIPOTENT STEM CELL CARDIOMYOCYTES

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The microtubule (MT) network density and detyrosination (dTyr) is increased in heart failure. Reduction of dTyr by overexpression of the tyrosinating enzyme tubulin tyrosine ligase (TTL) or knockdown of vasohibin 1, which catalyzes dTyr with its chaperone small vasohibin binding protein (SVBP), improved contractility of failing cardiomyocytes (CMs). However, the effects of chronic modulation of dTyr-MT are yet to be investigated. Human iPSC lines deficient in SVBP or TTL (KO) were generated with CRISPR-Cas9 and differentiated into CMs and engineered heart tissues (EHTs). Transcriptomics were performed by NanoString for 2D CMs and RNAseq for EHTs, while mass-spectrometry was employed for proteomic analyses in EHTs. EHT data were collected after 60d in culture. For assessment of proliferation, we employed immunofluorescence for KI-67 and flow cytometry of cells incubated with EdU (5-Ethynyl-2'-deoxyuridine). MTs were destabilized with nocodazole (500 nM/10 μM) or stabilized with taxol (10 μM). Hypertrophy of hiPSC-CMs was induced with 100 nM endothelin-1 (ET1) and cell area assessed via confocal imaging. TTL overexpression was achieved with AAV9. SVBP-KO EHTs exhibited lower dTyr-MT levels, higher force and faster relaxation WT EHTs, while TTL-KO EHTs showed enrichment of dTyr-MTs accompanied by lower force development and impaired relaxation. SVBP-KO EHTs presented a marked increase in cell cycle markers, while TTL-KO EHTs exhibited enrichment in hypertrophic markers, motor and cytoskeleton proteins. SVBP-KO CMs showed higher levels of cell cycle related transcripts at both d7 and d30, while TTL-KO CMs showed higher levels of hypertrophic markers ACTA1, NPPB and FHL1 at d7 but not d30. SVBP-KO CMs showed significantly more KI-67+ nuclei at d12 and d28 but not d4 in culture than WT and TTL-KO. EdU incorporation was significantly higher in SVBP-KO than in WT and TTL-KO CMs at d12-16 but not d2-6. Both nocodazole in low or high dosage and taxol lowered EdU incorporation rates. Furthermore, taxol induced CM elongation without increasing cell area. Finally, TTL overexpression and SVBP-KO both prevented ET1-induced hypertrophy in hiPSC-CMs.

This study provides evidence that chronic activation of MT tyrosination improves contractility, induces proliferation and reduces hypertrophy in hiPSC-CMs.

Keywords: microtubule detyrosination, cardiomyocyte proliferation, cardiomyocyte hypertrophy

5:45 PM – 6:45 PM

283

CHARACTERISATION OF HYPERTROPHIC RESPONSE AND THE EFFECT OF A GATA4-TARGETING COMPOUND ON THE PHENOTYPE OF DILATED CARDIOMYOPATHY PATIENT-DERIVED HIPSC-CARDIOMYOCYTES

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Dilated cardiomyopathy (DCM) is a prevalent cause of heart failure and is characterised as left ventricle enlargement and systolic dysfunction. Over 30 different genes are implicated in causing the genetic form of DCM, with the LMNA gene being the second most mutated. Several mouse models have been established to study the pathophysiology of LMNA-related DCM. However, patient-derived human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) provide a superior system for comprehending the genetic basis of human DCM. Our study aimed to determine the phenotypic differences between hiPSC-CMs derived from DCM patients with a mutation in the LMNA gene and hiPSC-CMs derived from healthy donors. In addition, we aimed to investigate the effect of the GATA4-targeting small molecule 3i-1262 on cell phenotype and response to mechanical and hormonal stimuli. We subjected hiPSC-CMs to mechanical stretch for 24 or 72 hours or endothelin-1 (ET-1) for 24 h to induce hypertrophic response. To investigate the effects of 3i-1262, we added the compound or vehicle to the cells 1 h before stimuli. We investigated the expression of hypertrophic markers and cytoskeletal proteins with qPCR. Furthermore, we subjected hiPSC-CMs to ET-1 for 24 h, and quantified proBNP expression using high-content analysis (HCA). Control and DCM hiPSC-CMs displayed differential expression of cytoskeletal genes. Moreover, when subjected to mechanical stretch, they showed different gene expression responses of hypertrophy marker genes (e.g., NPPB) and cytoskeletal genes (e.g., MYH6). HCA results indicate that ET 1 increased proBNP-positive hiPSC-CMs, but there was no difference between the DCM and control cells. In addition, 3i-1262 affected the baseline levels of cytoskeletal genes (e.g., ACTN2) after 72 h. Finally, 3i-1262 inhibited stretch-induced NPPB expression after 72 h of stretching in control hiPSC-CMs. Our results indicate that DCM hiPSC-CMs show differential gene expression and respond differently to hypertrophic stimuli compared to the control hiPSC-CMs. Moreover, 3i-1262 may have anti-hypertrophic effects in hiPSC-CMs, as shown by the down-regulation of NPPB expression. However, further studies are required to fully comprehend the mechanisms underlying DCM and the effects of 3i-1262 on the hiPSC-CM phenotype.

Funding Source: The Finnish Cultural Foundation The Finnish Foundation For Cardiovascular Research Research Council of Finland

Keywords: hiPSC-derived cardiomyocytes, dilated cardiomyopathy, GATA4

5:45 PM – 6:45 PM

285

TOWARD A IN VITRO MODEL FOR CARDIAC DISEASE MODELLING: CHARACTERISATION OF UHV-ALGINATE HYDROGELS

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In vitro models are used to model diseases and to develop novel therapies. To mimic in vivo-like conditions, hydrogels must be considered to model defined cell environments and to enable cell-matrix-interactions. Especially in cardiac tissue cell-matrix-interactions play a crucial role (e.g., in case of cardiac fibrosis). These interactions are lacking in current available cardiac in vitro models, such as cardiac organoids or microtissues. There are different types of hydrogel materials classified by their origin (natural, synthetic and hybrid). UHV-alginate combines necessary biomimetic properties (typical for natural source) with mechanical controlment (typical for synthetic source) due to a quality-controlled harvesting and purification process. In addition, it has already been shown that alginate as a growth surface has a positive influence on the contractility of cardiomyocytes. Beside stiffness and viscoelasticity, the diffusion properties are a crucial parameter, since e.g., bioprinted cells are immobilised in the hydrogel and the nutrient supply must be guaranteed via the diffusion properties of the hydrogel. Detailed studies of diffusion characteristics of UHV-alginate are lacking. In this work, a diffusion study was conducted to determine the accessibility of cell-relevant molecule sizes. To find a meaningful correlation, a variation of UHV-alginate viscosities and crosslinking densities was investigated. In detail: UHV-alginate was prepared as membrane. FITC-Dextran solutions were used as model substances on basal side. The range of used molecule sizes represent nutrients for cell cultivation (e.g. hormones and growth factors). After distinct time intervals, samples of apical side were quantified via fluorescence signal intensity. The data highlights that all relevant molecule sizes can pass all hydrogel

compositions. By changing UHV-alginate viscosity the diffusion rate and the pore formation can be controlled. In summary, this study shows that UHV-alginate ensures the supply of nutrients and can be used to control the availability of molecules to immobilised cells. This forms the basis for future development of an in vivo-like cardiac model, which allows a correlation to be established with the diffusion properties in addition to the mechanical correlation of the biomaterial.

Funding Source: This work was supported by the Bavarian Ministry of Economic Affairs, Regional Development and Energy.

Keywords: hydrogel, immobilisation of cells, cell-based models

5:45 PM – 6:45 PM

287

MATURING IMPRINTING STATUS OF HIPSC-CARDIOMYOCYTES IN CARDIAC MICROTISSUES TO MODEL LONG QT SYNDROME TYPE 1

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Long QT syndrome type 1 (LQT1) is a cardiac arrhythmia caused by mutations in one specific gene, KCNQ1, encoding an ion channel contributing to action potential duration. For this simple genotype-phenotype link, LQT1 was one of the first arrhythmias modelled with human iPSC-derived cardiomyocytes (hiPSC-CMs), supporting the use of this model for in vitro preclinical studies. Notably however, KCNQ1 is developmentally regulated by genomic imprinting in the heart, with the paternal allele initially repressed and both alleles expressed postnatally. Here we used hiPSC-CMs from a LQT1 patient carrying a mutation in KCNQ1 paternal allele and compared functional properties with the isogenic corrected line. Contrary to what typically observed in LQT1 CMs, patch clamp analysis showed no prolongation of action potential duration (APD). We then checked KCNQ1 imprinting status by ddPCR in different hiPSC-CM lines and found unbalanced KCNQ1 allelic expression with repression of paternal allele. To investigate whether maturing hiPSC-CMs could remove abnormal KCNQ1 imprinting, we included hiPSC-CMs in cardiac microtissues (MTs) with hiPSC-derived cardiac fibroblasts and endothelial cells, a system previously shown to promote overall CM maturation and upregulation of KCNQ1. In MTs, we observed an increase in KCNQ1 paternal allele expression accompanied by changes in the epigenetic regulation. LQT1 hiPSC-CMs dissociated from MTs showed prolonged APD compared to the corrected line and a reduced potassium ionic current mediated by KCNQ1, thus revealing the mutation causative effect. In conclusion, we showed that immature hiPSC-CMs carry an abnormal KCNQ1 imprinting status, leading to under- or overestimate effects of mutations associated with LQT1. Maturation of hiPSC-CMs in tri-cellular MTs promotes KCNQ1 bi-allelic expression, showing the utility and importance of using matured hiPSC models for preclinical evaluations.

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Keywords: hiPSC-cardiomyocytes maturation, imprinting, LQT1

5:45 PM – 6:45 PM

289

STRAIGHT-IN: A RAPID AND EFFICIENT PLATFORM FOR TARGETED LARGE GENE INSERTIONS IN HUMAN PLURIPOTENT STEM CELLS

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In mammalian cells, such as human induced pluripotent stem cells (hiPSCs), integrating large DNA payloads (e.g. >10 kb) into specific genomic sites or replacing genomic segments, presents a significant technical challenge. To overcome this, we developed STRAIGHT-IN (Serine and Tyrosine Recombinase Assisted Integration of Genes for High-Throughput INvestigation), a platform that leverages the strengths of different site-specific recombinases, along with CRISPR-Cas9-mediated homologous recombination. STRAIGHT-IN enables stringent, site-specific integration or replacement of large genomic fragments (≥50 kb), while minimizing the inclusion of unwanted DNA remnants that could lead to post-integrative silencing. We have successfully applied STRAIGHT-IN in various projects, such as: i) creating a multi-parameter biosensor hiPSC line for reporting cardiomyocyte excitation-contraction coupling; ii) developing inducible gene expression systems for disease modelling and gene editing, among other applications, and iii) simultaneously generating multiple isogenic hiPSC lines with specific disease variants for research. Recent developments include upgraded versions of the STRAIGHT-IN platform which facilitates the rapid generation of genetically modified hiPSC lines with ~100% efficiency within a two-to-three-week timeframe, and the orthogonal integration of dual DNA payloads. These advancements further improve the platform's utility for synthetic biology, disease modelling, and lineage tracing applications.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine reNEW through a Novo Nordisk Foundation grant (NNF21CC0073729)

Keywords: site-specific recombination, disease modeling, synthetic gene circuit

5:45 PM – 6:45 PM

291

PERP MUTATION CAUSED CARDIOMYOPATHY THROUGH ALTERING NOTCH SIGNALING PATHWAY AND CARDIAC METABOLISM

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PERP (P53 apoptosis effector related to PMP-22) is a tetraspan membrane protein implicated in desmosome assembly, facilitating adhesive junction formation between cells, and in the modulation of P53 phosphorylation for maintaining epithelial integrity. Its association with erythrokeratodermia and Olmsted syndrome has been documented. Recent clinical findings also link PERP defects to cardiomyopathy. This study aims to characterize cardiac omics in cardiomyocytes harboring mutant Perp and to elucidate the signaling pathways underlying cardiomyopathy. Orthologous mutant PERP knock-in mice (muPerp mice) were generated using the CRISPR/Cas9 method for omics analysis via RNAseq, phosphoproteome, and proteome profiling in ventricular cardiomyocytes. Human cardiomyocytes derived from the proband's iPSCs (hiPSC-CM) were utilized for functional validation. Transcriptomic analysis revealed a significant upregulation of genes associated with cardiac ventricle morphogenesis, particularly in the Notch signaling pathway (JAG2, HEYL, HEY1), in muPerp ventricular cardiomyocytes. This coincided with increased cell proliferation in human myocytes derived from the proband's iPSCs. Conversely, downregulation of genes involved in Notch signaling (DLL4, SPEN, HEY2), negative regulation of cardiac muscle cell apoptosis, and heart development was observed. E15.5 muPerp mice exhibited pronounced ventricular hypertrabeculation. Furthermore, examination of energy metabolism-related pathways, including glycolysis, the tricarboxylic acid cycle (TCA), fatty acid metabolism, and oxidative phosphorylation, revealed divergent trends in muPerp ventricular cardiomyocytes. Additionally, alterations in OCR/ECAR, stress response and reactive oxygen species (ROS) metabolism were evident in hiPSC-CM. In conclusion, this study highlights the contribution of Perp mutation to cardiomyopathy pathogenesis by modulating Notch gene expression and protein phosphorylation cascades involved in mitochondrial metabolism.

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Keywords: cardiomyopathy, PERP, Notch

5:45 PM – 6:45 PM

293

THE EFFECT OF RN-1734 ON LMNA-RELATED DCM PATIENT SPECIFIC IPSC-CMS

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LMNA-related dilated cardiomyopathy (DCM) is characterized by early-onset atrioventricular (AV) block, supraventricular and ventricular arrythmia (VA) and progressive DCM. A defect in nuclear lamina caused by mutation in LMNA gene could impaired nuclear stability and disrupts cardiac muscle mechanical signalling. Studies revealed cytoskeletal, such as actin and tubulin colocalized with the transient receptor potential vanilloid-4 (Trpv4) non-selective cation which regulate cellular volume and calcium (Ca^{2+}) influx. Trpv4 has recently been showed in relation to cardiomyocyte Ca^{2+} overload following stretch that may contribute to cardiac senescence and cardiac fibrosis. Although the role of LMNA and trpv4 in cell function have been widely investigated, the pathophysiological mechanisms of cardiac senescence and fibrosis caused by LMNA mutation are not yet fully understood. Research is ongoing to evaluate the effect of RN1734, Trpv4 antagonism in LMNA-related DCM patient specific iPSC-CMs for LMNA and Trpv4-specific pathways. We generated iPSCs (R225X/WT) from one patient with documented LMNA-related cardiomyopathy arising from one nonsense mutations in UGA at loci R225X for disease modelling. We differentiated those iPSCs into cardiomyocytes to evaluate RN-1734 on apoptosis in cardiomyocytes and nucleus, lamin A/C protein expression, transcriptome expression and chromatin expression among R225X/WT, R225X/WT and WT/WT in presence of stress. The condition of nuclear blebbing is also ameliorated with RN 1734 treatment under electrical stress. Moreover, RN1734 treatment not only lowered apoptosis, but also reduced senescence-associated secretory phenotypes (SASP) in presence of mechanical stress. We hypothesis that the increase in SASP in mutant due to the instability of nuclear membrane which may affect the calcium influx and induce cell apoptosis. Our results allow us to differentiate RN1734 responder from non-responder to enter the clinical trials though attenuation of cardiac senescence and cardiac fibrosis.

Keywords: hiPSC-CMs, LMNA-related dilated cardiomyopathy, TRPV4 channel

5:45 PM – 6:45 PM

295

MYOFILAMENT HYPERSENSITIVITY MEDIATED STRETCH-RESPONSIVE TRANSIENT RECEPTOR POTENTIAL VANILLOID 2 IN HYPERTROPHIC CARDIOMYOPATHY PATIENTS BEARING CARDIAC SARCOMERIC MUTATION IN TROPONIN I

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The patients bearing sarcomeric mutations have been well characterized with HOCM phenotypes. In recent decades, the causal link of sarcomeric dysfunction to the presentation of HOCM has not been well investigated. Cardiac myofilament has been well known to play a role in calcium buffering. Any alteration of its structural organization or calcium binding affinity will adversely affect intracellular calcium homeostasis, thus causing arrhythmia, impaired electrical-contraction (EC) coupling and structural remodeling of myocardium. Combined with CRISPR/Cas9 technology and iPSC-based platform, here we reported the human induced pluripotent stem cell (hiPSC) model of a novel HCM-associated cTnIR186Q/WT mutation to study the cellular mechanisms result in increased susceptibility of diastolic heart failure. Our study demonstrated induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) with cTnIR186Q/WT mutation recapitulated many key aspects of HCM, including enlarged cell size, sarcomere disarray, abnormal electrophysiological properties and irregular Ca2+ transients and compromised contractility. Correction of the mutation reversed the phenotypic abnormalities, which verified the causal relationship that the disease phenotypes were caused by cTnIR186Q/WT mutation. Our results revealed calcium overload in cTnIR186Q/WT iPSC-CMs possibly leads to activation of NFAT hypertrophy pathway causing NPPB upregulation. Pilot screening of stretch-responsive receptor type showed a significant elevated level of TRPV2 in two cTnIR186Q/WT lines. We hypothesize that the hyperactivation of stretch-responsive receptor, Transient receptor potential vanilloid type 2 (TRPV2), contributes to the HCM phenotype by the overstretched myofilament hypersensitive to calcium binding. Treatment by blockade of the hyperactivated TRPV2 in HCM could resumed diastolic Ca2+ homeostasis and restored diastolic function, which shed lights on TRPV2-channel based therapeutics for cardiomyopathy.

Keywords: hypertrophic cardiomyopathy, TRPV2, calcium homeostasis

5:45 PM – 6:45 PM

297

EVALUATING LONG-TERM CARDIOTOXICITY USING HUMAN HIPSC-DERIVED CARDIOMYOCYTES

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Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CM) were already validated as an in vitro model for evaluating acute, proarrhythmic cardiotoxicity in 2018. Consequently, electrophysiological assays using hiPSC-CM were incorporated into the existing ICH S7B/E14 regulatory guidelines in 2022. To date, there are no regulatory authority guidelines for evaluating long-term cardiotoxicity. This is a concern as numerous drug treatments, including chemotherapy drugs like anthracyclines and certain protein kinase inhibitors, are known to capable of causing significant cardiomyopathy via toxic actions on the myocardium. Members of the Cardiac Safety Committee Stem Cell Work Group of HESI (USA) have conducted a multi-site study designed to test a standardized chronic assay protocol. 12 compounds were tested, with known cardiotoxicity mechanisms in humans (doxorubicin, erlotinib, sunitinib, pentamindine, BMS-986094, milrenone, nilotinib, endothelin-1, vinblastine, vincristine, vinorelbine), in a blinded study examining chronic actions (up to 6 days exposure) on human iPSC-derived cardiomyocytes while assaying a range of biomarkers . This abstract shows the data from one participating laboratory using microelectrode array (MEA) recordings from human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) (iCell2 Fujifilm CDI) for their suitability to assess drug-induced long-term cardiotoxicity. Overall, 9 of 12 compounds were assessed correctly with clear long-term cardiotoxic effects on hiPSC-CMs and erlotinib showed no effects as expected. One compound appeared to be false negative, namely the PDE-3 blocker milrenone, which is known to have a low sensitivity on hiPSC-CM cardiomyocytes. Arsenic trioxide revealed no cardiac toxicity in the concentration range tested. MEA recordings on hiPSC-CM are a powerful in vitro assay system to detect long-term cardiotoxic actions.

Keywords: hiPSC-derived cardiomyocytes, long-term cardiotoxicity, microelectrode array recording

5:45 PM – 6:45 PM

299

BIOENGINEERING FOR MATURE CARDIAC DISEASE MODELS USING HUMAN INDUCED PLURIPOTENT STEM CELLS

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HiPSC-Cardiomyocytes (hiPSC-CMs) have translational potential, yet following differentiation, these cardiomyocytes (CMs) are akin to neonatal rather than adult CMs. Whilst conventional methods for maturing hiPSC-CMs in culture exist (like Engineered Heart Tissues), a bioengineered approach to producing in situ mature cardiomyocytes without extensive manipulation or duration and by mimicking a native microenvironment, has not yet been explored. In this study, for the first time, we have adapted our hiPSC-CM differentiation protocol for use on 3D aligned electrospun poly(ε-caprolactone) (PCL) nanofiber (NF) plates, and developed a maturation media that functions synergistically to mature hiPSC-CMs metabolically, structurally and electrophysiologically, while maintaining scalability and compatibility with a variety of assays. Cardiac differentiation of NCRM5-hiPSCs in 2D (tissue culture polystyrene, TCPS) and 3D (NF) plates produced CMs with >70% purity, and NF CMs progressed to a sustainable mature phenotype when subjected to an accelerated media regime containing small molecules in a galactose-supplemented fatty acid-rich medium (Maturation Medium, MM) for 14 days. Assessments considered structural, functional and metabolic maturity. Briefly, MM-treated NF CMs showed a significant ratiometric shift from TNNI1 (neonatal isoform) to TNNI3 (adult isoform) gene expression (8-fold) by qPCR; greater Maximum Diastolic Potentials (MDP) compared to TCPS CMs, at -80.4 ± 2.5 mV ($p=0.0005$); faster calcium kinetics in MM-treated NF CMs (time to peak amplitude in MM treated NF-CMs was 113 ± 31 ms versus 161 ± 39 ms in control TCPS CMs) ($p < 0.0001$); a neutral/positive force-frequency relationship and a dose dependant response to standard cardiac ion channel blockers. These CMs also showed a shift from glycolysis to oxidative phosphorylation for ATP production. Validation of maturity was performed on an adult-onset disease model for Arrhythmogenic Cardiomyopathy (ACM) using CRISPR-edited isogenic mutants (mutant for PKP2 gene), with consistency across multiple phenotyping approaches as a result of the maturation strategy. Data presented supports a novel combined bioengineering approach to accelerate maturity of hiPSC-CMs for bonafide cardiac disease models.

Funding Source: British Heart Foundation

Keywords: cardiac maturation, bioengineering, hiPSC disease modelling

5:45 PM – 6:45 PM

301

PRECLINICAL EVALUATION OF CRISPR-BASED THERAPIES FOR NOONAN-SYNDROME CAUSED BY DEEP-INTRONIC LZTR1 VARIANTS

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Gene variants in LZTR1 are implicated to cause Noonan syndrome associated with a severe and early-onset hypertrophic cardiomyopathy. Mechanistically, LZTR1 deficiency results in accumulation of RAS GTPases and, as a consequence, in RAS-MAPK signaling hyperactivity, thereby causing the Noonan syndrome-associated phenotype. Despite its epidemiological relevance, pharmacological as well as invasive therapies remain limited. Here, personalized CRISPR/Cas9 gene therapies might offer a novel alternative for a curative treatment in this patient cohort. In this study, by utilizing a patient-specific screening platform based on iPSC-derived cardiomyocytes from two Noonan syndrome patients, we evaluated different clinically translatable therapeutic approaches using small Cas9 orthologs targeting a deep-intronic LZTR1 variant to cure the disease-associated molecular pathology. Despite high editing efficiencies in cardiomyocyte cultures transduced with lentivirus or all-in-one AAVs, we observed crucial differences in editing outcomes in proliferative iPSCs versus non-proliferative cardiomyocytes. While editing in iPSCs rescued the phenotype, editing using the same approaches did not robustly restore LZTR1 function in cardiomyocytes, indicating critical differences in the activity of DNA double-strand break repair mechanisms between proliferative and non-proliferative cell types and highlighting the importance of tissue-specific screens for testing CRISPR/Cas9 gene therapies.

Keywords: gene therapy, CRISPR/Cas9, iPSC-Cardiomyocytes, Noonan syndrome, LZTR1

5:45 PM – 6:45 PM

303

INVESTIGATION OF TRIM55-ASSOCIATED HYPERTROPHIC CARDIOMYOPATHY IN HIPSC-CMS

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Hypertrophic cardiomyopathy (HCM) is the most common human inherited cardiomyopathy with no curative treatment available. HCM is commonly associated with variants in genes encoding sarcomeric proteins that compromise cellular homeostasis, thereby highlighting the importance of the protein degradation machinery in disease development and progression of HCM. TRIM55 is part of the Ubiquitin-Proteasome System (UPS), the major proteolytic system within cells, that has been shown to be altered in HCM. A number of variants in TRIM55 have been associated with HCM but the underlying disease mechanism is still unknown, limiting treatment or identification of novel therapeutic interventions. This project directly addresses this knowledge gap as TRIM55 Knockout (KO) and patient-derived TRIM55 variant hiPSC lines were generated using CRISPR/Cas9 gene editing. Our preliminary data revealed an HCM-like phenotype in 3D cardiac organoids with a higher force, lower beating rate, a longer activation and relaxation time for TRIM55 KO compared to healthy control organoids, indicating an alteration of contractile function. High-content image-based analysis was performed to unravel disease hallmarks of TRIM55 related HCM by confocal imaging, including cell size, sarcomere organisation and aggregation of sarcomeric proteins. Immunofluorescence staining was performed to validate TRIM55 localisation along the M-band of the sarcomere, as well as to verify the absence of TRIM55 protein in TRIM55-KO hiPSC-CMs. TRIM55 absence was confirmed by mass spectrometry analysis, and gene ontology analysis revealed alteration of sarcomere organisation and muscle contraction in TRIM55-KO hiPSC-CMs, suggesting a regulatory role of TRIM55 in sarcomere formation. This study establishes a suite of cell lines that will facilitate a better understanding of TRIM55 function as a basis for the development of novel therapeutic approaches for HCM.

Funding Source: This work is funded by the Novo Nordisk Foundation Center for Stem Cell Medicine, reNEW, supported by a Novo Nordisk Foundation grant (grant number NNF21CC0073729).

Keywords: hypertrophic cardiomyopathy, disease modeling, proteopathy

5:45 PM – 6:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

305

MELATONIN-INSPIRED 3D STROMAL CELLS WITH ROBUST PGE2 PRODUCTION DRIVE FETAL REPROGRAMMING IN THE INTESTINAL EPITHELIUM

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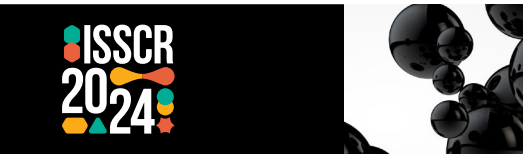
The intestinal epithelium exhibits remarkable regenerative capabilities following damage. Crypt-base-columnar stem cells (CBCs) are easily lost upon injury, such as irradiation or dextran sulfate sodium. However, the intestinal epithelium cells initiate a regenerative response through fetal reprogramming to repair the damaged tissue. Revival stem cells (RevSCs) express fetal genes like Clu and Sca-1, existing in a quiescent state under normal conditions and becoming activated in response to injury. To enhance the regenerative capacity mediated by revSCs, we employed a strategy of combining melatonin-loaded microspheres with 3D mesenchymal stem cells (3D-MSCs). 3D-MSCs exhibit high levels of Prostaglandin E2 (PGE2), which has been reported to act as an inducer of RevSCs. In the results from testing melatonin on intestinal organoids, it suppressed growth and budding without causing apoptosis, suggesting melatonin induces a reversible quiescence in organoids. Gene set enrichment analysis revealed an upregulation of gene signatures associated with fetal/regenerative state and yes-associated protein (YAP) activation in melatonin-treated organoids. Notably, the combined treatment of PGE2 and melatonin, compared to PGE2 only, led to an increase of Ly6a+ cells and significantly activated YAP signaling. Melatonin-incorporated 3D-MSCs (heterospheroid) were more effective in generating Ly6a+ cells in organoids compared to both 2D and 3D-MSCs. Furthermore, the administration of heterospheroids demonstrated the most pronounced protective effect against DSS-induced colitis in mice. In conclusion, these findings suggest a therapeutic strategy using heterospheroid, to induce cellular reprogramming towards a fetal-like state, thereby facilitating epithelial regeneration during tissue injury.



Funding Source: Korean Fund for Regenerative Medicine (KFRM, 22A0205L1-11) grant funded by the Korea government

Keywords: intestinal regeneration, revival stem cells, heterospheroid

5:45 PM – 6:45 PM

307

GENERATION OF SKIN ORGANOIDS FROM MULTIPLE INDUCED PLURIPOTENT STEM CELL LINES

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Induced pluripotent stem cells (iPSCs) are reprogrammed into an embryonic-like pluripotent state. Organoids differentiated from iPSCs are expected to be used in regenerative medicine, such as new drug development and tissue replacement through transplantation. We previously reported a minimally invasive method for generating iPSCs using cells isolated from plucked hair and urine. Recently, a new method has been developed to generate facial skin organoids by inducing differentiation of pluripotent stem cells to surface ectoderm and cranial neural crest cells. By combining these methods, we generated skin organoids from nine iPS cell lines (one Caucasian, one European-African, and seven Asian) to confirm their applicability. All iPS cell lines differentiated into skin organoids containing hair. Histological and molecular analyses revealed that the organoids comprised epidermis, dermis, and subcutaneous tissues, including epidermal keratinocytes, dermal fibroblasts, nerve cells, melanocytes, Merkel cells, and adipocytes, as well as skin appendages such as sebaceous glands and hair follicles. In addition, similar to human skin, UV irradiation induced skin inflammation in the skin organoids. This study shows that the method for generating skin organoids can be applied to multiple iPS cell lines, and may be used for drug screening. We believe that this study contributes to the discovery of new findings for the expansion and development of regenerative medicine.

Keywords: skin organoids, induced pluripotent stem cells (iPSCs), UV irradiation

5:45 PM – 6:45 PM

309

ADVANCING DRUG DISCOVERY: LEVERAGING CRISPR-READY MICROGLIA FOR FUNCTIONAL GENOMICS STUDIES

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Functional genomics screening plays a crucial role in drug target discovery by systematically assessing the impact of genetic perturbations on cellular functions. Methods such as CRISPR knockout screening enable both the identification of potential therapeutic targets, and the validation of these targets by elucidating their role in disease pathways. The use of physiologically relevant model systems is critical in these screening approaches to ensure the future clinical success of identified targets. Human induced pluripotent stem cell (hiPSC) derived cell types have emerged as a powerful tool to advance our understanding of complex human biology and accelerate drug discovery programs. Leveraging bit.bio's hiPSC derived ioMicroglia, we introduce CRISPR-Ready ioMicroglia for CRISPR/Cas9-based knockout screening. CRISPR-Ready ioMicroglia have been engineered to constitutively express Cas9, which is functional from day 1 post thaw. Using a guide RNA targeting beta-2 microglobulin (B2M), we demonstrate high knockout efficiency at protein level by flow cytometry analysis. Importantly, CRISPR-Ready ioMicroglia share the same features as ioMicroglia, expressing key markers including CD45, CD14, P2RY12, CX3CR1, CD11b and IBA1, and have comparable cytokine secretion profiles. Constitutive Cas9 expression does not impact the reprogramming potential, transcriptional profile, or functionality of CRISPR-Ready ioMicroglia. We performed a pooled single-cell CRISPR knockout screen targeting 110 genes involved in the modulation of innate immune signalling and the phenotypic consequences were profiled using a targeted single-cell RNA sequencing readout. Through subsequent bioinformatic analysis, 17 genes were identified that, when knocked out, altered responses to LPS stimulation and led to a reduction in microglial activation. Top screening hits will be brought forward for further validation using functional assays for cytokine secretion and phagocytosis activity. In conclusion, CRISPR/Cas9-based knockout screening using CRISPR-Ready ioMicroglia enables researchers to systematically interrogate healthy and diseased cell states for target identification and validation using physiologically relevant human cell types.

Keywords: iPSC-derived Microglia, forward programming, scCRISPR screening

5:45 PM – 6:45 PM

311

BIOENGINEERED GUT PERFUSABLE ORGANOIDS WITH IN-VIVO-LIKE COMPLEXITY AND FUNCTION FOR PRECISION MEDICINE APPLICATIONS

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Organoids and microphysiological systems have emerged as powerful tools for modeling human gut physiology and diseases in-vitro. However, although physiologically relevant, these systems often lack spatial organization, cell-type diversity, and maturity necessary for mimicking intestinal mucosa. We integrated organoid and organ-on-a-chip technologies to develop a primary human stem–cell-derived perfusable organoid model closely resembling the in-vivo cell-type composition and the native architecture of the intestine. This innovative platform facilitates access to both luminal and basal sides, promoting tissue longevity by removing shed cells and promoting enhanced cellular differentiation. Here we introduce a reproducible approach to generate a physiologically mature, human perfusable intestinal organoid system, cultured for up to 15 days. We confirm the cellular heterogeneity of the native intestine as well as the presence of a prominent brush border using immunofluorescence while our standardised barrier integrity assay show leak-tight and stable barrier. Our model can be used for a wide range of applications encompassing studies on nutrient absorption, disease modelling, gut microbia and drug screening. Our technology could be expanded to generate microtissues derived from other organs and incorporate additional microenvironmental components, thus emulating the intricate complexity of the native organ in an in-vitro setting. These bioengineered perfusable organoids provide a highly accurate, and functional platform to systematically study human organ physiology and pathology, and for the development of novel therapeutic strategies.

Keywords: organoids, perfusable organoid, 3D cell culture

5:45 PM – 6:45 PM

313

TOWARDS IMMUNITY ON CHIP - IMMUNE CELL PERFUSION OF AN IPSC-DERIVED INTESTINAL MODEL

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Understanding the complexities of the human intestinal epithelium and its interaction with the immune system is crucial to advance biomedical research. In this context, the development of a microfluidic chip-based intestinal model derived from induced pluripotent stem cells (iPSCs) holds immense significance. This study aimed to create an innovative model to closely mimic the intestinal epithelium, assess its characteristics and functionality and integrate immune cells to simulate the microenvironment of Peyer's patches. Following organoid differentiation, cells were dissociated and seeded onto transwells to establish a functional monolayer. Barrier integrity was evaluated via trans-epithelial electrical resistance (TEER) measurements and functional assays. To replicate Peyer's patches, a three-dimensional model was constructed, integrating a hydrogel between the epithelium and a leukocyte permissive membrane. B-cells were incorporated to simulate B-cell follicles within this 3D setup. Immunohistochemical characterization validated the similarity of the derived organoids to native intestinal epithelium. The monolayer demonstrated robust barrier integrity, confirmed by TEER measurements. The development of the 3D model successfully integrated B-cells within a simulated Peyer's patch microenvironment. Incorporation into a microphysiological system enabled immune cell migration from circulation into the model, facilitating interactions with the B-cells. This study presents the successful establishment of an immune-competent intestinal model derived from iPSCs. The model effectively replicates the native intestinal epithelium, exhibits barrier functionality, and recreates a specialized setup including simulated Peyer's patches. This advanced model holds promise for advancing our understanding of intestinal physiology, immune responses, and its potential applications in drug development and personalized medicine.

Funding Source: Funded by the European Union under Grant Agreement Nr. 101057438

Keywords: microphysiological systems, intestine, immunity

5:45 PM – 6:45 PM

315

MODELLING VIRAL INFECTION IN AN ENGINEERED WHOLE-LUNG

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To date, airways infections are one of the leading causes of death. Furthermore, the COVID-19 pandemic represented an unexpected exacerbation within these pathologies. This situation calls for the need of complex in-vitro model that can mimic viral infection and predict therapeutic outcomes, however, current modelling relies on standard 2D cell cultures which cannot recapitulate the three-dimensional lung structure and the interaction between cells and extracellular-matrix. To overcome these limitations, here we propose a tissue engineered lung model, based on decellularized whole-organ matrix, epithelial cells and an automated bioreactor system. The rationale is to provide an in-vitro model that features human cells in contact with the extracellular matrix within an anatomically correct three-dimensional structure. We developed an innovative and automated system able to deliver both decellularization and recellularization of the tissue by mean of a controlled and pulsatile flow which mimics natural breathing. Rat organ decellularization produced a scaffold which maintained the macro and micro-structure of the tissue. Whole rat lungs were re-epithelialized using Human Bronchial Epithelial Cells. Dynamic, bioreactor-based recellularization delivered an even and polarized coverage of the alveoli. To proof the feasibility of modelling viral infection, we administered into the re-epithelialized lungs RSV and treatment with Remdesivir. Results showed infection of cells and formation of the typical syncytial clusters. Supernatant collection showed drug effect on plaque-forming assays. Finally, we performed an assessment of immune response modelling by administering neutrophils in the infected engineered lungs and results showed migration and activation by IL-8 production. Overall, our engineered whole-lung model represents a powerful tool as drug-screening platform or as a gene therapy model, allowing modelling of human cells in a three-dimensional tissue specific environment

Keywords: tissue engineering, disease modelling, whole-organ

5:45 PM – 6:45 PM

317

ADVANCING MELANOMA RESEARCH USING HUMAN SKIN ORGANOIDS FOR IN VITRO MODELLING AND PRECLINICAL DRUG TESTING

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Malignant melanoma is the leading cause of skin cancer-related deaths. Therefore, it is crucial to understand its pathogenesis and explore new therapeutic approaches. Although model systems such as animal models and human in vitro melanoma cell cultures have been useful in clarifying the complexities of this disease, challenges persist in translating findings to human pathology and replicating an appropriate microenvironment in vitro. To address these challenges, we have successfully generated skin organoids from human pluripotent stem cells that closely mimic the characteristics of native human skin. These hair-bearing organoids provide a physiologically relevant platform for studying the progression of melanoma. Using this system, we aim to establish a comprehensive in vitro melanoma model. In order to create a model that accurately reflects the intricacy of the tumour microenvironment, we are currently investigating the co-cultivation of melanoma cells. This method allows for the examination of melanoma progression and the interactions between tumour cells and surrounding stromal

components. As part of our approach, we conducted tests using eight different melanoma cell lines with varying genetic defects. These cell lines were characterised and tested for tumour spheroid generation as part of our screening process. These tumour cell lines were then co-cultured with 80-day-old organoids. Immunofluorescence staining was performed on the resulting tumour skin organoids to elucidate the expression of tumour markers. This was combined with an analysis of microenvironmental changes, in particular the manifestation of cancer-associated fibroblasts. In conclusion, this work introduces a novel approach to melanoma research utilising stem cell generated human skin organoids, to create a physiologically relevant melanoma model. This model addresses existing limitations and enables the exploration of interaction between tumours and surrounding stroma. Findings from this translational investigation, involving human stem cells, are expected to advance the development of more potent melanoma therapies through the possible preclinical evaluation of medicinal compounds.

Keywords: melanoma, skin organoids, preclinical drug testing

5:45 PM – 6:45 PM

319

FROM BREAKTHROUGH TO BEDSIDE: HARNESSING HUMAN INTESTINAL ORGANOIDS FOR REGENERATIVE THERAPY

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The recent breakthrough in human organoid technologies represents a pivotal moment in personalized regenerative medicine, unlocking the potential to generate complex tissues from a patient's own stem cells for autologous transplantation. Among these advancements, Human Intestinal Organoids (HIOs) derived from pluripotent stem cells offer a significant promise for regenerative medicine by providing laboratory-grown tissues that faithfully replicate the intricate complexities of the human intestine. Our recent studies have demonstrated the remarkable regenerative potential of human intestinal organoid (HIO) fragments in a pre-clinical rat model subjected to mechanical and chemical damage. These HIOs, when introduced into the gut lumen, exhibited engraftment, proliferation, migration, and differentiation, effectively restoring both the epithelial and mesenchymal layers of damaged bowel tissue in an immunosuppressed rat model. To translate this exciting discovery into clinical application, we have assembled a multidisciplinary study team comprising gastroenterology experts, transplant surgeons, basic and clinical research scientists, and cell therapy manufacturing experts. The preclinical phase of our project has been dedicated to: (1) defining the specific indications for therapy and identifying the appropriate patient population (2) translating research protocols into cGMP-compliant manufacturing procedures for the large -scale production of clinical-grade material and (3) assessment of the product's safety in vivo animal models. Preliminary results have demonstrated the feasibility of generating clinical-grade HIOs, cryopreserving the final product, and establishing its safety profile in animal models. These findings pave the way for further advancements in regenerative therapy. Moreover, our work contributes significantly to the field by establishing a unique infrastructure for regenerative therapies and outlining a roadmap for the clinical translation of iPSC-derived organoids. The broader implications of this research extend beyond intestinal rejection, providing a foundation for efficient and safe organoid therapies across various organ systems.

Keywords: intestinal organoids, differentiation, translational medicine

5:45 PM – 6:45 PM

321

UNCOVERING THE PATHOGENESIS AND THERAPY FOR P63-RELATED STEM CELL DEFICIENCY

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The impact of disease-causing mutations on stem cell (SC) function and cellular dynamics is poorly understood. This gap of knowledge mainly stems from the technical limitation to follow adult SC function for long-term in their native niche in vivo. Here we utilized the cornea as a tissue model, as it allows monitoring of individual SCs and their progeny in real time. The corneal epithelium is constantly replenished by SCs that reside in the limbus niche. Limbal stem cell (LSC) self-renewal and proliferation is firmly controlled by the transcription factor p63. LSC loss or dysfunction results in LSC deficiency (LSCD), a devastating pathology accompanied by corneal opacification and blindness. However, the impact of p63 mutations on LSC dynamics in vivo is unknown and therapeutic options for the patients are limited. Here, we report that a single point mutation in p63 coding genes leads to ectodermal dysplasia accompanied by LSCD in human. We established a conditional mouse model carrying the same mutation and discovered that it recapitulates the LSCD phenotypes of patients. Quantitative lineage-tracing

reveals that mutated LSCs and corneal progenitor cells display abnormal activity and perturbed radial tissue renewal orientation. Computerized modeling of corneal tissue renewal suggests that mutated active LSC population is hyper activated and potentially linked with exhaustion. However, treatment of mutated animals with the small molecular weight compound, APR-246, substantially prevents LSCD development under different treatment modalities. In conclusion, we propose that APR-246 restores the activity of mutated P63 and LSC function, thereby maintaining and corneal tissue boundary intact. Future studies should further investigate the therapeutic potential of APR-246 in clinical trials.

Keywords: stem cell function, cellular dynamics, corneal epithelium

5:45 PM – 6:45 PM

323

NF-κB-MEDIATED HAIR FOLLICLE DAMAGE IN SOLAR UV-INDUCED PHOTODAMAGED SKIN ORGANOID

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Solar ultraviolet (sUV) exposure is known to cause skin damage. However, the pathological mechanisms of sUV on hair follicles have not been extensively explored. Here, we established a model of sUV-exposed skin and its appendages using human induced pluripotent stem cell-derived skin organoids with planar morphology containing hair follicles. Our model closely recapitulated several symptoms of photodamage, including skin barrier disruption, extracellular matrix degradation, and inflammatory response. Specifically, sUV induced structural damage and catagenic transition in hair follicles. As a potential therapeutic agent for hair follicles, we applied exosomes isolated from human umbilical cord blood-derived mesenchymal stem cells to sUV-exposed organoids. As a result, exosomes effectively alleviated inflammatory responses by inhibiting NF-κB activation, thereby suppressing structural damage and promoting hair follicle regeneration. Ultimately, our model provided a valuable platform to mimic skin diseases, particularly those involving hair follicles, and to evaluate the efficacy and underlying mechanisms of potential therapeutics.

Funding Source: - This study was partially supported by the Research Institute for Veterinary Science, Seoul National University, - Korean Fund for Regenerative Medicine(KFRM) grant (No. 23A0101L1)

Keywords: iPSC-derived skin organoid, photodamage, NF-κB pathway

5:45 PM – 6:45 PM

325

APICAL-OUT AIRWAY ORGANOIDS AND AIRWAY-IMMUNE CO-CULTURES ARE SUITABLE MODELS FOR ANTIVIRAL COMPOUND SCREENINGS AND INFECTIOUS DISEASES RESEARCH

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Advanced cell culture systems, such as airway organoids and co-cultures of epithelial cells with immune cells, could serve as a powerful platform to study viral infection and pathogenesis. Towards this goal, we describe the development of two new human lung culture systems and their use in host-pathogen interaction studies and in drug screening applications. In the first culture system, co-cultures of human bronchial epithelial cells (HBECs) and blood-derived macrophages were established in a complex air-liquid interface model in either PneumaCult™-ALI or ImmunoCult™-SF Macrophage Medium containing stimulating factors to specifically derive M0-, M1- or M2-like macrophages. Macrophages maintained their phenotype for the co-culture assay length of 72 hours, and the HBECs were unaffected by the specialized macrophage media. Subsequently, this system was used to determine the role of different macrophage phenotypes (M0, M1, and M2) following respiratory syncytial virus infection. Macrophage subtypes were shown to be influenced by their microenvironment, with M1-like macrophages considerably decreasing the initial infection,

whereas M0- and M2-like macrophages helped potentiate it. In the second culture system, HBECs were used to generate apical-out airway organoids (AOAOs) in an extracellular matrix (ECM)-free, serum-free, and easily scalable workflow. This novel 3D organoid culture model exposes the apical side of the epithelium to the environment. Infection of AOAOs with influenza A, influenza B, rhinovirus-A16, or enterovirus-D68 produced high viral RNA titers (approximately 5.5 log10 copies per sample) and strong cytopathogenic effects, whereas administration of two antivirals, Rupintrivir, and Itraconazole, significantly rescued the infection effects. In summary, we describe the generation and use of two new models to study infectious disease pathogenesis in vitro and assess antiviral drug effects.

Funding Source: The work described in this abstract was funded by Mitacs through the Mitacs Accelerate programme (Project Number IT11399) and OrganoVIR (Grant 812673) in the European Union's Horizon 2020 programme.

Keywords: apical-out airway organoids, lung epithelial cell-immune cell co-culture, infectious diseases

5:45 PM – 6:45 PM

327

PRENATAL MODELING OF CONGENITAL DIAPHRAGMATIC HERNIA USING AMNIOTIC AND TRACHEAL FLUIDS-DERIVED LUNG ORGANOIDS

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Congenital diaphragmatic hernia (CDH) is a rare condition associated with devastating fetal lung defects. The underlying disease mechanisms remain poorly understood due to the lack of patient-specific in vitro models. Fetal stem cell-derived organoids may provide a reliable tool to study CDH fetal lungs; however, they can currently only be generated after the termination of pregnancy. This work aimed to develop a human fetal lung organoid system to model CDH before birth, while allowing the continuation of pregnancy. Amniotic (AF) and tracheal fluids (TF) were collected from CDH fetuses pre- and post fetal surgery (FETO), and GA-matched healthy controls. Fetal fluid-derived lung organoids were expanded clonally and characterized by bulk and single-cell RNA-seq, as well as immunofluorescence and functional assays. We generated lung organoids from 16 CDH AFs and 7 CDH TFs. CDH organoids expanded for multiple passages and expressed lung epithelial stem/progenitor cell markers. SOX9 expression was downregulated in CDH organoids generated post-FETO, indicating enhanced tissue maturation. Notably, comparative analysis revealed a reduction in the number of differentially expressed genes (DEGs) between organoids generated before (380 DEGs) and after (102 DEGs) FETO when compared to controls. scRNAseq unveiled substantial differences in the cellular composition of CDH vs. control organoids, highlighting the unique characteristics of CDH patient-derived organoids. Lung organoids derived from fetal fluids successfully capture CDH features, offering potential for disease modeling, outcome prediction, and personalized prenatal drug testing.

Keywords: organoids, amniotic fluid, fetal stem cells

5:45 PM – 6:45 PM

329

VIROLOGICAL ANALYSIS OF RESPIRATORY SYNCYTIAL VIRUS USING HUMAN IPS CELL-DERIVED RESPIRATORY TISSUE SYSTEM

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The number of respiratory syncytial virus (RSV)-infected patients is rapidly increasing after the coronavirus disease 2019 (COVID-19) pandemic. There is an urgent need to elucidate the pathophysiology of RSV disease and conduct its pharmaceutical research. Therefore,

model development for conducting RSV research and its application in pharmaceutical discovery are essential. In this study, our human iPS cell-derived respiratory tissue model was infected with RSV, and then performed scRNA-seq analysis to analyze host responses. Our respiratory tissue model includes not only respiratory epithelial cells including ciliated cells, but also immune cells such as macrophages. After infecting a respiratory tissue model with 0.1 multiplicity of infection (MOI) RSV genotype A, scRNA-seq analysis was performed at 4 days post-infection (dpi). The high expression level of the RSV NS1 gene in ciliated cells and goblet cells suggests that RSV tends to infect ciliated cells and goblet cells. In addition, RSV infection increased the proportion of goblet cells from 14.4% to 23.1%. GO term analysis of genes with increased expression in RSV-infected alveolar macrophages showed that genes related to defense response to virus were enriched. To confirm the validity of the scRNA-seq data, we also reanalyzed scRNA-seq data using broncho-alveolar lavage fluid (BALF) collected from RSV-infected patients. In BALF of RSV-infected patients, we confirmed RSV infection tropism toward ciliated cells and goblet cells, an increase in the proportion of goblet cells, and increased expression of genes related to defense response to virus in alveolar macrophages. These results suggest that we were able to demonstrate the usefulness of the respiratory tissue model in RSV infection through a comparative analysis of the human iPS cell-derived respiratory tissue model and BALF. In the future, we will use respiratory tissue models to elucidate the pathophysiology of RSV disease and conduct pharmaceutical research.

Funding Source: This research was supported by the iPS Cell Research Fund and the Japan Agency for Medical Research and Development (AMED) (Grant Number: JP21gm1610005).

Keywords: scRNA-seq, respiratory tissue model, respiratory synthetical virus

5:45 PM – 6:45 PM

331

MODELING OF SPONTANEOUS PULMONARY FIBROSIS VIA CELLULAR SENESCENCE IN TYPE 2 ALVEOLAR EPITHELIAL CELLS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

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Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease that is more likely to occur in the elderly. Although the pathogenesis of IPF is not completely understood, telomere dysfunction in type II alveolar epithelial (AT2) cells causes pulmonary fibrosis in a subset of patients. TRF2 is a key component of the shelterin complex (SC) which prevents abnormally shortened and uncapped telomeres and genetic variants of SC were reported in some familial cases of IPF. Since it is difficult to culture AT2 cells derived from patients with such rare genetic variants, we conceived that in vitro senescence model of AT2 cells derived from human induced pluripotent stem cells (iPSCs) would be beneficial for elucidating the mechanism of IPF progression. Here, we generated an iPSC line using a doxycycline (dox) -inducible lentiviral vector encoding the dominant negative variant of TRF2 (TRF2-DN). We differentiated this iPSC line into AT2 (iAT2) cells and expressed TRF2-DN protein in a dox-inducible manner. The TRF2-DN-expressing iAT2 (TRF2-DN-iAT2) cells showed lower alveolosphere formation efficiency and smaller alveolospheres than the control iAT2 cells did in a colony-forming assay. TRF2-DN-iAT2 cells showed an increase in SA-β-gal activity. Consistent with the SA-β-gal activity, expression of CDKN1A, CDKN2A and markers of senescence associated secretory phenotype were upregulated in TRF2-DN-iAT2 cells. Bulk RNA sequencing suggested activation of p53 and NF-kB signaling in TRF2-DN-iAT2 cells. Alveolar organoids consisting of TRF2-DN-iAT2 cells and human fetal lung fibroblasts showed organoid contraction and presence of alveolar epithelial cells in abnormal differentiation states. In conclusion, we developed a new human in vitro model of spontaneous pulmonary fibrosis caused by dysfunction of a SC protein which serves telomere capping. This model could be useful for clarifying the pathogenesis of pulmonary fibrosis and thereby discovering the novel therapeutic agents.

Funding Source: Funded by Kyorin Pharmaceutical Co. Ltd

Keywords: iPSCs, senescence, pulmonary fibrosis

5:45 PM – 6:45 PM

333

SKIN CELL SELF ORGANOIZATION IS GUIDED BY PROTEIN CORONA-BEARING EXTRACELLULAR VESICLES

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Transport of functional protein cargo via extracellular vesicles (EVs) is an important mechanism in cell communication. We aim to understand the distribution of active cargo proteins between EV's inside

and outside and the implication for therapeutic applications in wound healing. Here we used skin organoids and a human-on-mouse skin model to investigate the contribution of EVs to this process. EVs from placenta-derived stromal (PLX) cell-conditioned medium or from human platelet lysate were enriched by tangential flow filtration (TFF) optionally followed by ultracentrifugation or size exclusion chromatography (SEC) to obtain EV preparations with different degree of protein corona preservation. Self organization capacity in presence or absence of different EV preparations was analyzed in a high throughput organoid assay. Contribution of EVs to wound healing was assessed in an in vivo model transplanting human skin cells on NSG mice. Skin organoid formation by keratinocytes together with endothelial cells and keratinocyte or spheroid formation of human skin fibroblasts was significantly superior in the presence of TFF-purified EVs bearing a functional protein corona. Soluble factors alone or corona-depleted EVs after SEC did not initiate 3d organization. In vivo, TFF-purified EVs were essential for proper stratified organization of human skin cells in wound areas and provided sufficient vascular suport. These findings allowed us to develop a self-organizing human skin model in mice and show the important role of corona proteins for EV function. The surface-to-bulk partition of EV cargo for small EVs < 180 nm is in favor of surface cargo loading supporting the new concept of a functional EV corona.

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Keywords: extracellular vesicles, EV, skin self organisation

5:45 PM – 6:45 PM

335

DEVELOPMENT OF A NOVEL CYSTIC FIBROSIS LUNG DISEASE MODEL BASED ON PATIENT-SPECIFIC HUMAN INDUCED PLURIPOTENT STEM CELLS

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Cystic fibrosis (CF) is caused by mutations of the epithelial chloride channel CFTR and results in a severe lung disease that is characterised by mucus accumulation, chronic infection and inflammation, and eventual lung failure. In vitro modelling of CF lung disease currently relies on the utilisation of primary respiratory epithelial cells and immortalised cell lines. However, these model systems show significant drawbacks due to altered physiology and accessibility, wherefore development of novel CF models is still needed. Recently, human induced pluripotent stem cells (hiPSCs) are emerging as a model system of CF. hiPSCs can easily be generated from healthy or diseased donors by reprogramming of somatic cells, show a virtually unlimited proliferation, support complex genome engineering and most importantly can be differentiated into various disease-relevant cell types. In our study, we developed a novel CF lung disease model based on CF patient-specific hiPSCs and demonstrated its utility as a tool in CF research. A multistep differentiation protocol was applied to WT and CF hiPSCs to generate respiratory epithelial cells in air-liquid-interface cultures (iALI cultures). Molecular and functional analyses verified the airway specification of iALI cultures and revealed high similarities compared to primary derived respiratory epithelial cells (pALI cultures). Moreover, we confirmed the manifestation of a CF-like disease phenotype in CF iALI cultures that was characterised by: an impaired CFTR protein expression, reduced transepithelial chloride conductance, reduced ciliary beating and an altered mucous layer ultrastructure shown by electron microscopy. Furthermore, the application of CFTR modulator drugs enabled to partially rescue the disease phenotype of CF iALI cultures. Our study provides a detailed description of our iALI cultures as a novel tool in pre-clinical CF research. A versatile set of analyses validated that iALI cultures closely resemble the established model system of pALI cultures and enable modelling of the CF lung phenotype. In the future, iALI cultures will support novel study approaches through complex genome engineering and innovative functional assessments that will augment the development of CF therapy approaches and facilitate clinical translation.

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Keywords: cystic fibrosis, lung disease, disease modelling

5:45 PM – 6:45 PM

337

AN IN VITRO CANINE COLONOID MODEL TO STUDY INTESTINAL INFLAMMATION AND DRUG RESPONSE

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Drug development is a lengthy and expensive process in human and veterinary medicine. The current paradigm in drug research and development is to use 2D cell cultures, which are typically first used to screen compounds. Products then move into live animal testing before human clinical trials. Despite undergoing rigorous and costly studies in the drug discovery and preclinical stage, most drug candidates fail in Phase II clinical trials. Only approximately 5% of the drugs that are tested are ultimately approved by the Food and Drug Administration (FDA), highlighting the critical need for a more reliable in vitro model. Inflammatory bowel disease (IBD), which refers to chronic inflammatory disorders of the gastrointestinal tract, is becoming increasingly diagnosed in both humans and dogs. Our laboratory has established canine colonoids that contain Goblet cells that produce mucin (Alcian Blue+) on their apical surface (lumen). 3D organoids were dissociated and seeded onto Transwell inserts to replicate cellular polarity. 2D monolayers took ~8 days to become confluent with Transepithelial Electrical Resistance (TEER) values increasing from ~19 Ω.cm2 on Day 3 to ~2,809 Ω.cm2 on Day 9. On the day of the experiment, 4 hours after TEER values were measured, 5 ng/mL of canine TNF-α was added to the basal chamber and incubated for an additional 6 hours. A significant drop in TEER (mean ± SD) was observed after TNF treatment (1898.4 ± 340.9 Ω.cm2 $p < 0.05$) compared to pre-treatment (3035.3 ± 265.5 Ω.cm2). Furthermore, there was a significant increase in secreted IL-8 (measured using a canine-specific ELISA) after addition of TNF in both the apical (Control 1318.4 pg/mL ± 130.6; TNF 1989.6 pg/mL ± 241.1) and basal (Control 128.7 pg/mL ± 18.1; TNF 2605 pg/mL ± 184.5) supernatants. RNA-sequencing of transwells revealed a global effect of TNF treatment on the canine colonoids, with a variety of inflammatory cytokines and regulators of barrier function (e.g., IL-7, IL-8, CXCL10, IL-17C, CCL20, IL-23A, ROCK1, ROCK2) being significantly upregulated. To conclude, canine colonoids displayed a pro-inflammatory response and altered barrier function to TNF-α, as confirmed by TEER, IL-8 ELISA, and mRNA expression which can be used to accelerate in vitro screening of anti-inflammatory drug candidates in both veterinary and human medicine.

Funding Source: University of Illinois at Urbana-Champaign - internal, unrestricted funds

Keywords: organoid, stem cell, canine

5:45 PM – 6:45 PM

339

HIPSC-DERIVED SKIN ORGANOIDS: A NEW APPROACH TO MIMIC VASCULARIZED COMPLEX SKIN STRUCTURES AND PATHOLOGIES

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Reproducing the complex vascular architecture of human skin in vitro remains a major challenge in tissue engineering and regenerative medicine. Human skin, an intricate organ comprising the epidermis, dermis and hypodermis, exhibits a sophisticated arrangement of different cell types and structures. One promising approach is to use human induced pluripotent stem cells (hiPSCs) to create skin organoids that mimic the multi-layered complexity of native human skin. These hiPSC-derived organoids provide a platform for modelling various skin pathologies and developmental processes. By integrating additional elements such as the vasculature and immune components such as macrophages. Our efforts have resulted in the cultivation of skin organoids that exhibit cystic structures with an inside-out orientation, mimicking certain aspects of native skin morphology. To create a suitable in vitro testing environment, these organoids were precisely sectioned and placed on different structure to allow air-liquid interphase. In collaboration with the Organ-on-a-Chip (OOAC) technology developed by Peter Loskill's laboratory, we successfully transferred these organoids onto a perfusable platform. This advance enabled dynamic perfusion, allowing more physiologically relevant nutrient exchange and waste removal within the organoid structure. Our comparative analyses between the cystic organoids with an air-liquid interphase and those integrated into the chip revealed nuanced differences, particularly in the maturation of the epidermal layers. In conclusion, the integration of various techniques, including hiPSC technology, innovative culture methods and OOAC platforms, has led us to more advanced and physiologically relevant skin models. These sophisticated skin organoids not only provide insights into basic skin biology, but also hold great promise for drug testing, personalised medicine and the development of novel therapeutic interventions for skin-related diseases.

Keywords: skin organoids, organ on a chip, air liquide interphase

5:45 PM – 6:45 PM

341

EVALUATION OF NANOFOBRIOUS ELECTROSPUN POLYCAPROLACTONE AS A SCAFFOLD FOR HUMAN IMMORTALIZED AIRWAY PROGENITOR CELL LINE TO MODEL ACUTE RESPIRATORY DISTRESS SYNDROME

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Acute Respiratory Distress Syndrome (ARDS) is a clinical syndrome involving classical constellation of acute onset of dyspnea, severe hypoxemia, diffuse lung infiltrates, and decreased respiratory system compliance. Clinically two-third of mild to moderate cases progress to severe with mortality rate up to 40%. The heterogeneous nature of the syndrome hinted that precision medicine approach is needed, and humanized in vitro bronchioalveolar model will be indispensable to reach that outcome. One challenge to design such a model is to ensure that the envisioned construct is compatible with pulmonary bronchial and alveolar epithelial, endothelial, and immune cells. Furthermore, such models need to have mechanical properties like a human lung, and poro-fibrous architecture to recapitulate the native lung extracellular matrix (ECM) condition. A lung-on-a-chip model from previously designed 3D-printed mold by the lab with few modifications was used as a starting point. Electrospun membrane of polycaprolactone (PCL) was explored given its favorable properties. We tested different surface modification methods through protein adhesion and found that 1% atelocollagen I coatings resulted in airway cell attachment and monolayer formation. Furthermore, fourier-transform infrared spectroscopy (FTIR) analysis on the treated membranes shows that lengthening treatment duration of protein coatings up to 12 hours is needed before protein deposition is detected despite already showing change on water-contact angle. Next, seeding of A549 or Calu-3 cells, a human immortalized cell line model for alveoli and airway cells respectively, on the pretreated scaffold is described with successful monolayer formation confirmed using FITC-dextran assay. In the future, compromised barrier function, which is a chief event in ARDS pathophysiology will be tested using LPS treatment with pre-and post-barrier function recorded using FITC assay, and coculturing with endothelial cells and macrophages will be considered.

Keywords: airway progenitor cells, electrospun scaffold, ARDS

5:45 PM – 6:45 PM

343

PATIENT-DERIVED CONJUNCTIVAL ORGANOIDS AS A MODEL FOR THE FRONT-OF-THE-EYE

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In the context of ocular research, dry eye disease (DED) poses a significant clinical challenge, affecting millions of patients worldwide. The conjunctiva, a vital tissue of the ocular surface, plays a critical role in maintaining ocular surface homeostasis and protection, contributing to the production of the aqueous and mucous components of the tear film. As a result, conjunctival damage causes instability of the tear film and sets off a cascade of processes, ultimately resulting in the development of DED, ocular discomfort, and, in more severe instances, vision impairment and blindness. Recent advances in stem cell research and three-dimensional (3D) in vitro culture have enabled the development of conjunctival organoids. Unlike traditional conjunctival epithelial cell monolayers, organoids have a 3D architecture, include multiple cell types, an extracellular matrix, and better recapitulate the physiology and function of the native conjunctiva. In addition, organoids provide a sustainable and reproducible platform for research as they can be cultured long-term and over multiple passages, which is not the case with primary cells or ex vivo tissue explants. Here, we initially focus on establishing organoids from mouse conjunctival tissue. By embedding mouse conjunctival stem cells into a niche-like extracellular matrix we developed organoids that express the conjunctival marker KRT19, the main transcription factor of eye development PAX6, the cellular marker for proliferation Ki67, the basal cell markers p63 and KRT14 and cytoplasmic mucus marker MUC5AC. Preliminary data reveal the presence of the main cell populations found in the mouse conjunctiva including conjunctival epithelial cells, proliferative basal cells and some mucus-producing goblet cells. Current work is ongoing to apply similar methods to generate conjunctival organoids from human tissue. Comprehensive characterization of the mouse and human conjunctival organoids, including molecular transcriptomic and structural analyses, will be applied to understand variability between species and among patients. By mimicking the human ocular environment, conjunctival organoids will enable better understanding of disease mechanisms, and hold promises for targeted therapies and personalized medicine in ophthalmology.

Keywords: organoids, ocular surface 3D model, conjunctiva

5:45 PM – 6:45 PM

347

HIGH-THROUGHPUT SINGLE ORGANOID SWELLING ASSAY FOR PERSONALIZED EVALUATION OF CFTR MODULATORS IN PATIENT-DERIVED RECTAL ORGANOIDS

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Cystic fibrosis (CF) is a genetic disorder characterized by impaired water and electrolyte transport resulting from malfunctioning cystic fibrosis transmembrane regulator (CFTR) channels. Over the past decade, CF has experienced a significant transformation in its standard of care, due to access to novel molecular entities that directly target the CFTR protein, known as CFTR modulators. Despite these advances, accurately representing CF disease states at the tissue level in-vitro remains a challenge. To address this, we developed optimal assay conditions for culturing patient-derived rectal organoids. Combined with an image-based high-throughput single organoid swelling assay, our approach allows the direct measurement of CFTR-dependent transepithelial fluid transport, overcoming the recurrently reported lack of sensitivity of the state-of-the art Forskolin-induced Swelling (FIS) assay used in CF. This novel method facilitates the study and prediction of treatment responses in CF patients to different CFTR modulators. Our results demonstrate that in-vitro analysis using this system enhances our understanding of the impact of various treatments on organoid epithelia, enabling the precise tailoring of therapies and supporting treatment decisions for complex CF phenotypes. Notably, our in-vitro results correlate with clinical responses for patients with various mutations, showing the potential to significantly improve patient outcomes and contribute to the ongoing transformation of CF care. We believe that our innovative approach will provide the CF community with new tools to establish robust functional assays using organoids and catalyze their establishments as routine tools in the clinics.

Keywords: organoids, cystic fibrosis, personalised medicine

5:45 PM – 6:45 PM

349

SINGLE CELL-GUIDED PRENATAL DERIVATION OF PRIMARY FETAL EPITHELIAL ORGANOIDS FROM THE HUMAN AMNIOTIC AND TRACHEAL FLUIDS TO MODEL DEVELOPMENT AND DISEASE

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Despite advances in prenatal diagnosis, it is still difficult to predict severity and outcomes of many congenital malformations. To this aim, isolation of tissue-specific fetal stem cells and derivation of primary organoids is limited to samples obtained from termination of

pregnancies. This hampering prenatal investigation of fetal development and congenital diseases. Therefore, novel patient-specific in vitro models are needed. To this aim, isolation and expansion of fetal epithelial stem cells during pregnancy, without the need for tissue samples or reprogramming, would be advantageous. The amniotic fluid (AF) is a source of cells shed from multiple developing organs. Using single cell analysis, we characterised the cellular identities in amniotic fluid (AF) and created a first atlas of the unperturbed human AF cells. While doing this, we identified and isolated viable epithelial stem/progenitor cells of fetal gastrointestinal, renal and pulmonary origin. With relevance for prenatal disease modelling, we investigated if these cells could be cultured to form primary clonal epithelial amniotic fluid-derived organoids (AFO) autologous to the fetus. Upon 3d culture these cells formed clonal epithelial organoids, manifesting small intestine, kidney tubule and lung identity. In this work we derived 423 AFO lines from 42 AF samples (16-34 GA weeks). AFO exhibits transcriptomic, protein expression differentiation potential, enzymatic activity and morphological hallmarks of their tissues of origin. Importantly, significant phenotypical alterations were detected in AFOs derived from congenital malformation cases recapitulating some pathological features of the conditions. This highlighted the potential use of our platform for disease modelling. This work shows the prenatal derivation of primary fetal organoids. AFO are autologous to the fetus, and derived with minimally invasive methods, in a timeline compatible with prenatal intervention. This paves the way for the investigation of therapeutic tools and regenerative medicine strategies personalised to the fetus at clinically relevant developmental stages.

Funding Source: MFMG held a H2020 Marie Skłodowska-Curie Fellowship (843265, AmnioticID). PDC received support by the NIHR (NIHR-RP-2014-04-046), H2020 (668294, INTENS) and NIHR GOSH BRC.

Keywords: primary organoids, amniotic fluid, fetal organoids

5:45 PM – 6:45 PM

351

DECIPHERING DRUG RESISTANCE IN ORAL SQUAMOUS CELL CARCINOMA USING PATIENT-DERIVED ORGANOIDS

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Oral squamous cell carcinoma (OSCC) is the most prevalent type of head and neck cancer and originates from different sites in the oral cavity. Half of the patients present to the clinic with advanced metastatic disease that has spread to regional lymph nodes and/or distant organs, such as the lungs. The response to therapy for OSCC is highly variable, and survival rates after first-line treatment remain low compared to other tumour entities. This is due to a lack of understanding of the molecular mechanisms underlying the inter- and intra-patient heterogeneity of OSCC. Therefore, it is crucial to overcome this knowledge gap to develop new and improved therapeutic options for OSCC, especially given its increasing incidence. To address this issue, we collected tissue samples from OSCC patients, including normal oral mucosa and primary, relapsed, and metastatic OSCC. We then established a living biobank of organoids and matched fibroblast lines using this tissue. Patient-derived organoids (PDO) recapitulate the histology of the tumour epithelium of origin and robustly express biomarkers such as CDH1, KRT14, MKI67 and P63. In drug screens, we found PDO-specific responses to inhibitors of key signalling cascades, reflecting inter-patient tumour heterogeneity. Using the patient-matched PDOs and fibroblasts, we are currently characterising the intra-patient drug responses of primary, relapsed, and metastatic OSCC and the influence of the stromal microenvironment. We aim to use our OSCC biobank to define inter- and intra-patient heterogeneity and identify potential new biomarkers and therapeutic targets.

Funding Source: The Kretzschmar group is funded by the German Cancer Aid (via MSNZ Würzburg/NG3), and the Interdisciplinary Centre for Clinical Research at the Medical Faculty of the University of Würzburg (IZKF project B-435).

Keywords: oral cancer, organoid technology, drug screening

5:45 PM – 6:45 PM

353

N-CARBAMYL GLUTAMIC ACID, A PROBIOTIC METABOLITE, UNVEILS PROTECTIVE AND THERAPEUTIC EFFECTS IN INTESTINAL MODELS

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Our comprehension of intestinal maturation and the therapeutic capabilities of microbial metabolites is an ongoing pursuit. This study delves into various models of intestinal influence, employing human pluripotent stem cell-derived human intestinal organoids (hIOs) as the fundamental platform. Building upon previous studies highlighting the robust maturation promotion by Limosilactobacillus reuteri strain DS0384, our

focus centers on the cell-free supernatant (CFS) of DS0384, enriched in N-carbamyl glutamic acid (NCG). Our findings illuminate the significant enhancement of intestinal stem cell (ISC) proliferation and the protective effect against cytokine-induced epithelial damage in ISC spheroids and inflamed hIO models attributed to NCG within the CFS. Additionally, NCG demonstrates its capacity to expedite epithelial wound healing and stimulate ISC growth in human intestinal epithelial cells (hIECs) by modulating Wnt signaling. Extending our research, we induced inflammatory bowel disease in mice, reaffirming the intestinal protective and therapeutic effects of NCG. Noteworthy is NCG's potential as an inflammatory bowel disease treatment, displaying efficacy without adverse effects such as weight loss at specific concentrations. These compelling outcomes underscore the potential of NCG as a valuable therapeutic agent for probiotic applications, not only in preventing intestinal barrier dysfunction but also offering hope for the management of inflammatory bowel disease.

Funding Source: This work was supported by the Korean Fund for Regenerative Medicine (KFRM) grant funded by the Korea government (21A0404L1), and a grant from the National Research Foundation of Korea (NRF) funded by NRF-2021M3A9H3016046.

Keywords: human intestinal stem cell, N-carbamyl glutamic acid, ulcerative colitis

5:45 PM – 6:45 PM

355

HIPSC-DERIVED DERMAL FIBROBLASTS HARBORING A PATIENT MUTATION IN XPA AS A MODEL TO STUDY XERODERMA PIGMENTOSUM

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Patients suffering from Xeroderma pigmentosum (XP) – a rare genetic disorder – are more than 1000 times likelier to develop skin cancers. This is due to mutations in genes encoding for components of the Nucleotide Excision Repair (NER) pathway, the predominant system that repairs DNA damage caused by ultraviolet radiation (UVR) which is present in sunlight and even some types of artificial lighting. NER is also responsible for repairing the toxic adducts formed between DNA and cisplatin – a common chemotherapeutic agent. Detection of bulky DNA lesions can occur via two distinct mechanisms which later converge into the same downstream processing and repair steps. The latter include a DNA excision step in which XPA protein plays a crucial role. XP is often diagnosed in the clinic by assaying NER capacity in patient-derived dermal fibroblasts. Here, we report the generation via CRISPR/Cas9 gene editing, and characterization of a human induced pluripotent stem cell (hiPSC) line carrying a mutation found in patients that introduces a premature stop codon in the XPA gene. We show that this mutation leads to the expression of a truncated yet stable isoform of the XPA protein lacking the 48 C-terminal amino acids of the full-length protein. Viability assays in dermal fibroblasts derived from the mutant XPA hiPSC line and its isogenic wild-type control show that the truncated XPA mutant protein confers extreme sensitivity to both UVR and cisplatin treatments compared to its wild-type counterpart. This hiPSC-derived system serves as an abundant source of disease-relevant cell types which we plan to use for high-throughput drug discovery screens in the near future in search of compounds that rescue the NER defects caused by the mutation in XPA. Furthermore, our disease model enables the development of DNA repair functional readouts to further validate the hits that emerge from the primary screens.

Funding Source: This work is funded by the Novo Nordisk Foundation Center for Stem Cell Medicine supported by the Novo Nordisk Foundation, Denmark (grant number NNF21CC0073729.

Keywords: Xeroderma pigmentosum, cancer, human induced pluripotent stem cells

5:45 PM – 6:45 PM

357

GRAFT VERSUS HOST IN A DISH AS AN ALTERNATIVE TO THE IN VIVO AGVHD MOUSE MODEL

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Acute Graft versus Host Disease (aGvHD) is an important complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Current aGvHD mouse models use radiation or chemotherapy followed by allo-HSCT, but are accompanied by high mortality. In order to decrease the need for large numbers of mice, we aimed to develop an aGvHD model in a dish using colon organoids (colonoids) that faithfully recapitulate the disease phenotype in vitro. For in vivo induction of aGvHD, we isolated CD3 T cells from C57Bl/6 spleens and stimulated the cells with CD3ε and CD28 for four days. 1x106 T cells were infused into MHC-mismatched Balb-c/Rag2-/- mice, pre-treated with Busulfan. Mice were monitored for 7 seven days and assessed for posture, weight loss and signs of diarrhea or bloody stool. Gastrointestinal permeability was assessed using FITC-Dextran. Infusion of T-cells resulted in rapid loss of intestinal integrity, widespread epithelial and crypt loss throughout the colon, as well as the presence of ulceration and areas of necrosis. For in vitro modelling of aGvHD, colonoids from Rag2-/- mice were co-cultured for 4 days with stimulated C57Bl6 T cells. Before and after T cell co-culture, colonoids were assessed using Hematoxylin/Eosin to visualize epithelial cell linings, anti-active caspase 3 to assess apoptosis, anti-cytokeratin 20 (CK20) to stain colonocytes and anti-MUC2 to reveal goblet cells. Healthy colonoids displayed CK20+ colonocytes and Muc2+ goblet cells that covered luminalized intestinal crypt surfaces, and low levels of caspase 3 positive cells. After co-culture with T cells, intestinal permeability increased, as evident from the presence of FITC-Dextran in colonoid lumens. In addition, culture in presence of T cells resulted in active destruction of the colonoids. In conclusion, co-culture of colonoids with T cells results in increased intestinal permeability, severe cellular damage and ultimately destruction of

colonoids in vitro, closely resembling the intestinal damage observed in mice after infusion of MHC mismatched T cells. We believe this in vitro aGvHD model can be used to test novel drugs or therapies for the treatment or alleviation of aGvHD as an alternative to in vivo mouse models.

Funding Source: TUBITAK 1001

Keywords: aGvHD, Rag2-/-, colonoid

5:45 PM – 6:45 PM

359

DEVELOPMENT OF A HUMAN IPSC-DERIVED INTESTINAL EPITHELIAL CELL/MACROPHAGE CO-CULTURE MODEL SYSTEM FOR DRUG TARGET IDENTIFICATION AND VALIDATION

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The homeostatic function of the intestine relies heavily on critical interactions between epithelial cells and the mucosal immune system. Dysregulation of these interactions leads to pathologies such the IBD spectrum of conditions, which are characterised by chronic inflammation and a disruption in the barrier function of the intestinal epithelium. This collaborative project between GSK and the University of Cambridge aims towards developing a translationally relevant in vitro co-culture model comprising an intestinal epithelial monolayer and a macrophage element, with both cell types derived from human iPSCs. As part of this work, we have developed a robust intestinal epithelial cell/macrophage co-culture system in which both cell types are derived from the same human iPSC line (autologous system). We have fully characterised the epithelial monolayer component in a “healthy” state and demonstrated that it displays epithelial barrier formation and consistent representation of various intestinal cell types. We have also successfully optimized a “diseased” (IBD) state for the epithelial monolayer component, where the intestinal epithelium exhibits a barrier disruption phenotype upon treatment with a pro-inflammatory cytokine cocktail. Moreover, we have validated the intestinal epithelial cell/macrophage co-culture model in a healthy state and have acquired preliminary data on modelling the IBD disease phenotype by challenging the co-culture system using our optimized IBD-associated cytokine cocktail. In future, this modular in vitro model system will be used by GSK for IBD target identification using CRISPR/Cas based screening, as well as for IBD target validation using tailored functional assays.

Keywords: iPSC-derived models for functional genomics, immune-competent disease models, complex in vitro models for drug discovery

5:45 PM – 6:45 PM

361

TOWARDS A HIGH-THROUGHPUT GUT-ON-CHIP MODEL WITH PATIENT-DERIVED COLON ORGANOIDS FOR PHENOTYPIC EVALUATION OF INTESTINAL EPITHELIAL RESPONSES

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The intestine plays a crucial role in nutrient absorption and immune defense. It consists of highly specialized cells, which form a barrier between the lumen and the underlying tissue. Traditional in vitro models often fail to replicate the complex microenvironment and physiological conditions found in the human gut. Although organoids have emerged as a promising technology to bridge this gap, several limitations persist. Matrix-embedded organoids present challenges in accessing the apical side of the epithelium, which limits sampling, transport studies, drug exposure, or co-culture with microbes. Moreover, the evaluation of barrier integrity - a key parameter in intestinal homeostasis - is difficult in such models. Here we addressed these limitations by using patient-derived colon organoids and the microfluidic OrganoPlate® platform to generate forty perfused membrane-free intestinal epithelial tubules on a single plate. We exposed these tubules to several pro-inflammatory triggers and evaluated the response by using trans-epithelial electrical resistance (TEER), protein detection, and immunostaining. We found that the different pro-inflammatory triggers had distinct effects on the gut tubules, such as modulation of barrier integrity, varying levels of cytokine release, and modulation of marker expression with changes in the levels and localization of specific proteins. In addition to addressing current limitations, our platform is scalable and allows the simultaneous evaluation of multiple samples, enabling rapid screening of compounds such as drugs or microbial metabolites. Furthermore, the use of patient-derived organoids opens avenues for personalized medicine. Collectively, these advancements pave the way for a better understanding of intestinal physiology and pathology, ultimately contributing to the development of novel therapies and personalized treatments for intestinal diseases.

Keywords: 3D gut model, high-throughput, intestinal disease modeling

5:45 PM – 6:45 PM

363

GASTRO-ESOPHAGEAL IN VITRO MODELLING WITH MULTI-ORGAN ASSEMBLOIDS

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Gastrointestinal pathologies such as esophageal atresia, neuromuscular disorders, or weak lower esophageal sphincter can contribute to gastro-esophageal reflux manifestation in pediatric patients. Long-term esophageal damage due to prolonged acid exposure may result in esophagitis, esophageal stricture, and Barrett's esophagus. The understanding of the mechanisms underlying these conditions caused by gastric acid exposure is crucial for improving life quality in affected patients. The aim of this study is to investigate the effect of acute and pro-longed acid exposure on the esophageal epithelium. To do so, we produced a novel in vitro multi-organ assembloid composed of pediatric tissue-derived esophageal and gastric organoids. Adult stem cells (ASCs) were isolated from the mucosa/submucosa of pediatric patients' biopsies of esophagus and stomach (fundus and antrum regions) to produce organoid lines. These lines were characterized to verify regional and organ identity. Fully-grown 7-days old whole organoids from each region were resuspended in extracellular matrix liquid pre-hydrogels (Collagen I and/or decellularized ECM gels). Organoids/pre-gel mixtures were seeded in custom-designed silicon culture wells, in the order of esophagus-fundus-antrum and allowed to gelate. Embedded organoids were cultured in suspension for 10 days, allowing the self-assembly and formation of a single assembloid with shared lumen. The assembloid was thoroughly characterized by whole mount immunofluorescence and transcriptomic to screen for regional and tissue specific functional markers. Each region maintained the identity and function of origin, increasing the level of differentiation compared to standard organoid culture. Control esophageal cells were exposed to increasing amount of gastric acid to validate the response and the level of damage. The assembloid was tested with acridine orange demonstrating gastric acid secretion. Stress cytokines were quantified to evaluate the acid-related damage. We successfully obtain a gastro-esophageal assembloid which are able to mimic a functional response to acid secretion. This novel in vitro model will be useful to investigate compromised acid secretion, simulate gastro-esophageal reflux in vitro, and test anti-reflux treatments.

Funding Source: OAK Foundation Award W1095/OCAY-14-191. NIHR Great Ormond Street Hospital Biomedical Research Centre (NIHR GOSH BRC). GOSH Children's Charity. UCL Therapeutic Acceleration Support (TAS) LifeArc Fund Rare Diseases Call (184646).

Keywords: gastro-esophageal organoids, assembloid, disease model

5:45 PM – 6:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

365

MYO-INOSITOL MITIGATES OXIDATIVE STRESS AND ENHANCES MITOCHONDRIAL FUNCTION IN PORCINE BLASTOCYSTS

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Myo-inositol (Myo-Ins), the most abundant type of inositol, serves as an antioxidant in various tissues and cells. Nonetheless, there is a lack of studies regarding its antioxidative role in porcine blastocysts during in vitro development. In this study, we investigated the antioxidative effect of Myo-Ins on porcine embryos derived from parthenogenetic activation (PA). We assessed various characteristics, including mitochondrial membrane potential (MMP), mitochondrial quantity, mitochondrial stress, and genes associated with mitochondrial function. Myo-Ins was added to the porcine zygote maturation media (pzm3) at different concentrations (5, 10, and 20 mM) during in vitro culture. The results demonstrated that Myo-Ins at 10 and 20 mM significantly improved the mitochondrial dysfunction by enhancing the MMP. Moreover, mitochondrial quantity also significantly increased in 20 mM treated group compared to the control group. In addition, mitochondrial oxidative stress was dramatically reduced in the 20 mM treated group compared to the control group. Furthermore, mitochondrial function related genes such as and solute carrier family 2 member 1(SLC2A1) and ATP synthase (ATP5F1A) were significantly increased in 10 and 20 mM treated groups compared to the control group. In short, these results indicate that Myo-Ins reduces the oxidative stress and improves the mitochondrial dysfunction in the porcine blastocysts.

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Keywords: porcine, blastocyst, oxidative stress

5:45 PM – 6:45 PM

367

NICHE-DERIVED STEMNESS SIGNALS ENSURE FUNCTIONALITY OF OVARIAN GERMLINE STEM CELLS VIA THE CONTROL OF MITOCHONDRIA

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The stem cell niche, a microenvironment formed by a group of cells, provides stemness signals to maintain stem cell fate. Changes in mitochondrial dynamics also influence stem cell fate; however, whether the stem cell niche controls stem cells' mitochondria and metabolism remains unclear. Here, we show that BMP stemness factors synthesized from the Drosophila ovarian germline stem cell (GSC) niche promote mitochondrial length and mass. Somatic overexpression of BMPs causes all germ cells to adopt a GSC fate with elongated mitochondria and increased mitochondrial mass, while disruption of BMP signaling in GSCs results in fragmented mitochondria with reduced mass. Strikingly, elongated mitochondria with increased mass are not observed in the ectopic GSCs generated by depleting the GSC differentiation factor. These results indicate that niche-derived BMP signals have a direct role in regulating mitochondria, regardless of GSC fate. We also report that BMP stemness signaling in GSCs increases the expression of mitochondria fusion regulator (Marf) and Spargel (Srl, a mammalian PGC1-alpha ortholog) to promote mitochondrial elongation and mass, respectively. Overexpression of marf does not impair fertility, while overexpression of srl increases the GSC division rate with decreased BMP signaling. On the other hand, depletion of marf causes fragmented mitochondria and GSC loss, while srl depletion does not result in obvious changes in GSC maintenance. We propose that under the control of BMP stemness signaling, mitochondrial elongation facilitates GSC maintenance, while mitochondria mass regulates GSC division. Our findings elucidate the intricate interplay between BMP signaling, mitochondrial dynamics, and content in regulating GSC fate, shedding light on mechanisms underlying stem cell maintenance and division in the Drosophila ovary.

Keywords: mitochondria, niche, BMP Signaling

5:45 PM – 6:45 PM

369

EFFECT OF FIBROBLAST GROWTH FACTOR 8 ON NUCLEAR AND CYTOPLASMIC MATURATION OF PORCINE OOCYTES

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Fibroblast growth factor 8 (FGF8), a cytokine directing outgrowth and patterning in the embryo, secreted from oocyte plays important roles in murine oocyte maturation. However, it is not yet known whether FGF8 is involved in porcine oocyte maturation. In this study, we investigated the effect of FGF8 supplementation during porcine oocyte *in vitro* maturation(IVM) via analysis of nuclear maturation, intracellular glutathione (GSH) and cumulus expansion. During IVM, the defined medium was supplemented with different concentrations of FGF8 (0, 1, 10, and 100 ng/mL). After IVM 42h, the nuclear maturation rate was significantly ($p < 0.05$) increased in 100 ng/mL FGF8-supplemented group compared to the control. The intracellular glutathione levels of oocytes, which are marker of cytoplasmic maturation, were significantly ($p < 0.05$) increased in 100 ng/mL FGF-supplemented group compared to the control. Additionally, we identified that supplementation of 100 ng/mL FGF8 during IVM significantly ($p < 0.05$) increased cumulus expansion, which helps oocyte maturation, compared to the control. Taken together, FGF8 supplementation during IVM enhances nuclear and cytoplasmic maturation of porcine oocytes. In further research, we will explore the mechanisms enhancing oocyte maturation by analyzing glycolysis and signaling pathways. Additionally, we will assess whether these mechanisms also improve embryo development rates.

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Keywords: porcine oocyte, In vitro maturation, FGF8

5:45 PM – 6:45 PM

371

STUDYING THE IMPACT OF PLOIDY ON HUMAN DEVELOPMENT AND TUMORIGENESIS USING HUMAN EMBRYONIC STEM CELLS

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Humans are diploid organisms and yet, 1-3% of human pregnancies are triploid, and triploidy is responsible for ~10% of all spontaneous miscarriages. Surprisingly, some of these triploid pregnancies proceed to term, while suffering from many fatal physiological and neuro-developmental disorders. Furthermore, polyploidy arise abnormally in cancer, and polyploid cancer cells are linked to tumorigenesis, metastasis and resistance to anti-cancer drugs. To investigate the impact of triploidy on human development, we generated triploid human embryonic stem cells (hESCs) by fusing isogenic haploid and diploid hESCs. A comparison of the transcriptome, methylome and genome-wide replication-timing show general similarity between diploid and triploid hESCs. However, triploid cells have a larger volume than diploid cells, demonstrating decreased surface area to volume ratio. This leads to a significant downregulation of cell surface ion channel genes, which are more essential in neural progenitors than in undifferentiated cells, leading to inhibition of differentiation, and affects the neuronal differentiation ability of triploid hESCs, both in vitro and in vivo. To investigate

the impact of triploidy on human tumorigenesis, we exposed our isogenic haploid, diploid and triploid hESCs to four different anti-cancer drugs. We have found that the ploidy of the cells dramatically affects their response to these agents. Moreover, we suggest that regulation of DNA damage response is playing a major role in the selective effect of ploidy, and genetic manipulation of hESCs with different ploidy supports our hypothesis. Notably, our research raises the possibility that polyploidy-related phenotypes may emerge due to their physical dimensions, affecting their initial developmental stages and their sensitivity to genotoxic agents. Overall, our results demonstrate the potential of our unique platform in studying the effects of ploidy on early human development and tumorigenicity.

Keywords: human pluripotent stem cells, ploidy, development and tumorigenicity

5:45 PM – 6:45 PM

373

HUMAN PLURIPOTENT STEM CELLS TO STUDY NEUROBLASTOMA

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The neural crest (NC) is a transient stem cell population during vertebrate embryogenesis. NC cells are multipotent and have the capacity to differentiate into a plethora of cell types. The trunk derivative of NC gives rise to cells constituting sympathetic ganglia and adrenal gland, tissues where childhood tumor form neuroblastoma (NB) arise. NB mainly affects young children under the age of 5, is the tumor form with lowest survival rates of all childhood cancers, and patients with high-risk tumors present with less than 50% overall survival. The most prevalent genetic anomaly in NB involves MYCN oncogene amplification, present in roughly 25% of cases, significantly influencing tumor progression. Moreover, there is a lack of embryonic models to study NB. This project aims to establish a human-chick chimera model, using human pluripotent stem cell (hPSC)-derived trunk neural crest transplantations with the chick embryo as host to study gene involved in NB development. Once established, the model will address fundamental biological inquiries by inducing MYCN oncogene expression at various stages pre- or post-transplantation to dissect its role in NB initiation. Human trunk NC cells are implanted on the top of chick embryo in ovo, at stages before endogenous NC stem cells undergo epithelial-to-mesenchymal transition and start to migrate within the embryonic body. We use the fluorescent label mscarlet, and staining for HuNu, a human-specific nucleus marker, to identify the human cells within the chick embryo. We demonstrate that the transplanted hPSC-derived trunk NC migrate along with the endogenous chick NC cells, suggesting that these human cells respond to environmental factors present in the chick. We are now optimizing the protocol by improving implantation matrix, cell numbers, pre-conditioning and cell survival. The role of MYCN will be established by downstream analysis including functional biological and spatial transcriptomics analysis. This human-chick chimera model is established by a non-invasive method, allows cell tracking throughout embryo development and provides the possibility to study any gene-of-interest. Elucidating the mysteries surrounding NB initiation holds promise for advancing our comprehension of this childhood cancer, potentially informing future therapeutic strategies.

Keywords: neuroblastoma, embryonic development, neural crest cells, human-chick chimera, cancer

5:45 PM – 6:45 PM

375

ADVANCED PEPTIDE HYDROGEL FOR SCALABLE 3D CULTURE OF PHYSIOLOGICAL RELEVANT HUMAN PLURIPOTENT STEM CELL ORGANOIDS: MAINTENANCE, EXPANSION, AND DIFFERENTIATION

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Monolayer (2D) culture of hPSCs including induced PSCs (hiPSCs) and hESCs severely limits stem cell capabilities and scalable production. Here, we reported a scalable peptide hydrogel platform for 3D culture of physiological relevant human iPSCs and ESCs. Novel 3D system was also developed for effective differentiation potential evaluations of three germ layers in early embryo development. Peptide hydrogel was prepared specifically suitable for PSCs 3D culture. Cell suspensions (hiPSCs derived from fibroblast and PBMC and hESC (WA09) were mixed with the peptide hydrogel to form 3D culture conditions. By day 5, 3D cell organoids were either fixed with 10% formalin or isolated by simple centrifugation. The isolated cell organoids and fixed organoids were characterized for proliferation, viability, and morphology, pluripotent biomarkers at mRNA and protein levels by RT-qPCR, immunostaining, flow cytometry, confocal imaging, and H&E staining for pathological structures. Differentiation potential in 3D system was determined using Trilineage kit. Aggregated hiPSC spheroids by using existing spheroids molding devices were used as comparison. The peptide hydrogel formed nanoweb networks mimicking the natural 3D extracellular matrix (ECM). Both hiPSCs and hESCs formed physiological relevant cell organoids in 3D culture and were easily harvested by centrifugation. Cell organoids size range was from 30-50 µm by day 5 and proliferated 10-20 folds with viability >95%. With seeding density of 200,000 cells/ml, about 80-120,000 cell organoids/ml were generated in 5 days. Pluripotency gene markers by RT-qPCR analysis showed stable expression levels of NANOG, OCT4, SOX2, DNMT3B, DPPA4, Myc, ESG1, hTERT, and UTF1. Flow cytometry results confirmed nearly 99% cells showed

Oct4 and SSEA4 expressions. Noteworthy, these hiPSC and hESC cell organoids possess lumen structure and showed superb differentiation potentials in three germ-layers. Conversely, the aggregated hiPSC spheroids formed solid spheres and had very limited differentiation capability. The nanoweb structure provides structural integrity for hiPSCs and hESCs forming cell organoids with physiological relevant lumen structures that sequesters signals for optimal cell performance and pluripotent integrity for various downstream applications.

Keywords: 3D model, pluripotent stem cells, organoid, phathological structure of hPSCs in 3D, differentiation efficiency in 3D model

5:45 PM – 6:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

377

CHARACTERISATION OF BLOOD-BRAIN BARRIER FUNCTION IN AN IN VITRO MODEL USING INDUCED PLURIPOTENT STEM CELLS IN FAMILIAL ALZHEIMER DISEASE

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Familial Alzheimer disease (FAD) accounts for only 1% to 5% of cases, typically striking before age 65 and progressing rapidly. The blood-brain barrier's (BBB) altered integrity and functionality are common in age-related neurodegenerative diseases, with endothelial cells playing a pivotal role. Our aim is to characterize the BBB in FAD, focusing on identifying specific proteins involved in FAD pathology and understanding associated signaling pathways. Using an established human in vitro model tailored for simulating FAD conditions, our primary focus is on understanding the role of amyloid precursor protein mutation in causing BBB dysfunction. Induced pluripotent stem cells (IPSCs) carrying an amyloid precursor protein mutation were employed to study BBB dysfunction. Three control IPSC lines are utilized for comparison. After differentiation into blood capillary endothelial cells (BCECs), barrier function is assessed TEER measurement and a sodium fluorescein assay. Cell maturity and purity are confirmed by morphological analysis, gene expression analysis, immunofluorescence analysis, and flow cytometry. As part of BBB examination, cell adhesion and migration are explored using electric cell-substrate impedance sensing (ECIS), and changes in tight junction proteins are monitored under cellular stress conditions with glutathione treatment. Flow cytometry indicated high BBB marker expression (>99% TJP1), while immunofluorescence confirmed typical barrier protein localization. TEER assessment showed stable barriers, with BCECs effectively preventing paracellular transport (indicated by low sodium fluorescein permeability coefficients). A pilot study showed altered diseased BCEC migration and adhesion capacity, enhancing our understanding of their resilience. Proteome analysis regulated proteins in FAD BCECs, that could support early FAD diagnosis or therapy development. We can conclude that we have established an in vitro model of the BBB specific to FAD, conducive to marker studies utilizing proteome analysis by mass spectrometry.

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Keywords: blood-brain barrier in vitro model, endothelial cells, familial Alzheimer disease

5:45 PM – 6:45 PM

379

INCORPORATING IPSC-DERIVED MACROPHAGES INTO CO-CULTURE SYSTEMS TO ASSESS IMMUNE-MEDIATED PHENOTYPES ACROSS ORGAN SYSTEMS

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Macrophages are key players in regulating organ homeostasis, tissue repair, and waste removal. In response to acute or chronic tissue injury, macrophages can contribute to tissue injury with diverse outcomes such cytotoxicity, inflammation, and fibrosis. Immune component co-culture is critical to understanding the mechanisms of immune-mediated toxicity. Human iPSC-derived iCell Macrophages 2.0 can be used to model drug-induced livery injury, cardiac fibrosis, and peripheral neuropathy through co-culture with other iPSC-derived cell types, including iCell Hepatocytes 2.0, iCell Cardiomyocytes^2 and Cardiac Fibroblasts, and iCell Sensory Neurons. While the practical application of incorporating macrophages into toxicology workflows can be technically and scientifically challenging, iCell Macrophages 2.0 provide an easy-to-use alternative to immortalized cell lines, without the inherent variability of sourcing and deriving primary macrophages. iPSC-derived

macrophages offer a consistent, renewable source of functional macrophages to interrogate acute and chronic tissue injury.

Keywords: macrophage, kupfer, coculture

5:45 PM – 6:45 PM

381

INVESTIGATING THE BIOLOGICAL PROPERTIES OF CIRCULATING HEMATOPOIETIC STEM/ PROGENITOR CELLS IN PEDIATRIC SUBJECTS AS NOVEL SOURCE FOR GENE THERAPY

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Conventional Hematopoietic Stem/Progenitor Cells (HSPC) harvesting procedures for clinical applications still present important technical difficulties for very young patients. Although most HSPC physiologically reside in the bone marrow (BM), significant numbers of circulating HSPC (cHSPC) are found in the peripheral blood (PB) during the first years of life. Nevertheless, no study has investigated the biological features and the clinical potential of pediatric cHSPC. Applying multi-parametric immunophenotyping to a cohort of 72 healthy donors (0-65 years-old), we observed enriched levels of the primitive circulating Hematopoietic Stem Cells (HSC) and Multi-Potent Progenitors (MPP) in pediatrics, which are reduced in adults. Moreover, we observed that primitive cHSPC display higher differentiation efficiency and increased multi-lineage hematopoietic production in pediatrics than in adults through in vitro multi-lineage differentiation assay on single circulating HSC and MPP. To investigate cHSPC molecular features, we transcriptionally profiled at single-cell level 32,978 HSPC isolated from different sources of pediatric donors, including PB (n=8), cord blood (CB, n=2), BM (n=2) and mobilized PB (mPB, n=2). PB-HSPC show low cell cycle activity compared to HSPC from the other sources. Notably, the fraction of HSC and MPP is comparable across the different HSPC sources, in line with the phenotypic characterization. Supervised and unsupervised analyses, performed on the cluster enriched for primitive HSC, highlighted similar levels of gene expression of important regulators of HSPC stemness across sources (CLEC9A, AVP, GPRC5C, MECOM). Finally, preliminary data suggest that pediatric cHSPC can be efficiently transduced with lentiviral vectors and ex vivo expanded at comparable rate with respect to CB-HSPC. These data will be integrated with in vivo experiments in mice, to evaluate the BM-homing and the hematopoietic reconstitution capabilities of pediatric cHSPC. Overall, our project will generate essential information to assess their potential as novel stem cell source for ex vivo and in vivo HSPC-GT strategies.

Keywords: hematopoietic stem cells, pediatric, gene therapy

5:45 PM – 6:45 PM

383

HUMAN IPSC-DERIVED MACROPHAGES WITH ENHANCED FUNCTIONALITY TO COMBAT MYCOBACTERIAL INFECTIONS

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Mycobacterium tuberculosis (Mtb), the main pathogen for the cause of lung tuberculosis (TB), still continues to be a global threat, being the 2nd leading infectious cause of death. Despite the effective standard treatment using a combination of antibiotics, the constant overuse is the main driver for antibiotic-resistant strains. Moreover, Mtb developed evasion strategies to escape the intra-cellular defense mechanisms of alveolar macrophages. Modern immunotherapeutic approaches aim to counteract these strategies via macrophage activation. An additional alternative would be the generation of enhanced macrophages, which could be used directly as an off-the-shelf cell-based immunotherapy. In our work, we investigated the immune response and functionality of primitive macrophages, which are derived from human induced

pluripotent stem cells (iMacs). Generation of iMacs has been performed from two healthy individuals and the response post-infection with the attenuated TB strain Bacillus Calmette-Guérin (BCG), heat-killed Mtb (HKMT) and Mtb has been analyzed. As comparison, classical monocyte-derived macrophages (MDMs) have been used as a control. Herein, we show that both types of macrophages start phagocytosis of HKMT and BCG shortly after contact, with iMacs demonstrating a stronger migration and an overall faster phagocytosis rate compared to MDMs. In addition, for both pathogens, iMacs showed a higher induction of autophagy as well as an increased production of reactive oxygen species and lysosomal enzymes, which are known to play a crucial role in the defense of mycobacterial infections. Furthermore, iMacs demonstrated a stronger increase of pro-inflammatory cytokine response upon infection with HKMT, BCG or Mtb compared to MDMs, including a subsequent downregulation of the pro-inflammatory state, thus, being able to return much faster to the baseline cell activation. In addition, iMacs showed a clear localization of BCG and V-ATPases, indicating a strong phagosome acidification post-infection. In fact, both types of macrophages were able to kill phagocytosed BCG and Mtb, with iMacs showing a stronger killing capacity. We here reveal iMacs of pluripotent origin with enhanced functionality to successfully eradicate mycobacteria, which may be considered for novel therapeutic applications.

Keywords: induced pluripotent stem cells, macrophages, tuberculosis

5:45 PM – 6:45 PM

385

INVESTIGATING THE ROLE OF APOPTOSIS IN CANCER THERAPY-INDUCED VASCULAR TOXICITIES USING HUMAN IPSC-ENDOTHELIAL CELLS

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Recent advancements in cancer treatment have improved the survival rate of patients in the clinic. However, cytotoxic and targeted therapies can inflict collateral damage on healthy tissues and trigger apoptosis (programmed cell death), leading to long-term toxicities. Cardiovascular toxicity is frequently diagnosed in cancer survivors and manifests as atherosclerosis, heart failure, acute thrombosis, venous thromboembolism, or others. Vascular toxicity is the second most prevalent clinical consequence for cancer patients and can be caused by chemotherapy or radiation therapy, yet the causative mechanisms are poorly understood. This is in part due to the lack of suitable in vitro model systems to study vascular toxicity development. Using human induced pluripotent stem cells (hiPSCs), we investigated cancer therapy-induced vascular toxicities in hiPSC-derived endothelial cells (ECs), and smooth muscle cells (VSMCs). We hypothesize that cell-type differences in apoptosis regulation affect their responses to cancer therapies, leading to a range of vascular toxicities. Using BH3 profiling, we find that ECs and VSMCs are both primed for apoptosis, which partly explains their high sensitivity to cancer therapies. Additionally, we treated ECs and VSMCs with a panel of chemotherapeutics, or radiation therapy, to determine drug sensitivity and differential apoptotic response. We show that treatments such as VEGF inhibitors cause greater toxicity in ECs than in VSMCs, corroborating previous observations. Interestingly, certain drugs - especially topoisomerase inhibitors - lead to higher toxicity in VSMCs, despite ECs being more primed for apoptosis. In mice treated with cancer therapies, ECs are also the weakest link in healthy tissues. To combat vascular toxicity, we blocked apoptosis through siRNA knockdowns, which is sufficient to protect ECs from undergoing cell death. In an orthogonal approach, we also found the commonly used drug dexamethasone lowers endothelial cell apoptotic priming. Altogether, we developed an in vitro human platform to investigate vascular toxicity, and demonstrate cell-type specific response to chemotherapy and radiation therapy. These results lay the groundwork for improved therapy regimens in the clinic, and decrease vascular toxicities in patients.

Funding Source: NCI Ruth L. Kirschstein F31 Fellowship Award #1F31CA275321-01

Keywords: apoptosis, vascular toxicity, endothelial cells

5:45 PM – 6:45 PM

387

A COMPARATIVE STUDY OF HUMAN PLURIPOTENT STEM CELL-DERIVED MACROPHAGES IN MODELING VIRAL INFECTIONS

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Macrophages play multiple roles in innate immunity, including phagocytosing pathogens, modulating inflammatory response, presenting antigens, and recruiting other immune cells. Tis-sue-resident macrophages adapt to the local microenvironment and can exhibit different immune responses upon encountering distinct pathogens. In this study, we generated human pluripotent stem cells (hPSCs) derived macrophages (iMACs) to investigate interactions between macrophages and various human pathogens, including hepatitis C virus (HCV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), or Streptococcus pneumoniae. IMACs can engulf all three pathogens. A comparison of the RNA-seq data of iMAC encountering different pathogens revealed that they activated distinct gene networks related to viral response and inflammation. Interestingly, in the presence of both HCV and host cells, iMACs would upregulate different sets of genes involved in immune cell migration and chemotaxis. Finally, we constructed an im-age-based high-content analysis system consisting of iMAC, recombinant GFP-HCV, and hepatic cells to evaluate the chemical inhibitor's effect on HCV infection. In summary, we developed a human cell-based in vitro model to study macrophage response to human viral infections; the comparative transcriptome analysis provided a useful resource to better understand the pathogen-macrophage-tissue microenvironment interactions.

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Keywords: macrophage, virus infection, human pluripotent stem cells

5:45 PM – 6:45 PM

389

CD19 CAR-EXPRESSING IPSC-DERIVED NK CELLS EFFECTIVELY ENHANCE MIGRATION AND CYTOTOXICITY INTO GLIOBLASTOMA BY TARGETING TO THE PERICYTES IN TUMOR MICROENVIRONMENT

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In cancer immunotherapy, targeting specific antigens with chimeric antigen receptors (CARs) has emerged as a potent tool for cell-based therapy. After being accessed by CARs, the selective anticancer lysis by CAR-natural killer (NK) cells through 'missing-self' and 'induced-self' mechanisms can mitigate off-tumor toxicity, a contrast to CAR-T cells. This specificity can be advantageous in the heterogeneous milieu of solid tumors. In the tumor microenvironment of glioblastoma (GBM), pericytes not only support tumor growth but also contribute to immune evasion, underscoring their potential as therapeutic targets in GBM treatment. Indeed, we examined and observed the presence of CD19-positive pericytes derived from GBM in patient samples, particularly within the perivascular niche, highlighting CD19 as a possible therapeutic target. Given this context, our study aimed to target the GBM tumor microenvironment, with a special focus on pericytes expressing CD19, to evaluate the potential effectiveness of CD19 CAR-iNK cells against GBM. In this study, we generated induced pluripotent stem cell-derived NK (iNK) cells with CD19 CAR. To determine whether CD19 CAR targets the tumor microenvironment pericytes in GBM, we developed GBM-blood vessel assembloids (GBVA) by fusing GBM spheroids with blood vessel organoids. When co-cultured with GBVA, CD19 CAR-iNK cells migrated towards the pericytes surrounding the GBM, inducing tumor lysis through NKp44 upregulation. Using a microfluidic chip, we observed that post-infused CD19 CAR-iNK cells targeted pericytes in a perfusion-like setting, facilitating an examination of CD19 CAR-driven migration and their tumor-specific cytotoxicity on the GBM. Furthermore, GBVAs were vascularized after transplantation into NSG mice and recapitulated the microenvironment including human CD19-positive pericytes, thereby enabling the application of an in vivo model for validating the efficacy of CD19 CAR-iNK cells against GBM. Compared to GBM spheroids, the presence of pericytes significantly increased the migration of CD19 CAR-iNK cells towards GBM and reduction of GBM proliferation. Therefore, we conclude that CD19 CAR-iNK cells may effectively target the pericytes in the GBM tumor microenvironment, suggesting their potential therapeutic value for GBM treatment.

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Keywords: tumor microenvironment, CAR-NK, glioblastoma

5:45 PM – 6:45 PM

391

EX VIVO EXPANSION AND LENTIVIRAL GENE MODIFICATION OF HUMAN HEMATOPOIETIC STEM AND PROGENITOR CELLS USING A83-01, POMALIDOMIDE, AND UM171

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Patients suffering from severe hematological diseases rely, in many cases, on the replacement of their defective hematopoietic system with a healthy graft. Finding an HLA-matched donor for hematopoietic stem cell transplantation can be cumbersome depending on the patient's family situation or ethnic origin. Umbilical cord blood (UCB) requires less stringent rules of HLA matching and causes fewer cases of the life-threatening graft-versus-host-disease, however, the low number of HSPCs within one UCB graft delays engraftment. Since pooling multiple UCB units did not reach sufficient hematopoietic reconstitution, we pursued the ex vivo expansion of HSPCs. Wataru Ebina from the Rossi lab reported that the TGFβ inhibitor A83-01, the Notch activator pomalidomide, and UM171 (APU) could expand HSPCs in the presence of SCF, FLT3-L, TPO, and IL-3 (SFT3). We could reproduce their findings and observed a 900-fold expansion of CD34+CD38-CD45RA-CD90+ cells in APU medium. Additionally, scRNAseq analysis verified the expression of HSC signature genes such as SPINK2, HLF, and AVP. Further, we used immune-deficient NBSGW mice, a xenograft mouse model mimicking transplantation of myeloablated patients, to determine the engraftment potential of expanded human HSPCs. The 7-day APU-expanded HSPCs reconstituted the bone marrow of the mice superior to SFT3-only, while a 14-day expansion in APU reduced the HSPC's engraftment potential. We hypothesized an impaired migration capacity of APU-expanded HPSCs due to a low expression of the bone marrow homing molecule CXCR4 in the respective scRNAseq cluster. Indeed, compared to SFT3-expanded HSPCs, we confirmed a lower CXCR4 expression on the surface of APU-expanded HSPCs although the LT-HSC marker EPCR is upregulated by APU. Currently,

we are investigating strategies to restore CXCR4 expression. Moreover, the presence of APU enhanced the cell modification with clinically applicable lentiviral vectors and maintained significantly more immune phenotypical HSPCs during ex vivo cultivation HSPCs compared to the minimal cytokine composition SFT3. In summary, the ex vivo expansion of HSPCs with APU could be purposeful for patients with low HSPC counts before gene therapy, such as Fanconi Anemia, or could facilitate the use of single UCB units.

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Keywords: hematopoietic stem cell expansion, umbilical cord blood transplantation, gene therapy

5:45 PM – 6:45 PM

393

PRODUCTION OF PATIENT SPECIFIC MACROPHAGES VIA PLURIPOTENT STEM CELLS

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Rare patient mutations are extremely difficult to study due to the very limited sample material available for functional studies. Especially in the case of monocyte-derived macrophages, which usually cover less than 10 % of the total white cell count of the blood and lack proliferative capacity in vitro. Current stem cell techniques allow reprogramming of almost any somatic cell type back to the pluripotent stem cell state. Moreover, human induced pluripotent stem cells (hPSCs) can be further differentiated into various cell types to study the effect of mutations on the behavior of various cells. Understanding the function of the unique characteristics of the individual phenotypes enables the development of patient-specific treatments and may reveal new roles for mutated proteins. In this study, human peripheral blood mononuclear cells were reprogrammed with a high-efficiency induction system into a pluripotent stem cell state. Produced patient-specific hPSCs were differentiated into monocytes and further into macrophages. Monocyte differentiation was achieved by mesoderm induction and hematopoietic differentiation with a STEMdiffTM monocyte Kit. The monocytes were further differentiated into macrophages by simultaneous stimulation of hematopoietic differentiation and myeloid specification using hu-IL-3 and hu-M-CSF and further into M1-type and M2-type macrophages. The resulting pluripotent stem cell-derived immune cells showed typical morphology and expression of common monocyte and macrophage markers. The obtained methodology allows extensive functional investigation of rare patients with dysfunctional macrophages.

Keywords: human pluripotent stem cells, monocytes, macrophages

5:45 PM – 6:45 PM

395

BACTERIAL INFECTION INDUCES ELEVATED VASCULAR INFLAMMATION IN ENDOTHELIAL-DERIVED DIABETES DONORS

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Patients with diabetes mellitus have increased risks of developing a new emerging infectious disease, mainly bacterial infection, and exhibit a higher risk of respiratory tract, gastrointestinal tract, urinary tract infections (UTIs), and skin/soft-tissue infections than healthy subjects in primary care practices. Endothelial cells (ECs) are one of the first cells in circulation to recognise microbial components, similar to innate immune system cells. They are equipped with pathogen/danger-associated molecular patterns (PAMPs/DAMPs). Because induced-pluripotent stem cells (iPSCs) preserve the memory from their generation, iPSCs-derived ECs (iPS-ECs) have emerged as a modality for studying disease mechanisms upon diabetes donors. We assessed the underlying mechanism involved in the severity outcome in diabetic-derived iPS-ECs (DBiPS-ECs) due to infection. The iPS-ECs were infected with E. coli, followed by incubation with antibiotics, and the cells were monitored for 48 hours. Sequencing and functional analysis revealed that DBiPS-ECs exhibit higher levels of inflammation, impair endothelial function as evidenced by less tube formation, and produce more LDH and ROS than non-diabetic (NDiPS-ECs). These findings suggested that endothelial dysfunction was exacerbated more damaging by infection. Furthermore, epigenetic regulation, such as histone post-translational modification, was potentially involved in this mechanism. Importantly, we have identified, an epigenetic modulator, which affect the severe outcome in DBiPS-ECs such as hyperinflammation and endothelial dysfunction. Inhibition of the activity and knock down of this epigenetic modulator markedly inhibits ROS, LDH production and suppresses inflammation. Nevertheless, research is ongoing to determine how this epigenetic modulator controls inflammation in E. coli -infected iPS-ECs. We conclude that an epigenetic modulator plays a role in inflammation in iPS-ECs-derived diabetes donors, providing insight into the signalling pathway underlying hyperinflammation and endothelial dysfunction. Importantly, our study provides new approach studying the mechanism of the environmental exposure, such as viral or bacterial infection, may worsen the clinical outcome of diabetes donors by affecting the epigenetic modulator.

Keywords: iPS-ECs, bacterial infection, epigenetic modulator inflammation

5:45 PM – 6:45 PM

397

UNRAVELING THE INFLUENCE OF CARDIOVASCULAR DISEASE ON HEMATOPOIETIC STEM CELL DYNAMICS THROUGH BONE MARROW ORGANOIDS

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Cardiovascular disease (CVD) remains the leading cause of death, despite expansive advancements in the field. The underlying mechanisms and pathophysiology of CVD are intricate and extend beyond the manifestations in the heart and vascular system. Indeed, CVD context was shown to influence bone marrow resident hematopoietic stem cells (HSCs) and ultimately lead to altered hematopoiesis that accelerates disease progression. Due to the complex nature of this specialized microenvironment, studying the role of CVD on human bone marrow has been limited to animal models and conventional 2D cell culture. Thus, the development of a human induced pluripotent stem cell (iPSC)-derived bone marrow organoid (BMO) mimicking the native human niche, offers a promising approach to investigate the effects of CVD context on human bone marrow. To determine whether BMOs are a useful tool for this purpose, the presence of hematopoietic cells and, in particular, hematopoietic stem and progenitor cells (HSPCs) is pertinent. Flow cytometry and advanced microscopy confirmed the presence of HSPCs (CD34+CD11b-) and myeloid cells (CD45+CD11b+) in BMOs. Moreover, functional hematopoiesis was demonstrated through colony forming unit assays (CFU) using sorted HSPCs from BMOs. The presence of CD31+ endothelial cells were also identified via FACS and microscopy revealed vascular-like structures throughout the organoids. Next, we tested the ability of BMOs to uptake low density lipoproteins (LDL), also known as bad cholesterol involved in CVD development. Live BMOs were treated with pHrodo-fluorescently labeled LDL and imaged using instant computational clearing fluorescence microscopy (Thunder). Interestingly, we found that while some CD45+ hematopoietic cells engulfed LDL, the vast majority of LDL positive cells were actually CD31+ endothelial cells. Altogether, our findings highlight the presence of the vascular and hematopoietic system within the BMOs as well as the uptake of LDL, which suggests that this model can be used to study changes within the bone marrow in CVD context. Further investigations will delve into the consequences of atherogenic conditions on HSCs.

Keywords: bone marrow organoids, hematopoietic stem cells, cardiovascular disease

5:45 PM – 6:45 PM

399

RUNX1 GERMLINE PREDISPOSITION TO MYELOID CANCERS: THROUGH THE LENS OF PATIENT-DERIVED IPSCS

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The transcription factor RUNX1 is vital for definitive hematopoiesis. It is one of the most commonly mutated genes in myeloid malignancies, where it is usually acquired as a secondary mutation following an initial disease-driving event. On the contrary, in individuals with RUNX1 germline mutations, primary RUNX1 mutations seem to significantly increase the risk of acquiring somatic disease-driving mutations and subsequent hematological malignancies. All RUNX1 germline patients present with FPDMM (Familial Platelet Disorder with Predisposition to Myeloid Malignancies), characterized by mild to moderate thrombocytopenia and an approximately 50% lifetime risk of developing hematopoietic malignancies, including myelodysplastic syndrome (MDS) and acute myeloid leukemia. The molecular mechanisms of the interactions between specific RUNX1 variants and different secondary mutations and how these lead to disease are unknown. We hypothesize that the somatic mutations present strongly influence disease type and account for the heterogeneity in phenotypes between patients. As per our knowledge, no comprehensive animal model of FPDMM has been established. Here, we use patient-derived induced pluripotent stem cells (iPSC) to investigate how germline RUNX1 variants interact with secondary mutations, promote malignancy development, and how RUNX1 deficiency subsequently leads to bleeding abnormalities. We have established and characterized iPSC lines from two biologically related individuals with FPDMM. In order to model different developmental and disease stages in vitro, we have successfully differentiated these iPSCs into hematopoietic stem and progenitor cells as well as megakaryocytes. Our current work is focused on using these workflows to investigate how RUNX1 variants affect hematopoiesis. Modelling of different disease stages will allow us explore how RUNX1 variants interact with other somatic mutations and lead to clinical heterogeneity of the disease. We believe that our system will be a crucial tool in unraveling the mechanism by which germline RUNX1 variants lead to disease and highlighting new targets to develop treatments and prevent disease progression.

Funding Source: Swedish Research Council

Keywords: myeloid malignancies, RUNX1 germline variants, iPSC

5:45 PM – 6:45 PM

401

HEMATOPOIETIC STEM CELL HEALTH AND IMMUNE CELL PROFILING OF ASTRONAUTS PRE-, DURING, AND POST-SPACEFLIGHT

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The International Space Station (ISS) offers a uniquely accelerating environment to study micro- and macro-environmental stressors in the context of modeling human diseases and simulating inflammation, aging, immune dysfunction, and pre-malignant cellular transformations. Studies done in low earth orbit (LEO) may offer accelerated insights into the human body, and specifically, human hematopoietic stem cells (HSC). HSCs are characterized by their resilience to environmental stressors: they have the capacity to self-renew without differentiating, the ability to differentiate into tissue-specific progenitors, and retain dormancy in protective environments. As a follow-on study for assessing HSC fitness in 4 iterative 30+ day SpaceX-CRS missions in ex vivo nanobioreactors, we sought to study these effects in astronauts conducting research in LEO. We designed a time course study to collect (under an IRB-approved protocol) peripheral blood from private astronauts on Axiom Missions 2 and 3 (Ax-2x Ax-3) to assess their HSC health and profile their immune subsets. Timepoints include 2 pre-flight, 1 inflight, and 7 post-flight. To investigate the effects of short-duration spaceflight on HSC health, we enriched for CD34+ hematopoietic stem and progenitor cells (HSPCs) from consenting astronauts and performed survival and self-renewal functional assays at each timepoint across the 2 missions. Additionally, HSPCs and total peripheral blood fractions were sent for whole genome sequencing (WGS), single cell RNA-sequencing (scRNA-seq) and whole transcriptome sequencing (RNA-seq) to identify pre-, in-, and post-flight genomic and transcriptomic changes. Results from functional assays suggest that HSPCs have reduced self-renewal capacity inflight; however, post-flight, HSPCs regain cloning capacity comparable to pre-flight levels. scRNA-seq results suggest that HSPC mobilization occurs post-flight. Results from WGS and RNA-seq are pending analysis. Finally, parallel analyses were performed on total peripheral blood fractions to profile the astronauts' immune cell subsets, which were also corroborated via FACS analysis. This longitudinal study has the potential to provide valuable insights into stem cell health and immune cell profiles associated with short-duration missions in LEO.

Keywords: hematopoietic stem cells, astronauts, macroenvironment

5:45 PM – 6:45 PM

403

TRACKING THE PROGENY OF LEUKEMIA-INITIATING STEM CELLS IN PRIMARY ADULT ACUTE LYMPHOBLASTIC LEUKEMIA AND RELAPSE BY SINGLE-CELL BARCODING

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The long-term prognosis for Acute Lymphoblastic Leukemia (ALL) is dismal, especially after relapse. Identification of molecular resistance mechanisms in leukemia-initiating cells (LICs) is a significant medical need, as LICs may be the source of therapy-resistant cells leading to relapse. However, there is still a debate about the existence, immunophenotype, and characteristics of LICs in ALL. To combine the analysis of subclonal architecture developing from LICs and the differentiation trajectories of single clones, also upon treatment, we created lentiviral barcoding technologies to track LICs and their fate at clonal and single-cell resolution in vivo. By transplanting our well-characterized model of patient-derived B-ALL cells (PDLTCs) from several donors, we showed that a majority of freshly transduced PDLTCs homed and engrafted in bone marrow and spleen of NSG mice. Dominant subclones demonstrated a strong local expansion accounting for most of the tumor mass, whereas hundreds of small clones persisted throughout the disease without expansion. Importantly, only 20% of subclones were in various organs within one recipient, while 60% of subclones expanded locally restricted. The clonal composition was highly variable between recipient mice receiving the same barcoded cells, suggesting

a high selective pressure by the local microenvironment. Nevertheless, cells with identical barcodes demonstrated consistent molecular patterns across animals, indicating clone intrinsic differentiation properties. Additionally, single-cell CITE-Seq revealed a proposed cluster of origin and distinct subpopulations of cells at various stages of differentiation in B-ALL (see abstract by A. Jolly et al.), which could be prospectively isolated based on their cell surface markers and transplanted into NSGs. Here we functionally demonstrated that stages of differentiation exist with a stable population of highly enriched LICs in ALL. Next, we will perform scCITE-Seq before and after treatment with chemical, immunological, and cell-based therapies to delineate how the subclonal architecture varies between therapeutic approaches. These results will allow us to find molecular targets and potential resistance mechanisms of LICs, to identify vulnerabilities to break resistance.

Keywords: BCP-ALL, barcoding, scCITE-Seq

5:45 PM – 6:45 PM

405

BRAIN DELIVERY OF PROTACS: STRATEGIES EXPLORED IN HUMAN IPSC-DERIVED BLOOD-BRAIN BARRIER MODELS

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The constantly increasing global burden of central nervous system (CNS) diseases is accompanied by a persistent lack of effective treatments. In neurodegenerative diseases, classical drug development based on small molecule inhibitors encounters limitations as disease-causing agents involve misfolded proteins and other toxic aggregates. Proteolytic-targeting chimeras (PROTACs) represent a promising therapeutic approach capable of addressing proteins previously considered as undruggable targets. In contrast to classical inhibitors of enzyme function, PROTACs facilitate the degradation of the target protein by harnessing the ubiquitin-proteasome system. However, the development of an effective medication for CNS diseases faces a considerable obstacle – permeation of the selective blood-brain barrier (BBB). This task is particularly challenging for PROTACs, given their complex physicochemical properties and higher molecular weight. To systematically assess BBB permeability of PROTACs, we established an in vitro platform composed of human-induced pluripotent stem cell-derived brain capillary endothelial cells (hiPSC-derived BCECs). Unlike conventional BBB models, these cells exhibit in vivo-like barrier properties, characterized by the expression of a broad spectrum of relevant marker proteins, limited transcellular transport, and high transendothelial electrical resistance. A robust workflow was developed to classify BBB permeability of various PROTACs by comparing them to reference substances and data obtained from conventional Caco-2 permeability assays. Further, our model serves as a valuable tool to explore novel strategies to improve brain delivery of PROTACs, such as encapsulation with cyclodextrin- or lipid-based nanoparticles. In conclusion, our hiPSC-based platform and systematic evaluation of BBB transport not only advances our understanding of PROTACs' BBB permeability but also contributes valuable insights for optimizing their delivery to the brain, offering new possibilities for the treatment of CNS diseases.

Keywords: PROTAC, blood-brain barrier, human induced pluripotent stem cells

5:45 PM – 6:45 PM

407

CHARACTERIZATION OF FACTORS INVOLVED IN IMMORTALIZATION OF MYELOID PROGENITORS AND OPTIMIZATION OF IN VITRO GENOTOXICITY ASSAYS

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Due to the occurrence of severe adverse events in some clinical trials using retroviral (RV) gene therapy, including gamma-retroviral (γRV) and lentiviral (LV) vectors, appropriate preclinical genotoxicity assessment is necessary to ensure patient safety. Our institute developed the In Vitro Immortalization Assay (IVIM) and Surrogate Assay for Genotoxicity Assessment (SAGA) to assess insertional oncogenesis risk. Here, murine hematopoietic stem and progenitor cells (mHSPCs) are transduced with RV vectors, and genotoxicity can be detected by a proliferation advantage under limiting dilution conditions (IVIM) or the activation of genes associated with oncogenesis and stem cell-like properties (SAGA). However, as mechanisms of insertional mutagenesis remain poorly understood, further investigation is required. We examined the role of genotoxicity predictors, identified as genes deregulated during transformation (SAGA genes), by inducible overexpression with LV vectors, resulting in a proliferation advantage of mHSPCs. The relevance of the SAGA genes in immortalization was further validated with SAGA-Q: a faster, simpler quantitative ddPCR readout as an alternative to the costly, time-consuming microarray-based SAGA. Preliminary results showed increased expression of the SAGA genes in samples transduced with mutagenic γRV and LV vectors, and immortalized clones

derived from these samples exhibited an even higher expression (2- to 6-fold over the parent sample). Also, SAGA-Q allowed us to detect transforming samples before the replating endpoint of our standard IVIM, providing a potential new readout. Moreover, we aimed to transfer the IVIM assay to human cord blood-derived HSPCs (hHSPCs), better reflecting the clinical stem cell source used in gene therapies for monogenic diseases and further supporting the 3Rs principle. The small molecules A83-01 and pomalidomide enabled a 4- to 8-fold expansion of hHSPCs while preserving their stem cell phenotype, reducing the required amount of cord blood. Despite achieving high transduction rates with γRV and LV, detecting immortalized samples remains challenging. The optimization of the preclinical genotoxicity assays will lead to more feasible and reproducible results, ensuring safer clinical translation of viral vector gene therapy products.

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Keywords: genotoxicity assay, gene therapy, hematopoietic stem and progenitor cells

5:45 PM – 6:45 PM

409

IPSC-DERIVED OSTEOCLASTS: A NEW TOOL TO STUDY MULTIPLE MYELOMA INDUCED OSTEOLYSIS

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Multiple myeloma (MM) is the second most common hematological malignancy in adults characterized by clonal proliferation of plasma cells. The use of novel therapeutic drugs has led to a substantial improvement in the overall survival of MM patients during the last 15 years. This extension of life expectancy has revealed the impact of secondary cancer-induced pathologies. More specifically, up to 90% of MM patients develop cancer-induced bone lesions throughout the course of their disease due to excessive bone resorption and suppression of bone formation, which is a major cause of morbidity and mortality. Whereas an increase in osteoclast (OC) numbers and activity has been identified to be responsible for the osteolysis in MM, the mechanisms resulting from the OC and MM cell interactions are very poorly understood. This is partly due to the difficulty to isolate and culture primary OCs, as well as the highly variable differentiation of OCs from CD14+ monocytes. Here we present induced pluripotent stem cells (iPSCs) as a valuable tool to efficiently and reliably generate OCs (iOCs), allowing to investigate the bidirectional interactions between OCs and MM cells. These iOCs are multinucleated, stain for TRAP by immunocytochemistry, and express typical genes, like NFATC1, CTSK, MMP9, CALCR, CA2, and TCIRG1, resembling primary OCs. Moreover, testing their resorption capacity, using bovine bone slices, they clearly show the ability to generate both pits and trenches in the bone surface. Next, we tested these iOCs in the context of MM and show that co-culture of iOCs with MM cells, using a wide variety of MM cell lines, results in increased MM cell proliferation, as well as drug resistance. In turn the MM cells promote the bone resorption mediated by iOCs when cultured on bone slices. Finally, we investigated whether interleukin 6 (IL-6), a key cytokine in the pathogenesis of MM, is regulated through this interaction. Indeed coculture with MM cells results in an increase in the expression of IL6, both mRNA and protein, in iOCs. Taken together, we demonstrate that iPSC-derived OCs can serve as an reliable platform to study the interactions between MM cells and OCs that result in osteolysis in more detail.

Keywords: iPSC, osteoclast, multiple myeloma

5:45 PM – 6:45 PM

411

PLASTICITY OF MACROPHAGES FROM HUMAN INDUCED PLURIPOTENT STEM CELLS FACILITATES THEIR USE IN DRUG-SCREENING AND-SAFETY BIOASSAYS

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Over the last decade, tremendous progress has been made in harnessing the immune system to fight a variety of diseases, introducing immunotherapies as innovative and highly promising medications. Among immune cells, macrophages are a key cell type that acts as important pro- and anti-inflammatory mediator in the onset and progression of various diseases, including fibrosis, cancer or autoimmunity. The availability of a reliable supply of macrophages is therefore a crucial point for the development of suitable disease modelling systems and new cell therapeutic approaches. We developed a GMP-compatible platform, that allows the production of human induced pluripotent stem cell (hiPSC)-derived macrophages (hiPSC-Mac) at different scales ranging from tissue culture plates to intermediate scale or industry compatible bioreactors. The cells exhibited convenient phenotypic properties, classical phagocytotic functionality and showed a transcriptional profile like blood-derived macrophages, though with a more primitive fingerprint. Given the importance of macrophages in various diseases, iPSC-Mac demonstrate a flexible adaptation into several

immunologic states in vivo and in vitro, enabling studies to investigate their impact on numerous disease-relevant processes. Of note, human hiPSC-Mac adopt towards an alveolar macrophage-like phenotype post intra-pulmonary transfer, demonstrating the suitability for cell-therapy studies. Using different cytokines, the hiPSC-Mac can be modulated to pro- or anti-inflammatory phenotypes, highlighting their immunogenic plasticity. IFNy-treated hiPSC-Mac demonstrated an enhanced inflammatory response after lipopolysaccharide (LPS) stimulation, whereas IL-4-primed iPSC-Macs maintained their stage of activation profile post inflammatory trigger. To further explore novel bioassays to evaluate drug safety or efficacy of novel immunomodulatory drug candidates, we have proven the use of these cells in regulatory approved assays and confirmed a suppressive effect of Dexamethasone in LPS-treated hiPSC-Mac. In summary, we present a scalable and efficient method to generate standardized human macrophages from hiPSC that can be used as therapeutic compound itself or for screening, identification or validation of new immunotherapeutic strategies.

Keywords: hiPSC, macrophages, polarization

5:45 PM – 6:45 PM

413

A HIGH THROUGHPUT AUTOMATED PLATFORM FOR DRUG SENSITIVITY SCREENING TO OPTIMIZE THERAPY IN RELAPSE ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is a cancer defined by an accumulation of immature and non-functional myeloid progenitors. The largest problem facing successful clinical management is the inability to decipher which combination of drugs will best treat a patient resulting in poor treatment responses and unacceptably low 5-year survival rates of < 20%. Genetic characterization only helps guide clinicians with use of targeted therapies such as FLT3 inhibitors and provides no information about chemotherapies or drug combinations that will be effective in the patient. We hypothesize that phenotypic ex vivo drug responses on primary tumour cells will provide a more robust approach for identifying effective clinical combinations in a concept called drug sensitivity screening (DSS). AML forms as a cellular hierarchy where the bulk of the tumour is sustained through the rare self-renewing leukemic stem and progenitor cells (LSCs & LPCs). LPCs are detected in >80% of patients using the in vitro colony forming unit (CFU) assay. Each CFU that forms originate from a single cell (i.e. clonal start) and harbor patient's leukemic mutations allowing us to define them as LPCs. We have previously demonstrated that LPC presence is a powerful predictor of overall survival indicating the powerful value the LPC assay holds as a tool for DSS. To date this assay has been limited in utility due to low throughput constrained by laborious protocols. We have overcome the limitations in handling viscous methylcellulose, drug incubation, and LPC quantification to develop a high-throughput LPC assay (HT-LPC) at 96 well density using fluorescent imaging and automated liquid handling allowing robust dose response profiling of drugs and combinations. Initial testing demonstrates it can retrospectively predict which patients would achieve remission or be refractory to AraC chemotherapy with 90% accuracy. Furthermore, resistance and sensitivity to targeted therapies and synergistic combinations can be identified. In two real-time studies, our assay was able to provide clinically actionable DSS data to clinicians within 8 days of diagnosis and provided more accurate results than genetic characterization. Based on these results we believe the HT-LPC platform can be utilized to immediately improve patient management in relapsed AML.

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Keywords: personalized medicine, leukemia, functional assay

5:45 PM – 6:45 PM

TOPIC: KIDNEY

415

AN HIPSC REPORTER PANEL FOR ORGAN-SPECIFIC CHEMICAL SAFETY EVALUATION

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The assessment of chemical toxicity has historically relied on animal studies and the use of human cell lines, both of which have limitations in accurately predicting adversities. Thus, there is a need for advanced in vitro testing platforms to effectively assess chemical-induced toxicity. Human-induced pluripotent stem cells (hiPSCs) provide a valuable resource for studying chemical-induced toxicities while maintaining a consistent genetic background. Here, we aimed to develop an advanced in vitro test system for chemical safety assessment using human induced pluripotent stem cells. Using CRISPR/Cas9-assisted protein tagging a panel of hiPSC reporter lines was generated for inflammatory signaling, DNA damage response, ER stress response, and oxidative stress response. Through endogenous tagging with enhanced green fluorescent protein, we monitored the activation of these pathways in hiPSCs differentiated into the major target organs of chemical toxicity, namely: hepatocyte-like cells, cardiomyocytes, and renal proximal tubule-like cells. Immunofluorescence staining confirmed the successful differentiation and maturity of the three cell types: CYP3A4 in hepatocyte-like cells, megalin and ZO-3 in proximal tubule-like cells, and troponin in cardiomyocytes. Upon exposure to reference compounds, our hiPSC reporter lines exhibited dose-dependent and stress-specific activation, detectable 24 hours later via confocal imaging. The different cell types exhibited varying sensitivities to chemical exposure. Since hiPSCs can be differentiated into various cell types and multicellular organoids, our iPSC-based reporter system can be used for disease modeling and drug screening in a wider context. This versatile system can help us identify early and late stress signals associated with many acute and chronic conditions. Additionally, our platform offers a valuable tool for unraveling the mechanisms underlying carcinogenesis and identifying novel causal factors contributing to poorly understood cancers.

Funding Source: This project receives funding from the European Union's Horizon 2020 project RISK-HUNT3R (Grant Agreement No. 964537) and the Horizon Europe project DISCERN (Grant Agreement No 101096888).

Keywords: induced pluripotent stem cells reporters, in vitro test systems for risk assessment, proximal tubule-like cells

5:45 PM – 6:45 PM

417

REVOLUTIONIZING RENAL RESEARCH: MICROFLUIDIC KIDNEY TUBULOIDS-ON-A-CHIP FOR IMPROVED DRUG TRANSLATION

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Current 2D in vitro models of renal epithelium lack the key features of the in vivo setting, such as tubular structure and perfusion, resulting in low translatability to human situations and failure in clinical translation. Microfluidic techniques are becoming increasingly recognized as a valuable tool for adding physiologically relevant cues to traditional cell cultures. These cues include long-term gradient stability and continuous perfusion. Microfluidic technology also allows for the patterning of cell layers as stratified co-cultures that are free from artificial membranes, enabling the capture of complex tissue architectures found in vivo. Here, we introduce the OrganoPlate microfluidic platform, which can accommodate up to 64 independent microfluidic chips in a microtiter plate format, allowing the growth of 64 independent kidney tubuloid-derived barrier tissues in the form of perfused tubules. These renal tubules can be formed in just four days of culture in the device showing rapid and reproducible cell polarization, tight junction formation and proper expression of renal markers. When integrated into an OrganoPlate system, kidney tubuloids form leak-tight, perfusable tubes with stable Trans Epithelial Electrical Resistance (TEER), and are suitable for high-throughput screening of compound effects through assessment of barrier integrity by use of OrganoTEER and by real-time imaging of transport. OrganoPlate grown kidney tubes treated with Pgp inhibitor Verapamil show significant reduction of Rhodamine123 transport through kidney tubule barrier which confirms show stable activity of Pgp transporter and usability of the model in studying renal drug clearance. Our results demonstrate the suitability of our in vitro microfluidic kidney tubuloid-on-a-chip model in mimicking key physiological aspects of the kidney and offer new ways for studying organ physiology and renal disease mechanisms and drug toxicity.

Keywords: 3D disease modeling, kidney tubuloids, renal disease mechanisms

5:45 PM – 6:45 PM

419

KIDNEY ORGANOID TRANSPLANTATION PROMOTES ENDOTHELIAL CELL SPECIFICATION TOWARD A FETAL KIDNEY ARTERIAL PHENOTYPE

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Kidney organoids (KORs) derived from human induced pluripotent stem cells hold promise for regenerative medicine and disease modeling. However, their applicability is hampered by the lack of a proper vasculature. During culture in vitro, progressive loss of endothelial cells (ECs) occurs, whereas in vivo transplantation promotes EC development,

enables chimeric vascularization and blood perfusion. This study aimed to characterize the molecular effects of KOR transplantation on endogenous ECs. KORs were transplanted into chicken embryos for 1 or 8 days, or cultured in vitro for the same time period. ScRNA-seq of FACS-sorted human CD31+ KOR ECs revealed that transplantation induced a response to hypoxia, associated with a metabolic transcriptome switch from oxidative phosphorylation to glycolysis in the ECs at day 1. After 8 days, ECs from transplanted KORs upregulated antigen presentation, angiogenesis, and blood coagulation/circulation genesets, indicating response to blood perfusion. Consistent with angiogenesis, EC proliferation was increased in this condition, as confirmed by Ki67 staining. In addition, ECs underwent a major vein-to-arterial phenotypic transition upon transplantation, with the emergence of arterial ECs. These arterial ECs, characterized by laminar shear stress response and Notch signaling, showed a similar transcriptome as human fetal kidney arterial/afferent arteriolar ECs. Transplantation-induced EC reprogramming involved proangiogenic and arteriogenic SOX7 transcription factor upregulation and regulon enrichment. In conclusion, KOR transplantation led to the development of arterial ECs resembling human fetal kidney arterial ECs, likely via SOX7 upregulation and blood flow exposure. These findings provide insights for the development of KOR vascularization strategies.

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Keywords: kidney organoid, vascularization, scRNAseq

5:45 PM – 6:45 PM

421

CORRECTION OF AN APDKD POINT MUTATION USING ADENINE BASE EDITORS IN HIPSCS AND KIDNEY VESSEL ORGANOIDS

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Autosomal dominant polycystic kidney disease (ADPKD) is a dominant genetic disorder caused primarily by mutations in the PKD1 gene, resulting in the formation of numerous cysts and eventual kidney failure. However, there are currently no gene therapy studies aimed at correcting PKD1 gene mutations. In this study, we identified two ADPKD mutation sites on the PKD1 gene, c.1198 (C>T) and c.8311 (G>A), in patients. The correction efficiencies of different adenine base editors (ABEs) were tested using the HEK293T-PKD1 c.1198 (C>T) and HEK293T-PKD1 c.8311 (G>A) reporter cell lines. The results showed that the ABE system can effectively repair the c.8311 (G>A) point mutation, but not the c.1198 (C>T) site, and that ABEmax has higher efficiency and minimal predicted off-target effects. We then generated induced pluripotent stem cells (iPSCsmut/WT) from the peripheral blood mononuclear cells (PBMCs) of the heterozygous patient to develop a disease cell model. Since the iPSCsmut/WT did not show a typical disease phenotype in stem cell status, we subsequently differentiated the iPSCs into kidney organoids in vitro, which expressed renal organ specific marker proteins, such as CDH1 and LTL. The addition of forskolin could stimulate cAMP signaling and lead to cystic expansion of renal epithelial tissue in iPSCmut/WT differentiated kidney organoids, similar to the vesicle phenotype in the kidney of ADPKD patient. However, the kidney organoids differentiated from ABE-corrected iPSCs did not show the cystic phenotype. Herein we tried to use this organoid to test the possibility of AAV-mediated ABE editing as a therapeutic strategy. The dual AAV split-ABEmax system was used to deliver ABEmax into the kidney organoids and the average editing efficiency was approximately 6.56%. Overall, this study lays the foundation for gene therapy targeting ADPKD using the ABE single-base editing tool.

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Keywords: gene editing, hiPSC, ADPKD kidney organoid

5:45 PM – 6:45 PM

423

UTILIZING KIDNEY TUBULOID MODEL TO STUDY COLLECTING DUCT (PATHO)PHYSIOLOGY

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The kidney consists of different segments that are essential in maintaining homeostasis throughout the body, including regulating electrolyte and water balance. The last segment of the kidney tubules is the collecting duct (CD), where finetuning of sodium and water reabsorption occurs. A dysfunction of this process can lead to nephrogenic diabetes insipidus (NDI) that causes the inability to concentrate urine which can lead to severe dehydration. It is known that NDI is caused by dysfunction of the water channel aquaporin 2 (AQP2) or the vasopressin 2 receptor (V2R) in the CD. Despite promising results of pre-clinical experiments, clinical trials often fail due to ineffectiveness or side-effects. Recent developments in organoid research promise to study kidney (patho)physiology with increased translational value compared to conventional in vitro research models. Tubuloids are kidney organoid models that are derived from adult stem cells and consists of epithelial cells from different segments of the kidney tubules. By utilizing these tubuloid models, our lab previously demonstrated functional CD-specific sodium regulation. In this study, we show that tubuloids are capable of physiological CD-specific water transport regulation by AQP2 and V2R. Results show that tubuloids can be differentiated towards the distal tubules, including CD. Further experiments revealed that the endogenously expressed AQP2 and V2R can be regulated shown by significantly increased expression of AQP2. Physiological stimulation of tubuloids resulted in phosphorylation of AQP2, which is an essential aspect of water homeostasis in CD. Furthermore, the upregulation and

phosphorylation of AQP2 resulted in the translocation towards the apical membrane where it increases water permeability. Finally, we performed 3D swelling assays which confirmed that CD tubuloids are indeed capable of increasing water transport and thereby respond to stimuli by swelling. These results show that kidney tubuloids endogenously express relevant markers for CD-specific water transport, and AQP2 signaling can be regulated through physiological stimuli which is measurable in 3D functional assays. These results further confirm that kidney tubuloid models hold great promise to study kidney (patho) physiology including NDI.

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Keywords: kidney, tubuloids, organoids

5:45 PM – 6:45 PM

425

KIDNEY-COLLECTING DUCT ORGANOIDS DERIVED FROM HUMAN PLURIPOTENT CELLS FOR DISEASE MODELLING OF HNF1B-MUTATION-INDUCED KIDNEY DYSFUNCTION

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The development of human collecting ducts from the ureteric bud during fetal development is a finely orchestrated process. Congenital anomalies of the kidney and urinary tract (CAKUT) often result from genetic mutations. Heterozygous (monoallelic) mutations in the HNF1B gene is a common cause without correlation between the type of mutation and patient phenotype. Conventional mouse models of human congenital diseases, while informative, often require bi-allelic mutation and have inherent limitations. A human model that shows promise for studying such developmental disorders of the kidney is pluripotent stem cell-derived organoids. Indeed, we have started to dissect the defects in the nephron caused by HNF1B mutation using such a model). However, this model though enriched for glomeruli and proximal tubules, lacks the collecting duct, known also to be affected in patients. Understanding the mechanistic underpinnings of these mutations in collecting ducts is vital for drug development and therapeutics. In this study, we utilise an alternative organoid model to evaluate the phenotype of gene-edited hESCs carrying a monoallelic HNF1B mutation in exon1, (het knockout) and patient-derived hiPSC with different HNF1B mutations. We create and characterise a collecting duct organoid model for kidney dysfunction induced by HNF1B gene mutation. The hESCs are guided through stages that mimic the development of UB and collecting duct using growth factors and small molecules, resulting in the successful generation of branching epithelia. Immunohistochemistry and gene expression analysis confirmed the presence of principal cells mirroring the in vivo collecting duct composition. Comparative analysis of the non-mutated and HNF1B gene-mutated organoid models provided critical insights into the role of HNF1B gene mutations in renal development and HNF1B pathogenesis. Our aim is to use this model for drug target discovery to facilitate the development of therapies for diseases caused by HNF1B- mutation and related conditions.

5:45 PM – 6:45 PM

427

STEPWISE DEVELOPMENTAL MIMICRY GENERATES PROXIMAL NEPHRON PRECURSORS IN ORGANOIDS

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The kidney maintains blood composition and body fluid homeostasis by reabsorbing essential compounds and excreting waste. Proximal tubule cells, crucial for renal nutrient reabsorption, are highly susceptible to damage, leading to pathologies that often necessitate dialysis and kidney transplants. Despite the use of human pluripotent stem cell-derived kidney organoids as platforms for modeling renal development, disease, and injury, the formation of proximal nephron cells in these 3D structures is inefficient. Here, we describe how to induce the uniform expression of proximal tubule precursors, utilizing developmental benchmarks to accurately model human nephrogenesis. Activating brief Notch signaling steers nascent organoid nephrons toward an early proximal state. Having been programmed to a nascent proximal state, these “proximalized” organoid nephrons proceed to generate proximal precursor cells. Single-cell transcriptomics across the organoid nephron differentiation timeline, comparing control and proximalized types, confirm the ability of transient Notch signaling driving proximal developmental trajectories. Proximalized organoids demonstrate dextran and albumin uptake, indicative of functional maturity akin to in vivo proximal tubules. Moreover, proximalized organoids are highly sensitive to nephrotoxic agents, display injury response, and drive expression of HAVCR1, a key proximal-specific marker of kidney injury. This response confirms the functional relevance of our model and underscores its potential for modeling mechanisms underpinning nephron injury and recovery. These advances strengthen the utility of iPSC-derived kidney organoids as a versatile tool for nephrology research, disease modeling, therapeutic testing, and for understanding human renal physiology.

Keywords: kidney, nephron, organoid

5:45 PM – 6:45 PM

429

A SCALABLE, COMPLEX MODEL OF ALIGNED KIDNEY PROXIMAL NEPHRONS

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With limited treatment options, chronic kidney disease (CKD) is a leading cause of death globally, arising from common conditions such as diabetes, hypertension, drug exposure, and genetic disorders that can damage the kidneys' critical blood filtration units (nephrons). While recent advances in generating kidney organoids from human pluripotent stem cells (hPSCs) have attracted attention as a disease model and a potential approach to supplement kidney function in CKD patients, applications remain challenged by the immaturity and spatial disorganization of the hPSC-derived tissue. This is particularly problematic for the proximal tubule (PT) segment of the nephrons. PT function is critical for maintaining body homeostasis, as well as hormone, vitamin, and energy production, making it a key target of disease research and treatment approaches. We recently refined hPSC differentiation to kidney progenitors by more accurately recapitulating the temporal signaling events occurring during embryonic kidney development. This resulted in kidney tissue with enhanced PT maturity and function, as well as nephron spatial organization that was influenced by localized WNT inhibition. Capitalising on this enhanced model, here we use CRISPR-tagged fluorescent reporter lines combined with 3D bioprinting, novel 3D tissue culture platforms, high-content confocal imaging, and transcriptional analyses to further regulate the maturation and spatial organization of proximal nephrons within bioengineered kidney tissue. Our results provide a comprehensive insight into the complex subdivision of engineered proximal nephrons into PT cell subtypes (S1/S2 – S3) and indicate improved metabolic identity when tissues are exposed to PT-relevant metabolic substrates. Additionally, the transferability of this model to organ-on-a-chip platforms was complemented by its amenability to scale-up approaches, both through 3D cellular bioprinting and cryopreservation of PT progenitors with retained differentiation capacity. Collectively, these developments have significantly refined current organoid technologies, improving our control of engineered tissue growth and organization, while enhancing the translational potential of stem cell-derived kidney proximal nephrons.

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Keywords: kidney organoid, proximal tubule, bioengineering

5:45 PM – 6:45 PM

TOPIC: LIVER

431

IPSC-DERIVED HEPATOCYTES AS A NOVEL TOOL FOR PROGRESSIVE FAMILIAL INTRAHEPATIC CHOLESTASIS 2 (PFIC2) MODELING AND DRUG SCREENING

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Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of autosomal recessive liver diseases, characterized by mutations in genes involved in hepatocellular bile acid secretion. Amongst these, PFIC2, caused by mutations in the ABCB11 gene, represents half of the total PFIC cases, and patients with PFIC2 present a range of symptoms, including liver failure, cirrhosis, and hepatocellular carcinoma. Despite the importance of the disease, there are currently no licensed treatments, mainly due to the lack of appropriate pre-clinical models in drug discovery. We developed a human hepatocyte model recapitulating the PFIC2 phenotype in-a-dish. In healthy induced pluripotent stem cells (iPSCs), the ABCB11 mutation D482G was introduced by CRISPR/Cas9 gene editing. Upon iPSC differentiation, hepatocytes (Opti-HEP) were cultured in transwell culture system to induce Opti-HEP polarization. Genotype confirmation was performed by Sanger sequencing, whilst iPSC pluripotency and differentiation of iPSCs towards Opti-HEP by qPCR and immunocytochemistry. ABCB11 expression and protein localization were determined by western blotting and immunocytochemistry, and alterations in bile acid transport by liquid chromatography-mass spectrometry. Both wild-type and ABCB11-mutant iPSCs were successfully differentiated towards Opti-HEP as evident by comparable mRNA levels of the hepatocyte maturity markers albumin, alpha-1-antitrypsin, and hepatocyte nuclear factor 4A to those seen in primary human hepatocytes. mRNA and protein levels of ABCB11 were lower in ABCB11-mutant Opti-HEP accompanied by disrupted ABCB11 membrane localization compared to wild type. Consistent with these data, bile acid transport was impaired in ABCB11-mutant Opti-HEP as shown by significantly lower secretion levels of taurocholic acid in the apical side of the transwell culture compared to wild-type. In conclusion, we have developed an iPSC-derived hepatocyte model that recapitulates human PFIC2 phenotype in vitro. This technology provides a framework for the development of liver disease-focused models and highlights the superiority of iPSCs as an effective platform for disease modeling and hit-lead drug screening.

Keywords: liver, hepatocytes, in vitro

5:45 PM – 6:45 PM

433

MOLECULAR MECHANISMS DRIVING CHOLANGIOCYTE ACTIVATION DURING PROGRESSION OF CHRONIC LIVER DISEASE AND LIVER CANCER

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Liver disease claims over two million lives annually, counting 4% of global mortality. Central to the pathobiology of chronic liver disease is Ductular Reaction (DR), a dynamic process consisting in the activation of intra-hepatic cholangiocytes following injury. Despite its association with liver fibrosis, liver cancer, and liver regeneration, the precise nature of DR—whether it represents a reparative response or exacerbates disease—remains enigmatic. The dearth of knowledge is especially notable in human, as most insights have been derived from animal models with limited translatability to chronic disease. Here, we describe new mechanisms driving DR in humans which elucidate in part the relationship between cholangiocytes and fibrosis in the context of disease progression and cancer development. To study DR in humans, we first generated the largest single-cell map of the human liver during Fatty Liver Disease progression, unraveling new markers for DR. Notably, we found that several receptors for ECM proteins are up-regulated by cholangiocytes in end-stage cirrhosis which prompted us to hypothesize that the interaction of cholangiocytes with ECM could be a pivotal mechanism for their activation. To test this hypothesis, we derived patient-derived cholangiocyte organoids and then grew the resulting cells in defined hydrogels to modulate the ECM composition. Using this approach, we were able to replicate DR in vitro and found that specific ECM proteins are necessary and sufficient for cholangiocyte activation. We then examined the downstream mechanisms and identified key signaling pathways as pivotal mediators. In addition, inhibition of ECM receptors blocks cholangiocyte growth and reverses DR in vitro thereby confirming the functional importance of these interactions. Finally, we found that a specific ECM-rich matrix can accelerate the growth of liver cancer organoids reinforcing the possibility that activation of cholangiocytes through DR could contribute to tumorigenesis. Our results validate that ECM modification during fibrosis and cancer serves as a permissive component for cholangiocyte growth. With these findings we shed light for the first time on the biology of DR in humans, providing potential avenues for therapeutic interventions.

Keywords: cholangiocytes, extracellular-matrix, liver disease

5:45 PM – 6:45 PM

435

CONSTRUCTION OF A LIVER TISSUE MODEL FOCUSING ON LIVER SINUDOIDAL ENDOTHELIAL CELLS

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Liver-on-a-chip is a system that enables reproduction of some liver functions by seeding cell types that constitute liver tissue on a microfluidic device to allow dynamic culture and reproduction of the microenvironment. The use of this system is expected to accelerate drug discovery by making drug metabolism and toxicity testing more predictive than conventional testing methods performed on single cells. Although several liver-on-a-chip systems have been developed in recent years, there are few systems that uses liver sinusoidal endothelial cells (LSECs), which are the first cell types to encounter many xenobiotics, are used. This is because they are sensitive to ischemia, and it is difficult to construct culture systems that guarantee their functions. Therefore, we attempted to differentiate human iPS cells into LSECs and hepatocytes. Using these cells, we aimed to construct a sinusoidal liver tissue model that recapitulates the sinusoids. In endothelial cells differentiated from human iPS cells, LSEC-specific gene expression and function, which were not observed in HUVECs, were identified. Therefore, we named these differentiated endothelial cells LSEChiPSC. Hepatocytes differentiated from human iPS cells were designated as HepatocytehiPSC, since hepatocyte-specific genes were identified. When LSEChiPSC and HepatocytehiPSC were co-cultured in two layers using cell culture inserts, specific gene expression of both cells was maintained for several days. To clarify the importance of the microenvironment, we used a membrane-based two-layered microfluidic device that can mimic the structure of liver sinusoidal tissue. Endothelial cells were seeded on the membrane and hepatocytes were seeded on the backside of the membrane and the bottom of the lower channel to reproduce the sinusoids in the upper channel and bile canaliculus in the lower channel. LSEChiSPC cultured in a flow environment on this microfluidic device showed different gene expression trends than in a static environment. This liver sinusoidal tissue model that mimics the hepatic sinusoidal structure is expected to have applications in drug metabolism research and antiviral drug screening.

Keywords: microfluidic device, hiPSC-derived cells, liver sinusoidal endothelial cells

5:45 PM – 6:45 PM

437

IDENTIFICATION OF NEUTROPHILIC AND FIBROGENIC INJURY PATHWAYS IN LIVER ORGANOIDS WITH INNATE MYELOID IMMUNITY MADE FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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The human liver primarily consists of hepatocytes, but nearly all chronic liver diseases involve myeloid innate immune cells, especially macrophages and neutrophil granulocytes, along with non-parenchymal cells (NPCs) – relevant in fibrogenesis – namely hepatic stellate cells (HSCs), biliary epithelial cells (BECs), and endothelial cells (ECs). Current human organoids lack a myeloid immune system, particularly neutrophils, which are the most abundant leukocyte. Here, we developed an integrated liver organoid system from human induced pluripotent stem cells, inclusive of syngeneic neutrophils, macrophages, hepatocytes, and NPCs (MyHLOs), as a platform to unveil innate immune-fibrogenic pathways. We co-developed endoderm and hemogenic mesoderm and observed the organization of AFP+ ALB+ ECAD+ hepatocytes, a self-organized reticular CD31+ EC system, CK19+ CK7+ EPCAM+ luminal BECs, PDGFRA+ aSMA+ HSCs, CD68+ IBA-1+ macrophages, and neutrophil-myeloid progenitors. The addition of IL34 and GM-CSF – modeling tissue-resident and bone marrow-derived signaling – completed macrophage polarization and yielded CD45+CD14-CD16+CD66b MPO+ segmented, reactive oxygen species-releasing bona fide neutrophil granulocytes. Under lipotoxic milieu conditions, MyHLOs exhibited a 5-fold decrease in HNF4a expression - indicating hepatocyte injury - and a 1.3-fold neutrophil cell expansion, along with a 2-fold increase in IL8 secretion. Blocking IL8 signaling with Reparixin mitigated HNF4a loss to 1.8-fold, suggesting that IL8-mediated neutrophil recruitment contributes to inflammatory hepatocyte injury. MyHLO-encapsulating fibrogenesis was observed after inflammatory cytokine provocation testing, further intensified by Notch-agonism via DLL1. In contrast, Notch-antagonism with DAPT prevented fibrogenesis. Inferring intercellular pathways from single-cell transcriptomic analyses supported Notch signaling across hepatocytes-to-NPCs, primarily HSCs, and ECs, in addition to autocrine NPC-signaling. Thus, MyHLOs are an innate immune organoid system recapitulating cell types relevant in most chronic liver diseases. Our findings highlight Notch as a key regulator in fibrogenesis and the druggable IL8 pathway for recruiting hepatocyte-injurious neutrophils.

Keywords: liver organoids, innate immune system, non parenchymal cells

5:45 PM – 6:45 PM

439

THE CRUCIAL INTERPLAY BETWEEN PARENCHYMAL AND MESENCHYMAL CELLS IN LIVER DISEASE

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The complex interplay between parenchymal and mesenchymal cell populations within the liver microenvironment exerts significant influence over liver homeostasis, regenerative capacities, and immune modulation. Dysregulations in these intercellular communications have been implicated across a spectrum of liver pathologies, encompassing viral hepatitis, metabolic fatty liver disease, fibrosis, cirrhosis, and hepatocellular carcinoma. In pursuit of deciphering these intricate communication networks, we have developed a multicellular liver culture platform incorporating human induced pluripotent stem cells (hiPSCs)-derived parenchymal and mesenchymal cells, such as hepatocytes, hepatic stellate cells, and macrophages. Furthermore, our innovative configuration allows for the integration of additional immune-related cell types, including neutrophils, endothelial cells, and natural killer cells. Leveraging this adaptable system, we have successfully modeled nonalcoholic fatty liver disease (NAFLD), alcohol-associated liver disease (ALD), and viral hepatitis induced by hepatitis B and hepatitis C viruses (HBV and HCV). Our investigations into NAFLD, ALD, and viral hepatitis have elucidated the significant contribution of macrophages, via soluble factors, to disease development and progression in NAFLD and ALD, as well as their roles in the interaction between HBV and HCV in the context of coinfection and HBV reactivation following HCV cure. This platform therefore offers a versatile tool for elucidating disease mechanisms and developing targeted therapeutic interventions for liver disease.

Keywords: liver disease, multicellular liver model, fatty liver disease and viral hepatitis

5:45 PM – 6:45 PM

441

EXOSOMES FROM HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED MESENCHYMAL STEM CELLS ALLEVIATE LIVER FIBROSIS IN A MOUSE MODEL OF WILSON'S DISEASE

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Mesenchymal Stem Cells (MSCs) are increasingly used as cell-based therapies in liver diseases. Previous works have demonstrated that MSCs administration could restore the hepatic function and ameliorate liver fibrosis by immunomodulation or via paracrine effects. And many studies reported that compare to MSC transplantation, exosome alone may exert similar therapeutic effects, while the potential risks are much lower. As such, in this study, we aimed to investigate the in vivo therapeutic potential of exosomes isolated from human induced pluripotent stem cell derived -MSCs (hiPSC-MSCs-Ex) in a Wilson's Disease mouse model with hepatic dysfunctions. In brief, Wilson's Disease is a rare hereditary disorder due to ATP7B gene mutation, which causes hepatic copper accumulation and progressive liver fibrosis. This was accompanied by injecting hiPSC-MSCs-Ex to the ATP7B Knockout mice every two weeks for 8 weeks. Our results showed that DiR-labeled hiPSC-MSCs-Ex was successfully targeted to the injured liver and maintained in the liver after systematic administration. Functionally, compared to the PBS-treated control, hiPSC-MSCs-Ex-treated mice showed reduced liver fibrosis as indicated by a decrease in collagen accumulation, enhanced liver functionality, and alleviation of inflammation. The gene expression of major contributors to fibrosis (Col1α, TIMP-1, and Fn-1) significantly decreased in the liver of hiPSC-MSCs-Ex treated mice. And the histopathological analysis revealed significant reduced collagen area and Ishak fibrosis score. The serum level of alanine aminotransferase (ALT) also significantly reduced in the hiPSC-MSCs-Ex-treated groups. Furthermore, increased secretion of anti-inflammatory cytokines (IL-10) and decreased pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) were also detected in the hiPSC-MSCs-Ex treatment group. These results demonstrated that hiPSC-MSCs-Ex could reduce inflammation and ameliorate hepatic fibrosis in a liver disease mouse model, which may provide an allogeneic cell-free MSC therapy for chronic liver fibrosis.

Keywords: exosomes, mesenchymal stem cells, liver fibrosis

5:45 PM – 6:45 PM

443

LIVER-ON-CHIP PLATFORM REPRODUCING METABOLIC ZONATION FOR STUDYING RECRUITMENT AND DIFFERENTIATION OF CIRCULATING MONOCYTES

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The spatial heterogeneity of liver cells, known as liver metabolic zonation, allows hepatocytes to execute diverse metabolic functions, crucial for liver homeostasis. Moreover, metabolic zonation is an important factor of liver disease development. However, in current protocols, the distinctive functions, metabolic traits, and phenotypes specific to each zone swiftly diminish during in vitro culture of primary human hepatocytes (PHH) and are hardly present in iPSC-derived hepatocytes like cells generated by published protocols. This represents significant limitations for drug testing and the use of in vitro human liver disease modelling. We developed a new scalable protocol allowed generating hiPSC-derived liver organoids with zone 3 (Z3-HLO)- and zone 1 (Z1-HLO)- specific phenotype and functionalities. Z1-HLO were characterized by albumin secretion and urea synthesis at the level of PHHs, as well as gluconeogenesis. Z3-HLO exhibited phase I metabolism (approaching the activity of PHHs), higher glucose uptake, glycogen accumulation and showed decreased expression of E-cadherin, when compared to control and Z1-HLO. Z3-HLO and Z3-HLO had a different oxygen consumption rate and β-oxidation. Z3-HLO were shown to be more susceptible to the hepatoxic effect of treatment with acetaminophen (APAP). Next, using a recently developed by our group pump-less recirculate on Organ-on-a-Chip (rOoC) platform, healthy and diseased zonated HLOs were co-cultured with human primary CD14+ monocytes. The functional interaction between zonated HLOs and circulating monocytes was analyzed in control and disease-mimicking conditions in presence and absence of endothelial barrier. We observed an increased migration of monocytes toward APAP-treated HLOs compared to untreated HLOs. The multiplex analysis of cytokines, in situ immunophenotyping and scRNAseq enabled a thorough characterization of deployment and disease-dependent alterations of monocytes and liver cells "on chip". In summary, presented here zonated HLO with post-natal zone-specific metabolic activity (as benchmarked to PHHs) in combination with immune component on a scalable rOoC platform can improve in vitro disease modeling, and offer a new tool for predictive toxicology studies.

Keywords: liver-on-chip, liver macrophages, liver metabolic zonation

5:45 PM – 6:45 PM

445

ESTABLISHMENT OF A LARGE-SCALE ORGANOIDS BIOBANK FROM LIVER CANCER PATIENTS

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Liver cancer ranks as the sixth most prevalent cancer and the fourth leading cause of global cancer-related deaths. There is currently no cure against end-stage liver disease and new treatments are urgently needed for the most predominant types of liver cancer: hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), and mixed HCC/CCA. To address this major challenge, our group at the Berlin Institute of Health is creating a unique biobank of liver tumoroids generated from cancer patients from the Charité – Universitätsmedizin Berlin, one of Europe's largest hospitals. The biobank is currently built by establishing "paired organoids" from each patient's tumoral and adjacent non-tumoral region, and by collecting the liver tissue in both snap-frozen and fixed samples. The resulting organoid lines are characterized using genotyping/karyotyping, RT-qPCR, and immunofluorescence and compared to the original tumors. For that, we aim to conduct single-nuclei RNA sequencing studies to further define the expression profiles of organoid cells with those of their corresponding parental tissues, providing an in-depth look at the molecular patterns driving tumorigenesis. This approach will underscore similarities and divergences between the cellular composition of organoids grown in vitro and the heterogeneity of their tumoral counterpart. We are particularly interested in defining the mechanisms by which cellular plasticity is established and maintained in liver cancer cells. Our liver tumoroids collection provides a unique platform for studying liver cancer and discovering new therapeutic agents. This living biobank not only represents a methodological breakthrough for tumor modeling in vitro but also provides a new resource to understand the molecular mechanisms underlying liver cancer development and progression.

Keywords: patient-derived-organoids, liver cancer, biobank

5:45 PM – 6:45 PM

447

GENERATION OF IPS-DERIVED HUMAN LIVER AND INTESTINAL ORGANOIDS WITHIN DEFINED EXTRACELLULAR MATRICES

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Human organoids are three dimensional structures that can recapitulate native cell architecture and functionality and have immense potential for evaluating new therapies for conditions such as Type 2 Diabetes (T2D). Liver and intestinal tissue, which are affected by T2D, can be generated in vitro by delivering a series of bioactive signaling molecules to guide the differentiation of induced pluripotent stem cells (iPSCs) in the presence of Matrigel, a gelatinous protein extract derived from mouse Engelbreth-Holm-Swarm tumor basement membrane. This process leads to the generation of iPSC-derived human liver organoids (HLO) and human intestinal organoids (HIO). Although Matrigel supports formation and maturation of both organoid types, it has several limitations. Matrigel's ill-defined composition of various extracellular (ECM) proteins and growth factors makes it difficult to determine what

matrix biosignaling cues are required to specifically form HLO and HIO. Furthermore, organoid generation relies on de novo iPSC differentiation, which can be manually intensive and costly. These limitations affect the implementation of organoids for wider use. In this study, we assessed the role that ECM biochemical signals have on the formation of both iPSC-derived liver and intestinal organoids. To further enable organoid generation, we have utilized cryopreserved endoderm intermediates that can directly be used at the start of the three-dimensional culture stage of organoid differentiation. By applying this strategy, we have shown that 3D ECMs containing only laminin/entactin or collagen type IV can be used to generate HLO with similar morphology to those generated in Matrigel. Furthermore, by controlling the concentration of these signals, we can control HLO yield and functionality. These matrices can also be used to generate HIO with comparable morphology and yield to those differentiated within Matrigel. We have also found that ECMs with no biochemical signals can also be used to form HIO with non-cyst like morphology. Together, this information can be used to guide the development of defined biomaterials to reliably generate organoids for high throughput screening applications.

Keywords: extracellular matrix, organoid, intestine

5:45 PM – 6:45 PM

449

NEEDLE BIOPSY-DERIVED LIVER ORGANOIDS ENABLE ALCOHOL-ASSOCIATED LIVER DISEASE MODELLING AND DRUG RESPONSE

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Alcohol-associated liver disease (ALD) is a major cause of liver disease worldwide. Despite its prevalence, the available therapeutic options are limited, and molecular mechanisms underlying the disease remain poorly defined. Organoids have emerged as a powerful human-based in vitro tool in the biomedical field. However, patient-derived liver organoids present a biliary phenotype and its generation still requires hepatic resections, thus limiting their use for ALD modelling. Here, we develop a method for needle biopsy-derived liver organoids (b-Orgs) generation to model ALD and drug response. b-Orgs were generated with an efficiency of 80% from both early and advanced stages of ALD. Moreover, b-Orgs showed an enriched liver-like phenotype and enhanced hepatocyte features when compared to the widely established standard organoids from liver resections, as assessed by immunofluorescence, functional studies, and bulk transcriptomics. Single cell RNA-sequencing revealed a heterogeneous composition comprising the different hepatobiliary populations found in ALD patients (i.e. cell populations with biliary, progenitor and hepatocyte features). Moreover, b-Orgs preserved disease-stage features at transcriptomic level, as b-Orgs derived from advanced patients showed increased genes related to epithelial-mesenchymal transition (EMT), angiogenesis or inflammation. Finally, b-Orgs recapitulated ALD features when stimulated with an ALD medium containing ethanol and pro-inflammatory mediators, showing upregulated ROS production, lipid accumulation, inflammation and decreased proliferation. Furthermore, b-Orgs responded to prednisolone, showing a reversion in oxidative stress production and cell cycle arrest. Overall, we have successfully developed a new methodology allowing the generation of patient-derived organoids from different stages of ALD. B-Orgs exhibit enhanced hepatocyte features and a cellular heterogeneity mirroring the epithelial composition of the liver tissue in ALD. Importantly, b-Orgs can mimic ALD features and respond to drugs, thus underscoring their potential as a personalized human-based in vitro tool to study the mechanisms of ALD and explore novel treatments.

Keywords: organoids, ALD, biopsy

5:45 PM – 6:45 PM

451

MODELLING SEX DIFFERENCES IN METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS USING STEM CELL DERIVED-LIVER TISSUE

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Metabolic dysfunction-associated steatotic liver disease (MASLD) affects more than a third of the world's population. MASLD encompasses a range of diseases characterized by the excessive fat accumulation within the liver (steatosis) that can develop into metabolic dysfunction-associated steatohepatitis (MASH). There are currently no specific therapeutics to treat MASLD/MASH. MASLD development is influenced by different factors such as obesity, insulin resistance and metabolic age. Sex hormones, notably estrogens and androgens, regulate liver function and disease development. Sex and age dependent hormone balance reduces the risk of developing insulin resistance, hyperlipidemia and cardiovascular disease. However, the mechanisms of the sex steroids in MASH remain under-explored. To study this we have developed male and female 3D liver models using human pluripotent stem cells (PSCs). Self-assembled liver spheres were comprised of hepatocytes, endothelial and stellate cells derived from PSCs. We used those to assess the sphere resemblance of that in human liver. We observed the time dependent increase in human serum albumin levels and the activity of cytochrome P450 enzymes, and decrease in alpha fetoprotein secretion, demonstrating the maturation of spheres. Then, we investigated our 3D model capacity to recapitulate the macrovesicular steatosis in vitro. After supplying the liver spheres with the high energy diet, we observed a significant increase in the lipid droplet size within the fat loaded liver spheres compared to the control. We further demonstrated the significant increase in intracellular and extracellular 3-hydroxybutyrate and pyruvate levels compared to the control, contributing to a dysregulated TCA cycle and saturation of β-oxidation pathway. Lastly, we assessed the effect of sex hormone signalling in disease progression using transcriptomics. The results of those studies were compared to a human database (SteatoSITE) covering the full spectrum of human liver fibrosis. Sex specific hormone addition showed a protective role against the development of cirrhosis 'in the dish' versus control spheres. We have since deconvoluted the gene regulatory networks and cell type dependent interactions important in MASH development and regression and those will be discussed at the meeting.

Funding Source: MRC Precision Medicine iCASE

Keywords: sex hormones, MASLD, MASH, metabolism, 3D PSC-derived liver model

5:45 PM – 6:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

453

SCALABLE XENO-FREE BIOREACTOR PRODUCTION OF MSC-DERIVED 3D MICROTISSUES

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The increasing demand for 3D in vitro models that replicate human tissues for studying physiological mechanisms, disease progression, and drug target validation, necessitates advanced manufacturing approaches. Although human mesenchymal stem/stromal cells (MSCs) offer a promising solution due to their ethical advantages and multipotency, the automated, standardized, and reproducible creation of MSC-derived 3D tissues that mimics human anatomy and function remains a significant obstacle. Therefore, we present a scalable, xeno-free 3D differentiation process tailored for generating microtissues derived from MSCs, specifically adipocytes, chondrocytes, and osteoblasts. Our approach involved utilizing micro-structured culture plates for high-throughput spheroid generation and establishing robust differentiation protocols for each lineage within physioxic conditions and xeno-free medium. Additionally, we encapsulated MSCs in alginate liquid-core shell capsules (LCSCs), inducing the formation of multiple spheroids within each capsule. These loaded LCSCs were cultured in a rotating wall vessel (RWV) bioreactor where differentiation was initiated under conditions established during preliminary static experiments. Differentiation was confirmed by histological sections, immunohistochemistry, and secretome analysis. Viability and proliferation of MSCs in the RWV was confirmed through viability assays, while ongoing efforts focus on evaluating the differentiation process using immunohistochemistry and secretome analysis. Our study introduces a robust, physiologic, xeno-free 3D differentiation protocol within physioxic conditions, seamlessly transferable to a scalable bioreactor. The innovative use of LCSCs for microtissue production allows for the generation of miniaturized adipogenic, chondrogenic, or osteogenic tissues derived from MSCs. This integrated system holds promise for high-throughput production of patient-specific microtissues, serving as tissue building blocks for 3D bioprinting or tissue models for drug discovery.

Funding Source: This project was supported by the Doctoral School "Biomaterials and Biointerfaces" of the BOKU Vienna and the program "zukunft.niedersachsen" by the Lower Saxony Ministry for Science and Culture.

Keywords: mesenchymal stem/stromal cells, microtissues, hypoxia

5:45 PM – 6:45 PM

455

PROTEOMIC ANALYSIS OF MESENCHYMAL STEM CELLS AND THEIR EXTRACELLULAR VESICLES FROM FERTILE AND INFERTILE WOMEN MENSTRUAL BLOOD

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Unexplained or idiopathic infertility accounts for 30 percent of couples with fertility problems worldwide and the diagnosis is confirmed if female is not able to get pregnant after at least 12 cycles of unprotected intercourse. But the pathogenesis is still poorly understood. In our study we were looking for differences in proteome of menstrual blood mesenchymal stem cells (MenSCs) and their extracellular vesicles (EVs) from fertile and infertile women menstrual blood and possible biomarkers of unexplained infertility. MenSCs were isolated from menstrual blood of 8 fertile female volunteers and 8 patients with unexplained infertility. Mesenchymal and other surface markers of MenSCs were detected using flow cytometry. Cell EVs were isolated using ultracentrifugation with density gradient. Cytosolic EV protein TSG101 and lipoprotein markers were analysed by Western blot. Proteins in both cells and their EVs were detected using mass spectrometry. Surface biomarkers CD9, CD10, CD13, CD14, CD44, CD54,CD63, CD73, CD90, CD105, CD146 and Notch1 were detected in MenSCs. Morphology of EVs population was confirmed using TEM, surface biomarkers CD9, CD63, CD81 and CD147 and cytosolic marker TSG101 were detected using flow cytometer Cytoflex LX and Western blot. Proteomic analysis showed that the highest number of proteins was detected in cells (3262 proteins in fertile group and 3236 in infertile), lower in cell EVs (1142 in fertile and 1119 proteins in infertile), while EV-free supernatant had the lowest number of proteins (343 in fertile and 287 proteins in infertile). The highest number of statistically significant differences in protein levels between fertile and infertile were detected in EV samples (47 high in fertile samples and 22 in infertile). Bioinformatic gene ontology analysis revealed different pathways that are regulated by significantly expressed proteins but cell adhesion proteins and collagens are the most abundant groups of proteins which differs in fertile and infertile groups. We demonstrated that MenSC EVs might appear a good source of infertility related biomarkers, and that altered cell adhesion might contribute to etiopathology of unexplained infertility.

Keywords: proteomics, fertility, mesenchymal stem cells

5:45 PM – 6:45 PM

457

MODULATION OF NAIVE MESENCHYMAL/ STROMAL STEM CELLS BY EXOSOMES DERIVED FROM INSULIN-PRODUCING CELLS

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The potential of MSC-derived exosomes for the treatment of diabetes has been explored in several experimental studies since they can avoid several problems associated with cell therapy. On this basis, an inquiry to determine the prospect of modulation of human naïve (undifferentiated) MSCs by exosomes derived from human iPCs was explored. Methods. Mesenchymal stem cells (MSCs) were isolated from liposuction aspirates obtained during elective cosmetic surgeries. MSCs were differentiated into iPCs. Exosomes were then collected from the culture media of undifferentiated and differentiated MSCs (uneducated and educated, respectively) and co-cultured with naïve MSCs. The spherical morphology of MSC-derived exosomes was confirmed by TEM. Their size ranged between 60-160 nm. The expression of specific associated proteins, CD9, and CD63 was verified by flow cytometry and Western blotting. Internalization of the isolated exosomes into MSCs was confirmed by the Exoria labelling. iPCs were positive for insulin (21.2%) and C-peptide (18.2%). The MSCs co-cultured with educated exosomes, also expressed insulin (16.15%) and C-peptide (14.25%) while those co-cultured with uneducated exosomes were 2.05% and 0.77% respectively. C-peptide nanogold, silver-enhanced immunolabelling revealed that iPCs contained-labelled C-peptide in the cytoplasm. Undifferentiated MSCs co-cultured with uneducated exosomes were negative. Undifferentiated MSCs co-cultured with educated exosomes showed labelled C-peptide in the cytoplasm. All pancreatic endocrine genes were expressed by the differentiated iPCs and the naïve MSCs co-cultured with educated exosomes. Gene expression among naïve MSCs co-cultured with uneducated exosomes was marginal. Their was an enormous increase in insulin and C-peptide in response to increasing glucose concentrations. This research demonstrates that educated exosomes can induce the differentiation of naïve MSCs into insulin-producing cells, while uneducated exosomes have no effect. This offers a potential alternative to traditional cell therapy, which can be limited by issues such as necrosis and immune rejection.

Keywords: diabetes, stem cells, exosomes

5:45 PM – 6:45 PM

459

IMMUNOMODULATION OF OCULAR SURFACE INFLAMMATION BY SUBCONJUNCTIVAL INJECTION OF ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Ocular graft-versus-host disease (GVHD) is a post-transplantation complication, impacting approximately 90% of individuals with chronic GVHD. Despite its common occurrence, available treatments are limited. Mesenchymal stem cells (MSCs), known for their low immunogenicity and immunoregulatory properties, have shown promise in the treatment of various inflammatory diseases, including systemic GVHD. In the pursuit of innovative therapies, we investigated the application of adipose-derived mesenchymal stromal cells (adMSCs) through subconjunctival injection within a murine model of chronic GVHD. At the onset of disease human adMSCs were injected, and the subsequent observation of disease progression spanned over three weeks. Our findings unveiled a significant enhancement in corneal integrity, marked by a reduction in epithelial damage, opacity, and maintaining of thickness. Intriguingly, adMSCs exhibited a distinct capacity to modulate immune responses, exemplified by the infiltration of regulatory T cells and the concurrent downregulation of TH1/TH17 cells in the ocular area. Supplementary in vitro wound healing assay experiments underscored the therapeutic potential of adMSCs in facilitating wound closure through their paracrine effects, further highlighting the multifaceted therapeutic capabilities of adMSCs. This dual impact suggests adMSCs as promising candidates for the treatment and mitigation of ocular chronic GVHD, offering a novel and potent approach to the very limited existing therapeutic ones. The observed enhancements in corneal integrity and immune modulation signify the potential of subconjunctival adMSC treatment in chronic ocular GVHD.

Keywords: adipose-derived stromal cells, graft-versus-host disease (GVHD), immunomodulation

5:45 PM – 6:45 PM

461

TRANSPLANTATION OF SCHWANN CELL-LIKE CELLS DIFFERENTIATED FROM HUMAN MESENCHYMAL STEM CELLS FOR DIABETIC NEPHROPATHY

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Diabetic neuropathy (DN) is the most common complication of diabetes, and approximately 50% of patients with this disease suffer from peripheral neuropathy. Nerve fiber loss in DN occurs due to myelin defects and is characterized by symptoms of impaired nerve function. Schwann cells (SCs) are the main support cells of the peripheral nervous system and play important roles in several pathways contributing to the pathogenesis and development of DN. We previously reported that human tonsil-derived mesenchymal stem cells differentiated into SCs (TMSC-SCs), named neuronal regeneration-promoting cells (NRPCs), which cells promoted nerve regeneration in animal models with peripheral nerve injury or hereditary peripheral neuropathy. In this study, NRPCs were injected into the thigh muscles of BKS-db/db mice, a commonly used type 2 diabetes model, and monitored for 26 weeks. Von Frey test, sensory nerve conduction study (SNCS), and staining of sural nerve, hind foot pad, dorsal root ganglia (DRG) were performed after NRPCs treatment. Von Frey test and SNCS results showed that the NRPC treatment group (NRPC group) showed faster responses to less force than the Vehicle group. Additionally, remyelination of sural nerve fibers also increased in the NRPC group. After NRPCs treatment, an improvement in response to external stimuli and pain sensation was expected through increased expression of PGP9.5 in the sole and TRPV1 in the DRG. Human NRPCs treatment may alleviate DN through the remyelination and the recovery of sensory neurons, could provide a better life for patients suffering from complications of this disease.

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Keywords: tonsil-derived mesenchymal stem cells, neuronal regeneration-promoting cells, diabetic neuropathy

5:45 PM – 6:45 PM

463

EFFICACY AND SAFETY OF MESENCHYMAL STEM CELL THERAPY FOR OVARIAN AGING IN MOUSE MODEL

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Ovarian aging is one of the major issues that impact female fertility potential. Mesenchymal stem cell (MSC)-based therapy has been making impressive progress in recent years. However, as a non-autologous component, before MSCs can be used as a routinely available clinical treatment, the efficacy and safety of MSC ovarian therapy remain to be adequately and systematically evaluated. In this study, clinical-grade adipose tissue derived MSCs (AD-MSCs) and umbilical cord derived MSCs (UC-MSCs) were cultivated under GMP-grade conditions in compliance with production and safety regulations. The cells were then injected into different mouse models to execute efficacy and safety verification. For validity verification, estrus cycle, ovarian weight, total/split follicle number and ratio, proliferation status and neovascularization assay were monitored for 3 weeks via a naturally-aging mouse model of 10-12 months old. And for safety, the tests of tumorigenicity (female BALB/c nude mice of 4-6 weeks old) for 16 weeks, as well as acute toxicity (body weight, organ pathology, etc.), immunogenicity (cellular and molecular), and in vivo migration for 1 week were performed. Furthermore, RNA sequencing of mouse ovaries after MSC transplantation was performed. After orthotopic transplantation of MSCs into the ovary, the estrous cycle, ovarian weight, the number and proportion of primary follicles, granulosa cell proliferation, and angiogenesis were improved. The effects of AD-MSCs were superior to that of UC-MSCs on several indexes, such as post-transplant granulosa cell proliferation, ovarian weight and angiogenesis. Moreover, safety validation experiments confirmed that both AD-MSCs and UC-MSCs were not tumorigenic, with no acute toxic reactions, low immunogenicity, and a small amount of non-deterministic distribution. Through RNA sequencing analysis, the enhancement of MAPK cascade was observed and long-term effects were mainly linked to the activation of immune function. In conclusion, orthotopic transplantation of MSC displays significant efficacy and high safety for the treatment of ovarian aging in mice. Our findings further demonstrate the feasibility and potential of MSC for ameliorating ovarian aging and advance the use of MSC for the treatment of clinical diseases.

Funding Source: This work was supported by the National Key R&D Program of China (2021YFC2700303) and the National Natural Science Foundation of China (82225019, 82192873, 82288102).

Keywords: mesenchymal stem cell, ovarian aging, safety

5:45 PM – 6:45 PM

469

THERAPEUTIC APPLICATION OF APOPTOTIC STEM CELLS IN RENAL INJURY MOUSE MODEL

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Feline is prone to kidney disease due to a lower number of nephrons. Several studies have explored the use of mesenchymal stem cells (MSCs) for treating chronic kidney disease in feline. Recently, apoptotic cells were highlighted for therapeutic effects in various animal models. In this study, feline apoptotic MSCs were applied to a renal injury mouse model to demonstrate their therapeutic effects. The MSCs were isolated from the feline amniotic membrane (AM) and apoptotic cells were generated using the self-developed freezing solution. The renal injury mouse model was induced by intraperitoneal (IP) injection of cisplatin and lipopolysaccharide. Viable or apoptotic cells were administered by IP or intravenous (IV) injection and the histological and hematological analysis, cell distribution, and gene expression in the kidney were evaluated. AM-MSCs showed high expression of MSC-specific markers and demonstrated differentiation into mesenchymal lineages. Apoptotic AM-MSCs exhibited high expression levels of apoptotic-related proteins, annexin V and cleaved caspase-3. Both apoptotic and viable cells displayed comparable global gene expression, including inflammation and fibrosis-related genes. However, the protein level of IL-10 was significantly higher in apoptotic cells. The IV and IP injection of viable or apoptotic cells in the mouse model restored histological damage and fibrosis in the kidney, further serum BUN (blood urea nitrogen) level was decreased to normal range. The localization of apoptotic bodies originating from the injected cells in kidney was confirmed by detecting NIR815- or PKH26-stained cells and the presence of mitochondrial genome. The injection of cells resulted in decreased expression of pro-inflammatory and pro-fibrosis genes and increased expression of anti-inflammatory factors in the kidney. In summary, apoptotic bodies were generated through IV or IP injection of viable or apoptotic AM-MSCs, and they were localized in the kidney of renal injury mouse model. The presence of apoptotic bodies induced an anti-inflammatory response, subsequently leading to inhibition of fibrosis and recovery of the kidney. These findings suggest that the application of apoptotic cells offers the potential for restoring renal function in treating renal disease, similar to viable cells.

Keywords: renal injury mouse model, mesenchymal stem cells (MSCs), apoptotic cell therapy

5:45 PM – 6:45 PM

471

INSULIN RECEPTOR BINDING TO GENE REGULATORY ELEMENTS ALTERS METABOLISM OF HPSC-ADIPOCYTES IN DIABETES

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Adipocytes are key regulators of body metabolism, and they play a central role in metabolic disease such as type II diabetes (T2D). However, the molecular progression of insulin resistance (IR) towards symptomatic T2D is still poorly understood. Traditional IR models fail to recapitulate the physiology of human insulin signaling, which has hampered our collective progress in therapeutically targeting IR mechanisms. We produced hPSC-adipocytes that mimic in vivo function, by creating a milieu resembling human physiology. Exposing these adipocytes to T2D patient insulin levels leads to IR, as demonstrated by reduced insulin response in protein phosphorylation, glucose uptake, lipolysis, and altered metabolic gene expression. Here we sought to understand how IR mechanistically affects the transcription and metabolism of diabetic adipocytes. Previous studies have shown that the insulin receptor (INSR) localizes to the nucleus and interacts with transcriptional machinery. In our hPSC-adipocytes, the INSR also localizes to promoters of active genes. It preferentially binds to highly expressed and insulin-responsive genes. Intriguingly, the INSR binding signal was exceptionally strong in the oxidative phosphorylation, glycolysis, and fatty acid metabolism gene sets; groups of genes we found to be transcriptionally dysregulated in insulin resistance. In accordance with all these findings, IR-inducing conditions also dysregulated the adipocyte metabolite composition. In our diabetes mimicking conditions, glycolytic metabolites are globally depleted, and there is a shift towards desaturation of fatty acids. However, while depleted TCA cycle metabolites had their corresponding enzyme's promoter bound by the INSR, elevated metabolites did not. This suggests that INSR promoter binding has repercussions on resultant metabolite levels. Additionally, our IR hPSC-adipocyte model mimicked metabolite findings of clinically used diabetes biomarkers. Altogether our findings indicate the INSR directly regulates the transcription of diabetes-relevant genes. Our hPSC-adipocyte model recapitulates physiological signaling and IR, and uncovered new insulin signaling biology. This demonstrates significant future potential for advancing therapeutic approaches targeting metabolic disease.

Keywords: adipocytes, diabetes, insulin

5:45 PM – 6:45 PM

473

EXTRACELLULAR VESICLES DERIVED FROM MESENCHYMAL STEM CELLS CURE MEMORY IMPAIRMENT CAUSED BY NEUROINFLAMMATION IN YOUNG BUT NOT AGED MICE

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Mesenchymal stem cells (MSCs) isolated from various sources are extensively studied as potential therapeutic tools for many pathological states including neuroinflammation and neurodegenerative diseases, but the possibility to avoid using the live cells is attractive, as it helps to prevent potential immune rejection. In this regard, a promising solution is the use of exosomes - secreted membrane-enclosed vesicles 30–100 nm in diameter containing proteins, lipids, and nucleic acids. The aim of our study was to compare the effects of MSCs and exosomes they produce on the brains of young and aged mice. Comparison of the therapeutic effect of exosomes and conditioned medium (CM) on young mice injected with LPS revealed that exosomes introduced simultaneously with LPS prevented memory impairment caused by LPS while CM provided a weaker effect which lasted two weeks only. In the second set of the experiments young (3months) and old (12 months) mice received 3 and 1 i.p. injections of LPS, respectively. Both MSCs and MSC-derived exosomes transplanted on the 14th day after the last LPS injection in young animals improved the memory of mice in two weeks and it remained good up to the end of experiment. In aged mice either MSCs, or exosomes slightly improved their memory after two weeks, but the effect was transient and went down one week after. Repeated injection of MSCs or exosomes performed five weeks after the first one improved the mice memory again in two weeks and, again, the effect lasted for one week only. The brains of LPS-treated young mice did not contain elevated amounts of IL-1β or IL-6 at the end of experiment (more than 100 days after LPS injection); however, both MSCs and exosomes decreased IL-6 levels in their brains. In contrast, the brains of aged mice treated with LPS still contained elevated amounts of IL-1β and IL-6, and MSCs as well as exosomes decreased them significantly. The results obtained demonstrate that a single injection of MSCs or exosomes stably improved the state of young mice chronically treated with LPS. In contrast, even repeated injections of MSCs or exosomes in aged

mice, which had obtained a single injection of LPS, provided a transient effect, lasting for about a weak only.

Keywords: MSCs, exosomes, memory impairment

5:45 PM – 6:45 PM

TOPIC: MUSCULOSKELETAL

477

UNDERSTANDING SKELETAL DEVELOPMENT AND DISEASE USING HUMAN PLURIPOTENT STEM CELLS AND SINGLE-NUCLEI RNA SEQUENCING

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The study of human growth plate cartilage is crucial for understanding and treating a spectrum of skeletal growth conditions. Here we use human pluripotent stem cells (hPSCs) and single-nuclei RNA sequencing (snRNAseq) technology to create a model that closely mirrors the complex processes of human embryonic cartilage development. Using snRNAseq we show our in vitro hPSC model successfully replicates human chondrogenic differentiation, providing insights into the cellular and molecular dynamics of the human growth plate. The resultant in vitro cartilage 'organoids/pellets' display typical cartilage structures and extracellular matrix composition. Furthermore, to uncover pathogenic mechanisms effecting the cartilage growth plate of skeletal growth conditions, we generated hPSCs with MATN3, COMP, KIF22 and PDE4D mutations using patient cells and CRISPR-Cas9 gene editing. Employing CRISPR-Cas9 gene editing to develop genetic reporters for key dysregulated signalling pathways, enabled 'medium-throughput' (96 well plate format) screening for future identification of compounds that promote differentiation and correct mutant phenotypes. Our findings reveal essential transcriptional regulators and pathways in chondrocyte differentiation and skeletal disease mechanisms, offering novel insights into skeletal growth conditions. The integration of pathway-specific reporters into our hPSC model represents a promising approach to drug discovery and new therapeutic interventions. This work significantly advances skeletal development and disease research, by focusing on disease mechanisms, it paves the way for more effective targeted therapies.

Keywords: human pluripotent stem cells, single-nuclei RNA sequencing, cartilage development

5:45 PM – 6:45 PM

479

ENGINEERING ORGANOID 3D STRUCTURE USING MULTIMATERIAL FRESH 3D BIOPRINTING

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Organoids have emerged as a powerful way to combine stem cell differentiation and self-assembly to form 3D tissue-like structures for in vitro studies. However, the size and complexity of organoids is limited in part by the lack of spatial cues that help control this organization, ultimately constraining the functional repertoire of these tissue models. Organoids often assume a spherical shape, which is dictated by cell-generated compaction forces and can differ from the internal spatial patterning in the native tissue. We asked if it is possible to achieve more complex organoid structure and function by guiding the magnitude and direction of compaction forces. Specifically, we hypothesized that an internal 3D extracellular matrix (ECM) scaffold could provide resistance to cellular compaction, as well as cell-ECM binding sites to control geometry. To create an internal organoid scaffold, we utilized the FRESH 3D bioprinting platform to simultaneously fabricate an internal ECM scaffold within a surrounding cell aggregate. A collagen bioink was used for internal ECM scaffold fabrication while surrounding this we printed a fibrinogen based C2C12 mouse myoblast cell bioink. Cell bioinks were printed in multiple geometries around internal rod shaped collagen scaffolds on the scale of 1-4 millimeter scale (diameter: 3408 ± 52 μm) and over time compacted to a size of < 1 mm (diameter: 952 ± 62 μm). The internal collagen scaffold was able to initially resist cell compaction forces and promote anisotropy in cell aggregates (Day 5 perpendicular diameters: 1800 ± 100 & 972 ± 95 μm) before cell compaction forces drove the aggregate towards a more isotropic, spherical geometry by Day 10 of culture. These results demonstrate that organoids can be 3D bioprinted using multiple bioinks and that an internal collagen scaffold can control cell aggregate or organoid geometry. Control over aggregate geometry can be further tuned through adjusting collagen concentrations to increase resistance to compaction or lowering the cell number to decrease the compaction force. Future works will focus on how the internal collagen scaffold affects cell patterning and the resulting physiological function, laying the groundwork to regulate physiologic structures within organoids.

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Keywords: organoids, tissue engineering, 3D bioprinting

5:45 PM – 6:45 PM

481

IDENTIFYING NEW THERAPIES FOR FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY USING A PHENOTYPIC SCREENING APPROACH

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Facioscapulohumeral muscular dystrophy (FSHD) is the most common autosomal-dominant neuromuscular disorder, with an estimated prevalence of up to 1 in 8,000 individuals. FSHD is characterised by a progressive degeneration and weakness of skeletal muscle in the neck, head and shoulders followed by lower extremities. Whilst the life expectancy of patients with FSHD is normal, the deterioration of muscle mass and function limits independent mobility, reduces quality of life and significantly increases the risk of secondary health complications. Epigenetic mechanisms control the expression of the transcription factor double homeobox 4 (DUX4) which is normally expressed during development and repressed in adult somatic tissue. Transcriptional reactivation of DUX4 in skeletal muscle is the primary cause of FSHD. The identification of targeted therapeutics for FSHD remain elusive. We have recently developed a model of FSHD via activation of the DUX4 transcription factor in bioengineered skeletal muscle. Expression of DUX4 in human micro-muscles produced a striking degenerative phenotype, evidenced by a functional decrease, and the disruption of myofiber integrity and viability. Using this model, we aim to identify treatments that will inhibit or prevent the functional decline in skeletal muscle after DUX4 expression.

Keywords: skeletal, muscle, dystrophy

5:45 PM – 6:45 PM

483

GENERATION OF POSITION-SPECIFIC NEUROMUSCULAR ORGANOIDS TO MODEL HUMAN DEVELOPMENT AND DISEASE (GPS ORGANOIDS)

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During embryonic development, spinal cord motor neurons organize along the anterior-posterior (AP) axis, crucial for coordinating voluntary movement through connections with skeletal muscles. Single-cell lineage analysis on developing embryos has revealed a common axial stem cell population known as neuromesodermal progenitors (NMPs), capable of giving rise to both neural and mesodermal lineages. NMPs can be efficiently differentiated from induced pluripotent stem cells (iPSCs) in vitro, offering a valuable tool for studying neural development. Building on this work, our lab has recently pioneered the generation of human neuromuscular organoids (NMOs) from iPSC-derived NMPs. NMOs represent the first in vitro model where spinal cord neurons and skeletal muscle cells develop simultaneously and self-organize to form functional neuromuscular junctions (NMJ) in 3D. Notably, NMOs are enriched for motor neurons corresponding to the lumbar segment of the spinal cord. Here we focus on establishing position-specific NMOs corresponding to more anterior spinal cord levels. Administration of extracellular signals at specific time points allows us to reconstruct aspects of the AP patterning occurring during human trunk development within an organoid framework. Through detailed molecular characterizations at both transcriptional and translational levels, we seek to elucidate the timing and dynamics of critical events such as muscle innervation and neural maturation within these organoids. Additionally, we conduct contraction and Multi-Electrode Array (MEA) recordings for functional analysis, aiming to elucidate potential variations in contraction patterns and NMJ functionality across different organoid models. Ultimately, our comprehensive approach aims to unravel the complexities of spinal cord development and neuromuscular circuit formation. These insights will not only enhance our understanding of developmental processes in the human spinal cord but also pave the way for translational research addressing axial-specific manifestations of neuromuscular diseases. Finally, our study holds great promise for advancing therapeutic interventions in the domains of human tissue engineering and regenerative medicine.

Funding Source: The Gouti lab is funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (101002689) and the European Molecular Biology Organization Young Investigator program award.

Keywords: neuromuscular organoids (NMOs), GPS organoids, development

5:45 PM – 6:45 PM

485

NERVE GROWTH FACTOR INDUCE CELL FIBROSIS AND HYPERTROPHY IN INDUCED PLURIPOTENT STEM CELL DERIVED CHONDROGENIC PELLETS

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Nerve growth factor (NGF) is a neurotrophic factor involved in the survival, differentiation, and growth of sensory neurons and nociceptive function. Additionally, it has been suggested to play a role in

osteoarthritis (OA) and chondrocyte hypertrophy. Previous studies have suggested a relationship between NGF and OA; however, the underlying mechanisms remain unknown. Therefore, we investigated the relationship between cartilage characteristics and NGF expression in the pathology of OA using human induced pluripotent stem cells (hiPSCs)-derived chondrogenic pellets. NGF treatment decreased the expression of chondrogenic markers (SOX9, aggrecan, and collagen type II) in chondrogenic pellets, whereas, the expression of hypertrophy (osteogenic) markers (collagen type X and vascular endothelial growth factor A) and fibrotic markers (collagen type III and α -SMA) was increased. The expression of inflammatory cytokines and matrix metallopeptidases was also increased in NGF-treated chondrogenic pellets. These findings suggest that increased NGF levels may induce cartilage hypertrophy and fibrosis, which might be responsible for chondrocalcinosis and osteophyte formation during OA progression.

Funding Source: This work was supported by the National Research Foundation of Korea grant funded by the Korea government (NRF-2020R1A2C3004123, NRF-2019R1A5A2027588, and NFR-2021R1C1C2004688) and the Catholic Institute of Cell Therapy in 2024.

Keywords: nerve growth factor, induced pluripotent stem cell, osteoarthritis

5:45 PM – 6:45 PM

487

THE ROLE OF PRIMARY CILIA IN CHONDROCYTE HYPERTROPHY UNDER INFLAMMATION EXPOSURE

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Primary cilia are single, slender extensions that emerge from the cell membrane of various mammalian cells. Structurally, a primary cilium consists of a microtubule-based axoneme (9+0), which is an extension of the cell's cytoskeleton. The axoneme is surrounded by a specialized membrane, and the ciliary base is anchored to the cell through a structure called the basal body. Initially, it was known as a vestigial organ created during the evolution of eukaryotic cells, but now, through various studies, its functions such as sensing and signal transmission in cells are being revealed. Osteoarthritis (OA) is a common degenerative joint disorder characterized by the progressive deterioration of the cartilage. OA progresses due to pro-inflammatory cytokines such as IL-1β, TNF, and IL-6. In previous studies, when IL-1β was treated in chondrocytes, it increased the length of Primary cilia and mediated the inflammatory response caused by IL-1β treatment. IL-1β is also known to induce chondrocyte hypertrophy, which can lead to cartilage destruction. However, there is limited information on the relationship between the changes in the primary cilia and chondrocyte hypertrophy when treated with IL-1β. In this study, we differentiated chondrogenic pellets using induced pluripotent stem cells (iPSCs) in the presence IL-1β and the changes in the cilia was observed during differentiation. The expression of hyaline cartilage markers (e.g. Collagen type II), hypertrophic cartilage markers (e.g. Collagen type X) and primary cilia-specific markers (e.g. ARL13B) were confirmed in the cells during in vitro chondrogenesis. This study further reveals the role of primary cilia, which has been insufficient to date, in chondrogenesis and OA-related inflammation. Also, the results suggest the primary cilia as a potential candidate for the development of an OA therapeutic.

Keywords: primary cilia, osteoarthritis (OA), induced pluripotent stem cells (iPSCs)

5:45 PM – 6:45 PM

489

USING BIOENGINEERED SKELETAL MUSCLE TO MODEL ATROPHY AND INFLAMMATION MEDIATED DYSFUNCTION ASSOCIATED WITH SARCOPENIA

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Age-related loss of skeletal muscle mass, strength, and function is referred to as sarcopenia. Sarcopenia is a progressive muscle wasting condition that affects the lives of many elderly people, putting them at increased risk of frailty and falls. The pathophysiology of sarcopenia is multifaceted and can be accelerated by a variety of factors, including extensive bed rest, chronic inflammation, poor nutrition, and age-related hormonal changes. Despite its prevalence in the community, there are no treatments for managing or preventing sarcopenia. Using novel in vitro modelling techniques, we aim to examine the impacts of mechanical unloading and inflammation on skeletal muscle biology. Using our bioengineered skeletal muscle system, we are able to generate miniaturised muscles tissues which can be assessed for strength and endurance. In this study, we aim to design and refine an in vitro model of mechanical unloading. This will mimic disuse atrophy that is typically seen with bed rest in the aged population. We will characterise this system by investigating the molecular and physical markers of skeletal muscle atrophy including, reduced fibre size, reduced protein synthesis, and induction of protein degradation pathways. Additionally, we will utilise a 2D screening platform using iPSC derived myoblasts to examine various cytokine cocktails that are able to mimic the inflammatory phenotype seen in sarcopenic adults. A larger scale pairwise cytokine screen will be undertaken to examine which combinations of cytokines are able to impair fibre formation and structure. Cytokines that induce atrophy will be taken forward into our 3D screening platform for functional and biochemical assessment. The merging of these two models will allow us to recapitulate sarcopenic stimuli in vitro and through multi-omics approaches, we aim to understand the molecular mediators of sarcopenia.

Keywords: sarcopenia, skeletal muscle, bioengineering

5:45 PM – 6:45 PM

491

INDUCED PLURIPOTENT STEM CELLS GENERATED FROM CALPAINOPATHY PATIENTS HARBOURING THE CAPN3 C.1746-20C>G VARIANTS

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Calpainopathy is a progressive autosomal recessive calpain-3-related limb-girdle muscular dystrophy (LGMD) R1 caused by mutations in the calpain 3 (CAPN3) gene. We have shown that the hypomorphic intronic mutation c.1746-20C>G, which is common in Latvia (MAF 0.237), causes incorrect splicing of the CAPN3 protein. Our project aims to establish calpainopathy patient-derived induced pluripotent stem cells (iPSCs) and create a disease model that recapitulates unique CAPN3 variants. Patients provided informed consent, and experimental procedures were approved by the Central Medical Ethics Committee of Latvia. Fibroblasts were isolated from a skin biopsy by explant-based dissociation technique. 2nd passage fibroblasts were transduced with CytoTuneTM-iPS 2.0 Sendai reprogramming kit. Emerging colonies were propagated in Essential 8 Flex medium and passaged manually until passage five. The continuing passages were lifted with 0.5 mM EDTA. At passage 10, iPSC clones were screened for Sendai virus (SeV) clearance. SeV-cleared iPSC clones were analysed for stem marker Oct-4, Nanog, Sox2, SSEA-4, and Tra-1-60 expression by fluorescence microscopy. The pluripotency of iPSC clones was assessed by directed trilineage differentiation. The expression of ectoderm, mesoderm, and endoderm markers was analysed by immunofluorescence analysis and TaqMan hPSC scorecard assay. Additionally, the genotype stability was assessed by CMA assay, and STR profiling was performed on hiPSCs and original fibroblast lines. Three iPSC clones from each patient were established showing PSC-like morphology with densely packed and round colonies with smooth edges. iPSC clones expressed Oct-4, Nanog, Sox2, SSEA-4, and Tra-1-60 markers. The trilineage differentiation confirmed the pluripotency of the iPSC clones. STR analysis showed matching STR profiles of hiPSCs and parental fibroblasts. Next, myoblast differentiation will be initiated to establish a model system for screening of novel calpainopathy therapies. LGMD R1 patient-derived induced pluripotent stem cells (iPSCs) will serve as a disease model to study the molecular mechanisms of the disease and facilitate the discovery of personalized therapies.

Funding Source: National Research programme Innovation Fund – Sectoral Research Programme Agreement No. VPP-EM-BIOMEDICĪNA-2022/1-0001. We thank COST CA20140 project CorEuStem for protocol assistance.

Keywords: iPS cells, CAPN3, disease model

5:45 PM – 6:45 PM

493

FUNCTIONAL CHARACTERIZATION OF A NOVEL GENETIC VARIANT IN DESMIN (P.GLU353DUP) CAUSING MYOFIBRILLAR MYOPATHY AND GENERATION OF PATIENT DERIVED INDUCED PLURIPOTENT STEM CELLS FOR DISEASE MODELING

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Desmin (DES) is a major intermediate filament protein crucial for the structural integrity and function of striated muscles. Mutations in DES have been associated with various forms of myopathies collectively known as “desminopathy.” In this study, we identified a novel heterozygous mutation (c.1059_1061dupGGA) in exon 6 of DES in an Argentine family with myofibrillar myopathy. This mutation leads to the duplication of a glutamic acid residue at position 353 (p.Glu353dup) of the DES protein. Clinical and myo-pathological evaluations of the index patient (IP) revealed characteristic features of myofibrillar myopathy, including muscle weakness, atrophy, and muscle fatty replacement. In-silico analysis of DES dimer assembly revealed alterations in the coiled-coil structure and a more stable complex conformation when one or both monomers contain the mutation. In addition, functional experiments over-expressing the mutated DES gene (DES_dupGGA) or the wild-type DES gene in HEK cells, revealed the formation of DES and vimentin (VIM) protein aggregates only in the presence of DES_dupGGA. Both results suggest that p.Glu353dup mutation impairs the formation of a normal DES network after affecting its polymerization. Moreover, patient-derived induced pluripotent stem cells (iPSCs) were generated from the IP and his two siblings (one sibling has the mutation and the other not) by peripheral blood mononuclear cells reprogramming using STEMCCA lentiviral system. Characterization of these iPSCs demonstrated normal pluripotency, karyotype and the ability to differentiate into cell types representing the three germ layers. Finally, we generated a DES_dupGGA homozygous iPSCs line derived from the IP-iPSCs using homologous recombination mediated by CRISPR/Cas9 edition. In summary, our study contributed to the understanding of the molecular basis of myofibrillar myopathy caused by the novel DES mutation

c.1059_1061dupGGA. The next step will be to differentiate these iPSCs lines into cardiomyocytes and skeletal myocytes in order to model the disease in vitro. The combination of clinical, molecular, and iPSC-based approaches offers insights into the pathogenesis of desminopathies and opens new possibilities for therapeutic development and precision medicine strategies.

Keywords: desminopathy, cell reprogramming, disease modeling

5:45 PM – 6:45 PM

TOPIC: NEURAL

495

THE OLIGODENDROCYTE BRAIN ORGANOID MODELS CHILDREN WITH LEUKODYSTROPHY AND ENABLES GENE THERAPY

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Leukodystrophies are genetic, neurological, degenerative disorders affecting myelin, associated with significant morbidity and mortality, primarily impacting children and occurring in approximately 1 in 7,663 live births. Mutations in aminoacyl-tRNA synthetases are responsible for these disorders, with particular mutations in DARS leading to Hypomyelination with Brain Stem and Spinal Cord Involvement and Leg Spasticity, or HBSL. Proposed molecular mechanisms contributing to HBSL pathology include variability in cell tolerance to the loss of aspartyl-tRNA synthetase, accumulation of misfolded proteins resulting in a toxic gain of function leading to ER stress, and subsequent activation of inflammatory and immune pathways. Despite studies on these molecular processes, evidence suggests that astrocytes and neurons, in addition to oligodendrocytes, actively contribute to HBSL pathogenesis. However, the sequence of events by which these specific cell types contribute to disease pathology remains largely unknown, hindering the development of rational treatments and early interventions to mitigate disease severity. We generated brain organoids enriched with oligodendrocytes from 4 iPSC lines derived from HBSL patients carrying different DARS mutations with varying disease severity. We observed differences in the growth rate of these organoids compared to control groups. Transcriptomic and cellular analysis revealed alterations in cortical neuronal layer specification and functional defects in these neurons as assessed by multi-electrode array (MEA), alongside enriched inflammation and arrested maturation of MBP-positive oligodendrocytes. Notably, brain organoids from HBSL2 patients exhibited reactive astrocytes. Delivering DARS using an AAV virus to MBP-positive cells promoted the maturation and further myelination of these cells. Collectively, these data indicate that different DARS mutations affect various neural cells in the brain, leading to HBSL pathology and hypomyelination; and secondly, that oligodendrocyte brain organoids are a validated approach to model leukodystrophy in children and enable gene therapy.

Funding Source: M.R.S. is supported by the Children Hospital Foundation (PCC0252021). E.J.W. is supported by the MRFF Leukodystrophy Flagship – Massimo's Mission (EPCD000034).

Keywords: leukodystrophy, organoids, oligodendrocytes

5:45 PM – 6:45 PM

497

HYALURONIC ACID GEL WITH PROTEOGLYCAN MIMICKS BRAIN CELL ORGANIZATION IN VIVO

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In recent years, it has become necessary to reproduce the complex network of the brain. To accurately model neuron-astrocyte interaction, there is a need for a model that contains both neurons and astrocytes and mimics the three-dimensional architecture of the brain. The purpose of this study is to construct a 3D culture gel, which induces a model containing neurons, astrocytes, and microglia, mimicking the 3D architecture of the brain. The importance of the ECM in the brain is well known as the perineuronal net (PNN), which exists at the relay point of the neural transmission pathway. In this study, we revealed that a hyaluronic acid-rich gel made from a combination of versican and neurocan (proteoglycans that are PNN components) induces in vivo similar differentiation of neurons and astrocytes when used as a 3D culture substrate. Tissue samples of developing brains (E12-P0) were extracted with 6 mol/L guanidine hydrochloride buffer, pH 7.5. Extracts were subjected to SEC using Sepharose CL-2B equilibrated and eluted with 4 M guanidine hydrochloride, and 50 mM Tris-HCl, pH 7.5. Dot blot analyses were performed. Hyaluronan-containing extracellular matrix (ECM) aggregates from the brain sample were characterized. Based on the analysis, promising proteins were determined. We made these recombinant proteins and a culture substrate prototype was created by changing these ECM composition, concentration, hyaluronic acid molecular weight, and hyaluronic acid concentration. Using prototype substrates, we investigated each substrate in the 3D culture of nerve progenitor cells derived from the developmental period of mice and human iPSC-derived neural progenitor cells. Culture data revealed that the hyaluronan-binding proteoglycans have a significant effect on inducing differentiation of brain neurons. Versican induces neuronal differentiation, and neurocan induces astrocyte differentiation. We made a prototype of a 3D culture substrate for brain cells. Astrocytes developed foot processes with S100β-positive perisynaptic astrocytic processes (PAP) in this gel shows the gel induces in vivo mimicking culture in vitro. Furthermore, in recent years, B27 supplements have been essential for most brain organoid cultures, but with this media system, in vivo mimic culture is achieved with a B27-free medium.

Keywords: hyaluronic acid, 3D culture, collagen

5:45 PM – 6:45 PM

499

MOLECULAR RESPONSE TO GLUCOCORTICOID ACTIVATION IN ASTROCYTES FROM HUMAN IPSC

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In-utero dysregulation of glucocorticoid receptors (GR) or stress hormone receptors due to glucocorticoid exposure has been linked to long-lasting effects on brain development and a high risk for psychiatric disorders. Therefore, it is important to decipher factors influencing glucocorticoid exposure at molecular and cellular levels that will enable us to identify stress-related pathological mechanisms of psychiatric disorders. In this project, we study the astrocyte-specific effect of GR activation by synthetic glucocorticoids like dexamethasone (Dex). Human iPSCs were used to derive in vitro astrocytes. Astrocytes differentiated for 30 days expressed astrocyte-specific markers Vimentin, GFAP, and S100β. Further, iPSC-derived astrocytes performed astrocytic functions like adult human astrocytes. For instance, the production of pro-inflammatory cytokines when treated with inflammatory cytokines TNF-α and iL1-β exhibiting astrocyte's activated state. Furthermore, Astrocytes exhibited intrinsic fluctuations of calcium signaling in response to ATP stimulation measured by calcium Fluo-4-based calcium-imaging. On day 30 of differentiation, astrocytes were exposed to 100 nM Dex or vehicle control (ethanol). Astrocytes were treated with dex at 3 different time points: (1) Day 30 astrocytes were exposed to dex for 24 hours or 7 days. (2) Glial precursor cells (GPCs) were also exposed to dex for 24 hours and further differentiated into astrocytes and at day 30 astrocytes were again exposed to dex for 24 hours or 7 days. (3) On Day 30 astrocytes were exposed to dex for 24 hours or 7 days and kept in culture for 14 more days in Astrocyte media. Then, we performed whole transcriptome RNA sequencing analysis on all the conditions. We also performed methylation sequencing. By RNA sequencing we observed differential expression of the known GR target genes such as FKBP5, ZBTB16, TSC22D3, and SGK1 after Dex treated compared to the vehicle control. These results show that our iPSC-derived astrocytes stress model system is robust and responds to the cortisone exposure shown by gene expression changes and the GR-responsive genes' methylation pattern. These results will also aid us in identifying GR-responsive genes after cortisone exposure in an astrocyte-specific manner.

Keywords: iPSC-derived astrocytes, glucocorticoid receptor**,** dexamethasone (Dex)

5:45 PM – 6:45 PM

501

ANALYSIS OF CELL PROGRAM INDUCTION COMPARING BETWEEN "ENDOGENOUS" AND "FAST" DIFFERENTIATION TECHNIQUES ON HUMAN INDUCED PLURIPOTENT STEM CELL (IPSC)-DERIVED GABAERGIC NEURONS

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Advances in human cell differentiation and reprogramming have opened up many new avenues for neuroscience research. The generation of induced pluripotent stem cells (iPSCs) and the subsequent development of complex human cell culture systems in particular have improved the translation of research from mouse models to human clinical trials. In order to maximize on the research being conducted with human iPSC technology, it is important to understand the advantages and disadvantages of the variety of differentiation methods that are being used. For example, "endogenous" differentiation techniques use chemical treatment, such as growth factors, to drive the iPSCs toward a specific cellular lineage. In contrast, "fast" differentiation techniques alter the iPSCs at the genetic level by transcriptionally activating a particular cellular program. We explored the effect of the "endogenous" versus "fast" differentiation method on GABAergic neuronal (iGABA) cell program induction. These iGABAs were analysed across morphological, transcriptomic and functional parameters. The purity of the cell cultures and the subtype specificity were examined using bulk RNA-sequencing and immunocytochemistry analysis. The functionality and maturation of the iGABA neurons was investigated by calculating neurite outgrowth, synaptic density and using calcium imaging. The batch-to-batch variability of the two differentiation methods was assessed by testing two separate Lots per cell line. In addition, two different time points were examined to provide information regarding the rate of maturation of the iGABAs. Differential gene expression analysis revealed that the "fast" differentiation method greatly reduced the inter-Lot, as well as Lot-to-Lot, variability of the iGABAs. The neuronal purity of the cell cultures was also greatly increased with the "fast" technique; however, the subtype specificity of the resulting neurons was not obtained. This in-depth comparison of cell program induction between the two main differentiation techniques provides essential information regarding the purity, as well as the functionality, of the iGABA cultures and has important implications for research on epilepsy and tauopathy models.

Keywords: differentiation, GABAergic Neurons, iPSCs

5:45 PM – 6:45 PM

503

CELL TRANSPLANTATION OF INDUCED NEURAL STEM CELLS INTO MULTIPLE SCLEROSIS MODEL ANIMALS

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Multiple Sclerosis (MS) is an autoimmune-related inflammatory CNS demyelinating disorder that affects multiple white matter tracts. The goal of current MS treatment is primarily to prevent recurrence without regenerating the affected nerve cells. The aim of this study is to investigate whether a novel cell-based therapy protects neurons from inflammation and replaces neurons lost in MS. We developed an induced neural stem cells (iNSCs) from human fibroblasts using the Sendai virus delivery system containing four Yamanaka factors in our neural induction medium. The iNSCs were induced in approximately 14 days and further expanded 3 weeks more for cell transplantation into the Experimental Autoimmune Encephalomyelitis (EAE) model mice. The iNSCs demonstrated immunopositivity against NSC markers and expressed NSC-associated genes such as Sox1, Sox2, Nestin and MSI1. These cells proliferated beyond 10 passages, maintaining a normal karyotype. After transplantation of the iNSCs, the therapeutic effects were observed in EAE model animals. In the experimental groups transplanted with the iNSCs, the EAE mice showed a superior ability of motor function recovery compared to control group, with recovery degree assessed through an established EAE scoring protocol. Immunostaining assays, H&E staining, LFB staining of spinal cord sections from EAE model mice showed that iNSC transplantation enhanced myelination and suppressed inflammation in both brain and spinal cord compared to control mice. In addition, quantitative RT-PCR results of spinal cord tissues showed that the expression levels of inflammatory cytokines decreased, while the expression levels of anti-inflammatory cytokines increased. Taken together, these results show that transplantation of the iNSCs dramatically improved the motor functions and exerted an anti-inflammation effect in the MS model animals, which suggests that the iNSCs may be feasible as a promising therapeutic cell source for MS treatment.

Funding Source: This work was supported by the Ministry of Science and ICT (2019M3E5D5065399) and the Ministry of Health and Welfare (RS-2022-00060247) of the government of the Republic of Korea.

Keywords: multiple sclerosis, induced neural stem cell, cell transplantation

5:45 PM – 6:45 PM

505

SCALABLE AND WELL DEFINED HUMAN GLUTAMATERGIC AND GABAERGIC CO CULTURE PLATFORM SUITABLE FOR STUDYING EXCITATORY INHIBITORY NEURON IMBALANCES AND THE DISCOVERY OF DRUGS TO TREAT ASSOCIATED DISEASES

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Neuronal circuits in the cortex consist of two main neuronal types, glutamatergic excitatory neurons and GABAergic inhibitory neurons (IN). The inputs of IN provide cortical networks with the ability to balance spontaneous and evoked excitatory activities, preventing runaway excitation. Abnormal IN function is associated with various neurological diseases including autism, epilepsy and schizophrenia. Scalable approaches are needed to generate reliable human in vitro models suitable for high-content drug screening to develop therapeutics to treat these neurological diseases. We have used our precision cellular reprogramming technology opti-ox™ (optimised inducible overexpression) to generate a highly pure (>95%) population of GABAergic neurons, named ioGABAergic Neurons, from human iPSCs, at scale, within 12 days post-revival. A deep molecular characterisation by immunocytochemistry, RT-qPCR and single-cell RNA-sequencing revealed that cultures consist of over 95% pure GABAergic neurons expressing the classical markers GAD1, GAD2, VGAT, DLX1, as well as DLX2 and are positive for GABA. Remarkably, SST was the only GABAergic subtype specific marker that was detected, further highlighting the purity of the ioGABAergic Neurons. Moreover, three independently manufactured ioGABAergic Neurons lots displayed highly equivalent transcriptomic profiles, confirming the consistency and scalability of the opti-ox technology. Functional assessment by MEA assays showed that ioGABAergic Neurons inhibit the excitatory activity of ioGlutamatergic Neurons in a ratio dependent manner, and that the inhibitory and excitatory balance can be further modulated by drugs targeting GABAergic signalling. Thus, the developed opti-ox driven co-culture platform can be used to study the principles underlying excitatory–inhibitory neuron imbalances and for the discovery of new drugs to treat for example epilepsy patients. In summary, opti-ox precision cellular reprogramming enables the manufacturing of highly pure (>95%), consistent, and functional GABAergic neurons that can serve as a high-quality human model to study both neurodevelopment and neurological disorders.

Keywords: gabaergic, excitatory–inhibitory, multi-electrode array

5:45 PM – 6:45 PM

507

GENOME EDITING STRATEGY FOR NORMALIZING FMR1 GENE EXPRESSION IN FRAGILE X-ASSOCIATED DISORDER PATIENT-DERIVED IPSCS AND NEURONS USING CYTOSINE BASE EDITOR

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Fragile X-associated disorders are a family of inherited disorders typically caused by abnormally expanded CGG trinucleotide repeat within the 5' UTR of the FMR1 gene. The degree of expansion of CGG repeats leads to various patterns of abnormal FMR1 gene expression, resulting in the different type of disease. Fragile X syndrome (FXS) with more than 200 CGG repeats (full mutation; FM) is caused by silencing of FMR1 gene expression, resulting in intellectual disability and autism spectrum disorder. Fragile X-associated tremor/ataxia syndrome (FXTAS) with 55-200 CGG repeats (premutation; PM) is caused by excessive FMR1 mRNA levels and the expression of FMRpolyG, a product of repeat-associated non-AUG (RAN) translation of CGG repeats. These changes in FMR1 mRNA expression result in a complete loss or lack of its encoding protein, fragile X messenger ribonucleoprotein (FMRP), crucial for neuronal interaction and synaptic activity. Here, we established a genome editing strategy to normalize expression of FMR1 gene in human induced pluripotent stem cells (hiPSC) derived from patient with fragile X-associated disorders by utilizing cytosine base editor (CBE), which replaces a cytosine base to thymine. We demonstrated that introducing CBE targeting CGG repeats of FMR1 gene into FXS-iPSCs resulted in the reactivation of FMR1 gene expression, which occurred as a result of CBE-induced R-loop formation leading to the contraction of CGG repeats under 200 copies. Interestingly, the edited FXS-iPSCs with PM (55 to 200 copies; so-called PM-like iPSCs) exhibited higher levels of FMR1 mRNA but lower levels of FMRP compared to WT-iPSCs, and showed FMRpolyG inclusion in the nucleus, mimicking the phenotype of PM carriers. Consecutively, we confirmed that cytosine base editing that targeted the upstream of CGG repeats in PM-like iPSCs resulted in decrease in FMRpolyG inclusion, rescuing FMRP expression by prematurely terminating +2 frame-shift RAN translation. Following cytosine base editing in PM-like iPSCs, restored FMR1 gene expression was sustained in neural progenitors and mature neurons, accompanying the rescue from disease-like phenotype. Our approach provides further insights into the molecular mechanisms of fragile X-associated disorders and future therapeutic strategy of trinucleotide repeat disorders.

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2022R1A2C2091165).

Keywords: Fragile X syndrome, FMRpolyG, cytosine base editor

5:45 PM – 6:45 PM

509

ENVIRONMENTAL TOXICANTS AND A GUT METABOLITE AFFECT NEURON-ASTROCYTE DIFFERENTIATION AND FUNCTIONAL ACTIVITY IN THE HUMAN NEURAL PROGENITOR TEST (HNPT)

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Humans are exposed to a large number of chemicals in daily life. This can be problematic, especially during development of the brain, and can have lifelong effects. Safety testing of compounds for developmental neurotoxicity is currently performed using animal experiments, which comes with scientific limitations and ethical issues. Human stem cell-derived cultures can be a solution to these issues, since they mimic many cellular events that occur during early human brain development. The goal of this study was to employ a human stem cell based in vitro model to study the effects of potentially toxic and/or beneficial compounds on neuronal differentiation. In this human neural progenitor test (hNPT), neural progenitor cells derived from human embryonic stem cells were differentiated to a neuron-astrocyte co-culture while being exposed to methyl mercury, sodium (meta) arsenite or trimethylamine-N-oxide (TMAO), a gut microbe-derived metabolite. Gene expression profiles were analysed using RNAseq; functional changes were measured as changes in spontaneous electrical activity using a microelectrode array. Mercury or arsenic exposure at concentrations that did not impair cell viability affected synaptic marker genes involved in excitatory or inhibitory neuronal signalling. Spontaneous activity was almost completely absent, showing clear toxicity of the compounds at a functional level. Exposure to TMAO regulated distinct sets of genes related to neuronal differentiation, specifically genes relating to synaptogenesis. On a functional level, there was a slight delay in the development of spontaneous electrical activity that did not ultimately affect final activity levels compared to the control condition. These results show that TMAO might subtly modulate the development of a human-based neuronal differentiation model including functional activity, while the environmental toxicants have a clearly harmful effect. The study also showed that the hNPT can contribute to human-relevant,

animal-free testing strategies to assess the effect of environmental toxicants and beneficial metabolites on the developing brain.

Funding Source: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 874583.

Keywords: developmental neurotoxicity, neuronal differentiation, transcriptomics

5:45 PM – 6:45 PM

511

ENHANCED REPLICATION OF SARS-COV-2 OMICRON BA.5 IN HUMAN ORGANOIDS AND SARS-COV-2 INDUCED DEAD-BOX RNA HELICASE INHIBITS VIRAL REPLICATION IN HUMAN MIDBRAIN ORGANOIDS

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Coronavirus Disease 2019 (COVID-19) is associated with many different neurological complications. Here, we studied the neuroinvasion and neurotoxicity of Omicron variants (BA.1, BA.2 and BA.5), and compared the findings with those of SARS-CoV-2 WT and Delta in human organoids. Our results showed that BA.5 replicated more efficiently while triggered lower levels of type I interferon response than that of WT and Delta in both human forebrain and midbrain organoids. Moreover, higher levels of apoptosis in the infected human forebrain and midbrain organoids were substantially triggered by BA.5. Next, we analyzed the transcriptional changes when the forebrain and midbrain organoids were transiently exposed to different variants of SARS-CoV-2, and revealed activation of several host genes related to cell survival, inflammatory response, or viral replication. Notably, a member of the DEAD-box RNA helicases was found upregulated upon viral infection specifically in the midbrain organoids. Mature neurons and astrocyte in midbrain organoids express more DEAD-box RNA helicase and expression of the RNA helicase could be induced by interferon lambda. Interestingly, knockdown of the RNA helicase resulted in even more robust viral replication in the midbrain organoids. Also, overexpression of the RNA helicase inhibits viral nucleoprotein level in Hela cells transfected with vRNA (segment of nsp1). Furthermore, DEAD-box RNA helicase could interact with both viral RNA and viral nucleoprotein. In summary, these findings suggest that the central neural system responds to SARS-CoV-2 infection by inducing specific host genes to restrict viral replication.

Keywords: brain organoid, SARS-CoV-2, DEAD-box RNA helicase

5:45 PM – 6:45 PM

513

HARNESSING CRISPR-READY GLUTAMATERGIC NEURONS FOR DRUG DISCOVERY IN NEURODEGENERATIVE DISEASES

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In the quest to unlock new therapies for neurodegenerative diseases, the insufficiency of traditional models, which rely on immortalised cell lines, has become a critical barrier. These models often fall short in replicating the intricacies of disease-affected human cells. Induced pluripotent stem cells (iPSCs) offer a solution, allowing for the recreation of disease-specific cell types, but they come with differentiation challenges, including variability and scalability issues. At bit.bio, we address these challenges with our innovative opti-oxTM technology, enabling rapid and consistent reprogramming of cell types at scale. Recognizing the transformative potential of CRISPR-Cas9 technology in functional genomics and its capacity to elucidate the genetic underpinnings of diseases, we have expanded the application of our cells by engineering CRISPR-ready ioCells. By integrating CRISPR-Cas9 into our cell models, we enable precise genetic manipulation, thereby enhancing our ability to identify and validate therapeutic targets. Using a guide RNA targeting SOX11, we demonstrate high knockout efficiency at DNA and protein level. CRISPR-Ready ioGlutamatergic Neurons form structural neuronal networks and express pan-neuronal markers (MAP2, TUBB3) and glutamatergic neuron specific markers (VGLUT2) by day 11. After thorough characterisation, CRISPR-Ready ioGlutamatergic Neurons were used in a scCRISPR screening workflow, to screen for genes integral to neurodegenerative processes. Single-cell transcriptomic analyses revealed disease-associated genes whose knockout caused notable transcriptomic signatures associated with the Charcot-Marie-Tooth Disease. Our findings underscore the value of combining innovative cell reprogramming technology with cutting-edge genetic tools to explore the complex landscape of neurodegenerative diseases, paving the way for future breakthroughs in the field

Keywords: Glutamatergic Neurons, forward programming, scCRISPR screening

5:45 PM – 6:45 PM

515

DECIPHERING THE MOLECULAR FUNCTION OF WWOX IN CNS MYELINATION

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Myelination is a crucial process for the proper functioning of the central nervous system (CNS), influencing neural circuitry and overall brain function. However, the molecular mechanisms driving myelination are not fully understood, representing a significant knowledge gap with implications for neurological disorder treatment. Our study aims to investigate the role of the WW domain-containing oxidoreductase (WWOX) gene in CNS myelination. We previously showed that murine Wwox deletion leads to hypomyelination and decreased mature oligodendrocyte numbers. Modeling the complete loss of WWOX in human embryonic stem cells (hESCs) and the generation of human oligocortical spheroids further confirmed the role of WWOX in hypomyelination. Remarkably, restoration of WWOX expression under the Syn1 promoter in our models rescues the hypomyelination phenotype, illustrating a complex neuron-glia cell interaction and critical function of the WWOX gene in myelination. Analysis of published single-cell RNA-sequencing data from human multiple sclerosis patients' chronic demyelinated lesions showed reduced WWOX expression in oligodendrocytes and neurons, prompting a deeper investigation into its function. Employing the Olig2-cre mouse model to specifically ablate WWOX in oligodendrocyte progenitor cells (OPCs), we demonstrated that WWOX deficiency impairs OPC differentiation, negatively affecting myelination and remyelination post-cuprizone treatment. By uncovering WWOX's molecular function in CNS myelination, our study not only fills a critical knowledge gap but also proposes potential therapeutic interventions, moving the field forward. Future studies will evaluate using human neural stem cell-derived OPCs and oligocortical spheroids, to better gain insights into WWOX's regulatory effects on myelin-related genes.

Keywords: myelination, WWOX gene, neural stem cell-derived OPC

5:45 PM – 6:45 PM

517

THE EFFECT OF HUMAN NEURAL STEM CELLS OVEREXPRESSING NRXN AND NLGN IN A SPINAL CORD INJURY MODEL

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In recent years, many researchers reported that therapeutic effect of stem cells for diverse diseases. However, unlike other tissue, brain has specific structure, called synapse. The synapses not only transmit, but also process and refine information. Therefore, synaptic regeneration is a key role for therapy of neurodegenerative disorders. Neurexins (NRXN) and neuroligins (NLGN) are synaptic cell-adhesion molecules that connect pre- and postsynaptic neurons at synapses, mediate trans-synaptic signaling, and shape neural network properties by specifying synaptic functions. In this study, we investigated to synaptic regeneration effect of human neural stem cells (NSCs) over-expressing NRXN (F3.NRXN) and NLGN (F3.NLGN) a spinal cord injury model. Over-expressing NRXN and NLGN in the neural stem cells upregulated expression of synaptophysin, PSD95, VAMP2, and synapsin, which are synaptic markers. In the BMS score test, the transplantation of F3.NRXN and F3.NLGN enhanced recovery of locomotor function in adult rodents following spinal cord injury. Transplanted F3.NRXN and F3.NLGN differentiated into neurons and formed the synapse with host cell in the spinal cord injury mouse. Also, F3.NRXN and F3.NLGN cells restored growth factors (GFs) and neurotrophic factors (NFs), and they induced the proliferation of host cells. This study suggested that NSCs overexpressing NRXN and NLGN cells could be a candidate for cell therapy in neurodegenerative disease by facilitated synaptic regeneration.

Keywords: synaptic regeneration, NRXN, NLGN, spinal cord injury

5:45 PM – 6:45 PM

519

ANKRD11, A CHROMATIN REGULATOR, REGULATES OLFACTORY BULB NEUROGENESIS IN MICE AND HUMANS

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Variants in ANKRD11 (Ankyrin Repeat Domain 11), a chromatin regulator, are associated with KBG syndrome, a rare neurodevelopmental disorder named after the first three families diagnosed. KBG syndrome patients display aberrant brain development, global developmental delay, autism, and intellectual disability. The brain is built by neural stem cells (NSCs), which generate neurons and glia (non-neuronal cells) in a strict spatio-temporal manner. Olfactory bulb (OB) is the part of the brain responsible for olfaction (sense of smell) and is maintained via adult OB neurogenesis. Reduction or loss of olfaction is linked to behavioural changes in patients with major depressive disorder, bipolar disorder and schizophrenia. Aberrations in OB development or olfaction in patients with neurodevelopmental disorders, including KBG syndrome, is not known. We used a mouse model where Ankrd11 is inducibly knocked out in NSCs using a Cre/Lox system (Ankrd11nscKO). Ankrd11 knockout was induced with tamoxifen injection at embryonic day (E) 14, a time point prior to the complete formation of the OB. Ankrd11nscKO mice showed aberrant postnatal olfactory bulb development and reduced size. We further showed this reduction in size was accompanied by defects in NSC proliferation, migration and differentiation. Finally, we corroborated these findings in vitro and showed that Ankrd11-deficient NSCs display reduced proliferation, migration, neurogenesis and aberrant global gene expression. OB phenotypes in KBG syndrome patients were recorded via magnetic resonance imaging (MRI) and/or clinical observations. We describe two clinically and molecularly confirmed KBG syndrome patients with anosmia (lack of smell) and OB structure perturbations, such as and hypo-dysgenesis/agenesis (reduction or lack of OB structures). Our work contributes significantly to the OB development and neurodevelopmental disorders fields and has important translational implications. From a basic science and mechanistic perspective, our results indicate a critical role of Ankrd11 in NSC biology in mice and humans. From a clinical perspective, our work suggests OB size or olfaction evaluations should be considered upon KBG syndrome diagnosis for appropriate genetic counselling and to improve clinical care.

Funding Source: This work was supported by private donations from families affected by the KBG syndrome, KBG Foundation seed funding and an award from The Scottish Rite Charitable Foundation of Canada.

Keywords: epigenetics, neurodevelopmental disorders, neural stem cells

5:45 PM – 6:45 PM

521

GENERATION AND FUNCTIONAL CHARACTERISATION OF MOTOR NEURONS DERIVED THROUGH TRANSCRIPTION FACTOR MEDIATED PROGRAMMING OF HUMAN PLURIPOTENT STEM CELLS

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Motor neurons consist of distinct neuronal subtypes that control the activity of muscles and glands in direct or indirect manners. Motor neurons form a large neuronal network that receives inputs from interneurons, sensory neurons or other motor neurons to control complex behaviours such as locomotion. Pathological perturbation of these motor circuits can lead to the development of motor neuron diseases (MNDs) such as spinal muscular atrophy and amyotrophic lateral sclerosis. Development of therapies to treat MNDs is hampered by the limited translatability of existing preclinical animal models as well as the lack of reliable and consistent sources of in vitro models. Human induced pluripotent stem cells (hiPSCs) can be used to generate motor neurons for in vitro applications, however current differentiation protocols are often lengthy, inconsistent, and difficult to scale. We have used opti-oxTM technology to rapidly reprogram hiPSCs into motor neurons, termed ioMotor NeuronsTM, which are a homogenous population of cells with classical neuronal morphology and neurite outgrowth. As early as 4 days in culture, cells express the pan-neuronal markers MAP2 and TUBB3, the cholinergic markers ChAT and VAChT and the motor neuron-specific markers MNX1 and ISL1/2, as assessed by both ICC and RT-qPCR. Bulk RNA sequencing of ioMotor Neurons demonstrates a rapid acquisition of a motor neuron signature, with an indicated spinal motor neuron identity (cervical region). A high-density microelectrode array (HD-MEA) system has been used to assess ioMotor Neuron functional activity, and has revealed spontaneous neuronal activity with

increasing firing rate over 40 days in culture. Finally, next generation sequencing methods have shown consistency between three different batches produced through opti-ox mediated reprogramming. opti-ox technology can be utilised for the scalable and consistent production of functional hiPSC-derived motor neurons. ioMotor Neurons have the potential to advance the development of new therapeutics for MNDs and to further our understanding of motor neuron development and maturation in vitro.

Keywords: motor neuron, iPSC-derived, MEA

5:45 PM – 6:45 PM

523

NOTCH INHIBITION ENHANCES SENSORY HAIR CELL REGENERATION AND RESTORES FUNCTION IN MICE WITH VESTIBULAR DISORDER

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The sense of balance relies on motion sensing vestibular hair cells within the inner ear. Hair cells accumulate damage with age, resulting in balance disorders, the number one health complaint of individuals over age 70. The lack of significant hair cell regeneration in the adult vestibular system makes balance disorders essentially irreversible, and patients have few treatment options. In this work, we employed pharmacological and genetic approaches to modulate Notch signaling in a murine model of damaged utricle. We targeted hair cells for ablation using a mouse line (Pou4f3-DTR) that expresses the diphtheria toxin receptor in hair cells. A gamma-secretase inhibitor was administrated locally via the cochlea and the extent of spontaneous vs drug-induced regeneration was compared between the treated and untreated (contralateral) ear. We also deleted Notch in supporting cells to explore the effect of Notch conditional knockout on hair cell regeneration. We employed lineage tracing and quantitative analysis to assess changes in cell fate in response to altered Notch signaling. Our findings reveal that targeted manipulation of Notch leads to a significant increase in cells expressing hair cell marker, Myo7A. Drug treatment resulted in regeneration of 48% of the normal number of hair cells after 1 month as compared to 25% restoration of hair cells in the damaged ear by spontaneous regeneration (without drug treatment). Drug-induced hair cells re-established afferent connections with the vestibular neurons as assessed by single unit recording, resulting in a significant functional improvement measured by the vestibulo-ocular reflex. Similar results were observed after conditional knockout of Notch. The treatment described here may pave the way toward a novel therapeutic approach suited for sensory hair cell replacement to restore vestibular function in patients with vestibular disorders.

Funding Source: This work was supported by NIH grant DC020322

Keywords: Notch signaling, sensory hair cells, balance disorder

5:45 PM – 6:45 PM

525

SCALABLE IPSC PROGRAMMING STRATEGY IN COMBINATION WITH OPTIMISED COCKTAILS OF NEUROTROPHIC FACTORS YIELDS FUNCTIONALLY DISTINCT NOCICEPTOR SENSORY NEURONS

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Nociceptive sensory neurons are a specialised subtype of somatosensory cells residing in the dorsal root ganglia. Nociceptors respond to diverse noxious and pruritic stimuli, and hence are critical for the study of pain mechanisms and neuropathies. Around 20% of adults suffer from chronic pain, but the current analgesics are limited by short duration and adverse events. Unfortunately, the efficacy of analgesics in animal pain are poorly translated to humans as clinical trials for pain therapeutics have only a 2% probability of success. Consequently, drug classes used to treat chronic pain have essentially not evolved over the past 40 years. Thus, there is an unmet need for reliable and scalable human in vitro models to develop new, efficacious, and safe pain therapeutics. However, conventional differentiation methods to generate nociceptors from pluripotent stem cells are complex, inconsistent, and characterised by protracted maturation times. By using our precision cellular reprogramming technology (opti-ox), we robustly expressed a combination of transcription factors in iPSCs to generate a homogeneous population of sensory neurons that display critical features of mature nociceptors. Bulk and single cell RNA-sequencing analysis together with immunocytochemistry showed that within 7 days after the induction of TFs, the neurons expressed the key sensory markers ISL1, POU4F1 and PRPH. At this early time point, the neurons also expressed the key nociceptor markers such as NTRK1, TRPV1, TRPM8, and SCN9A. Multi-Electrode Array and calcium assays demonstrated that reprogrammed sensory neurons are functional as displaying asynchronous spontaneous activity and responsiveness to diverse noxious stimuli. Neurotrophic factors play a critical role in sensory neuron subtype specification and by adapting culture conditions we were able to enrich for cells expressing key peptidergic nociceptor markers TAC1 as well as ADCYAP1 and substantially increase the responsiveness to specific noxious stimuli. In conclusion, with opti-ox precision reprogramming, iPSCs are rapidly converted into functional sensory neurons offering a robust and scalable source of human nociceptors that can be used as an in vitro model to study the biology of pain and to develop novel therapies for neuropathies.

Keywords: nociceptors, pain, sensory

5:45 PM – 6:45 PM

527

FUNCTIONAL ANALYSIS OF NEURONAL CELLS DIFFERENTIATION FROM IPS CELLS DERIVED FROM PATIENTS WITH DRAVET SYNDROME

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Dravet syndrome (DS), a devastating type of infantile-onset epilepsy that presents with cognitive deficits and autistic traits, is caused by a mutation in SCN1A, which encodes the α-subunit of the voltage-gated sodium channel, Nav1.1. Excitatory/inhibitory (E/I) imbalance in the cerebral cortex can cause central nervous system disorders, such as DS. However, the underlying cellular disturbance remains ill-defined owing to the reliance of available knowledge on animal models that are not readily transferable to the syndrome in humans. Recently, we generated induced pluripotent stem cells (iPSCs) derived from a DS patient (D1) with a c.4933C>T substitution in SCN1A predicted to cause truncation in the fourth homologous domain of the protein (p.R1645*). Moreover, to elucidate the mechanism of neurodegeneration in DS caused by c.4933C>T mutation, we performed gene correction in D1 iPSCs using TALEN (transcription-activator-like effector nuclease)-mediated genome editing, generating D1 TALEN iPSCs. In this study, we generated excitatory or inhibitory neurons by employing direct in vitro conversion of iPSCs through the overexpression of specific transcription factor cocktails as a novel approach for neuronal differentiation. These cells were seeded on multi-electrode array (MEA) systems, which is a measuring device with multiple electrodes integrated in a cell culture dish, and the spontaneous neuronal activity was recorded. We present data comparing excitatory or inhibitory neurons derived from healthy (WT), DS (D1), and isogenic control (D1 TALEN) iPSCs that were measured using MEA systems.

Keywords: Dravet syndrome, SCN1A, neuronal cells

5:45 PM – 6:45 PM

529

BUILDING A NEURON-MICROGLIA MICROVASCULAR NICHE TO PROBE THE REGULATORY EFFECTS OF MICROGLIA IN ALZHEIMER'S DISEASE

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Alzheimer's Disease (AD) is a progressive neurodegenerative disorder accompanied by vascular dysfunction and degeneration of the neurovascular unit (NVU). In particular, microglia and vascular changes in the NVU have been implicated as potential early drivers of neurodegeneration. The NVU is made up of microglia, astrocytes, neurons, endothelial cells (ECs), and pericytes that help to form the blood brain barrier and maintain oxygen and nutrient delivery to the brain. It has been challenging to study the human AD NVU due to the inaccessibility of the brain and a lack of post mortem brain samples. Current in vitro models of the NVU generally lack microglia, use immortalized or primary cells that cannot be used to study patient specific genetic backgrounds, and in 3D, lack perfusable vasculature with a complex in vivo-like geometry. Therefore, there is a need to develop in vitro NVU models that include induced pluripotent stem cell (iPSC)-derived brain cell types, including microglia, and perfusable vasculature with branching architecture. Here we developed a 3D multicellular NVU model containing perfusable vasculature made of brain ECs surrounded by microglia, neurons, and astrocytes to investigate the spatiotemporal dynamics of the NVU in AD. We also developed a complimentary 2D triculture model including neurons, microglia, and astrocytes that can be used to interrogate cell-cell interactions and cell type specific contributions to NVU dysfunction in AD. In 2D, we found that the presence of microglia in our triculture model improves neuronal heath compared to neurons alone. Through the temporal modulation of our 2D model, the addition of microglia showed a protective effect on neurons challenged for 24 hours with conditioned media (CM) from AD cells, although this protection seems to abate after 72 hours of CM treatment. In our 3D microfluidic model, we can support the survival of ECs, microglia, neurons, and astrocytes in simultaneous co-culture. We also see extensive spatial cell-cell interactions between the NVU cell types present in our model. Overall, we have successfully built complex, multicellular models of the NVU. These models provide an integrated neurovascular niche to characterize cell-cell interactions in the healthy and AD NVU and interrogate the protective role microglia play in NVU dysfunction.

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Keywords: neurovascular dysfunction, Alzheimer's Disease, 3D microvessels

5:45 PM – 6:45 PM

531

A NOVEL STEM CELL TOOLKIT TO ADVANCE THERAPEUTICS IN ANGELMAN SYNDROME

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Angelman syndrome (AS) is a rare and incurable neurodevelopmental disorder affecting approximately 1 in 20 000 individuals, characterized by a severe developmental delay, speech impairment, ataxia, a distinct happy demeanor and frequent laughter. AS arises from the loss of the maternally inherited copy of ubiquitin protein ligase E3A (UBE3A), which is only expressed from the maternal allele in mature neurons. Paternal UBE3A is silenced by transcriptional interference by an antisense long noncoding RNA, known as UBE3A-ATS, exclusively expressed from this allele due to epigenetic process of genomic imprinting. Defects on the maternal allele give rise to AS, and can appear from four (epi)genetic causes with varying degrees of severity: maternal megadeletions (MD) in chromosome 15q11-q13; UBE3A mutations; paternal uniparental disomy (UPD) for chromosome 15; and imprinting defects. Currently, the most promising therapeutic approach is the reinstatement of the silent, yet normal, paternal UBE3A by antisense oligonucleotides targeting UBE3A-ATS, a strategy with yet unclear applicability in all etiologies of the disease. While AS animal models have proven insightful, many important phenotypes are absent, thus encouraging the development of more human relevant models. As such, we aim to establish a stem-cell toolkit do advance therapeutics in AS, through the generation and characterization of patient-derived human induced pluripotent stem cells (hiPSCs) and familial controls, as humanized and personalized models to study AS. In collaboration with the Portuguese AS society (ANGEL), we recruited several families and generated hiPSCs using a Sendai virus reprogramming strategy. hiPSCs were characterized by flow cytometry, immunofluorescence (IF), quantitative PCR (RT-qPCR), methylation analysis and karyotyping. Pluripotency was assayed by tri-lineage commitment and analyzed by IF and RT- qPCR. We successfully generated several pairs of hiPSCs from patients with MD and UPD and will illustrate the quality control analysis performed. With these hiPSCs pairs of hiPSCs genetically matched controls, we lay the groundwork for developing brain organoids to unveil novel cellular and molecular features of AS. Ultimately, this organoid platform should be used to test and screen current and novel therapies for AS.

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Keywords: Angelman syndrome, human induced pluripotent stem cells, disease modeling

5:45 PM – 6:45 PM

533

ALS ORGANOIDS DISPLAY ALTERATIONS IN THE UBIQUITIN-PROTEASOME PATHWAY

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease that causes the death of the patient between 2-5 years after symptoms onset. Currently, ALS remains an incurable disease. Despite evaluating over 60 compounds in clinical trials, only three drugs with limited beneficial effects have been approved. Several drugs showed promising results in animal models but were not effective in clinical trials. This is likely due, in part, to the use of animal models that poorly predict human brain responses. Brain organoids are 3D models developed in the laboratory from human stem cells, commonly pluripotent stem cells, recreating tissue and organ structures more physiologically than conventional 2D cultures and allowing interactions between different cell types. We have generated cortical organoids from ALS patients with C9ORF72 hexanucleotide repeat expansions and from their corresponding isogenic controls where the mutation has been corrected using CRISPR/Cas9. Organoid maturation was favoured by adding motor cortex extracellular matrix (cECM). After 2 months of maturation, ALS organoids displayed alterations in neurons such as low intracellular expression of neurofilament proteins. Furthermore, ALS organoids showed a transcriptional deregulation of pathways previously related to ALS physiopathology, such as the ubiquitin-proteasome pathway. Deregulation of the ubiquitin-proteasome pathway was mainly observed at the level of de-ubiquitinating enzymes, especially ubiquitin-specific proteases (USP) with some transcripts being strongly downregulated. This deregulation was also observed in organoids derived from control iPSC lines when exposed to a combination of small molecules that induce chemical senescence (SBI-0206965, Lopinavir), suggesting that ALS organoids undergo a process of premature senescence. Furthermore, control organoids grown with cECM from ALS patients also showed pathological hallmarks and specifically, downregulation of several USP. Our results suggest that generation of cortical organoids with cECM is a powerful model to study ALS. This model could provide a deeper understanding of the underlying mechanisms of the disease and, in turn, become an unprecedented tool for the development of effective therapies against this devastating condition.

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Keywords: cortical organoids, ALS, brain

5:45 PM – 6:45 PM

535

A NOVEL INDUCED PLURIPOTENT STEM CELL (IPSC)-DERIVED 3D PLATFORM FOR MODELLING OF TDP-43 PATHOLOGY

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Synapticure's mission is to provide access to the best care and treatments possible for each patients' specific type of neurodegenerative disease regardless of where they live. We currently help >1600 patients across Dementia, Motor and Movement Disorders by tailoring today's best possible care to each person. Our vision in R&D is to help accelerate the development of targeted, disease altering therapies, including developing accurate in vitro models on a patient basis, to bridge the gap between therapeutic development and therapy access. TAR-DNA binding protein 43 (TDP-43) proteinopathy is a neuropathological feature observed in ~97% of amyotrophic lateral sclerosis (ALS), up to 57% of Alzheimer patients and ~45% of frontotemporal dementia (FTD) cases, including those carrying loss of function (LoF) mutations in the progranulin gene (GRN). TDP-43 proteinopathy is characterized by TDP-43 LoF and cytoplasmic mislocalization, together with TDP-43 hyperphosphorylated inclusions. Modeling TDP-43 pathology has been challenging in vivo and in vitro. Here we present a novel 3D-induced pluripotent stem cell (iPSC)-derived paradigm - mature brain organoids (mbOrg) - composed of cortical-like-astrocytes (iA) and neurons (iN). When devoid of GRN (GRN-/-), mbOrgs spontaneously exhibit TDP-43 mislocalization, aggregation, and LoF phenotypes, such as cryptic splicing of stathmin 2 (STMN2). Creating heterotypic mbOrgs by mixing different genotypes, showed that GRN-/- iA are drivers for TDP-43 pathology. Additionally, STMN2 cryptic splicing was rescued by the introduction of exogenous progranulin protein in the system, demonstrating a link between TDP-43 LoF and progranulin expression. This innovative iPSC-derived platform thus shows striking features of human TDP-43 pathology that can be partially rescued. We leverage this work to develop a patient-derived 3D platform for high-fidelity modeling and screening of TDP-43 proteinopathy and associated phenotypes for ALS and FTD. This platform includes mbOrgs developed using iPSCs from C9ORF72 and sporadic ALS patients that are compared with mbOrgs derived from healthy controls. This work can potentially uncover TDP-43 pathology-associated mechanisms and allow patient-tailored therapeutic screening for FTD and ALS.

Funding Source: NINDS, NIA

Keywords: organoid, ALS, neurodegeneration

5:45 PM – 6:45 PM

537

ASSAY DEVELOPMENT FOR FUNCTIONAL ANALYSIS OF IPSC-DERIVED NEURAL ORGANOIDS

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The flexibility and accessibility of induced pluripotent stem cell (iPSC) technology has allowed complex human biology to be reproduced in vitro at high throughput scales. Indeed, rapid advances in stem cell technology have led to widespread adoption for the development of in vitro models of neuron electrophysiology to be used in screening applications in drug discovery and safety. Furthermore, advanced cell preparations, such as spheroids or organoids, are under intense investigation with aims toward establishing mature physiologically relevant phenotypes in vitro. The objective of this work is to develop and optimize a functional assay of neural organoids in vitro. To that end, a customized multiwell microplate was designed specifically for neural organoid assays. The multiwell plate utilized a funnel design to target the neural organoids to a planar grid of microelectrodes embedded in the substrate of each well of the culture plate. Impedance measurements were used to quantify the attachment of the organoids to the substrate and microelectrodes, while functional activity was quantified via the electrophysiological measurements. Acute (no attachment) and chronic (surface coating mediated attachment) recording protocols were evaluated and compared. Specifically, the use of surface coatings (no coating, PEI, Matrigel) and centrifugation were evaluated for chronic recording protocols with regular measurements of attachment and network function over four weeks. PEI-coated wells exhibited the best performance, with neural organoid attachment in 100% of wells (38.7 +/- 4.2 Ohms for weighted mean resistance, 5.4 +/- 2.7 active electrodes per well) and the development of synchronous network activity over 2 weeks in culture. Matrigel and no coating conditions displayed fewer active electrodes overall, but consistent results across wells due to the funnel design targeting organoids to the array. These results support the continued development of quantitative assays of neural function with increased throughput for in vitro 3D neural organoid models.

Keywords: micro electrode array, iPSC-derived neural organoids, in-vitro model

5:45 PM – 6:45 PM

539

A BLOOD BRAIN BARRIER (BBB) MODEL TO TEST NOVEL THERAPEUTIC STRATEGIES FOR GLUT-1 DEFICIENCY SYNDROME

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Glucose transporter type 1 (GLUT1) is a critical protein allowing glucose efflux to the brain through the Blood-Brain-Barrier (BBB). Monoallelic or bi-allelic mutations in the GLUT1 encoding gene - SLC2A1 - result in GLUT1-deficiency (GLUT1DS) featuring intractable seizures, intellectual disability, ataxia, and dystonia. Pathogenic mechanisms are still unclear and specific therapeutic approaches lacking due to the difficulty to obtain appropriate human-derived in vitro models. In this study we aimed to generate a well-established in vitro Transwell model of BBB with brain endothelial cells derived from controls and GLUT1DS patients's induced pluripotent stem cells (iPSCs). We selected two patients carrying different mutations and showing a severe (p.Leu-124Trpfs*12) and a milder (p.Arg400Cys) GLUT1-deficiency phenotype. We characterized the BBB model with standard tests of BBB functionality, including transendothelial electrical resistance (TEER), GLUT1 expression, immunocytochemistry for endothelial and tight junction markers and paracellular transport across the barrier. The results indicated a different rate of BBB differentiation between the severe and the milder patients' cells with respect to the controls, probably due to the drastic impairment in the nutritional molecules uptake. To improve the BBB model, we are moving from a static to dynamic culture system ensuring optimal microenvironment conditions and mimicking the in vivo physiology. A standardized BBB model could be used to test novel therapeutical approaches aimed at enhancing BBB glucose permeability. In this regard, we will initiate the search of genetic (e.g. by CRISPRa technology) and pharmacological tools able to increase the expression of SLC2A1 at the transcriptional and translational levels.

Funding Source: GR-2021-12372966

Keywords: Blood Brain Barrier (BBB), iPSCs, CRISPRa

5:45 PM – 6:45 PM

541

CHARACTERIZATION OF PCARE GENE AND PROTEIN EXPRESSION IN RETINAL MODELS

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Inherited retinal dystrophies, such as retinitis pigmentosa (RP), which affects one in every 4,000 people, and cone-rod dystrophy (CRD), which affects one in every 30,000 people, cause progressive degeneration of retinal photoreceptor cells, ultimately leading to blindness. The C2orf71 gene, also known as the photoreceptor cilium actin regulator protein (PCARE), along with WASF3, is required for photoreceptor outer segment (OS) disk neogenesis. Mutations in this ciliary gene cause autosomal recessive RP type 54 and CRD, which are incurable. We have currently diagnosed a family carrying the C2orf71 mutation c.860dup, p.L288Afs*23 + c.1795T>C, p.C599R, which includes a patient with homozygous mutation and CRD diagnosis, as well as two asymptomatic carriers with heterozygous mutations. While these mutations are predicted as pathogenic, the underlying mechanisms are unknown. Here, we characterized the PCARE gene and protein expression and localization in human retinal cell lines (Y79, ARPE-19, and retinal pigment epithelial (RPE) cells), retinal organoids (ROs) derived from human induced pluripotent stem cells, and mouse and porcine retinas to confirm its exclusivity to the retina and ciliary location. In addition, we analyzed the in vitro pathogenic consequences of C2orf71 mutations and the cilium length in patient-derived urine cells. PCARE was highly expressed in the retinas, Y79, and RPE cell lines. Furthermore, we found PCARE expression in ROs from the start of retinal differentiation (day 40), with the highest pick at day 150, corresponding to OS formation. PCARE was found in the cilia of human cell lines and the OS of photoreceptor cells in ROs. Plasmid transfection with wild type, L288Afs*23, and C599R plasmids indicated the missense mutation (C599R) did not affect protein location, whereas L288Afs*23 produced a degraded truncated protein. Confirming PCARE expression in the cilium and OS highlights ROs' potential as a platform for PCARE disease modelling, as well as for testing functionality and pathogenicity of patient-specific PCARE mutations.

Keywords: cone-rod dystrophy, PCARE, retinal organoids

5:45 PM – 6:45 PM

543

CEREBRAL ORGANOID AS A TOOL TO EVALUATE THE NEURAL OPIOID TOXICITY

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Organoids represent a model of choice for the study of developmental processes, neurodegenerative and genetic pathologies but also the impact of molecules on brain development. If the use of analgesic treatment with opioids effectively relieves pain, their increasingly widespread and sometimes misused use now poses a public health problem with risks of addiction and adverse effects. The long-term impact on brain development has not yet been evaluated. By taking advantage of the development of cortical organoids during a long kinetics of more than 70 days, we evaluated the action of Tramadol at 3, 30 and 300 µM and its main metabolite (M1) at 3 and 30 µM) on both the structure of organoids and the expression of specific markers as well as on the global transcriptomic profiles. Following these treatments, it appears that structural alterations are observed, in particular with the disappearance of rosettes, specific to the three-dimensional organization of organoids as well as modification of the distribution of early and late markers such as SOX2, PAX6, NESTIN, OLIG2, etc. among others. Transcriptional analyzes confirm significant changes in gene expression profiles both at moderate doses, but also at quite low doses (3 µM) like those found in patients. With these new data, it appears that tramadol and its metabolite M1 very significantly modify and disrupt the development of cortical organoids, which could suggest a similar effect during the development of the fetal brain. These alterations could have a long-term impact on the brain and associated cognitive faculties.

Keywords: cerebral organoid, opioid toxicity, induit pluripotent stem cell “IPS”

5:45 PM – 6:45 PM

545

CONTRIBUTIONS OF GABAERGIC INHIBITORY NEURONS TO THE PATHOPHYSIOLOGY OF ALZHEIMER’S DISEASE

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Alzheimer’s disease (AD) is a highly prevalent neurodegenerative disorder characterized by extracellular plaques composed of amyloid β-protein (Aβ) and intraneuronal tangles consisting of altered forms of the Tau protein. In addition, synaptic dysfunction has been identified as a component of AD, suggesting that changes in electrophysiological communication between neurons may be contributing to the pathogenesis of AD. While much of the focus in AD research has traditionally been on excitatory neurons and their synaptic connections, emerging evidence suggests that inhibitory neurons, particularly those utilizing gamma-aminobutyric acid (GABA) as their primary neurotransmitter, play crucial roles in the pathophysiology of the disease, contributing to synaptic impairment and cognitive decline. Imbalances in GABAergic signaling contribute to the excitatory/inhibitory (E/I) imbalance hypothesis of AD, where excessive neuronal excitability due to impaired inhibition contributes to neuronal dysfunction and degeneration. A major unresolved question is: what are the contributions of inhibitory GABAergic neurons to the AD phenotype? Here, we examine neurons derived from iPSCs of patients harboring an AD-causing APP mutation (APPV717I) to quantify AD-relevant phenotypes following directed differentiation to glutamatergic (Ngn2) and GABAergic (Ascl1/Dlx2) neurons. We compare control and APPV717I subjects and find that APPV717I neurons directed to GABAergic neuronal fates generated less Aβ40 with a higher 42:40 ratio than control neurons. Further, we show that APPV717I neurons express higher levels of total and phospho-Tau proteins relative to control neurons when directed to a glutamatergic neuronal fate, but not when directed to a GABAergic neuronal fate. Additionally, APPV717I GABAergic iNs exhibit altered expression and secretion levels of the neurotransmitter GABA, compared to control GABAergic iNs, implicating the potential for network disruption. Taken together, our results suggest that inhibitory neurons may have an altered profile in AD. Understanding the contributions of inhibitory neurons, particularly GABAergic interneurons, could lead to the development of targeted therapeutic interventions aimed at restoring inhibitory balance and preserving synaptic function.

Keywords: Alzheimer’s disease, inhibitory neurons, disease modeling

5:45 PM – 6:45 PM

547

AN IPSC-DERIVED CEREBRAL ORGANOID MODEL FOR CEP290-ASSOCIATED NEURODEVELOPMENTAL CILIOPATHIES IDENTIFIES ANOMALIES IN CILIARY MORPHOLOGY

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Ciliopathies are a group of human Mendelian disorders caused by dysfunction of primary cilia, small ubiquitous sensory organelles protruding from the surface of most cells, required for signal transduction. These disorders are associated with central nervous system (CNS) anomalies, particularly exemplified by the neurodevelopmental disease Joubert syndrome (JBTS). JBTS is characterized by a highly specific mid-hindbrain malformation whose underlying pathomechanism remains unclear. Moreover, the presence of non-structural CNS

defects such as seizures or intellectual disability implies a role for cilia in neuronal function beyond transmission of developmental signaling pathways. To understand the role of cilia and of JBTS genes in the CNS, we are generating iPSC-derived in vitro models for JBTS. We established a biobank for JBTS through generation of CRISPR-engineered isogenic human induced pluripotent stem cell (hiPSC) lines that carry mutations in selected JBTS-associated cilia-related genes such as the ciliary gene CEP290. To elucidate the consequences of mutations in CEP290 on brain development, we differentiated control, CRISPR-edited and patient-derived hiPSCs into 3D cerebral organoids. We find that CEP290-mutated hiPSCs can differentiate into cerebral organoids and single-cell RNA sequencing confirmed that the overall generation of a variety of neuronal cell populations does not appear to be significantly impacted up to day 80. We do, however, observe an increased propensity to generate choroid plexus in mutant organoids compared to controls and a disorganization of cortical plate units. Furthermore, we observe striking morphological anomalies of a subset of primary cilia in the lumen of cortical plate units generated, which is a consistent finding throughout our genetically engineered and patient-derived cerebral organoids. Altogether, this study represents an advancement in understanding JBTS in the context of human brain development.

Keywords: cerebral organoids, primary cilia, Joubert syndrome

5:45 PM – 6:45 PM

549

CORTICAL ASSEMBLOIDS SUPPORT THE DEVELOPMENT OF FAST-SPIKING HUMAN PVALB+ CORTICAL INTERNEURONS AND UNCOVER SCHIZOPHRENIA-ASSOCIATED DEFECTS

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Defects in fast-spiking parvalbumin positive (PVALB+) cortical interneurons have frequently been reported in schizophrenia patients, yet our understanding of how these defects emerge and contribute to the disease is lacking. PVALB+ cortical interneurons undergo a protracted stage postnatal development making their derivation from human pluripotent stem cells (hPSCs) exceedingly difficult and placing a significant roadblock in the modeling of their role in neuropsychiatric diseases using hPSC-based systems. Here, we have developed a cortical assembloid system that supports the derivation and maturation of fast-spiking PVALB+ cortical interneurons. We have interrogated the development of these cells with single-cell RNA-sequencing and shown that their expression profiles align with developing primary cortical interneurons and that they co-express key markers of cortical interneurons, including LHX6. Functional characterization revealed that PVALB+ cortical interneurons in cortical assembloids display the distinctive features expected of fast-spiking cortical interneurons and that cortical assembloids containing PVALB+ cortical interneurons display network-level gamma-band oscillatory rhythms, a process linked to cognition that is often disrupted in schizophrenia patients. Finally, we characterized cortical interneuron development in a series of CRISPR-generated isogenic structural variants strongly associated with schizophrenia risk. Here we identified variant- and stage-specific cortical interneuron defects in both tangential migration and the molecular profile of PVALB+ cortical interneurons. Together, our findings highlight plausible mechanisms regarding how the disruption of cortical interneuron development may contribute the development of schizophrenia and provides an exciting human platform for the study of bone fide fast-spiking PVALB+ cortical interneurons.

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Keywords: cerebral organoids, cortical interneurons, schizophrenia

5:45 PM – 6:45 PM

551

ALTERED FUNCTIONAL AND METABOLIC PHENOTYPES IN HUMAN IPSC-DERIVED FOREBRAIN-SPECIFIC FRAGILE X SYNDROME ASTROCYTES

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Fragile X syndrome (FXS), a leading inherited cause of autism spectrum disorder and intellectual disability, has been studied extensively using rodent models. More recently, human stem cell-derived model systems have also been used to gain mechanistic insights into the pathophysiology of FXS. However, these studies have focused almost exclusively on neurons. Further, despite growing evidence for a key role for glia in neuronal function in health and disease, little is known about how human astrocytes are affected by FXS. Hence, in this study we describe differentiation paradigms for human induced pluripotent stem cells that generate healthy and FXS patient-derived astrocytic progenitor cells (APCs). CNTF exposure of APCs yielded forebrain-specific GFAP-expressing astrocytes. We first generated hiPSC-derived astrocyte progenitor cells (APCs) that are forebrain-specific. Characterization of healthy/FXS APCs, in turn, set the stage for exploring various functional properties of forebrain-specific astrocytes generated. First, we found a reduction in the number of GFAP-positive astrocytes in FXS lines relative to healthy astrocytes. Next, we examined changes in intracellular calcium responses triggered by ATP in these cells with slow initiation and significantly reduced number of calcium responses. Further, FXS astrocytes display dysregulated glycolytic and mitochondrial metabolism. We found enhanced rates of glycolysis, glycolytic capacity and glycolytic reserve in human iPSC-derived FXS astrocytes. FUNCAT assays, revealed altered protein synthesis in the glial progenitor cells which disappeared during the maturation phase. This platform also offers the added advantage of a renewable source of human glial cells with regional specificity, thereby overcoming challenges posed by primary human tissue that are not easily available for neurodevelopmental

disorders like FXS. Taken together, these findings provide a human platform for the investigation of cell-autonomous and non-cell autonomous consequence of astrocyte mutations in neurological disorders.

Keywords: human iPSC, astrocytes, neurological

5:45 PM – 6:45 PM

553

CHARACTERIZING FUNCTIONAL AND MOLECULAR SIGNATURES OF INFLAMMATORY SENSITIZATION IN HPSC-DERIVED SENSORY NEURONS

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Nociceptors are specialized sensory neurons that are critical for the detection and transmission of noxious stimuli. These cells display heightened excitability during inflammatory sensitization, a process crucial towards the development and maintenance of chronic pain. While various processes contributing to inflammatory sensitization have been extensively studied in rodents, differences between transcriptomics and physiological properties across species indicate that human nociceptors may exhibit distinct patterns of gene expression during sensitization, which are not well understood. Using a cocktail of inflammatory mediators, we performed multi-electrode array, patch-clamp recordings, and ribosome and proteomic profiling in human pluripotent stem cell-derived sensory neurons (hPSC-SNs) to characterize changes in functional and molecular signatures during the induction (30 min) and maintenance (24 hour) of sensitization. Our results reveal unique hPSC-SN firing properties and gene expression patterns at both phases, indicating significant modifications to functional and molecular pathways in response to acute and prolonged sensitization. The integration of these methods in a cellular model of pain will enable the discovery of novel biological pathways and potential drug targets relevant to human pain etiology.

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Keywords: sensory neurons, sensitization, pain

5:45 PM – 6:45 PM

555

CRACKING THE CODE: 7-DAY DIRECTED DIFFERENTIATION OF HIGH PURITY MOTOR NEURONS FROM ANY HIPSC LINE

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Motor neurons are involved in the voluntary control of muscular contraction and are the primary cell type affected in amyotrophic lateral sclerosis (ALS), an incurable and deadly neurological disease resulting in paralysis. Given their unlimited potential for self-renewal and differentiation, it has been a longstanding goal to model ALS specifically using human pluripotent stem cell (hiPSC)-derived motor neurons for drug discovery in order to develop life-saving treatments for this disease. While numerous methods have been published to differentiate motor neurons from hiPSCs, all protocols to date demonstrate substantial donor-to-donor variability, which results in a failure to produce motor neurons at high yields and purity from each hiPSC line. Here, we report a drastically accelerated, developmentally guided, directed differentiation protocol based on small molecules and growth factors to generate motor neurons in only seven days from three different hiPSC lines (wild type - female, wild type - male, TDP43 ALS patient - male). Developmental stages of the differentiation and motor neurons were characterized via immunocytochemistry, qPCR, and RNA sequencing. Several functional assays were developed, including multi-electrode array (MEA) plates and microfluidic co-cultures with human skeletal muscle (hSKM). Motor neurons generated from all lines expressed MNX1, ISLET1, ChAT, and TUJ1 at a purity greater than 80% as confirmed by immunocytochemistry. qPCR and bulk RNAseq results showed expression of key motor neuron genes and molecular similarity to primary human spinal motor tissues. On MEA plates, cultures exhibited rapid maturation with burst spiking within a week. Motor neurons co-cultured with hSKM in microfluidic devices formed neuromuscular junction morphologies, and controlled stimulation of the motor neurons resulted in muscle activation. In conclusion, we have demonstrated a rapid seven day protocol to generate motor neurons from multiple donor lines, along with their utility in various screening assays for disease modeling, ALS drug discovery, and basic research.

Keywords: motor neurons, ALS, co-cultures

5:45 PM – 6:45 PM

557

CEREBELLUM AND SOCIAL COGNITION: NEW INSIGHTS FROM MODELLING PENETRANT AUTISM VARIANTS IN BENCHMARKED CEREBELLAR ORGANOIDS

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The cerebellum is increasingly recognized as central to tasks that go well beyond the regulation of balance and motor coordination including emotion regulation, language processing, attention and social behavior. Consequently, cerebellar perturbations early in life are associated with intellectual disability and autism spectrum disorders (ASD). Patterned brain organoids, self-organizing 3D structures generated from pluripotent stem cells, enable the study of developmental trajectories associated with specific brain regions, recapitulating their salient cytoarchitectural features and their underlying molecular cascades. In this work, we introduce cerebellar organoids (CbOs) as new platform to mechanistically dissect the endophenotypes underlying ASD. We present a longitudinal characterisation of CbOs from multiple induced pluripotent stem cells lines, benchmarking their transcriptomic profile compared to the gene expression of the developing human fetal cerebellum. At early stages of CbOs differentiation, we identified an enrichment in the expression of canonical ASD-related genes, including the chromodomain helicase DNA-binding protein 8 (CHD8). We next derived CbOs from CHD8+/+ pluripotent stem cells and their isogenic counterpart CHD8+/E1114X, carrying a high-penentrance ASD-associated variant causative of cognitive, motor and sensory impairments. Bulk transcriptomics uncovered a major CHD8-dependent dysregulation, cascading onto downstream transcriptional alterations and impinging on the WNT pathway and tissue patterning. Single-cell transcriptomics revealed that the developmental trajectories of CHD8+/E1114X -derived CbOs diverge from their controls already at early stages of development. In particular, cerebellar granule cells progenitors and oligodendrocytes were dramatically affected in CHD8+/E1114X mutants, leading to functional alterations of the neuronal network activity at later timepoints. Building on a longitudinal transcriptomic benchmarking of CbOs as foundational resource for the field, our work provides a first demonstration of the specific molecular effects of ASD-related mutations in the cerebellum, offering a proof of concept for CbOs as tool to dissect cerebellar contribution to human sociability and neurodevelopmental conditions.

Keywords: cerebellum, organoids, ASD

5:45 PM – 6:45 PM

559

A NOVEL, DSMAD-INDEPENDENT INDUCTION PARADIGM FOR RAPID GENERATION OF HIPSC-DERIVED NEURONS AMENABLE TO REGIONAL PATTERNING

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Induced overexpression of Neurogenin 2 (iNGN2) is a valuable strategy to accelerate the conversion of human induced pluripotent stem cells (hiPSCs) into neurons for modeling of neurodegenerative diseases such as Alzheimer's Disease (AD). However, iNGN2 alone generates neurons of a mixed regional identity, potentially limiting their relevance for modeling AD. We developed an alternative neural induction strategy, which prepatterns hiPSCs before NGN2 induction to obtain a stronger cortical identity. This strategy involves inhibiting different pathways critical for self-renewal and meso-endodermal differentiation, independent of commonly applied dual SMAD inhibition (dSMADi). Combined with iNGN2, this induction paradigm quickly leads to homogeneous cultures of excitatory cortical neurons. Furthermore, the cells are responsive to regional patterning cues during the short neural induction pulse. This enables generation of neurons from different regions of both the central and peripheral neuron systems, including midbrain dopaminergic, motoneurons and sensory neurons, improving in vitro models for a range of neurological diseases and neurodegenerative disorders. Cortical neurons generated with this paradigm are suitable for an AD-relevant tau aggregation assay, similar in performance to cortical neurons derived from the dSMADi protocol, highlighting their applicability in

disease – relevant in vitro models. Disclaimer: CH, AW, MJH, NN, CS, LR, TL, CU, LNM, JK, DG, DS, HL, LB, BMS, MB, RW, JR, IW, JDM, MC, PR are employees of AbbVie. VH was employee of AbbVie at the time of the study. AK was employee of the Institute of Molecular and Cell Biology, Mannheim University of Applied Sciences at the time of the study and was funded by the Graduiertenkolleg TASCDT of the MWK Baden-Württemberg and the Albert und Annelise Konanz-Stiftung. R.R and M.H. are current employees of Center for Mass Spectrometry and Optical Spectroscopy, Mannheim University of Applied Sciences and Institute of Medical Technology, Heidelberg University and Mannheim University of Applied Sciences and have no funding to disclose. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

Keywords: neuroscience, regional patterning, neuron differentiation

5:45 PM – 6:45 PM

561

A HUMAN CORTICAL ASSEMBLOID MODEL OF DRAVET SYNDROME

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Animal models have been instrumental in advancing our understanding of DS pathogenesis and as preclinical models to test advanced gene therapies. However, important genomic differences in SCN1A regulation between rodents and humans and influences of the genetic background may hinder our ability to effectively translate preclinical findings in rodents onto the clinic. Patient-derived somatic cells can now be converted into human iPSCs, capable of differentiating into a variety of cellular lineages. Leveraging this, the generation of stem-cell derived 3D cell cultures – known as organoids – allows to model brain structures in remarkably complex ways. Cortical assembloids are generated from the fusion of a cortical and a subpallial organoid, which allows the generation of both excitatory and inhibitory cortical neurons and recapitulates the tangential migration of cortical interneurons from the subpallium into the cortex, as it occurs in vivo. Importantly, we observed Nav1.1 expression at later stages of development in this model. Therefore, we generated iPSC lines from three Dravet patients and corrected two of them using CRISPR. Using a protocol we have established to record evoked epileptiform activity in mature assembloids, using local field potential recordings we have observed for the first time a clear epileptic phenotype in DS assembloids. Furthermore, our data shows a novel early developmental phenotype associated with SCN1A loss-of-function. Our human DS cortical assembloids could be pivotal to better understand disease pathogenesis in a human context and to improve preclinical testing of novel advanced therapy strategies.

Keywords: Dravet syndrome, SCN1A, assembloid

5:45 PM – 6:45 PM

563

CHARACTERISATION OF CORTICAL NEURAL PRECURSOR CELL DEFECTS IN DLG2-/- HUMAN BRAIN ORGANOIDS

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Schizophrenia (SZ) is a neurodevelopmental, neuropsychiatric disorder, affecting 24 million people worldwide with positive, negative, and cognitive symptoms. Both environmental and genetic factors are involved in the disease aetiology. Through population studies, large numbers of genetic risk factors are revealed including de novo deletions in DLG2. DLG2 is a well-known postsynaptic density (PSD) protein linking receptors and intracellular signalling molecules in PSD of neurons. Our previous study highlighted its expression and function in human cortical neurons during the prenatal neurogenesis period, contributing to neuronal maturation in cell identity gene expression, morphology and action potential firing. Although neuronal phenotypes were investigated in that study, evidence points to the earlier disease process in neural precursor cells (NPCs). NPCs are a diverse population, and their correct number and mode of divisions is important for generating the right quantity and types of cortical neurons. 3D brain organoids offer a great platform to study NPC organisation and function. Here, we aim to study the effect of DLG2 deficiency in human cortical NPC types, their behaviour and their interaction with neurons in organoids. We also aim to identify spatial expression of DLG2 within NPCs as they don't have PSDs like neurons and find its interacting partners to reveal their signalling pathway during early neurogenesis. For this, we have generated DLG2-/- cortical organoids. We are investigating the percentages and morphologies of ventricular radial glial cells (RGs), outer RGs, truncated RGs and intermediate progenitor cells and their daughter cell fate/identity in WT and DLG2-/- organoids. We are also generating DLG2-TurboID knock-in hESCs to determine DLG2s-interacting partners using proximity labelling. The results will be shared in the meeting as the data is being generated now. By elucidating DLG2 function during prenatal development we will gain a better understanding of the very initial cellular pathways involved in SZ.

Keywords: DLG2, organoids, schizophrenia

5:45 PM – 6:45 PM

565

ADVANCING CIPN AND PAIN DRUG DISCOVERY USING HUMAN IPSC-DERIVED SENSORY NEURONS

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Sensory neurons of the peripheral somatic nervous system respond to various impulses from sensory organs including touch, position in space, temperature, and pain. Sensory neurons are of great value in pain research, aiding in the development of better chemotherapy drugs with reduced incidence of chemotherapy-induced peripheral neuropathy (CIPN) and hematologic toxicity. Differentiating human induced pluripotent stem cells (iPSC) enables access to authentic human sensory neurons, but challenges arise when attempting to consistently produce them at large scale. Here we report the large-scale, directed differentiation of human sensory neurons from both male and female iPSC lines. These iPSC-derived sensory neurons have high purity (>80% BRN3A+/UCHL1+) and express hallmark nociceptive channels (i.e., Nav1.7, Nav1.8) and receptors (i.e., TRPV1, P2RX3 and NTRK). We characterized sensory function of these neurons using electrophysiology and calcium imaging. Notably, we confirmed the presence of P2RX and TRP channels and their response to different stimuli including ATP, capsaicin and menthol using calcium imaging and multielectrode array. Sensory neuron purity was consistent across both iPSC donor and between production lots, while sensory neurons cultured in medium and high-throughput tissue culture plates showed uniform responses across-well and across production lots in response to sensory agonists. Additionally, these sensory neurons display cytotoxicity to chemotherapeutic drugs (i.e., paclitaxel and Vincristine) in a dose-dependent manner and release substance-P and CGRP peptides in response to pain mediators. Our data demonstrate a process for robustly generating iPSC-derived sensory neurons across different iPSC donor lines that recapitulate human known pharmacology related to pain and CIPN. Large-scale production and accessibility of human sensory neurons will expedite research of sensory neuron properties and translation into high-throughput screening platforms for pain drug discovery.

Keywords: sensory neuron differentiation, chemotherapy-induced peripheral neuropathy, pain

5:45 PM – 6:45 PM

567

CONVERGENT TRANSCRIPTIONAL DYNAMICS OF MICROGLIA IN THE MOUSE SPINAL CORD DURING AGING AND ALS

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease resulting in paralysis and death three to five years after diagnosis. It is a late-onset disease that typically presents in older populations, usually between the ages of 51 and 66, with aging being its greatest risk factor. However, cell models do not fully recapitulate aged conditions and often require external stressors to display disease phenotype. Thus, it is crucial to understand the transcriptomic events that occur during aging in order to modulate these effects in cell models. We have previously shown that aging pathways are disrupted in ALS spinal cords and spinal motor neurons. However, the anatomical and cell-specific aging within the spinal cord and its contributions to ALS pathogenesis are still unclear. In this study, we have created a single-nuclei transcriptomic atlas of mouse spinal cords from wild type (WT) and ALS mice carrying the SOD1G93A transgene. We profiled WT mice from embryonic day 13.5 up to 800 days old, and we profiled the ALS littermates from the same life stages up until they reached their paralytic endpoint at 160 days old. Our findings indicate that astrocytes and microglia express the most differentially expressed genes between wild type and ALS at the endpoint of the disease. However, differential expression analysis during the onset reveals a sharp increase in differentially expressed genes in the cervical region of the spinal cord in microglia, suggesting spinal region-specific effects that may drive disease progression. Clustering analysis revealed that younger ALS microglia resemble old WT cells at the transcriptomic level. Weighted gene network correlation analysis reveals that this accelerated aging in ALS microglia may be attributed to three gene networks. The network eigengene expression shows that the expression of these genes is also specific to the spinal region, with the cervical region expressing genes that may be protective against ALS. These insights provide a framework for further studying factors driving ALS disease progression and offer valuable targets for faithfully modeling aging in in vitro models, developing ALS therapies, and guiding comparative aging studies.

Funding Source: California Institute for Regenerative Medicine, Center for Research in Women's Health, Louis B. Mayer Foundation, National Institute on Aging, Winnick Family Foundation

Keywords: transcriptomics, ALS, aging

5:45 PM – 6:45 PM

569

A SINGLE-CELL CONSENSUS ATLAS OF IN VIVO AND IN VITRO MIDBRAIN DOPAMINE NEURONS

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Parkinson's disease (PD) is a movement disorder that involves the selective loss of midbrain dopamine (mDA) neurons in the substantia nigra. Human pluripotent stem cells (hPSCs) represent a powerful technology to study and potentially treat PD. Methods to generate mDA neurons from human stem cells have been pioneered by numerous groups, and enormous progress has been made to increase and refine the generations of highly pure mDA neurons. Despite such progress, it is difficult to determine how well these protocols compare in terms of cell composition and authenticity to the primary tissue. Furthermore, for the latter point, there is a strong necessity for a "ground truth" fetal and adult midbrain atlas that can be systematically queried to score these hPSC-derived mDA neurons. To address this bottleneck, we started to create a consensus fetal and adult human midbrain single-cell atlas to resolve nomenclature differences between studies and to define hallmark gene signatures, gene regulatory network and signalling pathways of each cell state. In parallel, we integrated single-cell data derived from mDA protocols raging form monolayer cultures and organoids to grafted cells, to create a compendium to address mDA authenticity and cell diversity by scoring and classifying cells to the consensus atlas. Preliminary analysis of different DA protocols reveals a large diversity both in terms of cell types generated and DA authenticity compared to the primary tissue, strengthening the importance of this integrative study. Overall, we believe, our standardized high quality in vivo and in vitro midbrain atlas will be of considerable benefit to the stem cell community and facilitate efforts to optimize differentiation strategies towards truly authentic mDA neurons.

Funding Source: NCI Cancer Center Support Grant (CCSG, P30 CA08748), BlueRock Therapeutics, 1R01 NS118067-01A1 (L.S., D.B.), ASAP initiative, ASAP-020370, Druckenmiller fellowship, Marie-Josée Kravis Women in Science Endeavor fellowship.

Keywords: single-cell consensus atlas, midbrain dopamine neurons, hESC

5:45 PM – 6:45 PM

571

CONDITIONAL STRATEGIES FOR TUNABLE GENETIC MODELING ON HIPSCS

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Human induced pluripotent stem cells (hiPSCs) have revolutionized disease modeling and scientific innovation. Their potential, along with advanced differentiation protocols and organoid technology, facilitates the recreation of complex human tissue structures. As complexity grows, sophisticated genetic control systems become paramount particularly for accurate disease modeling. Our research capitalizes on genetic engineering, using CRISPR/Cas9 and conditional site-specific recombination systems for flexibility and precision in hiPSC manipulation. Our focus is on strategies enabling precise spatiotemporal control over transgene expression, creating dynamic cellular systems. The adaptability of hiPSCs to genetic engineering enables the incorporation of complex genetic constructs, facilitating the generation of conditional and inducible hiPSC lines for tailored, controlled gene expression. This approach includes inducible expression of potent biomolecular tools for dissecting network intricacies. Here we show the utility of conditional strategies in generating hiPSC-based models, providing a versatile platform for exploring cellular mechanisms. Enabling precise control over gene expression has the potential to enhance targeted disease modeling, offering insights into genetic regulation and biological circuits. Our research leverages genetic engineering, using CRISPR/Cas9 and conditional expression for better flexibility and precision in manipulating hiPSC genetics. Our primary focus is on strategies enabling precise spatiotemporal control over transgene expression, opening possibilities for dynamic and responsive model systems.

Funding Source: The study is funded by SSMF Svenska Sällskapet för Medicinsk Forskning (Swedish Society for Medical Research) and Cancerfonden (Swedish Cancer Society)

Keywords: hiPSCs, modeling, tools

5:45 PM – 6:45 PM

573

ABERRANT CHCHD2-ASSOCIATED MITOCHONDRIOPATHY IN KII ALS/PDC ASTROCYTES

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Patients with Western Pacific Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex (ALS/PDC) exhibit clinical and pathological features that are characteristic of amyotrophic lateral sclerosis (ALS) or Parkinson's disease (PD). To study this complex neurodegenerative disease, the etiology of which remains elusive, we focused our study on the Japanese variant, which predominantly affects people living in the Kii Peninsula of Japan (Kii ALS/PDC). Why this rare disease only affects residents of certain areas is still a mystery, so we decided to differentiate patient induced pluripotent stem cells into astrocytes (Kii-iPasts), considering emerging evidence suggesting the potential involvement of astrocytes in the onset and progression of Kii ALS/PDC. To generate Kii-iPasts, we used our previously reported method to efficiently generate astrocytes from control iPSCs and cultured multiple lines (2 healthy and 5 Kii ALS/PDC cases) for study. In addition to the major neurological disorders represented in Kii-iPasts, gene ontology from RNA sequencing revealed pathways related to cilia dysfunction, which are known to play a role in neurodegeneration. Kii-iPasts exhibited abnormal mitochondrial cristae and a number of metabolic alterations, such as reduced glutamate uptake and reduced ATP production. In addition, we isolated a number of genes and proteins of interest whose expression is altered in Kii ALS/PDC, with a major finding that a mitochondria-related gene, CHCHD2, previously identified as a PD gene, is significantly downregulated in Kii-iPasts and in neuropathologic specimens from the thoracic spinal cord of a deceased donor. Lentiviral overexpression of CHCHD2 could partially rescue some of these defects in some Kii-iPasts lines, providing a promising therapeutic target for patients. In conclusion, our findings suggest a potential reduction in the support that Kii ALS/PDC astrocytes provide to neurons in the context of CHCHD2 and mitochondrial health, providing the first genetic explanation for this disease for which no causative gene has been identified.

Funding Source: JSPS Japan AMED Japan Intractable Diseases Research Foundation Kanae and Uehara Memorial foundations YUKIHIKO MIYATA MEMORIAL TRUST FOR ALS RESEARCH Okasan-Kato Foundation Research and Yoshio Koide grants Japan ALS Association

Keywords: Kii ALS/PDC, Parkinson disease, CHCHD2

5:45 PM – 6:45 PM

575

CIRCADIAN PROTEIN REGULATION OF VOLTAGE GATED SODIUM CHANNELS AS A NOVEL THERAPEUTIC TARGET IN PAIN

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Pain displays significant diurnal fluctuations, yet the underlying mechanisms remain elusive. In this study, we investigated the relationship between pain and the circadian system using hiPSC-derived nociceptors. We show molecular clock rhythms can be induced in nociceptors and that it governs time-dependent neurophysiological responses. We showed using patch clamp electrophysiology that voltage gated sodium currents exhibited substantial time-dependent variation. This matched findings in mouse primary nociceptors, indicating conserved mechanisms. We also found that human iPSC derived nociceptor firing rates as measured using multi-well multi-electrode arrays, correlated with the variation in sodium current. Administration of the cryptochrome CRY1 and CRY2 small molecule stabilizer KL001, rapidly reduced sodium currents and reversed nociceptor hyper-excitability. We then showed that in iPSC nociceptors derived from a patient with the chronic pain condition, erythromelalgia, KL001 was able to reverse the hyper-excitability phenotype. These findings underscore promising avenues for addressing chronic pain by directly modulating circadian mechanisms.

Keywords: cryptochrome, hiPSC-derived nociceptors, chronic pain

5:45 PM – 6:45 PM

577

CHARACTERIZATION OF SCN1A METHYLATION PATTERNS IN INDUCED PLURIPOTENT STEM CELL-DERIVED CELLULAR MODELS AND TARGETING THEM AS AN ALTERNATIVE THERAPEUTIC STRATEGY IN DEVELOPMENTAL EPILEPSIES

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Epilepsy is a disorder of the central nervous system, characterized by abnormal neural activity, resulting in chronic seizures. Approximately 40% of known epilepsy disorders are linked to genetic causes. SCN1A encodes for the subunit of the sodium channel and is crucial for synaptic plasticity in the healthy brain. Mutations in SCN1A cause Dravet syndrome, which is a severe form of epilepsy, impacting a child's development, often being resistant to antiepileptic drugs. Despite

identification of various genetic mutations associated with SCN1A, little is known about the impact of these mutations on gene transcription in epilepsy. Gene transcription changes in various epilepsies are driven by epigenetic modifications, like DNA methylation (DNAme). Two forms of DNAme, 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC) have been identified in the brain and are dysregulated in epilepsy. However, changes in 5-mC and 5-hmC and their association with SCN1A dysfunction have not been explored. Here, we sought to identify and characterize changes in DNAme in the presence of downregulated expression of SCN1A. Using an induced pluripotent stem cell (iPSC)-based neurodevelopmental model, we will trace 5-mC and 5-hmC epigenetic changes at different stages of cellular differentiation. Our preliminary findings suggest that SCN1A knockdown (KD) results in 5-mC and 5-hmC changes in iPSC and neural progenitors (NPC). These 5-mC and 5-hmC changes were localized to transcription start site and enhancer sequences. While we found that both 5-mC and 5-hmC marks were decreased in SCN1A KD iPSCs, SCN1A KD NPCs 5-hmC marks were significantly increased, suggesting that manipulation of SCN1A is sufficient to alter 5-mC and 5-hmC marks. Additionally, SCN1A KD resulted in downregulation of DNA methyltransferase 1 gene expression with no effect on 5-mC and 5-hmC levels in iPSCs and NPC. In summary, these findings suggest loss-of-function in SCN1A results in altered DNAme patterns, which might influence gene transcription mechanisms early in neuronal differentiation.

Keywords: SCN1A, DNA methylation, iPSC

5:45 PM – 6:45 PM

579

INVESTIGATING THE DYNAMIC ROLE OF ALPHA SYNUCLEIN IN NEURONAL INNATE IMMUNITY IN HUMAN EMBRYONIC STEM CELL-DERIVED CORTICAL NEURONS UNDER LIVE IMAGING

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Alpha synuclein (αSyn), and the modified, oligomeric and fibrillar disease state conformations it adopts, is a major pathological molecular driver of dementia with Lewy bodies (DLB), Parkinson's disease (PD), and other synucleinopathies. However, αSyn's physiological role is undefined, leading to poor understanding of pathophysiological disease mechanisms which could contribute to αSyn-mediated neurodegeneration. Recent evidence suggests αSyn has innate immune roles in protecting neurons from neurotrophic viruses, with mouse in vivo models showing increased susceptibility to viral-induced infection and death when αSyn is knocked-out. However, no functional studies in human disease models have investigated the innate immune role of αSyn in the CNS. We investigated αSyn transcription, protein expression, and protein localisation, in human pluripotent stem cell (hPSC)-derived cortical neurons stimulated with viral mimics of infection and innate immune activators. We showed that Type-I interferon (IFN) stimulation in human pluripotent stem cell (hPSC)-derived cortical neurons showed significantly modulated extracellular αSyn secretion into conditioned media, and increased nuclear localisation of αSyn in a subset of cortical neurons. This may elude to a dynamic change in subcellular localisation of αSyn when under acute interferon treatment. Thus, we have constructed a novel live-imaging aSyn-HaloTag live reporter construct in hPSC-derived cortical neurons, whereby observing live changes in subcellular localisation may shed light into αSyn's physiological and potentially protective role in cortical neurons. Furthermore, changes in αSyn structure and localisation due to innate immune stimulation may reveal links between infection and αSyn-mediated neurodegenerative diseases such as DLB and PD.

Keywords: neuroscience, neurodegeneration, immunity

TRACK: CLINICAL APPLICATIONS (CA)

Session I: Even

6:45 PM – 7:45 PM

TOPIC: CARDIAC

102

GMP-COMPLIANT HLA-HOMOZYGOUS IPS CELL-DERIVED CARDIOMYOCYTES FOR REPLACEMENT THERAPY: OVERCOMING THE ISSUE OF BATCH-TO-BATCH VARIABILITY

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Induced pluripotent stem (iPS) cell-derived cardiomyocytes (CMs) are emerging as a powerful novel therapeutic prospect to treat heart failure, as these cells can repopulate and replace the damaged myocardium. The use of good manufacturing practice (GMP) HLA-homozygous iPS cell lines presents one strategy to overcome the critical issue of immune incompatibility. However, current iPSC-CM workflows exhibit complexities, producing inconsistent results and low yield. Hence, there is an urgent need for developing workflows with enhanced robustness, efficiency, and compatibility with GMP standards. To address this need, we have been developing a GMP-compliant workflow for the conversion of HLA homozygous iPSCs into CMs using a revised and systematically optimized methodology. Hence, the 3D differentiation process was developed based on rational and titrated signaling pathway perturbations. By applying a co-stimulation strategy involving both WNT and BMP signaling to induce cardiac mesoderm resulted in improved differentiation efficiencies. However, true robustness in methodology was achieved only upon extending the co-stimulation strategy to include manipulation of FGF and TGFβ signaling pathways, in conjunction with adjustments to media changes. Importantly, this approach essentially eliminated bulk cell density as a critical process parameter and increased consistency to an extent that multiple high-efficiency differentiation runs could be performed in a row – a key pre-requisite for GMP translation and a hallmark that has not previously been shown to our knowledge. The CMs resulting from this new platform initially displayed a highly enriched but immature gene expression signature, as expected. Over time, however, early myosin light and heavy chain-encoding genes were replaced by late isoforms and the cells acquired a more pronounced sarcomeric structure, in line with the idea of maturation towards a ventricular default fate. Preliminary data

further suggest that this methodology is compatible with upscaling to larger culture volumes and can be coupled with a prior expansion of the iPSC using an independent 3D system, thus enabling a combined therapeutic platform.

Funding Source: This work was supported by EU HORIZON-HLTH-2021-TOOL-06-02 [Grant no. 101056712].

Keywords: GMP-compliant iPSC, iPS Cell-derived cardiomyocytes, replacement therapy

6:45 PM – 7:45 PM

104

SYSTEMIC ADMINISTRATION OF IPSC-MSC DERIVED EXOSOME FOR CARDIAC REGENERATION

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Myocardial infarction (MI) is the leading cause of death all over the world. The limited regeneration capacity of adult mammal heart leads to irreversible cardiomyocytes damages, causing pathological left ventricular remodeling and heart failure (HF). Induced pluripotent stem cell derived mesenchymal stem cells (iPSC-MSC) has been reported to have better proliferative capacity, survival, more immune privilege than bone marrow (BM)-MSC, making iPSC-MSC more effective in therapeutic application in cardiac repair after MI. Mounting evidence suggests that exosomes derived from MSC can promote cardiac regeneration and repair after MI via anti-inflammatory, anti-apoptotic, anti-fibrotic, immunomodulatory, and pro-angiogenesis. In this study, we extracted exosome from iPSC-MSC and evaluated its therapeutic potential in mouse model of MI. Mice were used to establish MI model by ligating left anterior descending coronary artery and randomized into 3 groups: intravenous injection of phosphate-buffered saline (PBS) (MI group), intravenous injection of iPSC-MSC-exosome immediately after MI (iPSC-MSC-Exosome once group), intravenous injection of iPSC-MSC-exosome immediately and weekly after MI (iPSC-MSC-Exosome 4 times group). Echocardiography was performed to evaluate cardiac function, and our results showed that in iPSC-MSC-Exosome 4 times group, left ventricle ejection fraction (LVEF) increased significantly compared with MI group. In iPSC-MSC-Exosome once group, LVEF increased compared with MI group at Day7 after MI, which could not be maintained in the following 4 weeks. Masson's trichrome staining showed that the infarct size in iPSC-MSC-Exosome once and 4 times groups significantly decreased compared with MI group. Meanwhile, immunofluorescence staining with α-smooth muscle actin (α-SMA) and inducible nitric oxide synthase (iNOS) showed that iPSC-MSC-exosome could increase microvascular density and decrease inflammation after MI. In addition, qPCR demonstrated that expression of angiogenesis related marker increases in iPSC-MSC-exosome groups. In conclusion, iPSC-MSC-exosome could improve cardiac function after MI via neovascularization, anti-fibrosis, and anti-inflammation and repeated administration has better therapeutic efficacy.

Keywords: iPSC-MSC, exosome, cardiac regeneration

6:45 PM – 7:45 PM

106

ENHANCED EXPANSION AND DIFFERENTIATION OF HUMAN IPSC AGGREGATES IN SUSPENSION CULTURE USING AN INNOVATIVE ACF SYSTEM

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Human pluripotent stem cells (hPSCs) have tremendous potential for cell therapy applications due to their self-renewal capacity and ability to differentiate into all three germ layers. However, generating enough cells for therapeutic applications requires scalable and reproducible culture methods. Aggregates suspension culture enables efficient expansion of pluripotent cells followed by directed lineage differentiation. Aggregates differentiation recapitulates better embryonic development and signaling milieu experienced by cells in vivo. Animal-Component-Free (ACF) culture medium is critical for clinical translation as it avoids immunogenic contaminants associated with animal and human-derived components. ACF systems supporting both expansion and efficient differentiation in controlled bioreactors are needed to facilitate regulatory approval of cell-based therapeutics. Nutri3D hPSC ACF, a novel ACF expansion medium, was previously shown to be an optimal medium for pluripotent aggregate suspension culture, supporting efficient aggregation and expansion of iPSC over multiple passages. Expanded aggregates preserve pluripotency and stable karyotype. A proof-of-concept (POC) evaluation of further directed lineage differentiation was performed, including differentiation along the mesoderm and endoderm lineages. iPSCs were differentiated into cardiomyocytes (CM) a representative cell from the mesodermal lineage. Nutri3D ACF-based differentiation medium and standard Wnt on Wnt off differentiation protocol resulted in a high efficiency of above 95% viable juvenile cardiac cells, expressing the structural proteins troponin-T and Sarcomeric α-actinin by day 10. Spontaneous beating was observed at day 8 of differentiation. For definitive endoderm (DE) lineage, the same Nutri3D ACF-based differentiation medium supplemented with Activin A and CHIR99021 resulted in a above 95% efficiency of DE cells, expressing the SOX17 and CD184 by day 5. In conclusion, Nutri3D hPSC ACF allows scalable ACF suspension culture of human iPSCs as pluripotent aggregates and a platform for endoderm and cardiac differentiation. This represents a promising platform for GMP-compliant production of hPSC-derived mature cells suitable for cell-based therapies and tissue engineering.

Funding Source: Horizon Consortium, Europe Union (EU)

Keywords: hPSC, aggregates, expansion, ACF, cardiomyocytes, endoderm, differentiation, ACF medium, ACF system

6:45 PM – 7:45 PM

108

INTRAMYOCARDIAL INJECTION OF HUMAN IPSC-CARDIOMYOCYTES/PROEPICARDIAL-LIKE CELLS ENHANCES VASCULARIZATION AND SALVAGES MYOCARDIAL THICKNESS IN CRYOINJURED RAT HEARTS

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The proepicardial organ derives cells which forms the epicardium for heart development but ceases after birth. A prior study demonstrated that proepicardial-like cells (PECs) from human induced pluripotent stem cells (hiPSCs) resemble embryonic epicardial cells. This study explores PECs functions in cardiomyocyte therapy for cryoinjured rat hearts. HiPSCs were produced from cord blood and subsequently differentiated into cTnT+ cardiomyocytes (CMs) and WT1+ PECs using previously established protocols. Sprague Dawley rats were immunosuppressed with Cyclosporin A (15mg/kg/day) for 3 days prior to cell transplantation and throughout the experiment. Hearts were cryoinjured using liquid nitrogen-cooled vanadium probe for 1 minute, followed by intramyocardial injection of phosphate-buffered saline (control, n=5), CMs (106, n=4), or CMs+PECs (CMPEC, 106 each, n=5) into the infarcted area. Cardiac functions were assessed using the Millar Pressure Volume System at 4 weeks post-cell transplantation, and heart sections were immune-stained for endothelial cells using von Willebrand Factor (vWF). Differences between groups were analyzed using ANOVA. Left ventricular ejection fraction (LVEF) was reduced to 48.2±4.6%, with increased end-systolic volume (155.4±7.5 µl) compared to sham-operated controls ($p < 0.05$). While CMs and CMPEC treatments showed a slight improvement in LVEF at 4 weeks (57.8% and 57.5%, respectively), this difference did not reach statistical significance compared to the control, despite the preservation of myocardial thickness ($p=0.0107$). However, CMPEC-treated hearts showed significantly greater vessel density (5.0±0.7 vs. 1.6±0.4 units/mm2, $p < 0.05$) and increased vWF expression (10.0±5.1% vs. 2.8±1.0% of infarct area, $p < 0.01$) compared to the control. In conclusion, CMPECs preserved myocardial wall thickness by promoting vascularization within the infarct region. An extended experimental duration may be required to examine the benefits of CMs or CMPEC on cardiac functions.

Funding Source: This project was funded by RBS Medical Research Grant 2022 and ALPS Global Holding Bhd. JJT was a recipient of Fundamental Research Grant Scheme (FRGS/1/2018/STG05/USM/03/3) and USM-CryoCord Matching Grant (1001/CIPPT/8011101).

Keywords: cardiac regeneration, human induced pluripotent stem cells, epicardium

6:45 PM – 7:45 PM

110

FEASIBILITY STUDY FOR THE DETECTION OF RESIDUAL UNDIFFERENTIATED HUMAN INDUCED PLURIPOTENT STEM CELLS BY MEASURING MICRORNA-302B IN CULTURE SUPERNATANT

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Detection of residual undifferentiated human pluripotent stem cells (hPSC) is important for the development of cardiac regenerative therapy, in particular a non-destructive method is required. A feasibility study was designed to evaluate the sensitivity of the detection method for undifferentiated hPSCs by measuring microRNA-302b (miR302b) as a biomarker in cell/tissue culture supernatant. This method consists of a unique extraction process and a quantitative reverse transcription polymerase chain reaction (RT-qPCR). Two types of human induced pluripotent stem cell (hiPSC) control lines (in-house generated ERC020 sv2452 and cGMP-manufactured TC-1133) and hiPSC culture systems at the Institute of Experimental Pharmacology and Toxicology UKE were utilized. For the sensitivity test, the detection sensitivity was evaluated with a spiking-in experiment. hiPSCs were spiked into other types of

human cells (primary dermal fibroblasts or HEK293T cells), 6 concentrations of hiPSCs were seeded at a percentage of 0, 0.001, 0.01, 0.1, 1, 10% among background cells. After 24 hours of incubation under hiPSC maintenance culture condition, the supernatant was harvested and frozen immediately before extraction. The miR302b copy numbers in 0.1 mL medium of each sample were examined by RT-qPCR. As a time course test, the alteration of miR302b level in the medium during cardiac differentiation of hiPSCs was examined. Directed growth factor-mediated cardiac differentiation was performed using a 2D protocol (attached culture) and a 3D protocol (embryoid bodies). The culture supernatant was collected at the time points of the stage changes according to the respective differentiation protocols, from d0 to d21. At the end of differentiation runs, sarcomeric cardiac troponin T expression level (% positive population) was determined by flow cytometry. For the sensitivity test, spike-in experiments resulted in a detection limit of 0.01% of 2 independent hiPSC lines in the 2 different types of background cells. In the time course test, 1000 fold decrease of miR302b level was observed during directed cardiac differentiation of 2 hiPSC lines in both 2D and 3D manner (N=3 each). The detection limit of 0.01% of hiPSCs was consistent with previous studies, demonstrating the robustness of this technology.

Keywords: non-destructive detection of residual hiPSC, cardiac differentiation, feasibility study

6:45 PM – 7:45 PM

112

ALL-IN-ONE CRISPR/CAS9 AAV TREATMENT RESTORES CONTRACTILE FUNCTION IN ENGINEERED MUSCLE TISSUES FROM DMD PATIENTS

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Duchenne muscular dystrophy (DMD) is a rare disease characterized by progressive muscle loss, which causes respiratory disabilities and cardiomyopathies during adulthood and ultimately premature death. This X-linked recessive disorder has an incidence of 1 in 5,000-6,000 males and is caused by mutations in the dystrophin gene. Dystrophin is a giant protein expressed mainly in muscle cells whose function is to stabilize the muscle fibers by connecting the cytoskeleton to the extracellular matrix. More than 5,000 different DMD mutations have been reported, most being out-of-frame deletions of at least one exon. Consequently, dystrophin expression is impaired. Interestingly, in-frame deletions resulting in truncated functional dystrophins can give rise to milder muscular dystrophies or even asymptomatic clinical phenotypes. In our study, we aimed to develop personalized CRISPR/Cas9-based gene therapy strategies in different DMD patients, converting out-of-frame deletions in in-frame deletions by inducing skipping of single or multiple exons. To achieve this, patient-specific iPSCs were produced, differentiated into cardiac and skeletal muscle cells, and phenotyped at the cellular and tissue level. Depending on which exon was skipped by CRISPR/Cas9 editing, stable or unstable dystrophin peptides were generated. Strikingly, engineered heart muscle derived from CRISPR-edited cardiomyocytes with robust dystrophin re-expression showed a restoration of the contractile function. Finally, functional restauration was achieved by the treatment of engineered skeletal muscle with all-in-one AAVs containing smaller Cas9 variants. Altogether, the use of patient-specific iPSC-engineered muscle tissues and all-in-one AAVs represents an excellent platform to test different clinically translatable CRISPR/Cas9-based exon skipping strategies for treating DMD.

Keywords: Duchenne muscular dystrophy, iPSC-engineered muscle tissues, CRISPR/Cas9-based gene therapy

6:45 PM – 7:45 PM

114

DEEP IMMUNE-PHENOTYPING OF IPSC-DERIVED HLA-HOMOZYGOUS CARDIOMYOCYTES

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Cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CM) are promising tools to regenerate damaged hearts. Donor-derived hiPS-CM constitute grafts with a distinct immune-phenotype and rejection risk. HLA homozygous (HLAho) hiPSC may reduce

this risk and increase matching frequencies. Here we performed a comprehensive immune-profiling of 3D-differentiated HLAho compared to HLA heterozygous (HLAhe) hiPS-CM. Cardiomyocytes were differentiated from HLAhe and HLAho iPSC strains in 3D cultures using Wnt pathway modulation in hiPSC spheroids. Day-10 hiPS-CM aggregates were analyzed for cardiac identity and purity. We performed 354 surface-marker LEGENDScreen immune-profiling of hiPSC and corresponding hiPS-CM. Unsupervised hierarchical clustering and pathway analysis identified differentially expressed immune recognition molecules and cell lineage identity. We designed an extensive marker panel for single-cell deep immune-phenotyping using spectral flow cytometry. HLAhe and HLAho iPSC expressed low levels of HLA-ABC, β2-microglobulin and MIC-A/B, and lacked HLA-DR/-DQ without differences between haplotypes. Resulting hiPS-CM purity was 89.0-98.6% cTNT+. We identified 102 differentially expressed immune-target molecules defined by log10-fold expression change ≤0.5 / ≥2 (hiPS-CM/hiPSC). Prominently upregulated markers included CD164, CD59, N-cadherin; most downregulated included SSEA-5, EpCAM, Thy1, TRA-2-54, and complement regulators CD46/CD55. All differentiated hiPS-CM downregulated HLA-ABC and MIC-A/B, and upregulated the 'don't eat me' signal CD47. Multiplex deep phenotyping recapitulated screening results and unsupervised clustering separated undifferentiated hiPSC from hiPS-CM. In two test populations of 561 vs 57,736 HLA-typed platelet donors we identified 109 (19.4%) vs 14,623 (25.3%) haploidentical and 5 vs 457 homozygous matched individuals at ABC 2-digit resolution. High resolution and HLA-DRB1 matching reduced matching frequencies by 1.7% and 31.3%, respectively. The hiPS-CM immune-phenotype indicates a privileged graft recognition profile. Preliminary HLA matching results encourage further functional validation of HLAho iPS-CM testing sensitivity to matched vs mismatched T and NK cells in vitro.

Funding Source: This work received funding from the European Union's Horizon 2020 research and innovation program (grant agreement 101056712 HEAL), by Land Salzburg, and Cancer Cluster Salzburg.

Keywords: iPS-cardiomyocytes, immune-phenotyping, HLA matching

6:45 PM – 7:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

116

HETEROGENEITY OF XENO-FREE CULTURED HUMAN PLURIPOTENT STEM CELLS (HPSCS) INDUCES VARIATION IN THE DIFFERENTIATION EFFICIENCY OF HPSC-DERIVED CORNEAL LIMBAL STEM CELLS

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Differentiation of corneal limbal stem cells (LSCs) from hPSCs presents a promising therapeutic approach to treat severe bilateral limbal stem cell deficiency. However, hPSC variation is known to challenge the robustness of the protocols and increase unwanted heterogeneity. Here, hPSCs were differentiated towards corneal epithelium, and the process was serially analyzed with real-time quantitative PCR (RT-qPCR) and immunofluorescence (IF), to reassess the cell line-specific sensitivity of an established, clinically applicable hPSC maintenance + differentiation protocol. Only the hPSC lines with appropriate pluripotent marker expression, multilineage differentiation capacity, and normal karyotype were used. The results from five hiPSC lines were compared with a hESC line showing a good historical performance in this protocol. Partial success in the stepwise differentiation towards LSCs was achieved, but it was indeed diminished by varying lineage commitment efficiency between the hiPSC lines and even between differentiation batches within individual cell lines. RT-qPCR results indicated high initial variation from line-to-line as well as from batch-to-batch within the undifferentiated hiPSCs maintained on Essential 8 Flex/laminin-521 system with a weekend-free routine. Interestingly, the variations of BMP4, LEF1, PAX6, and TGFB1 gene expressions, which represent some of the key factors in corneal epithelial induction, were notably high also in the control hESC line, suggesting that a standardization of the starting material is needed for this protocol. Some cell batches failed to upregulate PAX6 and developed a fibroblastic, mesenchymal morphology in association with elevated TGFB1 gene expression, which was linked to the decreased number of cells expressing LSC markers p63 and CK14 in IF in the later phases of the differentiation. These results emphasize the need for additional development and quality control to ensure reliable production of homogenous starting material for the LSC differentiations. Moreover, as underlined in our results, the difficulty to develop a universal differentiation protocol for distinct hPSC lines should further inspire the exploration of additional strategies to facilitate the development of a widely available, cost-effective corneal cell therapies.

Funding Source: This research was supported by the Research Council of Finland, Sigrid Jusélius Foundation, Finnish National Eye and Tissue Bank Foundation, Evald and Hilda Nissi Foundation, and Mary and Georg C. Ehrnrooth Foundation.

Keywords: human pluripotent stem cells, differentiation, corneal epithelium

6:45 PM – 7:45 PM

118

PSEUDO-TURBINATE REGENERATION: NOVEL INSIGHT FOR THE TREATMENT OF EMPTY NOSE SYNDROME

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Empty nose syndrome (ENS) is a rare and poorly understood iatrogenic condition occurring in 20% of patients undergoing turbinate resection. The main symptoms depicting this syndrome are paradoxical sensations of suffocation, nasal bleeding, crusting, sleep disorders, anxiety and depression with a high suicidal rate. Existing medical treatments tend to alleviate symptoms without providing a permanent solution. Surgical reconstructive methods commonly prioritize restoring nasal cavity volume over regenerating the respiratory mucosa, which plays a crucial role in air filtration, warming, and humidification. The primary objective of the present study is to create a nasal pseudo-turbinate, which includes a human nasal tissue-engineered cartilage graft (N-TEC) covered by a functional airway epithelium. Emphasis has been given to studying the interaction and coexistence of the two tissues during the in vitro regeneration process. Firstly, the preservation of parameters used as release criteria for the clinical application of N-TEC graft has been confirmed following culture with epithelial cells. Secondly, the ability of primary airway epithelial cells to stably adhere and proliferate onto the N-TEC, promptly regenerating a continuous epithelium, has been assessed. Efforts have been dedicated to identifying the culture conditions necessary to guarantee the retention of the highest airway epithelial clonogenic potential as well as the tissue-specific multilineage differentiation capacity, namely the ability to regenerate all the airway specialized cell types. Finally, essential for the translational prospects, the preservation of the epithelial cells stem cell pool has been investigated after culture onto the N-TEC through single-cell clonal analysis and quantification of biological and molecular parameters associated with this particular cell population. The successful regeneration of a 3D pseudo-turbinate holds promises not only for patients with ENS and for those suffering from more severe respiratory disorders but also propels the entire field of regenerative medicine forward. This achievement lays the foundation for developing successful combined ex vivo tissue engineering therapies, pushing the boundaries of personalized treatment to regenerate intricate 3D structures and organs.

Funding Source: This research has been funded by RESEARCH PROJECTS OF RELEVANT NATIONAL INTEREST (PRIN) prot. 2022CMNWCZ

Keywords: empty nose syndrome, epithelial and cartilage tissue engineering, airway stem cells-based therapy

6:45 PM – 7:45 PM

120

ADVANCING IMMUNOTHERAPEUTIC APPROACHES THROUGH CO-CULTURE OF GASTROINTESTINAL ORGANOIDS WITH CAR-T CELLS

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Developing investigational ex-vivo models that accurately replicate the intricate interactions between tissues and immune cells is crucial for advancing novel immunotherapeutic approaches. However, such co-culture systems are often challenging to establish and limited in their scope. Our study introduces a new method using healthy organoids to investigate the interactions between the gastrointestinal (GI) epithelium and Chimeric Antigen Receptor - T (CAR-T) cells. The primary aim is to assess any potential cytotoxic effects of CAR-T cells on the organoids, thereby evaluating the adverse effects CAR-T cell therapy may have on the healthy tissue. As CAR-T cell therapy relies on specific target interactions present in the native epithelium, it is crucial to maintain this specificity in the organoid model. The presence of the target of interest, as well as adherens and tight junction proteins, is assessed through immunohistochemistry (IHC) and immunofluorescence (IF) directly on the organoids. Our co-culture system uses microwells for the generation of GI organoids to which immune cells can be added. The interactions between the organoids and the immune cells are visualized using cell tracker dyes, allowing us to monitor the co-culture evolution over multiple days. Moreover, we utilize live imaging and image analysis techniques to quantify the cytotoxic effects and dose-limiting activity of CAR-T cells against organoids expressing the target of interest. The high level of cultures' homogeneity ensured by our system allows us to precisely control the effector-to-target ratio, demonstrating the potential of our approach for applications in immunotherapy toxicity and efficacy assessment. This novel approach has the potential to contribute to the development of improved immunotherapeutic strategies.

Keywords: organoids, CAR T-cell therapy, personalized medicine

6:45 PM – 7:45 PM

122

EN31-FNCAS9-BASED ADENINE BASE EDITORS FOR PRECISION EDITING IN PATIENT-SPECIFIC IPSCS AND CORRECTION OF TWO PATHOGENIC MUTATIONS LINKED TO LEBER CONGENITAL AMAUROSIS (LCA)

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The clinical success of CRISPR-based therapies depends on the safety and efficacy of Cas proteins. The Francisella novicida Cas9 (FnCas9) has negligible affinity for mismatched substrates enabling it to discriminate DNA off-targets with very high precision, thus offering better safety. However, the gene targeting efficiency of this high fidelity FnCas9 is low. To improve edit efficiencies, we carried out protein structure-guided engineering of the gene, to create engineered FnCas9 variants (en1, en15, en31). These variants show enhanced cellular edit efficiency, while retaining the inherent high-fidelity and target specificity. They also offer PAM flexibility (NGG to NGR/NRG) and supports gRNAs of variable lengths (g20-g28). We evaluated the enFnCas9 variants on a PAX6 target loci in human ARPE-19 cells and observed efficient gene knockout and loss of protein expression in transfected cells. Also, in hard to transfect iPSCs, we observed a significantly higher edit efficiency with en1FnCas9 (18.6%) and en15FnCas9 (23%), when compared to the regular spCas9 (13.8%). The en31FnCas9-based, cut-free, adenine base editing system (en31ABEmax8.17) with an expanded edit window (PAM distal A3-A14 position), enhances the genome accessibility and targeting range by ~3.5 folds. We further evaluated the target specificity and edit efficiency of en31ABEs at two mutant loci (RPE65 and RD3) in patient-specific iPSCs. We observed precise A>G edits and mutation correction within RD3 in 13% cells, using a 21 bp guide with NGG PAM. Similarly, we observed successful mutation correction within exon 9 of RPE65 in 21%, 13%, 8% of the cells, using 21 bp guides with NGG, GGA, AAG PAMs respectively (N=3 each). The edited cells were clonally expanded, and two clonal lines that showed >99% edits, with precise edits at the target locus were differentiated into neuro-retinal organoids and RPE cells. Detailed characterization of mature iPSC-RPE cells confirmed the successful restoration of RPE65 mRNA and protein expression in edited cells, as levels comparable to healthy control cells. Thus, we provide the proof-of-concept of mutation correction using high fidelity FnCas9-based Adenine Base Editing system, which offers significant translational potential towards developing cell-based therapies for various inherited eye disorders.

Funding Source: Department of Biotechnology (DBT), Department of Science and Technology-Science and Engineering Research Board (DST-SERB), Government of India.

Keywords: adenine base editing, engineered FnCas9, patient-specific iPSCs

6:45 PM – 7:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

124

THE OPTIMIZATION OF THE CRYOPRESERVATION WORKFLOW FOR PLURIPOTENT STEM CELLS

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Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), hold immense promise in regenerative medicine, disease modeling, and drug discovery. However, research and clinical applications heavily rely on development of efficient methods to ensure long-term storage, viability, and functionality. Many of the standard protocols vary from lab to lab and are not optimized for a simple effective methodology. Here we explore strategies for cryopreservation of PSCs, with a focus on optimizing protocols for both DMSO-containing and DMSO-free cryoprotective solutions. Workflow parameters including cell collection techniques, cryoprotective agents (CPAs), cooling and thawing protocols, and post-thaw recovery methods were examined. In addition, the use of drugs affecting Rho associated protein kinase(s) (ROCK) and associated signaling molecules were compared for optimal recovery of PSCs following cryopreservation. After testing these various factors, we provide an optimized protocol for PSC recovery following cryopreservation. Finally, we explore the potential of rapid-cooling and storage, from room temperature, to enhance cryopreservation efficiency and cell recovery. Best practices in data generation and analyses for determining optimal PSC cryopreservation, such as viability and recovery, morphological and functional characteristics, as well as genetic stability, are also addressed. Using optimized protocols in a unified cryopreservation workflow, we can achieve over 2-fold better long-term recovery of PSCs compared to conventional passive cooling methods. Critical steps identified include the method of cell dissociation, timing of loading and unloading of CPA solutions (washing steps), thawing temperatures and times, and centrifugation parameters (G-force and spin times). We aim to provide valuable insights into the current state-of-the-art and advance cryopreservation protocols for PSCs, ultimately improving their widespread discovery and clinical translation applications.

Keywords: cryopreservation, clinical applications, cGMP

6:45 PM – 7:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

126

DIFFERENTIATION OF IPSCS TO FUNCTIONAL INK CELLS IN SUSPENSION AND APPLICATIONS IN CANCER RESEARCH

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Pluripotent stem cells (PSCs) are a renewable cell source that could be used to generate cell therapies to treat many diseases including cancer. Numerous methods have been established to generate different cell types however significant challenges remain to ease the transition from discovery to commercial manufacturing. Recently we developed CTS-StemScale™, a PSC suspension culture medium designed for CGT manufacturing that enables the large-scale culture of PSCs. Natural killer (NK) cells are innate, cytotoxic lymphoid immune cells that can kill malignant cells without HLA matching and are a major focus for allogeneic therapy development. NK cell therapy clinical trials indicate that ~5x106 - 1x108 NK cells per kilogram body weight may be required for effective treatments. Yet requirements such as donor sourcing and successful expansion hamper the ability to efficiently generate large quantities of functional NK cells. Here, we describe a method to produce PSC-derived NK (iNK) cells, from CTS-StemScale suspension cultures enabling the generation of highly enriched, functional iNKs in a scalable culture format. PSCs grown in suspension as spheroids were induced using growth factor cocktails to differentiate into CD34+ hematopoietic progenitor cells and subsequently to CD56+ iNK cells without the addition of feeder cells. Further culture of iNK cells in CTS™ NK-Xpander led to significant enrichment of CD56+CD3- and CD56+CD16+ phenotypes. The cytolytic potential of these iNKs was further demonstrated by their ability to kill K562 cancer cells as well as patient-derived 3D colon tumoroids and showed similar degranulating activity as primary NK cells. In summary, the use of CTS-StemScale highlights the potential for feeder-free PSC suspension cultures to be differentiated into cytolytic iNKs at scale.

Keywords: pluripotent stem cell, cell therapy, natural killer cell

6:45 PM – 7:45 PM

128

CELL THERAPY SCALE-UP: BIOPROCESS DEVELOPMENT FOR THE PRODUCTION OF HEMATOPOIETIC CELLS

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Blood transfusion is currently the most common cell therapy applied worldwide to treat various medical purposes. One of the purposes is the treatment of severe anemias, as these diseases can only be treated with blood transfusions and stem cell transplantations. An attractive therapy that could overcome the risks involved with donor-derived transfusion products is in vitro-produced red blood cells (RBCs) or the corresponding stem cells, namely hematopoietic stem cells (HSCs). In vitro RBCs can be produced from induced pluripotent stem cells (iPSCs), which are pluripotent cells created by reprogramming human somatic cells. This reprogramming technique started a new era in regenerative medicine due to their self-renewing properties and multi-lineage differentiation potential to produce patient-specific progenitor or functional effector cells. However, hematopoietic cells and RBCs from iPSC are mostly produced via multiple differentiation steps using static adherent protocols which hinders scalability towards clinically relevant volumes. A dynamic shake flask cultivation was developed to produce hematopoietic stem/progenitor cells (HSPCs), which can further be differentiated into functional RBCs. The dynamic cultivation led to a ~16,000 fold increase in cell number compared to static cultivation. This project aims to transfer the developed protocol to a suspended stirred-tank bioreactor to produce HSPCs. Employing bioreactors will improve reproducibility and facilitate upscaling towards the desired volumes, as mini-transfusions (1011 RBC, required in phase I trial) could be generated with 3 to 4 three-litre bioreactors. The main challenges to be optimized are specific nutrient and oxygen requirements for each differentiation step, as well as shear stress effects.

Keywords: hematopoietic stem progenitor cells, bioreactor, scale-up

6:45 PM – 7:45 PM

130

AN 8-CELL(8C)-LIKE HUMAN INDUCED PLURIPOTENT STEM CELL PLATFORM FOR BIOENGINEERED MYELOID PROGENITOR THERAPIES

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Primary myeloid progenitors (MP) can reduce the severity and duration of neutropenia in animal and early-phase clinical studies. Following transient engraftment, MP can generate differentiated effector cells in vivo over several weeks, thus reducing the required cell numbers, and extending the duration effects, compared to neutrophil transfusions. However, primary MP rely on donations, are prone to spontaneous differentiation, and are poorly amenable to bioengineering (e.g., CAR myeloid cells). Although hiPSC can generate unlimited numbers of stably gene edited MP, clinical translation is hindered by poor in vivo persistence of differentiated progeny from conventional hiPSC following transfusion into adult hosts; which may be linked to the lineage-primed differentiation bias of primed iPSC and the pro-apoptotic state of their progeny. We have thus developed a chemical PARP-regulated stem cell culture system that confers hiPSC with 8C-like features and improved differentiation capacity (without epigenomic or karyotypic aberrations). These Tankyrase/PARP inhibitor-regulated (TIRN)-hiPSC directly differentiated to progenitor lineages at significantly greater efficiency and viability than conventional hiPSC, without a need for 'repriming' or capacitation steps, and with high in vivo engraftment potential. Here, we describe methods for generating bioengineered myeloid progenitors from these 8C-like TIRN-hiPSC in defined xeno/feeder-free (XF/FF) culture conditions to serve as versatile cGMP-compliant cell therapy platform. In teratoma assays, XF/FF 8C-like TIRN-hiPSC robustly generated three-germ layer structures with less differentiation bias than their isogenic primed hiPSC counterparts. In hematopoietic differentiation, XF/FF 8C-like TIRN-hiPSC cells efficiently generated CD34+CD45+ progenitors and myelocytes with improved cellular identity (CD15, CD11b expression) and morphology at 50-fold greater yields than primed hiPSC. These data provide the first functional 8C-like hiPSC platform for generating high-yield cellular immune therapies; a goal which has remained elusive using conventional hiPSC. The high production of viable MP's from these functional 8C-like human stem cells is uniquely amenable to large-scale production for early phase clinical studies.

Funding Source: NIH/NEI (R01EY032113; R01EY023962), NIH/NICHD (R01HD082098), The Maryland Stem Cell Research Fund (2023-MSCRFV-5995), and the Dr. Werner-Jackstädt Stiftung (0134-10.127).

Keywords: myeloid cell therapies, naive iPSC, cell engineering

6:45 PM – 7:45 PM

132

DUAL TARGETED ANTICD19 AND ANTICD22 CHIMERIC ANTIGEN RECEPTOR-NATURAL KILLER CELLS IN LIMPHOMA CELL LINE

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Entering chimeric antigen receptor (CAR) T cells into the field of treatment for patients with B-cell malignancies has become a promising treatment option, and to date, CAR-T cells against various CD markers have shown good results. However, the limitations of CAR-T cells, which are limited to autologous samples as a source and can also lead to complications such as graft-versus-host disease and cytokine release syndrome, have led researchers to other alternatives. Natural killer (NK) cells can be a good substitute for T cells due to their very cytotoxic nature, which has led CAR-NKs to be added to the studies. Despite the high remission rate following CAR therapies in lymphoma, disease relapse and the development of resistant mechanisms continue to account for a high percentage of disease cases. Therefore, new solutions are one of the main needs in the field of treating these diseases. We hypothesized that simultaneous targeting of two antigens may reduce the chance of antigen loss, so one strategy to prevent CD19-negative tumor escape is to simultaneously target CD19 and a second B-lineage antigen, CD22, which has The potential to be co-targeted in treatment with CD19 in CAR-NK cells. We simultaneously targeted the Raji cell line as a lymphoma model with two separate anti-CD19 and anti-CD22 constructs in vitro. Cytotoxicity of anti-CD19/CD22 CAR-NK was evaluated using flow cytometry and in terms of induction of apoptosis, CD107a granulation and secretion of GM-CSF, TNF, MIP and IFN-g, dual CAR NK was significantly superior to si-CAR-NK. These results demonstrate the potential of dual anti-CD19/CD22 CAR-NK cells and suggest that optimization of CAR constructs in NK cells can improve the efficacy of CAR therapy.

***(Author contributed equally to this research)*

Keywords: dual chimeric antigen receptor, immunotherapy, natural kill cell

6:45 PM – 7:45 PM

134

UNLOCKING THE POTENTIAL OF IPSC-DERIVED MACROPHAGES (IMACS) FOR CANCER IMMUNOTHERAPY

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Cancer presents a formidable global health challenge, urging the exploration of novel therapeutic avenues with reduced side effects compared to conventional treatments. Immunotherapy, harnessing the body's immune system, offers promise, especially given the limitations of T cell-based approaches, prompting investigation into alternatives like macrophage-based strategies. Macrophages, pivotal in immune function, serve dual roles as phagocytes and antigen-presenting cells. However, cancer cells often evade macrophage detection through upregulation of "don't eat me" signals, notably CD47. Despite challenges, a new generation of macrophage checkpoint inhibitors targeting the CD47-SIRPα axis shows promise. By leveraging recent advances in induced pluripotent stem cell (iPSC) technology, we aimed to pioneer effective cancer immunotherapy using iPSC-derived macrophages (iMACs). Our pilot objectives included phenotypic and functional characterization of iMACs, development of a screening assay for macrophage phagocytosis, and optimization of CRISPR gene editing of iMAC precursors. We successfully generated iMACs from human iPSCs and evaluated their phagocytic capacity in vitro and in vivo using a mouse model of Lymphoma. Our focus on CRISPR-mediated targeting of the SIRPα-CD47 axis aimed to enhance iMAC phagocytic function, furthering therapeutic development. In conclusion, this innovative approach harnessing iPSC-derived macrophages presents a promising avenue for advancing cancer immunotherapy. By leveraging the scalability and genetic engineering potential of iPSC technology, a foundation is laid for the development of a novel cellular therapy poised to tackle critical challenges in cancer treatment.

Funding Source: This study was funded by Cedars- Sinai 2023 CSC and RMI Joint Research Initiative Award.

Keywords: macrophage, iPSC, cell therapy

6:45 PM – 7:45 PM

136

SCALABLE GENERATION OF HUMAN IPSC-DERIVED NATURAL KILLER AND DENDRITIC CELLS USING A CONTINUOUS CONCURRENT PRODUCTION PLATFORM

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Cell-based immunotherapies using T cells, natural killer cells (NK), and dendritic cells (DC) in human clinical trials have been experiencing an outstanding rise. However, certain challenges persist, such as limited availability of desired cell type, inherent heterogeneity, donor variability, and others. As a potential solution, human induced pluripotent stem cells (iPSCs) offer an alternative approach to generate immune cell types at scalable numbers for future immunotherapies. Here, we report the continuous and scalable production of fully functional iPSC-NK (i-NKs) and -DC (i-DCs) applying a chemically defined differentiation protocol, which is initiated by 3D hematopoietic organoids (Hemanoids). Of note, Hemanoids exhibit the capabilities to generate various immune cells through adjustments only in the cytokine cocktail, facilitating the induction of different hematopoietic lineages. i-NKs were harvested

regularly in adherent and scalable suspension benchtop bioreactors with a purity of over 90%. Notably, the i-NKs exhibited stage-specific NK populations characterized by the immunophenotype profile CD56-CD16-, CD56+CD16-, CD56+CD16+, demonstrating their ability to degranulate and engage in killing against K562 cells. Transcriptomic and trajectory analysis confirmed a progenitor population CD56- that gives rise to CD56+ cell populations. Additionally, i-NKs could be further expanded, resulting in activation and thus driving i-NKs towards a maturation state, leading to an increased frequency of CD56+ cells and enhanced killing capacity. For i-DC generation, three media formulations were utilized to generate various DC subsets. Transcriptomic analysis confirmed various DC subsets such as cDC2, DC3, DC progenitors. Irrespective of the cytokine used, i-DCs were able to process antigens efficiently and stimulated T cells, demonstrating the capacity to induce effector memory phenotype in CMV positive patient cells, showing proof of concept for potential immunotherapy approaches for future vaccinations or development of antigen -specific T cell therapy. In summary, we introduce a new method for the continuous generation of fully functional i-NKs and i-DCs that would serve as an attractive immune cell-farming platform for clinical applications.

Keywords: dendritic cells and natural killer cells, bioreactors, cell therapy

6:45 PM – 7:45 PM

138

DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS (HPSCS) INTO HEMATOPOIETIC AND ENDOTHELIAL CELL TYPES USING A DEFINED, ANIMAL ORIGIN-FREE CELL CULTURE MEDIUM

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The development of fully defined cell culture systems to support differentiation of human pluripotent stem cells (hPSCs) into clinically significant cell types is crucial for advancing hPSC-based therapies. To address this need, we developed STEMdiff™ APEL™2—a fully-defined hPSC medium composed of animal origin-free components, based on Ng et al., (2008). STEMdiff™ APEL™2 is a flexible base medium designed to support multi-lineage hPSC differentiation and is adaptable to both 3D and adherent cell platforms. In this study, we demonstrate the utility of STEMdiff™ APEL™2 by generating two therapeutically-relevant cell types: hematopoietic progenitor cells (HPCs) and endothelial cells (ECs). For hematopoietic differentiation, embryoid bodies (EBs) were generated from three hPSC lines (H9, WLS-1C and SCTi003-A) using 2000 cells/well of round-bottom 96-well plates. EBs were then cultured in STEMdiff™ APEL™2 medium supplemented with SCF, VEGF, BMP4, and bFGF. After six days of culture, flow cytometry analysis revealed 27 ± 7% viable cells were CD34+ HPCs, yielding 4.7 x 10^5 ± 3.1 x 10^4 CD34+ cells per well of a 96-well plate (mean ± SD; n = 9, 3 cell lines). To assess the functionality of these HPCs, subsequent differentiation steps, performed as described by Kaufmann et al. (2020), produced robust natural killer (NK) cell differentiation, with 87% ± 7% of cells expressing NK markers CD56+/CD45+ after 30 days and yielding 5.4 x 10^6 ± 5.3 x 10^5 NK cells per well of a 6-well plate (n = 6, 2 cell lines). Cytotoxic killing and degranulation assays further demonstrated the functional properties of NK cells in vitro. Additionally, we established a STEMdiff™ APEL™2-based protocol for endothelial differentiation, adapted from Tan et al., (2013). Seeding three hPSC lines (H9, WLS-1C, and SCTi003-A) at 5 x 10^4 cells/cm2 in STEMdiff™ APEL™2 supplemented with BMP4, VEGF, and CHIR-99021 led to robust endothelial differentiation, with 63% ± 9% of ECs expressing CD31+/CD144+ and yielding 3.1 x 10^5 ± 1.8^4 ECs per cm2 (n = 9, 3 cell lines) after six days. In summary, STEMdiff™ APEL™2 is a defined, animal origin-free, and adaptable medium that supports hPSC differentiation into diverse, clinically-relevant cell types. This medium serves as a robust base for researchers to develop, customize, and optimize their differentiation workflows.

Keywords: pluripotent stem cells, cell therapy, hematopoietic differentiation

6:45 PM – 7:45 PM

140

INDUCTION OF IPSC-DERIVED GAMMA-DELTA T CELLS WITH IN VIVO ANTI-CANCER ACTIVITY USING A FEEDER-FREE/SERUM-FREE METHOD

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The cytotoxicity of gamma delta T (γδT) cells against various types of cancer cells in an MHC-unrestricted manner has led to their potential applications in allogenic immune cell therapy with no concern about GVHD. However, γδT cells represent only a few percent of peripheral blood mononuclear cells and cannot be expanded ex-vivo sufficiently for mass production. Induced pluripotent stem cell (iPSC) technology may be able to overcome these limitations and enable us to realize off-the-self allogenic γδT cell-based therapy. Previously, we established human iPSC lines from peripheral blood-derived γδT cells with a simple and clinically applicable method and successfully generated iPSC-derived γδT cells (iγδT cells) using xenogenic feeder cells and serum-containing media, and confirmed their cytotoxicity in vitro against several types of cancer cell lines in an MHC-unrestricted manner. In the present study, we aimed to generate iγδT cells using a feeder- and serum-free (FF/SF) protocol and to clarify whether FF/SF iγδT cells function in vivo. As a result, we have successfully established an FF/SF method for the differentiation of γδT cell-derived iPSCs into iγδT cells. In addition, several cytokines and a recombinant protein used in the novel protocol could be replaced by animal-origin-free (AOF) ones. The FF/SF iγδT cells killed three types of cancer cell lines in vitro in an MHC-unrestricted manner. Moreover, they also showed significant cytotoxicity

against patient-derived colon cancer organoids. Experiments using xenograft model showed that the administration of the FF/SF iγδT cells had significant anti-cancer efficacy with no apparent toxicity in vivo. Our current results will advance off-the-shelf γδT cell-based immunotherapy using genetically engineered cells such as CAR-γδT and TCR-γδT cells as well as non-engineered iγδT cells.

Keywords: iPS cell, γδT cell, cancer immunotherapy

6:45 PM – 7:45 PM

142

REPAIRING CHOROIDAL ISCHEMIA USING HUMAN PLURIPOTENT STEM CELLS DERIVED ENDOTHELIAL CELLS

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Choroidal atrophy is a common fundus pathological change closely related to the development of age-related macular degeneration (AMD), retinitis pigmentosa, and pathological myopia. Studies suggested that choroidal endothelial cells (CECs) that form the choriocapillaris vessels are the first cells lost in choroidal atrophy. Therefore, restoring the choriocapillaris vessels is crucial to the prognosis of the above diseases. We found that endothelial cells derived from human pluripotent stem cells (hPSC-ECs) through the MESP1+ mesodermal progenitor stage expressed CECs specific markers and can integrate into choriocapillaris. In a rat model of choroid ischemia (CI), transplantation of hPSC-ECs into the suprachoroidal space increased choroid thickness and vasculature density. Close-up examination showed that engrafted hPSC-ECs formed new blood vessels and integrated with existing rat vessels. Our work demonstrated that hPSC-ECs could be used to repair choroidal ischemia in the animal model, which may lead to a new therapy to alleviate choroidal atrophy implicated in AMD and other ocular diseases such as retinitis pigmentosa, and pathological myopia.

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Keywords: choroidal ischemia, human pluripotent stem cells, endothelial cells

6:45 PM – 7:45 PM

144

FEEDER-FREE EXPANSION OF IPSC-DERIVED NK CELLS TO CLINICAL SCALE

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Natural killer (NK) cells are a key part of the immune surveillance system, killing transformed cells before they can develop into life-threatening cancer. Unlike T-cells, NK cells can be given as an allogenic therapy without risking graft-vs-host disease, meaning large quantities of this cell therapy could be manufactured from a single donor for off-the-shelf use. We have developed a feeder cell-independent immune cell differentiation platform starting from GMP-grade iPSC lines. In a first stage, iPSCs are converted to hematopoietic precursor cells sharing characteristics of bona fide HSCs. Using a newly established protocol, these HSC-like cells are generated at high purity in a defined manner and may be cryopreserved as intermediate cell banks. Following recovery, the precursor cells may, in a flexible manner, be further differentiated to immune cell types of both the myeloid and lymphoid lineages. This approach via intermediate cell banking bears key advantages for GMP manufacturing and upscaling as it significantly simplifies and shortens the final manufacturing process. Here we show our defined iPSC-derived immune cell platform being used to generate and expand up to several billion NK cells per cm2 of seeded iPSC in a feeder-free process. This was accomplished by using defined media to maintain the expansion of NK precursor cells at small scale, before switching to traditional cost-effective media for the final large-scale expansion phase. This results in a high purity of mature NK cells featuring KIR receptors as well as NK activation marker expression, and highly potent killing ability against K562 cells. To our knowledge, this represents the first method of expanding iPSC-derived NK cells without feeder cells and in a GMP-compatible manner, to the scales necessary for clinical use.

Keywords: natural killer, upscaling, manufacturing

6:45 PM – 7:45 PM

146

SCALABLE PRODUCTION OF IPSC-DERIVED, HLA-UNIVERSAL MEGAKARYOCYTES TO EVADE ALLOGENEIC IMMUNE RESPONSES

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The clinical need of platelets (PLTs) for the treatment of thrombocytopenic patients is rising and faces various challenges, such as difficulties in

PLT storage, risk for bacterial contamination, a limited donor pool and a short shelf life. In addition, in hematological and oncological patients requiring long-term transfusion, anti-human leukocyte antigen (HLA) class I alloantibodies may cause life-threatening platelet refractoriness (PTR), which is characterized by the absence of a sufficient increase in PLT count after transfusion. In this study, we aimed at the scalable production of low immunogenic Megakaryocytes (MKs) and PLTs capable to evade allogeneic immune responses and survive under PTR conditions. We reprogrammed peripheral blood mononuclear cells (PBMCs) from female donors with blood type O and rhesus negative into iPSCs using non-integrative Sendai viruses. Expression of typical pluripotency markers was proven at mRNA and protein levels. The in vitro trilineage differentiation capacity was verified by specific marker detection. HLA class I silenced iPSCs were generated using lentiviral vectors encoding short hairpin RNAs (shRNA) targeting β2-microglobulin (shβ2m) and/or CIITA (shCIITA) respectively, and showed significantly reduced expression levels of β2m and CIITA compared to control. After differentiation of the iPSCs towards MKs using a 3D differentiation protocol, allogeneic CD4 and CD8 T cell responses were examined in CPD-based proliferation assays and showed that HLA-silenced MKs abrogated MK-induced allogeneic CD4 and CD8 T cell proliferation ($p< 0.001$) and cytotoxicity ($p< 0.05$). Granular T cell-mediated cytotoxicity was measured by quantifying Granzyme B secretion levels by ELISA and was found to be significantly reduced ($p< 0.01$) for both, HLA class I (24.5%) and HLA class I and II silenced (52%) MKs compared to controls. In order to be able to actually meet the clinical need for HLA-universal MKs, we have successfully upscaled the MK production in a bioreactor and were able to achieve a percentage of up to 50% MKs and a total cell number of 1.89 x108 ± 9 x107 MKs per differentiation batch. The use of in vitro produced HLA-universal MKs could bring enormous therapeutic benefits in the treatment of highly sensitized thrombocytopenic patients.

Keywords: reprogramming of PBMCs into iPSCs, HLA silencing, upscaling of differentiation towards megakaryocytes in bioreactors

6:45 PM – 7:45 PM

TOPIC: LIVER

148

ENCAPSULATED HUMAN PROLIFERATING HEPATOCYTE ORGANOIDS IN THE TREATMENT OF LIVER FAILURE

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Alginate-encapsulated hepatocyte transplantation is a promising strategy for the treatment of liver failure. However, its clinical application was impeded by the lack of primary human hepatocytes and the difficulty to control their quality. We previously addressed cell expansion by developing a dedifferentiation culture of proliferating human hepatocytes (ProliHH). Here, we produced ProliHH in mass with quality assurance. Importantly, ProliHH were engineered as liver organoids to improve viability and maturity. Encapsulated liver organoids (eLO) were intraperitoneally transplanted to treat liver failure animals. Notably, eLO treatment increased survival of mice with post-hepatectomy liver failure (PHLF) and ameliorated hyperammonemia and hypoglycemia in PHLF by providing liver function. Additionally, eLO treatment protected intestine from PHLF-augmented permeability and normalized the increased serum endotoxin and inflammatory response, which then facilitated hepatocyte proliferation and liver regeneration. The therapeutic effect of eLO was additionally proved in acetaminophen-induced liver failure. To ensure the clinical application, we performed extensive assessments of toxicity and biodistribution, demonstrating that eLO had no adverse effects on animals and remained non-tumorigenic. These findings guaranteed the clinical study of eLO transplantation in next step.

Funding Source: The National Natural Science Foundation of China (NSFC) (32221002), Shanghai Municipal Science and Technology Major Project, the National Key Research and Development Project (2019YFA0801503)

Keywords: liver organoids transplantation, proliferating human hepatocytes, liver failure

6:45 PM – 7:45 PM

150

LARGE SCALE PRODUCTION OF HIPSC DERIVED HEPATOCYTES FOR CELL BASED THERAPY

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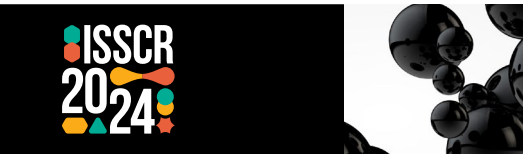
Liver disease is the only major disease where mortality is rising every year. The Covid-19 pandemic also had a major impact with a 21% death increase from liver disease in 2021 compared to 2019 in the UK. Thus, liver diseases are global health care challenge. The main reason of this situation lies in the absence of curative option for end stage liver disease with the only treatment being organ transplantation. Only a small fraction faction of patients can benefit from this option due to shortage of suitable organs whereas lifelong immunosuppression is necessary and carries associated risks. To tackle this unmet clinical situation, alternative therapies are urgently needed, and one promising strategy is cell-based therapy using hepatocytes. While there have been some encouraging results with this approach, the limited availability and quality of primary hepatocytes represent a major drawback. Thus, a new and unlimited source of cells is required to make cell therapy in the context of liver disease a clinical reality. Human induced pluripotent stem cell (hiPSCs) derived hepatocytes could provide an advantageous option. However current protocols are time and resource consuming while failing to produce fully functional cells. Here, we address this challenge by leveraging Forward Programming (FoP), a differentiation strategy, not solely relying on growth factors but, also on the overexpression of cell specific transcription factors (TFs) through the Opti-OX system. By identifying a suitable TFs combination, multiple iterations of protocol optimizations as well as adaptation to GMP compatible reagents in a 3D bioreactor setting, we created a scalable 20-day process with a high yield of ~10 FoP-Hepatocytes per hiPSC. These FoP-Hepatocytes are created at high purity as well as high quality. Indeed, the resulting cells exhibit functional characteristics



like albumin, A1AT and clotting factor secretion, Urea and CYP metabolism similar to primary hepatocytes. Finally, Fop-Hepatocytes have been successfully transplanted in mice models for liver failure. Taken together, these results demonstrate that large scale production of hepatocytes in vitro is feasible and that cells generated by forward programming can be used to counter liver disease symptoms thereby paving the way for future clinical developments in human.

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Keywords: liver disease, human induced pluripotent stem cells, cell-based therapy

6:45 PM – 7:45 PM

152

DERIVATION OF LIVER PROGENITORS FROM HUMAN PRIMARY HEPATOCYTES IN 2D AND 3D CONDITIONS

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The liver is a unique organ which fulfils a plethora of vital functions. Due to the importance of its tasks, diseases inducing liver dysfunctions are life-threatening and they currently account for more than 2 M deaths annually. The only treatment available is liver transplantation, which is associated with many drawbacks, especially a severe lack of organ donors. Cell-based therapy could provide an advantageous alternative as engraftment of functional hepatic cells could restore the functionality of the damaged organ. Accordingly, primary hepatocytes have already been used for cell therapy applications. However, these cells cannot be grown in vitro as they rapidly dedifferentiate and lose their metabolic activity. Thus, only a limited number of patients can benefit from this approach and the development of new methods to cultivate hepatocytes has been a major imperative for several decades. Here we describe our efforts to develop culture conditions to derive liver progenitors from adult hepatocytes under defined culture conditions in vitro. We have screened different conditions both in 2D and 3D to identify the best settings and media for the derivation of liver progenitors from human cells. To do so, we have employed human primary hepatocytes obtained from three different donors, and we have identified a cocktail of factors promoting the derivation of liver progenitors. The resulting cells can be maintained and expanded for several passages in vitro. They acquire the expression of biphenotypic markers such as ALB for hepatocytes and CK19 for cholangiocytes. Current work includes single-cell and functional analyses while the interest of these cells for cell-based therapy will be established using an animal model for liver failure. The development of a protocol to expand human liver progenitors from primary hepatocytes will have important implications not only for cell therapy but also as a model to investigate the mechanisms controlling hepatocyte dedifferentiation and redifferentiation during liver regeneration.

Funding Source: Einstein Foundation and Berlin Institute of Health

Keywords: cell therapy, liver disorders, primary hepatocytes

6:45 PM – 7:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

154

TENDON-DERIVED STEM CELLS: FROM THEIR IMMUNOMODULATORY POTENTIAL IN VITRO TO THEIR USE IN PRACTICE

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The repair of equine tendinopathies is presently a clinical challenge. These injuries often lead to an early end of the athletic career of the affected horses. Stem cells therapies aims to improve healing using different strategies like reparation, anti-inflammatory and immunomodulation actions by paracrine effects. This study aimed to further explore the immunomodulatory properties of tendon-derived mesenchymal stem cells (TDSCs) using a multiplexing immuno-assay approach by Luminex© - MagPix©. These tests will be carried out on freshly isolated cells and on cells after freezing. A specific freezing technique has been tested on the TDSCs in our laboratory. The freezing medium is a Dulbecco's Modified Eagle Medium (DMEM) dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) free medium enriched with 20% of glycerol as protective agent. This cryopreservation technique is in adequation with clinical therapies but we need to ensure that it preserves the immunomodulatory properties of our cells. The immunomodulatory properties of TDSCs are important for their use in clinical therapies. The paracrine action of TDSCs initially leads to a pro-inflammatory action (TNF-α, IL-1β, IL-6) which cleanses the lesion site. Next, anti-inflammatory molecules (IL-4, IL-10) modulate the inflammatory reactions to enable the recruitment of regenerative cells (fibroblasts, pro-regenerative macrophages) and to inhibit pro-fibrotic and catabolic markers (MMPs, TNF- α, IL-1, IL-12). Preliminary results showed that TDSCs are able to produce cytokines like IL-10, IL-4, IL-12p70, IL-6, TNF-α and IL-1β under inflammatory conditions. No significant difference between fresh and cryopreserved TDSCs was actually observed. These results need to

be confirm but are nevertheless promising for the creation of a bank of frozen TDSCs available for rapid use in the treatment of tendinopathies.

Keywords: tendon-derived mesenchymal stem cells, immunomodulation, cryopreservation

6:45 PM – 7:45 PM

156

A COMPARATIVE ANALYSIS OF ASC EXPANSION IN HOLLOW FIBER BIORACTORS AND TISSUE CULTURE POLYSTYRENE FLASKS: IMPACT ON IMMNUNOPHENOTYPES

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Adipose-derived stem cells (ASCs) hold significant promise for various regenerative applications. However, a large number of in vitro expanded ASCs is required for practical therapies. The immunophenotypical profiles, influenced by cell population times, play a pivotal role in shaping ASC potency. In this context, ASC cultures from the hollow fiber bioreactor (HFB) were compared with the traditional tissue culture polystyrene (TCP) flasks, with an emphasis on immunophenotypes. The ASCs for three donors were expanded in the two systems for an equivalent number of population doublings and analyzed for the co-expression of 13 epitopes including Panel A (CD73, CD90, CD105, CD166, and CD201) and Panel B (CD34, CD36, D146, CD200, CD248, CD271, CD274, and Stro-1) by multichromatic flow cytometry. The HFB supported faster growth rates, with an average doubling time (DT=1.24) being shorter than that in the TCP system (DT=1.52). Regarding lineage prevalence, the CD201+ clones were favored in the TCP conditions, whereas those lacking the CD274 had a predilection for the HFB environment. Furthermore, the HFB paradigm promoted more immunophenotypical heterogeneity and greater donor-to-donor variation. Samples from one of the donors appeared highly discrepant from the rest, mitigating the differences between the systems. HFB appears to provide for an accelerated expansion and less immunophenotypical selection pressure than the TCP with the caveat that some rare donors may yield unique lineages that can be propagated only in a specific system. Our study provides a useful basis for rational decision-making regarding the choice of in vitro ASC expansion approaches.

Funding Source: This project was supported by the Obelske Family Foundation and Svend Andersen Foundation.

Keywords: adipose-derived stem cells, immunophenotype, in vitro expansion

6:45 PM – 7:45 PM

158

HAIR FOLLICLE MESENCHYMAL STEM CELL SECRETOME AS A PROMISING CELL-FREE THERAPY IN WOUND HEALING AND CARTILAGE REPAIR

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The MSC (mesenchymal stem cell) secretome, known for its wound-healing and regenerative capabilities, offers a cell-free alternative devoid of the tumorigenic risks associated with other stem cell and MSC-based therapies. However, common MSC sources such as bone marrow are costly, invasive, and limit the production scale. The human hair follicle, rich in diverse cell types, presents a promising and accessible source for autologous stem cell therapies aimed at tissue repair and regeneration. Amongst MSC sources, the plucked hair follicle is the most accessible, non-invasive, cost-effective, and readily scalable. This study focuses on characterizing the HF-MSC secretome's protein composition and assessing its therapeutic efficacy in vitro and in vivo. MSCs were successfully isolated and expanded from plucked hair follicles, achieving scalable numbers and confirming their MSC phenotype. The cells exhibit adherence to plastic and are easily expandable, displaying the typical MSC immunophenotype with CD90+, CD105+, CD73+, and CD44+. Moreover, these MSCs have the capacity to differentiate into adipocytes, osteocytes, and chondrocytes. Cell secretome was collected and characterized in various biochemical assays such as cytokine arrays and ELISAs. High levels of growth factors, collagen, and hyaluronic acid were found, alongside its potent stimulation of tissue repair mechanisms. Its effectiveness in promoting wound healing and cartilage regeneration was demonstrated through in vitro scratch assays and an in vivo mouse model with a cartilage defect, revealing significant improvements in tissue repair. These insights contribute to advancing cell-free therapy, highlighting the HF-MSC secretome's potential as a safe, effective, and commercially viable treatment option for regenerative medicine, in both wound-healing and tissue regeneration.

Keywords: secretome, MSC, healing

6:45 PM – 7:45 PM

160

TRANSGENE FREE INDUCED MESENCHYMAL STEM CELLS FOR EQUINE REGENERATIVE MEDICINE APPLICATIONS

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The use of autologous Mesenchymal Stem Cells (MSCs) for tissue regeneration is well-established in equine veterinary practice. However, using autologous MSCs is time-intensive, costly, and subject to variable clinical efficacy depending on donor and cell extraction procedure. Alternatively, induced pluripotent stem cells (iPSCs) could provide an attractive source of 'off-the-shelf' induced MSCs (iMSCs) to address the limitations of naïve MSCs. While equine iPSCs have been successfully generated using transgene-integrating approaches, obtaining genetic footprint-free iPSCs, as would be required for an eventual therapeutic use, has to date met with extremely limited success. Our aim was to use a Sendai Virus (SV)-based system to generate equine iPSC as a source of genetic footprint-free iMSCs from horses. Infection of umbilical cord-derived MSCs with SV vectors coding for human OCT4, SOX2, KLF4, and c-MYC sequences resulted in colonies that displayed classic iPSCs morphology and exhibited positive staining for Alkaline Phosphatase. Colonies were expanded successfully for over 20 passages while retaining typical iPSC morphology and maintaining expression of key pluripotency markers including OCT4, SOX2, LIN28, DNMT3, TERT and NANOG. In addition, embryoid bodies (EBs) were generated that stained for markers of all three germ layers including TUBB3 (ectoderm), AFP (endoderm) and VIM (mesoderm). Finally, either using EB differentiation or through direct induction, mesenchymal cell populations were generated with a distinct immunophenotype (CD90+, CD44+, CD29+, CD105+) typical of naïve MSCs, while expressing almost negligible levels of SV. Moreover, these cells showed chondrogenic and adipogenic differentiation abilities, similar to naïve MSCs. In conclusion, we report for the first time the generation of footprint-free iMSCs from horses. Our results pave the way for the next-generation of cell-based regenerative therapies for veterinary medicine.

Funding Source: IC BIOSOLUTIONS

Keywords: equine iPSC, Sendai Virus, 'off-the-shelf' induced MSCs

6:45 PM – 7:45 PM

162

COGNITIVE EVALUATION OF CHILDREN WITH CEREBRAL PALSY FOLLOWING CELL THERAPY: A RANDOMIZED CLINICAL TRIAL

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Cerebral Palsy (CP) is characterized by lifelong motor dysfunction and cognitive impairment in 30-50% of the patients. Although the safety and efficacy of stem cell therapy in improving the gross motor function of children with CP have been widely evaluated, available data on cognitive improvement is insufficient. In this study, we aim to explore the effect of Umbilical Cord Tissue-derived Mesenchymal Stromal Cells (UCT-MSCs) and Umbilical Cord Blood Mononuclear Cells (UCB-MNCs) on the cognitive function of children with CP. In this double-blinded randomized clinical trial, 108 children aged between 4 to 14 years old with spastic CP were randomly and equally allocated to three groups. Experimental groups received a single dose intrathecal injection of either 5x106/kg UCB-MNCs or 2x107/kg UCT-MSCs. A valid and reliable researcher-made questionnaire (Cronbach's alfa=0.78) was used to assess the intellectual impairment of the children with CP. The participants' cognitive function was assessed at baseline, 6, and 12 months after the intervention in seven domains including language, perceptual-motor function, social cognition, complex attention, memory, motivation, and understanding of primary concepts. Results: All domains of cognitive function had significant progress at the 12-month follow-up (P-values< 0.05). The complex attention, memory, and perceptual-motor function were substantially improved during the follow-ups (P-value< 0.001). The mean difference of the total cognitive function score between the UCB-MNC and the control groups was 39.98 (95% CI: 14.1-65.8; P-value=0.002) and 44.63 (95% CI: 18.8-70.44; P-value= 0.001), respectively at the 6th and 12th months follow-ups. The mean difference of the total score of cognitive assessment between the UCT-MSC and control groups was 42.56 (95% CI: 13.37-71.75; P-value=0.004) and 49.8 (95% CI: 20.64-79.03, P-value=0.001), respectively at the 6th and 12th months follow-ups. This study demonstrated that the mean score of cognitive function was significantly improved during the 12-month

follow-up in the children with CP who received intrathecal cell transplantation in all domains, especially in attention, memory, and perceptual-motor function in comparison with the control group.

Funding Source: The Research Deputy of Tehran University of Medical Sciences and Royan Stem Cell Technology Company

Keywords: cognition in cerebral palsy, mononuclear cells, mesenchymal stromal cells

Clinical Trial ID number: NCT03795974

6:45 PM – 7:45 PM

164

FROM BENCH TO CLINIC: ROBUST 2D AND 3D MSC EXPANSION AT SCALE

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Human mesenchymal stem cells (hMSCs) hold excellent therapeutic potential due to their ability to self-renew, as well as differentiate into multiple lineages. However, one of the major challenges is obtaining the large number of cells required for clinical therapy applications. One of the key requirements is a robust, xeno-free culture media that can support large scale expansion of hMSCs in 2D and 3D, whilst maintaining their multipotency. To address this, we have developed a xeno-free, phenol red-free medium for maintenance and expansion of MSCs, available in both research and GMP grades. Here we show that Cellartis® MSC Xeno-Free Culture Medium supports robust expansion of both bone marrow- and adipose-derived hMSCs. Furthermore, MSCs could be flexibly expanded either with or without coating in Cellartis MSC medium in 2D and 3D formats which allows simpler and economical workflows. HMSCs were confirmed to be more than 98% positive for key markers including CD73, CD90 and CD105 by flow cytometry after 16-23 PDL. Multilineage differentiation of the cells into adipocytes, chondrocytes and osteocytes was further confirmed. These data show that Cellartis MSC xeno-free medium supports the expansion of hMSC while maintaining their multipotency, thus facilitating easy scale up for research and clinical applications.

Keywords: hMSCs, 3D expansion, xeno-free

6:45 PM – 7:45 PM

166

DEVELOPMENT OF A 3D SUSPENSION CULTUE SYSTEM FOR MESENCHYMAL STEM CELLS WITH CELLHESION-MS

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Cell therapy using mesenchymal stem cells (MSCs) is a potential modality for regenerative therapy. Large-scale production is required for further clinical applications, however conventional 2D culture system has some limitations mainly in lot size. 3D suspension culture system is attracting attention to overcome this problem. Microcarrier is now widely used as a scaffold in 3D culture system, but there are some problems such as complicated process control in seeding step and fusion. We focused on Cellhesion-MS, a chitin-based nanofiber, as a scaffold to develop a novel 3D culture system for MSCs expansion. Compared to conventional 2D culture, MSCs cultured with Cellhesion-MS have been demonstrated to have improved secretion of immunomodulatory and angiogenic factors and significant upregulation of stemness and migration related genes. However, these studies were conducted only static condition so there is much room for improvement to apply large-scale cell production.We applied Cellhesion-MS for adipose-derived MSCs expansion in dynamic condition. Cells formed aggregates including Cellhesion-MS and grow maintaining dispersed aggregates with single agitation control in seeding and expansion process in bioreactor. Then we tried scale-up to 5 L, as a result cells grow 80 times for 7 days and live cell density reached at 1.2 x 10^6 cells/mL same efficiency as in 30 mL scale. These results indicate that the 3D culture system with Cellhesion-MS has a potential for large-scale MSCs production.

Keywords: 3D culture, MSC, large-scale culture

6:45 PM – 7:45 PM

168

ENGINEERED BIOASSEMBLIES OF PRE-VASCULARIZED SOFT BONE CALLUS MICROTISSUES LEADS TO ACCELERATED BONE FORMATION

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Critical-size bone defects resulting in non-unions pose a significant challenge due to the limited success of current treatment strategies. Hence there is a need for the engineering of large tissue implants able to mimic native bone defect regeneration which is based on the formation of a transient cartilaginous tissue. Thus, it was explored the development of pre-vascularized soft bone callus microtissues from human periosteum-derived cells (PDCs) and endothelial cells (ECs). Meso-scale-size implants were engineered through self-assembly and their endochondral ossification potential was assessed in vivo. The incorporation of ECs into soft bone callus microtissues resulted in accelerated chondrogenic maturation. Microtissues differentiated in the presence of ECs exhibited higher sulfated glycosaminoglycan content, highly-organized Collagen I and Collagen II together with higher expression of hypertrophy-related genes IHH and COL10A. The origin of ECs whether umbilical vein (HUVECs) or skin microvascular (MVECs) did not exert a significant influence on the maturity of microtissues in vitro. The whole-mount two-photon imaging of microtissues revealed that the ECs were consistently patterned and compartmentalized within each microtissue. The presence of ECs initiated PDCs proliferation, which was stopped by day 14, leading to controlled proliferation-differentiation homeostasis and a stable EC:PDC ratio in the engineered construct. The MERFISH spatial transcriptomics for key 140 genes revealed important PDCs-EC interplay resulting in mini-marrow niche formation. Upon ectopic implantation in vivo, pre-vascularized implants facilitated earlier mineralization. Pre-vascularization in vitro supported the proper formation of the cortex and bone trabeculae as well as well-developed bone marrow compartments within ossicles in vivo. The importance of living microtissue-based implants was additionally confirmed by the presence of human cells after 4 weeks from implantation and secretion of human OPN in the sites with active bone remodeling processes. In conclusion, pre-vascularized soft bone callus implants support endochondral bone regeneration events, which makes microtissue-based implants a promising autologous solution to treat challenging boned defects.

Funding Source: MSCA4Ukraine-project ID: 101101923

Keywords: tissue engineering, biomanufacturing, cartilaginous microtissues

6:45 PM – 7:45 PM

170

ENHANCING EFFICACY OF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS VIA IFN-GAMMA PRIMING: A TRANSCRIPTOMIC AND FUNCTIONAL STUDY

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Mesenchymal stem cells (MSCs) are integral to regenerative medicine, offering promising therapeutic applications through their tissue regeneration and immunomodulatory abilities. Despite the great potential of MSC therapy in veterinary medicine, especially for treating canine immune-mediated diseases, optimizing and enhancing strategies such as priming with pro-inflammatory cytokines requires further understanding. In the present study, the immunomodulatory effects of canine adipose tissue-derived MSCs (cAMSCs) primed with interferon-gamma (IFN-κ), a pro-inflammatory cytokine, were evaluated and further complemented by comprehensive RNA sequencing analysis. To evaluate the immunomodulatory function, cAMSCs were treated with 50 ug/ml IFN-κ for 48 hours. RNA sequencing following IFN-κ priming showed upregulation in crucial genes for immunomodulation (IDO1, PD-L1), cellular communication (CD74, DLA-DQA1, DLA-DRA), and cell trafficking (CXCR4, CCL8, CX3CL1), resulting in an enhanced cAMSCs' response to inflammatory stimuli. PCR validation further supported the results by presenting increased expression of pluripotency markers (OCT4, SOX2, NANOG), immunomodulatory genes (IDO, COX2, PD-L1, HGF), migration factors (VCAM, ICAM, E-cadherin), and PGC-1α, highlighting in mitochondrial function enhancement. To evaluate the metabolic enhancement of IFN-κ-primed cAMSCs, the oxygen consumption rate, mitochondrial membrane potential, and oxidative stress levels were measured through mitochondrial function assays, TMRM staining, and MitoSOX assays, respectively. The primed-cAMSCs demonstrated a significant reduction in T-cell proliferation by co-culture with peripheral blood mononuclear cells (PBMCs), indicating a potentiated immunomodulatory capacity. Moreover, the motility of the primed-cAMSCs was improved as evidenced by migration assay, which is a critical attribute for targeted tissue repair. In summary, IFN-γ priming significantly enhances cAMSC functionality and upregulates crucial genes for immunomodulation, pluripotency, and cellular metabolism. The comprehensive approach underscores the potential of priming strategies for advancing MSC-based therapies in canines.

Keywords: IFN-γ primed canine MSCs, mitochondrial metabolism, immunomodulation

6:45 PM – 7:45 PM

172

INFLAMMATORY PRIMING ENHANCES THE SECRETION OF ANTI-INFLAMMATORY PROTEINS BY MESENCHYMAL STROMAL CELLS

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The therapeutic properties of Mesenchymal Stromal Cells (MSCs) are generally attributed to their paracrine activity. Priming approaches using various cytokines in the culture microenvironment of MSCs can optimize the secretions of therapeutic factors which may be used in the clinical development of cell-free therapies for target indications. We have harnessed this ability of MSCs by preconditioning them with a cocktail of proteins to mimic the inflammatory microenvironment to elicit anti-inflammatory response. MSCs isolated from adipose tissue and Wharton's jelly of umbilical cord were characterized referring to ISCT criteria, by analyzing the morphology, surface markers, and trilineage differentiation potential. MSCs cultured till 70-75% confluence were exposed to a cocktail of IL-1β, TNF-α, and IL-17 for 4, 24, and 48h. Cell surface, apoptotic markers, and cell cycle were analyzed post priming. The mRNA expression levels of IL-6, TSG-6, and CCL-20 were measured using qPCR, as an indicative of its anti-inflammatory potential. Vascular endothelial growth factor (VEGF), TNF-α, and IL-17 levels in the secretome were quantified using sandwich ELISA. In vitro macrophage polarization assay was performed to check the functionality of the preconditioned secretome towards resolving inflammation. Cell cycle analysis of primed MSCs confirmed an acceptable range of DNA ploidy with no significant cell death. Q-PCR revealed a statistically significant increase in the expression levels of IL-6, TSG-6, and CCL-20, which are indicative of anti-inflammatory function. ELISA showed < 2.0 ng/mL of TNF-α and IL-17 indicating that the inflammatory proteins were not retained in the secretome while harvesting MSCs post priming. VEGF levels quantified in the secretome (< 2.0 ng/mL) did not show enhanced angiogenic potential, which would increase inflammation. In vitro studies using secretome showed polarization of macrophages to anti-inflammatory M2 phenotype. These results show promising avenues for future therapies using primed MSC secretome tailored for inflammatory diseases. Compared to cell therapies, secretomes can be utilized as a preferred alternative as they are non-immunogenic, have prolonged shelf-life, show batch-to-batch consistency, and can be formulated to meet specific demands.

Keywords: anti-inflammation, MSC secretome, priming

6:45 PM – 7:45 PM

TOPIC: MUSCULOSKELETAL

174

EXPLORING DONOR HETEROGENEITY FOR THE CLINICAL APPLICATION OF AUTOLOGOUS CELLS TO TREAT KNEE OSTEOARTHRITIS

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Osteoarthritis (OA) is a common degenerative disease of the joints affecting millions of people worldwide. Critical-size osteochondral defects, in which self-healing is compromised, are recognized as one of the major risk factors. Previous studies highlighted the ability of human periosteum-derived cells (hPDCs) to repair this type of lesion, making them an interesting cell source for autologous therapies that aim to prevent the onset and/or delay the progression of OA. Nonetheless, the behavior and potency of hPDCs can be variable, complicating their clinical application. In this work, we aim to explore donor cell heterogeneity to unravel cell performance and address the reproducibility of using these cells in autologous therapies. To this end, we collected periosteum from the knees of 20 individuals, aged 15 to 45 years, and isolated hPDCs for extensive cell characterization. We found that donor cells showed similar viability, yet differences in proliferation rates and growth curves were observed. Moreover, while all donors differentiated to the chondrogenic lineage, the osteogenic and adipogenic differentiation profiles presented more variability, including failure to differentiate in some donors. Furthermore, donor-derived cells had different expression profiles of the mesenchymal and skeletal stem cell markers CD73, CD105, CD90, CD164, CD146 and PDPN, measured by flow cytometry. These findings show that hPDCs sourced from the same area of the knee can exhibit diverse cellular characteristics and behavior. Further exploration of these differences will offer insights into the consistency and potential outcomes of employing these cells in personalized stem cell therapies.

Keywords: osteochondral defects, periosteum, donor variability

6:45 PM – 7:45 PM

176

POTENTIAL MARKER EXTRACTION TO PREDICT AN EFFICACY OF ALLOGENEIC CHONDROCYTE CELL SHEET ON KNEE CARTILAGE REGENERATION

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Knee osteoarthritis (OA) is a disease whose symptoms progress with age and yet no fundamental treatment has been established. Dr. Masato Sato of Tokai University applied cell sheet engineering using polydactyly-derived chondrocytes on temperature-responsive cell cultureware to treat cartilage defects associated with knee OA. We are currently developing an allogeneic chondrocyte sheet (CLS2901C) based on his research. We established a master cell bank (MCB) of chondrocytes by confirming safety and efficacy and started a Phase III clinical trial in Japan in September 2023. For donor selection, we evaluate the efficacy of CLS2901C on cartilage regeneration using the nude rat osteochondral defect model. Although this model is useful for predicting the efficacy on cartilage regeneration, it is time-consuming, expensive and has animal welfare issues. In this study, we extracted potential in vitro markers to predict an efficacy on cartilage regeneration. We prepared chondrocyte sheets at different passages from seven donors and transplanted to nude rat model. The gene expression and the secreted proteins of the same sheets were analyzed by RNA sequencing and aptamer-based proteomics assay, respectively. We obtained the 21,121 gene expression data and the 7,596 protein expression data, and then correlated them with the in vivo tissue repair potency scored by the modified International Cartilage Repair Society (ICRS) histological grading system. Focusing on adhesion-related molecules, we identified nine genes with a high correlation with in vivo efficacy. In this study, we extracted potential markers to predict the efficacy on cartilage regeneration of chondrocyte sheet. In the future, we may replace the animal testing with these markers for donor selection and expansion of the cell bank for the large-scale production of CLS2901C.

Funding Source: Japan Agency for Medical Research and Development (AMED)

Keywords: efficacy marker, cell sheet, cartilage regeneration

6:45 PM – 7:45 PM

178

PRECLINICAL GENE THERAPY IN AN ENGINEERED MUSCLE TISSUE MODEL OF DUCHENNE MUSCULAR DYSTROPHY DERIVED FROM PLURIPOTENT STEM CELLS

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Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease caused by mutations in the DMD gene coding for dystrophin. Young DMD boys suffer from marked muscle degeneration which progresses into respiratory failure, cardiomyopathy and premature death. No curative treatment exists to date, but promising approaches are currently investigated in the clinic, such as gene therapy using vectors derived from the adeno-associated virus (AAV). In animal models of DMD, AAV gene therapy leads to long-term transgene expression and to an almost complete phenotypic rescue. Promising results have also been obtained in clinical trials, however several patients have experienced serious adverse events that were not anticipated in pre-clinical studies. Therefore, new experimental models are urgently required for the development of next generation therapeutic strategies with an improved efficacy and safety profile. In this context, human induced pluripotent stem cells (hiPSCs) derived from patient samples could provide an alternative to animal experimentation. Our main objective is to develop a preclinical testing platform for DMD using engineered muscle tissues derived from hiPSCs. We first optimized a protocol to generate large batches of myogenic progenitors from DMD and control hiPSCs, that we use as building blocks to generate engineered muscle tissues (EMTs) in fibrin hydrogels. We characterized the phenotype of DMD EMTs in vitro at the structural and functional level, and by RNA-Sequencing. In parallel, we optimized a gene transfer protocol in hiPSC-derived muscle tissues using a reporter AAV vector. We determined the most efficient vector embedding strategy for successful transduction, together with the minimal dose and the most efficient AAV serotypes. Transgene expression was detected with doses as low as 1.0E+3 vector genomes (vg) per cell, and could be maintained over 4 weeks, proving that the EMT model allows for durable monitoring of AAV efficacy. We are currently evaluating the efficacy of a therapeutic AAV vector expressing microdystrophin in DMD EMTs to demonstrate the relevance of in vitro gene therapy in a disease context. In the future, this preclinical testing platform will be used to screen libraries of next generation AAV vectors and accelerate the discovery of the most promising candidates.

Keywords: gene therapy, engineered muscle tissue, Duchenne Muscular Dystrophy

6:45 PM – 7:45 PM

180

ENGINEERING STEM CELLS AS FACTORY UNITS FOR THE MANUFACTURING OF HUMAN TISSUE GRAFTS

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The demand for tissue/organ replacement is growing substantially driving the need for innovative engineering strategies. Existing approaches rely predominantly on the design of living grafts, associated with unpredictable performance, limited availability, and autologous-restricted exploitation. To overcome these challenges, we propose a paradigm shift by engineering dedicated human stem cell lines, offering the standardized and customized generation of tissue grafts. Following in vitro tissue engineering, decellularization and lyophilization steps are expected to lead to an off-the-shelf biomaterial capable of guiding endogenous repair in vivo. We validated the approach in the context of bone repair, for which 2 million grafts are needed on a yearly basis. Human mesenchymal stem cell lines were genetically programmed to ensure the production of cartilage tissue enriched in osteoinductive factors. Effective decellularization and lyophilization was demonstrated and led to tailored immunogenicity in animal models and upon in vitro exposure to human immune components. The resulting acellular cartilage material further exhibited unprecedent bone repair capacity in a critical rat femoral defect. By encompassing both safety and performance, our work prompts a clinical phase-1 trial using our validated GMP-based protocol. Our findings exemplify the broader paradigm of harnessing human stem cell lines as biological factory units to engineer customized acellular tissues, self-sufficient to activate endogenous regenerative programs. Beyond bone regeneration, this work lays the ground for application in other tissue/organ contexts.

Keywords: endochondral ossification, human skeletal development, tissue engineering

6:45 PM – 7:45 PM

TOPIC: NEURAL

182

DEVELOPING HPSC-BASED DRUG SCREENING AND CELL THERAPY PLATFORMS FOR ENTERIC NEUROPATHIES

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Gastrointestinal (GI) motility disorders represent a significant medical challenge, as current therapeutic options are lacking. These disorders often result from dysfunction in enteric inhibitory motor neurons, particularly those producing nitric oxide (NO neurons), which play a pivotal role in regulating GI motility. Selective loss and dysfunction of NO neurons are associated with devastating dysmotility conditions including achalasia, gastroparesis, intestinal pseudo-obstruction, and colonic inertia. Here, we introduce a novel human pluripotent stem cell (hPSC)-based platform for the development of therapeutics targeting GI motility disorders. Leveraging our innovative hPSC-derived 2D enteric nervous system (ENS) cultures and 3D enteric ganglioids, we conducted an unbiased high-throughput screening approach to identify drug candidates capable of inducing NO neuron activity. Furthermore, we demonstrated the efficacy of these candidates in promoting motility in murine colonic tissue ex vivo. Moreover, our transplantation studies, where engraftment of ENS ganglioids into the colon of NO neuron-deficient mice led to significant improvement in GI motility. This xenograft model not only provides a platform for studying human ENS dynamics in vivo but also holds potential for the development of cell-based therapies for neurodegenerative GI disorders. Our findings offer insights into fundamental aspects of the human ENS and lay the groundwork for the design of effective therapeutic strategies to address enteric neuropathies.

Funding Source: We thank UCSF PBBR, Sandler Foundation, UCSF QCRG, NIH Director's New Innovator Award DP2NS116769, NIDDK R01DK121169, Takeda Development Center Americas, Inc, NIH T32-DK007418, LLHF postdoctoral fellowship.

Keywords: enteric nervous system (ENS), enteric neuropathy, cell therapy

6:45 PM – 7:45 PM

184

INJECTION OF HUMAN NEURAL STEM CELLS INTO THE CISTERNA MAGNA, A NOVEL APPROACH FOR AMYOTROPHIC LATERAL SCLEROSIS (ALS), DEPENDS ON CONCENTRATION & VOLUME OF CELL SUSPENSION

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ALS is a lethal neurodegenerative disease caused by the death of motoneurons (MNs) & loss of skeletal muscle innervation, resulting in paralysis & respiratory failure. We previously demonstrated that human neural stem cells (hNSCs) are neuroprotective in the SOD1 mouse model of ALS based on their constitutive secretion of neuroprotective factors & their spontaneous differentiation into trophic (rather than toxic) glia (astrocytes, gray matter oligodendrocytes). Survival was dramatically prolonged (up to 300%) in 25% of mice. Survival correlated with the extent to which the SOD1 spinal cord (SC) was rendered chimeric with hNSCs. We are trying to maximize hNSC distribution along the SOD1 neuraxis. We synthesized a peptide agonist of the binding pocket of CXCR4 on hNSCs, enabling us to direct their migration & increase their spread. We co-administer this peptide with hNSCs in the SC to increase hNSC coverage & chimerism. Furthermore, we now transplant hNSCs less traumatically into the cisterna magna (CM), a route which itself enhances hNSC distribution (by co-mingling with CSF flow) while positioning hNSCs in proximity to cervical respiratory control centers. Once in the thecal space, hNSCs migrate along ventral roots into the ventral horn (VH) juxtaposed to MNs. The peptide, injected minimally-invasively into the VH (like an LP), augments that process. Because intra-CM injections are novel, we report methodological insights we've gained to ensure success in the SOD1 mouse. hNSCs suspended in volumes >10 µL overwhelm the mouse CM capacity, resulting in reflux of the injectate. Increasing hNSCs concentration >2x105/µl leads to clumping & obstruction of needle flow. These observations define the upper parameters for a single hNSC dose: 2x105 hNSCs/µL in 10µl HBSS. Recognizing that, if a greater hNSC number were desired, that an additional hNSC injection within those parameters would be required after a time interval, we devised a chronically-indwelling transcutaneous port into the CM. Note: such considerations are pertinent only to preclinical rodent models of ALS because the CM capacity in humans is vast & accessible via non-surgical, minimally-invasive, repeatable needle injections using anatomical landmarks or ultrasound. However, successful preclinical studies are crucial precursors to clinical trials.

Funding Source: Supported by California Institute for Regenerative Medicine (CIRM) & SENS Research Foundation

Keywords: human neural stem cells, ALS, cell therapy

6:45 PM – 7:45 PM

186

OVERCOMING SOME OF THE PITFALLS OF RETINAL ORGANOID (RO) CULTURING: TOWARDS LARGE SCALE PRODUCTION

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Multiple groups have demonstrated the importance and of pluripotent cells-derived retinal organoids (RO). As ROs are laminated and highly structured, they faithfully replicate complex cell:cell interactions in the human retina in vivo. Therefore, ROs are ideal models for disease modelling. In addition, individual cell types can be isolated from ROs and used as an unlimited source for cell transplantation. However, a main challenge of working with ROs is the length of cell culture time and laborious differentiation protocols. ROs replicate human development, consequently the cultures times are long, with differentiation times of 120-260 days. Additionally, the initial steps of most published protocols involve a considerable amount of manual labour, with individual picking of neuro-epithelial vesicles, which, eventually, develop into ROs. These challenges impede easy upscaling and automatization of RO production and applications in therapeutic areas. The highly manual-dependent steps are especially problematic, when aiming to achieve GMP compliance for cell therapeutic applications. Here, we show that our protocol overcomes those main challenges, allowing for robust production of thousands of ROs per differentiation, with the possibility for easy further upscaling and automatization. Our protocol provides robust ROs, which are consistent across differentiations and iPS cell lines, as shown by gene expression, histology, and single nuclei RNA sequencing. We can also demonstrate enrichment for photoreceptor cells, which show mature markers, such as abundant expression of opsins, from day 100 in culture. Taken together, our results show that our RO platform is robust, leads to a reduction of culturing days and improves

the scalability of the process. Evotec aims to use ROs as a valuable and more efficient and available tool in drug discovery and cell therapy to treat retinal degeneration.

Keywords: photoreceptor replacement, retina, cell therapy and production upscaling

6:45 PM – 7:45 PM

188

RAS PATHWAY ACTIVATION FOR ENHANCED SURVIVAL AND NEURITE GROWTH OF INDUCED DOPAMINERGIC NEURONS

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Recent advances in stem cell research enabled researchers to generate and transplant induced pluripotent stem cells (iPSCs)-derived dopaminergic neurons to the brains of Parkinson's disease (PD) patients. Due to significant improvements in motor functions in vivo, we and others have started the first clinical trials. However, due to increased cell death and restricted neurite growth of transplanted cells within the host brain's inhibitory environment, the full potential of these cell replacement therapies has not yet been unleashed. Therefore, as cell transplantation alone cannot solve this problem, our research focuses on modulating signaling pathways in transplanted induced dopaminergic (iDA) neurons to overcome limitations and enhance therapeutic outcomes for PD. Previously, it was shown in vitro and in vivo that H-RAS (Harvey-Rat sarcoma) is able to promote neurite growth, enhance cell survival, protect from neurotoxic insults, and more. Here, the constitutive-active mutant H-RAS V12 is introduced into iPSCs that are subsequently differentiated into iDA neurons. A doxycycline-inducible promoter ensures the temporally controlled H-RAS V12 expression in differentiated cells. To enhance neuronal survival, H-RAS V12 expression is induced before transplantation, while a sustained H-RAS V12 expression aims to promote integration and increased neurite length of transplanted cells within the host brain. Preliminary in vitro experiments demonstrated that modified iPSCs expressing H-RAS V12 show increased neurite length. Moreover, iDAs were transplanted by stereotactic injection into the striatum of mice. Expression of H-RAS V12 for a duration of three months was confirmed. Behavioral tests after transplantation in hemi-Parkinson rats and subsequent histological analyses are currently performed to investigate the effects in vivo. By harnessing the potential of H-RAS signaling, we aim to unlock the full capabilities of induced dopaminergic neurons, thereby improving the quality of life for PD patients. The outcomes of this study not only hold promise for PD but also pave the way for advancements in cell replacement therapies for other central nervous system disorders like stroke and brain injuries, significantly impacting healthcare strategies.

Keywords: cell transplantation, RAS signaling pathway, Parkinson's disease

6:45 PM – 7:45 PM

190

BIOREACTOR PRODUCED, IPSC-DERIVED DOPAMINERGIC NEURON CONTAINING NEURAL MICROTISSUES INNERVATE AND RESTORE MOTOR FUNCTION IN A DOSE-DEPENDENT MANNER IN A PARKINSON RAT MODEL

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A breadth of preclinical studies is now supporting the rationale of pluripotent stem cell-derived cell replacement therapies to alleviate motor symptoms in Parkinsonian patients. Replacement of the primary dysfunctional cell population in the disease, i.e. the A9 dopaminergic neurons, is the major focus of these therapies. To achieve this, most therapeutical approaches involve grafting single-cell suspensions of DA progenitors. However, a considerable number of cells die during the transplantation process, as cells face anoïkis. One potential solution to address this challenge is to graft solid preparations, i.e. adopting a 3D format. Cryopreserving such format remains a major hurdle and is not exempt from causing delays in the time to effect, as observed with the use of cryopreserved single-cell DA progenitors. Here, we used a high-throughput cell-encapsulation technology coupled with bioreactors to provide a 3D culture environment enabling the directed differentiation of hiPSCs into neural microtissues. The proper patterning of these neural microtissues into a midbrain identity was confirmed using orthogonal methods including qPCR, RNAseq, flow cytometry and immunofluorescent microscopy. The efficacy of the neural microtissues was demonstrated in a dose-dependent manner using a parkinsonian rat model. The survival of the cells was confirmed by post-mortem histological analysis, characterised by the presence of human dopaminergic neurons projecting into the host striatum. The work reported here is the first bioproduction of a cell therapy for Parkinson's disease in a scalable bioreactor, leading to a full behavioural recovery 16 weeks after transplantation using cryopreserved 3D format.

Keywords: cell therapy, off-the-shelf, 3D cell format

6:45 PM – 7:45 PM

192

MODELLING THE IMMUNE EFFECTS OF EMBRYONIC STEM CELL-DERIVED DOPAMINE CELL THERAPIES FOR PARKINSON'S DISEASE

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Parkinson's disease (PD) is a common, progressive neurodegenerative disorder, with a core pathology being the loss of a population of midbrain dopaminergic neurons which project to the striatum. Dopamine cell-based therapies to replace this population of lost neurons are being investigated in a number of clinical trials, including a European study between Lund in Sweden and Cambridge in the UK (the STEM-PD trial), which is using an allogenic dopamine cell product, derived from the RC17 human embryonic stem cell (hESC) line. These cells have been tested in pre-clinical animal models but their interaction with human immune cells in the brain, in particular with microglia and infiltrating T-cells, is not well characterised. We have previously shown little immunogenicity of the cells when co-cultured with primary T-cells from healthy and PD donors but have not previously investigated the innate immune response to the cells, or the NPC-T-cell interaction, in the presence of human microglia, which have the capacity to present foreign antigen to T-cells. In this study, we sought to model this interaction of the hESC-derived dopaminergic cell product in-vitro with human microglia, with and without T-cells. This work has involved looking at both primary human and induced pluripotent stem cell-derived microglia. The work is ongoing and the preliminary results work will be presented.

Keywords: Parkinson's disease, cell therapy, microglia

6:45 PM – 7:45 PM

194

A GDNF RICH TRANSPLANTATION SITE PROMOTES THE SURVIVAL AND MATURATION OF DIRECTLY REPROGRAMMED DOPAMINE PRECURSORS AND RESTORS MOTOR DEFECTS IN A 6 OHDA RAT MODEL OF PARKINSONS DISEASE

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Parkinson's disease is a neurodegenerative disease characterized by the loss of A9 dopaminergic neurons in the substantia nigra. Cell replacement therapy (CRT) for Parkinson's disease involves transplanting dopamine precursor cells into the brain to replace the lost neurons. Our laboratory has created dopaminergic precursors (hiDAPs) directly reprogrammed from human dermal fibroblasts using non-viral chemically modified mRNA expressing SOX2 and LMX1A with a two-week reprogramming protocol. This results in a clinically viable source of dopamine precursors for CRT. We have refined this protocol by investigating the timing of addition of key lineage-specific small molecules Sonic Hedgehog (SHH) and Fibroblast Growth factor 8 (FGF8) during reprogramming. Results showed the addition of SHH only at the start of reprogramming (D4-D7) and FGF8 only during the second week of reprogramming (D7-14) produced a greater number of neurons expressing the dopaminergic specific markers tyrosine hydroxylase (TH), NURR1 and neuronal marker TUJ1 compared to the standard protocol. This improved reprogramming protocol was chosen to assess the potential for hiDAPs to survive, generate dopaminergic neurons and improve motor function impairment following transplantation into a 6-OHDA lesion model of PD. The effect of the neurotrophic factor GDNF administered by intrastriatal AAV-GDNF injection on hiDAP transplant survival and dopaminergic differentiation was also assessed. 6-OHDA-lesioned rats were separated into the following groups (n=12 per group); 1)AAV-GDNF injection 2 weeks prior to hiDAP transplant; 2) AAV-GDNF injection and hiDAP transplant at the same time; 3) hiDAPs only; 4) AAV-GDNF only; and 5) saline control. Motor function was assessed using the corridor test. Corridor test data indicated that rats injected with AAV-GDNF prior to hiDAP transplantation showed a restoration of motor function to baseline at 8-weeks post-transplantation . Transplanted hiDAPs in this group also co-expressed the human-specific marker STEM121 and the dopaminergic marker TH. Our results demonstrate the benefit of a GDNF-rich environment for the survival and differentiation of transplanted hiDAPs and the therapeutic potential of our directly reprogrammed hiDAPs for CRT for PD.

Funding Source: Our work is supported by the Marsden Fund from the Royal Society of New Zealand and Jayarjun is funded by a University of Auckland Doctoral Scholarship

Keywords: Parkinson's disease, cell replacement therapy, cell reprogramming

6:45 PM – 7:45 PM

196

SINGLE CELL RNA-SEQ FOR QUALITY CONTROL OF PATIENT-DERIVED DOPAMINE NEURONS FOR AUTOLOGOUS CELL REPLACEMENT THERAPY

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The primary cause of motor dysfunction in Parkinson's disease (PD) is the decline in dopamine levels in the striatum due to the loss of dopaminergic neurons in the substantia nigra. There is currently no effective therapy that could prevent the onset of the disease or slow down the progressive degeneration of dopaminergic neurons in PD. Targeted regenerative therapies, specifically aimed at restoring lost dopaminergic neurons via cell transplantation, represent a major advance in the treatment of PD. Ongoing clinical trials are investigating the potential of allogeneic stem cell-based therapies as a treatment option, but an even more promising approach may be the use of patient-specific autologous induced pluripotent stem cells (iPSCs), which could bypass the immunosuppression currently required and reduce the risk of rejection by avoiding the immunological barriers and presumably improving cell survival. For allogeneic therapies where cells are produced in big batches for large patient groups, safety and efficacy is evaluated in extensive in vivo studies, which is not feasible for patient-specific therapies due to cost and time. One key step in the development of patient-specific treatments is therefore to identify new methods such as single cell transcriptome analysis as a quality control to assess batch-to-batch variation, rule out contaminating populations and to minimize the safety and efficacy studies performed in vivo. Towards this aim, we have generated sequencing data from DA progenitor preparations to provide a target transcriptional profile at the single cell level and linked this to cell differetiation and maturation in vitro and in vivo. This enables us to predict safety and efficacy and limit inter-batch variation of patient-derived dopamine neurons which will serve as a benchmark for future developments of autologous cell replacement therapies.

Keywords: cell therapy for Parkinson's disease, patient derived iPSCs, single cell RNA-seq for quality control

6:45 PM – 7:45 PM

198

HUMAN LEUKOCYTE ANTIGEN-HOMOZYGOUS IPS CELLS-DERIVED NEURAL PROGENITOR CELLS SHOWED NEURONAL DIFFERENTIATION, NEURITES EXTENSION, AND SYNAPTIC STRUCTURE FORMATION IN RODENT ISCHEMIC STROKE BRAINS

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Ischemic stroke is a major cerebrovascular disease with a high morbidity and mortality rate, but effective treatments to recover ischemic stroke caused-neurological functions have remained to be developed. One of novel and hopeful strategies to restore damaged central nervous system functions is regenerative medicine using cell-based therapy, and feasibility of cell-based therapies for ischemic stroke are studying using various types of cells or tissues. In this study, we generated neural progenitor cells from human leukocyte antigen-homozygous iPS cells (HLA-homo hiPSCs-NPCs), and evaluated their therapeutic effects for ischemic stroke. HLA-homo hiPSCs-NPCs were

intracerebrally transplanted into rat ischemic brains made by transient middle cerebral artery occlusion at either the subacute or acute stage, and evaluated in vivo survival and differentiation, and efficacy against functional improvement of neurological dysfunctions caused by ischemic stroke. HLA-homo hiPSCs-NPCs were histologically identified in host brain tissues showing neuronal differentiation to vGLUT-positive glutamatergic neurons, extending neurites into both ipsilateral infarct and contralateral healthy hemispheres, and forming synaptic structures at the point after 12 weeks from both acute and subacute stage transplantation. They also made some positive effects for improving neurological functions when were transplanted at subacute stage with g-secretase inhibitor-pretreatment. but their effects were modest and not significant, and showed a possible risk of remaining cells in their undifferentiated and immature status in acute stage transplantation. These findings suggest HLA-homo hiPSCs-NPCs show cell replacement effects in ischemic stroke damaged neural tissues, but their efficacy is insufficient for neurological functional improvement in the use of acute or subacute transplantation. Further examination and optimizing of the timing of cell transplantation, and preparation methods of transplanted cells should be needed considering balance of efficacy and safety of hiPSCs-NPCs transplantation.

Funding Source: This research was supported by AMED under Grant Number JP15bm0204001, and JP23bk0104161.

Keywords: Ischemic stroke, neural progenitor cells from human iPS cells, intracerebrally transplantation

6:45 PM – 7:45 PM

200

PRE-CLINICAL EVALUATION OF CLINICALLY RELEVANT IPS CELL DERIVED NEUROEPITHELIAL STEM CELLS AS AN OFF-THE-SHELF CELL THERAPY FOR SPINAL CORD INJURY

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Preclinical transplantations using human neuroepithelial stem (NES) cells in spinal cord injury have exhibited promising results and demonstrated cell integration and functional improvement in transplanted animals. Previous studies have relied on the generation of research grade cell lines in continuous culture. Using fresh cells presents a huge hurdle for clinical transition regarding time and resources while maintaining high quality standards. In this study, we generated a good manufacturing practice (GMP) compliant human iPS cell line in GMP clean rooms alongside a research grade iPS cell line which was produced under standardized protocols with GMP compliant chemicals. These two iPS cell lines were differentiated into human NES cells, from which six batches of cell therapy doses were produced. The doses were cryopreserved, thawed on demand and grafted in a rat spinal cord injury model. Our findings demonstrate that NES cells can be directly grafted post-thawing with high cell viability, while maintaining their cell identity and as well as their differentiation capacity. This opens the possibility of having off-the-shelf cell therapy products in the future. Moreover, this manufacturing process yields stable cell doses with minimal batch-to-batch variability, characterized by consistent expression of identity markers as well as similar viability of cells across the two clinically relevant cell lines. These cryopreserved cell doses exhibit sustained viability, functionality, and quality for at least 2 years. These results provide proof of concept that cryopreserved NES cells present a viable alternative to using freshly cultured cells in future cell therapies and exemplify a platform from which cell formulation can be optimized and facilitate the transition to future clinical trials.

Keywords: NES cells, cell therapy doses, spinal cord injury

6:45 PM – 7:45 PM

202

CHARACTERIZING INDUCIBLE OLIGODENDROGENICALLY-BIASED NEURAL PROGENITOR CELLS IN SPINAL CORD INJURY: A TRANSCRIPTOMIC STUDY USING RNA-SEQ ANALYSIS

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Spinal cord injury (SCI) is associated with the loss of oligodendrocytes, which play key roles in myelination and in modulating interactions between glia and neurons. Neural progenitor cells (NPCs) are a promising source of cells for SCI treatment, due to their ability to replace the lost oligodendrocytes, neurons and astrocytes. However, the differentiation of NPCs into oligodendrocytes is often inefficient, whereby the majority of the cells differentiate into astrocytes following transplantation. Therefore, we aimed to enhance oligodendrocyte differentiation by generating an inducible oligodendrogenic NPCs (ioNPCs) in which the extent of oligodendrocyte differentiation could be carefully regulated. Human ioNPCs were prepared by engineering NPCs to express Olig2 under the control of the conditional doxycycline-inducible tet-ON promoter, in which doxycycline administration regulates Olig2 expression. The cells were characterized in vitro using a combination of qRT-PCR analysis, immunostaining, and bulk RNA sequencing, which confirmed that the ioNPCs had higher expression of oligodendroglial lineage genes, including OLIG1, OLIG2 and PDGFRA, and differentiated into a greater proportion of O1+ oligodendrocytes (39.44 ± 16.5%) compared to NPCs (24.73 ± 6.5%). To assess the cells in vivo, athymic Rowett nude rats were subjected to a 23 g cervical clip-compression injury and half were transplanted with ioNPCs one week after the injury. After eight weeks the rats were sacrificed, RNA was isolated from the spinal cord and bulk RNA sequencing was performed. Preliminary RNA sequencing analyses suggest that a total of 181 genes were differentially expressed following ioNPC transplantation, including several genes related to extracellular matrix proteins, cell substrate adhesion and detoxification. In conclusion, our study aims to assess the therapeutic potential of ioNPCs in cervical SCI.

Funding Source: CIHR

Keywords: spinal cord injury, oligodendrocyte, neural progenitor cell

6:45 PM – 7:45 PM

204

THE PRECLINICAL AND PHASE-I CLINICAL STUDY OF IPSC-DERIVED FOREBRAIN NEURAL PROGENITOR CELL INJECTION-HNPC01 IN TREATING CHRONIC ISCHEMIC STROKE

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The hNPC01 injection, is a cell injection containing Human Forebrain Neural Progenitor Cells (hFNPCs) derived from induced Pluripotent Stem Cell (iPSC) and manufactured under GMP condition. This allogenic cell therapy product contains more than 95% hFNPCs and its progeny cells, which was able to differentiate further into functional cortical neurons and glia cells both in vitro and in vivo. In previous mechanism and preclinical studies, hNPC01 showed potential in promoting functional recovery of ischemic stroke rat and cynomolgus monkey models and forming neural circuitry in vivo. The preclinical safety study programs including tumorigenicity study in NOG mice up to 52 weeks indicated a good safety profile of hNPC01. The phase I open-label dose-escalation clinical study of hNPC01 injection treating chronic ischemic stroke by intracerebral injection approved by NMPA showed good early-stage safety and sign of efficacy in subjects with ischemic stroke onset 17 to 40 months prior to the enrollment. It is expected that the long-term safety observation and exploration of evaluation tools will provide more valuable information for the future development of iPSC cell therapy for stroke or traumatic brain injury patients with moderate to severe sequalae.

Keywords: iPSC, neural progenitor cell, stroke

6:45 PM – 7:45 PM

206

A CELL THERAPY APPROACH BASED ON HESC-DERIVED MIDBRAIN DOPAMINERGIC PROGENITORS FOR PARKINSON'S DISEASE

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Parkinson's Disease (PD) is a common age-related neurodegenerative disorder with a rising prevalence. Human pluripotent stem cells have emerged as the most promising source of cells for midbrain dopaminergic (mDA) neuron replacement in PD. This study aimed to generate transplantable mDA progenitors for treatment of PD. Here, we optimized and fine-tuned a differentiation protocol using a combination of small molecules and growth factors to induce mDA progenitors to comply with good manufacturing practice (GMP) guidelines based on our clinical-grade human embryonic stem cell (hESC) line. The resulting mDA progenitors demonstrated robust differentiation and functional properties in vitro. Moreover, cryopreserved mDA progenitors were transplanted into 6-hydroxydopamine-lesioned rats, leading to functional recovery. Furthermore, scalability was demonstrated via transplantation in a parkinsonian monkey model. Our data suggest a prospective future for cell-based therapy in PD that is quickly approaching the initial clinical trial phase.

Keywords: Parkinson's disease, human embryonic stem cells, dopaminergic neurons

6:45 PM – 7:45 PM

208

PATIENT-DERIVED NEURONS AS A PLATFORM FOR IDENTIFYING MECHANISMS OF ANTIDEPRESSANT RESPONSE AND THEIR TIME-COURSE

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Major depressive disorder (MDD) is a widespread global issue, affecting an increasing number of individuals annually. Current antidepressants prove ineffective in over 30% of patients and often require several weeks for symptom relief even in responsive cases. The delay in clinical response may be attributed to neuronal plasticity processes, such as synapse modulation, crucial for therapeutic outcomes. However, limitations in studying antidepressant effects exist due to animal models' disparity with human biology and the inaccessibility of brain tissue. Stem cell technology, particularly patient-derived neurons, offers a promising avenue to investigate central nervous system (CNS)-specific responses in depression. In this study, we utilized induced pluripotent stem cell (iPSC)-derived neurons to examine the cellular and molecular effects of citalopram, a common antidepressant, in depressed patients. Patient-derived lymphoblastoid cell lines from the Sequenced Treatment Alternatives to Relieve Depression (STARD) study were utilized to efficiently generate cortical neurons. Following exposure to citalopram or vehicle, RNA was extracted at three time points. Comparative analysis of RNAseq data between responders (n=3) and non-responders (n=2) revealed time-dependent molecular effects associated with clinical efficacy. Specifically, an incremental increase in differentially expressed genes (DEGs) was observed in responders compared to non-responders at 6h, 24h, and 72h post citalopram dosing (FDR< 0.05). Functional enrichment analysis of Gene Ontology (GO) highlighted significant enrichment of molecular and cellular pathways related to neuronal plasticity, such as axonal branching and synaptic signaling, particularly at 24h and 72h post-treatment (FDR< 0.05). The augmentation of pathways associated with neuronal plasticity aligns with the delayed clinical response to citalopram. These findings underscore the value of patient-derived neurons in elucidating the mechanistic basis of antidepressant response, thus advocating for precision medicine strategies in drug development and treatment selection.

Keywords: depression, neurons, citalopram

6:45 PM – 7:45 PM

210

SECRETED PROTEINS IN CULTURE MEDIUM SUPERNATANT AS MARKERS FOR QUALITY CONTROL OF DONOR CELLS IN CELL THERAPY FOR PARKINSON'S DISEASE

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In cell therapy for Parkinson's disease using pluripotent stem cells, controlling the variation in induction efficiency of dopaminergic progenitors remains challenging. This study aims to establish a method for monitoring the differentiation efficiency using secreted proteins in the culture medium supernatant for quality control in cell production. In this study, dopaminergic neural progenitors were induced from several pluripotent stem cell lines in several differentiation protocols. The medium was sampled at each time of the medium change. We identified secreted proteins dynamically changed during differentiation by unbiased liquid chromatography-mass spectrometry (LC/MS). The list of candidate biomarker proteins was validated in vitro analyses. A large list of candidate proteins secreted into the culture medium supernatant was obtained. After narrowing down the list using an algorithm, approximately 20 highly specific secreted proteins were finally identified as dopaminergic biomarker candidates. Detailed immunostaining and gene expression analyses of these candidate markers revealed that many of them were specific for mesencephalic dopaminergic differentiation. All candidates could be quantified in culture medium with commercial ELISAs, and selected markers were detectable as early as day 4 of differentiation. The stability of the proteins after secretion into the culture medium was also confirmed. Finally, we have identified a collection of over 10 secreted biomarkers for non-invasive monitoring for induction of dopaminergic neural progenitors. The result suggested that real-time monitoring of identified marker proteins could be used to optimize the induction protocol and to control the quality in donor cell production. As it is real-time and non-invasive, this approach is compatible with the concept of quality by design (QbD) for cell production in clinics in future.

Funding Source: AMED JP20bm0704054, MRC Regenerative Medicine Grant Mr/V00560x/1, MRC Confidence-in-Concept award, Wellcome Trust Institutional Translational Partnership Award (iTPA), and Cure Parkinson's project grant.

Keywords: dopamine neuron, biomarker, secreted protein

6:45 PM – 7:45 PM

212

AAV VECTORS TRIGGER DNA DAMAGE RESPONSES AND STING-DEPENDENT INFLAMMATION IN HUMAN CNS CELLS

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Adeno-associated viral (AAV) vector-based gene therapy is gaining foothold as a treatment option for a variety of genetic neurodegenerative and neuromuscular diseases with encouraging clinical results. Nonetheless, dose-dependent toxicities and severe adverse events have emerged in some recent clinical trials through mechanisms that remain unclear. We have modelled here the impact of AAV transduction in the context of cell models of the human central nervous system (CNS), taking advantage of induced pluripotent stem cell-based technologies. Our work uncovers vector-induced cell-intrinsic innate immune mechanisms at the single-cell level that contribute to neurotoxicity in 2D and 3D models of the human CNS. The AAV genome triggered p53-dependent DNA damage responses across CNS cell types followed by induction of IL-1R- and STING-dependent inflammatory responses. In addition, transgene-expression led to MAVS-dependent signalling and activation of type I interferon (IFN) responses. Cell-intrinsic and paracrine neurotoxicity could be prevented by inhibiting p53 or acting downstream on STING- and IL-1R-mediated activation of inflammatory responses. Together, our work sheds significant light on the cell-autonomous innate immune mechanisms of vector sensing that can contribute to AAV-associated neurotoxicity.

Keywords: AAV gene therapy, iPSC-derived CNS models, innate immunity

6:45 PM – 7:45 PM

214

PRECLINICAL AND CLINICAL DEVELOPMENTS OF PERIPHERAL NERVE REGENERATION BY SCHWANN CELL-LIKE CELLS DIFFERENTIATED FROM HUMAN TONSIL-DERIVED MESENCHYMAL STEM CELLS FOR CHARCOT-MARIE-TOOTH DISEASE

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Charcot–Marie–Tooth disease (CMT) is a hereditary disease with heterogeneous phenotypes and genetic causes. CMT type 1A (CMT1A) is a type of disease affecting the peripheral nerves and is caused by the duplication of the peripheral myelin protein 22 (PMP22) gene. Human tonsil-derived mesenchymal stem cells (TMSCs) are useful for stem cell therapy in various diseases and can be differentiated into Schwann cell-like cells (TMSC-SCs). We investigated the potential of TMSC-SCs called neuronal regeneration-promoting cells (NRPCs) for peripheral nerve and muscle regeneration in C22 mice, a model for CMT1A. We transplanted NRPCs manufactured in a good manufacturing practice facility into the bilateral thigh muscles of C22 mice and performed behavior and nerve conduction tests and histological and ultrastructural analyses. Significantly, the motor function was much improved, the ratio of myelinated axons was increased, and the G-ratio was reduced by the transplantation of NRPCs. The sciatic nerve and gastrocnemius muscle regeneration of C22 mice following the transplantation of NRPCs downregulated PMP22 overexpression, which was observed in a dose-dependent manner. These results suggest that NRPCs are feasible for clinical research for the treatment of CMT1A patients. Clinical trial Phase I for patients with CMT has been conducted in Korea.

Keywords: Charcot–Marie–Tooth disease type 1A, tonsil-derived mesenchymal stem cells, Schwann cell-like cells

6:45 PM – 7:45 PM

TOPIC: NO TISSUE SPECIFICITY

216

DEVELOPMENT OF A NEW ADHERENT PSC CULTURE MEDIUM ENABLING ROBUST PERFORMANCE IN CELL THERAPY WORKFLOWS

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Culture systems for human pluripotent stem cell (PSC) expansion enable generation of a nearly unlimited pool of cells capable of differentiation to all three germ lineages with potential for cell-based regenerative therapies. To advance PSC therapy research to the clinic, there is a need for high-quality ancillary materials with GMP-compliant manufacturing, quality and safety testing, raw material traceability and supporting regulatory documentation. To ease this transition, we have developed Cell Therapy Systems (CTS™) StemFlex™ Medium based on research use StemFlex Medium, but without raw materials directly of human or animal origin. CTS StemFlex Medium supports expansion of high-quality PSCs as well as superior outcomes for challenging applications in the PSC workflow. Here we demonstrate PSCs cultured long-term in CTS StemFlex Medium maintain normal PSC characteristics including morphology, pluripotency, trilineage differentiation potential and karyotype. This new medium allows for versatility in culture conditions: a flexible feed schedule with a weekend-free option and compatibility with multiple defined matrices and passaging reagents. CTS StemFlex Medium is shown to provide critical support of stressful PSC applications including single-cell passaging and recovery from cryopreservation as well as challenging parts of the gene editing workflow including electroporation and clonal expansion.

Keywords: GMP PSC culture medium, gene editing, clonal expansion

6:45 PM – 7:45 PM

218

MAPPING THE PLURIPOTENT "BREAKOME": INSIGHTS INTO THE ORIGINS OF GENETIC VARIANTS IN HUMAN PLURIPOTENT STEM CELLS

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Genome Instability drives oncogenic transformation in cancer, and the same phenomenon occurs in human pluripotent stem cells. Human pluripotent stem cells (hPSC) are a promising source of cellular material for regenerative medicine applications. However, over prolonged in vitro culture, hPSC are prone to acquiring recurrent genetic changes. Many of the changes observed in hPSC are common to cancers, raising concern over the safety of hPSC-derived products in therapy. DNA damage, followed by unfaithful repair, precedes many of the genetic changes observed in hPSC. Pluripotent cells harbour higher constitutive levels of DNA damage than their differentiated counterparts, however, the causes of this damage remain poorly characterised. We have used INDUCE-seq to map genome-wide endogenous DNA double-strand breaks in pluripotent and differentiated cells. From these data, we have discerned differences in the distribution of DNA breaks between isogenic pluripotent and differentiated cell types and have identified pluripotent-specific DNA damage hotspots, several of which fall within regions of recurrent genetic change in hPSC. By Integrating published sequencing datasets, and contextualising damage hotspots, we identify DNA replication stress as a putative cause of DNA damage and ultimately recurrent chromosomal translocations on chromosome 1q. We hope, ultimately, this work will inform modified culture conditions to minimise the occurrence of such variants in hPSC cultures.

Funding Source: This work was supported by the UK Regenerative Medicine Platform, MRC reference MR/R015724/1

Keywords: human pluripotent stem cells, DNA damage, genetic variants

6:45 PM – 7:45 PM

220

AN AUTOMATED METHOD TO CHARACTERIZE AMINO ACID DEGRADATION IN THE CELL CULTURE MEDIA

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Glutamine (Gln) metabolism is essential to sustain stem cell viability, proliferation, specification, and differentiation. In this sense, exogenous Gln in the nutrient environment is indirectly involved in cellular processes and stem cell fate regulation, such as redox homeostasis and epigenetic modifications in cancer stem cells. We aimed to evaluate long-term amino acid degradation in Dulbecco's Modified Eagle's Medium (DMEM) utilizing a fully automated amino acid analysis system with post-column detection. Amino acids concentrations in DMEM (type 6046; Sigma-Aldrich) were analysed using Biochrom Bio 30+ Amino Acid Analyser. In brief, DMEM samples were examined in an ion exchange (IEX) liquid chromatography with a dilution (1:1) in lithium citrate-based loading buffer. A lithium high-performance program (at a wavelength of 570 nm), with an injection volume of 20 µL (for both sample and standard), was run on days 0, 33, 68, and 96. Data was calibrated against a sodium hydrolysate (standard) buffer. The IEX technique was free of interference from the inorganic salts, sugars and other additives found in the media. From the 15 amino acids assessed in each chromatogram, only Gln showed a significant fall in measured concentrations (25%) during the studied period. Gln concentrations were 0.499 mg/L (day 0), 0.428 mg/L (day 33), 0.387 mg/L (day 68), and 0.370 mg/L (day 96). Data was fit to an exponential decay function to estimate the half-life (53.47 days) of Gln in DMEM stored at 4oC. The amino acid analyser system (Biochrom Bio 30+) is an accurate, automated, and fast technique for characterizing amino acid concentrations in cell culture media. Gln degradation in the nutrient environment increases over time, limiting the long-term usage of DMEM. Therefore, care should be taken to always utilize sequentially fresh DMEM bottles for longitudinal studies.

Keywords: glutamine metabolism, stem cell viability, amino acid degredation

6:45 PM – 7:45 PM

222

ENSURING GMP STANDARDS: A CLOSED-SYSTEM APPROACH TO EXPAND HUMAN PLURIPOTENT STEM CELLS

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Human pluripotent stem cells (hPSCs) hold great promise for disease modeling, drug discovery and clinical applications. Therefore, working with highly pluripotent, well characterized and quality controlled cell stocks during development is crucial to ensure reproducible experimental conditions. Here, we established a workflow encompassing stable expansion of hPSCs using a xeno-free and GMP compliant cultivation medium. For quality control of cultivated hPSCs, the pluripotency was assessed using a defined AB flow based marker cocktail, additionally, genetic stability was evaluated. Furthermore, functional pluripotency was also identified after differentiation in all three germ layers by multicolor flow cytometric analysis using markers for the three embryonic lineages . Following this workflow, hPSCs could be stably expanded over 10 passages with persistent, high expression of pluripotency markers TRA-1-60, SSEA-4, Oct3/4 and Nanog as well as almost no expression of the differentiation maker SSEA-1 (CD15). PSCs revealed a homogenous morphology and also retained genetic stability. Quantitative, flow cytometry–based analysis reproducibly proved their differentiation potential into ectoderm, mesoderm, and endoderm. Thus, the workflow shown here assures a standardized, robust expansion of hPSCs and includes characterization as well as quality control of the expanded cells. The flow based QC strategy was

also successfully applied for characterization of cells cultivated under standardized, automated, closed system conditions using the CliniMACS Prodigy Instrument which is of major relevance for generation of master cell banks (MCB) and working cell banks (WCB) for future clinical cell manufacture.

Keywords: hPSC expansion, standardized automated closed cultivation, ensuring GMP cell culture

6:45 PM – 7:45 PM

224

SCALING IPSC PRODUCTION IN DIFFERENT 3D PLATFORMS IN SUSPENSION CULTURE FOR THEIR USE IN ALLOGENEIC REGENERATIVE THERAPIES

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Human induced pluripotent stem cells (iPSCs) exhibit significant potential for advancing regenerative medical treatments owing to their pluripotency and expansive proliferation capacity. However, in order to utilize this type of cells for large-scale allogeneic regenerative therapies, a significant quantity of these cells is required, thus emphasizing the importance of studying large-scale production methods. Conventional two-dimensional static culture techniques are inadequate to meet the demand for large-scale cellular therapy production, prompting exploration into innovative bioreactor-based expansion techniques such as microcarrier-based or suspension aggregate cultures. In this investigation, we conducted a direct comparison of PSC expansion and quality metrics across microcarrier, and aggregate cultures in spinner flasks, aiming to ascertain which 3D growth configuration yields superior yields while maintaining cellular product quality. We demonstrated comparable expansion of iPSCs on microcarriers Cytodex 1 and Cultisphere G, as well as in aggregates, by day 6, achieving a cell density of 2.64 × 10^6 cells/ml and 5.62 × 10^6 cells/ml on microcarriers respectively, and 7.58 × 10^6 cells/mL as aggregates, with fold-expansions of 4- and 8-fold on microcarriers and 11-fold as aggregates. The efficiency in the recovery of iPSCs in single-cell form was 72% from Cytodex 1, 64% from Cultisphere G, and 70% from aggregates, with a cell viability over 95% after harvesting for all cultures. The energetic efficiency of iPSCs to reproduce in the different cultures yielded values of 1.2, 1.7, and 2 respectively, indicating that the primary metabolic pathway used by cells to obtain energy is lactic fermentation. “iPSCs cultured on microcarriers and as aggregates exhibit OCT3/4, SSEA4, TRA-1-60, and SOX2 pluripotency marker expression, as well as retention of differentiation potential into three germ layers. This analysis supports the utilization of microcarrier and aggregates as a 3D expansion platform for suspension cultures of iPSCs, aiming to scale production to bigger stirred-tank bioreactor systems. This approach leverages efficient cellular metabolism while preserving the phenotypic characteristics and differentiation potential of iPSCs.

Keywords: iPSC, scaling, microcarrier

6:45 PM – 7:45 PM

226

TECHNOLOGY TRANSFER PROCESS TO ASSESS EXPERIMENTAL REPRODUCIBILITY

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Manufacturing processes for cell therapies are inherently complex and evolve from research to commercialisation. Successful replication of the process and analytical methods is critical but according to a survey conducted by Nature 70 % of scientists experienced problems while trying to reproduce published research results. Unsuccessful replication of methods leads to wasted time, resources and acts as a barrier to the commercialisation of cell therapeutics. Cell and Gene Therapy Catapult (CGTC) has established a robust Technology Transfer (TT) process to identify and circumvent challenges in the replication of experimental methods between different sites. Recently, this TT process has been used to successfully transfer two processes for human induced pluripotent stem cell (hiPSC) expansion from their Technology and Process Innovation (TPI) group (Guys Hospital, London) to the Pre-manufacturing Development (PMD) group (Edinburgh). TT was initiated by compiling historical data generated at TPI to identify key criteria with specific value ranges to determine the success of the TT. These included factors such as viability, confluency and pluripotency marker expression. In addition, detailed experimental documentation was shared and a gap analysis identifying critical differences in equipment, materials and analytical requirements between both sites was conducted. A training strategy for the project team was designed to identify key process and analytical training opportunities to observe and perform runs at the CGTC sites. These training sessions were identified as a major factor in enabling PMD to successfully reproduce the process from TPI and meet the technical acceptance criteria. Major difficulties encountered during the project evolved around supply chain delays for reagents and equipment. Based on our experience communication between both parties, detailed experimental documentation and anticipation of supply chain issues are crucial factors to consider when performing a TT.

Keywords: technology transfer, reproducibility, expansion

6:45 PM – 7:45 PM

228

DONOR SEQUENCE OPTIMIZATION ENABLES TARGETED INSERTION OF COMPLEX STEALTHING CONSTRUCTS IN MRNA-REPROGRAMMED INDUCED PLURIPOTENT STEM CELLS

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Cellular therapies are an emerging field of medicine that involves the administration of cells as living agents to repair or replace damaged cells and tissues. While traditional cell therapies, such as autologous and donor-derived allogeneic therapies, hold great potential in combating intractable diseases, their clinical utility has been limited by several factors, including manufacturing complexities, high costs, safety considerations, scalability, and batch-to-batch consistency. Next-generation allogeneic cell therapies derived from mRNA-reprogrammed induced pluripotent stem cells (iPSCs) can directly address many of these challenges. However, iPSC-derived cells can still be subject to elimination by host immune cells, which can greatly reduce their efficacy. To address this challenge, we generated mRNA-reprogrammed iPSCs engineered to avoid elimination by host T cells and NK cells, primary drivers of immune-mediated rejection to allogeneic cell therapies. The engineered iPSCs do not express B2M but instead express a B2M-HLA-E fusion protein. UltraSlice™ gene-editing mRNA was used to insert donor DNA containing a (G4S)4 linker and HLA-E gene sequence upstream of the endogenous B2M stop codon. Incomplete insertion of a linker sequence with high GC content (83.3%) was observed in edited cells, with one edited iPSC line missing 68 nucleotides from the transgene. We hypothesized that the high GC content of the linker sequence may promote secondary structure formation and lead to aberrant splicing during genomic insertion. Interestingly, a template containing a sequence-optimized linker with lower GC content (61.7%) resulted in full-length biallelic insertion in 2 of 16 colonies screened and exhibited detectable HLA-E expression via flow cytometry, while templates containing linkers with higher GC content (71.7% and 83.3%) did not (0 of 16 colonies in each case). We show that sequence optimization of donor DNA enabled the generation of mRNA-reprogrammed iPSCs with stealthing features. These cells may prove useful in the development of a broad range of engineered cell therapies.

Keywords: induced pluripotent stem cells (iPSCs), allogeneic cell therapies, gene editing

6:45 PM – 7:45 PM

230

KEY REQUIREMENTS FOR EFFICIENT AND LINEAR SCALE-UP OF SHEAR SENSITIVE PLURIPOTENT STEM CELL AND OTHER CELL THERAPY PRODUCTS USING THE VERTICAL-WHEEL BIOREACTOR FAMILY

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Pluripotent stem cells (PSCs) hold immense potential to enable life-saving therapies, yet the lack of robust, reproducible, and scalable production processes presents a significant obstacle to their clinical adoption. While there have been attempts to scale up stem cell processes, the variation in published results shows that key scale-up challenges such as bioreactor mixing characteristics, medium exchange methods, gas transfer efficiency, and cell harvest remain insufficiently understood. There is an industry-wide need to develop reliable methods for scaling the manufacturing processes of cell therapies to reproducibly yield clinically relevant lot sizes. Through systematic process development, we have addressed these challenges and identified key requirements to linearly scale up PSC manufacturing processes: versatile and scalable bioreactors with well-characterized hydrodynamics, efficient and scalable MX methods, well-characterized gas transfer in large-scale bioreactors, and scalable, low-shear methods for cell harvest, wash, and concentration. Here we describe studies used to identify and implement these key requirements and develop a platform for scalable production of shear-sensitive PSCs and PSC-derived products. This platform incorporates two stages of planar seed train cell expansion and scalable 3D cell expansion and/or differentiation phases. The bioreactor stages are followed by scalable cell harvest methods involving downstream concentration and wash devices suitable for shear-sensitive cell therapies. We demonstrate the effectiveness of this platform by linearly scaling the production of undifferentiated iPSCs from the 0.1L to the 15L scale. Moreover, using this versatile platform, we have produced clinically relevant lot sizes of cell products from various cell types (PSCs, PSC derivatives, MSCs, and other primary cells), growth modalities (microcarriers, aggregates, and single cells), and physiological cell processes (expansion and differentiation). By proactively addressing scale-dependent challenges impacting yield and quality, we have developed reliable large-scale processes for shear-sensitive cell therapy production, advancing their translation from research to the clinic, and enabling widespread access for the population.

Keywords: cell therapy manufacturing, scale-up, pluripotent stem cells

6:45 PM – 7:45 PM

232

GENERATION OF JAWBONE ORGANOIDS FROM HUMAN PLURIPOTENT STEM CELLS VIA FIRST PHARYNGEAL ARCH ECTOMESENCHYME

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Jawbones, the maxillary and mandibular bones, are the largest pair of biomineralized organs of the oral region that not only play a central role in mastication with teeth, but also are essential in maintaining respiration, pronunciation, and facial esthetics. However, jawbones are highly susceptible to irreversible damage due to diverse pathological factors such as oral bacterial infection, excessive mastication, traumas, tumors, and congenital malformations1, and sustaining jawbone injury seriously affects the quality of life. Nevertheless, engineering jawbone tissues from pluripotent stem cells is challenging. This is due to the lack of protocols for selective induction of the jawbone progenitor, first pharyngeal arch (PA1) ectomesenchyme, as well as for recapitulation of three-dimensional (3D) osteocyte networks. Here, we report a mean for generating jawbone-like organoids from human induced pluripotent stem cells (iPSCs) via PA1 ectomesenchyme of mandibular prominence (mdEM). A 3D culture system enables to sequentially differentiate iPSCs into neural crest cells and mdEM. The mdEM exhibited proximal-distal patterning from the center outwards similar to mandibular development. The addition of exogenous pharyngeal epithelial signals induced mandibular prominence-specific several regional patterning in the mdEM. When grown under osteogenic conditions, the mdEM formed jawbone-like organoids composed of osteoblasts and network-forming osteocytes embedded in self-produced mineralized bone matrixes. Furthermore, the organoids facilitated bone regeneration when transplanted into jawbone defects and recapitulated phenotypes of osteogenesis-imperfecta, a genetic disorder characterized by fragile bones, using patient-derived iPSCs. Our comprehensive protocols provide a foundation for studying human jaw embryology and pathophysiology and developing therapeutics.

Keywords: jawbones, first pharyngeal arch, osteocyte

6:45 PM – 7:45 PM

234

DENDRITIC CELL REPROGRAMMING PROGRESSES IN IMMUNOSUPPRESSIVE TUMOR SPHEROIDS

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Immunotherapy effectiveness varies across patients and cancer types, and the tumor microenvironment (TME) plays a crucial role in outcome variability. Previous studies demonstrated that PU.1, IRF8, and BATF3 (PIB) transcription factors reprogram cancer cells into tumor-antigen presenting cells (tumor-APC) eliciting anti-tumor immunity. Modeling immunotherapy responses using cancer spheroids recapitulating the immunosuppressive TME is essential for identifying reprogramming determinants within tumors and assessing therapeutic efficacy. Here, we generated tumor spheroids from the human brain, breast, tonsil, pancreas, and melanoma cancer cell lines, and evaluated reprogramming efficiency mediated by PIB-encoding lentiviral vectors at day 9. Reprogrammed cells in spheroids acquired a tumor-APC phenotype (CD45+HLA-DR+) at rates comparable to 2D monolayers. High-content fluorescence imaging revealed a correlation between increased reprogrammed cell numbers and smaller spheroid sizes, indicative of reduced proliferation and loss of tumorigenicity during tumor-APC reprogramming. Single-cell transcriptomic analysis demonstrated accelerated reprogramming in spheroids. To mimic the immunosuppressive TME, spheroids were generated with cancer-associated fibroblasts (CAFs), monocytic myeloid-derived suppressor cells, pericytes, or in the presence of suppressive cytokines at increasing ratios. Surprisingly, immunosuppressive components did not significantly impact reprogramming efficiency and, in some cases, enhanced it indicating that the human immunosuppressive TME does not impede but rather favors reprogramming. Functional antigen presentation assays in the presence of CAFs and matched peripheral blood mononuclear cells confirmed immune cell activation and cytotoxicity against cancer cells. This study challenges the notion that the immunosuppressive TME impedes immunotherapy and advances our understanding of immune cell reprogramming-based therapeutic strategies in the complex TME context.

Keywords: tumor microenvironment (TME), tumor spheroids, reprogramming

6:45 PM – 7:45 PM

236

ALLOGENIC NAÏVE AND PRIMED EMBRYONIC STEM CELLS ELICIT A CANCER-SPECIFIC IMMUNOTHERAPEUTIC RESPONSE IN C57BL6 MICE

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Despite notable advancements in cancer treatment, metastatic solid tumors remain an unmet medical need. Immunotherapy has emerged as a leading-edge approach for advanced tumors resistant to the existing therapies, with spectacular success creating widespread interest and high expectations. Nonetheless, accurate antigen loading, and precise tumor targeting is limiting the effectiveness of current strategies. Patient-derived induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESC) exhibit significant similarities with cancer (stem) cells, sharing a plethora of known and unknown tumor-associated antigens and tumor-specific antigens (TAAs/TSAs), suggesting a potential to stimulate an immune response against cancer. In fact, it was previously successfully described an iPSC related cancer vaccine in distinct mouse models of cancer. Here we explored the TNG-A ESC transcriptome and proteome and their similarities with a breast cancer cell line (E0771). TNG-A were able to trigger a sustained anti-breast cancer immunogenic response in a syngeneic and orthotopic mouse model of breast cancer involving the inoculation of E0771 breast cancer cells, a pre-clinical model where breast tumor cells are transplanted into the mammary fat pad to establish primary tumor nodules. Additionally, TNG-A growth in suboptimal culture conditions towards a primed state exacerbated the similarities to cancer at the proteome level. Through a candidate approach we highlight the role of Claudins in this process. In particular, we demonstrate that absence of Claudin 6 is sufficient to change the properties of the TNG-A cells, leading to a loss of immunogenic potential. Our results support the use of ESCs as reservoirs of cancer antigens, emphasizing the role of Claudins in this process, and raising hopes to treat advanced stages of disease.

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Keywords: cancer immunotherapy, ESC, Claudin6

6:45 PM – 7:45 PM

238

THREE MAKE A WHOLE - ASSESSING DIFFERENTIATION POTENCY OF INDUCED PLURIPOTENT STEM CELLS AFTER LARGE-SCALE EXPANSION

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Cell therapies constitute the great promise of the 21st century with the potential to provide novel, effective treatment approaches for various diseases. Allogenic cell therapy is based on large-scale expansion of induced pluripotent stem cells (iPSCs), which subsequently can be stimulated to differentiate into almost any desired cell type of the body to address a wide range of therapeutic needs. Therefore, the differentiation potential of iPSCs constitutes a critical quality attribute for their suitability as a base material to generate consistent cell therapy products. However, common large-scale expansion systems bear various stress factors for iPSCs, such as suspension culture or shear force, which might impair the cell's differentiation potency and lead to product failure at the differentiation stage. Therefore, a small-scale test to assess the differentiation potency of an iPSC production batch is critical before performing large-scale differentiation. A common method for this purpose is the trilineage differentiation assay, which consists of targeted stimulation to trigger lineage specification into the three germ layers ectoderm, mesoderm, or endoderm. Nowadays, trilineage differentiation kits are commercially available but often come at a high cost with limited or no insights into medium composition or underlying mechanistic details. Here, we describe a cost-effective and robust trilineage differentiation assay that provides full control over the assay procedure and enables a quantitative measure to monitor the preservations of iPSC differentiation potential throughout a large-scale expansion process. Culture medium compositions were optimized for effective and specific induction of differentiation and RNA sequencing was used to identify robust marker genes for each germ layer. Routinely, expression analysis of these genes via quantitative real-time PCR provides a quantitative measure for iPSC differentiation capacity. Subsequently, the trilineage differentiation assay was used for characterizing the impact of different bioreactor culture-associated stress factors on iPSC differentiation potential, showing clear impairment of certain differentiation responses by shear stress.

Keywords: cell therapy, iPSC, differentiation

6:45 PM – 7:45 PM

TOPIC: PANCREAS

240

ADVANCING STEM CELL DIFFERENTIATION FOR DIABETES MELLITUS: EVALUATING MICROCARRIER-MEDIATED EXPANSION OF PANCREATIC PROGENITORS AND IPS DIFFERENTIATION

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Diabetes mellitus is a highly prevalent global disease, currently affecting 560 million individuals. The current treatment involves exogenous insulin administration; however, achieving strict glycemic control remains elusive. An alternative treatment for type 1 diabetes comprises replacing insulin-producing β-cells through the transplantation of Langerhans islets; nevertheless, one of the main limitations is the scarcity of islets for transplantation. Due to the high cell requirement, around one billion cells per patient, scalable cellular sources become imperative. Induced pluripotent stem cells (iPS) represent a valuable tool in regenerative medical therapies due to their capacity to differentiate into different tissues, including pancreatic endocrine cells, opening the possibility of generating islet-like structures in vitro. Several differentiation protocols to β-like cells via pancreatic progenitors have been developed in the past years, yet the production of desired cells remains low. Given the high demand for functional cells, there is a need to study new differentiation and expansion methods to scale up production. An innovative alternative is to use microcarriers for large-scale iPS expansion and differentiation because of their high surface area-to-volume ratio and scalability to bioreactors. This study aims to evaluate the use of microcarriers for iPS differentiation into β-like cells and for the expansion capacity of pancreatic progenitors on microcarriers. Initial results demonstrate the feasibility of the process, allowing for the seeding and expansion of pancreatic progenitors while retaining characteristic markers such as PDX1 and NKX-6.1, with a higher fold-expansion than the control in 2D culture. Regarding the complete differentiation process on microcarriers, which showed a lower percentage of cells maintaining characteristic differentiation markers during the process, suggests that microcarriers are a better platform for expanding already differentiated cells than for the differentiation itself. This initial analysis suggests the need for further investigations to establish process parameters, as an opportunity for scaling up the process, providing flexibility in research and therapeutic production.

Funding Source: Funding is gratefully acknowledged from the Centre of Biotechnology and Engineering (CeBiB) at the University of Chile and the Institute of Diabetes and Regeneration Research at the Helmholtz Centre Munich headed by Dr. Heiko Lickert.

Keywords: induced pluripotent stem cell differentiation, microcarrier-mediated expansion, diabetes mellitus

6:45 PM – 7:45 PM

242

A CO-CULTURE SYSTEM TO STUDY THE ACTIVATION OF NOTCH PATHWAY IN PANCREATIC PROGENITORS

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Type 1 diabetes (T1D) is an autoimmune disorder characterised by loss of beta-cells, resulting in hyperglycemia. If left untreated, this disorder leads to the death of the patient. Among the different therapeutic approaches to treat T1D, the transplantation of human embryonic stem cell (hESC) derived beta-cells could prove to be a definitive treatment. However, the current differentiation protocols still have room for improvement. It has been shown that modifying Notch signalling at late stages of differentiation can increase beta-cell yield, but in embryonic pancreas development Notch signalling is crucial already from the early stages of organ formation. We hypothesize that modulation of Notch signalling earlier at the pancreatic progenitor stage, would result in a more efficient expansion of this cell type and ease the downstream differentiation to beta-cells. Here, we report the findings from co-culture systems with inducible Notch ligand expressing Flp-In 293 T-REx cells. Dll1, Dll4, and Jag 1 are the three Notch ligands found to be expressed during pancreatic development in mice. Co-culture with a Notch1 reporter cell line confirms that Dll4 shows strong activation of Notch, Dll1 less so, and Jag1 has the least activation potential. Preliminary data from co-culturing the Dll1 expressing Flp-In 293 T-REx cells with hESC derived pancreatic progenitor cells (hESC-PPs) for 24 hours, suggests that there are changes in expression of some Notch pathway related proteins, most prominently in Jag1 expression. Currently, we are investigating this further by looking at mRNA levels of relevant Notch pathway components. We anticipate that results from this study will eventually elucidate whether manipulation of Notch signalling at early stages of pancreatic differentiation ultimately can contribute to enhanced expansion of hESC-PPs and an increase in beta-cell yield. Finally, this work will enable us to move one step closer to the goal of increasing the efficiency of beta-cell generation for cell therapy.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) is supported by a Novo Nordisk Foundation grant (NNF21CC0073729)

Keywords: Notch signaling, Notch ligands, pancreatic progenitors, co-culture

TRACK: DISEASE MODELING AND DRUG DISCOVERY (DMDD)

6:45 PM – 7:45 PM

TOPIC: CARDIAC

244

MODELING ABERRANT LIPID METABOLISM AND CARDIAC DYSFUNCTION IN IPSC-DERIVED HUMAN ENGINEERED HEART TISSUE

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Adipose triglyceride lipase (ATGL) encoded by patatin-like phospholipase domain containing 2 (PNPLA2) catalyzes the first step of the hydrolysis of triglycerides. Mutations in the PNPLA2 gene can cause neutral lipid storage disease associated with severe cardiomyopathy. In our previous study using a pnpla2-deficient mouse model, we observed massive accumulations of neutral lipids and diastolic dysfunction in the heart. The aim of this study is to set up a human induced pluripotent stem cell (iPSC)-derived engineered heart tissue (EHT) model to investigate the relationship between aberrant lipid metabolism and cardiac dysfunction. A PNPLA2 knock-out (KO) iPSC line was generated by CRISPR/Cas9 genome editing. Two guide RNAs (gRNAs) were designed to target the catalytic domain of ATGL, resulting in a truncated protein with deletion from Val91 to Thr119. Isogenic control and KO iPSCs were differentiated into cardiomyocytes (CMs) and cast into fibrin-based 24-well format EHTs. After cultured in a serum-containing medium, the KO CMs showed more neutral lipid accumulation as compared with the WT CMs, monitored by BODIPY staining. The contractility of the EHTs was assessed by a magnetic sensing platform. The WT and KO EHTs had a similar contractile force development until reaching a plateau stage. Subsequently, the KO EHTs displayed a decreasing force and slower kinetics with both longer contraction and relaxation time as compared to the WT EHTs. Moreover, by evaluating the COL1A1 protein levels, we detected excessive collagen deposition in the KO EHTs. Thus, we successfully generated a human EHT model derived from iPSCs and recapitulated the phenotypes of aberrant lipid metabolism and cardiomyopathy in patients and our animal model. In the future, this model can be utilized to get more mechanistic insights into how abnormal lipid metabolism affects cardiac function.

Keywords: engineered heart tissue, lipid metabolism, cardiac dysfunction

6:45 PM – 7:45 PM

246

NAT10 CHEMICAL INHIBITION RESTORES CARDIAC EXCITABILITY OF IPSC-DERIVED LAMINOPATHIC CARDIOMYOCYTES

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Dilated cardiomyopathy (DCM) is a primary disease of the heart and a major cause of heart failure. Approximately 30-50% of cases are familial, with several genes involved; among these, mutations of LMNA gene, encoding the nuclear envelope proteins Lamin A/C, are associated with a severe phenotype and a worse prognosis, typically manifesting with conduction disorders and arrhythmias. Remodelin – a chemical inhibitor of N-acetyltransferase 10 (NAT10) - has been shown to improve the survival of laminopathic mice and to rescue nuclear abnormalities of Lamin A/C-depleted cells. However, studies on cardiac cells are still lacking. Our aim is to fill this gap, by testing Remodelin on Induced Pluripotent Stem Cells-derived cardiomyocytes (CMs) obtained from patients carrying LMNA mutations (LMNA-CMs). Previous electrophysiological data by our group indicated a significant impairment of electrical excitability of these cells, characterized by reduced peak sodium current density and conduction velocity. Here we show that Remodelin treatment in LMNA-CMs is able to restore peak sodium currents density and the related action potential parameters, concurrently boosting the junctional conductance. We also found that the treatment enhances translocation at the plasma membrane of sodium channel Nav1.5 and gap junction component Connexin 43, that were found improperly localized in LMNA-CMs. A positive drug-response, in terms of cardiac conduction and action potential, was also observed in control CMs (CNTR-CMs). This might be explained by the modulation of other cardiac biological processes, including microtubule stability and metabolism, as emerged from RNA sequencing experiments. Notably, genes involved in CMs metabolic switch have been found significantly modulated by Remodelin in both LMNA- and CNTR-CMs. Testing of Remodelin on these pathways is currently ongoing. In conclusion, although some of the mechanisms underlying Remodelin beneficial effect are still to be investigated, our study reinforces the evidence indicating NAT10 inhibition as a promising therapeutic target for LMNA-dependent DCM.

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Keywords: genetic cardiomyopathy, NAT10 inhibition, LMNA mutations

6:45 PM – 7:45 PM

248

VASCULAR PHENOTYPE SWITCHING IN INDUCED PLURIPOTENT STEM CELL-DERIVED MODELS OF SPONTANEOUS CORONARY ARTERY DISSECTION

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Spontaneous coronary artery dissection (SCAD) accounts for ~25% of myocardial infarcts in women under 50 years old. It is characterised by haematoma development within the coronary artery wall, likely from microvessel rupture, causing the coronary to dissect from within. As patients lack traditional cardiovascular risk factors, the cause of this potentially lethal disease is poorly understood, and no specific therapeutics are available for prevention. Recent studies indicate onset is influenced by genetic background and environmental stressors. To best capture this complexity and study SCAD aetiology at the cellular level, we have generated a cohort of 18 genetically different induced pluripotent stem cell (iPSC) lines from SCAD survivors, and age- and sex-matched controls, including familial groups. Using these iPSCs, we have generated coronary artery wall cells including vascular smooth muscle cells (VSMC; TAGLN+, αSMA+, CNN1+, SM-MHC+) and endothelial cells (ECs; CD31+, VWF+, VE Cadherin+). Phenotypic analysis of these cells suggests SCAD may arise due to dual dysfunction in ECs and VSMCs. Proteomic analysis of SCAD patient-derived ECs demonstrated an upregulation in a thrombus formation associated protein (adjusted P=0.04) compared to controls. Live cell image tracking (via Incucyte) showed significantly increased proliferation rates in SCAD ECs (4.5±1.1 h) compared to controls (5.6±1.6 h) (P=0.05). SCAD patient-derived VSMC proteomic analysis revealed alterations in phenotype switching pathways, including the TGF-β pathway, consistent with morphological changes detected with immunofluorescent imaging of proteins belonging to these pathways. Together, these results suggest that cellular activation underpins SCAD. Importantly, this process of activation can be therapeutically targeted. These models will be fundamental in assessing therapeutic efficacy in future drug screening, critically needed to identify the first SCAD-specific therapeutics for disease prevention.

Keywords: spontaneous coronary artery dissection, vascular smooth muscle cells, endothelial cells

6:45 PM – 7:45 PM

250

POOLED PERTURBOME IN CARDIAC ORGANOIDS TO DISSECT CONGENITAL HEART DISEASE PATHOGENESIS

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Congenital heart disease (CHD) is the most common congenital malformation, affecting 1% of live births. Whilst nearly half of CHD patients carry damaging coding variants, a definitive association between most candidate CHD-genes and the pathology is lacking. Moreover, knowledge of the molecular mechanisms and the cell types affected by these mutations remain elusive. Here we report our progress on developing a gene function atlas for putative CHD genes. We leverage on two emerging transformative technologies: human induced pluripotent stem cells (hiPSC)-derived chamber specific cardiac organoids, self-assembling multilineage models of the developing heart; and iPS2-seq – iPS-optimized inducible Post-transcriptional Silencing deconvoluted by single cell sequencing – an approach we recently developed to enable robust pooled perturbome analyses in hiPSC models. We are implementing two screens: first, a focused assessment of 18 genes for which there is a definitive association to CHD based on a meta-analysis of de novo mutations in trios of CHD patients and their parents. We are studying the transcriptional signatures induced in cell types from four key sections of the heart (left ventricle, right ventricle, atria, and outflow tract). Single cell transcriptomes are analyzed using catcheR, our dedicated computational pipeline to identify clonal and treatment-controlled effects of LoF perturbations. Secondly, we are performing a larger scale assessment of a comprehensive list of over 100 genes which have been associated with de novo CHD in isolation or in the context of a more complex syndrome, but for which a definitive disease association is still lacking. Here we are proceeding in three steps: (1) an initial assessment of LoF perturbations enrichment/depletion in cardiomyocytes, endothelial cells, and stromal cells from the various cardiac chambers - to select for effective perturbations in genes involved in cell fate decisions during cardiogenesis; (2) single cell genomics assessment of a subset of the perturbations; (3) small scale arrayed LoF assessment in the top hits. In all, this work is refining our understanding of LoF perturbations in diverse cell types contributing to CHD, paving the way to improved diagnosis and possibly to targeted therapies.

Funding Source: Giovanni Armenise-Harvard Foundation Career (A.B.) Development Award 2021 (A.B. and S.M.); Additional Ventures Single Ventricle Research Fund 2021; ERC Starting Grant (TRANS-3; 101076026 – A.B.)

Keywords: organoids, heart development, single cell genomics

6:45 PM – 7:45 PM

252

CELL-SPECIFIC MITOCHONDRIAL AND NUCLEAR DYSFUNCTIONS IN FRIEDREICH ATAXIA IPSC-DERIVATIVES

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Friedreich ataxia (FRDA) is a genetic disease that affects multiple systems in the body. It leads to a progressive loss of motor coordination, neural and musculoskeletal degeneration, and dysfunction in the heart and other organs. The disease is caused by an intronic elongation of a tandem repeat (GAA) in the gene encoding the protein frataxin. This results in lower levels of frataxin in all cells, particularly in cardiomyocytes and neurons, the cells most affected by FRDA. Frataxin participates in the biosynthesis of Fe-S clusters in the mitochondria. Although several methods to increase frataxin levels were developed, these methods are not cell-specific and can only partially reverse cellular dysfunctions, causing side effects in some cell populations. We hypothesized that decreased frataxin levels only cause dysfunctions in cells where the adaptive mechanisms cannot compensate for the loss. The compensatory mechanisms are cell-specific and dynamic, involving mitochondria, the nucleus, and other cellular components. We compared FRDA patient iPSC-derived cells (cardiomyocytes, neural crest cells, peripheral sensory neurons, spinal motor neurons, neuroepithelial cells, telencephalic neurons, and cerebellar nuclei neurons) with control cells. We applied various stressors such as mitochondrial stressors, hypoxia, increased iron, oxidative stress, and forced aging to these cells. We studied the changes in mitochondria and cellular metabolism, transcriptomic profiles, DNA repair, and cell death. We found that all cell types showed increased glycolysis in normoxia, but there were cell-specific differences in hypoxia, paralleled by gene expression differences. Increased iron and oxidative stress mainly affected the survival of FRDA peripheral and cerebellar neurons. We also detected cell-specific expression of several transcription factors with known protective roles (such as ATF3 and ATF4), which suggest cell-specific compensatory pathways in FRDA. By manipulating these compensatory mechanisms, more effective therapeutic approaches can be developed.

Funding Source: The Austrian Science Fund (FWF), Project ZFP268860

Keywords: frataxin deficiency, cardiomyocytes and neurons, cell-specific compensatory mechanisms

6:45 PM – 7:45 PM

254

HIGH-THROUGHPUT SCREENING OF FDA-APPROVED COMPOUNDS TO REPURPOSE FOR PREVENTION OF ANTHRACYCLINE-INDUCED CARDIOTOXICITY

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Anthracyclines are the front-line therapy for many paediatric cancers; however, their clinical usage is limited by significant dose-dependent and irreversible cardiac damage in many patients. To address this concern, we developed an in vitro model of anthracycline-induced cardiotoxicity using human pluripotent stem cell-derived cardiomyocytes. Using this model, we performed an unbiased, high-throughput drug screen of 2,906 FDA-approved compounds to identify clinically available compounds that may be repurposed for cardioprotection. From the screening library, two candidates were identified that demonstrated protection against anthracycline-induced cardiotoxicity in vitro. Chemical analogues of these candidates were then generated with the aim of increasing cardioprotective efficacy. Subsequent validation of the two candidates and their analogues was then performed in a 3D cardiac organoid model of anthracycline toxicity, however, we ultimately these compounds to be inappropriate for continued development. Overall, our study revealed a lack of potential cardioprotective agents within currently clinically available drugs. This outcome emphasises the need to assess novel chemical space in order to identify and develop new compounds that may protect against anthracycline-induced cardiotoxicity.

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Keywords: cardiotoxicity, drug screening, drug repurposing

6:45 PM – 7:45 PM

256

GLA-MODIFIED RNA TREATMENT LOWERS GB3 LEVELS IN IPSC-DERIVED CARDIOMYOCYTES FROM FABRY-AFFECTED INDIVIDUALS

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Fabry disease, a rare X-linked lysosomal storage disorder caused by mutations in the GLA gene coding for the α-Galactosidase A (α-Gal) enzyme, often leads to impaired diastolic function of the heart and can ultimately result in death due to heart failure. At present, enzyme-replacement therapy is considered the best standard of care for persons with Fabry disease. However, despite alleviation of clinical symptoms, some individuals still suffer from cardiac manifestations. Moreover, enzyme-replacement therapy has been reported to induce an immune response in some individuals, resulting in reduced enzymatic activity or infusion-associated reactions. In addition, the high cost of enzyme-replacement therapy limits the widespread use of this approach and emphasizes the urgent need for alternative therapeutic approaches. Recent studies in non-human model systems have shown therapeutic potential of nucleoside-modified messenger RNA (modRNA) treatments for lysosomal storage diseases. Here, we assessed for the first time the efficacy of a modRNA treatment to restore the expression of α-Gal in a human cardiac model generated from induced pluripotent stem cells (iPSCs) derived from two individuals with Fabry disease. Consistent with the clinical phenotype, cardiomyocytes from iPSCs derived from Fabry-affected individuals showed an accumulation of the glycosphingolipid Globotriaosylceramide (GB3), which is an α-Gal substrate. Furthermore, the Fabry cardiomyocytes displayed a significant upregulation of lysosomal lumen-associated proteins. Upon GLA modRNA treatment, a subset of these lysosomal proteins were partially restored to wild-type levels, implying the rescue of the molecular phenotype associated with the Fabry genotype. Importantly, a significant reduction of GB3 levels was observed in GLA modRNA-treated cardiomyocytes, demonstrating that α-Gal enzymatic activity was restored. Together, our results validate the utility of iPSC-derived cardiomyocytes from affected individuals as a model to study disease processes in Fabry disease and the therapeutic potential of GLA modRNA treatment to reduce GB3 accumulation in the heart.

Funding Source: Marie Skłodowska-Curie Actions

Keywords: Fabry disease, cardiomyocytes, induced pluripotent stem cells

6:45 PM – 7:45 PM

258

STRESS RESPONSE OF MOUSE EMBRYONIC STEM CELLS AND THEREOF DERIVED ENDOTHELIAL CELLS TO DOXORUBICIN AND DNA DAMAGE RESPONSE (DDR) MODULATORS

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The clinical use of the chemotherapeutic agent doxorubicin (Dox) is limited by a cumulative and irreversible cardiotoxicity. It is believed that both the inhibition of topoisomerase II isoforms and iron-dependent ROS formation leading to DNA damage contribute to the pathophysiology of Dox. However, underlying molecular mechanisms and the pathophysiological relevance of different cardiac cell types are still unclear. The present study aims to characterize stress responses of murine embryonic stem cells (mESC), mESC-derived cardiac progenitor cells (CPC) and differentiated endothelial cells (EC) to Dox and two inhibitors of the DDR. To this end, cytotoxic effects of Dox and the DDR modulators B02 (Rad51i) and Entinostat (HDACi), on mESCs, CPCs and ECs were evaluated. In more detail, cell cycle progression, proliferation activity, apoptosis, DNA damage formation and gene expression of susceptibility factors were analyzed. Moreover, the differentiation potential of CPCs after treatment was examined followed by functional analysis of thereof derived ECs. We found that Dox impairs proliferation of mESCs and induces apoptosis. At the same time pro-survival DDR pathways are activated as reflected by checkpoint activation and promotion of ATR-related replication stress response pathways. Western Blot analysis and qPCR data indicates that Dox-induced DNA damages can either be repaired or appear to trigger senescence in surviving cells. Similar response was observed for mESCs treated with Entinostat, but not with B02. Hence, surviving mESCs might be able to compensate for loss of Rad51 activity, but are sensitive to epigenetic changes. CPCs reveal higher Dox sensitivity compared to mESCs and differentiated ECs. In line with this, mRNA expression of susceptibility factors (i.e. DNA repair, apoptosis etc.) change in course of differentiation. CPCs that survived Dox treatment were able to continue differentiation into phenotypical EC-like cells. However, functional analysis showed an impaired tight junction formation after Dox but not after inhibitor treatment. Based on the data, Dox exposure of sensitive cardiac progenitor cells might compromise regenerative processes of the heart by impairing the functionality of differentiated progeny.

Funding Source: Deutsche Forschungsgemeinschaft (GRK2578)

Keywords: anticancer drugs, DNA damage, cardiotoxicity

6:45 PM – 7:45 PM

260

INVESTIGATING SPONTANEOUS CORONARY ARTERY DISSECTION (SCAD) PATHOGENESIS USING IPSC-DERIVED VASCULAR SMOOTH MUSCLE CELLS

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Spontaneous coronary artery dissection (SCAD) is a potentially fatal disorder of unknown etiology. It primarily affects women, with a median age of 51 years, who have few traditional cardiovascular risk factors. SCAD is caused by a bleed in the intermediate layer of the coronary artery wall, which results in the formation of an intramural hematoma. This leads to obstruction of coronary blood flow, causing an acute coronary syndrome or death. There is no current treatment to prevent SCAD or its recurrence. As coronary artery tissue is not readily available, we utilized iPSC-derived vascular smooth muscle cells (VSMCs) to model the disease. Here, we generated iPSCs from first-degree cousin SCAD survivors harboring a predicted pathological coding-sequence variant in the low-density lipoprotein receptor-related protein 1 (LRP1) gene. We also generated iPSCs from an unaffected sister in this SCAD family, who has a wild-type LRP1 genotype. CRISPR/Cas9 gene editing was used to correct the variant in SCAD-iPSCs and restore the wild-type genotype. Resulting isogenic VSMCs harboring the LRP1 variant or native LRP1 gene were differentiated into contractile VSMCs, and the phenotypes of these cells compared using high-throughput confocal imaging (n=4) and analyzed by one-way repeated measures ANOVA ($P< 0.05$). CRISPR/Cas9-edited and -unedited VSMCs derived from both SCAD cases showed similar levels of VSMC markers alpha smooth muscle actin (α-SMA) and myosin heavy chain 11 (MYH11) expression, as the unaffected sister iPSC derived VSMCs. Morphometric features were also similar across the VSMC lines. Immunocytochemistry showed that LRP1 expression is predominantly localized in the early endosome compartment. Ongoing phenotypic characterization of iPSC-derived VSMCs, along with base editing to introduce the LRP1 gene variant into the unaffected sister-derived iPSCs, aims to demonstrate a link between genotype and phenotype, which would provide compelling evidence for a role of LRP1 gene variant in SCAD pathogenesis in this family and may indicate a potential therapeutic target for preventing SCAD recurrences.

Keywords: iPSC-derived vascular smooth muscle cells, CRISPR/cas9-editing, cytosine base editing, acute coronary syndrome, LRP1 gene

6:45 PM – 7:45 PM

262

ADVANCING DISEASE MODELING TO DECIPHER THE CARDIORENAL PHENOTYPE OF FABRY DISEASE USING ORGANOIDS FROM PATIENT-DERIVED INDUCED PLURIPOTENT STEM CELLS

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Fabry disease is a lysosomal storage disorder caused by mutations in the GLA gene resulting in a multisystemic disorder affecting the kidney, heart and nervous system. This complex disease can currently not be modelled accurately in rodent systems. Our project aims to develop advanced human in vitro systems to improve our molecular understanding of the cardiorenal phenotype of Fabry disease. To establish these informative systems, we use human induced pluripotent stem cells (hiPSCs) and differentiate them into different organoid systems. We reprogrammed hiPSCs from primary urinary cells collected from Fabry patients with various mutations and created isogenic control lines through CRISPR Cas9 gene editing. Using published protocols, we differentiated these cell lines into heart and kidney organoids as well as cardiomyocytes for the generation of engineered heart tissues (EHTs). We confirmed the loss of alpha-galactosidase A protein, deficiency of alpha-galactosidase A enzyme activity and the accumulation of globotriaosylceramide in patient-derived hiPSC lines, organoids and EHTs. Heart organoids exhibited robust contractions and showed several cardiac cell types. Kidney organoids reflected the cellular composition of different nephron segments. We implemented treatment with enzyme replacement therapy and, when applicable, chaperone therapy. In the hiPSC monolayer and both organoid systems, treatment resulted in a significant increase in enzyme activity and a consequent reduction in substrate accumulation. Fabry EHTs were prone to arrhythmias, reflecting the clinical phenotype of patients. Here, administration of enzyme replacement therapy again reduced substrate accumulation but did not alter the functional impairment. Furthermore, we confirmed the published pathological accumulation of synuclein alpha in Fabry nephropathy in kidney organoids. We have established novel, unique discovery platforms for Fabry disease in humans using in vitro disease models. Ongoing experiments focus on single-cell analyses of the established systems in combination with deep proteomic and spatial phenotyping together with the administration of available therapies.

Funding Source: Amicus Therapeutics

Keywords: disease modeling, Fabry disease (lysosomal storage disorder), kidney and heart organoids

6:45 PM – 7:45 PM

264

TESTING OF PERSONALIZED CRISPR THERAPIES FOR TITIN-TRUNCATING VARIANTS

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Truncation mutations in the gene TTN have been shown as strongly associated with familial Dilated Cardiomyopathy (DCM). Many of these TTN-truncating variants (TTNtv) are found within the A-band, the largest sarcomeric component encoded by TTN. Patients with such mutations may experience haploinsufficiency of protein expression, an accumulation of poison-peptides, and sarcomere deficiency. To date, the patient's only option is to undergo a heart transplant. The use of a targeted CRISPR-Cas9 therapeutic strategy to employ gene-editing at the site of the mutation may prove a viable alternative. Using a preclinical platform of patient-specific induced pluripotent stem cell (iPSC) models harboring selected TTNtv, we tested different clinically translatable CRISPR/Cas9-based gene therapy strategies: a) exon skipping, in which the entirety of the exon is removed; and b) reframing, in which the required number of base pairs is inserted to shift the genomic sequence back in frame. We demonstrate that these techniques are feasible for editing in proliferative iPSCs and non-proliferative iPSC-CMs by restoring titin protein expression, sarcomere assembly and cardiac function. While further efforts must be made to ensure the safety of such therapies, our proof-of-concept research presents a favorable alternative to current DCM treatment.

Keywords: genome editing, hiPSC-CM, TTN

6:45 PM – 7:45 PM

266

PRECISE GENOME EDITING OF INHERITED CARDIOVASCULAR DISEASES IN HUMAN CARDIAC MICROTISSUES

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Inherited cardiovascular diseases (CVDs) are a major cause of sudden cardiac death and the most common conditions comprise cardiomyopathies and channelopathies. With an incidence of 1 per 200 adults, they are a substantial burden to the healthcare services of all nations. Although a few medications can slow the progression of these diseases, today there is no cure for inherited CVDs. Hereditary CVDs are mainly caused by individual point mutations spread across multiple genes, and several different pathological mutations can be found in each gene. For these reasons, gene editing approaches designed to precisely induce gene edits offer exciting options to permanently treat these diseases. Due to the lack of mutational hotspots, a versatile and robust in vitro model of mature human cardiomyocytes would be required to test new strategies to treat different inherited CVDs. Here, we combined the CRISPR-Cas9 precision genome editing technology of base editing with three-dimensional human induced pluripotent stem cell-derived cardiac microtissues (CMTs) as a platform to demonstrate the utilizations of base editing in an advanced in vitro model that recapitulates the cellular complexity of the human heart. Base editing can permanently edit point mutations of genes, making base editors ideal tools to permanently treat these diseases. We deployed base editors in CMTs to: i. Introduce different patient-specific point mutations in the FLNC gene to study their pathological molecular pathways leading to dilated or hypertrophic cardiomyopathy; ii. Precisely correct point

mutations in the KCNQ1 gene that cause the channelopathy long QT type 1 syndrome; iii. Develop a universal strategy to permanently treat dilated cardiomyopathy that affects Duchenne muscular dystrophy patients. The utilization of base editing in CMTs permits a rapid and solid screening of the efficacy, delivery systems, and therapeutic safety of genome editing components and we anticipate it can be adopted as a crucial and important pre-clinical step towards therapeutic genome editing of CVDs.

Funding Source: The Novo Nordisk Foundation grants (NNF21CC0073729); European Union's Horizon 2020 research and innovation programme under European Research Council (ERC-CoG Mini-HEART no. 101001746); HE-MSCA-PF-2021-101063293- Edit-hCOs

Keywords: cardiac diseases, gene editing, cardiac microtissues

6:45 PM – 7:45 PM

268

SINGLE-CELL AND SPATIAL TRANSCRIPTOMICS OF THE CARDIAC NEURAL CREST REVEAL A DUAL ROLE OF VINCULIN IN TGFB SIGNALING AND CELL-EXTRACELLULAR MATRIX INTERACTIONS DURING CARDIAC OUTFLOW TRACT DEVELOPMENT

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Cardiac outflow tract (OFT) defects are commonly observed in neurocristopathies. Vinculin (VCL), a focal adhesion and adherens junction adaptor protein, has been associated with these complex clinical conditions, but the underlying pathological mechanisms remain largely unclear. Here, we showed that mouse mutants with neural crest cell (NCC)-specific deletion of Vcl exhibit malformation of pharyngeal arch arteries, cardiac outflow tract septation defects and hyperplastic semilunar valves, due to defects in migration and vascular smooth muscle (VSMC) lineage differentiation of cardiac NCCs (CNCCs), which prohibited the subsequent myocardialization, perturbing the OFT and valve remodeling. Combining high-resolution transcriptomic and immunohistochemistry analyses and human pluripotent stem cell (hPSC)-based in vitro differentiation assays, we further demonstrated that CNCC-to-VSMC differentiation is orchestrated by Vcl-dependent p38 and TGF-β signaling pathways. Multimodal analysis integrating the single-cell and spatial transcriptomes of OFT cells further resolved various OFT cells by their spatial profiles and highlighted another role of Vcl in mediating cell-extracellular matrix interactions during OFT morphogenesis. In sum, Vcl is required for TGF-β, p38 pathways and cell-ECM interactions to modulate the VSMC lineage differentiation and migration of CNCCs. Timely coordination of these cellular processes and OFT cell interaction dynamics is essential for triggering myocardialization to drive OFT remodeling.

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Keywords: cardiac neural crest cell, outflow tract, single cell and spatial transcriptomics

6:45 PM – 7:45 PM

270

IN VITRO MODEL OF ATRIAL FIBRILLATION: INVESTIGATING ATRIAL REMODELING MECHANISMS USING HIPSC-DERIVED ATRIAL CARDIOMYOCYTES

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Each year hundreds of thousands of new cases worldwide are annually diagnosed with atrial fibrillation (AF). However, despite its clinical importance, the initiation mechanisms of atrial remodeling during AF is still unclear. Furthermore, really few experimental models have the power to investigate these mechanisms in a human in vitro two-dimensional (2D) monolayer of atrial cardiomyocytes. The purpose of this work is to use human induced pluripotent stem cell (hiPSC)-derived atrial cardiomyocytes to develop a new human in vitro atrial model of AF able to reproduce and evaluate the arrhythmia initiation and maintenance mechanisms. Two hiPSC lines were differentiated into atrial cardiomyocytes, comparing the protocol with ventricular hiPSC differentiation. Seeded in plates of different surface area in order to control reentries formation, characteristic of AF. After 10 days, the genetic remodeling of cells under fibrillating rhythm were compared with those from AF patients. Patch clamp recordings revealed that atrial cardiomyocytes had significantly shorter action potential duration at 90% repolarization (182.3 ± 68 ms) compared to vetricular cardiomyocytes (371.6 ± 82 ms), along with a faster depolarization rate and higher spontaneous beating frequency. Optical mapping experiments showed larger surfaces activated at 1.6 ± 1.9 Hz, while smaller surfaces had 0.9 ± 0.5 Hz activation rate. Larger surfaces exhibited a higher number of reentrant wavefronts (11.0 ± 8.2 reentries/cm2) compared to smaller ones (0.9 ± 0.5 reentries/cm2), indicating an AF phenotype. RNAseq studies revealed expression alteration of genes related to extracellular matrix remodeling (CDH1, SDC4, MMP1, VTN) and inflammation (IL16, IL11, CXCL12, IL18) in larger surfaces, consistent with RNAseq analysis of isolated tissue from AF patients. We conclude that atrial cardiomyocytes obtained from hiPSC can recapitulate the remodeling that atrial

tissue undergoes during AF initiation in patients. Providing a unique opportunity to study the initiation of fibrillatory activities and their maintenance in a human-relevant in-vitro model.

Funding Source: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No.860974.

Keywords: atrial fibrillation, atrial remodeling, cardiac arrhythmia

6:45 PM – 7:45 PM

272

MATURATION OF CARDIAC ORGANOIDS USING ELECTRICAL STIMULATION

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Cardiovascular diseases are the world's leading cause of death and their prevalence continues to increase with the aging population. Modern human cardiac three-dimensional in vitro models, such as cardiac organoids, have successfully been used to study cardiac diseases. However, current cardiac organoids often lack the proper maturation to accurately mimic aging conditions. We therefore set out to increase the maturation of induced pluripotent stem cell (iPSC)-derived cavity containing cardiac organoids through noninvasive electromechanical cues. We first optimized the vascularization of established cardiac organoid protocols and found that early administration of vascular endothelial growth factor (VEGF) resulted in an endothelial lining and a physiologically representative amount of endothelial cells (6.4%), while still retaining a high amount of cardiomyocytes (77%). Next, immunofluorescent imaging confirmed the presence and spatial distribution of three of the main cell types of the heart: cardiomyocytes, endothelial cells and cardiac fibroblasts. Following characterization, we embedded the vascularized cardiac organoids within a hydrogel and exposed them to electrical pulses during maturation. Our findings revealed that exposure to electromechanical cues increased the expression of several functional markers related to maturation, conduction and calcium handling when compared to non-stimulated cardiac organoids. These findings suggest that exposure to electromechanical cues can improve the maturation of cardiac organoids. So far, only cardiac microtissues, engineered heart tissues and two-dimensional cardiac cultures have been matured using electromechanical stimulation. This strategy has not been applied to more complex organoid models, such as those generated through the directed differentiation of iPSCs into cavity containing cardiac organoids. Hence, these cardiac organoids typically still lack the required maturation levels to accurately mimic events in mature tissues. Here, we demonstrate an approach to achieve higher maturation level in iPSC-derived cavity containing cardiac organoids, thereby facilitating their use in modeling diseases that require mature tissue, such as aging-related diseases.

Keywords: cardiac organoid, maturation, electrical stimulation

6:45 PM – 7:45 PM

274

PRE-CLINICAL TESTING OF ALL-IN ONE CRISPR/CAS9 AAV GENE THERAPY FOR DMD IN PATIENT-SPECIFIC IPSC-CARDIOMYOCYTE AND SKELETAL MUSCLE

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Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder caused by mutations in the DMD gene, resulting in the loss of the dystrophin protein. This essential protein stabilizes the muscle sarcolemma by providing a link between the extracellular matrix and the actin cytoskeleton. With an incidence of 1 in 3500 males, DMD is as a predominant dystrophy that presents with respiratory disabilities and cardiomyopathies in adulthood and premature mortality in the patients 20s to 30s. Over 5000 causative mutations resulting in a premature termination of dystrophin translation by disruption of the reading frame have been identified, underscoring the complexity of therapy development. Here, we aim to develop a personalized CRISPR/Cas9-based gene therapy for DMD patients, focussing on the restoration of the disrupted reading frame through exon skipping strategies. As a platform, patient-specific iPSCs were generated, differentiated into cardiac (iPSC-CM) and skeletal muscle cells (iPSC-SM), and phenotyped at the molecular and cellular level. Patient-derived iPSC-CM and -SM cells confirmed the lack of dystrophin protein. In order to induce exon skipping and reframing of the coding sequence, different CRISPR/Cas9 genome editing approaches were evaluated. Excitingly, CRISPR-based exon skipping demonstrated restoration of dystrophin expression and contractile function. Finally, gene correction in cardiomyocytes was achieved by the use of all-in-one AAVs containing smaller Cas9 variants and the guide RNA cassettes within a single construct. In conclusion, the use of patient-specific iPSC-CMs and -SM represent an excellent platform for the exploration and validation of various clinically translatable CRISPR/Cas9-based exon skipping strategies for treating DMD.

Keywords: Duchenne Muscular Dystrophy, patient-specific iPSC-muscle cells, CRISPR/Cas9 therapy

6:45 PM – 7:45 PM

276

CARDISCOVER-IL: A NOVEL HUMAN CARDIOVASCULAR ORGANOID DRUG DISCOVERY SYSTEM BASED ON INTEGRATED MECHANO-SENSORS AND OPTOGENETIC CONTACTLESS CONTROL

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Human-induced pluripotent stem cell-derived cardiovascular organoids (hIPSC-CVOs) are a widely used tool in the investigation of cardiovascular diseases and drug screening. While high-throughput platforms offer robust statistical power, they often lack the intricate multicellularity and pacing capabilities inherent in high-content platforms. Here, we present a novel approach within cardiovascular drug screening platforms. Optogenetic hiPSC-cardiomyocytes (CMs) were combined with hiPSC-derived endothelial cells (ECs), smooth muscle cells (SMCs), and epicardial derived cardiac fibroblasts (CFs), embedded in a collagen matrix. Subsequently, the mixture was seeded around pairs of mechano-sensors fabricated from PDMS within a 96-well optical plate. By day 4, the generated CVOs began contracting and deflecting the mechano-sensors. Pacing was achieved by illuminating the optogenetic CVOs in a specific pattern. The CVOs were monitored in high-speed camera 100 Frames per second and analyzed via a tailor-made automated image analysis program. Deflections of the mechano-sensors were detected by the program and parameters such as active force, time-to-peak, relaxation time, contraction time, peak-to-peak time, and the relaxation constant (τ) were calculated. Immunofluorescent staining was conducted to investigate the multicellularity of the developed organoids. Immunofluorescent staining revealed vessels-like structures formed by the ECs in the CVOs. Exposure to 0.5 μM isoprenaline resulted in increased active force (139.9 vs 117.2μN), shortened time-to-peak (78 vs 113 msec) and shortened relaxation (147 vs 184 msec). The platform enabled monitoring under spontaneous pacing conditions and within a frequency range of 0.5 Hz to 5 Hz (the tested frequency range). The contact-less pacing of human multicellular organoids, coupled with the integrated mechano-sensors, achieves a synergy between high content and high throughput. This platform holds promise for high-throughput, high-content drug screening and patient-specific drug testing.

Keywords: drug screening, high throughput, cardiovascular organoids

6:45 PM – 7:45 PM

278

GENETIC VARIANT RISK ASSESSMENT FOR LONG QT SYNDROME THROUGH MACHINE LEARNING AND MULTIELECTRODE ARRAY RECORDINGS ON IPSC-DERIVED CARDIOMYOCYTES

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Long QT syndrome (LQTS) is a life-threatening disease characterized by a prolonged QT interval on the electrocardiogram and by an increased sensitivity to drugs affecting cardiac repolarization. Despite extensive efforts to evaluate the pathogenicity of LQTS-associated genetic variants, the vast majority of them represent variants of unknown significance and remain poorly characterized, limiting the possibility of appropriate patient risk-stratification and personalized therapy. Patient-specific human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are promising tools to provide precise insights into variant-specific alterations in cardiac biology and electrophysiology. However, generating hiPSCs from every subject is virtually impossible at the current state of technology and more advanced strategies which make use of cohort data are required to predict variant-specific risk. Here, we propose a novel approach which integrates electrophysiological measurements of patient-specific hiPSC-CMs with a machine learning (ML) pipeline to predict the variant risk level. We utilized healthy hiPSC-CMs alongside those derived from 10 patients with LQTS

subtypes, including LQT1, Jervell and Lange-Nielsen Syndrome (JLNS) and LQT2, representing variable disease severities. Multielectrode array (MEA) measurements and drug responses to 12 compounds with known proarrhythmic effects were recorded to establish a comprehensive MEA measurements database (> 12000 data points). Strong correlation between variant severity, hiPSC-CMs electrophysiology and drug-response profile was revealed. Through integration with a ML classification algorithm, we found that model trained on electrophysiological data demonstrated high predictive accuracy (>90%), providing a promising strategy for assessing variant-associated risk levels. Furthermore, this pipeline could be easily expanded to incorporate other LQTS subtypes, including acquired forms of LQTS such as drug- or exercise-induced LQTS to collect representative data from broader cohorts. By combining hiPSC-CMs, medium-throughput electrophysiological readouts with ML, our approach yields high quality data that could represent a significant advanced variant risk classification.

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Keywords: precision medicine, Long QT syndrome, iPSC-derived cardiomyocytes

6:45 PM – 7:45 PM

280

DEVELOPMENT OF A ENGINEERED IPSC REPORTER SYSTEM FOR MONITORING HUMAN CARDIOMYOCYTE PROLIFERATION

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Cardiovascular disease remains the foremost cause of mortality worldwide. A primary contributing factor to this trend is the limited regenerative potential of mammalian cardiomyocytes (CMs). Augmenting the intrinsic capacity for CM proliferation stands as a pivotal objective in regenerative medicine. Yet, the absence of a human translational test platform constrains the screening of potential drug candidates aimed at promoting cardiac regeneration.To address this challenge, we established a human induced pluripotent stem cell (hiPSC)-derived cardiomyocyte system for live-cell monitoring of CM proliferation. To generate a stable hiPSC cell line, we used a lentiviral vector carrying a cell cycle regulated destruction signal (N-destruction box of cyclinB1) fused to an eGFP (cyclinB1-eGFP), which can efficiently label cells in the S/G2/M phase of the cell cycle. Immunofluorescence staining, flow cytometry and RT-qPCR analyses demonstrated that lentiviral transduction, cell passage, expansion and sorting did not compromise the pluripotency and trilineage differentiation potential of the reporter hiPSC line. Finally, the hiPSC reporter cell line was further differentiated into functional and beating CMs. The benefit of using our reporter line for the identification and screening of other novel pro-proliferative compounds is highlighted by the stimulation of CM proliferation with the Wnt pathway activator CHIR99021. These findings were further validated by live time-lapse imaging of eGFP+ CMs. Furthermore, large-scale CM differentiation facilitated sorting into cyclinB1-eGFP+ and cyclinB1-eGFP- subpopulations, followed by whole transcriptome RNA sequencing. Gene set enrichment analysis (GSEA) highlighted a substantial upregulation of genes associated with DNA replication, and cell cycle progression in proliferating CM, accompanied by a downregulation of genes involved in cardiac muscle contraction, oxidative phosphorylation and fatty acid metabolism. These findings suggest a metabolic and structural reprogramming in proliferating CMs, reminiscent of an embryonic/neonatal molecular signature that allows CMs to divide. In summary, we have devised an innovative and sophisticated tool for large-scale in vitro screening of pro-regenerative compounds.

Keywords: cardiomyocyte proliferation, drug screening, cardiac regeneration

6:45 PM – 7:45 PM

282

A NEW BIOSENSOR PLATFORM FOR HIGH THROUGHPUT SIGNALING ANALYSIS IN IPSC-DERIVED CARDIOMYOCYTES AND CARDIAC FIBROBLASTS

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Dysregulated signaling pathways such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases (PI3K)/AKT have been shown to be involved in cardiac disease. Consequently, many therapeutic approaches aim at the modulation of signaling pathways to restore signaling activity. However, manipulation of these highly dynamic signaling circuits is challenging as intra- and extracellular crosstalk increases complexity. Compensatory mechanisms, like signaling crosstalk, have been described to appear not only intracellularly but also intercellularly through cell-cell crosstalk. Due to their highly dynamic and adaptive characteristics, new tools are needed that allow for signaling pathway visualization in a live setting and over longer time periods. Here, we describe a biosensor-based analysis platform in iPSC-derived cells that allows for high throughput measurement of prominent signaling pathways (ERK, JNK, p38, PKA, and AKT). The genetically-encoded biosensors translate kinase activity into quantifiable nucleus-cytoplasm translocation. We transduced iPSC-derived cardiomyocytes and fibroblasts with lentiviral encoding biosensors and analyzed live cell imaging data with a trained algorithm. The biosensors respond highly sensitive and specific to small-molecule inhibitors and stimulants in a concentration-dependent manner. Moreover, we could record signaling dynamics over 24h revealing also later drug responses. The established iPSC biosensor platform can be used for high throughput signaling analysis, drug screening, and testing.

Keywords: biosensor-based signaling pathway measurement, iPSC-Cardiomyocytes and cardiac fibroblasts, drug screening

6:45 PM – 7:45 PM

284

DEVELOPING IPSC MODELS OF INFLAMMATION INDUCED ATRIAL FIBRILLATION

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Atrial fibrillation (Afib) is associated with a 5-fold increased risk of stroke, greater susceptibility to cognitive decline, and increased risk of heart failure. Current treatment options can have adverse side effects and become ineffectual over time, as Afib evolves from a paroxysmal to permanent arrhythmia, due to progressive physical and electrical remodeling of the heart. Current studies are hindered by limited human samples, and animal models that have differences in heart size, beat rate and electrophysiology. To overcome these limitations, we have developed human induced pluripotent stem cell (iPSC) models of atrial arrhythmias. Additionally, we are utilizing a cytokine storm to induce rapid new on-set Afib that occurs during severe COVID or sepsis. By doing so we have recreated the rotor-style arrhythmia that underpins Afib pathology, which we are investigating through a combination of electrophysiology and OMICs techniques to uncover novel disease mechanisms, and to establish an anti-arrhythmic drug screening platform. With a novel atrial differentiation protocol, we are investigating the effects of Afib-linked inflammatory cytokines, on tri/quad-culture models, including atrial cardiomyocytes, cardiac fibroblasts, smooth muscle cells and endothelial cells. A multi-electrode array (MEA) system demonstrated the ability of select inflammatory cytokine conditions to induce arrhythmic activity, promoted by conduction slowing, and field potential prolongation. Additionally, we created large vascularized (up to 1.5 x 1.5 cm) engineered heart tissues situated around a network of 3D printed pillars, incubation with inflammatory cytokines induced conduction slowing and facilitated the formation of rotor style arrhythmia that could be tracked and quantified including, rotor formation, phase singularity lifetimes, complexity, and movement patterns across the tissue. The ability to induce a pro-arrhythmic phenotype through cytokine addition demonstrates these models will be a useful tool for undercovering mechanisms underlying induced atrial arrhythmia and for drug screening.

Funding Source: This study was supported by a St Vincent's Clinic Research Grant, Perpetual Philanthropy Grant, NSW Office of Health and Medical Research Cardiovascular capacity building grant, NHMRC Principal research Fellowship

Keywords: arrhythmia, atrial cardiomyocyte, inflammation

6:45 PM – 7:45 PM

286

2D AND 3D HIPSC-CARDIAC MODELS OF A MYBPC3 MUTATION REVEAL MODEL-SPECIFIC HYPERTROPHIC CARDIOMYOPATHY DISEASE PHENOTYPES

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To study cardiac disease, both 2D and 3D models derived from human induced pluripotent stem cells (hiPSCs) are used, with each system offering different insights. However, the complexity of diseases like hypertrophic cardiomyopathy (HCM) challenges the effectiveness of these models. HCM is a life-threatening condition caused by mutations in cardiac sarcomeric genes, and manifests through a spectrum of symptoms including cardiac hypertrophy, myofibrillar disarray and myocardial hypercontractility. The disease's unpredictable progression complicates treatment, underlining the need for better research models. In this study, we developed and compared 2D and 3D models of HCM using hiPSCs derived from a patient carrying the MYBPC3-2373insG mutation, alongside genetically corrected controls. While the 2D hiPSC-cardiomyocyte (hiPSC-CM) models revealed metabolic changes and disorganized sarcomeres when aligned, they lacked the ability to uncover contractility issues. In contrast, 3D engineered heart tissues demonstrated reduced contraction force. Expanding our investigation to include more recently developed 3D multicellular models, namely cardiac microtissues (cMTs) and cardioids, we observed that mutant cMTs exhibited a hypocontractile phenotype, altered calcium transients, and MyBP-C haploinsufficiency. Similarly, mutant cardioids displayed both hypocontractility and signs of hypertrophy. In sum, this study underscores the importance of model selection based on the biological question and highlights how the maturity of the hiPSC-CMs in the different models affects the detectability of

disease phenotypes. Our results confirm that more complex, multicellular models that improve hiPSC-CM maturation are more effective in revealing the functional impairments associated with HCM. This insight provides valuable tools to further explore HCM disease mechanisms and to identify novel therapeutics.

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Keywords: hypertrophic cardiomyopathy, cardiac myosin binding protein c, 3D hiPSC models

6:45 PM – 7:45 PM

288

SUSPENSION CULTURE-DERIVED ORGANOID MODELS OF CARDIAC DEVELOPMENT

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Self-assembly of hiPSCs enables the development of tissue-like structures called organoids. Initial cardiac organoid (CO) models demonstrated the ability to mimic aspects of human heart development, including myocardial walls, fibroblasts, and endothelial cells (ECs) surrounding one or more cavities, and an epicardium. These COs were predominantly grown in static culture in multiwell dishes and induced through complex sequences of chemical cues. Here we used Wnt modulation and suspension culture to generate suspension-derived COs (sCOs), which enabled upscaling of organoid production and led to the discovery that small changes in conditions create unique developmental niches and induce novel tissues, such as a structure resembling the proepicardium. We used directed differentiation by Wnt modulation and stirred spinner flasks to produce sCOs. Spinner flasks were inoculated with iPSCs and spontaneously formed embryoid bodies (EBs) were differentiated and after 4-5 days in culture we observed a secondary sphere emerge on sCOs. This secondary sphere consisted of an outer epithelial layer enclosing mesenchymal (VIM+) and multipotent (SOX2+) cells, resembling a proepicardium. The proepicardial-sphere (PS) continued to grow out of the initial EB by forming a "stalk". Over time the stalk bent thereby enabling contact and fusion of the PS to the initial EB, which by this time point had developed into a spontaneously beating myocardium enclosing a cavity. Fusion of the PS to the myocardium resulted in the spread of mesenchymal cells (VIM+) over its surface, which subsequently differentiated into epicardial cells (WT1+). At later time points, non-CMs and ECs (CD31+) populated the CO, and the ECs formed networks resembling vasculature. This process mimics normal heart development, in which epicardial cells undergo epithelial-mesenchymal transition, migrate into the myocardium, and differentiate to form non-cardiomyocytes. Here we present a novel cardiac organoid model, sCOs, that is fully derived in suspension and can be readily scaled. We show that typical Wnt modulation, coupled with appropriate physical culture conditions, creates developmental niches that spontaneously generate proepicardial-like structures, which subsequently form the epicardium and epicardium-derived lineages.

Funding Source: Additional Ventures SVRF

Keywords: cardiac organoid, suspension culture, epicardium

6:45 PM – 7:45 PM

290

IN VITRO MODELLING OF MACROPHAGES AND CARDIOMYOCYTES CROSSTALK IN HEART FAILURE

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Heart failure, characterized by the inability of the heart to maintain organ perfusion at a rate commensurate with the needs of the body, ranks among the foremost causes of global mortality. Intriguingly, emerging research on patients who recovered following left ventricular assist device therapy highlighted the correlation between an increase in tissue resident macrophages and improved heart functions; however, the underlying mechanisms remain elusive. To elucidate the contribution of macrophage-cardiomyocyte crosstalk to cardiac recovery, we established an in vitro model system using cardiomyocytes and macrophages derived from induced pluripotent stem cells. Our findings show that LPS-induced macrophages provoke changes in cardiomyocyte energy dynamics and contractility, while IL4/IL13-stimulated macrophages preserve cardiac function and cellular energetics. This research sheds light on the intricate interplay between macrophages and cardiomyocytes, offering insights into potential therapeutic avenues for mitigating cardiomyopathies and enhancing cardiac healing mechanisms.

Keywords: heart failure, macrophages, cardiomyocytes, cell-cell Interactions, cardiovascular homeostasis

6:45 PM – 7:45 PM

292

HUMAN IPSC-DERIVED SENSORY NEURONS AND CARDIOMYOCYTES INTERACT IN MULTIELECTRODE-INTEGRATED COMPARTMENTALIZED MICROFLUIDIC CHIPS

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Understanding the cellular and molecular basis of neuro-cardiac interactions is crucial for elucidating the systemic mechanisms in healthy and diseased states. Sensory neurons play a vital role in transmitting sensory information- such as pressure and pain- from the heart to the brain and provide regulatory feedback to the autonomic neurons for cardiovascular homeostasis. While the interaction between the heart and autonomic neurons is documented well, human sensory neuron and cardiac cell interactions in vitro were not reported in the literature at the cellular, molecular, or functional levels. Here, we investigated the interaction between induced pluripotent stem cell- (hiPSC) derived sensory neurons (iSensN) and hiPSC-derived cardiomyocytes (iCM) on the fabricated multielectrode array (MEA)-integrated microfluidic system. We manufactured a microfluidic system using a PDMS chamber with two separate compartments interconnected by microchannels placed on a fabricated 32-gold electrode array. The molecular and functional characterization of healthy individual-originated iSensN and iCM was done by qRT-PCR, immunocytochemical analysis, electron microscopy, and Fluo4-AM calcium imaging. The molecular validation of iSensNs and iCMs was done by mRNA and protein expression of neuronal Tuj1, peripherin, Vglut2 markers, and cardiac cTnT marker. Importantly, the axons of iSensNs projecting through microchannels interacted with iCMs that were revealed to be by synaptic connections evident by Synaptophysin protein immunoreactivity. Further characterization by scanning electron microscopy coupled with immunostaining confirmed synaptic zones and cellular interactions at high resolution. At the functional level, simultaneous recordings of electrical activity from both iSensNs and interacting iCMs were obtained by using MEA recordings. The iCMs displayed spontaneous rhythmic firings, while spontaneous spikes with low amplitude were measured in human iSensNs. Taken together, we demonstrated and evaluated the interaction between human iSensN and iCMs in vitro for the first time by developing an MEA-integrated compartmentalized microfluidic system, that could be used for future pharmacological and functional investigations.

Funding Source: This study was supported by The Scientific And Technological Research Council Of Türkiye (TUBITAK) under the 1001 funding Program by project number 119S132, and Istanbul Medipol University BAP project number 2023/18.

Keywords: iPSC-derived cardiomyocytes, iPSC-derived sensory neurons, MEA-integrated microfluidic systems

6:45 PM – 7:45 PM

294

MOSAIC-LIKE EXPRESSION OF CMYBP-C AS CONTRIBUTING FACTOR IN THE HCM DEVELOPMENT - INSIGHTS FROM HIPSCS CELL CULTURE MODEL CARRYING A HETEROZYGOUS MUTATION IN MYBPC3

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Hypertrophic cardiomyopathy (HCM) is an inherited heart muscle disease mostly caused by a mutation in sarcomere protein genes. HCM patients suffer from diastolic dysfunction, myocardial remodeling, and heart failure. Nearly half of HCM cases involve mutations in the MYBPC3 gene, encoding the thick filament-associated cardiac myosin-binding protein C (cMyBP-C), an important modulator of cardiac contractility. HCM patient ventricular cardiomyocytes with a heterozygous truncation mutation in MYBPC3 showed strongly varying force generation and calcium sensitivity as well as unequal cMyBP-C expression. Since truncated cMyBP-C fragments are undetectable, it is assumed that they undergo degradation, resulting in haploinsufficiency. We hypothesize that variable expression of cMyBP-C protein from cell-to-cell causes contractile dysfunction and HCM progression. For disease modeling, we used genetically modified human induced pluripotent stem cells (hiPSC) derived cardiomyocytes with a heterozygous, truncating mutation within the N-terminus of cMyBP-C. Cultivated up to 85 days on different surfaces mutant cardiomyocytes revealed disrupted morphology, decreased cMyBP-C levels, enhanced mosaic pattern, and altered contractility. Notably, during short-term culture, mutant cardiomyocytes demonstrated faster contractility and calcium transients, however, slower contractility and reduced calcium transients were observed in long-term culture. Despite continued cMyBP-C protein synthesis from the intact wild-type allele in the heterozygous mutant cardiomyocytes, overall quantity of cMyBP-C might be insufficient to sustain normal contractility. We hypothesize that with reduced cMyBP-C levels, contractility is probably preserved initially, but prolonged deficiency leads to gradual deceleration. We are currently examining if potential changes in Ca2+ sensitivity are linked to the observed slower calcium transients. Future perspectives involve the examination of these mechanisms and studies of potential therapeutic approaches.

Keywords: hypertrophic cardiomyopathy (HCM), cMyBP-C, stem cells, cardiomyocytes, long-term culture, contractility, calcium handling

6:45 PM – 7:45 PM

300

HIGH-THROUGHPUT SCREENING OF 3D HIPSC CARDIAC MICROTISSUES WITH INHERITED ARRHYTHMIA DISORDERS TO IDENTIFY NOVEL THERAPEUTIC COMPOUNDS

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Current therapeutic approaches often involve applying the same treatment to all patients, yet this is often ineffective as only some patients respond positively. This is particularly true for conditions like catecholaminergic polymorphic ventricular tachycardia (CPVT), where the standard treatment of beta-blockers alone or in combination with flecainide, is effective for some patients but fails for others. Recent FDA regulatory changes now permit the use of in vitro hiPSC-derived disease models to identify potential therapeutic compounds, underscoring the importance of developing reliable, high-throughput hiPSC models. However, hiPSC-derived cardiomyocytes (CMs) alone often lack the maturity and intricacy required to act as accurate models of cardiac disease. To overcome this, 3D cardiac models have been developed, and we have shown previously that combining CMs, endothelial cells, and fibroblasts into tri-cellular 3D cardiac microtissues (MTs) enhances CM maturity. Here, we have generated a CPVT MT model in which we can trigger the arrhythmic phenotype, and monitor the acute rescue of these arrhythmias. We have also automated this process for high-throughput screening, showing that CPVT MTs can be generated and maintained in 384-well plates with minimal variation. Endpoint acute flecainide treatment effectively halted arrhythmias in ~97% of arrhythmogenic CPVT MTs. Moreover, we have developed a custom application for analysing these results. Overall, our results demonstrate that using robotics we can streamline the production of high-quality MTs, allowing for the rapid evaluation of therapeutic effects on an arrhythmic phenotype in a high-throughput manner. This confirms the potential of 3D cardiac MTs for modelling cardiac disease and facilitating high-throughput drug screening.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine reNEW through a Novo Nordisk Foundation grant (NNF21CC0073729)

Keywords: high-throughput drug screening, arrhythmic disease modeling, cardiac microtissues

6:45 PM – 7:45 PM

302

THE SECRETOME OF CARDIAC MESENCHYMAL STROMAL CELLS PROTECTS HUMAN CARDIOMYOCYTES FROM HYPOXIA/ REOXYGENATION INJURY

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Myocardial infarction remains one of the main causes of death worldwide, despite undeniable progress in reperfusion and revascularisation strategies. Unfortunately, they do not reverse the molecular damage that has already occurred. Preventing cardiomyocyte loss is an important therapeutic target, as infarct size predicts the risk of heart failure and one-year mortality. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) are gaining wide acceptance as a platform for cardiac disease modelling; however, their functional immaturity is considered a major limitation. Here, we test paracrine protection mediated by cardiac mesenchymal stromal cells (cMSC) in a novel in vitro model of ischaemia-reperfusion injury. Metabolically mature hiPSC-CMs produced according to a recent protocol by Feyen et al. were compared to those produced with standard protocols to develop the injury model. Time-course experiments of hypoxia/reoxygenation were carried out to establish conditions that cause 50% cell death. Subsequently, the protective effect of medium conditioned by cMSCs was tested. Finally, a novel proximity biotinylation strategy using TurboID was implemented to carry out mass spectrometry profiling of the cMSC secretome to characterise protective mediators. We noted a significant increase in cell death of mature hiPSC-CMs in response to hypoxia (0.1% [O2]) in combination with nutrient deprivation, when compared to hiPSC-CMs obtained with the standard differentiation protocol. Addition of reoxygenation following hypoxia resulted in nearly 2-fold increase in cell death, compared to hypoxia alone. Notably, medium conditioned by cMSCs added at the time of reoxygenation significantly reduced cell death, while also preserving mitochondrial membrane potential. Proteomics of the cMSC secretome allowed to identify 44 putative mediators of the observed effect. Altogether, a novel in vitro model of myocardial ischaemia-reperfusion injury was developed using metabolically mature hiPSC-CMs and hypoxia-reoxygenation. The translational potential of the model in the development of cardioprotective therapies is confirmed by testing cMSC secretome. Using proximity biotinylation and mass spectrometry, potential mediators of the protective effect were identified for further validation.

Keywords: human iPS-derived cardiomyocytes, ischaemia/reperfusion injury, paracrine protection

6:45 PM – 7:45 PM

304

ARRHYTHMOGENIC CARDIOMYOPATHY: MATURE IPSCS-DERIVED CARDIOMYOCYTES SHOWED DISRUPTED INTERCALATED DISCS IN ABSENCE OF PKP2 OR PKG DESMOSOMAL PROTEINS

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Arrhythmogenic cardiomyopathy (ACM) is a genetic heart muscle disease in which the ventricular myocardium is progressively substituted by fibro-adipose tissue. ACM is associated with severe ventricular arrhythmias, sudden death, and progressive heart failure. ACM is principally associated with genetic mutations of desmosome proteins, including among others, Plakoglobin (PKG) and Plakophilin-2 (PKP2). Desmosomes are multiprotein structures localized within the intercalated discs (IDs) that allow mechanical coupling of adjacent cardiomyocytes (CMs). Desmosomes form connections with other ID components, such as ion channels, gap junctions and adherens junctions. The objective of this work was to determine the functional importance of PKP2 and PKG in iPSCs-derived CMs in order to elucidate cellular and molecular causes of the ACM in the future. To achieve this, we differentiated two CRISPR/Cas9 edited human iPSCs lines (PKP2-Knock out and PKG-Knock out) in addition to a wild-type (WT) control, into CMs. We carried out a monolayer differentiation protocol followed by a maturation protocol to obtain CMs with a structure more similar to the adult cardiac tissue. To determine the effect of PKP2 or PKG absence in these cells, we performed immunofluorescence staining of structural cardiac proteins (cTNT, MYL2, CX43, PKP2, PKG and DES) and we evaluated F-actin filaments alignment (a key property of mature CMs) using phalloidin staining. Differences in MYL2 and CX43 organization were observed in both PKP2-KO and PKG-KO mature CMs with respect to WT mature CMs. Quantitative bioinformatic analysis of the alignment degree of F-actin cytoskeleton revealed a more anisotropic (not-aligned) pattern in the PKP2-KO and PKG-KO CMs with respect to the WT CMs, obtaining an order parameter value of 0.37, 0.55 and 0.67, respectively (an order parameter near 1 indicates an isotropic pattern). In addition to these experiments, transmission electron microscopy of IDs and functional analysis will be performed. In conclusion, both PKP2 and PKG absence triggers structural alterations in the CMs, observing even less cytoskeletal organization in PKP2-KO CMs with respect to PKG-KO CMs.

Keywords: mature cardiomyocytes, cardiac desmosomes, arrhythmogenic cardiomyopathy

6:45 PM – 7:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

306

SURFACE CONJUGATION OF MICROSPHERES CARRYING RAPAMYCIN ON MESENCHYMAL STEM CELLS EXERTS IMPROVED ANTI-FIBROTIC EFFECTS AGAINST PULMONARY FIBROSIS

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Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease with a poor prognosis characterized by progressive pulmonary fibrosis. Current treatments for pulmonary fibrosis have been reported to slightly delay the progression of fibrosis, therefore, promising drugs that can significantly reduce or reverse fibrosis should be developed. Mesenchymal stem cells (MSCs) have been reported to have an anti-fibrotic effect by producing various paracrine factors, and MSCs therapy could be an alternative treatment. Rapamycin has been demonstrated to have anti-fibrotic activity in animal models of lung fibrosis by regulating cell growth and the fibrosis progression through the inhibition of the mTOR signaling pathway. In this study, we aimed to investigate the anti-fibrotic efficacy of MSCs with rapamycin using surface microsphere-conjugating techniques. Lung fibroblasts were co-cultured with MSCs using trans-well in the presence or absence of rapamycin, and the expression of markers related with myofibroblast differentiation in fibroblasts were determined by western blotting. Production of anti-fibrotic paracrine factors from MSCs were analyzed by ELISA. In addition, rapamycin microspheres were conjugated to the surface of MSCs using polydopamine coating, then added to fibroblasts treated with TGF-β1, and the markers of myofibroblast differentiation were measured. We found that MSCs reduced TGF-β1-induced fibrosis of lung fibroblasts, determined by the expression of α-SMA, collagen and fibronectin. Furthermore, we found that rapamycin elevated the production of MSC-derived anti-fibrotic factors such as HGF and PGE2. And rapamycin-mediated enhancement of anti-fibrotic abilities of MSCs was dependent on PGE2 production. Co-treatment of rapamycin with MSCs reduced fibrosis in vitro. In addition, MSCs conjugated with rapamycin-loaded microspheres exhibited higher inhibitory effect against fibrosis compared to

sole treatment of MSCs or rapamycin. These results suggest that MSCs conjugated with rapamycin-loaded microspheres on their surface might exert improved therapeutic efficacy against pulmonary fibrosis.

Funding Source: Bio & Medical Technology Development Program of National Research Foundation (RS-2023-00223591) and Korean Fund for Regenerative Medicine (KFRM, 23A0205L1), funded by Korean government.

Keywords: idiopathic pulmonary fibrosis, mesenchymal stem cell, microsphere, rapamycin

6:45 PM – 7:45 PM

308

PATIENT-DERIVED ALVEOLAR ORGANOIDS TO MODEL STAT3-HYPER IGE SYNDROME AND GENE EDITING THERAPY

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The transcription factor STAT3 is involved in many different physiological processes from embryogenesis to inflammation control. Consequently, heterozygous dominant-negative STAT3 mutations cause a multisystem disease, known as STAT3-Hyper IgE Syndrome (STAT3-HIES). Without rigorous treatment, recurrent infections due to immunodeficiency and defective wound healing lead to chronic destruction of the alveolar walls. Assessment of human lung tissue of STAT3-HIES patients revealed a reduced number of alveolar type II (AT2) cells. Since AT2 cells play a major role in regeneration as stem cell of the alveolus, we sought to unravel the molecular mechanisms within AT2 cells leading to epithelium destruction to pave the way for regenerative therapies. Fibroblasts isolated from STAT3-HIES patients were reprogrammed into induced pluripotent stem cells (iPSCs) by transient transgenic expression of reprogramming factors. We used CRISPR-Cas9 mediated adenine base editing to repair the STAT3 p.R382W hot-spot mutation in STAT3-HIES patient iPSCs. Patient cells and repaired cells were successfully differentiated into iAT2 organoids, expressing the markers NKX2.1 and Pro-SPC, and displaying Lysotracker-stained lamellar bodies. Further differentiation into AT1-like cells expressing the markers PDPN and AGER was also possible. Stimulation of the JAK/STAT3 pathway with human recombinant LIF in the repaired patient iPSCs showed a significant increase in the expression of the STAT3 target genes CCL2 ($p< 0.0001$) and SOCS3 ($p< 0.005$) comparable to that of healthy controls, but only after gene editing, and not in untreated patient iPSCs. SOCS3 expression was also increased in repaired iAT2 compared to untreated one. To conclude, patient iPSCs-derived alveolar organoids are a suitable human model to study the therapeutic effect of gene repair in a monogenic disease such as STAT3-HIES. Furthermore, our results point toward a substantial role of STAT3 in AT2 cell proliferation and a positive effect of gene repair to rescue AT2 functions.

Funding Source: This project is funded by the Job Research Foundation.

Keywords: iPSCs-derived alveolar organoids, disease modelling, gene repair

6:45 PM – 7:45 PM

310

TGF-BETA2-MEDIATE FERROUS ION IMBALANCE DRIVES FERROPTOTIC CELL DEATH OF SALIVARY GLAND ACINUS IN ESTROGEN-DEFICIENT CONDITIONS

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Xerostomia, also known as dry mouth, is a salivary gland (SG) dysfunction characterized by reduced saliva secretion or changes in saliva composition. The prevalence of xerostomia is particularly high among elderly women, indicating a correlation between SG function and estrogen. To investigate the pathogenesis of menopause-related xerostomia, we investigated changes in SG function using ovariectomized (OVX) mice. After surgery, we observed lower estrogen levels, reduced salivation, dilated ducts, and reduced acinar rates. Microarray analysis revealed that genes related to the TGF-β signaling pathway, particularly TGF-β2, and ferroptosis drivers were increased upon OVX. To assess the impact of TGF-β2, we treated the SG-derived organoids (SGOs) with TGF-β2. Interestingly, a dose-dependent decrease in organoid number and proliferation was observed. Furthermore, TGF-β2 treatment increased ferroptosis marker Tfr1 expression and lipid peroxidation, while co-treatment with SB431542, a TGF-β signaling inhibitor, prevented this phenomenon. Finally, treatment of ferroptosis inhibitor restored saliva secretion and reduced ferroptosis marker expression in OVX mice. These findings suggest that TGF-β2 induces SG dysfunction by promoting ferroptosis-induced oxidative stress and cell death.

Funding Source: Bio & Medical Technology Development Program of National Research Foundation (RS-2023-00223591) and Korean Fund for Regenerative Medicine (KFRM, 22A0205L1-11), funded by Korean government

Keywords: xerostomia, salivary gland organoid, ferroptosis

6:45 PM – 7:45 PM

312

COMPARATIVE SINGLE-CELL RNA-SEQ ANALYSIS OF AIR-LIQUID INTERFACE CULTURE IN ADULT TISSUE-DERIVED HUMAN LUNG ORGANOIDS REVEALS KEY REGULATORS OF EPITHELIAL DIFFERENTIATION

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Ciliated cells, descendants of club cells or goblet cells, play a pivotal role in respiratory health and susceptibility to viral infections. The air–liquid interface (ALI) culture method is advantageous for promoting differentiation of ciliated cells, which enables in vitro studies on human respiratory diseases having potential to replace animal experiments. Although clarifying the factors involved in the cell differentiation is important for the development of in vitro respiratory models, studies on differentiating human lung cells into airway epithelium containing ciliated cells have been limited. Here, we comprehensively analyzed differentiation effects of the ALI culture method on adult tissue-derived human lung organoids and examined the similarity with in vivo human airway epithelium compared to the submerged cultures using single-cell RNA sequencing. We observed a pronounced shift in the composition of cell types in the ALI model, marked by a notable increase in ciliated cells and a decrease in proliferative basal cell subtypes. The cell type composition and transcriptional profile of the ALI model most closely resembles that of in vivo human lung parenchymal airway epithelium. Numerous genes and signaling pathways were altered in the ALI model. Through the analysis of gene regulatory networks in basal and club cells, we identified key regulatory signaling pathways, including the downregulation of HIF1A-VEGFA associated with oxygenation and the upregulation of TP53-CDKN1A associated with reactive oxygen species. Furthermore, we suggest chemicals that have the potential to replace the differentiation effect of the ALI culture method by modulating the activity of key regulators. Our study highlights the efficacy of the ALI culture method in replicating human lung parenchymal airway epithelium, providing valuable insights for investigating crucial factors in human ciliated cell differentiation.

Funding Source: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant number: 2021K1A4A7A0209775712)

Keywords: airway epithelium, air-liquid interface culture, single-cell RNA-seq

6:45 PM – 7:45 PM

314

GENERATION OF A LUNG CANCER ORGANOID MODEL USING C-MET MUTATED INDUCED PLURIPOTENT STEM CELLS (IPSC)

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Lung cancer is the most common cancer worldwide. In a minority of lung cancers, a driving molecular event allows the development of targeted therapies. The tyrosine kinase receptor c-Met can be involved in 5-20% of non-small cell lung cancer (NSCLC) either by mutation or amplification. We report here the development of a lung organoid model from an iPSC line expressing an oncogenic c-Met mutation. The c-Met-mutated iPSC line has been cultured and maintained in E8 media. To induce differentiation toward pulmonary lineage, we have first generated lung progenitor cells (LPC) using 18-day culture

protocol including endoderm induction using Activin A (100 ng/ml) and CHIR9901 (5 µM) (days 1-4) followed by anterior foregut induction (days 4 - 7) until the generation of LPC stage at day+18 with cells expressing NKX2-1. In this stage, control and c-Met-mutated cells expressed characteristic LPC markers (NKX2-1, FOXJ1) and could be maintained in culture for > 50 days. Day+18 LPCs were then used to generate mature lung cells using air-liquid interface cultures using Matrigel and 3D organoid induction medium. At day +38, 3-D structures reminiscent of alveolar structures were detected in both control and c-Met-mutated LPCs. Interestingly, the maturation marker surfactant protein C (expressed alveolar type II epithelial cells) was highly expressed and abnormally clustered in c-Met mature lung organoids as compared to controls. c-Met-mutated-iPSC derived lung organoids have also been found to exhibit high expression of CD31+ endothelial cells as compared to controls. Immunostaining results showed that the expression of NSCLC markers (TP63, MUC1, PD-L1, KRT5/6, CD73, C1QTNF6) was significantly higher in c-Met mature lung organoids as compared control organoids. To confirm the malignancy potency, LPCs were subcutaneously transplanted in NOD/SCID mice. c-Met -mutated LPCs gave rise macroscopic tumor-like structures revealed by histology performed 16–20 weeks later. Transcriptome and single cell RNA sequencing experiments are currently in progress. Thus, these data demonstrate for the first time to our knowledge, the development of an off-the shelf lung cancer model from c-Met mutated iPSC-derived lung organoids with the potential to be used as a preclinical model for discovering novel targets.

Funding Source: INGESTEM, INSERM, ANR

Keywords: lung organoid, IPSC, cancer

6:45 PM – 7:45 PM

316

ESTABLISHING HUMAN RESPIRATORY ORGANOID CULTURE SYSTEM FOR MODELING AND DISSECTING VIRAL INFECTIONS

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We have established the first human respiratory organoid culture system. Organoids are derived from adult stem cells in primary lung tissues with high efficiency and can be stably expanded over half a year. We have developed differentiation protocols and generated airway organoids and alveolar organoids that faithfully simulate the native airway and alveolar epithelium, respectively. Apart from a noninvasive procedure to procure readily accessible nasal cells for organoid derivation, nasal organoids more adequately simulate the upper airway epithelium than airway organoids. Thus, the respiratory organoid culture system allows us to rebuild and propagate the entire human respiratory epithelium in culture plates with excellent efficiency and stability. The high transmissibility of SARS-CoV-2 Omicron subvariants was generally ascribed to immune escape. We demonstrated that BA.5 exhibited a dramatically increased replicative capacity and infectivity than B.1.1.529 and an ancestral strain WT in human nasal and airway organoids. Notably, we observed prominent syncytium formation in BA.5-infected nasal and airway organoids. Collectively, the higher entry efficiency and fusogenic activity of BA.5 spike potentiated viral spread through syncytium formation in the human airway epithelium, leading to enhanced replicative fitness and immune evasion. The lack of a robust system to reproducibly propagate HRV-C, a family of viruses refractory to cultivation in standard cell lines, has substantially hindered our understanding of this common respiratory pathogen. we developed an organoid-based system to reproducibly propagate the poorly cultivable HRV-C, which allowed us to elucidate HRV-C infection and innate immunity in an unprecedented manner. More importantly, our study has opened an avenue for propagating and studying other uncultivable human and animal viruses.

Keywords: epithelial organoids, modeling viral infections, uncultivable viruses

6:45 PM – 7:45 PM

318

INTERSPECIES HUMAN-MOUSE NEURAL CREST CHIMERAS TO STUDY HUMAN MELANOMA

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Limited persistence of xenogenic cells in human-mouse interspecies chimeras is an essential obstacle to overcome for the creation of disease models which allow the functional consequences of disease-associated mutations to be studied in the most relevant context of in vivo human cells within their appropriate niche. This work presents a novel approach to efficiently increase interspecies chimerism in human-mouse embryo chimeras by combining proliferative advantage and pre-lineage biasing to establish the first evidence that genetically-defined human melanoma has the capacity to robustly generate post-natal human-mouse chimeras when injected into the gastrulating embryo. In short, we injected primary human melanocytes carrying four targeted mutations known to drive melanoma (CDK2NA-null, BRAFV600E, TERT-124 C>T, APC-null; 'CBTA' melanocytes reported in Hodis et al. 2022) into E8.5 gastrulating IGS-CD1 immune-competent mouse embryos to determine whether they gain an neural crest-like phenotype and have the capacity to contribute to development. Indeed, CBTA melanocytes have the impressive capacity to invade the mouse embryo and generate pigmented melanocytes on the heads of post-natal animals similar to neural crest chimeras generated with primary mouse neural crest cells. CBTA melanocytes localize to the lower to middle dermis of chimera skin but not to the hair follicle, and persist for upwards of 4 months despite residing in an immune-competent host. The capacity for genetically defined melanoma to persist in immune-competent chimeric animals opens new possibilities for in vivo modeling of human melanoma.

Keywords: melanoma, interspecies chimera, neural crest

6:45 PM – 7:45 PM

320

HAIR-BEARING SKIN ORGANOIDS DERIVED FROM HUMAN IPSC FOR MODELLING MPOX VIRUS INFECTION AND DRUG TREATMENT

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In 2022, widespread Mpox outbreaks affected non-endemic areas, spreading to over 100 countries, with most of the cases in Europe and the Americas. The Mpox virus (MPXV) primarily infects human skin, causing lesions that were globally reported to be more severe in terms of pain and body distribution and frequently associated with secondary bacterial infections during the 2022 outbreak. Therefore, there is an urgent need for a better understanding of the pathophysiology of MPXV infection in the skin and the rapid development of therapeutics. However, complex models that recapitulate skin infection by MPXV are currently lacking. Here, we took advantage of the hair-bearing skin organoids derived from hiPSC in their original inverted version and upon maturation in an air-liquid interface culture system, to study MPXV infection in conditions that closely resemble human skin physiology. Our findings demonstrated that hiPSC-derived skin organoids are susceptible to MPXV infection and support infectious virus production. Using transmission electron microscopy, we visualized the four stages of intracellular virus particle assembly: crescent formation, immature virions, mature virions and wrapped virions. Transcriptional analysis revealed that MPXV infection rewires the host transcriptome and triggers abundant expression of viral transcripts. Early treatment with the antiviral drug tecovirimat effectively inhibits infectious virus production and prevents host transcriptome rewiring. Delayed treatment with tecovirimat also inhibits infectious MPXV particle production, albeit to a lesser extent. Overall, our study establishes human skin organoids as a robust experimental model for studying MPXV infection, mapping virus–host interactions and testing therapeutics.

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Keywords: hiPSC, skin organoids, viral infection

6:45 PM – 7:45 PM

322

MODELING GENETIC INNER EAR HEARING LOSS: DEVELOPMENT OF HIPSC-DERIVED INNER EAR ORGANOIDS HARBORING GJB2 MUTATIONS

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Hearing loss is the foremost common sensory disorder globally, impacting approximately 5% of the population. The current treatment options are limited to hearing aids or cochlear implants, which mitigate symptoms but are not a permanent solution. In the context of genetic hearing loss, gene therapy could be the ultimate solution. In Europe, the most common mutations occur in the GJB2 gene, which encodes Connexin 26 (Cx26), a component of gap junctions, which facilitate ion transport between cells. The exchange of ions is an essential process for hearing as it functions as a message between sound detection by the hair cells and transmission of this message to the brain. Disease-causing mutations in GJB2 lead to aberrant or non-functional protein, probably leading to improper exchange of ions between cells and thereby reduced hearing. The only available human model for inner ear research is the inner ear organoid model, generated from human induced pluripotent stem cells (hiPSCs). This model recapitulates the diverse cell types found within the inner ear, providing insights into hearing-related mechanisms. We are employing this model system to study how GJB2 mutations result in hearing loss. To select the most relevant GJB2 mutations, we performed a literature search to identify mutations associated with hearing disorders. Multiple sequence alignment and AlphaFold 2 predictions were performed to assess mutations at conserved protein sequence positions. Two selected mutations, c.35delG and c.269T>C/p.L90P, commonly found in European patients, induce a shortened Cx26 protein and a missense mutation with a structural change, respectively. Guide RNAs for these mutations are undergoing efficiency testing. We outline selection criteria for mutations that can be studied in hiPSC-derived inner ear organoid models. This will facilitate the study of GJB2 mutations on essential hearing cells, enhancing understanding of the disease and guiding future therapy development.

Funding Source: This research is funded by the Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), which is supported by a Novo Nordisk Foundation grant, number NNF21CC0073729.

Keywords: disease modeling, inner ear organoids, gene editing

6:45 PM – 7:45 PM

324

EXPLORATION OF THE EFFECT OF RETINOIC ACID ON THE DIFFERENTIATION POTENTIAL OF THE SALIVARY GLAND ORGANOIDS

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The salivary glands play a crucial role in upholding oral health by facilitating digestion and maintaining oral hygiene through saliva production. Despite their significance, the limited regenerative capacity of salivary glands presents a substantial challenge in addressing dysfunction. Tissue-specific organoids have emerged as a powerful tool for exploring organogenesis, tissue regeneration, and disease modeling. Retinoic acid, a derivative of vitamin A renowned for its pivotal role in embryonic development, is systematically tested to discern its impact on the differentiation of salivary gland organoids. This study aims to delve into the differentiation potential of salivary gland organoids, specifically focusing on the regulatory role of retinoic acid in their development. To establish the foundation of salivary gland organoids, we isolate and culture tissue-specific stem cells within a three-dimensional environment. The organoids undergo thorough verification to ensure their ability for self-organization and the manifestation of morphological and functional similarities to native salivary glands. Our investigation explores the effects of retinoic acid treatment on the differentiation and functional specialization of salivary gland organoids. The factors influencing this process are meticulously evaluated to ascertain their role in the formation of acinar and ductal structures within the organoids. Additionally, we examine the functional aspects of salivary gland organoids affected by retinoic acid, with a focus on synthesis of saliva-related proteins. Furthermore, the molecular mechanisms underlying retinoic acid-mediated differentiation in salivary gland organoids were investigated, including comprehensive analyses of gene expression to unveil the effects mediated by retinoic acid on cell fate determination, proliferation, and morphogenesis. These findings promise to yield valuable insights into the regulatory mechanisms governing the development of salivary gland organoids, emphasizing the potential of retinoic acid in guiding their differentiation and maturation. The results obtained may pave the way for innovative regenerative therapies aimed at addressing dysfunctional salivary glands.

Keywords: salivary glands organoid, retinoic acid, differentiation

6:45 PM – 7:45 PM

326

DEVELOPMENT OF A CHEMICALLY DEFINED, XENO-FREE PLATFORM TO GENERATE TYPE 2 ALVEOLAR EPITHELIAL CELLS FROM HUMAN PLURIPOTENT STEM CELLS

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Type 2 alveolar epithelial cells (AECs) of the distal lung are responsible for the synthesis and secretion of pulmonary surfactant to prevent alveolar collapse during respiration. They also serve as the progenitor cells of the lung alveoli, capable of self-renewing and differentiating into type 1 AECs to maintain alveolar integrity during homeostasis and in response to lung injury. However, repeated damage to type 2 AECs disrupts alveolar epithelial regeneration and can drive diseases of the lung such as idiopathic pulmonary fibrosis (IPF). Recent advances in directed differentiation of human pluripotent stem cells (hPSCs) to type 2 AECs has afforded further investigation into the mechanisms of human alveolar development and disease. However, current differentiation protocols rely on animal-derived components in culture media and poorly defined basement membrane matrices like Matrigel®. This can introduce undesired biological variability during differentiation, limit the faithfulness of respiratory models of disease and hinder translation of derived type 2 AECs to the clinic. To address these limitations, we have developed a novel, xeno-free platform to generate type 2 AECs from human induced PSCs (hiPSCs). We direct the differentiation of hiPSCs to definitive endoderm followed by lung epithelial progenitor cells expressing NKX2.1 in chemically defined medium on human recombinant laminin, and isolate cells using expression of the cell surface marker carboxypeptidase M (CPM). By aggregating isolated lung epithelial progenitors and culturing in suspension with a 2% extracellular matrix (ECM) mix comprising human recombinant laminin-111 and a self-assembling peptide hydrogel, we show the formation of spheroids comprising a near homogenous population of self-renewing type 2 AECs that express transcripts including SFTPC, SFTPA and NKX2.1, as identified by RT-qPCR and bulk-RNA sequencing, and can be isolated via the sodium-dependent phosphate transporter 2B (NAPI2B). We demonstrate the application of our platform to model interstitial respiratory fibrosis caused by a mutation in SFTPC, and importantly for the efficient and reproducible production of clinically compatible type 2 AECs.

Funding Source: Asthma and Lung UK The Masonic Charitable Foundation NC3Rs UKRI

Keywords: type 2 alveolar epithelial cells, xeno-free, pluripotent stem cell differentiation

6:45 PM – 7:45 PM

328

ADVANCED SKIN ORGANOID MODELS: COMBINATION OF HIPSC VASCULAR ORGANOIDS AND SKIN ORGANOIDS FOR INFECTION & DISEASE RESEARCH

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Human skin, a complex organ comprising the epidermis, dermis and hypodermis, exhibits a sophisticated arrangement of different cell types and structures. Reproducing the complex vascular architecture of human skin in vitro remains a major challenge in tissue engineering and regenerative medicine. Skin organoids (SO) based on human induced pluripotent stem cells (hiPSCs) mimicking the skin are remarkable in their ability to recapitulate the intricate architecture of the human skin, including skin layers and the formation of hair-like structures. The organoids have been utilized to study infectious diseases such as human sleeping sickness, caused by the African parasite Trypanosoma brucei, and to model cancer, providing a comprehensive simulation of complex skin tissues. In a unique experiment, skin organoids were exposed to tsetse flies infected with Trypanosoma brucei on day 140. We observed selective infiltration of the parasite, which expressed the fluorophore tdTomato, into the dermal layer of the organoids, while the epidermal layer remained largely unaffected. The skin layers were identified using markers for different cell types: keratinocytes (CK5), dermal cells (vimentin), adipocytes (Nile red) and complex hair peg formation (indicated by the expression of CK5 and CK17). Seven days after infection, the parasites had spread throughout the organoid and accumulated in specific areas of the skin layer. In parallel, the development of a vascularized, perfusable hydrogel using a scaffold of PcycloPrOx by Melt Electro Writing and a hydrogel composed of GelMA and ColMA in a bioreactor is realized. Vascularization will be realised with hiPSC derived vascular organoids. We identified a vascular network (VE-Cadherin), with endothelial cells (CD31), pericytes (NO-GC, PDGFRß) and smooth muscle cells (SMMHC). This complex skin model will be used to further investigate the mechanism of invasion of the parasite into the human circulation as well as other pathologies. In conclusion these sophisticated skin organoids not only provide insights into basic skin biology, but also hold great promise for drug testing, personalized medicine and the development of novel therapeutic interventions for skin-related diseases.

Keywords: skin organoid, vasculature, infection

6:45 PM – 7:45 PM

330

REPRODUCING INFLAMMATION AND FIBROSIS USING A HUMAN INDUCED PULIPOTENT STEM CELL-DERIVED AIRWAY TISSUE SYSTEM

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Inflammation and fibrosis in the respiratory tracts are crucial pathophysiological processes accompanying respiratory diseases that often determine clinical outcomes. It is thus essential to develop airway models that can replicate these processes to fully recapitulate in vivo disease conditions to study the pathogenic mechanisms and employ such models for pharmaceutical research. However, it is difficult to reproduce inflammation and fibrosis using conventional airway models, including airway organoids. In addition to airway epithelial cells, the human airway contains immune cells and fibroblasts, each play critical roles in inflammation and fibrosis. In this study, after simultaneously differentiating definitive endoderm and mesoderm cells from human iPS cells, we developed a human airway tissue system that includes not only airway epithelial cells but also immune cells and fibroblasts. Through scRNA-seq and histological analyses, we confirmed the cell composition of the human airway tissue system to be similar to that of human airway tissues. To investigate whether it is possible to reproduce the inflammation and fibrosis caused by viral infections, we

conducted experiments using a variety of viruses with different pathogenicity. Inflammation and fibrosis, consistent with clinical findings, were induced by SARS-CoV, MERS-CoV and highly pathogenic SARS-CoV-2 mutants, but not HCoV-229E, HCoV-OC43, and less pathogenic SARS-CoV-2 mutants. We hope that our human airway tissue system will contribute to the understanding of inflammation and fibrosis in the airway and to drug discovery.

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Keywords: human iPS cells, SARS-CoV-2, inflammation

6:45 PM – 7:45 PM

332

AN INTESTINAL ORGANOID STUDY ABOUT WHAT BROUGHT BOWEL PROBLEMS IN MULTIPLE SCLEROSIS MODEL

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Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation in central nervous system (CNS). Almost half of MS patients suffer by gastro-intestinal problems, as proper gut function relies on CNS feedback. While not life-threatening, these side effects can cause discomfort and embarrassment for patients. Our research focuses on understanding the mechanisms and effects of MS progression on the intestine. We used the murine Experimental Autoimmune Encephalomyelitis (EAE) model to mimic the MS conditions in vivo. We evaluated intestinal inflammation through histological features and cell composition and confirmed the presence of changes in the EAE model. Later, we developed intestinal organoids from mice with MS-like symptoms to point out the underlying mechanisms of action. This allows us to understand the development of the disease through the analysis of the stem cell population, its surrounding environment, and the associated pathways for proliferation and differentiation. However, we debated whether the changes caused by the condition were specific to MS or a general pattern resulting from inflammatory processes. Hence, we tested the intestinal organoids in a nonspecific inflammatory environment to identify common pathways. This study presents opportunities for deeper exploration into the interactions between the intestine and multiple sclerosis, potentially leading to new treatment options for managing gastrointestinal issues in MS patients.

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Keywords: intestinal organoids, multiple sclerosis model, gastro-intestinal inflammation

6:45 PM – 7:45 PM

334

IN VITRO MODELS OF HUMAN UROTHELIUM FOR DISEASE MODELING AND BLADDER RECONSTRUCTION

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Urinary tract infections (UTIs) and Bladder Pain Syndrome (BPS) are common conditions affecting the bladder urothelium. The key to improving these conditions lies in understanding the cellular and molecular mechanisms maintaining homeostasis, as well as the mechanical dynamics of the urinary cycle. However, in vitro models fully recapitulating the complexity of the native human urothelium are missing. We have developed different urothelium models starting from commercially available cell lines, and deriving human primary cells. Our stratified models have a multilayered architecture and express markers of stem cells and progenitors, intermediate cells and differentiation. The in vitro urothelium has a stem cell basal layer characterized by the expression of keratin 14 (K14) and Integrin alpha 6 (Itga6), several intermediate layers that are p63 positive, and an apical umbrella cell layer expressing keratin 18 (K18), keratin 20 (K20) and ZO-1. Additionally, we have developed an innovative apical-out spheroid model, featuring an internal layer expressing Itga6+/P63+ and an external layer with K20+ umbrella cells. These models can give important insights into understanding homeostasis and disease states, and they may contribute to the development of more effective treatment strategies and interventions aimed at promoting urothelium regeneration.

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Keywords: in vitro models, bladder, urothelium

6:45 PM – 7:45 PM

336

IPSC-BASED AIRWAY EPITHELIAL PLATFORM FOR INVESTIGATING PERSONALIZED RESPONSES TO CFTR MODULATORS

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Developing cell-based in vitro model systems for identifying people with Cystic Fibrosis (PwCF) likely to benefit from modulators represents an important objective for CF "precision" therapies. Several factors complicate such analysis. For example, native regulatory elements or non-CFTR associated polymorphisms can influence CFTR mRNA levels and impact "theratype" in vivo, but are poorly represented by traditional cDNA-based expression cell models. On the other hand, chronic infection/inflammation within CF tissues may epigenetically alter mRNA levels in patient primary airway cells or intestinal organoids; in this regard, removal of the epigenetic marks via iPSC reprogramming may be beneficial. In the current project, we investigated iPSC-derived airway epithelial monolayers as a tool for testing patient-specific modulator response. First we established novel and robust differentiation protocols that generate airway basal stem cells from CF iPSCs. We then further differentiated into airway epithelium [via air liquid interface (ALI) culture]. 42 study subjects were recruited on the basis of CFTR genotype, clinical phenotype, and/or known responses to approved modulators. CFTR in iPSC-derived airway epithelium was tested by short circuit current (Ussing chamber) analysis, mRNA measurements, and western blot. In initial studies, Ussing chamber analysis demonstrate levels of CFTR function [in the absence or presence of elexacaftor/tezacaftor/ivacaftor (ETI)] that are consistent with CFTR genotype. For example, G542X show minimal ion transport activity with or without ETI whereas G551D activity can be rescued with ETI to 70-80% wild type function. Several CF genotypes studied to date have demonstrated ETI rescue of ≥60% wild type levels. The iPSC-based assay therefore appears highly sensitive for detecting ETI-dependent CFTR modulation. This new model will be useful for addressing key questions for CF, including the extent to which CFTR activity in vitro correlates with clinical changes in sweat chloride or lung function among PwCF carrying particular genotype(s). Our data indicate the new model system will provide quantitative insight regarding CFTR function and modulator rescue for rare disease-causing variants.

Funding Source: Supported by National Institutes of Health (R01 HL139876)

Keywords: patient-derived iPSCs, personalized medicine, airway stem cells

6:45 PM – 7:45 PM

338

REAL-TIME CHEMOSENSITIVITY OF CANINE MUSCLE-INVASIVE BLADDER CANCER ORGANOIDS

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Spontaneously occurring muscle-invasive bladder cancer (MIBC) in dogs closely resembles human MIBC in biological behavior and histological and genetic heterogeneity, and therefore, represents an excellent model for comparative oncology research. We recently established a biobank of canine MIBC-derived organoids and previously showed that these organoids accurately recapitulated the histology and molecular phenotypes of the original tumor. A real-time chemosensitivity assay was developed to monitor cell viability in a way that allows for the determination of the onset of cell killing and efficacy estimates and provides a higher signal-to-background ratio compared to traditional assays. We have evaluated the chemosensitivity of three organoid lines for four clinically used MIBC chemotherapies, including gemcitabine (0.1-100 µM), cisplatin (0.1-100 µM), vinblastine (0.01-10 nM), and mitoxantrone (0.1-100 ng/mL), either alone or combined with piroxicam (10 µM), using a real-time MT cell viability assay. Tested concentrations were selected based on the pharmacokinetic studies of the drugs in vivo in canines. We observed substantial differences in the sensitivity of organoid cell lines to the tested chemotherapies. Overall, the organoid cell lines displayed high resistance to cisplatin, vinblastine, and mitoxantrone, with the concentrations used failing to inhibit 50% of organoid viability in all three lines. For gemcitabine, the IC50 was achieved at 60 h with 100 µM in one organoid line, while in the other line, it was achieved with 10 µM at 62 h and 100 µM at 46 h. The combination treatment with piroxicam showed synergism with some drugs. In conclusion, analyzing real-time cell viability can help to determine the onset of cell killing and provide personalized estimates of chemotherapy effectiveness and potency. The preliminary findings in this pilot study emphasize

the importance of conducting further studies on larger numbers of MIBC-derived organoids of canine and human patients to establish the use of real-time chemotherapy testing as a tool for making treatment decisions at the patient's bedside.

Keywords: muscle-invasive bladder cancer, organoids, real-time cell viability

6:45 PM – 7:45 PM

340

ELUCIDATING THE IMPACT OF ASCITES ON HIGH GRADE SEROUS OVARIAN CANCER STEM CELLS THROUGH IN VITRO PATIENT-SPECIFIC MULTI-MODEL PLATFORMS

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High Grade Serous Ovarian Cancer (HGSOC) is the fifth cause of cancer death among women due to late diagnosis and tumor recurrence. Growing evidence relates relapse to the persistence of cancer stem cells (CSC), a model corroborated by the prevalence of peritoneal ascites in OC patients. Indeed, ascites acts as a source of soluble factors, providing a pro-inflammatory and tumor promoting micro-environment that supports the survival of CSC, abundant in ascites, and mediate metastatization. Understanding the downstream effects of CSC exposure to ascitic fluid (AF) would thus reveal the pathogenetic mechanisms of stemness, metastasis, and chemoresistance. We supplemented AF to three culturing systems of HGSOC ascites-derived cells, namely 2D tumor cells, bulk spheroids (BS) and single-cell metastatic OC spheroids (sMOCS) and analyzed its effect on proliferation and stemness. We had already introduced sMOCS as a new model able to capture clonal features at high throughput and resolution to expose inter/intra-patient CSC variability and that relies on supplementation with AF, pointing to soluble factors able to sustain growth from single cells. We found that AF increases proliferation for both 2D cells and spheres. 2D cells and BS cultured with AF could be propagated for higher passages, improving culture conditions. Also, we scored an increased sphere forming efficiency (SFE) in BS in AF, suggesting its ability to sustain the CSC niche. Interestingly, when deriving BS from 2D cells with or without AF, we could not score differences in SFE, showing that AF activates culture-method specific gene programs that support CSC growth only in the 3D condition. Bulk RNAseq analyses on the different in vitro systems highlighted an AF-dependent model-specific regulation of gene expression and allowed to identify CSC-related, AF-dependent genes as upregulated in spheres and de-regulated in ascites. These results shed new light on the effect of AF on different tumor subpopulations, highlighting endophenotypes compatible with metastatization and chemoresistance. While also affording a major improvement in culture conditions for a more streamline and systematic capturing of patient-specific features, these studies offer an internally comparative experimental platform to advance the HGSOC field towards precision oncology.

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Keywords: ascites, cancer stem cells, in vitro models

6:45 PM – 7:45 PM

342

CO-CULTURE OF ALVEOLAR EPITHELIUM AND MACROPHAGES TO MODEL LUNG DISEASE IN VITRO

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There is an urgent need to develop improved therapy of complex lung ailments that lack efficient treatment, such as pulmonary fibrosis. Alveolar organoids and other iPSC-derived alveolar epithelial cell cultures have been used as in vitro models for drug screening of various lung disorders, but most of the established alveolar organoid systems lack an immune component, making it difficult to truly model lung disease in vitro. Macrophages are the most abundant immune cells in the alveolar space, are crucial for alveolar homeostasis, and play a large role in the development of respiratory disease. To improve in vitro lung disease modelling, our work is focused on the establishment of systems for co-culture of macrophages and alveolar epithelium. We have successfully differentiated both iPSCs and patient derived Peripheral Blood Mononuclear Cells (PBMCs) to macrophages expressing alveolar macrophage markers including CD206 and CD169, and these macrophages can be polarized to both the pro-inflammatory M1 type and the anti-inflammatory/pro-fibrotic M2 type. We have optimized the culture conditions for co-culture of macrophages with fibroblast-supported alveolar organoids formed by iPSC-derived alveolar epithelial cells cultured in Matrigel. Using a bleomycin-induced pulmonary fibrosis model we have found that co-culture of alveolar organoids with macrophages inhibit bleomycin-dependent myofibroblast induction and fibrotic Matrigel contraction, and that this phenomenon seems to be affected by the type of co-cultured macrophages. We are currently examining the mechanisms behind these results. This work may lead to better understanding of the processes behind the development of various lung diseases and the potential discovery of novel therapeutic drugs.

Keywords: macrophage, lung, fibrosis

6:45 PM – 7:45 PM

344

ADVANCED IPSC-BASED PLATFORM FOR RAPID GENERATION AND GENETIC ENGINEERING OF HUMAN ORGANOIDS IN DEVELOPMENTAL AND DISEASE RESEARCH

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Induced pluripotent stem cells (iPSCs) represent a revolutionary tool for developmental biology, disease modeling and regenerative medicine, offering unprecedented opportunities for the generation of human organoids. Unlike adult stem cells (ASCs), which exhibit limited differentiation capabilities restricted to the lineage of their tissue origin, iPSCs possess the remarkable ability to differentiate into a wide array of cell lineages. This is achieved through the application of tailored differentiation protocols, facilitating the production of complex organoids that mimic the structure and function of human organs more accurately by incorporating diverse cell types. Here we introduce an advanced iPSC-based platform designed for the efficient and rapid generation of genetically engineered human organoids. This platform is comprised of two core components: a versatile genetic toolkit and a series of specially engineered iPSC lines. The genetic toolkit, named the Toolbox for Rapid Assembly of Integration Templates (TRAIT), integrates the strengths of both Golden Gate and Gateway cloning methodologies. Its modular architecture allows for the swift assembly of intricate genetic constructs, enhancing the platform's versatility and efficiency. The engineered iPSC lines within our platform are optimized for stable, inducible expression of the CRISPR-Cas9 system. These lines also harbor additional genetic modifications that facilitate cell tracking, conditional expression of transgenes or guide RNA (gRNA). A notable feature of our system is its capability to perform multiplex parallel genetic modifications. This makes it exceptionally suited for applications involving gRNA or cDNA libraries, enabling the exploration of gene functions and interactions within the context of human organoid models.

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Keywords: genetic engineering toolkit, CRISPR-Cas, lung organoid

6:45 PM – 7:45 PM

346

MODELING INFLAMMATION AND FIBROSIS IN SALIVARY GLANDS USING SALIVARY GLAND ORGANOIDS FOR ADVANCED STUDY AND DRUG SCREENING

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Inflammation and fibrosis of salivary glands, often triggered by factors such as salivary duct obstruction, infections, or radiotherapy, lead to debilitating symptoms like swelling, pain, and dry mouth, adversely impacting patients' quality of life. Despite the prevalence of these conditions, proper in vitro models for research remain limited. Our goal was to replicate clinical diseases within organoids to enhance our understanding of the involved pathologies for a comprehensive understanding of disease progression and to develop diverse biomarkers enabling screening efficacious therapeutics based on the organoid model.
We employed activin A to induce fibrosis in the salivary glands, while inflammation was induced by applying LPS and TNF. Activin A treatment resulted in reduced expression of pro-acinar cell marker (AQP5), increased expression of mesenchymal cell markers (ACTA2, CDH2, FN1), and induced fibrosis, evident in H&E morphology. Treatment with LPS and TNF prompted acino-ductal metaplasia and elevated pro-inflammatory cytokines, such as TNF, and IL6, and chemokines, such as CCL2, CXCL5, and CXCL12. Additionally, dexamethasone, a widely used glucocorticoid, demonstrated efficacy in mitigating both inflammation and fibrosis, validating the feasibility of these models. Additionally, exposure of organoid culture media to macrophages showed a noticeable shift in macrophage polarity, with inflamed organoid-derived media inducing pro-inflammatory M1 markers (Tnf, Il6, Il1b, Cd86). In contrast, dexamethasone-treated media reduced inflammation and promoted an anti-inflammatory M2 phenotype (Ym1, Il10, Cd163, Klf4). Salivary gland organoids offer a straightforward means to monitor epithelial cell responses to stimuli and reveal immune system modulation, which is a function not observable in 2D cell cultures. The findings suggest that the organoid model exhibits potential as a robust platform for systematically screening novel pharmaceutical agents, as dexamethasone can be used as a positive control in salivary gland fibrosis and inflammation.

Funding Source: This work was supported by the National Research Foundation (NRF) of Korea funded by the Ministry of Science and ICT to J-Y.L. (2020M3A9I4039045).

Keywords: salivary gland, organoid, modeling

6:45 PM – 7:45 PM

348

USING MULTI-REGIONAL GASTRIC ASSEMBLOIDS FOR ANTRAL FOVEOLAR HYPERPLASIA MODELLING

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Stomach-related pathologies, congenital or postnatally acquired, pose health issues in children, and often lack definitive therapeutic solutions. There is an unmet need to model genetic pathological conditions, to understand their molecular causes and propose personalized treatments. The aim is to investigate the differential cellular behaviour of paediatric patients affected by Phosphomannomutase 2 (PMM2) deficiency and autosomal recessive polycystic kidney disease (HIPKD), who exhibit unconventional foveolar antral polyps. In order to accurately recapitulate the specific regional manifestation of the polyps, we developed a protocol to culture paediatric patient-derived epithelial organoids from fundus, body, and antrum biopsies, recreating a continuous mucosal multi-regional assembloid. This innovative approach allowed us to model the paediatric disease of interest from patient derived cell cultures. First, the organoids were characterized for regional markers, and we examined their ability to self-organize into complex tubular-shaped assembloids. In this set up, we observed how the interaction of organoids from different regions increased the maturation state of the cell sub-types, according to their origin. Using HNF4a/PMM2 patient-derived organoids, we generated assembloids replicating the pathology in vitro, showing increase in proliferation at antral level, and the formation of polyp-like structures. A reduced presence of MUC5AC observed in patients, was confirmed in the disease model. Moreover, we also demonstrated reduced functionality of ATP4b, with increased non-glycosylated protein in the mutant assembloid, and reduced acid production on the fundus side. We demonstrated the derivation of multi-regional stomach organoids, and we successfully obtained a gastric mucosal assembloid in vitro. Each assembloid region maintained its identity and function, enhancing differentiation compared to standard patient organoid culture. We investigated the antral foveolar hyperplasia from a molecular viewpoint, recapitulating patient's condition and offering a reliable laboratory model. The assembloid is a useful tool to recapitulate features of the disease and it offers further insights into the disease's molecular mechanism, uncovering new therapeutic targets.

Funding Source: OAK Foundation Award W1095/OCAY-14-191. NIHR Great Ormond Street Hospital Biomedical Research Centre (NIHR GOSH BRC). GOSH Children's Charity. UCL Therapeutic Acceleration Support (TAS) LifeArc Fund Rare Diseases Call (184646).

Keywords: regional gastric organoids, assembloid, disease modelling

6:45 PM – 7:45 PM

350

FULL AUTOMATION OF 3D ORGANOID CULTURE AND ASSAYS WITH THE NEW INSTRUMENT SOLUTION

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Organoids show great promise in disease modeling and drug screening, however, challenges associated with assay complexity, reproducibility have limited their adoption as a primary screening method in drug discovery. We developed a cell culture automation solution CellXpress.ai. The instrument contains automated imager, liquid handler, and incubator, connected by AI- powered software. Here we present results from successful and efficient automation of 3D culture of healthy intestinal organoids or patient-derived colorectal cancer organoids in matrix domes. Organoid culture started with automated seeding cells mix in organoid domes, using liquid handler unit, then organoids were cultured with automated media exchanges every 24hours and monitored by imaging every 48h. After 5-8 days organoids were automatically harvested, collected, and purified from Matrigel by centrifugation, then dispersed by pipetting, mixed with fresh Matrigel and re-pated into 24 well or 96 well plates. Organoids self-organized during culture and developed complex crypt structures of expected phenotype. Machine learning-based image analysis allowed determining organoids number, size (by area), density (by area sum), and complexity (by number of crypts). Machine learning-based protocols allowed to trigger passaging steps based on organoid density and maturity. For endpoint assay (96well or 384well), organoids were treated with compounds, then stained for viability assessment by imaging with CalceinAM/EtHD. We monitored the concentration -dependent effects of the panel of anti-cancer compounds on healthy intestinal organoids (toxicity evaluation), or patient-derived colorectal cancer organoids (drug screening workflow). Image analysis allowed to define the ratio of live/dead cell numbers and determined effective concentrations of compounds. All steps, with exception of external

centrifugation step, were done as fully automated process. The process automation allows to alleviate the bottlenecks that come with labor-intensive manual protocols and enables automation of the entire organoid culture for prolonged complex workflow. Cell culture automation powered by imaging and machine-learning -controlled decision making has a great potential to bring 3D biology of organoids into another level.

Keywords: automation, intestinal organoids, machine learning

6:45 PM – 7:45 PM

352

THE HUMAN IPSC-DERIVED INNER EAR ORGANOID AS A MODEL FOR TOXICITY STUDIES

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Hearing loss and balance disorders can result from inner ear damage after treatment with antitumor drugs or antibiotics. Existing animal and in vitro models, while valuable, lack comprehensive representation of the intricate human inner ear, thereby posing challenges in safety testing and elucidation of pathophysiological mechanisms. This pioneering study introduces the application of the human induced pluripotent stem cell (hiPSC)-derived inner ear organoids (IEO) as an innovative model for ototoxicity screening. These organoids contain cochlear or vestibular hair cells, which are morphologically and physiologically similar to their counterparts in the human inner ear. The primary objective of this study is to validate these IEOs as an effective model for assessing the ototoxic effects of the antitumor drug cisplatin and the antibiotic gentamicin. To this end, IEOs were generated from hiPSCs in three-dimensional culture and, next, 200 µm thick vibratome sections were obtained at day 75 to access the hair-cell-containing inner ear vesicles within the organoids. The ototoxic compounds were applied for 24 hours; cisplatin doses ranged from 0-100 µM and gentamicin doses from 0-1000 µM. Evaluation techniques included immunofluorescence for assessing cell morphology, protein expression, and apoptosis, along with quantitative PCR for analyzing RNA expression of otic markers, apoptotic pathways, and stress-related responses. Preliminary results seem to indicate increased apoptosis within the inner ear vesicle epithelium of the vibratomed IEOs. Both cisplatin and gentamicin treatment appear to impact cellular and nuclear morphology. Ongoing work focusses on the time course of these changes and the ototoxic effects of both compounds on the human IEOs. This study underscores the potential translational impact of our human inner ear organoid model for ototoxicity, and not only contribute to our understanding of these toxicity mechanisms but also pave the way for the development of targeted interventions and personalized therapies in mitigating inner ear damage induced by antitumor drugs and antibiotics.

Funding Source: The Novo Nordisk Foundation for Stem Cell Medicine (reNEW)

Keywords: induced pluripotent stem cells, inner ear organoids, toxicity studies

6:45 PM – 7:45 PM

354

ADVANCING IMMUNOTHERAPIES THROUGH BI-SPECIFIC ANTIBODY REDIRECTED LYSIS ON COLORECTAL 3D TUMOROIDS

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Developing investigational ex-vivo models that accurately replicate intricate interactions between tissues and immune cells is crucial for the development of novel immunotherapies. Conventional co-culture systems are often challenging to establish and limited for capturing complex cellular events. Our study introduces a new method using tumoroids to investigate the interaction between T cells and cancer cells when exposed to T cells bi-specific (TCB) antibodies. We first aimed at assessing the efficacy and toxicity of Cibisatamab on colorectal cancer tumoroids and healthy colorectal organoids. Cibisatamab (CEA-TCB) is a novel bispecific antibody targeting the carcino-embryonic antigen (CEA) receptor on cancer cells and CD3 on T cells1. In a second step, we aimed at demonstrating the relevance of 3D models to better capture heterogeneity of response to TCB therapy, thereby validating our models as tools to screen novel immunotherapies and identify resistances. Our method consists in using high-density microcavity arrays for the generation of tumoroids in high-throughput to which immune cells together with TCBs can be simultaneously or sequentially added. The interactions between cancer cells and immune cells are monitored over multiple days in culture using live-tracker dyes. We utilize live imaging and image analysis techniques to quantify the cytotoxic effects of immune cells in presence of Cibisatamab against CEA-expressing tumor cells versus healthy cells. Moreover, we multiplex our image-based workflow with endpoint analyses of immune cells by flow cytometry and cytokine level measurments allowing the determination of the T cell activation status and cytokine release activity. Our highly standardized workflow allows a precise control on the effector-to-target ratio, demonstrating its pertinence for immunotherapy toxicity and efficacy assessment. Taken together, these results demonstrate the potential of our innovative approach to catalyze the development of improved immunotherapeutic strategies.

Keywords: organoid models, pmmuno-oncology, pre-clinical drug development

6:45 PM – 7:45 PM

356

INTESTINAL ORGANOIDS AS A MODEL FOR SMALL INTESTINAL EPITHELIUM ALTERATIONS IN ALCOHOL USE DISORDER (AUD)

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Excessive alcohol intake is a public health burden with more than 3 million related deaths worldwide. Evidence indicates that alcohol abuse associated gut barrier dysfunction contributes to alcohol-associated liver disease. Although some aspects of this process have been elucidated, the pathophysiology of alcohol-associated gut failure remains poorly understood. In particular, the role of intestinal epithelium, a major component of the gut barrier, and its alterations in subjects with an Alcohol Use Disorder (AUD) are poorly studied. There is need for new models that allow studying the impact of alcohol specifically on the intestinal epithelium under conditions as close as possible to the in vivo situation in AUD patients. Our preliminary data on duodenal epithelium in humans indicate a profoundly disturbed proliferation-differentiation program in AUD patients associated with increased intestinal permeability. In this context, we first designed an Intestinal Organoid (Enteroid) model susceptible to reproduce at least partially the alterations found in AUD. We plan to investigate morphological changes as well as signaling pathways alterations observed in vivo. Finally, the model will be used to establish a cause-effect relationship between alcohol and the epithelium modifications in AUD. To generate Enteroids, crypts were isolated from control and AUD patients' duodenum biopsies by enzymatic digestion and grown in specific culture medium on matrigel. These 3D models grow, differentiate and later develop a crypt and villi architecture mimicking in vivo condition. Compared to control, AUD Enteroids displayed morphological alterations including the absence of well-defined crypt and villi architecture. Using Propidium Iodide staining assay to assess membrane integrity and cell death, we found increased cell death with rising ethanol concentrations. In addition, the gene expression profile of epithelial cells assessed by qPCR was modified in AUD compared to controls. LGR5, a marker of stem cells, was downregulated whereas Muc2, representing Goblet cells, was upregulated. These results are in accordance with the observations made in human duodenal biopsies. Therefore, duodenal biopsy derived Enteroids seem to be a valuable model to investigate alcohol-induced epithelial changes ex vivo.

Funding Source: FNRS (Fonds de la Recherche Scientifique) in Belgium - NIH-NIAAA (National Institute on Alcohol Abuse and Alcoholism) in the US

Keywords: AUD (alcohol use disorder), intestinal organoids, stem cells

6:45 PM – 7:45 PM

358

LGR5 AND ITS LIGANDS ARE REDUCED IN PLACENTAS FROM PREGNANCIES COMPLICATED BY PREECLAMPSIA

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Establishment & maintenance of trophoblast populations are crucial to placentation and a successful pregnancy. Preeclampsia is a serious pregnancy complication resulting from aberrant placentation. Within placenta, a subset of epithelial mononuclear trophoblasts serve as progenitors, giving rise to two main trophoblast lineages: extravillous trophoblasts (EVTs) and multinucleated syncytiotrophoblasts (STBs); via mononuclear cytotrophoblast intermediates. LGR5 regulates progenitor cell function in adult epithelial tissues, however its expression & function in placenta is unknown. We investigated LGR5 in human placenta with and without preeclampsia, in 2D human trophoblast stem cells (hTSCs), and organoids. LGR5 expression was assessed in human placenta using in situ hybridisation and was localised to a subset of mononuclear trophoblasts underlying terminal STBs in placental villous folds. LGR5 mRNA co-localised with MKI67, suggesting specificity to proliferative trophoblasts. Next hTSCs were differentiated to EVTs or STBs, confirmed by transcriptional analysis. LGR5 expression was lost with differentiation ($p< 0.0215$ EVT, $p< 0.0350$ STB). Analysis of publicly available data (Shannon et al., Development 2022) from single cell RNA sequencing of hTSC-derived organoids, supported that LGR5 was unique to subsets of undifferentiated cells, including (proliferative) mononuclear trophoblasts. We next demonstrated LGR5 was significantly reduced in placentas from pregnancies complicated by preeclampsia compared to controls ($p=0.0046$). In the same samples, expression of LGR5 ligands, R-Spondin (RSPO)1, -4 were also reduced ($p=0.0005$, $p=0.0003$ respectively). RSPO3 was unaltered. Transcriptomic analysis suggests RSPOs may have a non-trophoblast source.

In preeclampsia, there is placental hypoxia/inflammation. However, neither hypoxia, (1% O2 vs 8% O2), or inflammation (TNFα or IL6) altered LGR5 expression in mononuclear trophoblast cells. The cause of aberrations in LGR5 expression within preeclamptic placenta remains elusive. This study is the first to demonstrate LGR5 and its ligands are consistently reduced in placentas from pregnancies complicated by preeclampsia. These reductions may contribute to the poor placentation that drives preeclampsia.

Keywords: placenta, LGR5, WNT pathway

6:45 PM – 7:45 PM

360

EXPLORING THE MOLECULAR MECHANISMS UNDERLYING RADIATION-INDUCED THYROID DAMAGE USING AN ORGANOID MODEL

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Head and neck cancer (HNC) represents one of the most diagnosed cancer types. To date, radiotherapy is among the main approaches for the HNC treatment. Radiation of tumors unavoidably results in off-side effects on the surrounding tissues, such as the thyroid, causing hypothyroidism. Previous studies have revealed that radiation can damage thyroid cells directly, impair their mitosis, or induce an auto-immune response. Here, we investigate thyroid stem-like cells' faith following radiation damage by exploiting mouse-derived thyroid organoids. This study shows that irradiation-induced damage of single cells isolated from mice thyroid glands impairs their ability to grow organoids in a dose-dependent manner, compromising their stemness. Furthermore, irradiation of completely-formed thyroid organoids increases cell death and decreases cell stemness, inducing an in vitro differentiation process, as observed by gene expression analysis. This also revealed that, following irradiation, several interferon-related genes mainly involved in the innate immune response to viral infections are activated, such as cytokines, chemokines, and pattern recognition receptors (PRRs). The molecular events observed underline some of the radiation-dysregulated pathways, factors leading to tissue damage and misfunctioning, giving more information about the causes underlying hypothyroidism. Knowing the molecular mechanisms responsible for healthy tissue damage occurring with radiotherapy is fundamental to counteract them and prevent side effects, promoting stem-like cells' activity and improving the patient's post-cancer treatment quality of life.

Keywords: radiotherapy-induced hypothyroidism, stem-like cells, thyroid organoids

6:45 PM – 7:45 PM

362

INSIGHTS INTO AMELOGENESIS: HARNESSING TOOTH ORGANOIDS AS AN INVESTIGATIVE TOOL

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The tooth is a complex, multi-structure organ composed of highly mineralised tissues, enamel, and dentin. Approximately 60-90% of all children and adults experience tooth decay, highlighting the crucial significance of dental health for our overall well-being. Molar Incisor Hypomineralization (MIH) is a dental developmental defect primarily affecting the permanent first molars and incisors in young children. It is marked by a qualitative enamel defect resulting in reduced mineral content, discoloured plaques, and heightened tooth sensitivity. In the process of tooth development, the dental epithelium generates ameloblasts, responsible for enamel production. Following the finalisation of amelogenesis, these cells undergo apoptosis, and the dental epithelial stem cell population experiences significant decline during adulthood, posing challenges for their study. Our research group recently succeeded in generating epithelial tooth organoids (ETO) derived from the epithelial cell rests of Malassez found within the dental follicle of extracted human wisdom teeth. This cutting-edge organoid model closely replicates the composition, function, and structure of the dental epithelium, including its differentiation towards ameloblasts. Therefore, these 3D structures can be used as a starting point to study the aetiology and causes of enamel-related pathologies. In the first approach, we subjected the ETO during ameloblast differentiation towards various factors known to be correlated to MIH, such as the antibiotic amoxicillin and bisphenol A. Our findings show that amoxicillin severely reduced gene expression of ameloblast markers compared to control conditions in a dose-dependent manner. In addition, the ETO served as a dependable in vitro disease model, as consistent effects were generally observed across multiple patients. In the second approach, ETO are developed from MIH-infected teeth to serve as a platform to search for genetic causes, which are presently unclear. This research project strives to enhance the comprehension of the amelogenesis process regarding MIH pathophysiology. Moreover, this knowledge may advance the refinement of this reliable in vitro model for enamel regeneration, which can be used for cell-based dental tissue regeneration strategies in the long term.

Keywords: tooth organoids, amelogenesis, molar incisor hypomineralization

6:45 PM – 7:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

364

UNRAVELLING THE PATHOGENESIS OF MOLAR PREGNANCY: EXPLORING THE ROLE OF NLRP7 IN MODULATION OF BMP4 AND HIPPO PATHWAYS DURING HUMAN EARLY EMBRYOGENESIS

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Familial biparental complete hydatidiform mole (FBCHM) is a gestational trophoblastic disease, characterized by excessive growth of trophoblast cells with no embryo formation. Currently, the sole option for achieving a healthy pregnancy in FBCHM patients is oocyte donation. FBCHM primarily results from mutations in the NLRP7, which is a cytosolic pattern recognition receptor implicated in inflammasome formation in innate immune cells. Yet, the mechanism by which mutations in the maternal-effect gene impair early embryogenesis and lead to molar pregnancy remains elusive. To unravel how FBCHM associated with NLRP7 mutations arises, fibroblasts from a patient with compound heterozygous NLRP7 mutations and a healthy donor were reprogrammed into induced pluripotent stem cells (iPSCs). When exposed to the cocktail of BMP4, A83-01 (inhibitor of Activin/NODAL pathway) and PD173074 (Inhibitor of FGF pathway), HM-patient derived iPSCs exited from pluripotency earlier and exhibited expedited expression of trophoblast markers compared to WT iPSCs, validating the establishment of HM iPSC disease model. Further experiments revealed that inhibition of Activin and FGF signaling (AP cocktail) was adequate to prompt the differentiation of HM iPSCs into trophoblasts, likely due to elevated levels of endogenous BMP4. Moreover, BMP4 pathway is activated earlier through SMAD1/5/8 phosphorylation in HM cells during AP-induced trophoblast differentiation. Further inhibition studies targeting ALK2/ALK3 or directly BMP4 during AP-induced trophoblast differentiation underscored the essential role of the BMP4 pathway in trophoblast commitment. Although comprehensive transcriptomic profiling revealed upregulation of trophoectoderm-related YAP/TEAD target genes in HM cells, inhibiting the interaction between YAP and TEAD4 using TED-347 and verteporfin during trophoblast differentiation decelerated the expression of trophoblast markers, albeit not entirely prevented, suggesting that YAP/Hippo pathway is downstream of BMP4 pathway during trophoblast differentiation. Overall, NLRP7 mutations direct iPSCs to selectively trophoblast differentiation via BMP4 pathway once Activin and FGF pathways are inhibited, suggesting a role for NLRP7 in modulation of signaling pathways during early embryogenesis.

Funding Source: TUBITAK

Keywords: mole pregnancy, BMP4 pathway, NLRP7

6:45 PM – 7:45 PM

366

RECONSTITUTING THE HUMAN FETAL TESTIS TO MODEL GERM CELLS AND THE TESTICULAR SOMATIC NICHE

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Infertility is prevalent in one out of six people of reproductive age worldwide. To understand and model infertility, we and others are aiming to develop culture systems for in vitro gametogenesis (IVG). Research efforts toward male IVG concentrate on germ cells (GCs), but also on the testicular somatic niche (TSN). The TSN is comprised of AMH+ Sertoli cells (SCs), which are in direct contact with the GCs inside the seminiferous cords. These cords are in turn surrounded by NR2F2+ interstitial cells (ICs). Current testis culture systems in mice and humans are still facing challenges regarding either GC maturation or TSN organization. This work developed a 3-dimensional (3D) culture system for second trimester human fetal testis. For that, we investigated whether the number of DDX4+ GCs maintained after culture correlated with the TSN organization in 3D reconstituted human testes (rhTestes). Human fetal testes were digested into single cells and aggregated in ultra-low attachment (ULA) plates to create rhTestes. rhTestes were subsequently cultured in suspension for 7 days. We compared the effect of different ROCK inhibitors, including Y27632, during the aggregation step in the formation of rhTestes. We found that the organization of SCs and ICs in rhTestes did not correlate with the number of DDX4+ GCs observed. Next, using magnetic-activated cell sorting (MACS) to separate ITGA6+ SCs and GCs from ITGA6- ICs allowed us to recombine them in rhTestes in different proportions of ITGA6+ and ITGA6- cells. However, although this resulted in different organizations of SCs relative to ICs in the rhTestes, the MACS did not influence the number of DDX4+ GCs present after culture. Together, our results indicate that the number of DDX4+ GCs maintained after 7 days in culture did not correlate with the TSN organization in rhTestes. This work contributes to establishing a culture system of rhTestes, which could be used as platform to study human IVG.

Keywords: human fetal testis, 3D culture system, somatic niche

6:45 PM – 7:45 PM

368

LARGE SCALE ANALYSIS OF LOSS OF CHROMOSOME Y IN HUMAN PLURIPOTENT STEM CELLS: IMPLICATIONS FOR TURNER SYNDROME AND RIBOSOMOPATHIES

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Human pluripotent stem cells have high incidence of gaining chromosomes, but the only viable full monosomy in human pluripotent stem cells (hPSCs) in culture is X0, resulting from the loss of one of the sex chromosomes. Loss of chromosome Y (LOY) occurs in various cancers in males, but the extent and implications of LOY on hPSCs was not thoroughly studied. In this study, we performed a large-scale analysis of RNA-seq data from over 2,700 samples from about 200 independent studies, containing both hPSCs and their differentiated derivatives. We show that 12% of the samples have lost chromosome Y, either partially or fully, across different studies and cell lines. The LOY samples do not significantly differ from wildtype samples in other technical or genomic irregularities, such as read coverage, autosomal aneuploidies and TP53 mutations. Enrichment differential expression analyses revealed that the undifferentiated LOY samples show downregulated expression of pluripotent markers, X-degenerate tumor suppressors and ribosomal protein genes. In addition to the downregulation of the Y-linked ribosomal protein gene RPS4Y1, a significant decrease in expression of the majority of the autosomal ribosomal protein genes, both small- and large-ribosomal subunit proteins, was observed in LOY samples. We performed differential expression analysis on samples from Turner syndrome and certain ribosomopathies, known for losing one allele in an autosomal ribosomal gene, and observed similar trend of down-regulation in the expression of ribosomal protein genes, supporting our hypothesis of ribosomal haploinsufficiency. Overall, we present an extensive analysis of LOY in hPSCs and identified the downstream molecular effects of this chromosomal aberration, mainly downregulation in pluripotency and in ribosomal protein genes. Thus, we elucidate on the implications of LOY, and provide a possible explanation for the phenotypes underlying other ribosomal-deficiency disorders.

Keywords: human pluripotent stem cells, chromosome Y, Turner syndrome

6:45 PM – 7:45 PM

370

MURINE TROPHOBLAST ORGANOIDS AS A MODEL FOR TROPHOBLAST DEVELOPMENT AND CRISPR-CAS9 SCREENING

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The placenta becomes one of the most diversified organs during placental mammal radiation. The main in vitro model for studying mouse trophoblast development is the 2D differentiation model of trophoblast stem cells, which is highly skewed to certain lineages and thus hampers systematic screens. Here, we established culture conditions for the establishment, maintenance, and differentiation of murine trophoblast organoids. Murine trophoblast organoids under the maintenance condition contain stem cell-like populations, whereas differentiated organoids possess various trophoblasts resembling placental ones in vivo. Ablation of Nubpl or Gcm1 in trophoblast organoids recapitulated their deficiency phenotypes in vivo, suggesting that those organoids are valid in vitro models for trophoblast development. Importantly, we performed an efficient CRISPR-Cas9 screening in mouse trophoblast organoids using a focused sgRNA (single guide RNA) library targeting G protein-coupled receptors. Together, our results establish an organoid model to investigate mouse trophoblast development and a practicable approach to performing forward screening in trophoblast lineages.

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Keywords: trophoblast organoid, CRISPR-Cas9 screening, placenta

6:45 PM – 7:45 PM

372

QUANTITATIVE MODELLING OF HUMAN EMBRYO IMPLANTATION ON-CHIP

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Repeated Implantation Failure (RIF) is a multifactorial condition characterized by the persistent inability to achieve clinical pregnancy, despite the transfer of high-quality embryos. Typically diagnosed within the context of assisted reproductive technology (ART) treatment, RIF arises due to embryo and/or endometrial factors, the interaction between the endometrium and early embryo, or both. The ability of the endometrium to accept and support embryo implantation is crucial, but factors influencing this process remain elusive due to ethical and technical limitations of in-patient research. Here, we developed a personalized implantation-on-chip platform using in vitro models of the human endometrium (organoids) and the embryo (blastoids) to quantify functional embryo adhesion. Organoids were transformed into endometrial monolayers within custom-made microfluidic chips. Endometrial monolayers on-chip were cultured for 6 days with or without EPCX; β-estradiol (E2), progesterone (P4), 8-Br-cAMP (C) and XAV939 (X), for which EPCX showed increased receptivity-related gene expression (by RT-qPCR) and reduced expression of mucin and ciliated cell markers (through immunofluorescence). To assess functional receptivity of the endometrium, large numbers of blastoids (>100), surrogates for human embryos, were infused per chip followed by 48 hours of co-culture. Then, the rate of adhered blastoids was quantified after exposure to a step-wise increase of controlled flow rates. The EPCX condition showed a significantly higher adherence profile compared to the control (up to 86% vs 16%, in mid flow rate), which could be carefully modulated by step-wise decrease of hormonal exposure. Within 48 hours of adhesion, trophoblast cells actively displaced endometrium epithelial cells, indicating further implantation steps. In conclusion, this platform provides a means to obtain quantitative insights into factors affecting embryo implantation and may provide a diagnostic tool for improving embryo receptivity in patients.

Funding Source: Maastricht University Medical Centre

Keywords: endometrium-on-chip, blastoids, embryo implantation

6:45 PM – 7:45 PM

374

GENOME-WIDE SCREENING IN HUMAN EMBRYONIC STEM CELLS TO IDENTIFY GENES INVOLVED IN DNA DAMAGE PATHWAYS

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The DNA damage response (DDR) is a complex network of signaling cascades that activate pathways that induce cell cycle arrest, DNA repair or apoptosis. This response consists of proteins that sense DNA damage and activate transducer kinases that intercede the signal to DDR mediators who in turn activate the appropriate cellular response. One pivotal protein among those activated by DDR mediators and transducers is p53, which plays a crucial role in determining cell fate following various stresses, including DNA damage. In this study, we utilize genome-wide CRISPR-Cas9 screening conducted on haploid human embryonic stem cells (hESCs) to investigate various genes and pathways involved in the DDR. The genetic loss-of-function library is made up of approximately ~180,000 gRNAs, targeting virtually all protein-coding genes. We have conducted three positive selection screens using different treatments: Nutlin - a p53 activating molecule; Neocarzinostatin - a radiomimetic drug; and ionizing radiation (IR). Screen results highlight the roles of various prominent genes that take part in the DDR and apoptosis such as p53, PTEN, and CHEK2. Moreover, many genes that are not known to have a prominent role in these processes are enriched in our screens. Notably, screen results show that the p53 gene network is at the center of the response to these different stress-inducing agents. Furthermore, through thorough analysis and comparison of these screens, we aim to illuminate the contributions of genes that may have been overlooked in previous studies. By shedding light on these genes, we strive to deepen our understanding of the DDR identify novel targets for potential therapeutic interventions in the treatment of cancer.

Funding Source: The Neubauer Foundation

Keywords: DNA damage response, cancer, CRISPR Screen

6:45 PM – 7:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

376

A HUMAN STEM CELL-DERIVED IN VITRO MODEL OF THE BLOOD-BRAIN BARRIER SHOWS PATHOLOGICAL HALLMARKS OF FRONTOTEMPORAL DEMENTIA PATIENTS

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Altered integrity and functionality of the blood-brain barrier (BBB) is a common feature for many age-associated neurodegenerative diseases. However, little is known about the BBB in frontotemporal dementia (FTD). We therefore aim to characterise the BBB in FTD more precisely. We want to understand which proteins in BBB endothelial cells are involved in FTD and draw conclusions about the signalling pathways they regulate. For this purpose, we used an in vitro model of the BBB based on human induced pluripotent stem cells (hiPSCs). hiPSCs were obtained from FTD patients carrying a disease-causing mutation in the progranulin gene. Healthy control hiPSCs served as matched controls regarding age, sex, and ethnicity. We differentiated brain capillary endothelial cells (BCECs) with BBB-specific properties. Maturity was shown by gene expression analysis, morphological and immunofluorescence analysis for localisation of barrier proteins. A flow cytometry analysis was used to validate purity. For functional barrier evaluation, transendothelial electrical resistance (TEER) and a sodium fluorescein (NaF) transport assay were applied. With regard to the BCECs resilience against mechanical stress, we examined their adhesion and migration capacity. After comprehensive characterisation of the model, the BCECs were subjected to mass spectrometry. The BCECs showed a cobblestone-like endothelial morphology. The obtained morphologically homogenous cell cultures already suggested high purity, which could be confirmed by flow cytometry analysis (>99% TJP1). The localisation of the barrier proteins displayed a typical reticular structure in the immunofluorescence analysis. Functional analyses showed a stable barrier in all models (TEER >300 Ωcm2). Paracellular transport measured by the permeability coefficient of NaF (PCNaF < 1,5 µm/min) was prevented as a result of the barrier's integrity and tightness. To better understand the resilience of diseased BCECs, the analysis of cell adhesion and migration by electric cell-substrate impedance sensing (ECIS) and a wound-healing assay were initialised in a pilot study. We have established an FTD BBB in vitro model which is suitable for marker studies using proteome analysis. This may be used to help develop or improve strategies for diagnosis of prodromal factors in FTD.

Funding Source: Budget funds, University Clinic and Polyclinic for Neurology University Medicine Halle (Saale) Ernst-Grube-Straße 40 06120 Halle (Saale)

Keywords: frontotemporal dementia, blood-brain barrier in vitro model, BCEC-derived biomarkers

6:45 PM – 7:45 PM

378

A TOOLBOX OF HUMAN IPSC-DERIVED MICROGLIA IN DIFFERENT GENETIC BACKGROUNDS AND DISEASE MODELS FOR NEURODEGENERATION DRUG DISCOVERY

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Microglia are the tissue-resident macrophages of the brain. They survey neuronal function, play roles in neurogenesis and synaptic remodelling, and are the first responders to infection. Microglia have been implicated in a number of neurodegenerative diseases including Alzheimer's disease (AD). Due to the importance of microglia during development and disease, new models that can replicate the human phenotype are important to advance drug discovery. Using opti-ox, we have generated human induced pluripotent stem cells (iPSCs)-derived microglia from both male and female backgrounds in a consistent manner, with the derived microglia expressing CD45, P2RY12, CD11b, CD14, IBA1,

and TREM2. Transcriptomically, male and female-derived ioMicroglia show high similarity, and with a signature similar to primary microglia. Functionally, whilst both male and female-derived microglia demonstrate capacity to phagocytose various particles and exhibit a cytokine response to LPS and INFɣ stimulation, background specific responses can be observed. We have also demonstrated that ioMicroglia from both male and female-backgrounds can be co-cultured with excitatory neurons (ioGlutamatergic Neurons), highlighting their ability to be used to form more complex cellular model systems. With an aim to provide a platform for investigation into mechanisms involved in neurodegeneration, we employed CRISPR/Cas-9 gene editing to introduce specific point mutations in TREM2 (R47H), and APOE (4/3 C112R) into the ioMicroglia male background; both genetic risk factors associated with late onset AD. The derived ioMicroglia maintain a microglia phenotype and remain functionally active, providing a platform for further phenotypic characterisation. In conclusion, we have demonstrated that opti-ox mediated cellular reprogramming can generate microglia from iPSCs of different genetic backgrounds, and can be used as a platform to create physiologically relevant disease model systems.

Keywords: microglia, iPSC-derived, Alzheimer's disease

6:45 PM – 7:45 PM

380

UNCOVERING REGULATORY REGIONS FOR FETAL HEMOGLOBIN REACTIVATION FOR BETA -HEMOGLOBINOPATHIES

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Reactivation of fetal hemoglobin (HbF) at adult stage offers a promising therapeutic approach for Sickle Cell Disease (SCD) and β-thalassemia (BT). Consequently, comprehending the regulation of HbF reactivation is crucial. This study aims to unravel the role of cis-regulatory elements (CREs) in governing HBG gene expression, providing insights into both fundamental gene expression mechanisms and potential therapeutic targets. The cis-regulation of the β gene cluster remains poorly understood, leading us to hypothesize that the Hereditary Persistence of Fetal Hemoglobin (HPFH) deletion region may harbor putative HbF regulatory regions (PHRR). To investigate this hypothesis, we generated 33 overlapping deletions within a 110 kb region of the PHRR using CRISPR/Cas9 in erythroid progenitor cell line. Flow cytometric analysis of differentiated reticulocytes revealed 11 deletions surpassing a 20% HbF threshold, with 7 consistently exhibiting significantly high HbF levels through HPLC analysis. Subsequently, the PHRR with highest level of HbF (PHRR 29) was chosen and evaluated for its therapeutic potential. Traditional studies involving patient-derived hematopoietic stem/progenitor cells (HSPCs) faced limitations due to restricted donor access and renewal capacity, especially for experimental therapeutic approaches like the present one. To overcome these challenges, disease cellular models SCD and BT were developed using BEL-A cells. In-depth analysis of cellular disease models, BEL-A SCM (BEL-A sickle cell mutation) and BEL-A BTM (BEL-A β-thalassemia mutation), demonstrated comparable differentiation profiles, globin expression, and proteome dynamics to patient-derived HSPCs. Further, these cellular models recapitulate pathological conditions associated with both the diseases including sickling in SCD and globin chain imbalance in BT. Evaluating PHRR 29 in these models demonstrated a reversal of disease phenotype with reduced sickling, ROS in BEL-A SCM, and reduced ROS and globin chain balance in BEL-A BTM. Targeted proteomics for PHRR 29 is currently underway to identify potential protein targets for small-molecule interventions. Overall, this study sheds light on previously unexplored regulatory regions, providing valuable insights into potential targets for genome editing.

Keywords: fetal hemoglobin reactivation, beta-hemoglobinopathies, cis-regulatory elements

6:45 PM – 7:45 PM

382

DEVELOPMENT OF A PIG BIOREACTOR SUPPORTING ROBUST HUMAN HEMATOPOIESIS FOR LARGE-SCALE PRODUCTION OF FUNCTIONAL HUMAN IMMUNE CELLS

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There is an urgent need for large animal models to study human hematopoiesis and regenerate functional human immune cells in vivo. Herein, we generated gene-edited immunodeficient pigs that lack T, B and NK cells (RG pig) and have attenuated macrophage xenoreactivity (RGD pig) and tested their potential to support human hematopoietic engraftment and differentiation. In RG pigs, human CD34+ cell transplantation achieved human hematopoietic engraftment in bone marrow, but poor chimerism in blood and spleen. However, human CD34+ cell-transplanted RGD pigs showed high levels of human hematopoietic chimerism composed of T, B, NK, and myeloid cells. These RGD pigs had robust human hematopoiesis in bone marrow and ongoing thymopoiesis in thymus. Furthermore, human T and B cells developing in RGD pigs were functional and expressed broad receptor repertoires. Thus, the RGD pig offers a useful preclinical model for investigating human normal or diseased hematopoiesis and therapies, and a powerful bioreactor for large-scale production of human immune cells.

Funding Source: This work was supported by grants from the

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Keywords: pig bioreactor, human hematopoiesis, human immune cell production in large-scale

6:45 PM – 7:45 PM

384

UNCOVERING HETEROGENEITY IN HIPSC-DERIVED ENDOTHELIAL PROGENITORS FROM DIFFERENT DIFFERENTIATION PROTOCOLS

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Human induced pluripotent stem cell-derived endothelial progenitors (hiPSC-EPs) have shown considerable promise as an easily expandable and patient-specific cell source for vascular tissue engineering. Considerable work has focused on differentiating these cells and characterizing their vasculogenic potential both in vitro and in vivo. However, most research in the field does not consider the heterogeneity that exists within endothelial cells. To that end, we investigated differences in hiPSC-EPs produced from two differentiation protocols that drive differentiation using either small molecules (SM Protocol) or growth factors (GF Protocol). Following encapsulation of SM- and GF-derived CD34+-hiPSC-EPs in Collagen/Norbornene-modified hyaluronic acid interpenetrating polymer network hydrogels, we found that although cells from both protocols were able to form vasculature within 7 days of culture, GF-derived cells formed denser and more interconnected vasculature. In addition, we observed that GF-derived cells secreted lower levels of matrix metalloproteinases and Collagen IV, indicating decreased extracellular matrix remodeling relative to SM-derived cells. This was corroborated by RNAseq, which also showed that GF-derived cells are significantly more migratory. Taken together, this suggests that GF-derived cells have a higher propensity for undergoing an endothelial-to-mesenchymal transition. This demonstrates that although both SM- and GF-derived cells were selected for the same surface marker and successfully undergo angiogenesis in 3D hydrogels, there exists significant differences on both the gene and protein level. If this cell type is to be used for tissue-engineered vasculature, researchers need to perform similar characterization to better understand the cell types that they have generated.

Funding Source: National Heart, Lung, and Blood Institute of the National Institute of Health (R01HL15829)

Keywords: tissue engineering, angiogenesis, 3D in vitro models

6:45 PM – 7:45 PM

386

DEVELOPMENT OF A ROBUST PRECLINICAL MODEL FOR HUMAN AGE-RELATED CLONAL HEMATOPOIESIS USING COMPANION ANIMALS

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Clonal hematopoiesis (CH), also called age-related CH (ARCH), describes the abnormal expansion of hematopoietic stem and progenitor cells (HSPCs) carrying acquired somatic mutations, most frequently in DNMT3A, TET2, and ASXL1. Patients with CH are associated with hematopoietic malignancy, including acute myeloid leukemia, as well as a higher risk for cardiovascular disorders. Advanced age and hyperinflammation, the fundamental characteristics of CH, have led to extensive investigation of the relationship between CH and various inflammatory and age-related diseases such as COVID-19 and HIV. However, due to the lack of hematological and clinical diagnostic biomarkers for this precancer stage, predictive animal models have been unavailable that reliably recapitulate the longitudinal spectrum of human CH. Rhesus macaques (RMs) are the only model to date found to have naturally occurring human-type ARCH, but it is difficult to secure a sufficient number of aged RMs in captivity. Companion animals, such as domestic dogs (Canis familiaris), serve as a robust translational model to study age-related human disease from a long-term perspective, considering relatively long lifespan, shared lifestyle, and similarity in hematopoietic tumor morphology. Utilizing the advanced methodologies used in the RM study, we sought to elucidate whether aged dogs develop spontaneous CH equivalent to the human spectrum and its correlation with specific diseases. So far, paired germline and peripheral blood mononuclear cells have been obtained from more than 40 canine patients over 8 years old with or without various comorbidities. More than half (53%) of patients visiting Jeju National University Animal Hospital suffered from either inflammatory diseases or cancer, whereas only 16% had cardiovascular disorders. Blood cancer accounted for about 28% of all cancer patients, and most cases were lymphoma rather than leukemia. We analyzed the expression level of major CH driver genes and found that the expression of TET2 was generally reduced in tumor patients, unlike DNMT3A. Now, we are testing both hybridization capture- and PCR-based library prep methods simultaneously to optimize the error-corrected ultra-deep whole exome sequencing in canine samples. This will soon lead us to more solid conclusions.

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (RS-2023-00212960).

Keywords: hematopoietic stem and progenitor cells, age-related clonal hematopoiesis, hyperinflammation

6:45 PM – 7:45 PM

388

PERFUSABLE VASCULAR ORGANOIDS FOR MODELING HIERARCHICAL CEREBROVASCULATURE

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Engineering complex tissues holds promise for tissue regeneration and disease modeling. However, developing organ-specific vasculature, which plays a critical role in mediating inter-tissue communication and maintaining unique metabolic homeostasis in each organ, remains a critical challenge. Human induced pluripotent stem cell-derived vascular organoids (hVOs) offer the potential to mimic in vivo blood vessels with extensive remodeling capacity that, upon implantation in vivo, can integrate with the host vasculature and remodel into hierarchical vessels. However, in vitro perfusion of the hVOs nor the development of blood-brain barrier (BBB) phenotype in hVOs have been achieved. Here, we combine hVOs, microvascular engineering, and co-culture models to develop a perfusable neurovascular niche in vitro. To investigate vascular remodeling in hVOs under intraluminal perfusion, we established anastomosis between hVOs with pre-patterned microvasculature seeded with primary human brain microvascular endothelial cells (HBMECs), evidenced by dextran perfusion of hVO microvessel (hVOMV). Capillary extension from hVOs connected with angiogenic sprouts from HBMECs within the microchannel to generate vascular networks with lumen diameters ranging from 100um to 10um. Moreover, distinct remodeling features occur within hVOs when pressure drop and a constant flow were applied across hVOMV system. Finally, astrocytes and cortical neurons co-cultured with hVOs interacted with the capillary vessels within hVO and enhanced endothelial tight junction and glucose transporter expression. Ongoing work involves utilizing single-cell RNAseq to understand cell population and transcriptomic profile of perfusable BBB-mimicking hVOs. This advancement would hold significant promise for disease modeling and tissue engineering applications in the realm of neuroscience and regeneration medicine.

Funding Source: AHA Predoctoral Fellowship (23PRE1014179 to Y.J.S)

Keywords: vascular organoid, neurovascular unit, vascular development

6:45 PM – 7:45 PM

390

ACCELERATED HEMATOPOIETIC STEM CELL AGING IN SPACE

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Hematopopietic stem cell (HSC) fitness declines in response to macroenvironmental and microenvironmental stressors, including aging and inflammation. While the NASA Twins study revealed inflammatory cytokine upregulation, chromosomal alterations, and telomere changes indicative of hematopoietic defects, the direct impact of spaceflight on reduced HSC fitness and accelerated aging were not studied. To investigate the effects of spaceflight on human HSC fitness and aging, our NASA-supported Integrated Space Stem Cell Orbital Research (ISSCOR) team developed human HSC fitness-detecting bone marrow niche nanobioreactors with lentiviral FUCCI2BL fluorescent cell cycle reporters in automated CubeLabs, each equipped with a confocal fluorescence microscope and AI algorithms for real-time clonal tracking. These space nanobioreactors were flown in four separate 30 to 45-day missions to the International Space Station (ISS) (NASA SpX-24, 25, 26, 27) and compared with ground controls followed by functional colony survival, replating and stromal co-culture recovery assays combined with whole genome sequencing and whole transcriptome sequencing analyses to detect mutations and transcriptomic alterations. After a month in space, we observed reduced clonal dormancy, decreased telomere length, reduced ADAR1p150 self-renewal gene expression and decreased replating (self-renewal) capacity as well as

mitochondrial DNA amplification, APOBEC3-induced C-to-T mutagenesis, and repetitive element alterations typical of accelerated aging and pre-leukemic disorders. Space-induced HSC fitness deficits were partly reversible on HS27 stromal co-cultures. Space-associated accelerated and pre-malignant HSC aging may be predictable and preventable with appropriate countermeasure implementation.

Keywords: aging, microenvironment, hematopoietic stem cell

6:45 PM – 7:45 PM

392

IN VITRO MODELING OF ATHEROSCLEROSIS USING IPSC-DERIVED BLOOD VESSEL ORGANOIDS

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As modeling of atherosclerosis requires recapitulation of complex interactions with vasculature and immune cells, previous in vitro models have limitations in that they lack atherosclerotic phenotypes due to their insufficient 3D vascular structures. However, iPSC-derived blood vessel organoids (BVOs) are applicable for modeling vascular diseases, as they contain multiple cell types, including endothelial cells, vascular smooth muscle cells self-assembled into a blood vessel structure. Here, we successfully developed atherosclerotic BVOs with microenvironment associated with atherogenesis, such as disturbed flow, low-density lipoprotein, proinflammatory cytokine TNFα, and monocyte coculture. In atherosclerotic BVOs, representative atherosclerotic phenotypes, including endothelial dysfunction, inflammatory responses, formation of foam cell and fibrous plaque, and moreover, calcification of the plaques were observed. To verify the drug response in this BVO model, we treated it with clinically used lovastatin and confirmed the phenotype attenuation. Furthermore, by using atherosclerotic BVOs, we evaluated the therapeutic efficacy of nanosized graphene oxides (NGOs) on atherosclerosis. Due to their anti-inflammatory effects, NGOs effectively alleviated the pathologic lesions in atherosclerotic BVOs by ameliorating endothelial dysfunction as well as promoting macrophage polarization toward M2. These results suggest that atherosclerotic BVOs are an advanced in vitro model that is suitable for drug discovery and further elucidation of therapeutic mechanisms.

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Keywords: blood vessel organoid, atherosclerosis, monocyte

6:45 PM – 7:45 PM

394

INVESTIGATION OF MITOCHONDRIAL FUNCTION IN HIPSC-DERIVED NEUROVASCULAR IN VITRO MODELS OF PARK2 COPY NUMBER VARIATION CARRIERS WITH ADULT ADHD

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Copy number variations (CNVs) in the PARK2 gene have been shown to be associated with attention deficit hyperactivity disorder (ADHD). Parkin is involved in mitochondrial quality control, indicating that PARK2 CNVs might impair mitochondrial energy metabolism. Blood-brain barrier (BBB) disruption has been implicated in ADHD, since changes in BBB functionality and metabolic activity can lead to barrier opening and a disrupted microenvironment, disturbing neuronal function. While treatment with stimulant medication can alleviate symptoms of ADHD, about 50% of adult patients are non- or only partial-responders to pharmacological therapy, demonstrating that development of novel medication is inevitable. Therefore, an isogenic microfluidic in vitro co-culture model of the neurovascular unit (NVU) based on human induced pluripotent stem cell (hiPSC)-derived brain capillary endothelial-like cells (BCECs) and cortical neurons was established to provide a standardized platform for drug screening in ADHD treatment. To investigate PARK2 CNV-associated functional and metabolic changes at the NVU, we analyzed BCECs derived from hiPSC lines of ADHD patients with PARK2 CNVs compared to wildtype carriers with (and without) ADHD. Mono-cultured BCECs of PARK2 CNV carriers with ADHD did not display signs of disrupted barrier integrity and impaired cellular functionality under baseline conditions. However, preliminary Seahorse extracellular flux analysis revealed that both mitochondrial-related and glycolytic ATP production of BCECs with PARK2 CNVs was slightly diminished compared to wildtype controls. Within BCECs, mitochondrial oxidative phosphorylation accounted for just over a third of total ATP production, suggesting that BCECs utilize primarily glycolysis for ATP synthesis. Furthermore, co-culture and microfluidic culture conditions were established for future studies. Our work will promote drug screening in ADHD treatment, thereby improving drug-related therapy approaches at the BBB and NVU. Additionally, novel therapeutic approaches will be validated to restore cellular and mitochondrial function.

Funding Source: Interdisciplinary Center for Clinical Research (IZKF) Würzburg

Keywords: attention deficit hyperactivity disorder (ADHD), neurovascular unit (NVU), mitochondrial dysfunction

6:45 PM – 7:45 PM

396

GENETIC CONTRIBUTION TO MICROGLIAL ACTIVATION IN SCHIZOPHRENIA

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Several lines of evidence indicate the involvement of neuroinflammatory processes in the pathophysiology of schizophrenia (SCZ). Microglia are brain resident immune cells responding toward invading pathogens and injury-related products, and additionally, have a critical role in improving neurogenesis and synaptic functions. Aberrant activation of microglia in SCZ is one of the leading hypotheses for disease pathogenesis, but due to the lack of proper human cell models, the role of microglia in SCZ is not well studied. We used monozygotic twins discordant for SCZ and healthy individuals to generate human induced pluripotent stem cell-derived microglia to assess the transcriptional and functional differences in microglia between healthy controls, affected twins and unaffected twins. The microglia from affected twins had increased expression of several common inflammation-related genes compared to healthy individuals. Microglia from affected twins had also reduced response to interleukin 1 beta (IL1β) treatment, but no significant differences in migration or phagocytotic activity. Ingenuity Pathway Analysis (IPA) showed abnormalities related to extracellular matrix signaling. RNA sequencing predicted downregulation of extracellular matrix structure constituent Gene Ontology (GO) terms and hepatic fibrosis pathway activation that were shared by microglia of both affected and unaffected twins, but the upregulation of major histocompatibility complex (MHC) class II receptors was observed only in affected twin microglia. Also, the microglia of affected twins had heterogeneous response to clozapine, minocycline, and sulforaphane treatments. Overall, despite the increased expression of inflammatory genes, we observed no clear functional signs of hyperactivation in microglia from patients with SCZ. We conclude that microglia of the patients with SCZ have gene expression aberrations related to inflammation response and extracellular matrix without contributing to increased microglial activation.

Keywords: microglia, schizophrenia, induced pluripotent stem cell

6:45 PM – 7:45 PM

398

SCALABLE GENERATION OF ALVEOLAR MACROPHAGE-LIKE CELLS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Tissue-resident macrophages (TRMs) are crucial for tissue homeostasis, with certain subsets originating from embryonic progenitors and possessing self-renewal capacity. Alveolar macrophages (AMs), as TRMs of the lungs with embryonic origin play an essential role in lung health. They serve as the frontline defense against airborne pathogens, contribute to surfactant metabolism and are involved in lung regeneration. TRM dysregulation contributes to various lung diseases, highlighting the significance of AMs as targets for cell therapy and drug testing. However, investigating human AM biology and developing new therapies is hampered by the scarcity of primary AMs, particularly from healthy individuals. In this study, we aimed to address this limitation by establishing a standardized and scalable protocol for generating AM- like cells from human induced pluripotent stem cells (hiPSC; iAML), enabling the generation of both healthy and patient-specific iAML cells. Inspired by recent advancements in differentiating peripheral blood monocytes into AML cells, we generated iAML cells from hiPSC-derived macrophages (iMacs) in the presence of a defined cocktail comprising surfactant, GM-CSF, TGFβ, and IL-10. Our results revealed distinctive morphological changes in the iAML cells compared to reference iMacs cultivated in the absence or presence of M-CSF only. While phenotypical changes in the typical surface marker expression of e.g. CD64, MARCO and CD36 towards an AML phenotype were minor, gene expression patterns resembled those reported in AML cells derived from peripheral blood monocytes. Notable changes included upregulation of SPI1, PPARG, and MRC1, and downregulation of MMP9. In summary, our findings suggest the feasibility of inducing iMacs to adopt an AML phenotype, providing a valuable model for studying AM biology in health and disease. This new cell type could further support the establishment of completely new immunocompetent lung models for disease modelling and drug testing.

Keywords: alveolar macrophage-like cells, induced pluripotent stem cells, disease modelling and drug testing

6:45 PM – 7:45 PM

400

INVESTIGATING PARKINSON'S DISEASE CELL-AUTONOMOUS PHENOTYPES IN IPSC-DERIVED MICROGLIA

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Microglia are the tissue-resident macrophages of the central nervous system, and have important roles in brain development, homeostasis, and disease. The concentration of microglial cells is particularly high in the substantia nigra pars compacta of the midbrain, where dopaminergic neurons die in Parkinson's Disease (PD), and microglial activation and neuroinflammation is a hallmark of disease. In this study, we used isogenic PD-model iPSC lines carrying an Snca (alpha-synuclein) gene triplication (a rare cause of aggressive, early-onset parkinsonism) and a Lrrk2 G2019S mutation to evaluate microglia-intrinsic PD phenotypes. After small molecule-mediated differentiation, we assayed the iPSC-derived cells for microglial identity markers and functionality through FACS, immunocytochemistry, cytokine secretion and phagocytosis. The protocol generated CD11c-positive erythromyeloid progenitors (EMPs) that infiltrated neurospheres. EMPs matured when co-cultured with neuronal cells, exhibiting strong Iba-1 signals and characteristic microglial morphodynamics. To simulate microglial activation, we stimulated the cells with lipopolysaccharide (LPS). HLA-DR expression patterns indicate distinct subclusters of microglia upon LPS-induced activation. Both Snca and Lrrk2 variants influenced immune response in iPSC-derived microglia, with mutated cells having a distinct IL-6 release profile from their respective controls. LPS treatment increased phagocytosis rate and total capacity in Snca control and knock-out but had no effect on Snca triplication cells. This suggests that microglia carrying Snca triplication exhibit a strong reactive state marked by phagocytic exhaustion and increase cytokine release. Lrrk2 control microglia increased their total phagocytic capacity in response to LPS treatment, while cells carrying the G2019S mutation failed to respond to stimulation. Our next steps include the investigation of microglial responses to alpha-synuclein monomers and pre-formed fibrils (PFFs). Our results show the value of iPSC-derived microglia as a model to elucidate cell-autonomous contributions to health and disease. Leveraging these cell type-specific assays allows us to investigate cell-to-cell interactions in co- and tri-cultures with neurons and astrocytes.

Funding Source: This research was funded, in part, by Aligning Science Across Parkinson's [Grant number: ASAP-000472] through the Michael J. Fox Foundation for Parkinson's Research (MJFF).

Keywords: iPSC-derived microglia, Parkinson's disease, neuroinflammation

6:45 PM – 7:45 PM

402

ADMINISTRATION OF UMBILICAL CORD BLOOD PRIMED BY OP9 STROMAL CELLS AMELIORATES NEUROLOGICAL DEFICITS IN MOUSE CEREBRAL INFARCTION MODEL

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Umbilical cord blood (UCB) transplantation shows proangiogenic effects and contributes to symptom amelioration in animal models of cerebral infarction. However, the effect of specific cell types within a heterogeneous UCB population are still controversial. OP9 is a stromal cell line used as feeder cells to promote the hematoendothelial differentiation of embryonic stem cells. Hence, we investigated the changes in angiogenic properties, underlying mechanisms, and impact on behavioral deficiencies caused by cerebral infarction in UCB co-cultured with OP9 for up to 24 h. In the network formation assay, only OP9 pre-conditioned UCB formed network structures. Single-cell RNA sequencing and flow cytometry analysis showed a prominent phenotypic shift toward M2 in the monocytic fraction of OP9 pre-conditioned UCB. Further, OP9 pre-conditioned UCB transplantation in mice models of cerebral infarction facilitated angiogenesis in the peri-infarct lesions and ameliorated the associated symptoms. In this study, we developed a strong, fast, and feasible method to augment the M2, tissue-protecting, pro-angiogenic features of UCB using OP9. The ameliorative effect of OP9-pre-conditioned UCB in vivo could be partly due to promotion of innate angiogenesis in peri-infarct lesions.

Funding Source: JSPS KAKENHI Grant-in-Aid for Scientific Research (C) [Grant Number JP22K09221]

Keywords: cerebral infarction, umbilical cord blood, angiogenesis

6:45 PM – 7:45 PM

404

HUMAN PLURIPOTENT STEM CELLS-DERIVED CELLS FROM DISCORDANT MONOZYGOTIC TWINS: MODELLING ENDOTHELIAL DYSFUNCTION TO SMOKING AND NICOTINE PRODUCTS

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Smoking is widely recognized as a threat to vascular health and linked to coronary artery disease (CAD), stroke, and peripheral artery disease. Despite being touted as safer alternatives, vapes and e-cigarettes may also pose risks. In this study, we aimed to investigate the effects of nicotine and e-cigarette liquid (ECL) on human pluripotent stem-derived endothelial cells (hiPSC-EC). hiPSC lines were reprogrammed with Sendai viral transduction from a pair of discordant monozygotic twins diagnosed with diabetes mellitus and an age-matched healthy control participant. One of the twins was a smoker, diagnosed and treated for severe CAD, whilst the other was a non-smoker without CAD. No differences were found in the exome sequences of the reprogrammed twin hiPSC lines. Human iPSC lines were differentiated by two distinct differentiation protocols into CD31-positive endothelial cells, resulting in capillary and artery endothelial phenotypes. The cells were treated with nicotine and ECLs (with nicotine and nicotine-free variant) and analyzed with high-content fluorescent microscopy (Perkin Elmer Opera), using Hoechst, vital apoptotic marker caspase3/7, necrotic marker TO-PRO3, mitochondrial metabolic activity marker TMRM and fluorescent CD31 antibody for toxicological, and functional assays. Image and data analysis was performed using machine learning assisted workflow. The results revealed differences in endothelial cell responses to both nicotine and ECL. Cells from patients who chronically smoke exhibited decreased apoptosis response to nicotine. The signal of the mitochondrial membrane potential marker was increased, and a slight disruption in the dynamics of mitochondrial filaments was also observed in all three cell groups treated with nicotine. The wound healing potential of ECL treated hiPSC-ECs was reduced. These findings shed light on how endothelial cells from smoking and non-smoking twins autonomously respond to nicotine exposure and suggest a potential adaptation mechanism leading to decreased apoptosis in smokers. We have developed a high-fidelity patient-specific in vitro hiPSC model where an alteration in nicotine exposure is associated with distinct endothelial phenotypes. This approach proves to be a useful tool in both clinical and environmental toxicology research.

Funding Source: Hungarian National Research, Development and Innovation Fund (RRF-2.3.1-21-2022-00003, TKP2021-EGA-23, NKFIH 146125)

Keywords: twin research, cardiovascular disease model, smoking pathomechanism

6:45 PM – 7:45 PM

406

INVESTIGATING THE ROLE OF SF3B1 MUTATION-MEDIATED MIS-SPLICING OF UBA1 IN MDS

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Myelodysplastic syndromes with ring sideroblasts (MDS-RS) are a low-risk MDS subtype that originate in hematopoietic stem cells and are predominantly defined by mutations in the SF3B1 splice factor and a clinical profile of refractory anemia. Treatment of MDS-RS is largely limited to mitigating the anemia through transfusion or use of erythropoiesis-stimulating agents, and allogeneic stem cell transplants remain the only curative option. An incomplete understanding of the mis-splicing landscape and its molecular consequences in shaping the disease phenotype, as well as the lack of accurate model systems, has hampered the development of new treatments. To identify novel RNA mis-splicing events, we employed full-length RNA sequencing of bone marrow CD34+ cells from MDS-RS patients, followed by functional characterization using a patient-derived induced pluripotent stem cell (iPSC) model. Cohort analysis among splice factor-mutant patients identified significant, SF3B1 mutation-exclusive mis-splicing of the gene ubiquitin-like modifier activating enzyme 1 (UBA1), which encodes the master enzyme of the cellular ubiquitination cascade. To evaluate the effect of UBA1 mis-splicing on protein function, K562 cells were transfected with cDNA constructs encoding mis-spliced or wild-type UBA1. While both led to mRNA production, the mis-spliced UBA1 construct failed to generate protein. Further, iPSC lines generated from mutated and wild-type cells from MDS-RS patients were differentiated into hematopoietic cells in vitro. RNA sequencing identified the same mis-splicing event of UBA1 in the SF3B1-mutated line and its absence in SF3B1 wild-type cells. To determine whether lower levels of functional UBA1 protein sensitize cells to UBA1 inhibition, hematopoietic cells from both iPSC lines were treated with the selective UBA1 inhibitor TAK-243. Interestingly, cell death was induced in SF3B1-mutated cells at significantly lower doses compared to wild-type SF3B1. In conclusion, we have identified the novel and significant mis-splicing of UBA1 in SF3B1-mutated patients

which increases mutant cell sensitivity to UBA1 inhibition, providing a new avenue to improve treatment of MDS-RS.

Keywords: myelodysplastic syndromes, induced pluripotent stem cells, disease modelling

6:45 PM – 7:45 PM

408

CAPSID-ENGINEERED AAV-VEC-MEDIATED GENE THERAPY IN IPSC-DERIVED ENDOTHELIAL CELLS FROM FABRY DISEASE PATIENTS

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Fabry disease (FD) is a rare X-linked lysosomal storage disease affecting multiple organs like the heart, kidney, and brain. FD is caused by variants in the GLA gene that lead to reduced α-galactosidase A (α-gal A) activity and thus to accumulation of the enzyme's substrates like globotriaosylceramide (Gb3) in cell types like cardiomyocytes or endothelial cells (ECs). FD patients typically receive either enzyme replacement therapy (ERT) or pharmacological chaperone therapy (PCT). As only patients with certain GLA mutations are amenable to oral PCT and the ERT is cumbersome and expensive, gene therapy potentially addresses unmet needs by a single treatment. Since FD is a rare disease with limited primary patient material, FD patient-derived iPSCs offer a promising tool to test potential therapies on disease-relevant cell types. We have successfully generated iPSCs from three FD patients (two male, one female) and detected lower α-gal A activity and substantial Gb3 accumulation in only the male FD iPSCs. Since ECs are among the most affected cell types in FD patients, we differentiated FD iPSCs to ECs and observed the same biochemical features as in FD iPSCs, confirming that our system recapitulates key findings of FD. Transduction of iPSC-derived ECs using the EC-targeting capsid-engineered adeno-associated virus (AAV) vector AAV-VEC resulted in at least 10-fold higher α-gal A activity levels within cells and in cell culture supernatants compared to healthy control iPSC-derived ECs. Further, Gb3 accumulation in the male FD iPSC-derived ECs was reduced by up to 9-fold 14 days post-transduction to a level similar to healthy control and female FD iPSC-derived ECs. These findings indicate that Gb3 accumulation in FD iPSC-derived ECs can be efficiently reduced to physiological levels using the capsid-engineered AAV-VEC vector. The current gene therapy trials for FD mainly rely on GLA delivery to the liver and enzyme secretion into the circulation. By using AAV vectors engineered to also transduce ECs, we envision to reduce Gb3 accumulations directly in the ECs and thereby remove the cause of EC dysfunction in addition to secretion into the blood to reach other affected cells. Moreover, our differentiation system presents a platform for further disease modeling studies and testing of additional therapeutics for FD patients.

Funding Source: European Research Council (ERC) under Grant agreements No. 819531

Keywords: Fabry disease, gene therapy, iPSC-derived ECs

6:45 PM – 7:45 PM

410

INVESTIGATING THE EFFECTS OF NOTCH3 ASIAN-SPECIFIC MUTANT ON BLOOD-BRAIN-BARRIER USING IPSC-DERIVED CELL TYPES

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Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), is the genetic form of vascular dementia leading to vascular impairments and cognitive decline. CADASIL is caused by mutations in the NOTCH3 gene which encodes a single-pass transmembrane receptor and expressed in smooth muscle cells (SMC). The NOTCH3 p.R544C is reported to be the most common risk variant in Asia with almost 1% of community controls in Singaporean Chinese are carriers. Despite clinical observations, p.R544C has yet to be listed as a "pathogenic" variant due to the lack of functional studies done. Several reports indicated that Blood-Brain-Barrier (BBB) dysfunction can be the driving mechanism for synaptic dysfunction in CADASIL. Our work aims to model p.R544C pathogenesis and study its effects on vascular function with patient-derived induced pluripotent stem cells (iPSC)-derived BBB cell types. In our study, p.R544C iPSC-derived endothelial cells (EC) displayed decreased angiogenesis. In addition, bulk RNA sequencing of EC and SMC revealed dysregulated pathways involved in branching and angiogenesis, in line with our observations from the angiogenesis assay. Using a 3D in vitro platform, iPSC-derived BBB cell types are able to spontaneously form constructs with vessel-like and lumen-like structures 7 days after seeding. NOTCH3

p.R544C constructs displayed lower expression of cell specific markers and tight junction markers indicating possible defects to cell survival and vessel formation which is being explored. To our knowledge, the exact disease mechanism and effects of NOTCH3 p.R544C in CADASIL is still unknown. Studying these genes and pathways could help us unravel new mechanisms leading up to CADASIL neuropathology. Moreover, these target genes are an opportunity to develop or repurpose drugs to intervene in CADASIL progression. Ultimately, unravelling effects of vascular deregulation in CADASIL will contribute to understanding its disease pathophysiology.

Keywords: vascular dementia, blood-brain-barrier modelling, 3D in vitro platform

6:45 PM – 7:45 PM

412

ENGINEERING ADVANCED ORGANOID STRUCTURES THROUGH THE DEVELOPMENT OF STROMAL VASCULAR ORGANOIDS

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Blood vessels are a critical component in organ development and tissue homeostasis, as they facilitate the exchange of oxygen and nutrients. Organotypic structures such as organoids faithfully recapitulate numerous in vivo-like functions. However, the lack of vasculature in many of these systems hinders their capacity to fully capture cell-stroma interactions. In this study, we develop vascular organoids with built-in mature stromal components to use them as vascularized building blocks that can be combined with healthy and diseased organ models. These stromal vascular organoids (SVOs) are generated using primary mesenchymal stromal cells (MSCs) and umbilical cord blood-derived endothelial colony-forming cells (ECFCs) assembled as spheroids in microwells. After harvesting, the spheroids are embedded in collagen-CultrexTM where they form vascular sprouts. After 7 days, the pre-vascular network is transferred to an ultra-low attachment plate for further maturation and the SVOs can be cultured for >40 days. Vessel-specific markers have been found to be expressed by each cell types: CD31 for ECFCs, aSMA, PDGFRb and Nestin for MSCs. Collagen IV, an essential protein of the vessel basement membrane, is also expressed, constituting ~20% of all ECM proteins readily produced by the SVOs, as detected via metabolic labelling experiments. Overtime, the SVOs reach a diameter of 1,9±0,22mm at day 7. The ease of production and scalability of these SVOs make them suitable vascularized building blocks to increase the complexity in engineering multiple organoids. In preliminary studies, hematopoietic stem and progenitor cells (HSPCs) engrafted in the SVOs at a depth of at least 500µm over 7 days of culture. Additionally, SVOs could be kept in culture in a full hematopoietic-supporting medium, and can be loaded onto microfluidic systems for dynamic culture. More studies are underway to investigate the potential of using SVOs as a replica of the hematopoietic stem cell niche. Additionally, the assessment of their potential in the formation of vascularized assembloids with iPSC-derived pancreatic endocrine islets to improve insulin sensitivity is ongoing. We believe that these novel structures will pave the way for accessible as well as more complex engineering of tissues comprising key interactions with vascular cells.

Keywords: vascularization, organoids, assembloid

6:45 PM – 7:45 PM

414

EX-VIVO TRACING OF HEMATOPOIETIC STEM CELL FATES UPON ACQUISITION OF PRELEUKEMIC MUTATIONS

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Myeloid malignancies (MMs) are clonal hematologic disorders that originate in hematopoietic stem and progenitor cells in the bone marrow. Strikingly, although MMs usually carry a relatively small number of driver mutations, phenotypically they are very heterogeneous diseases, even across tumors with the exact same mutations. Until recently, research predominantly focused on MMs genetic mutations, neglecting the role of non-genetic factors in tumor evolution and clonal selection. In our group, we aimed to bridge this gap by employing high-throughput single-cell barcoding techniques to unravel the intricate interplay between genetic and epigenetic heterogeneity in MMs clonal fitness and fate. By leveraging single-cell RNA sequencing, ex vivo HSC cultures and transgenic mouse models of preleukemic mutations, we tracked ~1000 HSC clones over 27 days of ex vivo culture from wild-type, Dnmt3aR882H/+ and Npm1cA/+ cells. Remarkably, our findings suggest that clonal expansion is epigenetically hardcoded in HSC clones, resulting in a highly heterogeneous response in clonal fitness following the acquisition of mutation. Interestingly, we observed that low-fitness clones exhibited a pronounced benefit upon acquisition of Dnmt3aR882H/+ and Npm1cA/+ mutations, contrasting with high-fitness clones that did not demonstrate a significant increase in clonal expansion. Our data also revealed that Npm1cA/+ mutations stimulated HSC expansion significantly more than Dnmt3aR882H/+. Interestingly, a subset of Npm1cA/+ also acquired a HSC aged-like phenotype with increased self-renewal, megakaryocyte bias and upregulation of aging transcriptomic signatures. The acquisition of this phenotype was heterogeneous across Npm1cA/+ clones and associated to specific cell-of-origin transcriptional states. Overall, our data revealed how different initial HSC states can drive highly heterogeneous responses upon the acquisition of the exact same preleukemic mutations. These findings highlight the relevance of HSC heterogeneity as a potential driver of MMs phenotypic heterogeneity.

Keywords: hematopoietic stem cell, lineage tracing, fate perturbation

6:45 PM – 7:45 PM

TOPIC: KIDNEY

416

NEW TRANSLATIONAL SCREENING ASSAY WITH PHENOTYPIC AND TRANSCRIPTOMIC READOUTS USING IPSC DERIVED CELLS AND ORGANOIDS FROM A NEPHROTIC SYNDROME PATIENT

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Prof. Nishinakamura's group previously generated induced pluripotent stem cell (iPSC) derived kidney organoids from a patient suffering from a congenital nephrotic syndrome caused by the mutations (c.G1379A, c.2515delC) in the nephrin (NPHS1) gene. The patient iPSC derived kidney organoids show impaired nephrin phosphorylation and localization. Despite the advantages of iPSC derived models in disease modeling and drug discovery, scalability of generation and robustness of phenotypic readouts are often challenging and prevent its application in drug discovery. To overcome this, we further developed this system into a robust, reproducible, and high-throughput organoid assay useful for the progression of drug discovery programs. To enable readouts in 384w, we scaled up the production of iPSC-podocytes and iPSC-kidney organoids. 3D whole mount immunostaining readout and automated western blot have been developed to quantify the phenotypic signature of iPSC-derived podocytes and kidney organoids. Bulk ScreenSeq™ and single-nucleus sequencing were applied to characterize the transcriptomic profiles of both systems. Molecular disease signature has been identified in 2D patient podocytes, while a specific lack of nephrin phosphorylation phenotype was confirmed via automated western blotting only in patient kidney organoids, but not in 2D podocytes. Besides, patients kidney organoids completely lacked nephrin signals in synaptopodin-positive regions of glomerular structures. We further quantified the general nephrin deficit with a new script-based whole mount imaging analysis. Finally, transcriptomic analysis revealed molecular disease signatures in kidney organoids. Our results indicate kidney organoids can recapitulate the nephrotic disease phenotype and molecular disease signature, highlighting the translational relevance of these kidney organoid models for drug discovery in the field of podocytopathies. We successfully developed and characterized a patient-derived kidney organoid assay and made it available in a high-throughput format, thereby overcoming current challenges in scalability and robustness of readout. This translational in vitro system will prove useful in the identification and validation of candidate targets and for the progression of new kidney therapeutics.

Keywords: iPSC-derived kidney organoids, nephrotic disease, high-throughput screening

6:45 PM – 7:45 PM

418

AN INTEGRATED PROTEOME-TRANSCRIPTOME ORGANOID ATLAS ILLUMINATES CORE CONCEPTS OF KIDNEY DISEASE

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The use of kidney organoids as a model for studying kidney disease shows great promise, but their potential is limited by our limited understanding of the proteins they express and their functional profiles. In this study, we aimed to address this limitation by examining the proteome and transcriptome of organoids throughout their culture period and in response to TNFα, a cytokine stressor. In our study, we employed proteomic analysis to compare kidney organoids with other established model systems and native tissues, including native glomeruli and cultured podocytes. We examined the developmental trajectory of organoids and explored their innate immune responses, thereby expanding the applicability of organoids as a valuable model system in the field of nephrology. Additionally, we performed a comprehensive comparison of our proteomic data with both bulk and single-cell transcriptomics data, providing a more comprehensive understanding of the molecular landscape of kidney organoids. We found that older organoids displayed increased accumulation of extracellular matrix while showing decreased expression of glomerular proteins. By integrating single-cell transcriptome data, we discovered that most changes in the proteome were localized to podocytes, tubular cells, and stromal cells. Treatment of the organoids with TNFα resulted in the differential expression of 322 proteins, including cytokines and complement components. Importantly, the transcript expression of these 322

proteins was significantly higher in individuals with poorer clinical outcomes in proteinuric kidney disease. Notably, key proteins associated with TNFα (C3 and VCAM1) were found to be increased in both human tubular and organoid kidney cell populations, indicating the potential of organoids to advance the development of biomarkers. VCAM1 was localized to the descending thin limb (DTL) of proteinuric patients with kidney disease. By integrating various "omic" layers of kidney organoids, incorporating a relevant cytokine stressor, and comparing with human data, we highlight the significance of kidney organoid modeling in understanding and studying complex human kidney disease.

Keywords: proteomics (mass spectrometry), kidney disease (FSGS), gene expression analysis

6:45 PM – 7:45 PM

420

MEK/ERK AND CDK INHIBITORS MITIGATE CYST FORMATION IN AUTOSOMAL DOMINANT POLYCYSTIC KIDNEY DISEASE PATIENT-DERIVED TUBULOIDS

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Autosomal dominant polycystic kidney disease (ADPKD), a hereditary kidney disorder caused by mutations in the PKD1 or PKD2 genes, is characterized by fluid-filled cysts growing in the glomeruli. Tolvaptan, a vasopressin receptor 2 antagonist, is the approved drug, but its limited efficacy and side effects side effects necessitate the need for new drugs. In this study, we utilized kidney tubuloids to establish the dual drug screening platform for ADPKD. Healthy control and ADPKD patient tubuloid strains were generated from urine-derived adult stem cells, with ADPKD tubuloids showing increased susceptibility to cisplatin-induced nephrotoxicity and larger size compared to healthy controls. Stimulation of cystogenesis by forskolin or desmopressin resulted in a 2-fold increase in ADPKD tubuloids compared to the controls. Our platform integrated imaging-based and multiplex single-cell RNA sequencing, providing insights into the pharmacologic mechanisms of drugs at the molecular level. We evaluated 15 drugs related to cystogenesis using this platform. We performed single-cell transcriptomics on 26,132 cells collected from tubuloids of 1 control and 2 ADPKD patients that expressed various renal markers including distal convoluted tubules, loop of henle, and collecting duct. Comparative analysis of each cluster revealed upregulation in cell cycle, proliferation, mTOR, and cAMP-related response pathways in ADPKD tubuloids compared to controls. Furthermore, drugs associated with the MEK/ERK and CDK pathways exhibited efficacy in reducing cystogenesis. Our drug screening platform contributes to understanding the pathogenesis of ADPKD and developing therapeutic agents.

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT)(RS-2023-00225239).

Keywords: autosomal dominant polycystic kidney disease, kidney tubuloids, drug discovery

6:45 PM – 7:45 PM

422

INCREASED FIBROSIS AND CYSTS IN CEP290-MUTANT RENAL ORGANOIDS RECAPITULATING NEPHRONOPHTHISIS

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Nephronophthisis (NPHP) is a fibro-cystic renal disease part of the ciliopathy spectrum, a group of disorders caused by dysfunctional primary cilia. These quasi-ubiquitous cellular organelles play a role in signal transmission, thereby regulating cell behavior. While mutations in ciliopathy genes frequently cause cystic kidney disease, the underlying mechanism remains unclear. A plethora of signaling pathways have been implicated in various animal and renal organoid models, the latter focusing mostly on Autosomal Dominant Polycystic Kidney Disease (ADPKD). Given important differences in the pathological findings between ADPKD and NPHP, it is likely that distinct mechanisms underlie the cysts in these two disorders. The aim of this work was to elucidate the pathomechanism underlying NPHP caused by dysfunction of CEP290, a frequently mutated centrosomal protein. We found that CEP290 expression in iPSC-derived organoids peaks at the nephron progenitor stage. We next created isogenic iPSC lines harboring frameshift mutations in CEP290, which led to minimal residual CEP290 protein due to exon-skipping of an in frame exon. Since this reproduces findings in patients with CEP290 mutations, we next generated renal organoids to model NPHP. Lack of CEP290 did not interfere with differentiation into all renal cell types but caused elongated cilia in mutant intermediate mesoderm cells and decreased ciliation rates in nephron progenitor cells and in distal tubules of mature organoids. We observed increased fibrosis in mature mutant organoids compared to controls. Treatment with Forskolin to increase cAMP led to a dramatic increase in cysts, arising predominantly from distal tubules in mutant vs control organoids, without a significant increase in proliferating cells. RNA sequencing with/without Forskolin of whole organoids and of Epcam-purified tubular cells identified alterations in multiple pathways. This work establishes a new model for CEP290-mediated NPHP presenting with the combination of fibrosis and cysts.

Keywords: primary cilia, renal cysts, renal organoid

6:45 PM – 7:45 PM

424

EFFICIENT PODOCYTE-SPECIFIC GENOME EDITING IN CRISPR/CAS9 HIPSC LINE-DERIVED KIDNEY ORGANOIDS

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Human induced pluripotent stem cell (iPSC)-derived kidney organoids offer valuable resources for disease modelling, drug screening, and tissue engineering applications. However, specific gene editing strategies for targeting podocytes in iPSC-derived kidney organoids have not been developed. In this study, we designed a model to precisely target genes in iPSC-derived podocytes employing the Campylobacter jejuni CRISPR/Cas9 (cjCAS9) system. Our approach involved the design of a lentiviral plasmid incorporating a podocyte-specific promoter (NPHS2) to target renal podocytes. The cjCAS9 is small in size offering opportunities for using lentiviral systems with limited carrying capacity and was utilized to sustain high on-target gene editing. Additionally, incorporation of the puromycin resistance gene with mPGK promoter facilitated selection of 'pure' modified iPSCs. Following plasmid isolation from E.coli and purification, plasmid DNA was validated through restriction enzyme digestion and sequencing. Lentivirus was packaged in HEK293T cells and transduced into iPSCs to generate a novel iPSC cell line suitable for genetic editing targeting podocytes in kidney organoids. The modified iPSCs were able to differentiate into kidney organoids displaying glomerular-like structures containing podocytes, as well as proximal and distal tubular structures. cjCAS9 integration in the genome was confirmed by PCR on both DNA and RNA samples isolated from kidney organoids. In conclusion, we developed an iPSC line capable of efficiently and specifically targeting podocytes in kidney organoids, presenting an innovative approach that holds promise for in-depth exploration of the role of diverse genes in podocyte biology using iPSC-derived kidney organoids.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW, supported by Novo Nordisk Foundation grants (NNF21CC0073729)).

Keywords: iPSC-derived kidney organoids, CRISPR/Cas9, podocytes

6:45 PM – 7:45 PM

426

NEW INSIGHTS INTO DISEASE MECHANISMS IN HNF1B-ASSOCIATED DYSPLASTIC KIDNEY MALFORMATIONS USING HPSC-DERIVED KIDNEY ORGANOIDS

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Hepatocyte nuclear factor 1B (HNF1B) encodes a transcription factor expressed in developing human kidney epithelia. Heterozygous HNF1B mutations are the commonest monogenic cause of dysplastic kidney malformations (DKMs). To understand their pathobiology, we generated heterozygous HNF1B mutant kidney organoids from CRISPR-Cas9 gene-edited human ESCs and iPSCs reprogrammed from a family with HNF1B-associated DKMs. Mutant organoids had abnormal anatomical features, such as enlarged malformed tubules displaying deregulated cell turnover. These mutant tubules also exhibited abnormal physiology, as they resisted cAMP-induced dilatation seen in controls. Bulk and single-cell RNAseq experiments showed downregulation of genes implicated in Mendelian kidney tubulopathies and ion transport and abnormal WNT, calcium, and glutamatergic pathways, the latter hitherto unstudied in developing kidneys. ScRNAseq further revealed abnormal populations of tubular epithelial and glomerular cells in the mutant cells without an equivalent in the controls. Conversely, normal proximal tubule epithelial cells were almost completely absent in the mutants. Intriguingly, Glutamate ionotropic receptor kainate type subunit 3 (GRIK3) was one of several glutamate receptor subunits upregulated in malformed nephron tubules of mutant organoids and was also detected in HNF1B mutant fetal human dysplastic kidney epithelia. Moreover, scRNAseq analyses showed that it was expressed largely in the same cell populations as the mutant version of HNF1B. These results reveal morphological, molecular, and physiological roles for HNF1B in human kidney tubule morphogenesis and functional differentiation and illuminate the developmental origin of mutant-HNF1B-causing kidney disease.

Funding Source: Majority of his work was funded through grants from Kidney Research UK project grant JFS/RP/008/20160916; a Kidneys for Life pump priming projects 2021 and Engineering and Physical Sciences Research Council (EPSRC)/Medical Research Council (MRC) Centre forof Doctoral Training grant EP/L014904/1.

Keywords: human pluripotent stem cells, organoid, kidney

6:45 PM – 7:45 PM

428

ASYNCHRONOUS DIFFERENTIATION DURING IN VITRO KIDNEY ORGANOID GENERATION DRIVES OFF-TARGET PATTERNING

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Human pluripotent stem cell-derived kidney organoids represent an accessible model system for studying the developing kidney, disease modelling and tissue engineering. However, such in vitro models are imperfect. Thorough microscopic and transcriptional approaches have identified both lack of nephron maturation in kidney organoids and the presence of 'off-target' populations not present in vivo. These differences between organoids and the fetal kidney need to be addressed to advance the application of such differentiation protocols. We performed a single-cell transcriptional analysis of the complete kidney organoid time-course from the equivalent of the gastrulation stage to mature organoids to further understand the cellular complexity and origin of 'off-target' endpoints during the protocol. This includes protocol modifications to shift patterning along mediolateral and rostrocaudal axes. Each protocol variation was sampled longitudinally to produce a complete temporal series. Analysis of this data set provided insights into the origins and timing of 'off-target' populations. We then connected the temporal series into a developmental trajectory using diffusion mapping, RNA velocity and network analysis. While a clear lineage divergence emerged between nephrons and non-nephrons after two weeks of culture, a population of cells remained behind or moved ahead of the predominant differentiation state at all time points. This suggests a component of heterogeneity results from asynchronous differentiation, contributing to 'off-target' differentiation. A connectivity network of all populations across time was generated by applying the Cluster Alignment Tool to compare and calculate the Euclidean distance between populations. Comparing all populations generated a network that predicted population trajectory across the entire protocol. This network supported the inference that asynchronous differentiation contributed to 'off-target' patterning. By generating a temporal kidney differentiation atlas, we show that asynchronous differentiation occurs during in vitro differentiation to kidney organoids. Such asynchrony is likely to occur in other directed differentiation protocols. Controlling this process provides an avenue through which organoid protocols can be improved.

Funding Source: This work was supported by the Australian Research Council (SR1101002; DP190101705), the National Institute of Health (DK107344) and the National Health and Medical Research Council (GNT1136085).

Keywords: kidney organoid, asynchrony, bioinformatics

6:45 PM – 7:45 PM

430

IPSC-DERIVED PROXIMAL TUBULE-LIKE CELL MATURATION IN ADME-CHIP FOR TOXICITY TESTING

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Complex human in vitro absorption, distribution, metabolism and excretion (ADME) models involving co-culture of key organs to mimic certain exposure routes, establishing functionally relevant as well as physiologically based pharmacokinetic (PBPK) distribution behaviour in the culture environment present a challenge. In our recent study, we developed a PBPK-compliant ADME-Chip (HUMIMIC Chip4) with a downscale factor of 1:100.000 of the human body. The co-culture of an intestinal barrier model for absorption, liver for the main metabolism, a kidney model with proximal tubular-like cells (PTL) and podocytes for excretion, and neuronal spheroids were optimized in the chip. We studied the maturation and functionality of the kidney cells and exposed the Chip to Haloperidol, an antipsychotic medication in the butyrophenone family and to Carbamazepine, a tricyclic compound with anticonvulsant properties through different routes with a repeated dose to observe their metabolite induced toxic effects on an organ-specific level. We initially optimized iPSC-derived PTL cells and the podocyte co-culture in the ADME-Chip and tested their barrier integrity via FITC-Inulin assay and functionality by Albumin uptake and CalceinAM release. Following the 21-day perfused culture of kidney cells, P-gp (MDR1) mediated release of Calcein AM was increased indicating transporter upregulation while repeated dose application of Haloperidol inhibited P-gp mediated Calcein AM release. We were able to observe podocyte stress response in transcriptomic levels as well as a decrease in barrier integrity of PTL in a dose-dependent manner. Carbamazepine on the other hand showed cytotoxicity for the kidney cells. These results indicate that physiological flow supports the iPSC-derived PTL cells in the chip to mature and respond to compounds in relation to humans in vivo.

HUMIMIC-Chip4 is a unique human systemic in vitro testing tool as a potential new approach methodology to replace animal models for predictive testing of compounds. Establishing (multi) organ culture in chips through iPSC-derived cells allows us to study patient-specific disease backgrounds on the chip with the aim of personalized medicine.

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Keywords: proximal tubule, microphysiological systems, ADME-Chip

6:45 PM – 7:45 PM

TOPIC: LIVER

432

UNCOVERING HEPATOBLASTOMA SUBPOPULATIONS USING PATIENT-DERIVED ORGANOIDS

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Hepatoblastoma, the most prevalent pediatric liver cancer, almost always carries a WNT-activating CTNNB1 mutation, yet exhibits notable molecular heterogeneity. To characterize this heterogeneity and identify novel targeted therapies, we performed comprehensive analysis of hepatoblastomas and tumor-derived organoids using single-cell RNA-seq, spatial transcriptomics, single-cell ATAC-seq and high-throughput drug profiling. We identified two distinct tumor epithelial signatures: hepatic 'fetal-like' and WNT-high 'embryonal-like' signatures, displaying divergent WNT signaling patterns. The liver-specific WNT targets were enriched in the fetal-like group, while the embryonal-like group was enriched in canonical WNT target genes. Gene regulatory network analysis revealed enrichment of regulons related to hepatic function such as bile acid, lipid and xenobiotic metabolism in the fetal-like subgroup but not in the embryonal-like subgroup. In addition, the dichotomous expression pattern of the transcription factors HNF4A and LEF1 allowed for a clear distinction between the fetal- and embryonal-like tumors. We also performed high-throughput drug screening using patient-derived tumor organoids and identified sensitivity to multiple inhibitor classes, most notably HDAC inhibitors. Intriguingly, embryonal-like tumor organoids, but not fetal-like tumor organoids, were sensitive to FGFR inhibitor treatments, suggesting a dependency on FGFR signaling. In summary, our data uncover the molecular and drug sensitivity landscapes of hepatoblastoma and pave the way for the development of targeted therapies.

Keywords: hepatoblastoma, organoids, liver cancer

6:45 PM – 7:45 PM

434

DRUG-TESTING IN A PATIENT SPECIFIC IPSC BASED LIVER MODEL FOR ADULT AND PAEDIATRIC ALPHA-1-ANTITRYPSIN DEFICIENCY

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Alpha-1-antitrypsin (AAT) is a serine protease inhibitor which is produced and secreted from hepatocytes. It plays a crucial role for attenuating neutrophil elastase mediated inflammatory processes, especially in the lungs. Severe Alpha-1-antitrypsin deficiency (AATD) is in 95% of the cases a result of an autosomal-dominantly inherited mutation in the SERPINA1 gene resulting in a homozygous Glu342Lys substitution (Z-AAT). This leads to miss-folding of AAT and accumulation of the Z-variant within the endoplasmic reticulum (ER) of hepatocytes. Besides a severe lung phenotype, which is common in adults, AATD can manifest in two distinct liver phenotypes: Liver fibrosis/cirrhosis in adults and cholestasis in children. Up to now, there is no approved treatment for the liver phenotypes. Moreover, it is not known, why it varies between children and adults and why there is a dichotomy of disease onset. In this project, we collect and bank urine derived mesenchymal stem/stromal cells (MSCs) from adult and paediatric AATD-patients, who are homozygous for the Z variant of AAT (Pi*ZZ genotype), and reprogram them into iPSCs. In order to model AATD, these iPSCs are then differentiated into hepatocyte-like cells (HLCs) via an established 3-step protocol. Healthy as well as patient derived iPSCs could be successfully differentiated into HLCs as documented by immunofluorescence staining and qRT-PCR for hallmark hepatocyte markers. Healthy and diseased lines could be distinguished by comparing AAT and Z-AAT levels in immunofluorescence and Western Blots. Treatment of HLCs with the potential therapeutical drugs Carbamazepine (CBZ), SAHA, Kifunensine (KIF) and Cysteamine (CYS) had direct positive effects on Z-AAT accumulation in the hepatocytes and/or on AAT secretion. These results give an early insight into the potential of patient-specific HLCs generated from iPSCs as a suiting model for general research on the individual genetical and molecular background of AATD. This would further allow its functionality as a drug testing system for various compounds as potential therapeutical treatments to decrease hepatocyte damage due to high Z-AAT accumulations and to enhance secretion of correctly folded AAT.

Funding Source: Ministry of Culture and Science North Rhine-Westphalia: iPSC_AATD Else Kröner-Fresenius-Stiftung: 2020_EKEA.64 Medical Faculty HHU Düsseldorf

Keywords: alpha-1-antitrypsin deficiency (AATD), iPSC derived hepatocyte-like cells, endoplasmic reticulum stress

6:45 PM – 7:45 PM

436

OPTIMIZATION OF 3D IPSC ORGANOID CULTURE FOR ENHANCED HEPATOCYTE DIFFERENTIATION

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Induced pluripotent stem cell (iPSC)-derived hepatocytes hold promise for applications in disease modelling, drug screening and cell therapy. However, current differentiation protocols yield hepatocytes with immature phenotypes. This study aims to optimise hepatocyte differentiation from iPSCs using the clinostat-based bioreactor system ClinoStar, which utilises a clinostat principle to generate large, uniform 3D constructs from iPSCs closely mimicking the in vivo liver microenvironment. Human iPSCs were cultured in the ClinoStar system to form organoids under varying conditions of seeding density, static vs dynamic media flow, and culture duration. Differentiation was carried out using a hepatocyte differentiation protocol. Organoid size, cell proliferation, ATP levels and pluripotency marker expression were analysed. Hepatocyte differentiation was monitored by measuring levels of albumin, HNF4α, and CYP3A4. Proteomics analysis characterised the protein expression profiles, allowing comparison of hepatocyte phenotypes over time and optimising differentiation conditions to promote maturation. Preliminary results suggest maintaining high cell density and pluripotency enhances hepatocyte differentiation. Optimising 3D organoid differentiation may provide a robust platform for generating mature hepatocytes.

Keywords: hepatocytes, differentiation, organoids

6:45 PM – 7:45 PM

438

ANALYSIS OF SUPPRESSION EFFECTS OF IPS CELL-DERIVED EXTRACELLULAR VESICLES ON MOUSE MELANOMA METASTASIS

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Metastases are still incurable and result in the development of clinical complications and decreased survival for cancer patients. Recently, several studies have shown that extracellular vesicles (EVs) play important roles in the regulation of tumor progression. Here, an induced pluripotent stem (iPS) cell line was focused as a more anaplastic cell line, potentially producing EVs with higher metastasis-suppressive effects. The iPS-EVs were introduced into the tail vein nine times before introducing Nanog+F10 cells. Two weeks later, the liver and lung were

resected, and metastatic colonies were quantified. The involvement of macrophages (invasion inhibiting ability, phagocytic activity) and cytotoxic T cells (cytotoxicity) was evaluated using J774.1 and CTLL-2 cell lines. iPS-EVs showed high-level effects in every item relevant to metastasis suppression. Differential expression analysis of miRNAs in EVs and functional network database analysis revealed that dominant regulatory miRNAs were predicted. The candidate hub genes most highly associated with the metastasis suppression mechanism were predicted as ten genes, including Ins1 and Kitl, for iPS-EVs. Our findings elucidate that iPS-EVs suppress melanoma metastasis. We will investigate the predicted dominant regulatory miRNAs and hub genes in the future.

Keywords: iPS cells, extracellular vesicles, metastasis suppression

6:45 PM – 7:45 PM

440

MODELLING CHOLESTASIS USING IPSC DERIVED HEPATOCYTES

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Cholestasis, a liver pathology characterised by impaired bile formation and flow, results from genetic mutations in canalicular transporters or drug-induced causes. Drug-induced cholestasis (DIC) can be life threatening and often causes clinical trial failures or withdrawal of licenced drugs. Current pre-clinical models inadequately replicate human cholestasis due to species-specific differences and rapid loss of bile production in primary human hepatocyte ex vivo culture. This has hampered our mechanistic understanding of this disease, limiting our ability to identify DIC-causing drugs before they reach patients. iPSC-human hepatocytes (iHEPs) and advanced ex vivo culture techniques offer a potential solution to these problems. Indeed, recent advances in iHEP technology have resulted in an ability to phenocopy key features of metabolic and virally mediated pathology. To date, however, there has been less success in generating iHEPs with functional bile canaliculi (BC) and canalicular transporters as is critical for modelling cholestasis. This study aimed to overcome this roadblock by developing multipolar iHEPs that possess functional canalicular transporters and could model DIC. iPSCs underwent an optimised 25-day hepatocyte differentiation protocol. DIC was induced with bosentan, a BSEP inhibitor, or by feeding excess chenodeoxycholic acid for 6 days. Functional, immunological and gene expression assays, along with confocal and transmission electron microscopy, were employed to validate iHEP physiology and characterise the induced cholestatic phenotype. The new differentiation cocktail in combination with sandwich culture significantly improved the functionality, polarisation, and ultrastructural phenotype of iHEPs. BC expressed canalicular transporters, were sealed by tight junctions and developed a brush border, closely resembling normal liver tissue. BC functionality was confirmed by active excretion of a fluorescent MRP2 substrate into the canalicular lumen. DIC caused by BSEP inhibition and bile acid overload demonstrated changes in BC and mitochondrial morphology/ultrastructure, like canalicular dilatation and mitochondrial cristae curling, as seen in patient tissue samples, as well as mitochondrial dysfunction, ER stress and inflammatory cytokine secretion.

Keywords: cholestasis, disease modelling, hepatocytes

6:45 PM – 7:45 PM

442

PPAR- AND DPP4 SIGNALING ARE POTENTIAL TARGETS FOR TREATING NAFLD - REVEALED WITH AN IPSC-DERIVED HLC MODEL

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Non-alcoholic fatty liver disease (NAFLD) has an estimated global prevalence of 30 % and manifests by hepatocyte ballooning from excessive lipid storage, called steatosis. Chronically elevated fatty acid (FA) flux hampers the normal FA metabolism, resulting in lipotoxicity, apoptosis and inflammation. This initiates the progression to non-alcoholic steatohepatitis (NASH), which can develop into life-threatening liver cirrhosis or hepatocellular carcinoma. Regardless of the high prevalence, the multifactorial character and low public awareness of NAFLD complicate studies and drug development. We derive hepatocyte-like cells (HLCs) from several iPSC-lines (2 patient-derived/ 2 controls) and treat them for one week with oleic acid (OA), one of the most abundant monounsaturated fatty acids in the human body. With our relatively long incubation time, we try to mimic the chronic effects of life-long elevated FAs. Interestingly, we found overlapping differentially regulated pathways upon NAFLD-induction throughout all individuals, indicating their importance during the disease. In line with clinical studies, we detected the upregulation of lipid metabolism (PPAR pathway) and downregulation of the glutathione pathway – emphasizing the relevance of our model. Furthermore, we detected genes of inflammatory pathways upregulated, among others TLR-4, CCL20, ACE2 and ICAM1. Interestingly, these genes are associated with dipeptdidyl peptidase 4

(DPP4) which was secreted at significantly higher levels upon OA treatment. DPP4 is a serine protease involved in the hormonal regulation of the metabolism. Elevated DPP4 levels are associated with several metabolic diseases including NAFLD. Taken together, these findings suggest inflammatory signaling and corresponding progression of the disease. By manipulating PPARα and DPP4 signaling with Vildagliptin, a DPP4-inhibitor as well as Pemafibrate, a selective PPARα agonist, we are able to identify target pathways reducing the hepatic lipid load and respective progression of the disease. Together, our findings not only help to identify potential novel NAFLD biomarkers and anti-NAFLD drugs, but also provide essential information on the hepatocytes` contribution to the disease.

Funding Source: Else Kröner-Fresenius-Stiftung – 2020_EKEA.64 Medical faculty of Heinrich-Heine University Düsseldorf

Keywords: non-alcoholic fatty liver disease, iPSC-derived hepatocyte-like cells, dipeptidyl peptidase 4

6:45 PM – 7:45 PM

444

PLURIPOTENT STEM CELL-DERIVED HEPATOCYTES AND BETA CELLS TO ADVANCE DRUG DISCOVERY

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Effective drug discovery requires representative cell models to accelerate development and increase the number of compounds reaching pre-clinical trials. A major challenge in using human induced pluripotent stem cell (hiPSC)-derived cells is ensuring that the cells accurately reflect the native phenotype and functionality, as well as availability of large quantities of cells with minimal batch-to-batch variability. To address this, we have developed hiPS Beta Cells (hiPS-beta) and hiPS-hepatocytes (hiPS-HEP) for drug discovery applications. By cryopreserving the hiPS-derived cells during the last maturation step, we ensure that the thawed cells are mature with the expected phenotype and genotype. We show that hiPS-beta cells express key maturation markers including C-peptide, UCN3 and NKX6.1, and that over 30% of the cells are insulin positive by flow cytometry. In addition, hiPS-beta cells exhibit similar expression of mRNA to primary human islets, particularly for PDX1, GLUT1 and NeuroD1. hiPS-beta cells produced from 3 iPSC lines were able to undergo glucose-stimulated insulin secretion, reaching up to 10-fold increased secretion after exposure to 20 mM glucose, demonstrating their functionality. Insulin secretion was also upregulated after incretin exposure. Our hiPS-HEP cells display key hepatocyte markers including HNF4α and α1AT, as well as comparable or higher albumin secretion (1.5-3.5 µg/mg protein) to primary human hepatocytes (1.0-2.0 µg/mg). The cells show functional metabolic characteristics of mature hepatocytes such as LDL and fatty acid uptake and respond to insulin with phosphorylation of AKT. Importantly, hiPS-HEP demonstrate stable CYP activity for 20 days in culture and have comparable mRNA expression levels of eight common drug-metabolising CYP450 genes to primary hepatocytes. Finally, hiPSC-HEP show increased sensitivity to 4 known hepatotoxic compounds after chronic exposure and perform similarly to HepaRG cell line and primary 3D spheroids. Taken together, this data demonstrates that hiPS-beta and hiPS-HEP cells are effective and robust tools for drug discovery, with both cell types displaying similar metabolism and gene expression profiles and levels to primary cells.

Keywords: hiPS-derived hepatocytes, hiPS-derived beta cells, disease modelling

6:45 PM – 7:45 PM

446

SAFETY EVALUATION OF ADENO-ASSOCIATED VIRUS VECTORS USING HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED LIVER TISSUE MODELS

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Recently, gene therapy using adeno-associated virus (AAV) vectors has been widely performed. The safety evaluation is essential for the development of gene therapy products. It is necessary to evaluate their safety in the liver because AAV vectors are known to accumulate in the liver. In this study, we evaluated the safety of three serotypes of AAV vectors (AAV2, 8, and 9 vectors) using human induced pluripotent stem (iPS) cell-derived liver tissue models. This human liver tissue model is considered to be useful for the safety evaluation of AAV vectors in the human liver because this model has hepatic functions including drug-metabolizing activities. The transduction efficiency of AAV vectors in human iPS cell-derived liver tissue models was evaluated by immunofluorescent staining and RT-qPCR. Among the AAV2, 8, and 9 vectors, AAV2 vectors showed the highest gene transduction efficiency. Severe cytotoxicity was not observed in AAV-transduced human iPS cell-derived liver tissue models. In addition, AAV vector transduction did not affect the expression levels of hepatic markers and secretion capacity of blood coagulation-related factors in human iPS cell-derived liver tissue models. Furthermore, innate immune and inflammatory responses were not observed in AAV-transduced human iPS cell-derived liver tissue models. However, the CYP3A4 activity was decreased by approximately 20%, 40%, or 51% in human iPS cell-derived liver tissue models by AAV2, 8, or 9 vector transduction, respectively. The decreases in the CYP3A4 activity were consistently observed in the human liver S9 fraction incubated with AAV2, 8, or 9 vectors. These findings indicate that AAV vectors did not show a negative effect on hepatic functions, while they reduced the CYP3A4 activity. Because CYP3A4 plays a crucial role in the metabolism of various commercially available drugs, it is recommended to evaluate drug-vector interactions when administering AAV vectors. In the future, we aim to clarify the

mechanism by which AAV vectors decrease the activity of CYP3A4. In addition, we will improve AAV vectors which do not decrease the CYP3A4 activity.

Funding Source: This research was supported by the iPS Cell Research Fund and the Japan Agency for Medical Research and Development (AMED) (JP21gm1610005, JP23bm1323001).

Keywords: adeno-associated virus vectors, safety evaluation, drug-vector interactions

6:45 PM – 7:45 PM

448

DEVELOPMENT OF A COMPUTATIONAL-TO-LABEL FREE DRUG REPURPOSING SCREENING STRATEGY IN AN HIPSC-BASED MODEL OF COMMON METABOLIC LIVER INJURY

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Human induced pluripotent stem cell (hiPSC)-derived models carry potential for drug discovery. However, immature and simplified models and laborious screening read-outs limit clinical relevance, efficacy, and scalability. Focusing on the unmet need for steatotic liver disease (SLD)–the most common liver disease–we integrated independent computational compound predictions, label-free hepatocyte injury evaluation of drug candidates in a hiPSC-derived injury model of Hepatocyte-like cells (HLCs), and experimental validation of their effect on disease hallmarks. Through Weighted Gene Co-expression Analysis (WGCNA), we pinpointed targets and pathways associated with SLD and predicted potential drug candidates affecting these functions. Knowledge Graph link prediction and reverse transcriptomic signature mapping provided a framework to generate hypotheses of potential drug-target interactions. Comprehensive Omics Analysis, utilizing single hepatocyte datasets, enabled us to construct and interrogate biological networks, employing techniques such as Random Walk with Restart (RWR), node centrality algorithms, and drug proximity analysis to filter for key proteins and identify repurposed FDA-approved compounds. Converging these methodologies yielded candidates. To test these, we subjected HLCs to SLD injury. Combining HLC exposure to a steatotic and lipotoxic milieu, muscle and adipose inter-organ SLD signaling molecules, and PBMC-co-culture for inflammatory modeling, activated SLD hallmarks, namely steatosis and mitochondrial dysfunction. Neural network label-free imaging analysis could discriminate injured from healthy cells, permitting label-free scoring of hepatocyte injury. In a proof-of-principle, we tested two predicted repurposing candidates for anti-SLD effects. Candidate 1–involved in mitochondrial respiration–reduced injury scores via label-free imaging, which was validated by decreased steatosis of the cells in conventional staining. In sum, we establish an in silico to in vitro pipeline using hiPSCs for discovering anti-SLD drugs.

Keywords: steatotic liver disease, drug repurposing, hepatocytes

6:45 PM – 7:45 PM

450

COMPARATIVE EFFECTS OF EXTRACELLULAR VESICLES DERIVED FROM NANOG-OVEREXPRESSING MELANOMA AND COLON CANCER CELLS ON THEIR METASTASIS

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Extracellular vesicles (EVs) derived from Nanog+F10 suppressed the metastasis of Nanog+F10. To examine such effects of Nanog in other cell lines of cancer, we generated an Nanog overexpressing mouse colon26 (Nanog+col26). In vitro tests of Nanog+col26 revealed that the cell proliferation rate and migration activity were higher levels than those of colon26. However, the migration activity was same level and the invasion ability was a lower level as compared to colon26. These contrasted Nanog+F10 that showed higher levels than F10 in every test. These suggested that the functional role of Nanog in Nanog+col26 was different from that in Nanog+F10. Nevertheless, metastasis suppression effect was also observed with Nanog+col26-EVs. The metastasis suppression effect was suspected to be brought about via immune cell systems. Here, we focused on the involvement of phagocytic activity and analyzed it using a cultured macrophage cell line J774.1 by a fluorescent nanobead uptake method. J774.1 cells were loaded with PKH26-labeled EVs. The number of beads taken up by Nanog+col26-EVs-loaded cells was 1.5 times greater than col26-EVs-loaded cells, suggesting the suppression of the proliferation of cancer cells. The differential expression analyses of miRNAs were conducted to predict miRNAs that were most relevant to the metastasis suppression effects. The number of miRNAs whose expression was statistically significantly ($p < 0.05$) increased (up: Fold change (Fc)>2) and decreased (down: Fc< 1/2) was one (miR-122-5p) and two (miR-22-3p, miR-379-5p), respectively. On the other hand, the number of those increased and decreased in Nanog+F10-EVs as compared to F10-EVs were four and six, respectively. In both cases, the number of miRNAs that changed significantly was lower than expected. Contrarily, the numbers of miRNAs that varied in Nanog+col26-EVs versus Nanog+F10-EVs were remarkably large (up: 43, down: 38), suggesting different functional effects of Nanog on both EVs.

Keywords: colon26, extracellular vesicles, metastasis suppression

6:45 PM – 7:45 PM

452

MODELING NAFLD: PATHOLOGICAL FEATURES IN IPSC-DIFFERENTIATED HEPATOCYTES WITH AN OBESOGENIC PROFILE

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Obesity is not solely dictated by genetics but is instead a complex interplay of genetic and environmental factors. This intricate relationship characterizes obesity as a blend of genetic and environmental disruptions, often traceable back to early developmental stages. Environmental factors, such as consuming energy-dense foods and maintaining a sedentary lifestyle, are recognized contributors to obesogenicity. However, the synergistic impacts of gene-environment interactions remain elusive. Investigating the influence of multiple genetic variations leading to subtle changes in gene expression proves challenging in monogenic or high-fat-fed animal models of obesity. In this study, we harnessed the potential of human induced pluripotent stem cells (hiPSCs) derived from both normal and genetically obese individuals. These hiPSCs were differentiated into hepatocytes using an established protocol. The hepatocytes originating from obese iPSCs displayed notable characteristics, including increased lipid accumulation, reduced E-CAD expression and excessive cell death, pointing towards the intricate nature of obesity-related pathophysiology. Furthermore, transcriptome analyses revealed distinctive pathways enriched in obese iPSCs, including those associated with hepatic fibrosis and liver cancer. Further validation of specific genes identified potential contributors to altered lipid accumulation and differential expression of genes linked to hepatic fibrosis—an established hallmark of non-alcoholic fatty liver disease (NAFLD). This study underscores the potential of iPSCs derived from genetically obese individuals as a valuable model for delving into the complexities of this multigene disorder and its impact on organ development. Furthermore, it offers insights into the pathologies associated with NAFLD, providing a broader understanding of the health implications connected to obesity.

Keywords: iPSC-hepatocyte differentiation, obesity, non-alcoholic fatty liver disease modeling

6:45 PM – 7:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

454

BEYOND TRADITION: ADVANCING REGENERATION THROUGH 50% ETHANOL-PRESERVED AMNIOTIC EXTRACTS

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The human amniotic membrane (hAM) has garnered significant attention in regenerative medicine due to its unique properties, including anti-inflammatory, anti-scarring, and wound-healing capabilities. Various preservation methods have been explored to maintain the integrity and functionality of hAM for clinical applications. In this context, this research explores an innovative preservation strategy for the HAM using 50% ethanol (EtOH) as an alternative to conventional methods like DMEM-Glycerol or phosphate buffered saline (PBS). The primary objective is to evaluate the supportive potential of hAM extract on adipose stem cells (AMSCs) regenerative capacities. Our data show that AMSCs treated with hAME from 50% EtOH-preserved amniotic membranes exhibited a significantly higher proliferation rate ($p < 0.05$) compared to their counterparts, cultured on DMEM-Glycerol and PBS-preserved hAM. However, migration rates were uniform in cells cultured following preservation in 50% EtOH and DMEM-Glycerol, and each demonstrated significantly higher migration rates compared to the negative control and PBS group. Furthermore, the hAME showed a significantly greater protective effect on ASCs as compared to the negative control group, when exposed to H2O2-induced oxidative stress. This was evidenced by a lower apoptosis priming rate of 16-17% compared to DMEM-Glycerol ($p < 0.0001$), along with enhanced proliferation. These findings highlight the promising potential of 50% EtOH-preserved hAME in improving the cellular characteristics of ASCs. Encouraged by these findings, further investigations were conducted using a rat model with full-thickness skin wounds. Application of the extracts on the wounds showed an equivalent rate of wound healing compared to traditional methods. Our data show that using 50% EtOH as a preservation method for hAM as a novel preservation protocol significantly enhances regenerative properties, offering a viable alternative to conventional preservation techniques. This suggests that hAME preserved with 50% EtOH can be used as a better, more cost-effective alternative to traditional preservation method.

Funding Source: ASRT JESOR #5275, funded by the Egyptian Academy of Scientific Research and Technology (ASRT), Egypt. The Science and Technology Development Fund (STDF) (FLUG grant No. 46721).

Keywords: amniotic membrane, preservation techniques, skin regeneration

6:45 PM – 7:45 PM

456

ENGINEERING 3D MICROENVIRONMENTS FOR THE GENERATION OF VASCULARIZED HUMAN BEIGE ADIPOSE TISSUES: FROM ORGANOIDS TO TRANSPLANTABLE MICRO-TISSUES

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Beige adipose tissues are key regulators of human physiology through their unique plasticity and ability to dissipate energy as heat according to the needs. Activating beige adipose tissues represents a major therapeutic opportunity to address the rise of obesity and diabetes worldwide. Pathological conditions such as severe burns and cancer-related cachexia are also associated with hyperactivation of these tissues, extending their implications beyond the scope of metabolic diseases. However, in vitro models of human beige adipose tissue remain highly limited, hindering research on these tissues and the development of biotherapies. Here, we propose an innovative engineering approach enabling the generation of vascularized and functional human beige adipose tissue organoids using the stromal vascular fraction of human white adipose tissue as a source of adipose-derived mesenchymal stem cells (ASCs) and endothelial progenitors. To this aim, we engineered a defined chemical and biomechanical environment, applicable at multiple tissue scales, to guide the self-organization of ASC spheroids in a photopolymerizable gelatin hydrogel (GelMA). The chemical environment was fine-tuned to promote beige adipogenesis and vascular formation, while a defined biomechanical setting facilitated cell reorganization, leading to the development of a tissue architecture resembling native beige adipose tissue. Resulting organoids replicate key functional features of native beige adipose tissue, such as inducible expression of uncoupling protein-1 (UCP1), associated with increased uncoupled mitochondrial respiration and batokines secretion. The controlled assembly of these spheroids allows the morphogenesis of organoids at a centimeter scale, enabling the creation of transplantable vascularized beige adipose micro-tissues. Our approach represents a major advancement in the creation of in vitro 3D models of human beige adipose tissue, enabling a variety of applications ranging from fundamental research to biotherapies, including the development of relevant bioassays for screening thermogenic drugs.

Funding Source: Main funding: French National Agency for Research (hiPSC-Adipospheres ANR-18-CE18-0006, Printiss Project, grant ANR21 CE1941), LAAS-CNRS micro and nanotechnologies platform, a member of the French Renatech network.

Keywords: mesenchymal stem cells, adipose tissue organoid, vascularization

6:45 PM – 7:45 PM

458

HARNESSING THE POTENTIAL OF MESENCHYMAL STROMAL/STEM CELLS AS NOVEL TOOLS FOR HOST DIRECTED THERAPY AGAINST INFECTION

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Mesenchymal Stromal/Stem Cells (MSCs) and their products have emerged as a potential tool against infection, owing to their antimicrobial properties and ability to activate host immune, repair, and regeneration pathways. However, studies on application of MSC in livestock are scarce and host-directed therapies remain unexplored. In bovine mastitis, an inflammatory disease of the udder typically caused by intra-mammary infection, Mammary Epithelial Cells (MECs) act as sentinel cells, being the first to encounter invading pathogens and initiate the antimicrobial and immune response. As such, MECs represent a prime target for prospective MSC-based therapies, and are the focus of this study. To determine whether MSC products could potentiate the mammary epithelial response to injury, conditioned medium was generated from primary MSC cultures derived from bovine adipose tissue (n=3 calves, 5 months old). To explore whether TLR3 activation of MSCs would augment the production of factors which may enhance the epithelial response, MSCs were transiently stimulated with TLR3 agonist Poly(I:C). This resulted in a distinct secretome with elevated levels of anti-inflammatory marker IDO-2,3-dioxygenase ($p = 0.016$) and chemokine CCL5 ($p = 0.0175$). Next, primary MECs were isolated from abattoir tissues and exposed to Lipopolysaccharide (LPS) from Escherichia coli a common pathogen in mammary infection. As expected, LPS treatment upregulated expression of innate defence (MUCIN1 and Lactoferrin) and inflammatory (TNFα and IL-6) genes in MECs. Importantly, it was found that exposure to MSC-conditioned media (CM), prior to LPS stimulation resulted in enhanced ($p< 0.01$) epithelial cell expression of defence and immune response genes, an effect that was most pronounced when using CM from Poly(I:C)-activated MSCs, as was the case for TNFα (4-fold increase relative to no pre-treatment with CM, $p< 0.001$), IL6 (4-fold $p< 0.0001$), Lactoferrin (2-fold, $p = 0.01$) and MUCIN1 (6-fold, $p = 0.0003$). These data suggest that, in the context of bovine mastitis, MSC products (CM) may be beneficial in a prophylactic capacity, enabling the cells of the mammary gland to launch an augmented immune response upon infection.

Funding Source: EASTBIO

Keywords: infection, MSC, cell therapy

6:45 PM – 7:45 PM

460

THERAPEUTIC EFFECTS OF SKELETAL MUSCLE CELL SPHEROIDS DIFFERENTIATED FROM MESENCHYMAL STEM CELLS IN NEUROMUSCULAR DISEASES

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The dominant features of neuromuscular diseases (NMDs) are muscle weakness and loss. So, many studies have been conducted for finding of effective treatment to muscle loss in NMDs. Mesenchymal stem cell-based therapeutics using 3D cultured cells such as spheroid is one of the spotlighted fields in muscle regeneration. In previous studies, we have confirmed that tonsil-derived mesenchymal stem cells (TMSCs) can be differentiated into skeletal muscle cells (SKMCs), and these cells have characteristics of skeletal muscles. In this study, we established two types of neuromuscular disease rat models such as dexamethasone (Dex)-induced sarcopenia and volumetric muscle loss (VML). Spheroids formed from TMSC-derived skeletal muscle cells (TMSC-SKMC-spheroids) using microwell were transplanted into these two animal models, respectively. To evaluate the treatment effects, grip test, gait analysis and electromyography test were performed after TMSC-SKMC-spheroids implantation into rat models. Both rat models were improved in the motor functions of hind limbs and muscles were regenerated after TMSC-SKMC-spheroids transplantation. Furthermore, neuromuscular junctions of gastrocnemius muscle in both rat models were restored after transplantation. These results indicate that therapeutic potential of the TMSC-SKMC-spheroids transplantation for NMDs such as sarcopenia and VML.

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Keywords: tonsil-derived mesenchymal stem cells, skeletal muscle cells-derived spheroid, neuromuscular diseases

6:45 PM – 7:45 PM

462

EXTRACELLULAR VESICLES DERIVED FROM MESENCHYMAL STEM CELLS CONTAINING MIR-186-5P ALLEVIATEDS APAP-INDUCED LIVER INJURY BY THE SUPPRESSION OF CXCL1

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Acetaminophen (APAP) overdose is one of the major cause of drug-induced liver injury (DILI) and acute liver failure (ALF), and patients with advanced APAP toxicity rarely benefit from N-acetylcysteine (NAC), which is the first-line agent used in the clinic. Mesenchymal stromal cells (MSCs) and their extracellular vesicles (MSC-EVs) have shown promising effects in the treatment of DILI by decreasing neutrophil infiltration. However, the specific mechanism underlying the therapeutic effects of MSCs or MSC-EVs still needs to be elucidated. In this study, by using RNA-seq, we found that CXCL1, which is a chemoattractant for neutrophils, is a key molecule in MSC-mediated amelioration of DILI, and by luciferase reporter assay, we verified that MSC-EVs-derived miR-186-5p binds to the 3'-UTR of CXCL1 to inhibit its expression in hepatocytes. Neutralizing CXCL1 reduces APAP-induced liver damage in vivo, and the agomir miR-186-5p shows excellent potential in the treatment of DILI. Overall, these findings suggest that the use of MSC-EVs may be a promising novel strategy for preventing DILI and that targeting the miR-186-5P/CXCL1 axis is a feasible approach for improving the efficacy of MSCs in the treatment of DILI.

Keywords: drug-induced liver injury, mesenchymal stromal cells, extracellular vesicles

6:45 PM – 7:45 PM

464

CANINE ENDOMETRIAL AND ADIPOSE MESENCHYMAL STEM/STROMAL CELL PRODUCTS CAN SUPPRESS THE GROWTH OF ANTIBIOTIC-RESISTANT STAPHYLOCOCCUS PSEUDINTERMEDIUS

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Bacterial resistance to antibiotics is a major concern worldwide. In different species, we and others have shown that Mesenchymal Stem/Stromal cells (MSCs) actively produce antimicrobials. In this project, we assessed the antimicrobial capacity of cell-free conditioned medium (CM) obtained from MSCs of different tissue origins, endometrium (EM-CM) and adipose tissue (AT-CM), against a clinical isolate multidrug-resistant bacterial strain from Staphylococcus pseudintermedius (Staph), a bacterium species commonly associated with unresolved lethal infections in dogs. MSCs were harvested from tissues and characterised

following ISCT guidelines. Both MSC types produced antimicrobial transcripts including lipocalin-2, elafin, βdefensin-1, and LL37. The antimicrobial activity of CM, both alone and in combination with antibiotics, was measured by bacterial culture OD and live bacteria by CFU/ml. The results showed that treatment with either EM- or AT-CM significantly attenuated Staph growth ($p < 0.01$ for both CM types), in contrast to the antibiotic erythromycin to which the bacterium displays resistance, suggesting that CM could serve as a tool against antibiotic-resistant infection. Moreover, a higher antibacterial effect was detected when EM-CM and erythromycin were applied together ($p < 0.001$). In addition, the effect of either EM- and AT-CM in combination with chloramphenicol, an antibiotic to which Staph is sensitive, was assessed. Results showed a significant reduction of live bacteria numbers when a sub-lethal dose of chloramphenicol was combined with CM compared to antibiotic alone ($p < 0.001$ for EM-CM and $p< 0.01$ for AT-CM), indicating that CM could play an important role in reducing the dose or length of antibiotic treatment in infection. Our findings provide novel evidence of the antimicrobial activity of canine AT- and EM-MSCs and the potential of MSC-CM as a novel tool against antimicrobial resistance and towards the reduction of antibiotic usage in infection.

Funding Source: Dog Trust

Keywords: multipotent mesenchymal stem cells, infection, secretome

6:45 PM – 7:45 PM

466

TNF-ALPHA AND INF-GAMMA PRIMED CANINE STEM CELL-DERIVED EXTRACELLULAR VESICLES FOR POTENTIAL USE IN LUNG REPAIR

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Acute lung injury is a disorder of acute inflammation that causes disruption of the lung endothelial and epithelial barriers. In this study, we investigated whether the Extracellular vesicles (EVs) obtained by stimulating inflammatory cytokine on canine adipose mesenchymal stem cells (cASC) improved anti-inflammatory and/or immunosuppressive potential of EVs, and/or their ability to alleviate LPS induced lung injury in vitro. We also explored the correlation between Epithelial to mesenchymal transition and the inflammatory repressive effect of primed EVs. Through small RNA-Seq, we confirmed the changes caused by inflammatory cytokines to stem cell-derived EVs. Moreover, by analyzing pro-/anti-inflammatory cytokines in LPS induced lung injury model, we found that the anti-inflammatory effect of EVs was improved through pretreatment with inflammatory cytokines. Importantly, EVs obtained from primed stem cells effectively suppressed endothelial-to-mesenchymal transition in lung injury model. Our results provide a new and effective therapy for the EVs obtained from ASC stimulated with TNF-α and IFN-γ against not only lung inflammation, but also endothelial to mesenchymal transition.

Funding Source: The National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2023-00240708) and Research Institute for Veterinary Science, Seoul National University

Keywords: canine, stem cell, extracellular vesicle

6:45 PM – 7:45 PM

468

ELUCIDATING THE ROLE OF MESENCHYMAL STEM CELLS IN RECOVERY OF EXTRAOCULAR MUSCLE HYPERTROPHY IN A THYROID EYE DISEASE MODEL

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Thyroid-associated ophthalmopathy (TAO) is primarily characterized by fat accumulation and inflammation-related hyaluronic acid deposition and fibrosis in the extraocular muscles and connective tissues due to thyroid-related autoimmunity. Inflammation, hypertrophy, and fibrosis of extraocular muscles, as well as inflammation of orbital fat and lacrimal glands, lead to proptosis, eyelid retraction, ophthalmoplegia, and diplopia, causing severe functional impairment. Currently, various immunotherapeutic agents are under development, yet treating extraocular muscle hypertrophy remains the most challenging aspect. Hence, understanding the pathogenesis and researching effective treatments for this are imperative. We successfully isolated myoblasts from both healthy individuals and TAO patients' extraocular muscles, followed by induction of inflammation by treating the isolated myoblasts with IL1β. Subsequently, hyaluronic acid synthesis inhibitor 4-methylumbelliferone (4-MU) and human placenta-derived mesenchymal stem cells (hPMSCs) were separately administered, and the expression of hyaluronic acid, fibrosis, and inflammation-related transcription genes was assessed. Additionally, hPMSCs were injected into the left orbit of TAO animal models, and changes in extraocular muscle volume were measured. As a results, hPMSCs effectively suppressed the expression of hyaluronic acid-related gene HAS2, inflammation-related gene TGFβ2, and fibrosis-related genes α-SMA and Fibronectin in IL1β-induced TAO muscle cells compared to 4-MU. Additionally, in TAO animal models injected with hPMSCs, there was a significant decrease in the cross-sectional area of the extraocular muscles compared to the control group. Additionally, target proteins were identified via single-cell RNA sequencing analysis. In this study, we established an in vitro model of TAO-related inflammation using extraocular muscle cells and successfully reproduced extraocular muscle hypertrophy in TAO animal models. hPMSCs effectively regulated hyaluronic acid synthesis and inflammation-related fibrosis processes in both extraocular muscle cells and thyroid animal models, demonstrating their potential as a cellular therapeutic agent.

Keywords: mesenchymal stem cells, extraocular muscle, thyroid eye disease

6:45 PM – 7:45 PM

470

VARIOUS CELLULAR MODELS FOR THE STUDY OF THE DEVELOPMENT OF ATHEROSCLEROSIS AND AORTIC ANEURYSM

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The global aim of our research is to track the development pathways of human aortic cells in conditions of aortic aneurysm development combined with atherosclerotic damage. Despite being vastly different diseases in outcome, both mentioned pathologies exhibit similar cellular level changes, particularly affecting smooth muscle cell phenotype alterations. In this study, our goal was to explore approaches to studying the functioning characteristics of certain cells within the human aorta intima under conditions similar to those in atherosclerosis development, as comorbid pathology. We chose two cell groups, endothelial and smooth muscle cells, as our model. The pathological stimulus was the stimulation of phagocytosis with native LDL particles and latex beads. For endothelial cells, this stimulation was preceded by treatment to induce giant multinucleated cells, which some sources suggest provoke atherosclerosis development on the vessel endothelium. The results showed that the secretion of pro-inflammatory cytokines (IL1b, TNFα, IL6, IL8) in aortic smooth muscle cells of patients was significantly higher under the influence of native atherogenic LDL particles and latex beads compared to control cells from non-aneurysmal individuals. This could be explained by these cells acquiring a phenotype associated with pro-inflammatory cytokine secretion. This confirms that the phenotypic transition of smooth muscle cells may be a key event in the development of pathological processes within the vessel wall, with atherogenic LDL exposure potentially being a trigger. Regarding giant multinucleated endothelial cells, it was shown that under similar atherogenic LDL exposure, these cells accumulate 1.5 times more cholesterol than typical endothelial cells and have increased secretion levels of pro-inflammatory cytokines and chemokines. The data suggest that circulating atherogenic LDL particles can influence smooth muscle cell trans-differentiation pathways and insufficient endothelial barrier efficiency can accelerate these processes.

Funding Source: Supported by the Russian Science Foundation (Grant #22-65-00089)

Keywords: models, aorta, aneurysm

6:45 PM – 7:45 PM

472

HUMAN VASCULARIZED SPHEROIDS DERIVED FROM DENTAL PULP STEM CELLS AS IMPROVED MODEL FOR IN VITRO CHARACTERIZATION OF CHIMERIC 3D STRUCTURES

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The use of 3D brain organoids has gained importance as an improved in vitro model with higher structural and physiological accuracy with respect to traditional in vitro approaches. Furthermore, 3D brain organoids represent an alternative to in vivo use of animals for the study of neurological pathologies and pharmacological therapy screening. Historically, brain organoids have been generated using embryonic stem cells (ESCs) and/or induced pluripotent stem cells (iPSCs) promoting a neural induction using neural maturation protocols. Thus, the main limitation of this model is the absence of vascularization, limiting the size and missing crucial cellular interactions for the study of neurological pathophysiology and brain cell homeostasis. In our novel approach we describe the coculture system to generate patient tailored vascularized 3D brain organoids. We describe for the first time the ability of a subset of human neural crest stem cells from the human dental pulp, known as human dental pulp stem cells (hDPSCs) to differentiate to neural precursors (GFAP or doublecortin positive cells) as well as to endothelial cells (CD31 and vWF) in the generated 3D spheroid. We have been able to correlate the initial cell quantity with the final spheroid size to adapt them eventually to a given use (brain organoid assembly, use in a microfluidic device...). Furthermore, we also document for the first time the use of hDSPCs 3D spheroids as a valid tool to provide human vascular endothelial cells for 3D nerve tissue structures and assembloids . Our model is a proof of concept that paves the way to generate vascularized mature 3D brain organoids by hDPSCs. The generation of patient-derived vascularized 3D brain organoids will represent a step further in the validation of 3D in vitro avatar models of neurological pathologies.

The broader implications of our vascularizing spheroids could be the fully autologous generation of patient-derived brain assembloids to mimic cell interactions between the vascular and neural compartments in 3D culture models alternative to in vivo animal testing.

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Keywords: stem cells, spheroids, vascularization

6:45 PM – 7:45 PM

TOPIC: MUSCULOSKELETAL

474

GENERATION OF HUMAN IPSC-DERIVED DUCHENNE MUSCULAR DYSTROPHY SKELETAL MYOCYTES SUITABLE FOR 3D FUNCTIONAL STUDIES AND INVESTIGATING METHODS FOR DYSTROPHIN RESTORATION

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Skeletal myocytes play a number of roles in many biological processes ranging from limb movement to the regulation of nutritional homeostasis and are implicated in the pathophysiology of a variety of diseases such as muscular dystrophies and metabolic disorders. There is a pressing need for reliable models of mature human skeletal muscle to permit investigations into physiological and disease mechanisms, and to facilitate the generation of new therapeutics. We have developed an optimised inducible system (opti-ox™) that enables tightly controlled expression of transcription factors (TF) improving cellular reprogramming approaches for the differentiation of hiPSCs. Through opti-ox mediated TF overexpression, we have generated ioSkeletal Myocytes that homogeneously express the key proteins of the myofilaments, desmin, dystrophin and titin, and form striated and multinucleated myocytes that are functionally active. Furthermore, ioSkeletal Myocytes can form functional 3D skeletal muscle microtissues using the MUSbit™ microchip, where twitch and tetanus responses can be observed and become stronger over time, and contraction in response to electrical stimulation can be inhibited or stimulated by the addition of compounds. We employed CRISPR/Cas-9 gene editing to introduce deletions in the DMD gene (dEx44 and dEx52) of the wild type ioSkeletal Myocytes to recapitulate the genetics of Duchenne Muscular Dystrophy (DMD). The generation of DMD deletions in the ioSkeletal Myocytes results in inhibited dystrophin expression at both the transcript and protein level, but does not impair their ability to form myocytes. Restoration of dystrophin mRNA and protein expression has been demonstrated using antisense-mediated exon skipping. Utilising the MUSbit microchip, we have observed impaired functional activity of DMD ioSkeletal Myocytes including reduced twitch and tetanus contraction in response to electrical stimulation. Overall we have demonstrated the ability to generate a reproducible isogenic system for the investigation of Duchenne muscular dystrophy. This novel system has the potential to reveal new in vitro functional phenotypes to improve drug screening specificity and accelerate drug development.

Keywords: Duchenne Muscular Dystrophy, skeletal muscle, disease modelling

6:45 PM – 7:45 PM

478

DEVELOPING AN ENGINEERED INDUCED PLURIPOTENT STEM CELL-DERIVED 3D MODEL OF OSTEOSARCOMA

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Osteosarcoma (OS) is a rare, metastatic, and lethal bone cancer of adolescents defined by chromosomal instability (CIN), including structural and copy number variation (CNV). Treatment options have stagnated for decades, highlighting the need for improved models. To address previous models limitations, we engineered iPSCs to model OS initiation using CRISPR/Cas9-based knockout (KO) of TP53 and RB1, followed by differentiation to mesenchymal stem cells (iMSC) and overexpression of cMYC or cMYC and hRAS. Cells in 2D culture exhibited in vitro transformation and formed tumors in immunodeficient mice, but failed to capture the complex pathology of primary OS and did not metastasize. We thus developed a 3D “iOS” organoid model involving osteogenic differentiation of engineered iMSCs in hyaluronan-based 3D culture. Mutant organoids exhibited anaplastic, less organized structures with an osteoid matrix - a hallmark of OS. Immunohistochemistry (IHC) confirmed similarity to primary OS, validating iOS organoids as a robust model to replicate OS development. iOS organoids implanted subcutaneously formed tumors that were histologically consistent with primary OS, displaying increased complexity compared to tumors formed from 2D-cultured cells. Notably, pulmonary metastases were observed in 70% of mice bearing tumors from 3D iOS organoids. Low-pass (5X) whole genome sequencing (WGS) revealed significant CNV in 3D iOS-derived tumors, consistent with primary OS. To examine CIN in the

context of chemotherapy resistance, iOS organoids and 2D cells were treated with methotrexate, doxorubicin, and cisplatin (MAP) chemotherapy. Half maximal inhibitory concentration values were considerably lower for 2D cells, indicating heightened sensitivity compared to the iOS organoids, which showed increased resistance (106-260x higher). We are currently evaluating organoids in the presence and absence of MAP chemotherapy using low-pass WGS and RNAseq to uncover recurrent patterns linked to drug resistance. In summary, our 3D iOS organoids are the first model to recapitulate the entirety of tumor initiation, development, and metastasis, and should facilitate previously impossible studies on the process of genome instability and its role in resistance to chemotherapeutic and immunotherapeutic interventions.

Keywords: Osteosarcoma, induced pluripotent stem cells, tumor organoids, cancer modeling

6:45 PM – 7:45 PM

480

PROLIFERATION INDEPENDENT YAP1 ACTIVITY PROMOTES MYOFIBRIL GROWTH AND MATURATION IN HUMAN CARDIOMYOCYTES

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Rising occurrence of heart failure is an inevitable consequence of the aging world population. Attempts to regenerate the adult heart by reactivation of developmental programs are actively pursued. Most promisingly, ectopic expression of Hippo effector YAP1 improves short term survival through reactivation of cardiomyocyte proliferation in animal models. However, the benefits of pro-proliferative effects of YAP1 re-expression come with a downside. Uncontrolled growth and insufficient maturation of newly formed cardiomyocytes result in their poor integration and arrythmias. On the other hand, YAP1 has also been indicated in compensatory hypertrophic response and maintenance of proper calcium homeostasis in vivo. It is therefore possible that proliferation independent effects of YAP1 activity could be leveraged to improve heart function in absence of cardiomyocyte proliferation. Control and YAP1 deficient human cardiomyocytes were differentiated in vitro from human embryonic stem cells. Cell, myofibril, and sarcomere morphology were characterized using super resolution imaging. Dramatic morphological changes (shorter, disorganized, and less mature myofibrils) were observed in YAP1 deficient cardiomyocytes during maturation. Interestingly, some of these changes could be reversed by YAP1 ectopic expression in post-mitotic cardiomyocytes. Moreover, whole genome expression and electrophysiological measurements revealed less mature excitation-contraction coupling and calcium metabolism in absence of YAP1. Finally, compensatory hypertrophic response in response to mechanical stimulation was lost in YAP1 deficient cardiomyocytes. Taken together our data show the previously unknown key role of YAP1 on myofibril generation, maturation, and compensatory hypertrophic response, which could be utilized for future therapy of chronic heart disease without the adverse effects of cardiomyocyte proliferation.

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Keywords: sarcomere maturation, mechanosensing, YAP1

6:45 PM – 7:45 PM

482

NOVEL PLATFORM FOR BIOENGINEERING SKELETAL MUSCLE FOR HIGH RESOLUTION IMAGING AND FUNCTIONAL ANALYSIS

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Contractile function of skeletal muscle is assumed to be tightly linked to muscle structure. Combining tissue level contractility measurements with myotube or even sarcomere level imaging would confirm this hypothesis and investigate in more detail how and under what conditions function follows form. Recently introduced in vitro engineered culture models allow for creating macroscale muscle tissue from primary cells (myoblasts) and induced pluripotent stem cells (iPSCs) from healthy and diseased patients. Although highly versatile in their application, available culture platforms are incompatible with high resolution live microscopy due to unfavorable object-lens distances. Here, we present a new platform that allows high-resolution 3D microscopy in living human engineered skeletal muscle (ESM) with parallel non-invasive quantification of tissue level contractility. Using immortalized myoblast cell lines or iPSC differentiation protocols, we demonstrate the engineering of millimeter-sized (4.92 ± 0.02 mm length, 0.28 ± 0.01 mm2 cross-section; n=38) ESMs between two elastic poles (39 µN/

µm bending constant) positioned directly above a thin glass coverslip. Tissue to coverslip distance is 80 ± 9 µm. By this design, we can perform deep tissue imaging down to the sarcomere level (in a model of stable expression of ACTN2-Citrine). By integration of electrodes, we can apply electrical stimuli to introduce controlled tetanic contractions (up to 100 Hz). By video-optic pole deflection analyses we report tetanic contractile forces at 100 Hz of up to 0.6 mN. Finally, we report that ESMs from patients suffering from Duchenne muscular dystrophy develop overall thinner myotubes (13.1 ± 0.44 µm; n=24) and show weaker tetanic forces (0.098 ± 0.01 mN, 0.2 ± 0.02 mN/mm2; n=80) compared to ESMs from healthy patients (14.7 ± 0.3 µm myotube diameter, n=157 and 0.24 ± 0.03 mN, 0.41 ± 0.05 mN/mm2 (n=42); p< 0.05 unpaired, two-sided t-test). Taken together, the combination of standard pole bending analysis with tissue imaging creates new opportunities to discover muscle structure-contractile function relationships. More specifically, live deep tissue imaging can be applied to study developmental or pathological processes as well as the consequences of novel therapies such as for example genome editing in muscle dystrophies.

Keywords: muscle tissue engineering, high resolution microscopy, Duchenne muscular dystrophy

6:45 PM – 7:45 PM

484

MODELLING THE HUMAN NEUROMUSCULAR JUNCTION IN VITRO

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Neuromuscular junctions (NMJs) are affected early on in amyothropic lateral sclerosis (ALS). Developing an in vitro model that is able to reproduce the in vivo NMJ pathology and dysfunction in ALS could significantly improve our understanding of this important disease event. Towards this goal, we have generated human in vitro NMJs by deriving and co-culturing spinal motor neurons and skeletal muscle fibers from pluripotent stem cells (PSCs) from a healthy donor. Muscle expressed the expected proteins conferring contractility such as titin and myosin heavy chain, while motor neurons expressed neurofilaments, MNX1, ISL and VACHT. Motor neuron processes were in close apposition to acetylcholine receptor (ACHR) clusters on myotubes, as shown by immunostaining against beta3-tubulin, SV2A, and BTX, indicating the formation of NMJ-like structures. To validate the generation of functional human NMJs in our co-culture system, we used human primary myoblast-derived myotubes expressing the fluorescent calcium indicator MHCK7::G-CaMP and co-cultured these with PSC-derived motor neurons. Functional connectivity was detected by an increase in fluorescence intensity produced by the calcium indicator of the myotubes after glutamate stimulation of motor neurons. The investigation of functional connectivity was paralleled by transcriptional profiling of both compartments across various stages of co-culture. Further understanding will be obtained by comparing NMJ structures and transcriptomic responses of in vitro generated NMJ co-cultures in health and disease. This study employs a biologically relevant model to investigate the interaction between motor neurons and muscle compartments, aiming to enhance our understanding of ALS.

Keywords: neuromuscular junction, amyotrophic lateral sclerosis, spinal motor neurons

6:45 PM – 7:45 PM

486

DEVELOPMENT OF ADVANCED 3D IN VITRO HUMAN SKELETAL MUSCLE ORGANOIDS DEMONSTRATING STRUCTURALLY WELL-ORGANIZED MYOTUBE AND PATHOPHYSIOLOGY OF SARCOPENIA

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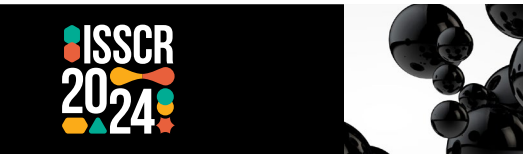
Sarcopenia, characterized by progressive and generalized loss of skeletal muscle mass, strength, and function, is overall decreased quality of life. Most of previous study to understand the underlying mechanism of muscle regeneration are relied on animal models and 2D cell culture systems, which have limitations in mimicking human-specific pathophysiology. We have previously developed human pluripotent stem cell (hPSC)-derived three-dimensional (3D) skeletal muscle organoids recapitulating the skeletal muscle development and possessing muscle regenerative capacity. Here, we report a reproducible method for generating advanced 3D human skeletal muscle organoid (hSkMO), demonstrating robust skeletal muscle production. hPSCs were initially induced to paraxial mesoderm stem cells (PMSCs) homogeneously expressing maker genes, such as MSGN1 and TBX6. The PMSCs were then aggregated and further differentiated to undergo myogenesis in 3D culture platform. Upon long-term maturation, hSkMOs exhibited typical skeletal muscle tissue-like signatures including mature myofiber structure, existence of quiescent satellite cells under basal lamina of myofibers and diverse cell types such as motor neurons and fibro-adipogenic progenitors (FAPs), which is closely resembling in vivo muscle tissue. Notably, neuromuscular junctions (NMJs) were detected in hSkMOs with spontaneous contraction, indicating neuron-to-muscle connections. By chemotherapy (cisplatin) treatment in hSkMOs, we could model the sarcopenic muscle. Immunofluorescence assay revealed the recapitulation



of sarcopenia-related phenotype, such as muscle atrophy, apoptotic myofibers, senescence of myocytes, damaged DNA of myocytes, exhaustion of muscle stem cells in chemotherapy-induced hSkMOs. Furthermore, the hSkMOs demonstrated the spontaneous muscle recovery in long-term culture, indicating retaining intrinsic regenerative capacity. Collectively, the hSkMOs represent a potentially useful model for human skeletal muscle development and its related disorders, such as sarcopenia. They may also serve as a versatile platform for investigating pathophysiological mechanisms of sarcopenia and developing therapeutic interventions for sarcopenic patients.

Funding Source: This work was supported by the Korean Fund for Regenerative Medicine (23A0206L1) of MSICT and MHW, the Basic Science Research Program (2019R1A6A1A03032888 and 2022R1A2C1004718) and K-Brain Project (RS-2023-00262332) of NRF of ROK.

Keywords: human skeletal muscle organoid, sarcopenia, muscle regeneration

6:45 PM – 7:45 PM

488

OVARIAN FUNCTION IS REQUIRED FOR THE REGENERATIVE EFFECT OF HUMAN EMBRYONIC STEM CELL-DERIVED MESENCHYMAL PROGENITOR CELLS (HESC-MPCS) IN MOUSE MODEL OF CHEMOTHERAPY-INDUCED SARCOPENIA

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The ovary plays a crucial role in female hormone production, and contributes not only to reproductive regulation but also to homeostatic regulation such as maintenance of skeletal muscles. Chemotherapy presents the potential advantage of extending the lifespan of cancer patients; however, it may be linked to potential drawbacks, such as the risk of premature ovarian insufficieny (POI) and sarcopenia. To rescue from adverse effects of chemotherapy, numerous studies using mesenchymal progenitor cells (MPCs) sourced from various tissues are underway. In our previous researches, the administration of hESC-MPCs demonstrated the ability to restore ovarian function and body weight in mouse model showing chemotherapy-induced POI. However, it has not been elucidated whether the ovarian axis plays a direct role in the therapeutic effect of hESC-MPCs on muscle regeneration in chemotherapy-induced sarcopenic mice. In the present study, we confirmed that continuous injection of cisplatin for 10 days induces sarcopenia in normal and ovariectomized mice, characterized by a decrease in muscle fiber size and body weight. When hESC-MPCs with hyaluronic acid (HA) gel were transplanted subcutaneously on the dorsal part of nearby ovaries in cisplatin-induced sarcopenic normal mice (non-OVX group), a positive effect was observed in the restoration of sarcopenic conditions, evidenced by an increase in body weight and muscle mass. However, in the case of ovariectomizd mice (OVX group), therapeutic effect did not exhibited, as evidenced by the absence of increase in body weight and muscle mass restoration. The expression of Pax7 and MyoD, factors related to muscle regeneration, was not up-regulated in the OVX group compared to the non-OVX group. Furthermore, the absence of ovaries could not reduce intramuscular collagen fibrosis in cisplatin-induced sarcopenic mice. Also, intramuscular lipid infiltration was increased in OVX group. From these results, we may suggest that the maintenance of ovarian function is essential for the prevention of sarcopenia by regenerative medicine using stem cell therapy.

Funding Source: This work was supported by the Korean Fund for Regenerative Medicine (KFRM, 23A0206L1) of the Korean government (MSICT and MHW), the Basic Science Research Program (2019R1A6A1A03032888 and 2022R1A2C1004718) of NRF of Korea.

Keywords: human embryonic stem cell-derived mesenchymal progenitor cell, ovarian function on muscle regeneration, sarcopenia

6:45 PM – 7:45 PM

490

A PARALLELIZED CULTURE PLATFORM FOR SCREENING OF CONTRACTILE SKELETAL MUSCLE ORGANOIDS

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Skeletal muscle organoids (SMO) recapitulate development and physiological function of human muscle in vitro. To utilize SMO for phenotypic screens, we aimed to establish a platform for parallelized analysis of skeletal muscle organoid development and function. To generate contractile skeletal muscle organoids, undifferentiated human iPSCs were seeded in a collagen/Matrigel™ hydrogel and subjected to a serum-free directed differentiation protocol. For parallelized analysis, SMO were generated in a specialized 48-well culture platform (myrPlate) that allows electrical stimulation and optical analysis of muscle function. Development of SMO was monitored by transcript analysis at various steps of differentiation and confirmed a significant

overlap with characteristic signatures of paraxial mesoderm formation, somitogenesis, and muscle maturation. After 50 days of cultivation, SMO contraction could be tracked by optical analysis of 48 SMO in parallel. SMO showed an organotypic response to increasing electrical stimulation frequencies with tetanic contractions. As proof-of-concept for its application in drug screening, SMO in myrPlate were treated with several substances to enhance maturation. We found that the ERK1/2 inhibitor SCH772984 improved the contractile function of SMO. At a stimulation frequency of 100 Hz, tissues treated with 1 µM SCH772984 showed a ~4-fold increase in twitch tension compared to the control group (0.8±0.07 vs. 0.2±0.03 mN, n=11). As a further sign of maturation, the twitch to tetanus ratio was enhanced and the force-frequency response was shifted to higher frequencies. The functional changes were associated with substantial changes in expression of sarcomeric genes in SCH-treated SMOs. Gene expression analysis of tissues revealed a 2.8-fold increased expression of ACTN2 in tissues treated with SCH772984 (3664±212 vs. 1328±67 RNA counts, n=3). In addition, expression of myosin heavy chain isoforms 7 and 8 were elevated 5.8- and 7-fold, respectively (191.7±23.8 vs. 1119.6±56.7 RNA counts, n=3; 2231.8±176.3 vs. 15606±1872.8 RNA counts, n=3). SMO represent a scalable muscle model that closely recapitulates organotypic skeletal muscle development. The parallelized culture system of SMO may be utilized as a screening platform or for disease modelling applications.

Keywords: skeletal muscle organoids, drug screening, tissue engineering

6:45 PM – 7:45 PM

492

HUMAN NEUROMUSCULAR ORGANOIDS MODEL SPINAL MUSCULAR ATROPHY

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Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality. Motor neurons (MNs) are considered the primary target in SMA but retrograde signals from skeletal muscles and neuromuscular junctions (NMJs) can also be crucial players of the MN degeneration. Muscle atrophy is a hallmark of SMA, and a growing body of evidence suggests that muscle pathology could have an important role in this disease. However, the series of events that lead to MN degeneration in humans remain poorly understood. Here, we used induced pluripotent stem cells (iPSCs) from three different patients diagnosed with SMA type 1 to generate neuromuscular organoids in 3D. SMA-NMOs could be efficiently generated and self-organized into the neural and muscle compartments. However, we observed a loss in muscle function characterized by the inability to contract, largely recapitulating the phenotype observed in patients. To reveal temporal aspects of the disease phenotype, we analyzed the SMA-NMOs at different developmental stages using single nuclear RNA sequencing. This approach allowed us to identify the different cell types affected with temporal resolution. Additionally, we performed a small-molecule drug screening to identify potential treatments that rescue aspects of the disease phenotype. Our findings demonstrate the potential of patient-specific NMOs for drug screening approaches, paving the way for developing treatments that target the early stages of the disease and thus improving the lives of patients.

Keywords: neuromuscular organoid, spinal muscular atrophy, neuromuscular diseases

6:45 PM – 7:45 PM

TOPIC: NEURAL

496

ZIKA VIRUS INFECTION FACILITATE HD-ASSOCIATED PATHOLOGICAL PHENOTYPES IN HUNTINGTON'S DISEASE IPSC DERIVED NEURAL PROGENITOR

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The Zika virus (ZIKV) has been extensively studied for its preferential infection of human neural progenitor cells (hNPCs) and its association with microcephaly in neonates. However, the impact of ZIKV infection on neurodegenerative diseases such as Huntington's disease (HD) has been largely overlooked. Using HD-induced pluripotent stem cells (iPSC) as an in vitro differentiation model, we demonstrate that HD-iPSC derived NPCs are more susceptible to ZIKV infection and exhibit increased DNA damage and apoptotic cell death. This susceptibility may arise from impaired function of the Non-homologous end joining (NHEJ) DNA repair pathway, specifically through downregulation of Ku70 in the HD-iPSC derived NPCs. Additionally, ZIKV infection enhances mHtt expression, leading to mHtt-induced cellular toxicity. We observe an increased interaction between mHtt and Ku70, which promotes the ubiquitination of Ku70 protein. This disruption of the NHEJ DNA repair complex impairs DNA repair activity. Together, these results suggest that ZIKV infection exacerbates pathological changes in HD during neurodevelopmental processes and may influence the progression of Huntington's disease even at the pre-symptomatic stage. By shedding light on the cross-interaction between ZIKV infection and HD, our study contributes to a better understanding of the complex mechanisms underlying neurodegenerative diseases and highlights the importance of considering viral infections as potential modulators of disease progression.

Keywords: Zika virus, Huntington's disease, induced pluripotent stem cells

6:45 PM – 7:45 PM

498

DECIPHERING RETINOBLASTOMA MULTI-STEP TUMORIGENESIS THROUGH DEEP SINGLE CELL RNA-SEQUENCING OF RB1 KNOCKOUT CONE REPORTER RETINAL ORGANOIDS

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Most retinoblastomas are thought to originate from maturing cone photoreceptor precursors with biallelic RB1 inactivation. The maturing cone precursor origin is supported by studies of explanted fetal retina, in which pRB-depleted post-mitotic cone precursors re-enter the cell cycle and proliferate over ~2 months, dependent on intrinsically high MYCN and MDM2, followed by a 4-5 month indolent premalignant phase and emergence of retinoblastoma-like masses at tissue ages similar to perinatal retinoblastomas in vivo. To define the cell state transitions that underlie these steps with a more tractable in vitro model, we produced and characterized RB1-null cone reporter retinal organoids (ROs) that recapitulate multi-step retinoblastomagenesis. We first generated cone-reporter iPSCs that can be differentiated into ROs in which EGFP specifically, robustly and innocuously labels cones throughout their maturation. A second round of CRISPR produced homozygous RB1 knockout. Chimeric organoids generated from RB1-null cone reporter iPSCs mixed with unedited parental iPSCs recapitulated pRB loss in a subset of retinal cells in an otherwise healthy retina and enabled live-imaging cell tracking in intact hydrogel embedded ROs. Bi-weekly live confocal imaging of EGFP+ RB1-/- cones from d85 to d238 captured their initial proliferation followed by a heterogeneous indolence phase marked by low-level proliferation, quiescence, and cone-like morphologic differentiation. Nascent retinoblastoma-like foci that co-expressed EGFP, cone markers, and proliferation marker Ki67 formed after d281, equating to the first post-natal month when early retinoblastomas typically emerge. Deep, full-length scRNA-seq on FACS-isolated EGFP+ cones from RB1+/+ and RB1-/- organoids revealed divergent cell state trajectories of human cone development and tumorigenesis. RB1-/- cones transitioned from proliferation to differentiation and p53-related stress states implicating p53 pathway activation as a driver of indolence entry and inactivation as an enabler of escape. In summary, we generated a human stem cell derived organoid model that recapitulates the cell-of-origin and timing of multi-step retinoblastomagenesis, and identified cell state transitions underlying malignant progression.

Keywords: retinoblastoma, retinal organoid, scRNA-seq

6:45 PM – 7:45 PM

500

COMPARISON OF HIPSC-DERIVED DOPAMINERGIC NEURONS

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Mutations in the alpha synuclein gene (SNCA) cause autosomal dominant Parkinson's disease (PD), with loss of dopaminergic neurons in the substantia nigra, and aggregation of alpha synuclein. The generation of midbrain dopaminergic neurons (mDA) aids the modelling of neurodegenerative diseases such as PD in vitro. Alongside ongoing efforts to refine differentiation methods, we optimised a protocol in adherently cultured cells that produces a cell population highly enriched (80%) in mDA neurons from patient human induced pluripotent stem cells (hiPSCs). Adherent cultures enable modelling of aspects of the midbrain environment, which, aside from mDA neurons, also contains other cell types, such as astrocytes. However, the role and function of these non-neural cells in synucleinopathies is less well understood. To better model the mDA neurons in their three-dimensional environmental niche, we utilised our small molecule-based differentiation protocol to produce enriched mDA precursor cells (NPCs) and subsequently midbrain organoids. Employing single cell multiome sequencing, we characterised cell types in our three-dimensional organoid model. The comparison of these organoid cell populations versus the adherently cultured mDA neuronal cell populations highlights differences and commonalities in cellular diversity, cellular maturity, and regulatory landscapes. In addition, contrasting hiPSCs from patients with and without SNCA mutations not only aids the identification of gene expression changes underlying pathology, it also highlights differences in the regulatory landscapes. Taken together, this in-depth approach from 2D to 3D models allows us to delineate the role of non-neuronal cells and their interaction with neurons in SNCA associated PD.

Funding Source: This work is supported by Aligning Science Across Parkinson's [ASAP-000509 and ASAP-000463]. The Francis Crick Institute receives funding from the UK Medical Research Council, Cancer Research UK, and the Wellcome Trust.

Keywords: neurodegenerative disease modelling, multiome single cell analysis, iPSC-derived midbrain neurons

6:45 PM – 7:45 PM

502

PHYSIOLOGICALLY RELEVANT MEDIA UNMASKS SEVERE MITOCHONDRIAL DYSFUNCTION IN A PRECISION REPROGRAMMED IPSC-DERIVED MODEL OF HUNTINGTON'S DISEASE

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Huntington's disease (HD) is a devastating disease characterised by degeneration of the medium spiny neurons (MSNs) in the striatum. HD patients suffer from uncontrollable movements as well as severe mental problems and currently no disease modifying treatments are available. HD is an autosomal dominant disorder caused by a CAG repeat expansion encoding an elongated polyglutamine (PolyQ) stretch in the Huntingtin (HTT) protein. Although the precise pathogenic mechanisms remain poorly understood, the mutant aggregation prone HTT protein has been reported to affect various cellular processes, including the biogenesis, fission, transport and respiration of mitochondria. HTT is ubiquitously expressed in the brain and, albeit MSNs are the most susceptible neurons to the toxic effects of mutant HTT protein, other neuronal subtypes such as the cortical glutamatergic neurons are affected during later disease stages. We developed a novel iPSC-derived HD model based on our opti-ox reprogrammed ioGlutamatergic Neurons. These ioGlutamatergic Neurons HTT 50CAG/WT contain a genetically engineered heterozygous 50 CAG repeat expansion in exon 1 of huntingtin. To investigate mitochondrial function in our HD model, cells were cultured in Neurobasal medium for 11 days and analysed with a Seahorse assay. The HD model showed relative to the wild type isogenic control a significant but modest reduction in basal and ATP-linked respiration. Unexpectedly, at day 25 oxygen consumption rates in both genotypes were highly similar as neurons switched from mitochondrial respiration to glycolysis. Interestingly, culturing cells in a more physiologically relevant medium supported mitochondrial respiration at day 25 and unmasked a dramatic and significant mitochondrial dysfunction in the HD model. As neuronal firing is energy demanding, we assessed by high-resolution MEA recordings whether mitochondrial dysfunction in the HD model affects neuronal activity relative to wild type cells. Culturing the cells over 30 days in a physiologically relevant media significantly decreased the firing amplitude and rate as well as network activity in the HD model. Overall, we have developed a scalable and consistent human HD model that recapitulates critical disease aspects and enables disease mechanistic and drug discovery studies.

Keywords: HTT Huntington's disease CAG repeat PolyQ iPSC-derived disease model iPSC reprogramming glutamatergic neurons, Seahorse assay, mitochondrial dysfunction, iPSC-derived model glutamatergic neurons

6:45 PM – 7:45 PM

504

EFFECT OF NEURAL STEM CELLS OVER EXPRESSING NEUROGLOBIN IN PHOTOTHROMBOSIS MODEL

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Stroke is a major cause of death and disability worldwide. In the stroke, lack of oxygen due to abnormal blood flow generates ROS and thereby causes infarction, which may cause sequelae. Meanwhile, neuroglobin (NGB) scavenges reactive oxygen/nitrogen species, prevents apoptosis, and promotes cell proliferation. So, in this study, we investigated that the recovery effect of human neural stem cells overexpressing NGB (F3.NGB) in photothrombosis model. F3.NGB cells increased cell proliferation more than their parent naïve cells (F3). And F3.NGB cells upregulated the expression of PTEN, AKT, and ERK, which are cell proliferation markers. F3.NGB exhibited a significant cell proliferation effect in the wound healing assay. In the photothrombosis model, transplantation of F3.NGB facilitated cell proliferation via PI3K/AKT/mTOR signaling pathway and restored the infarcted area faster than transplantation of the F3 cells. Especially, after intravenous transplantation of F3.NGB, the cells distributed damaged site and differentiated to neuron and astrocytes. Also, the cells expressed the Ki-67, the cell proliferation marker. This study suggests that human neural stem cells overexpressing neuroglobin (F3.NGB) have significant recovery effects after stroke models by promoting cell proliferation.

Keywords: neuroglobin, stem cell, photothrombosis

6:45 PM – 7:45 PM

506

FUSIDIC ACID AND TRIMETHOPRIM ENHANCE CELL DEATH AND CAUSE CELL CYCLE ARREST IN FOLATE-DEPLETED NEUROBLASTOMA CELLS

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As early neural development is susceptible to changes in folate homeostasis, a reduction in folate levels can lead to neural tube defects. Maintaining adequate folate levels can be affected by numerous factors, including concomitant treatment with drugs that interfere with folate metabolism, known as antifolates. The use of prescribed and over-the-counter medications during pregnancy and childbearing age is increasing. Pharmacotherapy with antifolates may have effects on the folate cycle and may be detrimental to neural development. Therefore, we aimed to investigate the antifolate activity of drugs approved by the European Medicines Agency for their effect on the mechanisms of cell proliferation under normal and folate-depleted conditions using in vitro cell models for neural development. First, we created a library of all approved drugs (N=2575) and performed a virtual screening for their ability to bind to the folate cycle enzymes of interest, namely DHFR, SLC19A1, MTHFD1, and MTHFR. The »top hits« with the highest binding score in our in silico experiment were unanimous among different folate enzymes and comprised: trimethoprim (TMP), dexamethasone, canagliflozin, fusidic acid (FUS), apixaban, vorapaxar, nebivolol, dabigatran, dasabuvir, domperidone, vemurafenib, olmesartan, cefpirome and irinotecan. Next, metabolic activity and apoptosis were measured and cell cycle characteristics of the neuroblastoma cell line SH-SY5Y were analysed after exposure to the identified compounds. Cells were treated under regular and folate-depleted conditions, methotrexate was used as a positive control. We found that FUS and TMP decreased relative metabolic activity, especially in combination with folate-depleted medium (FUS: IC50+FA = 159.8 mM, IC50-FA = 194.8 µM, and TMP: IC50+FA = 18.5 M, IC50-FA = 117.9 µM). This was accompanied by an arrest in the S-phase of the cell cycle in folate-depleted media (pFUS = 0.0001 and pTMP = 0.0008). Regular conditions in the medium showed no significant difference in the cell cycle profile, compared to the control. Our results show the importance of folate supplementation in child-bearing age and during pregnancy, as even supposedly safe drugs can have significantly increased toxicity to neurons in folate-depleted environments.

Keywords: folates, neurotoxicity, neural development

6:45 PM – 7:45 PM

508

UNRAVELING THE MOLECULAR BASIS OF DLG4-RELATED SYNAPTOPATHY

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DLG4-related Synaptopathy, also known as SHINE syndrome, is a newly identified neurological disorder associated with variants in the Discs Large Homolog 4 (DLG4) gene. It encodes for the post-synaptic density 95 (PSD-95) protein, the most abundant scaffolding MAGUK protein in the excitatory post synaptic density (PSD) of glutamatergic synapses. Owing to the diversity of signaling pathways associated with PSD-95, one hundred and fifty DLG4 variants have been identified, with disease manifestations consistent with those of its numerous interacting partners. De novo heterozygous pathogenic mutations in 160 individuals are associated with moderate to severe intellectual disability, developmental delay, autism spectrum disorder, epilepsy, ataxia, hypotonia, ADHD, language delay and vision problems. No genotype-phenotype correlations have been established thus far, with treatment being restricted to management of symptoms and supportive care. We employ patient-derived induced pluripotent stem cell lines (hiPSCs) for in vitro characterization of glutamatergic NGN2-induced neurons and 3D forebrain organoids for disease modeling. To this end, we aim to establish the different mutational effects of three different variants on the gene expression levels while elucidating the primary affected pathways by studying the domain-specific protein interactions. Preliminary data shows a trend of haploinsufficiency for all mutations. Functional characterization of both 2D and 3D culture models is carried out through a high-density microelectrode array (HD-MEA) system, through which we observe mutation-specific neuronal excitability patterns and maturation kinetics. Lastly, we strive to generate patient-specific platforms for testing possible drug combinations, working with an AAV9-hSyn-hDLG4 gene therapy approach for loss-of-function (LOF) mutations. The research holds promise for establishing the groundwork for future studies on other DLG4 variants and insights for gene therapy approaches for other monogenic brain disorders.

Funding Source: SHINE syndrome and Hope for Harvey foundations

Keywords: DLG4-related Synaptopathy, AAV9 gene therapy, mutation modeling

6:45 PM – 7:45 PM

510

ELEVATED CRMP2 & RESULTING NETWORK DYSFUNCTION IN SCHIZOPHRENIA AS A DRUG TARGET

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Schizophrenia (SZ) is a debilitating mental disorder characterized by disruptions in thought processes, perception, emotional responsiveness, and social interactions. While SZ affects one percent of the world's population, patients with SZ have an elevated risk for premature mortality, including elevated suicidality. Increasingly, across research modalities, researchers are identifying large-scale brain network dysfunction in patients with SZ which may contribute to complex symptomatology. Our lab has shown that Collapsin Response Mediator Protein 2 (CRMP2), a cytoskeleton modulator that serves as a master regulator of neural network formation and function, is dysregulated in SZ. CRMP2 activity, pivotal to dendrite morphometrics and function, is dependent on its phosphorylation state, which, under normal circumstance, is in dynamic equilibrium. By differentiating SZ patient induced pluripotent stem cell (iPSC) into cortical neurons, our lab has identified imbalances in the ratio between phosphorylated (inactive) and non-phosphorylated (active) CRMP2 and we have shown that this imbalance promotes neuronal hyperexcitability. Counterintuitively, this leads to neural network hypofunction by promoting overly-synchronous circuits and impairing the transfer of patterned information. First, we have shown elevated active CRMP2 in SZ relative to healthy patients. We then investigated how dynamic dysregulation of CRMP2 results in corresponding network dysfunction in SZ and found that elevated active CRMP2 is associated with hyperactive and overly synchronous neural circuits in calcium imaging and microelectrode array (MEA). The final pathway of many cognitive disorders is neural network dysfunction that results from the linking of hyperexcitable neurons (regardless of how they become hyperexcitable), including bipolar disorder and Alzheimer's disease. In this way, network-modifying drugs may have both applicability in treating schizophrenic patients and broad applicability across cognitive disorders. Elucidating how CRMP2 regulates the molecular machinery underlying network dysfunction and identifying novel drug targets implicated in this dysfunction may unlock powerful alternatives to treat network hypofunction across cognitive disorders.

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Keywords: schizophrenia, psychiatric disease, network dysfunction

6:45 PM – 7:45 PM

514

PROTEIN INTERACTOMES OF RARE VARIANT SCHIZOPHRENIA RISK GENES IN IPSC-DERIVED NEURONS CONCENTRATE GENETIC, TRANSCRIPTOMIC, AND PROTEOMIC PERTURBATIONS LINKED TO SCHIZOPHRENIA

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Schizophrenia (SCZ) is a severe psychiatric disorder with hundreds of genetic variants contributing to its risk across the allele frequency spectrum. We performed interaction proteomics for 13 genes identified by the Schizophrenia Exome Sequencing Meta-Analysis (SCHEMA) study, in iPSC-derived neural progenitor cells (NPC) and excitatory neurons (ExN). The resulting protein-protein interaction (PPI) networks reflect cell-type-specific biology in NPC vs. ExN and are the convergence point for genetic, transcriptomic, and proteomic perturbations associated with SCZ. To assess whether this type of convergence is also manifested in the proteome, we profiled iPSC-derived neurons from 22q11.2 deletion carriers with SCZ vs. controls. Several ExN-specific networks that were downregulated in postmortem brains of SCZ individuals with varied genotypes were also downregulated in the neuronal proteome of 22q11.2 deletion carriers. Our findings recapitulate the genetic correlations between SCZ and neurodevelopmental disorders and implicate the ExN-specific networks as a convergent point of transcriptional and translational dysregulation in SCZ.

Keywords: Schizophrenia rare variants, iPSC-derived cortical neurons, proteomics

6:45 PM – 7:45 PM

516

A VASCULAR ENDOTHELIAL CELL-DERIVED FACTOR ENHANCES THE ABILITY OF IPSC-DERIVED BRAIN PERICYTES TO DEGRADE ALPHA-SYNUCLEIN

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Parkinson's disease (PD) is characterized by widespread distribution of Lewy bodies, which are composed of phosphorylated and aggregated forms of α-synuclein (α-Syn), in the brain. Accumulation and propagation of α-Syn contribute to the development of PD. We previously revealed that primary cultured rat brain pericytes, one of the blood-brain barrier (BBB) -constituting cell types, exerted the ability to degrade α-Syn. Pericytes are located at the wall of capillaries to be responsible for the formation, maturation, and stabilization of the cerebral microvasculatures. Decline in pericyte number and coverage with aging are associated with disruption of BBB and neurodegeneration. Therefore, we intend to establish pericyte replacement therapy to maintain BBB function and prevent PD. In this study, we established brain pericytes from human iPS cells (hiPSCs) and evaluated the degradation of α-Syn in hiPSC-derived pericytes (iPericytes). iPericytes were produced by differentiating hiPSCs (strain: 201B7) through mesoderm cells and subsequently culturing in pericyte medium with or without a vascular endothelial cell-derived factor (named herein as "Factor A"). Expression of pericyte markers in iPericytes was confirmed by Western blot, RT-PCR, and immunostaining. iPericytes were treated with α-Syn for the indicated time, and then the intracellular α-Syn level was measured by Western blot. Commercially available human brain pericytes (HBVP) were used as control. We obtained brain pericytes from hiPSCs, which expressed pericyte markers and improved barrier function in rat brain endothelial cells (RBECs). Although iPericytes exerted the ability to take up α-Syn, α-Syn degradation activity was insufficient compared with HBVP. The expression levels of pericyte markers were increased when iPericytes was cocultured with RBECs, suggesting that RBEC-derived soluble factors could enhance the maturation of iPericytes. Cultured with brain endothelial cell-derived Factor A during the differentiation phase, iPericytes acquired the ability to degrade α-Syn. These results suggested that a vascular endothelial cell-derived factor is important for the functional maturation of the α-Syn degrading ability of brain pericytes.

Keywords: blood-brain barrier, Parkinson's disease, brain pericytes

6:45 PM – 7:45 PM

518

TOWARDS THE OPTIMIZATION OF HUMAN MEDIUM SPINY NEURON GRAFTS IN THE QA-LESION HD RAT MODEL

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Neurotransplantation represents a promising alternative for the treatment of certain localized neurodegenerative pathology, such as Huntington's disease (HD). The survival, maturation and integration of medium spiny neurons (MSN) progenitors in the lesioned striatum is a key requirement for a successful cell therapy. The toxin-based HD model, obtained with unilateral quinolinic acid (QA) injection into the striatum of athymic rats, recapitulates the cell loss of MSN seen in HD, and allows to investigate the efficacy of the graft. We formerly showed that first generation (fg) hESC-derived striatal grafts survive up to 6 months post-transplantation in the HD model and differentiate into key striatal cell types including relevant fractions of matured MSNs that become integrated into the host circuits. However, fg-grafts yielded a limited fraction of human MSN (hMSN) that matured very slowly and with limited integration in vivo. This prompted the development of a second-generation (sg) in vitro differentiation protocol that significantly enhanced the MSN yield and maturity. Here, we present the first data on short- and long-term grafts of sg-hMSN progenitors that were transplanted into the QA lesioned rat striatum. Immunohistological analyses show that sg-hMSNs display features not found in fg-graft at corresponding time points. Namely, despite a similar degree of neurochemical maturation, sg-hMSNs displayed relevant integration already at 2-month post-transplantation with established extrastriatal inputs and efferents to direct striatal targets. Moreover, long-term sg-grafts revealed progression towards gliogenesis and efficient colonization of the spared host parenchyma by human grafted cells. Single nuclei transcriptional profiling and innovative tracing approaches will provide further insight on the specific properties of sg-graft and their therapeutic potential.

Funding Source: NSC-Reconstruct H2020, GA no. 875758

Keywords: hESC-derived medium spiny neurons, striatal grafts in HD rat model, neuroreplacement strategies

6:45 PM – 7:45 PM

520

ESTABLISHMENT OF HUMAN LEBER'S HEREDITARY OPTIC NEUROPATHY MODEL USING IPSC-DERIVED RETINAL ORGANOIDS

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Mitochondria are key intracellular organelles that have the important role of ATP synthesis and the production of reactive oxygen species. Mitochondria have their own DNA called mitochondrial DNA in addition to nuclear DNA. Mitochondrial diseases are diseases in which mutations of mitochondrial DNA. Leber's hereditary optic neuropathy (LHON) is one of the mitochondrial diseases which is characterized by blindness due to degeneration of retinal ganglion cells (RGCs) and their axons, which form the optic nerve. Idebenone is widely used to treat LHON patients, but only a few patients respond to treatment. Therefore, there is an urgently need to elucidate the pathogenesis of the disease and to develop novel therapeutics. However, there is no human retinal model that mimics the pathology of LHON, which limits to develop a novel therapeutics. In this study, we aimed to establish the retinal organoid (RO) model with the pathogenesis of LHON utilizing patient-derived iPSCs. First, we investigated RO differentiation protocols to establish the LHON-RO model. Next, RGCs and nerves were evaluated using RO after 35 days of induction. We examined the number of RGC-like cells, axonal density, and expression level in the RO by immunostaining, and mRNA levels of BRN3, a RGC marker, and TUJ1 (TUBB), a neuronal marker, by RT-PCR. We succeeded in differentiating iPSCs derived from LHON patients into retinal organoids with a layered structure. The number of Brn3+ cells, TUJ1+ axon density, and expression were decreased in LHON-RO compared to Health-RO at 35 days after differentiation. In addition, the mRNA levels of BRN3 and TUBB were found to decrease in LHON-RO compared to Health-RO under similar conditions. Our established LHON-RO model mimics the clinical findings of LHON patients, i.e., reduced RGC and axonal loss. Taken together, this study provides a human retinal model for elucidating the pathogenesis of LHON and for developing of the new therapeutics.

Keywords: mitochondrial disease, retinal organoid, neuron

6:45 PM – 7:45 PM

522

UTILIZATION OF HUMAN IPSC-NEURAL SPHEROIDS FOR ASSESSING SIRNA-MEDIATED KNOCKDOWN

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Human induced pluripotent stem cells (hiPSCs) are cells that have the ability to be differentiated into numerous cell types including cells of the CNS like neurons and astrocytes. Once differentiated, hiPSC-derived cells exhibit many of the morphological and functional characteristics of in vivo cell types. As such, over the past decade, the utilization of hiPSCs-cell for 2D and 3D modeling of cellular development and drug discovery has expanded rapidly. RNA interference (RNAi) is an emerging technology utilizing small interfering RNAs (siRNAs) to target messenger RNA (mRNA), resulting in gene silencing. This platform allows for therapeutic intervention of targets that were once deemed undruggable with traditional drug moieties. hiPSC-derived cells provide an exciting model system for testing the potency and durability of emerging RNAi drugs. A gap that currently exists for siRNAs is the correlation between in vitro and in vivo (IV/IV) activity, specifically for the CNS. This gap has led to an increase in animal studies to screen siRNAs for drug efficacy. One way to address this problem is to utilize a model that more closely represents the in vivo tissue of interest. As such, in this study 3D-iPSC neural spheroids were tested for their capacity to serve as a CNS in vitro screening tool to increase IV/IV correlation and better represent tissue uptake and siRNA knockdown in vitro. Results demonstrate uptake and retention of lipid and non-lipid siRNAs in culture as well as dose-dependent gene knockdown that correlated to historical in vivo trends. Additionally, spheroids were tested for their capacity to accommodate various delivery of oligonucleotides to CNS cell types including non-lipid siRNA designs, ASOs, and LNPs in an effort to reduce lipid-mediated toxicity. Lastly, a library of 19 target sequences were screened in spheroids to improve IV/IV correlation for sequence-specific knockdown. HiPSC spheroids were successful in the uptake of lipid and non-lipid siRNAs, novel siRNA designs, ASOs, and LNPs and yielded improved correlation for knockdown compared to previous screening methods including neuroblastoma cell lines. As a result of improved IV/IV correlation, there is the opportunity in the future to reduce animal studies needed to screen siRNA drugs in early discovery and help get medicines to patients.

Keywords: siRNA, spheroids, neural

6:45 PM – 7:45 PM

524

CHD4 MUTATIONS DISRUPT THE DEVELOPMENT OF COCHLEAR ORGANOIDS

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Sensorineural hearing loss (SNHL) is a significant cause of functional disability worldwide affecting millions globally. The development of targeted therapies requires an in depth understanding the underlying molecular mechanisms of cochlear development and its disruption by mutated genes affected in SNHL patients. Recently, pluripotent stem cell-derived inner ear organoids were established as a scalable and high-fidelity alternative for studying human auditory biology . Chromodomain helicase DNA-binding protein 4 (CHD4) is an ATP-dependent chromatin remodeler that plays a central role in epigenetic gene regulation, DNA repair, and cell cycle progression. Several mutations were identified in CHD4, resulting in patients manifesting hearing loss under the syndrome of Sifrim-Hitz-Weiss (SIHIWES). In this study, we investigated the impact of CHD4 mutation on cochlear organoid development derived from human pluripotent stem cells (hESCs). To achieve this, we initially established a PAX2-mCherry reporter hESC line, allowing us to isolate otic progenitors efficiently. Subsequently, CRISPR / Cas9 technology was employed to generate hESC lines from mutant CHD4 p.G1003D and CHD4 knockout (KO) hESC lines. Mutant cochlear organoids show reduced number of hair cells, increased proliferation in both pluripotent state and during differentiation, and increased apoptosis in KO lines at pluripotent state. RNA-seq data on day 20 otic progenitors show disruption of developmental, survival, cell death and proliferation pathways. Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) data on otic progenitors show disruption of similar pathways. This study provides valuable insights into the specific role of CHD4 during the development of cochlear organoids.

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Keywords: inner ear, 3D organoids, epigenetic

6:45 PM – 7:45 PM

526

IPSC-BASED DISCOVERY OF NEURONAL RESCUE MECHANISMS IN MPS II: EFFICACY OF GSK-3B INHIBITION

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Mucopolysaccharidosis type II (MPS II) is an X-linked recessive genetic disorder characterized by the deficiency of iduronate-2-sulfatase, leading to the accumulation of glycosaminoglycans and severe neurodegeneration. To date, efficient therapies for MPS II neurodegeneration are still lacking. This study utilized induced pluripotent stem cells (iPSCs) from four MPS II patients to model neurodegeneration, explore the underlying molecular mechanisms, and identify potential new drugs. By differentiating these iPSCs into cortical neurons, we identified novel MPS II neuropathology, including significant axonal beading with phosphorylated tau accumulation and distinctive electrophysiological dysfunctions. The RNA sequencing analysis highlighted dysregulated Wnt/β-catenin, p38 MAP kinase, and calcium signaling pathways in mature neurons derived from MPS II iPSCs, with elevated glycogen synthase kinase-3β (GSK-3β) activity playing a potentially important role in neurodegeneration. Notably, intervention with a GSK-3β inhibitor not only improved neuronal survival but also restored neurite morphology and electrophysiological properties. These findings underscore the potential of iPSC-based models for unraveling disease mechanisms and testing therapeutic interventions in neurodegenerative diseases like MPS II. Our work proposes GSK-3β inhibition as a promising therapeutic strategy for MPS II neurodegeneration.

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Keywords: iPSC-derived neurons, neurodegeneration, GSK-3β inhibition

6:45 PM – 7:45 PM

528

MEF2C PROMOTES THE MATURATION OF ASCL1-INDUCED BASKET CELL-LIKE GABAERGIC NEURONS

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Fate conversion of non-neuronal cells by overexpression of neurogenic transcription factors (TFs) holds promise as a source of induced neurons for basic and translational research purposes. While neural progenitor-specific TFs such as Ascl1 can efficiently reprogram neural and non-neural cells to induced neurons (iNs), our ability to instruct the acquisition of molecular, morphological and functional properties typical of endogenous neurons is still limited. Promisingly, overexpression of a phosphorylation-defective mutant form of Ascl1 (Ascl1SA6) has recently been shown to convert cortical glia to an inhibitory neuron-like identity with marker expression (e.g. Parvalbumin) and functional features typical of cortical fast-spiking interneurons such as Basket cells, while retaining broadly immature morphological characteristics. While encouraging, these observations suggest that expression of TFs with endogenous roles at later stages of neuronal differentiation may be necessary to promote iN maturation. To test this hypothesis, we overexpressed Mef2c, a TF essential for Basket cell differentiation and whose expression is maintained in mature neurons. Intriguingly, albeit overexpression of Mef2c alongside Ascl1SA6 did not affect the abundance of Parvalbumin-expressing iNs, these neurons displayed more complex morphologies and progressively acquired the ability to extensively decorate the soma and dendrites of nearby iNs, resembling endogenous Basket cells. Interestingly, such morphologies could not be associated with markers of mature GABAergic synapses in vitro, suggesting that complex morphological and cell-cell interaction programs can be activated independently of synaptogenesis in the course of reprogramming. These observations highlight the modular nature of neuronal differentiation following fate conversion.

Keywords: reprogramming, interneurons, Ascl1 and Mef2c

6:45 PM – 7:45 PM

530

AUTOMATED TAGGING OF PARKINSON'S DISEASE ISOGENIC IPSC LINES FOR LIVE VISUALIZATION OF CELLULAR COMPARTMENTS IN DISEASE-RELEVANT CELL TYPES

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Defining novel phenotypes in neurodegenerative diseases such as Parkinson's disease (PD) is becoming increasingly tractable using iPSC-derived brain cell types in combination with high-content imaging techniques. Most studies, however, use fixed staining techniques that only yield endpoint data, despite the great potential for discovery in dynamic assays. Live imaging using fluorescently tagged iPSC lines is mostly done with single model lines, since reporter engineering and QC is laborious and slow. Studying a disease like PD with many reported risk alleles by live imaging of tagged lines representing all risk alleles in various genetic backgrounds has thus not been possible to date. To address this challenge, we used the NYSCF Global Stem Cell Array® automated platform to generate a bank of tagged clones from two cohorts of isogenic-quadruplet iPSC/ESC lines, including the well-characterized KOLF2.1J and H9 lines, each consisting of WT, GBA N370S, LRRK2 G2019S, and SNCA A53T. We created clones for each isogenic line with monoallelic GFP fusion tags of ACTIN-B, LAMININ B1, TOMM20 or LAMP1 from the Allen Institute Cell Collection. The automated platform at NYSCF enabled us to generate these lines in batches of 12-24 reactions; therefore, we can edit the same isogenic sets simultaneously, ensuring identical conditions, passage numbers, cell stress, reagents, etc, throughout the entire gene editing and clone generation process. We characterized each clone by flow cytometry, imaging, and sequencing to ensure proper integration and reporter localization without unwanted on-target effects. We are now differentiating these tagged lines into PD-relevant cell types such as NGN2 neurons, dopamine neurons, astrocytes, and microglia, and characterizing disease phenotypes by profiling baseline differences in organelle composition and dynamics, as well as their interactions with disease-relevant toxic cues such as alpha-synuclein pre-formed fibrils. All tagged PD-iPSC lines representing the 4 genotypes and 4 tags may be made available to collaborators to advance research across the field; we anticipate they will help to better understand cellular PD phenotypes and explore the potential of novel drugs to reverse these phenotypes.

Keywords: high-throughput tagging, gene tagging, Parkinson's disease

6:45 PM – 7:45 PM

532

CRB1 MUTATION ALTERS PHOTORECEPTOR DEVELOPMENT IN HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED RETINAL ORGANOIDS

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Inherited retinal diseases (IRDs) cause progressive and irreversible blindness in around two to five million people globally. Crumbs cell polarity complex component 1 (CRB1 mutations) account for approximately 10% of all genotypes associated with two common IRDs, causing severe, uncurable vision loss in children and young adults. This vision loss is caused by a decrease in number of light-sensitive photoreceptors (PRs), and some animal studies report potential Hippo/YAP and NOTCH pathways' involvement. However, detailed knowledge about the timing and mechanisms underlying PR loss in human CRB1-associated retinal disease (CD) is limited. As such, we hypothesize that CD leads to a prolonged proliferative state and increased cell death, lowering the PR population. To study human CD, we generated retinal organoids (ROs) from induced pluripotent stem cell lines derived from a healthy donor and a patient with a CRB1 mutation (p.Q120X; p.Q120X). CD ROs showed more disruptions in the outer neuroblastic layer compared to healthy ROs, suggesting structural disorganization as early as week 8. Immunofluorescent staining in healthy and CD ROs from weeks 8-20 showed sequential differentiation of early retinal cells (ganglion cells: BRN3a; retinal progenitors: PAX6, CHX10; PR/ bipolar progenitors: OTX2, CRX) at week 8, and later cells (rods: NRL) by week 13. However, CD ROs were positive for cone outer segment marker ARR3 unlike healthy ROs at week 8. Moreover, early assessments of mature CD ROs showed less rhodopsin staining and shorter brush borders compared to healthy ROs by week 30. These results suggests that CRB1 mutation may alter the number and/or development timing of PRs. Additionally, qRT-PCR of bulk RNA revealed significantly lower expression of genes in the NOTCH and Hippo/YAP pathways in CD ROs compared to healthy ROs at week 8. In conclusion, disease-specific retinal organoids showed alterations to PR development and revealed the NOTCH and Hippo/Yap pathways as potential targets for novel therapies.

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Keywords: organoids, CRB1-associated retinal disease, retinal development

6:45 PM – 7:45 PM

534

BRAIN EXTRACELLULAR MATRIX PROMOTES THE MATURATION OF DOPAMINERGIC PROGENITORS DERIVED FROM HUMAN EMBRYONIC STEM CELLS

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Acquiring authentic ventral midbrain dopamine (vmDA) neurons, which are focal targets of degeneration in Parkinson's disease (PD), is crucial for investigating the disease pathogenesis and developing novel therapeutics. Various methods have been developed for differentiating human pluripotent stem cells into vmDA progenitors, which highly express vmDA progenitor markers such as LMX1A, OTX2, FOXA2, and EN1. However, despite the high purity of vmDA progenitors, their maturation into vmDA neurons in vitro and the yield of vmDA neurons in vivo after transplantation remain comparatively low. In this study, we investigated the differentiation pathway from vmDA progenitors to vmDA neurons by conducting whole transcriptomic analysis of LMX1A-positive vmDA progenitors and PITX3-positive vmDA neurons derived from LMX1A-eGFP and PITX3-mCherry double knock-in human embryonic stem cells. Consequently, we found that extracellular matrix (ECM) was critical for maturation of vmDA neurons. To provide brain-specific extracellular matrix in current culture system, we utilized brain extracellular matrix (BEM) decellularized from pig cerebrum and midbrain tissue. The BEM-based culture system promoted the maturation of vmDA neurons and their axonal projection compared to Matrigel, indicating that brain-specific environmental factors including ECM have supportive effect on the differentiation of neuronal population. Our approach provides an efficient method for obtaining mature vmDA neurons, which could profoundly contribute to in vitro disease modeling and drug development for PD.

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Keywords: midbrain dopaminergic neuron, neural differentiation, brain extracellular matrix

6:45 PM – 7:45 PM

536

A TRIGEMINAL PEPTIDERGIC NOCICEPTOR MODEL TO STUDY MIGRAINE

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As the second largest cause of disability worldwide, migraine is a complex neurovascular disease involving the trigeminovascular system. Experimental models of migraine are challenging with the uncertain translational power of rodent models and a lack of robust human functional peptidergic models in vitro. Therefore, we aimed to create an iPSC-derived nociceptor (iNociceptor) model that (1) is embryologically canonical to the trigeminal ganglia, (2) produce and secret neuropeptides such as CGRP, and (3) express functionally responsive TRPV1. We first derived trigeminal placodes that contain neural progenitors of both cranial placode and neural crest origins, similar to in vivo development. Nociceptors arising from these progenitors naturally secreted CGRP, the level of which was further enhanced two-three folds by astrocyte co-cultures. We identified that the successful CGRP-secreting competency required both innate differentiation potentials of progenitors and the presence of glia during development. Further characterisation showed high expression of sensory neuron markers including multiple neuropeptides and ion channels and robust functional responses to noxious stimuli. Using migraine patient-derived iPSC lines, we are able to document differential CGRP release in response to inflammatory stimuli and migraine treatments. Overall, our study provided a robust and reproducible peptidergic nociceptor models which improved upon all existing methods with respect to accuracy of embryological origin, multi-neuropeptide expression and secretion, and functional TRPV1 responses. We also demonstrated the translational relevance of our model using iPSC lines from migraine patients with differential treatment responsiveness. We believe our work has provided insights on critical factors for human nociceptor cell fate specification and migraine pathobiology.

Keywords: nociceptor, calcitonin-gene related peptide, migraine

6:45 PM – 7:45 PM

538

DEVELOPING A HUMANIZED ASTROCYTIC CALCIUM IMAGING PIPELINE FOR COMPOUND SCREENING

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Astrocytes are crucial mediators of diverse aspects of brain function such as energy metabolism and synapse formation and maturation. They form a vast network in the brain and signal both with each other and with neurons. Calcium is the primary information carrier in astrocytes and can be measured using fluorescent indicators. Therapeutic approaches for cognitive diseases are commonly designed to influence neurons, but neglect astrocytes, despite them accounting for half the mass of the brain. Furthermore, screenings in murine cells result in low confidence of potential drugs that translate to humans. Here we use astrocytic calcium signals and recent advances in analysis to develop an astrocytic compound screening pipeline in a humanized model. Astrocytic calcium signals were measured using virally expressed calcium sensors based on GCaMP. Benchmarking was performed with a set of compounds of known effect in mouse hippocampal neuron-glia cultures. We then established an induced pluripotent stem cell-derived human astrocyte culture system and again measured calcium signals using GCaMP-based sensors. Human induced astrocytes showed wave-like events similar to those seen in mouse cells, and stimulation with ATP caused an increase of calcium events as expected. These preliminary data demonstrate that our humanized astrocyte cultures could replace murine cells in screens of compounds that affect astrocytic function. In the future, we will validate the pipeline with compounds of unknown effect such as LSD. Calcium imaging data from this humanized model can be used to investigate potential drug effects on neuronal and astrocytic function and predict efficacy in treating cognitive diseases with greater confidence.

Keywords: pipeline, astrocytes, calcium

6:45 PM – 7:45 PM

540

AD ASTRA: THE STELLAR GENERATION OF ASTROCYTES FROM HUMAN FIBROBLASTS

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A major gap in studying the molecular basis of age and age-related disorders stems from the inherent lack of efficient human model systems that capture age. Stem cell-derived models of neurons, astrocytes, and other cell types have been consistently shown to reflect fetal-like gene expression as well as DNA methylation signatures; and with increasing risk for age-related brain disorders, it has become increasingly necessary to develop better models that correlate with cells of the adult brain. Direct reprogramming approaches generating neurons from fibroblasts have been considered widely successful in this regard, and yet, research on glial cells, such as astrocytes, has been limited. Based on prior conversion approaches generating astrocytes from embryonic fibroblasts, we devised a strategy that can efficiently generate mature astrocytes from human adult fibroblasts using small molecules. Our approach yields GFAP/S100B/ALDH1L1 positive cells at >40% efficiency across multiple cell lines independent of age and sex of starting fibroblasts; and exhibit astrocyte-like glutamate uptake capacity, ATP-induced calcium signaling, and neuroinflammatory properties. Additionally, transcriptomic analysis and correlation to previously published datasets indicate an astrocytic population that resembles adult brain states. Comparison with iPSC-derived astrocytes from the same individual shows fibroblast-derived induced astrocytes to have higher oxidative stress and cellular senescence, indicative of an aging phenotype. Further studies are in progress to assess DNA methylation of these cells which would help establish epigenetic markers of age, in addition to further validating astrocyte-cell specificity. This method will complement current in vitro models of neurons and other cell types, and therefore serve as a useful tool in improving our understanding of age and disease-related processes in the adult brain.

Keywords: direct conversion, astrocyte modeling, lineage reprogramming

6:45 PM – 7:45 PM

542

A DRUG DISCOVERY PLATFORM FOR NEURODEVELOPMENTAL DISEASES BASED ON PATIENT-SPECIFIC CELLULAR DISEASE MODELS

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Neurodevelopmental disorders (NDDs), such as epilepsy, autism spectrum disorder or intellectual disability affect millions of patients worldwide, yet the development of novel therapeutics has not yielded significant progress. Induced pluripotent stem cells (iPSCs) create an opportunity for the development of disease- and patient-specific models that replicate the genetic complexity underlying NDDs. Recognizing the recent technical progress in patient-specific in vitro disease modeling as well as the huge unmet medical need, Neurolentech is building an innovative platform that combines patient-specific disease models for NDDs with biologically-relevant physiological and molecular assays allowing us to probe disease mechanisms in order to identify and validate drug targets as well as drug candidates. We have developed an optimized pipeline for generating neuronal models derived from patient blood samples building a commercially consented living cell biobank. For this, low amount peripheral blood mononuclear cells (PBMCs) are reprogrammed into iPSCs, which are subsequently converted into excitatory and inhibitory neurons using an inducible expression cassette with neural transcription factors (TFs). The resulting patient-specific neuronal models display physiological and molecular maturation, as well as, comprehensive expression of neurodevelopmental disease-associated genes. Our platform employs physiological and molecular assessment of cellular and network characteristics combined in disease-relevant assays using high-density multi-electrode arrays and transcriptomics, respectively. In summary, our optimized platform offers the resources and technological basis to tackle the discovery of novel targeted therapeutics for NDDs.

Funding Source: FFG, AWS, Xista Ventures

Keywords: disease model, drug discovery, neurodevelopmental disorders

6:45 PM – 7:45 PM

544

A POLYGENIC RISK SCORE-BASED IPSC MODEL OF BIPOLAR DISORDER TYPE 1

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Bipolar disorder type 1 (BD1) is a severe neuropsychiatric condition with a lifetime prevalence of about 1%. It has a heritability of around 70%, with common alleles explaining 20-27% of the variance in disease susceptibility. In the majority of cases, multiple genetic variants with small individual effect sizes are assumed to conspire in disease pathogenesis – a scenario difficult to address with classic genetic disease models. Here we set out to generate an induced pluripotent stem cell (iPSC)-based neuronal disease model based on polygenic risk scores (PRS) for BD1. Specifically, we generated 18 iPSC lines from three BD1 patients with high PRS and three healthy individuals carrying low PRS. Quality control based on high-resolution single nucleotide polymorphism analysis revealed a complex copy number variant architecture that is of particular relevance for studies where expected phenotypes are mild and have a polygenic nature. Since multiple reports point to a neurodevelopmental origin of BD1, we focused our analysis on the early stages of neuronal differentiation. Specifically, patient and control-derived iPSCs were differentiated into cortical neuronal progenitors (NPCs) and early postmitotic neurons using dual SMAD inhibition. Samples were collected at four different time points (days 0, 12, 22, and 32) and subjected to RNAseq. A number of differentially expressed genes (DEGs) were identified, including NBPF3, a gene of the NBPF gene family carrying an Olduvai domain which has greatly expanded during primate evolution and is associated with the severity of symptoms in schizophrenia. Another DEG identified in our study, IFITM2, has been associated with the innate immune response and found upregulated in schizophrenia. Our results suggest that patient selection based on PRS represents a realistic strategy for modeling BD1. We expect our experimental system to provide valuable insights into disease-associated changes in gene expression and signaling pathways during early human brain development.

Funding Source: This project is supported by a grant of the German Research Foundation and Swiss National Science Foundation

Keywords: bipolar disorder 1, neuronal progenitors, polygenic risk scores

6:45 PM – 7:45 PM

546

COMBINATION OF IPSC-DERIVED MOTOR NEURON PROGENITOR CELLS WITH IRRADIATED BDNF OVER-EXPRESSING EMSCS ENHANCED RESTORATION OF AXONAL REGENERATION IN A CHRONIC SPINAL CORD INJURY RAT MODEL

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Spinal cord injury (SCI) is a disease that causes permanent impairment of motor, sensory, and autonomic nervous system functions. Stem cell transplantation for neuron regeneration is a promising strategic treatment for SCI. However, selecting stem cell sources and cell transplantation based on experimental evidence is required. Therefore, this study aimed to investigate the efficacy of combination cell transplantation using the brain-derived neurotrophic factor (BDNF) over-expressing engineered mesenchymal stem cell (BDNF-eMSC) and induced pluripotent stem cell-derived motor neuron progenitor cell (iMNP) in a chronic SCI rat model. A contusive chronic SCI was induced in Sprague-Dawley rats. At 6 weeks post-injury, BDNF-eMSC and iMNP were transplanted into the lesion site via the intralesional route. At 12 weeks post-injury,

differentiation and growth factors were evaluated through immunofluorescence staining and western blot analysis. Motor neuron differentiation and neurite outgrowth were evaluated by co-culturing BDNF-eMSC and iMNP in vitro in 2-dimensional and 3-dimensional. Combination cell transplantation in the chronic SCI model improved behavioral recovery more than single-cell transplantation. Additionally, combination cell transplantation enhanced mature motor neuron differentiation and axonal regeneration at the injured spinal cord. Both BDNF-eMSC and iMNP played a critical role in neurite outgrowth and motor neuron maturation via BDNF expression. Our results suggest that the combined transplantation of BDNF- eMSC and iMNP in chronic SCI results in a significant clinical recovery. The transplanted iMNP cells predominantly differentiated into mature motor neurons. Additionally, BDNF-eMSC exerts a paracrine effect on neuron regeneration through BDNF expression in the injured spinal cord.

Funding Source: A grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, & Future Planning (grant number: NRF-2020R1AC3004123 and NRF-2021R1C1C2004688).

Keywords: BDNF over-expressing engineered MSCs, IPSC-derived motor neuron progenitor cell, chronic spinal cord injury

6:45 PM – 7:45 PM

548

DEFICIENCY IN THE AUTISM CANDIDATE GENE CNTNAP2 PROMOTES HYPERACTIVITY IN FORWARD PROGRAMMED HUMAN SENSORY NEURONS

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Sensory abnormalities and chronic pain are severe comorbidities in a number of neurological, neuropsychiatric and neurodevelopmental disorders, including autism spectrum disorder (ASD). While abnormal sensory responses in ASD have been linked to altered connectivity in higher brain regions, recent findings suggest that ASD-related mutations also impact the functionality of peripheral sensory neurons (SNs). Here we explored the function of CNTNAP2, a gene that encodes the transmembrane protein CASPR2. Loss-of-function mutations of CNTNAP2 and autoantibodies against CASPR2 have been found to be associated with ASD and neuropathic pain. We employed human induced sensory neurons (iSNs) generated by inducible overexpression of NGN1, BRN3A, and ISLET1 to study the role of CNTNAP2 in pain sensation. This forward programming paradigm yields iSNs which express characteristic markers such as e.g. PRPH, NTRK1, Nav1.7, Nav1.8 and P2RX3, generate TTX-resistant sodium currents, and form functional synapses with human CNS neurons. We found that CNTNAP2-deficient iSNs efficiently differentiate into functional sensory neurons. However, in multi-electrode array recordings CNTNAP2-deficient iSNs displayed increased firing rates and numbers of bursts, accompanied by a shorter burst duration. Furthermore and in line with previous reports indicating that CNTNAP2-deficiency can disrupt membrane localization of the voltage-gated potassium channel Kv1.2 in rodent sensory neurons, voltage clamp analysis of iSNs revealed a significant reduction in voltage-gated potassium currents in mutant cells. Thus, our data suggest that CNTANP2-deficiency impairs Kv channel function, resulting in sensory neuron hyperactivity. We expect our iSN platform to further unravel pathomechanisms connecting CNTNAP2 deficiency and hyperactivity and to serve as a tool for drug discovery for affected patients.

Keywords: sensory neurons, CNTNAP2, autism spectrum disorder

6:45 PM – 7:45 PM

550

DECIPHERING THE COMPLEXITIES OF RETINITIS PIGMENTOSA: AN INTEGRATED ORGANOID MODEL APPROACH

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This study leverages advanced in vitro models, combining retinal and brain organoids to elucidate the impact of PRPF31 mutation on the development and survival of retinal ganglion cells (RGCs) and photoreceptors, offering insights into the pathology of Retinitis Pigmentosa (RP) and related neurodegenerative diseases. By integrating iPSC-derived organoids, we aim to create a comprehensive model that reflects the human visual system's complexity, focusing on the progression of inherited retinal dystrophy (IRD) and its effects on RGC development. iPSCs derived from severe RP subjects with PRPF31 mutation, an unaffected controls and a AAVS1::CAG-P_EGFP iPSC line, were differentiated into retinal and cortical brain organoids, then integrated into assembloids. Utilizing immunofluorescence, confocal microscopy, live imaging, qPCR, micro electrode array (MEA) systems, and single-cell RNA sequencing (scRNA-Seq), this study investigates the molecular and functional implications of the PRPF31 mutation, emphasizing photoreceptor degeneration and RGC axonal outgrowth. The assembloids demonstrated successful integration, with enhanced survival of RGCs and photoreceptors, and replicated the spatiotemporal organization of the human visual system. Functional assessments revealed significant electrophysiological activity changes, highlighting the mutation's impact on cellular functionality and gene expression patterns at the single-cell

level, providing a deeper understanding of RP's progression. By combining retinal and brain organoids into a unified model, this research offers a novel perspective on studying inherited retinal degenerations and neurodegenerative diseases. The study not only advances our understanding of the genetic and molecular mechanisms underlying RP but also proposes a versatile platform for developing vision restoration strategies and personalized therapeutics for retinal and neurological disorders.

Keywords: assembloid, retinal organoids, cortical organoids

6:45 PM – 7:45 PM

552

CRACKING THE CODE: 7-DAY DIRECTED DIFFERENTIATION OF HIGH PURITY SCHWANN CELL PRECURSORS FROM ANY HIPSC LINE

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Schwann cells, the major glial cell type within the peripheral nervous system, are implicated in a myriad of human diseases including neuropathic pain, Charcot-Marie-Tooth disorder, and diabetic peripheral neuropathy. A rapid, scalable, and reproducible protocol to produce Schwann cells from multiple human induced pluripotent stem cell (hiPSC) lines would aid in the ability to study these disorders in a patient-relevant context. SCPs, motor neurons, and sensory neurons were differentiated from the same hiPSC lines. The identity of all three cell types were confirmed by immunocytochemistry, qPCR, and RNA sequencing. Syngeneic SCPs and motor neurons were co-cultured and evaluated for alignment and myelination via immunocytochemistry and transmission electron microscopy. SCPs and sensory neurons were evaluated by the same methods and these experiments were replicated with multiple donor lines. Lastly, electrical activity of neuron-Schwann cell co-cultures from each line was tracked on multi-electrode array (MEA) plates to investigate the impact of donor-source on the electrophysiology of co-cultures. Manufactured SCPs expressed the classical markers for the Schwann cell lineage SOX10, S100b, and OCT6 by qPCR and immunocytochemistry. Time course bulk RNA sequencing of matured SCPs demonstrated increasing similarity to primary human Schwann cells. The co-cultures undergo rapid SCP-axon alignment within 48 hours, and myelination was observed via transmission electron microscopy and expression of the myelin-associated proteins–myelin basic protein (MBP) and myelin protein zero (MPZ) as early as 5 weeks in culture. Functional studies on MEA plates showed co-cultures with Schwann cells have higher levels of baseline activity in a shorter period of time than neuron cultures alone. These findings show that iPSCs from multiple donors can be successfully differentiated into functional Schwann cell precursors in a rapid and reproducible manner. This process allows for researchers to develop patient-specific models to investigate the different diseases affecting Schwann cells and myelination within the peripheral nervous system.

Keywords: Schwann cell progenitors, peripheral myelination, co-cultures

6:45 PM – 7:45 PM

554

DEEP LEARNING ANALYSIS ON IMAGES OF IPSC-DERIVED MOTOR NEURONS CARRYING FALS-GENETICS REVEALS DISEASE-RELEVANT PHENOTYPES

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Amyotrophic lateral sclerosis (ALS) is a devastating condition with very limited treatment options. It is a heterogeneous disease with complex genetics and unclear etiology, making the discovery of disease-modifying interventions very challenging. To discover novel mechanisms underlying ALS, we leverage a unique platform that combines isogenic, induced pluripotent stem cell (iPSC)-derived models of disease-causing mutations with rich phenotyping via high-content imaging and deep learning models. We introduced eight mutations that cause familial ALS (fALS) into multiple donor iPSC lines, and differentiated them into motor neurons to create multiple isogenic pairs of healthy (wild-type) and sick (mutant) motor neurons. We collected extensive high-content imaging data and used machine learning (ML) to process the images, segment the cells, and learn phenotypes. Self-supervised ML was used to create a concise embedding that captured significant, ALS-relevant biological information in these images. We demonstrate that ML models trained on core cell morphology alone can accurately predict TDP-43 mislocalization, a known phenotypic feature related to ALS. In addition, we were able to impute RNA expression from these image embeddings, in a way that elucidates molecular differences between mutants and wild-type cells. Finally, predictors leveraging these embeddings are able to distinguish between mutant and wild-type both within and across donors, defining cellular, ML-derived disease models for diverse fALS mutations. These disease models are the foundation for a novel screening approach to discover disease-modifying targets for familial ALS.

Keywords: amyotrophic lateral sclerosis (ALS), machine learning, high-content imaging

6:45 PM – 7:45 PM

556

CRACKING THE CODE: 7-DAY DIRECTED DIFFERENTIATION OF HIGH PURITY SENSORY NEURONS FROM ANY HIPSC LINE

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More effective, personalized treatment options are urgently required for chronic pain which affects one in five people worldwide. A scalable, rapid, and reproducible protocol to produce sensory neurons from multiple human induced pluripotent stem cell (hiPSC) lines would aid in better understanding the genetic basis of pain, and accelerate drug discovery platforms for novel analgesics. In this study, three different hiPSC lines (wild type - female, wild type - male, pain agnosia - female) were differentiated using a novel seven day directed differentiation protocol composed of daily combinations of small molecules and growth factors to recapitulate sensory neuron developmental stages. Initially, single cell seeding density titrations were carried out in 6-well plates to establish the best density for optimal differentiation before sensory neuron production was scaled up in flask format and banked. Sensory neurons were then thawed and matured before being molecularly and functionally characterized. Sensory neurons generated from all lines expressed BRN3A, PRPH, ISLET1, and TUJ1 at a purity of greater than 95% via immunocytochemistry. The three different donor lines were found to have similar gene expression profile via qPCR for the pan-sensory neuron ion channels: NaV1.7, NaV1.8, NaV1.9, TRPV1, CAV3.2, P2RX3, and TRKA. Lastly, sensory neurons derived from each line show morphological similarities when matured on multi-electrode array plates, but have functional differences in baseline activity through 4 weeks. These findings show a rapid seven day directed differentiation protocol can be applied to multiple hiPSC donor lines and successfully produce functional, sensory neurons for downstream assays. This process would allow researchers to test many biological samples with diverse backgrounds to better understand the genetic differences in pain and develop more effective, personalized treatment options.

Keywords: pain, sensory neurons, voltage gated sodium channels

6:45 PM – 7:45 PM

558

CRISPR-ENHANCED IPSC DIFFERENTIATION AND ENRICHMENT FOR THE ASSEMBLY OF NOVEL BIOPRINTED RETINAL MODELS IN AMD RESEARCH

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Age-related Macular Degeneration (AMD), a prevalent global cause of vision loss in the elderly, necessitates the development of efficacious treatments. Traditional approaches in preclinical research, including the use of laboratory animals and simplified 2D in-vitro cultures, have been limited in their ability to fully model the complexity and intricacies of AMD. Animals do not fully replicate human disease physiology, and 2D cultures fail to capture the sophisticated architecture and interconnectivity of retinal cells, which are essential for maintaining retinal homeostasis. For these reasons, researchers are now aiming to develop novel multilayered 3D bioprinted retinal tissues that could be used for disease modeling and in-vitro preclinical pharmacological testing. However, the construction of accurate 3D retinal models faces significant hurdles, primarily due to the reliance on prolonged and inefficient differentiation protocols, such as retinal organoid differentiation, and the absence of methods for purifying specific retinal cell types from the mixed cell populations produced during differentiation. To overcome these obstacles, our project introduces a novel strategy designed to enhance the efficiency of retinal differentiation and facilitate the isolation of specific cell types for subsequent incorporation into 3D bioprinted models. We have engineered induced pluripotent stem cell (iPSC) lines with fluorescent reporters for key retinal lineages: photoreceptors and bipolar cells. Additionally, in these same cell lines, we have implemented CRISPRa constructs to activate the expression of retinal differentiation genes, like RCVRN, upon doxycycline treatment, enhancing differentiation efficiency and reducing culture time. Using our approach, we have successfully generated iPSC lines that differentiate more efficiently into targeted retinal cell types and have managed to purify specific retinal cell progenitors that can now be used for 3D bioprinting. This paves the way for the construction of complex 3D retinal models that could offer a more reliable platform for preclinical pharmacological testing and the exploration of novel therapeutic interventions.

Funding Source: Marie Skłodowska-Curie Actions - Postdoctoral Fellowship (European Comission)

Keywords: age-related macular degeneration, disease modeling, CRISPR/Cas9

6:45 PM – 7:45 PM

560

BUILDING A FUNCTIONAL THALAMOCORTICAL CIRCUIT FROM REGIONALIZED HUMAN BRAIN ORGANOIDS

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The thalamocortical system is essential for important brain functions including sensory and motor processing, sleep, and attention, and has been implicated in neurological and neuropsychiatric disease. Central to the function of this circuit is its ability to generate and sustain brain-wide rhythms. Recent advances in pluripotent stem cell (PSC) technologies have made it possible to generate PSC-derived three-dimensional cultures, or "organoids", that resemble regions of the human brain that contain the essential components of the thalamocortical rhythm generator circuit. Initial attempts to reconstruct a model of the thalamocortical circuit by fusing separately patterned, regionalized PSC-derived organoids to form human thalamocortical assembloids (hTCOs) have enabled generation of the excitatory cell components of the thalamocortical circuit. A recent report has shown that ventralization of human thalamic organoids (hTOs) can produce thalamic inhibitory cell populations. Here, using ventralized hTCOs (vhTCOs), we study the role of oscillatory activity in thalamocortical circuit development and maturation. After culturing individual human cortical organoids (hCOs) and ventralized hTOs (hvTOs) for 30 and 100 days, we performed fluorescent immunohistochemistry and qPCR-based assessments. hCOs and hvTOs expressed key region-specific markers and maturation markers while maintaining appropriate cell identity markers. After these initial investigations, we interrogated vhTCO network activity via calcium imaging using fluorescent wide-field and two-photon microscopy. Here, we have observed spontaneous oscillations as well as evoked oscillations following electrical stimulation. We found that 10Hz and 20Hz electrical stimulations elicited the greatest number of events and resulted in greater event frequency post-stimulation. Altogether, these experiments are aimed at establishing an in vitro model of the thalamocortical circuit for use in studies of development and thalamocortical circuit disorders.

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Keywords: thalamocortical, assembloid, imaging

6:45 PM – 7:45 PM

562

APOE GENOTYPES DRIVES DIFFERENTIAL NEUROLOGICAL INFECTION OF SARS-COV-2 THROUGH MODULATED HOST SUSCEPTIBILITIES IN ALL-IN-ONE BRAIN ASSEMBLOIDS

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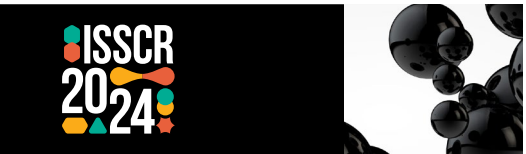
Amid the pronounced disparities of clinical spectrum upon SARS-CoV-2 infection, numerous studies have aimed to pinpoint the potential contributors that implicate the different degree of susceptibility to SARS-CoV-2 and heterogeneity of COVID-19 outcomes, yet the underlying mechanism is still insufficiently understood. Particularly, more than 1/3 of the hospitalized patients exhibited neurological manifestation to varying extend. In this study, we showcased that apolipoprotein E (APOE), a well-known risk gene factor for Alzheimer's disease, confers to variant-dependent host susceptibility for SARS-CoV-2 in APOE isogenic all-in-one multilineage brain assembloids comprising hiPSC derived neuron, microglia, pericyte and endothelial cells. Quantitative PCR confirmed that organoids with APOE2/2 and APOE4/4 genotypes are more susceptible to SARS-CoV-2 infection, displaying higher viral loads relative to APOE3/3, which is aligned with the UK Biobank epidemiological study. Intriguingly, APOE2/2 cells exhibited even higher viral loads compared to APOE4/4, suggesting APOE2/2's typical neuroprotective role in AD does not extend to SARS-CoV-2 infection but rather increases the infection susceptibility. Through extensive data mining of publicly available RNAseq and literature, we identified a list of genes potentially mediating APOE-variant dependent susceptibility, which were further validated on the transcriptomic level and protein level with our brain organoids. We reasoned that elevated expression of viral entry and receptor genes in APOE2/2 and APOE4/4 cells compared to APOE3/3 leads to higher infection susceptibility. Such augmented



susceptibility resulted in abnormal angiogenesis and vascular injury signaling, quantified by the cytokine and chemokine levels, implying an increased risk of brain hemorrhage and stroke for APOE 2/2 and APOE4/4 COVID-19 patients. Our findings bridge the gap between different APOE genotypes and the heterogeneity of COVID-19 outcomes, and further suggesting the potential for personalized treatments and preventative interventions for COVID-19 patients with different APOE genotypes. This necessitates further research into how SARS-CoV-2 infection might predispose individuals with various APOE isoforms to different extent of neuropathogenesis.

Funding Source: NIH/NIA/Alzheimer's Disease Research Center Developmental Award, P30 AG066507

Keywords: brain organoid, APOE, SARS-CoV-2

6:45 PM – 7:45 PM

564

A COMPREHENSIVE RESEARCH PLATFORM LEVERAGING IPSC-DERIVED 3D SPHEROIDS AND SINGLE-CELL RNASEQ TECHNOLOGY TO EXPLORE NEURONAL AND GLIAL CONTRIBUTIONS IN LEUKODYSTROPHIES

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Leukodystrophies (LDs) are a group of rare genetic brain disorders typically appearing in early childhood. They are characterized by progressive white matter (WM) degradation. While conventionally attributed to glial dysfunction, recent studies point to significant neuronal involvement in the WM pathology of LDs. To fully explore these disorders, we developed an innovative research platform leveraging human induced pluripotent stem cells (iPSCs) technology and long-term 3D spheroids containing neuronal, astro- and oligodendroglial cells ("myelinoids") coupled with single-cell RNA sequencing (scRNAseq) for the comparative analysis of overarching and disease-specific pathomechanisms. Collaborating in the context of the European Joint Programme on Rare Diseases (EJP RD; project NG4leuko), we analyzed scRNAseq data from iPSC-derived 3D spheroids of healthy donors (n=5), patients with 4H syndrome (n=2) and Globoid leukodystrophy (GLD; n=3), as well as CRISPR-engineered Galactocerebrosidase (GALC) and aspartoacylase (ASPA) knockout cells as models of GLD and Canavan disease (CD), respectively. We implemented stringent quality control regimens and conducted all experiments in a centralized location to ensure reproducibility and comparability. Spheroids propagated for up to 150 days underwent scRNAseq and immunofluorescence analyses at different time points. ScRNAseq data revealed a spectrum of glial and neuronal progenitor cell populations and their emerging differentiated progeny. Immunofluorescence data reflected the advanced maturity of the evolving cell types, including MBP-positive oligodendrocytes. In addition, we identified changes in gene expression profiles over time and across different pathologies. Integrating scRNAseq data with immunophenotypic analyses enhanced our understanding of the relationship between gene expression changes and disease-related cellular alterations, including changes in oxidative phosphorylation and E2F targets. We expect our platform to provide valuable insights into the molecular signature associated with LDs, providing temporal dynamics of gene expression, details on critical molecular pathways, and mechanistic insight into the early pathogenesis of these disorders, thereby providing a basis for therapeutic intervention.

Funding Source: European Joint Programme on Rare Diseases (EJP RD JTC 2019)

Keywords: genetic brain disorder, brain organoid, single-cell RNAseq

6:45 PM – 7:45 PM

566

ASSAY DEVELOPMENT FOR THE CHARACTERIZATION OF SPINE FORMATION AND NETWORK MATURATION OF IPSC-DERIVED NEURONS

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Induced pluripotent stem cell (iPSC)-derived neurons hold much promise as tools for in vitro disease modeling and drug discovery. However, synaptic maturation has been reportedly limited in iPSC-derived neurons, which made them difficult to use in cell-based assays compared to rodent neurons. Recent methods of transcription factors (TF)-based iPSC differentiation achieve accelerated and synchronized

neurogenesis, offering the opportunity to address further progression into late developmental stages. We have previously shown that TF-induced iPSC-derived neurons reached advanced synaptic maturation and spinogenesis reproducibly in about 70 days of culture. These neurons gained postnatal brain features as revealed by RNA-sequencing and immunocytochemistry, suggesting that they undergo an intrinsic maturation program after neurogenesis. Such mature neurons would be valuable as models to study synapse formation and alterations predictive of drug responses. For this purpose, a further challenge is to establish quantitative methods to assess functional maturation and phenotypic changes in cell-based assays. We report herein two approaches of functional characterization made possible by using mature iPSC-derived neurons. First, we developed an artificial intelligence-based imaging assay of synaptic marker density, which allows the automated screening of confocal images from 96- and 384-well plate cultures. We demonstrated the feasibility of establishing a dose-response curve to a 10-minute glutamate exposure by detecting the delocalization of the postsynaptic spine marker drebrin, which is indicative of spine functional maturation. Second, we cultured iPSC-derived neurons onto high-density multielectrode arrays to monitor their activity from week 3 to week 12. Changes in network burst profiles correlated with a sharp increase in axonal propagation from around week 9, which coincided with the typical period when dendritic spines start to be detected by imaging, thereby allowing us to extract distinctive electrophysiological features associated with network maturity. Overall, this work contributes insights into the potential of mature iPSC-derived neurons for developing powerful assays that are relevant to human brain functions and cognitive disorders.

Keywords: iPSC-derived neurons, synapse, neurodevelopment

6:45 PM – 7:45 PM

568

BRAIN ORGANOIDS: NAVIGATING THE COMPLEXITIES OF CONSCIOUSNESS

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Brain organoids are three-dimensional cellular systems that are pivotal in advancing neuroscience. They replicate the brain's cellular diversity and architecture, enabling the formation of neural networks crucial for simulating brain functions. These organoids can form synaptic connections, generate electrical activity, and respond to stimuli, providing valuable insights into neural functionality and the potential for drug testing and toxicity screening. Despite these advances, brain organoids lack the full functionality of the human brain, including consciousness, due to their simplified nervous system architecture and other current technological limitations. Unlike empirical investigation, which relies on direct observation and experimentation, theoretical investigation involves using conceptual frameworks, philosophical inquiry, and computational models to explore the possibility, nature, and implications of consciousness or consciousness-like phenomena in these complex structures. Theoretical investigations complement empirical research by exploring the potential for consciousness in organoids through conceptual frameworks, philosophical inquiry, and computational models. So far, the overarching consensus is that brain organoids lack the necessary attributes for consciousness in their current form and with current technology. This consensus is informed by the current understanding of neuroscience, ethics, and the philosophical underpinnings of consciousness. As the field evolves, these discussions remain critical for guiding responsible research and addressing potential ethical dilemmas. Also, while not explicitly focusing on organoid consciousness, ethical discussions emphasize the importance of ethical communication and consider neuroscience's moral dimensions carefully. These investigations and ethical considerations are essential for guiding realistic research aims, and the scientific community must remain vigilant in monitoring these developments to ensure responsible research practices

Keywords: organoids, consciousness, ethics

6:45 PM – 7:45 PM

570

DEVELOPING IN VITRO MODEL OF PARKINSONS DISEASE FOR PHENOTYPIC DRUG SCREENING

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Parkinson's disease (PD) is an incurable neurodegenerative condition associated with debilitating motor symptoms caused by progressive loss of dopaminergic (DA) neurons in the substantia nigra. While disease-modifying treatments are desperately needed, success rate in drug discovery for PD and neurological diseases in general has been alarmingly low. Some underlying reasons for this may be the use of poorly representative animal models, as well as a focus on single target-based screening approaches which may not be suitable for highly complex and sporadic diseases like PD. Phenotypic assays hold greater promise but are challenged by the inherent difficulty in coupling pathologically relevant disease models with clinically relevant high-throughput readouts. Here, we propose a novel screening assay based on a stem-cell derived model of PD pathology using alpha-synuclein overexpression together with preformed fibrils in co-cultures of authentic human DA neurons, microglia, and astrocytes. The read-out for the disease model relies on a fluorescence-based neurotransmitter detection assay using dopamine sniffer cells (GRAB-DA 2M). The detection assay provides a simple quantitative readout of spontaneous dopamine release from cultured neurons through sampling of culture medium. Notably, the sampling is not terminal and therefore allows monitoring of neuronal function at multiple different timepoints. Using this platform, we aim to screen small molecules to identify candidates which not only improve DA neuron survival upon alpha-synuclein-induced pathology, but also support functional DA release. The platform is unique in being target-agnostic and can thereby identify compounds which work through actions on either neurons, astrocytes, or microglia – or potentially through all three.

Keywords: Parkinson's disease, drug screening, in vitro modeling

6:45 PM – 7:45 PM

572

AN INDUCED PLURIPOTENT STEM CELL-DERIVED HUMAN TO MOUSE XENOTRANSPLANTATION MODEL FOR STUDYING THE EFFECTS OF TAU FILAMENTS IN FRONTOTEMPORAL DEMENTIA

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Frontotemporal dementia (FTD) is a cause of early-onset dementia with no effective treatments. FTD is partially pathologically characterized by the accumulation of cytotoxic protein aggregates including hyper-phosphorylated forms of microtubule-associated protein tau, encoded by the MAPT gene. The exact mechanisms of toxicity conferred by protein dysregulation are incompletely understood but represent an attractive therapeutic target. There is a need for accurate and reproducible FTD models that overcome translatability concerns of animal models or immortalized cell lines. Here we developed a model of FTD using human induced pluripotent stem cell (iPSC)-derived neurons reprogrammed from either healthy controls or patients harboring the causal FTD mutation MAPT N279K (henceforth termed FTD neurons). iPSC-derived FTD neurons recapitulate disease hallmarks including tau pathology with increased numbers of phospho-tau positive neurons, increased oxidative stress, metabolic alterations, and impaired neurite outgrowth. The addition of tau filaments purified from either healthy brain tissue or tissue from patients with MAPT-mediated FTD to FTD neurons induced upregulation of genes associated with apoptosis, oxidative phosphorylation, proteasome, and unfolded protein response. Additionally, genes related to neuroinflammation were upregulated including secreted phosphoprotein 1 (SPP1), a multifunctional cytokine which is also upregulated in the brains and cerebrospinal fluid of FTD patients. In parallel we have developed a novel xenotransplantation paradigm where purified filaments from FTD patients or healthy brain extract were co-injected with either control (wild-type) or FTD neural progenitor cells into the forebrains of immune-deficient mice. Cells were allowed to differentiate in vivo for six months, followed by histological characterization for graft size, extent of graft integration into the parenchyma, and interactions at the graft-host interface including measures of reactive astro- and microgliosis. Findings from these analyses will be presented in detail at the conference. The results of this study will help to clarify the effect of disease-associated protein aggregates on cell physiology in vitro and in vivo using a highly translatable patient stem cell-derived model.

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Keywords: xenotransplantation, disease modeling, frontotemporal dementia

6:45 PM – 7:45 PM

574

A NOVEL PROTOCOL FOR THE GENERATION OF PSC-DERIVED BRG CELLS IN 2D ALLOWS TO STUDY THE MECHANISMS OF BRG CELL BEHAVIOR IN VITRO

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Crucial steps during primate evolution led to the development of a structurally and functionally complex human brain, setting Homo sapiens apart from all other species and posing a persistent challenge for investigation. The expansion of basal radial glial (bRG, also termed outer radial glia) cells, a primate-amplified progenitor population, is believed to contribute to the increase in size and complexity of the human brain. Until recently, our knowledge about bRG cells was based on human fetal cortex ex vivo studies. However, such studies are limited due to the lack of tissue availability. Basal RG cells have also been identified in brain organoids generated from human pluripotent stem cells (PSC). Here, we focus on developing a protocol for the generation and expansion of PSC-derived bRG cells as homogenous 2D cell

cultures. Specifically, we have identified culture conditions comprising a cocktail of small molecules, designed to emulate the in vivo neural stem cell niche of bRG cells in vitro. Immunocytochemical analysis and transcriptional profiling of our cells identified the presence of a plethora of genes recently associated to bRG fates. During mitosis, bRG cells showed characteristic mitotic somal translocation (MST), a hallmark of bRG cell behavior. When studying the molecular mechanism of MST in more detail we identified a major role of YAP1 signaling in this cell type-specific movement. Furthermore, the in vitro generated bRG cells demonstrate the capability to differentiate into various cortical layer neurons and astrocytes. Finally, co-culture experiments with fore-brain-type organoids indicate the inclination of bRG cells to colonize and proliferate in the subventricular zone-like area, giving rise to neurons and glia. Consequently, our 2D protocol opens up new avenues to investigate bRG cell dynamics and human brain development in vitro.

Keywords: basal radial glial cells, neurodevelopment, mitotic somal translocation

6:45 PM – 7:45 PM

576

DELINEATING NEURONAL CIRCUITS FORMATION IN 7Q11.23 CNV USING BRAIN ASSEMBLOIDS

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Neuronal circuits enable our cognitive capacities and emotional processing. Assembly of neuronal circuits consist of the integration of excitatory pyramidal neurons and inhibitory interneurons that are generated at distant locations of the developing brain. The majority of inhibitory interneurons migrate large distances towards the cortical plate and integrate into the nascent circuits. Defects in interneuron migration disrupt the formation of neuronal circuits and are implicated in neuropsychiatric disorders. Here we tackle the dosage-dependent architecture of migratory alterations by focusing on Williams-Beuren (WBS) and 7q microduplication (7Dup) syndromes, caused by, respectively, deletion or duplication of the 7q11.23 region. While WBS and 7Dup individuals share intellectual disability, WBS have an over-gregarious personality and hypersociability. In contrast, 7Dup exhibit language deficits along with ASD traits. Currently, nothing is known about interneuron migration and the formation of neuronal circuits in 7q11.23 copy number variation (CNV). To understand these fundamental neurodevelopmental processes, we used 7q11.23 patient-derived iPSC to generate organoids that recapitulate dorsal (pallium) and ventral (subpallium) telencephalon and fused them to generate brain assembloids. To quantify interneuron migration from ventral to dorsal part of the assembloid, we virally labelled interneurons and tracked them using time-lapse microscopy. Furthermore, to reveal the molecular underpinnings of interneuron migration, we transcriptionally profiled assembloids 50 days post fusion. We revealed a 7q11.23 dose-dependent robust migration phenotype in WBS with increased velocity, distance covered and interneuron saltation frequency. Single cell RNAseq profiling of assembloids uncovered genotype and lineage-specific differences in CNS development and cell-cell communication. Trajectory inference analysis of inhibitory lineage from cycling progenitors to mature neurons uncovered distinct terminal states related to translation and neuronal excitability. These findings reveal a novel aspect of 7q11.23 CNV neurodevelopmental defects and underscore the potential of brain assembloids in illuminating patient-specific neurodevelopmental functional endophenotypes.

Funding Source: European Research Council Fondazione Umberto Veronesi Telethon

Keywords: brain organoids, disease modeling, interneurons

6:45 PM – 7:45 PM

578

BETTER CHANCES FOR BRAIN CANCER PATIENTS USING HUMAN CEREBRAL ORGANOIDS TO STUDY CNS SIDE EFFECTS AND TUMOR CONTROL AFTER RADIATION EXPOSURE

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Radiotherapy is the standard of care for the management of brain tumors such as glioblastoma (GBM), but the prognosis remains poor and radiation can induce severe side effects including brain necrosis (BN), which can be fatal if left untreated. BN occurs weeks or even months after irradiation, more frequently in pediatric patients, and is diagnosed as contrast-enhancing lesions on MRI scans. The initiating pathways are largely unknown, impeding early diagnosis and treatment. We studied the response of human cerebral organoids to different

radiation qualities (standard photon versus state-of-the-art proton and carbon ion irradiation) to identify the onset and progression of BN. Cerebral organoids responded to photon (X-ray), proton, and carbon irradiation with general growth retardation and decline in neural/glial progenitors. However, they showed increased formation of liquid-filled cavities similar to choroid plexus (CP), the blood-cerebrospinal-barrier and site of cerebrospinal fluid (CSF) production. Morphologically, CP formation (ZO1+/AQP1+/CLN3+) resembled contrast-enhancing lesions attributed to BN. Yet, irradiation did not correlate with an increase of extracellular lactate dehydrogenase (LDH), a marker of necrosis, but it led to alterations in the expression of members of NOTCH (NOTCH 1, NOTCH2, NGN2, HES1 and HES5) and WNT (WNT5a, LEF1) pathways, which were causative for radiation-induced CP formation. This effect was more pronounced in immature than in mature organoids, and protons and carbon ions were more effective than X-rays at the same dose. Our results suggest aberrant CP formation and subsequent excessive CSF production as a novel mechanism leading to contrast-enhancing BN lesions post radiotherapy, and point towards new potential therapeutic targets (i.e., members of Notch and WNT pathway or CSF inhibitors).

Funding Source: This project is supported by the Federal Ministry of Education and Research (02 NUK 049A) and NIH grant 1RO1CA256848-01. Irradiation was performed at the GSI Darmstadt (FAIR Phase-0, SBio08_Schroeder) and at HIT, Heidelberg, Germany.

Keywords: radiation-induced brain necrosis, cerebral organoids, aberrant choroid plexus formation

6:45 PM – 7:45 PM

580

THE GENERATION OF MOSAIC ORGANOID FOR RETT SYNDROME

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Rett syndrome (RTT) is a complex neurological disorder primarily caused by mutations in the Methyl CpG-binding protein 2 (MECP2) gene, which is located on the X chromosome. This genetic location contributes to a higher prevalence of RTT in females due to X chromosome inactivation (XCI), resulting in mosaic expression of MECP2 in affected individuals. Previous studies investigating RTT pathophysiology predominantly utilized mouse and hiPSC-derived neural models harboring hemizygous or homozygous mutations in MECP2. However, replicating the mosaic expression pattern observed in female RTT patients remains a challenge with these models. In this study, we addressed this challenge by generating mosaic neural organoids through the combination of wild-type (WT/WT) MECP2 homozygous and R133C (MT/MT) MECP2 homozygous isogenic human-induced pluripotent stem cells (hiPSCs) generated from heterozygous RTT patient iPSC (WT/MT). We also tagged wild-type MECP2 homozygous hiPSCs with eGFP and compared neuronal phenotypes between homozygous and mosaic contexts to identify any non-cell autonomous effects. Beyond insights garnered from conventional RTT disease models, our mosaic model provides further insights into the pathology of Rett syndrome and demonstrates the potential for recapitulating other X-linked or mosaic diseases. This expanded understanding enhances our capacity to investigate the complexities of such disorders

Funding Source: KFRM (Korean Fund for Regenerative Medicine) BK21 (Brain Korea 21) LIM Foundation

Keywords: hiPSC and disease modeling, Rett syndrome (RTT), organoid for mosaic disease

THURSDAY, 11 JULY, 2024

Session II: Odd

3:45 PM – 4:45 PM

TOPIC: NEURAL

101

ALTERED E/I BALANCE IN HIPSC-DERIVED NEURONAL CO-CULTURE MODELS FROM PATIENTS DIAGNOSED WITH SCHIZOPHRENIA

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Schizophrenia, a complex neuropsychiatric disorder, is often associated with disruptions in synaptic transmission and alterations in neuronal activity patterns. A key aspect implicated in its pathology is the imbalance between excitatory and inhibitory signaling within the prefrontal cortex, which contributes to cognitive deficits observed in patients. However, understanding the underlying morphological and functional changes, especially in a human context, remains limited. In this study, we introduce an approach utilizing defined neuronal co-cultures of glutamatergic and GABAergic neurons derived from human induced pluripotent stem cells (iPSCs) of schizophrenia patients, providing a humanized in vitro model to explore disease phenotypes. Through quantitative immunocytochemistry, we observed an increased density of glutamatergic excitatory synapses on inhibitory neurons within glutamatergic/GABAergic co-cultures from schizophrenia patients. Patch clamp analysis of GABAergic neurons revealed heightened excitatory postsynaptic current (EPSC) frequency and amplitude, along with elevated sodium current densities and action potential amplitudes, suggesting an altered network integration and behavior of GABAergic neurons. Furthermore, functional single-cell calcium imaging revealed decreased neuronal activity parameters in glutamatergic neurons, potentially linked to augmented GABAergic input. These findings were supported by increased network synchronicity observed in schizophrenia patient-derived co-cultures through Micro-Electrode-Array (MEA) recordings. In summary, our study establishes a human 2D in vitro model to investigate the morphological and functional characteristics of glutamatergic and GABAergic neurons, as well as their reciprocal interactions, in the context of schizophrenia. Our observations underscore disturbances in neuronal activity, synaptogenesis, and cell-specific network integration in co-cultures derived from schizophrenia patients. This model holds promise as a valuable platform for mechanistic exploration of schizophrenia pathology in vitro, potentially leading to insights into novel therapeutic strategies.

Funding Source: This work received financial support from the State of Baden-Wuerttemberg (grant no. AZ 35-4223.10/8) and the German Federal Ministry of Education and Research (grant no. 01EK2101A).

Keywords: schizophrenia, excitatory-inhibitory imbalance, iPSC-derived co-cultures

3:45 PM – 4:45 PM

103

LONG-TERM IPSC-DERIVED RETINAL ORGANOID MATURATION IS REQUIRED TO MODEL AUTOSOMAL DOMINANT RETINITIS PIGMENTOSA CAUSED BY THE COMMON P23H RHODOPSIN VARIANT

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Retinitis Pigmentosa (RP), an inherited retinal disorder, is characterized by progressive vision loss through the degeneration of rod photoreceptors. Rhodopsin (RHO) is a photopigment expressed in rod photoreceptors and is crucial for cell homeostasis and survival. The dominant-negative c.68C>A (p.Pro23His, known as P23H) RHO variant is the most recurrent mutation underlying autosomal dominant RP in the Caucasian population, with no therapy currently available. To test novel therapeutic strategies, especially genetic therapies, human-derived disease models are needed, as these therapies require sequence-specific approaches. We generated iPSC-derived retinal organoids from control, and CRISPR/Cas9 gene-edited P23H-RHO iPSCs, to create a human disease model for this variant and study the molecular and cellular phenotype. We found that P23H-RHO retinal organoids followed a similar developmental pattern as control organoids and did not display any disease phenotype up to 210 days in vitro (DIV210). Interestingly, during this time, RHO protein was not localized at the outer segment-like structures both in control and P23H-RHO retinal organoids. Only after DIV280, we detected proper localization of RHO in control retinal organoids. Contrarily, in P23H-RHO organoids, the majority of RHO remained mislocalized. This resembled the mislocalization pattern that was previously reported in several knock-in rodent models and postmortem P23H-RHO human retinas. We also investigated the presence of stress and apoptotic markers at different time points. To confirm that the different phenotypes are directly caused by the genetic defect,

we are reducing the mutant transcript expression in P23H-RHO retinal organoids using an allele-specific gapmer antisense oligonucleotide previously described in literature. In summary, we developed a human-based retinal model for autosomal dominant RP. Our results highlight the importance of timing in modeling RP and other degenerative disorders using retinal organoids and the challenges to fully recapitulate the disease phenotype. These findings contribute to the generation of novel in vitro retinal disease models that will help to further understand the underlying molecular mechanism, and accelerate the development of novel therapeutic interventions.

Funding Source: This work is supported by the strategic research programme: "Human Measurement Models" (grant nr. 18958) from NWO, ZonMw, SGF, LSH, and Health~Holland. Additional funding was obtained from Proefdiervrij.

Keywords: retinal organoids, rhodopsin, Retinitis Pigmentosa

3:45 PM – 4:45 PM

105

EPIGENETIC INSIGHTS INTO GABAERGIC DEVELOPMENT OF DRAVET SYNDROME IPSC AND THERAPEUTIC IMPLICATIONS

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Dravet syndrome (DS) is a devastating early onset refractory epilepsy syndrome caused by variants in the SCN1A gene. The underlying pathophysiological mechanisms are largely unknown but prior studies have shown that DS is associated with a compromised function of GABAergic interneurons. Our previous studies employing induced pluripotent stem cells (iPSCs) derived from DS patients and healthy donors also indicated aberrant changes in chromatin architecture along GABAergic development in DS. Therefore, we aimed to investigate the dynamics of chromatin architecture following GABAergic development in DS. Employing ATAC-Sequencing, we assessed chromatin accessibility at several time points (day 0, day 19, day 35, and day 65) of directed GABAergic differentiation. Analysis revealed distinct chromatin dynamics of DS cells when compared to control cells, predicting accelerated early GABAergic development. The differences in chromatin accessibility between DS and controls increased with continued GABAergic differentiation, indicating impaired interneuron development in DS. Additionally, we investigated the epigenetic effects of the commonly used anti-seizure drug valproic acid (VPA) in GABAergic neurons at day 65 of differentiation. Exposure to VPA for 6 days reshaped the chromatin landscape in GABAergic neurons to a variable extent in different lines; and VPA rescued the aberrant chromatin pattern in individual DS GABAergic neurons. Our study provides the first comprehensive investigation on the chromatin landscape of GABAergic development in our DS-patient model, suggesting early epigenetic changes in the progression to DS. Moreover, our detailed analysis of the chromatin changes induced in GABAergic neurons by VPA holds the potential to improve development of personalized and targeted anti-epileptic therapies.

Funding Source: Swedish Research Council (2020-01947, 2022-00658), Hjärnfonden (FO2020-0171; FO2022-0042), Swedish Cancer Foundation (211449Pj, 220491JIA), Stiftelsen Sävstaholm, Stiftelsen Margarethahemmet, Science for Life Laboratory.

Keywords: GABAergic differentiation in Dravet Syndrome, epigenetic profiling using ATAC sequencing, valproic acid treatment in epilepsy

3:45 PM – 4:45 PM

107

FUNCTIONAL DISSECTION OF PRC2-DEPENDENT DYSREGULATION IN WEAVER SYNDROME THROUGH CORTICAL BRAIN ORGANOIDS REVEALS CELL MIGRATION DEFECTS AND IMPAIRED DIFFERENTIATION TRAJECTORIES

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Weaver syndrome (WVS) is a rare multisystem disorder characterized by macrocephaly and intellectual disability. As of today, WVS remains a clinical unmet need due to the experimental challenge of accessing patient biological samples. WVS is caused by heterozygous mutations in polycomb repressive complex 2 (PRC2), which catalyzes the tri-methylation of Lysine 27 on histone 3 (H3K27me3), promoting transcriptional repression during corticogenesis. Nevertheless, our knowledge on the impact of such mutations on the landscape of H3K27me3 and on the transcriptome is still incomplete and several questions remain unsolved. To gain insight in molecular circuits underpinning the WVS phenotype, we profiled patient-derived cortical brain organoids (CBOs). The intersection of differentially expressed genes between WVS-CBOs and control CBOs, single cell RNA-seq data, H3K27me3 genomic distribution, PRC2 occupancy and DNA methylation profiles, revealed a dysregulation in neuronal differentiation timings and neuronal migration

processes, at relevant developmental stages. This finding is consistent with clinical data from WVS patients, whose MRI profiles show an alteration of the cortical development due to defects in neuronal migration. Moreover, from the intersection of omics data, AJAP1 resulted one of the most significantly and strongly upregulated genes in WVS-CBOs. AJAP1 is an adherens junction associated protein involved in cell migration and associated with intellectual disability. Therefore, to elucidate the role of AJAP1 in WVS, we established a CRISPR-based synthetic system to perturbate the expression of AJAP1, aiming to revert the phenotype in WVS-CBOs and to recapitulate it in controls, mimicking the disorder. In conclusion, our findings support impaired differentiation trajectories and cell migration defects throughout corticogenesis due to PRC2 misfunction in CBOs and shed light on cellular biology processes that were not known to be tightly regulated by PRC2.

Funding Source: The project was supported by the Telethon grant GGP19295 awarded to AT.

Keywords: corticogenesis, PRC2, cell migration

3:45 PM – 4:45 PM

109

PHARMACOLOGICAL RESCUE OF DEVELOPMENTAL AND SYNAPTIC PHENOTYPES IN HUMAN SHANK3- DEFICIENT-STEM-CELL-DERIVED NEURONAL MODELS

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Phelan-McDermid syndrome (PMDS) is a rare genetic disorder characterized by developmental delay, hypotonia, intellectual disability, speech impairments and autism spectrum disorder (ASD). In most PMDS affected individuals, the terminal region of chromosome 22q13 is often impacted by larger deletions or frameshift truncations in exon 21 of the gene SH3 and multiple ankyrin repeat domains 3 (SHANK3), which encodes a protein of the postsynaptic density (PSD) of excitatory. This work aims to validate a relevant PMDS stem cell derived cellular system as a tool to elucidate developmental and synaptic phenotypes and their modulation by chemical compounds for drug discovery efforts. The developmental and synaptic disturbances were investigated by high content/ high throughput microscopy in neurons derived from CRISPR-engineered stem cells with a heterozygous SHANK3 deletion. Morphological features were extracted from the images using an in-house software PhenoLink (https://github.com/Ksilink/PhenoLink). Validated phenotypic profiles were then used to screen 7,120 chemical compounds with known Mode-of-Action and to identify the small molecules that shift the PMDS phenotypic profile towards a healthy-like phenotypic profile. SHANK3-deficiency leads to reduced proliferation, increased differentiation of human, increased neuronal network length and the number of synapses, while decreasing neuronal activity. Automated image-based screening was performed and three compounds that rescued SHANK3-dependent neuronal hyperdifferentiation were identified. In particular, Benproperine increased the colocalization of Actin Related Protein 2/3 Complex Subunit 2 with ß-actin and rescued increased synapse formation when administered early in the differentiation. Surprisingly, there was no improvement in neuronal activity. We provide proof-of-concept that small molecule compounds are able to reverse both, developmental and synaptic phenotypes can be identified in human neuronal PMDS models. Our results thus contribute to the growing evidence that human PMDS stem cell models provide tangible opportunities to foster investigations into disease biology and enabling drug discovery approaches for the development of highly needed pharmacological interventions.

Funding Source: Ksilink is supported by the “Programme d’investissements d’avenir” (PIA) of the French government.

Keywords: Phelan McDermid syndrome, high content/ high throughput screening, disease modelling

3:45 PM – 4:45 PM

111

GENE EDITING AND STEM CELL MODELING TO UNRAVEL AND TREAT EYS-ASSOCIATED RETINITIS PIGMENTOSA

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Retinitis pigmentosa (RP) represents a collection of hereditary, degenerative eye disorders marked by a progressive deterioration of the retina. Despite advancements in understanding its genetic landscape, the precise cellular mechanisms involved in RP largely remain elusive, due primarily to the lack of appropriate animal models. To address this, retinal organoids and retinal pigment epithelium cells (RPE) derived from patient-specific induced pluripotent stem cells (iPSCs) provide a robust platform for unraveling disease mechanisms and serve as a viable asset for preclinical validation of prospective therapies. In this study, we utilized iPSCs derived from an RP patient carrying two

heterozygous mutations in the Eyes Shut Homolog (EYS) gene, associated with a prevalent type of autosomal recessive RP. We then used these iPSCs to produce both retinal organoids (ROs) and RPE, which enabled us to explore disease mechanisms and identify phenotypic changes compared to healthy-derived cells. To further our understanding, we employed CRISPR/Cas9-mediated Homology-Directed Repair (HDR) to correct one of the mutant alleles in the EYS iPSC line. We then differentiated this corrected line and compared its transcriptional, protein, and functional profiles with those of healthy and unedited mutant cells. Our findings revealed compromised functionality and a decrease in photoreceptor precursors in the unedited EYS mutant cells. However, the CRISPR/Cas9-corrected line showed significant improvements, suggesting that correcting one mutant allele could partially rescue the phenotype. Our work highlights the disruptive impact of EYS mutations on retinal architecture and demonstrates the potential of CRISPR/Cas9 and iPSC technologies for understanding and treating EYS-associated RP.

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Keywords: retinitis pigmentosa, retinal organoids, CRISPR/Cas9

3:45 PM – 4:45 PM

113

PROTEOSTASIS DEFICIENCY IN IPSC DERIVED ASTROCYTES EXACERBATES NEURODEGENERATION IN A MODEL OF EARLY ONSET PD FROM A53T ALPHA SYNUCLEIN PATIENTS

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Parkinson's disease (PD), the second most common neurodegenerative disorder is characterized by progressive loss of midbrain dopaminergic neurons resulting in motor and non-motor symptoms. The histopathological disease hallmark is the presence of intraneuronal protein inclusions of α-synuclein, termed Lewy bodies and Lewy neurites. Approximately 10% of PD cases are associated with mutations in specific genes, such as the p.A53T α-synuclein (αSyn) mutation (G209A in the SNCA gene) that causes a familial form of PD with early onset and severe phenotype. Despite intensive research, PD is still incurable. While neuron-intrinsic dysfunction has been extensively studied, research on the role of astrocytes, the most abundant cells in the human brain, has lagged behind, regardless of their critical roles in maintaining neuronal health and their capacity to exert neuroprotective or neurotoxic effects upon disease. Here, we employed our previously-established model of p.A53T-αSyn-PD patient-derived induced pluripotent stem cells (iPSC) to generate human ventral midbrain astrocytes and investigate their contribution in PD neuropathology. PD iPSC-derived astrocytes displayed cell-intrinsic pathological phenotypes such as intracellular protein aggregates, including accumulation of pathological phospho(Ser129)αSyn, and dysregulation of Ca2+ homeostasis. Proteomic analysis revealed perturbed protein catabolic processes, autophagy, and endocytosis, with dysregulation of lysosomal properties and the mammalian target of rapamycin (mTOR) pathway. Furthermore, iPSC-derived dopamine neurons co-cultured with PD astrocytes displayed exacerbated neurodegenerative phenotypes, including Lewy-related pathology. Notably, neurodegeneration was reversed by control astrocytes, at least partially, due to their capacity to uptake and resolve neuronal aSyn aggregates – a process impaired in PD astrocytes. Our findings underscore a critical impact of the p.A53T-αSyn mutation in astrocytic proteostasis and clearance mechanisms, rendering astrocytes important contributors to PD neuropathology. These results provide valuable insights into potential disease targets for the development of novel PD therapeutics.

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Keywords: alpha-synuclein, astrocyte, Parkinson's disease

3:45 PM – 4:45 PM

115

PHENOTYPIC HETEROGENEITY AND DRUG RESPONSES IN GRIN2B-RELATED NEURODEVELOPMENTAL DISORDER: A COMPREHENSIVE ANALYSIS

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GRIN2B-related neurodevelopmental disorder poses a significant challenge due to its diverse range of symptoms, encompassing intellectual disability, delayed speech, motor skill impairment, seizures, autism spectrum features, hyperactivity, and structural brain abnormalities. This study aims to provide a comprehensive understanding of the mechanism underlying clinical manifestations, neurological abnormalities, and associated challenges of the GRIN2B-related neurodevelopmental disorder. In this research, a comprehensive analysis of GRIN2B-related neurodevelopmental disorder is carried out, focusing on five patients with different mutations and examining the neurophysiological and molecular changes in GRIN2B mutant cortical neurons derived from the patients and their siblings as controls using induced pluripotent stem cell (iPSC) technologies. The study evaluates the efficacy of 15 drugs that were chosen for their known interactions with the NMDA receptors. Whole-cell patch clamp, Calcium Imaging, and calcium imaging-based Network Analysis were employed to investigate neuronal activity and connectivity and measured parameters such as Time Delay Stability (TDS), Link Density, Average Degree, Average Strength of the connections, and more. Patch clamp recordings were analyzed to assess excitability, Na and K currents, and synaptic connectivity. Patch clamp analysis revealed an accelerated development of GRIN2B mutant neurons from all 5 patients, whereas mature mutant neurons were hypoexcitable with less synaptic connections compared to neurons derived from the healthy siblings. The mutant neurons also exhibited lower synchronization but a higher connectivity, potentially indicating compensatory or inefficient pruning mechanisms. In addition to this, the network analysis revealed that GRIN2B mutant neurons exhibited lower node strengths, larger clustering coefficients, smaller network diameters, and more connections between modules. Furthermore, the study found that a few drugs demonstrated efficacy in treating GRIN2B-related neurodevelopmental disorders. This research contributes to the mechanistic understanding of the disorder and suggest novel drugs as a possible treatment.

Funding Source: 2Bcured

Keywords: GRIN2B, iPSC based neuromodel, drug screening

ABSTRACT WITHDRAWN

3:45 PM – 4:45 PM

119

DISSECTING THE ROLE OF CHROMOSOMAL COPY NUMBER ALTERATIONS IN NEUROBLASTOMA USING HUMAN EMBRYONIC STEM CELLS

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Paediatric cancers typically arise in utero following impaired differentiation and uncontrolled proliferation of transformed embryonic cells. Unlike their adult counterparts, they are driven by sparse genetic aberrations, predominantly large genomic rearrangements and chromosomal copy number alterations (CNAs). CNAs are particularly prevalent in neuroblastoma (NB), the most common extra-cranial solid childhood tumour. NB tumours are typically found in the adrenal gland/sympathetic ganglia and are thought to arise during early embryonic development due to oncogenic transformation of the embryonic precursors of these structures, namely multipotent trunk neural crest (NC) cells and their sympathoadrenal derivatives. Most NB tumours carry CNAs such as gains of the long arms of chromosomes 17 (chr17q) and 1 (chr1q) whose emergence appears to be an early tumourigenesis "priming" event. Chr17q/1q gains often co-occur with amplification of the MYCN oncogene suggesting they may jointly contribute to tumourigenesis. However, it remains unclear to date how CNAs disrupt NC differentiation and lead to NB initiation. Here, we used a human embryonic stem cells (hESC) to experimentally dissect the links between NB-associated CNAs, MYCN amplification, and tumour initiation. We interrogated the stepwise specification of trunk NC and sympathoadrenal lineages using directed differentiation of isogenic hESC lines with chr17q/1q gains and inducible MYCN overexpression. We found that NB-associated CNAs alter the differentiation trajectories of hESC-derived trunk NC lineages by promoting transitional progenitor states at the expense of mature sympathoadrenal cell types. In conjunction with MYCN elevation, they block differentiation and trigger atypical transcriptional programmes incompatible with normal trunk NC development. CNA-carrying trunk NC cells acquired altered cellular properties reminiscent of cancer hallmarks, such as increased proliferation, clonogenic and tumour formation capacity under the influence of MYCN overexpression. These changes were found to correlate with a stepwise corruption of developmental transcription factor networks. Together, our results sketch a mechanistic framework for the CNA-driven initiation of embryonal tumours.

Funding Source: European Union Horizon 2020 Framework Programme (H2020-EU.1.2.2; project 824070), Children's Cancer and Leukaemia Group/Little Princess Trust (CCLGA 2019 28, CCLGA 2020 19)

Keywords: neuroblastoma, neural crest, copy number alterations

3:45 PM – 4:45 PM

121

SENDAI POSITIVE REPROGRAMMED IPSC SHOWED INADEQUATE DIFFERENTIATION TO NPC AND CORTICAL NEURONS

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Reprogramming patient fibroblasts and PBMCs with the CytoTune iPS 2.0 Sendai reprogramming kit has become the most reliable method for producing iPSCs. iPSCs are used in a variety of applications, including disease modeling, drug screening, transplantation therapy, and differentiation into any desired terminal cell types. The Yamanaka factors Klf4-Oct3/4-Sox2-cMyc are delivered via Sendai particles in this kit to enable the effective generation of iPSCs. Even though the Sendai virus does not integrate during the reprogramming, Sendai clearance can be an issue. In one of our recent studies, 41.6% (15/36) of clones reprogrammed from PBMCs are positive by RT-PCR for one or more Sendai transcripts. KOS and hc-MYC are both on temperature-sensitive backbones to aid clearance. Still, not all patient lines are cleared of Sendai after heat shock. Sendai virus expresses the HN protein on the host cell surface, and Sendai-positive cells can be identified by using live-cell anti-HN antibody staining and imaging. We report that only a small fraction of cells in a Sendai-positive iPSC clone are in fact HN protein-positive. We differentiated Sendai-positive iPSC cultures to NPCs and Cortical neurons and observed defective differentiation by Sendai-positive cells. In conclusion, it is necessary to clear iPSCs of the residual Sendai genome. HN protein staining, followed by single-cell sorting or physical scraping, is a simple and effective method to aid the speedy clearance.

Keywords: Sendai virus, reprogramming, differentiation

3:45 PM – 4:45 PM

123

GENERATION OF A PANEL OF IPSC LINES FROM MOWAT-WILSON SYNDROME PATIENTS CARRYING HETEROZYGOUS ZEB2 MUTATIONS

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ZEB2 (Zinc Finger E-Box Binding Homeobox 2) encodes a transcription factor, mainly acting as a transcription repressor, expressed in various tissues and involved in different functions including, among others, neuro- and glio-genesis, hematopoiesis, and tissue repair. The best-known clinical picture associated with ZEB2 mutations is Mowat-Wilson syndrome, which is caused by ZEB2 haploinsufficiency and characterized by possible multi-organ malformations, dysmorphic features, intellectual disability, and epilepsy. To date, most of the knowledge on ZEB2 arise from studies carryout in mice, as, for example, the ZEB2-dependent modulation of Nkx2-1 during the differentiation and migration of GABA-ergic interneurons. However, the complete knockout of ZEB2 is required to model the Mowat-Wilson syndrome in mice, indicating that humanized models of the disease are necessary to fully understand the underling pathological mechanisms. Accordingly, we generated a panel of iPSC lines to be used for pathophysiological studies and therapeutics testing. PBMC of four patients carrying different heterozygous mutations in ZEB2 were used: p.Leu894Phe Fs*36, p.R218R fs*21, p.Leu273 – c.817del, pAsp527Thr Fs*17. The iPSC lines were generated with the non-integrating Sendai virus delivering the Yamanaka transcription factors: OCT4, SOX2, cMYC, and KLF4. Emerging colonies with typical iPSC-like morphology were observed after 8 days post-transduction. Loss of Sendai viral genes was confirmed by PCR at passage number 10. The pluripotency was confirmed by immunostaining for cell surface markers SSEA4 and TRA-1-60, and nuclear markers OCT4 and SOX2. The genomic stability was confirmed using the KaryoStat+ Assay. To demonstrate the differentiation ability into the three germ layers we performed an in-vitro direct differentiation and found positive stainings for different markers or the three germ layers, including the ectodermal markers PAX6, NESTIN and TUBB3, the mesodermal markers TBX6 and Brachyury, and the endodermal markers FOXA2 and SOX17. These iPSC lines may lay a basis for the development of brain-specific or multi-organ in vitro models of ZEB2 haploinsufficiency to be used for pathophysiological studies and therapeutics testing.

Funding Source: Fondazione Epilessia LICE

Keywords: PBMCs, ZEB2, Mowat-Wilson syndrome

3:45 PM – 4:45 PM

125

DEVELOPMENTAL EXPOSURE OF HUMAN IPSC-DERIVED CORTICAL CULTURES TO METHYLMERCURY CAUSES A PERSISTENTLY ALTERED HOMEOSTATIC STATE

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Developmental exposure to methylmercury (MeHg) is known to result in long-lasting effects in the developing and mature cortex. To understand the mechanisms behind altered function of cortical neurons, we developed a human induced pluripotent stem cell (hiPSC) model of differentiating cortical neurons to assess persistent MeHg-induced neurotoxicity. Cultures were exposed for 6 days during a developmental stage with neural epithelial and radial glia cells to in vivo relevant MeHg concentrations (0.1 and 1 µM). Following cessation of exposure, differentiation was continued till cultures reached mature stages. To ensure that observed effects did not result from residual MeHg in cells, MeHg concentrations in cell pellets were measured. It was found that the in vitro t1/2 is approximately 2 days, indicating no MeHg is present >14 days after cessation of exposure. At day 40, 150 and 280 of differentiation, cells were assessed for persistent effects using single cell RNA sequencing. Radial glia, glutamatergic and GABAergic neurons were identified across all days. However, intermediate progenitor cells were not present at D280 and astrocytes were absent at D40. Relative cell populations were not affected by exposure. Exposure to 0.1 and 1 mM MeHg significantly altered ($p < 0.05$) pathways related to neuronal function, e.g., axonal guidance signaling, calcium signaling, glutamate receptor signaling, synaptic long-term depression and potentiation and synaptogenesis signaling pathway in all cell types. No clear concentration dependency was observed, consistent with some clinical data. The highest number of pathways was altered in the glutamatergic neuron clusters. From day 110 onwards, mature cultures were grown on micro-electrode arrays and persistent effects on spontaneous neuronal network activity were assessed. We found that MeHg significantly alters spike and (network) burst frequency. Increasing exposure concentrations resulted in decreasing network organization. Taken together, our data demonstrate that developmental exposure to MeHg results in an altered transcriptomic and functional state in mature hiPSC-derived cortical cultures. Notably, we demonstrate that these effects manifest well after cessation of exposure.

Funding Source: This research was supported by NIH [R01 ES07331 (ABB/MA) and AG080917 (ABB)]

Keywords: human induced pluripotent stem cell-derived neurons, developmental methylmercury exposure, aging

3:45 PM – 4:45 PM

127

MODELING ADLD PATHOLOGY WITH HUMAN IPSC-DERIVED GLIAL CELLS: ALTERED PHENOTYPES AND RESCUE STRATEGIES

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Autosomal Dominant Leukodystrophy (ADLD) is a rare genetic disease associated with white matter loss in the CNS and characterized by autonomic dysfunction and motor impairment. The genetic cause is the presence of three copies of the lamin B1 (LMNB1) gene, which encodes for a structural protein located in the nuclear lamina. Pathogenic mechanisms in ADLD have only initially been explored and a therapy to treat this disease is currently not available. Based on evidence showing glial pathology in ADLD patients, we generated human glial cells from both ADLD patient- and healthy donor (CTRL)- derived human-induced pluripotent stem cells (hiPSCs), and specifically investigated ADLD astrocytes. Compared to CTRL cells, ADLD astrocytes displayed increased LMNB1 expression, at both RNA and protein level, and morphological and neurochemical cell alterations. Transcriptional profiling of the disease astrocytes pointed to functional defects in a number of key astrocytic functions comprising extracellular matrix composition, calcium signaling and mitochondrial metabolism. The analysis further revealed the acquisition of signs of cellular senescence and abnormalities in RNA processing. Importantly, ADLD Astrocyte Contidioned Medium affected murine oligodendrocytes survival and maturation, suggesting a detrimental effect of ADLD astrocytes on oligodendroglia. Moreover, we successfully reduced the elevated levels of LMNB1 protein and reversed the associated cellular abnormalities by using a specific RNA interference technique called Allele SPecific (ASP) RNAi, which selectively silenced the non-duplicated LMNB1 allele. Our “disease-in-a-dish” platform reveals previously unknown ADLD astroglial dysfunctions, shedding light on their potential contribution to the white matter loss observed in the disease. Moreover, our results provide direct evidence that ASP RNAi can effectively target and alleviate ADLD pathology in human glial cells.

Funding Source: European Leukodystrophy Association (ELA) International

Keywords: astrocytes, disease modeling, demyelinating disease

3:45 PM – 4:45 PM

129

UNDERSTANDING THE EFFECTS OF IN UTERO METFORMIN EXPOSURE USING HUMAN EMBRYONIC STEM CELLS

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Metformin is a first-line drug for the treatment of type 2 diabetes that cannot be controlled by diet and lifestyle changes alone. Increasingly, due to its high safety profile and low cost, it is being prescribed during pregnancy for women with pre-existing diabetes and gestational diabetes, as well as women with polycystic ovary syndrome (PCOS). This is concerning because metformin can cross the placenta and enter the fetal circulation. While there are no obvious teratogenic effects of metformin, the long-term effects of in utero metformin exposure on the offspring remain unclear. Data from clinical trials suggest that metformin exposure in utero may affect offspring adiposity and metabolism, but the sample sizes of these trials are small. Similarly, there is limited clinical data on the effect of metformin exposure on neurodevelopment. As such, we wanted to study the effect of metformin exposure on neurodevelopment using a human in vitro model. To this end, we differentiated hESCs into cerebral organoids in the presence or absence of metformin. We found that transcript levels of selected cerebral organoid markers were similar in both groups at the end of the differentiation protocol. Interestingly, mRNA expression of the metformin importers organic cation transporter 1 and 2 (OCT1 and OCT2) decreased over the differentiation time course, while that of the metformin extrusion transporters multidrug and toxin extrusion 1 and 2 (MATE1 and MATE2) increased during cerebral organoid differentiation. Further work is required to assess the expression of all the metformin transporters during cerebral organoid differentiation. Nevertheless, these preliminary data suggest that offspring may be more vulnerable to the effects of metformin at the earlier stages of development and calls for caution in prescribing metformin during pregnancy.

Keywords: cerebral organoids, neurodevelopment, metformin

3:45 PM – 4:45 PM

131

MODELLING SHASHI X-LINKED INTELLECTUAL DISABILITY SYNDROME

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X-linked intellectual disability (XLID) are heritable genetic disorders characterized by impaired cognitive abilities, primarily affecting males. Shashi-XLID syndrome (OMIM #300238) is caused by mutations in the RBMX (RNA binding motif protein X-linked) gene on the X chromosome. RBMX encodes for an RNA-binding protein with widespread expression regulating alternative splicing and consequently gene expression. RBMX is highly conserved across species and has many retrocopies on autosomes, with the most recent one, RBMXL1 on human chromosome 1, being likely functional. To provide a tool for investigations on the functional consequences of mutations in RMBX on brain development, we reprogrammed patient-derived fibroblasts into iPSCs using a synthetic self-replicating RNA vector. Fibroblasts were obtained from three male patients of independent families carrying different mutations in the RMBX gene. The iPSC lines underwent a panel of assays for rigorous characterization, including assessment of morphology, surface and nuclear pluripotency marker gene expression, differentiation potential, karyotype stability, cell line identity by STR analysis, mycoplasma, and virus testing. In addition, we demonstrated the gain of human telomerase reverse transcriptase (hTERT) activity in iPSCs using a fluorescent telomere repeat amplification protocol. Protein and mRNA expression levels of RBMX were elevated in the generated iPSC lines when compared to parental fibroblasts. However, assays and antibodies used could not discriminate between RBMX and RBMXL1 expression. By now, such reagents became available and analyses will be repeated to specifically attribute changes in expression in iPSCs to RBMX and/or RMBXL1. In addition, specific expression of RBMX and RBMXL1 will be determined in cortical neurons that were differentiated from the generated iPSCs. The three independent iPSC lines are the first patient-derived iPSC lines to carry mutations in the RBMX gene and will therefore be of great value for further studies on Shashi XLID and brain development.

Keywords: X-linked intellectual disability, reprogramming, neural differentiation

3:45 PM – 4:45 PM

133

MODELLING CONSEQUENCES OF THYROID HORMONE TRANSPORTER MCT8 DEFICIENCY IN CEREBRAL ORGANOIDS

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Thyroid hormone (TH) transporter MCT8 plays an important role in local TH action during human brain development. Inactivating mutations of MCT8 lead to the Allan-Herndon-Dudley syndrome (AHDS), a disease associated with neurological pathologies, developmental delay and hypothyroid conditions in the brain. Yet, it remains unclear how early human neurodevelopment is affected by dysfunctional MCT8. hiPSC-derived cerebral organoids (hCOs) and CRISPR/Cas9 offer the valuable opportunity to model effects of pathological MCT8 mutations on human neurogenesis. Here we aimed to characterize cell type-specific effects of MCT8 deficiency on cortical development in an hCO model by combining immunofluorescence (IF) analyses with single cell RNA sequencing (scRNASeq). Using CRISPR/Cas9-based gene editing, we generated three pairs of isogenic hiPSC lines carrying either WT or non-functional MCT8 variants. Two hiPSC lines contained the pathological Gly401Arg MCT8 mutation and one hiPSC line harbors a frameshift mutation resulting in MCT8 knock-out. Cerebral organoids derived from all six hiPSC lines were cultured in the presence of two different TH levels (0 nM and 2 nM TH). Quality of hCO cultures was stringently controlled by IF staining of cortical patterning and cell type markers and assessment of cytoarchitectural features at DIV 21, 46 and 70. MCT8-deficient hCOs showed no gross defects in cortical cytoarchitecture. However, when using scRNAseq to assess effects of MCT8 deficiency on cell type-specific gene expression at DIV 70, we observed both TH-dependent and TH-independent response patterns. Particularly for deep layer neurons, we observed changes in cell type abundances that were not related to TH availability suggesting effects of MCT8 deficiency that are caused by mechanisms unrelated to impaired TH transport. A key finding of our studies was that stringent

quality control measures allowed for detection of highly reproducible effect patterns among three isogenic pairs of WT-variant carrier hiPSC lines. In particular scRNASeq data analysis greatly benefitted from the triplicate study design. Our experimental strategy (isogenic pairs, triplicate analyses) provides us with robust data to dissect the molecular mechanisms linking MCT8 deficiency to neurodevelopmental pathogenesis.

Funding Source: DFG Collaborative Research Centre / Transregio 296 Local Control of Thyroid Hormone Action (LOCOTACT)

Keywords: disease modeling using cerebral organoids and CRISPR/Cas9, defect of thyroid hormone transport- effect on human cortex development, single cell RNA sequencing cerebral organoid

3:45 PM – 4:45 PM

135

EXPLORING THE RELEVANCE OF APOE POLYMORPHISM AND G206D-PSEN1 MUTATION ON IPSC-DERIVED HIPPOCAMPAL NEURONS FROM PATIENTS WITH SPORADIC AND FAMILIAL ALZHEIMER´S DISEASE

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Sporadic and familial Alzheimer´s disease (AD) is characterized by progressive neurodegeneration of the main brain areas involved in memory function, such as the entorhinal cortex and hippocampus. The human APOE polymorphism, more specifically the presence of the ε4 allele (coding for the APOE4 protein isoform), constitutes the main genetic risk factor for late-onset AD, while mutations in the PSEN1 gene are responsible in many cases for early-onset familial AD. There is increasing evidence of APOE4 being involved in many aspects of AD pathogenesis, but the impact of APOE alleles on human neuron differentiation, maturation and function remains to be fully elucidated. Furthermore, the effect of G206D-PSEN1 mutation on human neurons has been little explored. To address these questions, we obtained induced pluripotent stem cells (iPSCs) from fibroblasts of AD patients carrying the ε3 and ε4 alleles (in homozygosis) or having the G206D-PSEN1 mutation, and from healthy patients. Then, iPSCs were differentiated into hippocampal neurons by adding small molecules and growth factors, and their cellular, molecular, functional and neuropathological characterisation was performed. iPSCs-derived neurons expressed hippocampal markers and showed a functional profile determined by glutamate release, electrical activity and synapse formation visualized by electron microscopy and synaptic bouton analysis. In addition, the role of APOE4 in neurodegeneration was confirmed by determining amyloid-beta 42/40, total Tau and phosphorylated Tau in the culture medium, as well as by the presence of an increased number of extracellular amyloid-beta plaques and intracellular p-Tau181 aggregates in the neurons. Overall, our results point to specific actions of APOE polymorphism and G206D-PSEN1 mutation affecting neuronal differentiation, dysfunction and neurodegeneration.

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Keywords: iPSC-derived hippocampal neurons, APOE, Alzheimer's disease

3:45 PM – 4:45 PM

137

DRUG SCREEN AND MACHINE LEARNING PREDICT NEUROPROTECTIVE AGENTS FOR CHILDHOOD-ONSET DEMENTIA IN A PATIENT-DERIVED IPSC MODEL

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Sanfilippo syndrome, a leading cause of childhood-onset dementia, results from autosomal recessive genetic mutations. Gene therapy

shows promise in children under two, but most are diagnosed when the symptoms appear after four years old, leaving many without treatment. We reprogrammed induced pluripotent stem cells (iPSCs) from affected children and controls to create mature cortical neurons and astrocytes. These patient-derived SGSH-mutant neurons revealed lysosomal dysfunction and Heparan sulphate build-up, characteristics of the syndrome. The neurons were also particularly prone to stress, astrocytic reactivity, and neurodegeneration over time. Screening 63 existing drugs, we found six that prevented these adverse effects within two weeks of treatment. Validation through machine learning and single-cell transcriptomics provided insights into the drugs' mechanisms. Our study introduces a novel stem cell model for childhood-onset dementia, and the results may help address the pressing demand for treatments slowing the chronic neurodegeneration in Sanfilippo children. The work also validates the benefit of the iPSC drug screening models to expedite clinical solutions in rare diseases.

Keywords: drug screen, neurodevelopmental disorder, dementia

3:45 PM – 4:45 PM

139

HUMAN FMRP MRNA TARGETS DRIVE THE ALTERED DEVELOPMENTAL TRAJECTORIES OF HIPPOCAMPAL ORGANOIDS DERIVED FROM FRAGILE X SYNDROME PATIENTS

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Fragile X syndrome (FXS) is a leading genetic cause of intellectual disability and autism spectrum disorder, which typically caused by >200 CGG trinucleotide repeat expansion within the 5' UTR of the FMR1 gene (also known as FMR1 full mutation) that leads to the loss of its encoding protein – Fragile X Messenger Ribonucleoprotein (FMRP). Hippocampus, a key brain region that plays critical roles in mood and cognition, has been found to be significantly impacted in FXS patients. However, human hippocampus-specific mRNA targets of FMRP have not yet been identified and how FMR1 full mutation affects human hippocampal development is still largely unknown. To address these questions, we have developed human hippocampal organoids from both FXS patient and healthy control induced pluripotent stem cells. Interestingly, we observed increased neurogenesis but decreased gliogenesis in FXS hippocampal organoids during development. Single cell transcriptomic analyses revealed that developmental trajectory was altered in FXS hippocampal organoids. To further identify brain region-specific mRNA targets of FMRP, we performed enhanced crosslinking and immunoprecipitation followed by high-throughput sequencing (eCLIP-seq) in hippocampal organoids. Strikingly, we found that the altered developmental trajectory of FXS hippocampal organoids is mediated by brain region- and developmental stage- specific FMRP mRNA targets, which perturb the gene regulatory networks driving neurogenesis/gliogenesis and disrupt the neuron-astrocyte communication. In conclusion, our findings suggest the substantial molecular and cellular repercussions of FMRP deficiency on hippocampal development and function, potentially unveiling new avenues for therapeutic strategies.

Funding Source: NIH (P50HD104458; R21MH123711)

Keywords: fragile x syndrome, FMRP, hippocampal organoids

3:45 PM – 4:45 PM

141

UNLOCKING THE DARK KINONE TO TREAT ALS

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Despite the crucial role of protein kinases in cellular communication and disease progression, much of the kinome, particularly the "dark kinome," remains a mystery, largely because these kinases haven't been directly linked to known diseases, their substrates are hard to identify, or they pose significant challenges for research. Kinases are key to regulating processes such as cell division, growth, metabolism, and cell death, making them vital for understanding cellular functions and the mechanisms behind diseases. The study of the kinome opens possibilities for drug discovery, especially as targeting specific kinases can lead to innovative treatments. The Illuminating the Druggable Genome (IDG) program aims at investigating the "dark kinase" role and potential for therapy. This is particularly important for conditions like Amyotrophic Lateral Sclerosis (ALS), where our research has begun to explore the connection between certain IDG kinases and disease progression using CRISPR technology on stem cells. By focusing on kinases such as NEK1, DYRK2, STK36, and TTBK2, and observing their impact on cell differentiation into neural progenitors and motor neurons focusing on primary cilia formation. Primary cilia are small, hair-like structures that detect and relay external molecular signals to the nucleus, influencing critical pathways that control growth, differentiation, and cell division. Defects in cilia function can lead to a variety of developmental abnormalities and diseases. We found that the loss of a single understudied kinase such as DYRK2 or TTBK2 had a profound effect on primary cilia formation and elongation and demonstrated that chemical inhibition of those kinases in WT iPSCs and NPCs mimics the KO phenotype. Similar results were observed with the DNA damage response in iPSCs and NPCs, distinct mechanistic pathways lead to similar outcomes. We demonstrate that the identification and characterization of new kinases as potential drug targets for ALS create opportunities for the development of CNS drugs. The absence or inhibition of these lesser-known kinases has shown profound effects, indicating their potential as targets for drug development.

Keywords: dark kinases, IPSCs, ALS

3:45 PM – 4:45 PM

143

DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS TOWARDS BASAL FOREBRAIN CHOLINERGIC NEURONS AS A REGENERATIVE THERAPY FOR DEMENTIA

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Alzheimer's disease, dementia with Lewy bodies and Parkinson's disease with dementia are common neurodegenerative diseases, mainly caused by the presence of intracellular neurotoxic aggregates. In the basal forebrain, this leads to degeneration of acetylcholine-releasing neurons and cholinergic denervation, and thereby the typical disabling symptoms of dementias. Current available treatments merely provide temporary symptomatic relief. In this context, cell therapy is a promising approach to replace the lost basal forebrain cholinergic neurons (BFCNs) as a curative treatment option. In this study, we optimized the patterning of human pluripotent stem cells towards authentic BFCN progenitor cells, suppressing the differentiation of non-BFCN neuronal populations with similar developmental origin, such as the GABAergic interneurons. We validated the terminal maturation to a bona fide BFCN fate by transplanting BFCN progenitors to the rat hippocampus and cortex. We also generate a rat model of dementia by injecting in the basal forebrain a saporin toxin which targets only basal forebrain cholinergic neurons. In summary, we optimized the patterning of BFCNs progenitor cells, which upon transplantation leads to mature grafted cells and integration in the structure of the hippocampus.

Keywords: basal forebrain cholinergic neurons, dementia with Lewy bodies, stem cell therapy

3:45 PM – 4:45 PM

145

MODELLING GLUCOSE NEUROTOXICITY USING HUMAN IPSC-DERIVED CELLS TO INVESTIGATE THE THERAPEUTIC POTENTIAL OF P75NTR TARGETING

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Glucose is the main source of energy in the brain. However, glucose concentration should be tightly regulated to maintain brain homeostasis since persistent episodes of high glucose, a common feature of Diabetes Mellitus, cause complications including neuronal damage and inflammation. To date, most studies address glucose neurotoxicity in animal diabetic models. Here, we aim to study the neurological manifestations of high glucose in a human iPSC – based model with emphasis on the role of p75 neurotrophin receptor (p75NTR). p75NTR belongs to the TNF-receptor superfamily and signals apoptosis in different settings. We use mono- and co-cultures of human iPSC-derived neurons and astrocytes in 2D and porous collagen scaffolds based-3D conditions to investigate the involvement of p75NTR signaling in the direct and inflammation-mediated effects of hyperglycemia on neurodegeneration. Our results show that p75NTR is expressed in iPSC-derived neurons. Hyperglycemia triggers neuronal cell death in a dose-dependent manner accompanied by an up-regulation of p75NTR expression. Inhibition of p75NTR activity rescues neuronal cell death highlighting p75NTR as a mediator of glucose neurotoxicity. RNA-seq analysis of hyperglycemic neurons showed great transcriptional changes triggered by high glucose unraveling new pathways involved in this pathology. Furthermore, we found that high glucose downregulates key synaptic proteins in neurons indicating a deregulation of synaptic plasticity. Intriguingly, high glucose does not show any effect on iPSC-derived astrocyte survival or activation in contrast to previous findings in rodents. This finding suggests that microglia is probably the primary source of inflammation that is reported in rodents and human patients in hyperglycemic condition. Collectively, our study provides insights into the brain deficits caused by hyperglycemia and suggests the therapeutic potential of p75NTR targeting.

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Keywords: neurodegeneration, hyperglycemia, p75NTR

3:45 PM – 4:45 PM

147

EXPLORING IN VITRO MODELS OF PARKINSON'S DISEASE: A PLATFORM TO SCREEN PATIENT-DERIVED CELLS VULNERABILITY IN PD

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Parkinson's disease (PD) is a common neurodegenerative disorder affecting millions of people worldwide. The main hallmark of PD is the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta and thus degeneration of the nigrostriatal pathway. The majority of PD patients display the presence of Lewy bodies, which are characterized by misfolded and aggregated alpha-synuclein (a-syn) protein. As a result, dysfunction of a-syn is a critical event which leads to progressive deficits and ultimately to cell death in PD although the exact underlying mechanisms remains unclear. The advent of induced pluripotent stem cells (iPSC) revolutionized the applications with patient-derived cells that can be differentiated to affected neurons, yet physiologically relevant models for studying pathological progression and mechanisms of PD in human cells are limited. In this study, we aim to develop a physiologically relevant, fully humanized 3D in vitro model of PD. The model is based on a-syn overexpression and delivery of pre-formed fibrils to seed aggregation. In this model, differentiated DA neurons from patient-derived iPSC can be utilized for studying pathological events related to a-syn. Taking advantage of iPSC line derived from a patient carrying the triplication of the SNCA gene (AST18), we will investigate the development of the a-syn pathology in differentiated DA neurons. We will compare disease features displayed by AST18 to the same line genetically modified with either a copy of the SNCA (gene corrected) or with deletion (SNCA knockout). This model can be utilized for disease modelling to better understand a-syn related pathology in PD. As autologous transplantations in PD are becoming a reality, the platform provides a robust model to screen patient-derived lines to evaluate them for disease susceptibility. Moreover, it can function as a stage to evaluate strategies to prevent pathological development in cells after grafting, which may be necessary to fully exploit patient-specific cell-therapy in PD.

Keywords: Parkinson's disease, SNCA, autologous transplantation

3:45 PM – 4:45 PM

149

THE IMPORTANCE OF HIGH-DENSITY MICROELECTRODE ARRAYS FOR RECORDING MULTI-SCALE EXTRACELLULAR POTENTIAL AND LABEL-FREE CHARACTERIZATION OF NETWORK DYNAMICS IN IPSC-DERIVED NEURONS

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Advances in the development of microelectrode arrays (MEAs) for in-vitro electrophysiological recordings have enabled the characterization of multi-scale behavior in neuronal networks, ranging from subcellular level to network dynamics. Such devices are fundamental for studying the phenotype of neurological disorders and for drug discovery, providing unique insights into the complexity of neuronal networks. Electrode density, spacing, and size influence the signal quality, noise level, and sensitivity. To properly characterize the full behavior of neuronal networks, MEAs must combine single-cell and subcellular resolution with high-throughput assays, while maintaining sensitivity to small extracellular action potentials to describe the full range of network dynamics. In this study, the MaxOne and MaxTwo high-density (HD) MEA systems (MaxWell Biosystems, Switzerland) were used to record activity from induced pluripotent stem cell derived neurons, demonstrating the advantages of having 26,400 electrodes per well, which is key to increasing the statistical power of data collected longitudinally. HD-MEA recordings were compared with simulated low-density recordings, in which larger, low-density electrodes were mimicked by clustering adjacent electrodes on HD-MEAs. Additionally, the AxonTracking Assay, an automated tool for recording and analyzing individual axonal arbors from many neurons in parallel, was used to characterize the function and axonal structure of recorded cultures. Results indicated that higher density and smaller electrodes provided greater sensitivity, enabling the detection of smaller spikes, and covering the full spectrum of network behavior. The high-resolution analysis of network dynamics, coupled with the AxonTracking Assay's subcellular insights, provide powerful insights into drug screening and disease modelling.

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Keywords: high-density microelectrode array, iPSC, disease modeling

3:45 PM – 4:45 PM

151

TOWARD A BETTER UNDERSTANDING OF ITM2B PATHOGENICITY IN A SPECIFIC RETINAL DYSTROPHY

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Our team identified a missense variant in ITM2B in the C-ter cleaved peptide BRI23, underlying a novel autosomal dominant retinal dystrophy with ganglion cell (RGC) loss, inner retinal and photoreceptor (PhRs) dysfunction. The function of ITM2B in the retina and its physiopathological mechanisms remain poorly understood.Our recent work suggested that ITM2B may interact with proteins, implicated in oxidative stress as well as in vesicle trafficking in the human retina. This project aims to model the disease using patient-derived induced pluripotent stem cells (IPSCs) and retinal organoids (ROs) in order to investigate ITM2B function and associated pathogenic mechanisms. Three induced IPSC derived from an affected subject (Mut), his unaffected brother (NA), and a CRISPR-corrected (Cor), were established and differentiated into ROs. Immunostaining showed that ITM2B is mainly localized in PhRs and that the synaptic layer and bipolar cell layer are structurally modified in Mut ROs compared to NA and Cor ROs. To further investigate this area, we compared the transcriptomic profile of NA, Mut and Cor ROs. The data showed a down regulation of several genes implicated in cone and rod pathways, retinoic acid cycle and cell and synaptic ion homeostasis (amino acid transporters, SLC family...). Furthermore, proteomic analysis showed a higher interaction between BRI23-Mut and some retinal protein partners compared to BRI23-WT. The concerned proteins are involved in the mitochondrial electron transport, as well as ion transports across the plasma membrane involved in neurotransmission (SLC family). In addition, metabolic activity was explored in ROs using a dynamic full-field optical coherence tomography method and showed a lower metabolic activity in the outer and inner segments of photoreceptors from Mut ROs compared to those from the other two conditions. Taken all together, these data suggest a role of ITM2B in the energetic metabolism homeostasis of the photoreceptors through the regulation of amino acid and solute carrier transporters activity through the cell membrane.

Funding Source: Fondation de France

Keywords: retina, retinal organoids, ITM2B

3:45 PM – 4:45 PM

153

TRANSCRIPTOMIC EFFECTS OF MOOD STABILIZING DRUGS ON THE GENE REGULATORY NETWORK IN CELL VILLAGES OF HUMAN IPSC-DERIVED CORTICAL NEURONS

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Lithium (LiCl) and valproic acid (VPA) are first-line treatments for bipolar disorder (BD). Extensive research has been done on the effects of these medications but data on the molecular mechanisms of action that mediate mood regulation remain elusive. Historically, pharmacological studies used animal models, established cell lines, or postmortem tissues. Our study used renewable human iPSC-derived neuronal cells and a “cell village” culture approach to determine the impact of mood stabilizers on gene expression and transcriptional regulation. iPSCs from four healthy, unrelated individuals were differentiated into neural progenitor cells and then pooled together into a “cell village,” and subsequently differentiated into cortical neurons. Neurons grown post differentiation up to six weeks were divided into three treatment groups: a) untreated, b) treated with 1 mM LiCl for two weeks, and c) treated with 1 mM VPA for 72 hours. Accessible chromatin was analyzed using ATAC-seq and analysis is ongoing. Transcriptomic analysis of untreated versus lithium treated neurons showed ~70 genes that were significantly differentially expressed (p adj< 0.05). VPA-treatment revealed ~5900 genes that were significantly differentially expressed (p adj< 0.05) in untreated versus VPA-treated neurons, and g:Profiler analysis of these genes showed enrichment in synapse organization, axon guidance, and nervous system development. VPA treatment also revealed downregulation of several genes previously implicated in neuropsychiatric disorders including CACNA1C, which lies within a well-known BD and schizophrenia (SCZ) GWAS locus and encodes a voltage-dependent calcium channel; AKAP11, a risk gene for BD and SCZ; and SETD1A where loss of function variants has been associated with SCZ. VPA treatment showed upregulation of several HDAC genes, which are involved in chromatin remodeling. This study shows the strong effect of VPA on gene regulation in living neurons that may be relevant to its mood stabilizing action. The “cell village” strategy applied to human iPSC-derived neurons provides a potentially effective model to investigate drug mechanisms in the central nervous system. Additionally, differentially expressed genes and enriched pathways are potential targets for future investigations into novel therapeutics.

Funding Source: This research was funded by the National Institute of Mental Health Intramural Research Program, National Institutes of Health.

Keywords: human iPSC-derived neurons, lithium and valproic acid, cell village

3:45 PM – 4:45 PM

155

INVESTIGATION OF BLOOD-BRAIN BARRIER TRANSPORTER DYSFUNCTION IN SPORADIC ALZHEIMER'S DISEASE: INSIGHTS FROM PATIENT IPSC-DERIVED MODELS

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Alzheimer's disease (AD) is a leading cause of global dementia, primarily impacting individuals aged 65 and older, known as late-onset or sporadic AD (sAD). It is characterized by the accumulation of βeta-amyloid protein aggregates and neurofibrillary tangles formed by tau protein. Recent research underscores the critical association between AD and blood-brain barrier (BBB) dysfunction. The BBB, primarily composed of brain endothelial cells (BECs), pericytes, and astrocytes, serves as a guardian for the brain by regulating substance passage. Transporter-mediated regulation at the BBB is pivotal, with dysregulation implicated in neurodegenerative diseases, including AD. Non-invasive methods, such as focused ultrasound (FUS) mediated BBB opening have been investigated as a new therapeutic to potentially modulate transporter activity to enhances drug delivery. The present study used human-induced pluripotent stem cells (hiPSCs) from the apolipoprotein E (APOE4) high-risk and (APOE3) low-risk sAD patients to derive brain endothelial-like cells (iBECs) and astrocytes (iAstrocytes). The expression of key BBB transporters in these BBB cells were investigated. FUS combined with microbubbles (MBs) was used to modulate BBB transporters based on promising results from previous studies. The results demonstrated notable differences in BBB transporter expression between APOE4 and APOE3 BBB cells, particularly those involved in amyloid beta clearance. Interestingly, following treatment with FUS+MB, iAstrocytes in APOE4 exhibited higher expression levels of BBB transporters compared to APOE3 immediately after treatment. Notably, untreated samples showed initially lower expression of BBB transporters in APOE4 compared to APOE3, suggesting a potential compensatory increase in the levels of dysregulated transporters due to FUS treatment. The findings highlight the potential of using hiPSC-derived iBECs and iAstrocytes in elucidating phenotypic disparities in the BBB of high-risk and low-risk sAD patients. Moreover, hiPSC-derived BBB models also allow to study the potential therapeutic effects of FUS, allowing for translatable research outcomes to AD patients.

Funding Source: QUT Postgraduate Research Award and QUT Higher Degree Research Tuition Fee Scholarship 2020 to J.C.S.C. QIMR Berghofer SEED grant to L.E.O and NHMRC Project grants APP1125796 and Senior Research Fellowship APP1118452 to A.R.W.

Keywords: sporadic Alzheimer's disease, blood-brain-barrier (BBB), drug modulation via focused ultrasound (FUS)

3:45 PM – 4:45 PM

157

PATIENT DERIVED CORTICO-STRIATAL ASSEMBLOIDS FOR MODELING MITOCHONDRIAL DISEASE

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Defects in the mitochondrial respiratory chain (RC) underlie a spectrum of human conditions typically affecting the central nervous systems (CNS). One of the most severe manifestations of mitochondrial disease in children is Leigh syndrome (LS) that can affect 1/36,000 newborns. LS causes symmetric lesions in CNS and specifically in the striatum, leading to psychomotor regression. Among RC components, complex I (CI) is the most frequently affected in LS. Here, we focus on two mutations in the CI nuclear gene NDUFS4. We generated iPSCs from two LS patients carrying nonsense NDUFS4 mutations (c.316C>T and c.20C>G). Using CRISPR-Cas9, we introduced these two mutations into a control iPSC line. In all mutant iPSCs (patient- and CRISPR-derived), we observed a decrease in expression of the NDUFS4 protein and lower CI activity. We previously found that LS impairs the development of unguided cerebral organoids by disrupting neuro-morphogenesis. We now aim to address the region-specific CNS defects of LS by generating cortical (COR) and striatal (STR) brain organoids. We confirmed that COR and STR organoids express mature cortical and striatal markers by day 70. Bulk RNA sequencing revealed an up-regulation of inflammatory markers in mutant COR and STR organoids compared to isogenic control organoids, with particular accumulation within STR organoids. To investigate the connectivity between these different brain regions, we established COR-STRI assembloids. We added a synapsin reporter adeno-associated virus to COR from day 65-70 before co-culturing with STR. After 2 weeks of culture, we observed neural projections from COR to STR, and we are currently addressing potential differences in mutant organoids. We next plan to use high-density multi-electrode array (HD-MEA) to investigate the functionality of cortico-striatal assembloids, and to add iPSC-derived microglia to address their role in driving the neuronal pathology. Combining these various techniques, we hope to gain a deeper understanding of the striatal-specific defects and the overall neuronal pathology of LS to possibly identify innovative targets of interventions.

Keywords: neuroinflammation, assembloids, rare disease

3:45 PM – 4:45 PM

159

IDENTIFICATION OF PROTECTIVE FACTORS IN OCULOMOTOR NEURONS CONFERRING RESISTANCE TO SPINAL MUSCULAR ATROPHY

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Spinal Muscular Atrophy (SMA) is a neuromuscular degenerative disease caused by homozygous mutations or deletion of the survival motor neuron gene 1 (SMN1) gene, resulting in the loss of the ubiquitously expressed SMN1 protein. This leads to progressive and selective degeneration of spinal motor neurons and their innervation to the muscle fibers, causing muscular atrophy, immobility, and death. Although there are three FDA-approved treatments, Nusinersen, Zolgensma, and Risdiplam for restoration of SMN loss, they are not curative. Therefore, it is crucial to identify new therapeutic targets for effectively treating SMA. Interestingly, oculomotor neurons, responsible for eye movements, remain unaffected in SMA patients, implying a unique intrinsic mechanism to protect them from degeneration in response to the loss of SMN1. Previous studies revealed preferential expression of factors in oculomotor neurons of SMA mice but not in spinal motor neurons, suggesting their protective role. To investigate if similar factors are expressed in human ocular motor neurons, we utilized the expression of PHOX2A as a critical determinant of these neurons to generate a knock-in of the tdTomato reporter into its endogenous locus, enabling the enrichment of ocular motor neurons from induced pluripotent stem cells derived from healthy individuals- and SMA patients' urine samples for transcriptomic profiling analysis. This approach holds promise for identifying protective factors in oculomotor neurons that confer resistance to SMA and may serve as new therapeutic targets to enhance the efficacy of SMA treatments.

Funding Source: General Research Fund 17114619 Research Impact Fund R7018-23 Seed fund for collaborative research 2023-2024

Keywords: Spinal Muscular Atrophy (SMA), oculomotor neurons, PHOX2A reporter

3:45 PM – 4:45 PM

161

IMPACT OF TRAUMA ON NEURAL STEM CELL PROLIFERATION AND FUNCTIONAL MATURATION

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Traumatic Brain Injury (TBI) affects millions of individuals. Despite progress in understanding TBI mechanisms using rodent models, translating these findings to human tissue validation remains a critical need. Previously we found an increase in neural stem cell (NSC) proliferation in the hippocampal dentate gyrus after experimental fluid percussion injury (FPI) in rodents. Suppressing post-TBI neurogenesis has shown promise in reducing long-term neurogenic loss and the development of epilepsy. Additionally, our research indicates that blocking Toll-like receptor 4 (TLR4) can mitigate memory deficits and reduce the risk of epilepsy after TBI. We hypothesize that TLR4 signaling contributes to altered neurogenesis and epileptogenesis after TBI can be therapeutically targeted. In the mouse model of experimental TBI, our studies examining local field potential (LFP) recording confirm that the hippocampal dentate gyrus of mice subjected to FPI is more excitable than sham controls. To explore the role of TLR4 signaling, we used CLI-095 (a.k.a Tak 244) an FDA-approved TLR4 antagonist at increasing doses (0.5, and 3 mg/kg intraperitoneally) 2 hours post injury and identified 0.5 mg/kg of CLI-095 administered as the most effective in mitigating dentate gyrus excitability after FPI. In studies using EdU pulse labeling after FPI, we find an increase in adult neurogenesis in the dentate gyrus after FPI. We are investigating if CLI-095 reduces early posttraumatic neurogenesis. Considering potential differences in adult neurogenesis between rodents and humans, studies are underway to validate these findings in human tissue. To achieve this goal, we have generated NSCs from human induced pluripotent stem cells (iPSCs) using dual Smad inhibition. Characterization of these NSCs in the rosette stage indicated the expression of Zo-1, Nestin, Sox2, and Ki67. These well-characterized NSCs are now being adapted for use in vitro stretch injury to evaluate the effect of injury on human iPSC-derived NSC proliferation in vitro and to assess the role of TLR4 signaling on NSC proliferation and maturation. These studies aim to enhance our understanding of mechanisms of adult neurogenesis, specifically, the role of TLR4 in neuronal differentiation, migration, and maturation, and how they are perturbed after TBI.

Funding Source: Special thanks to CIRM and the Transcend Grant Agency for their support, which made this research possible.

Keywords: neurogenesis, neural stem cells (NSCs), Toll-like Receptor 4 (TLR4)

3:45 PM – 4:45 PM

163

MORPHOGEN-INDUCED DIFFERENTIATION OF STEM CELLS INTO OCULOMOTOR NEURONS TO MODEL RESILIENCE IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the loss of motor neurons (MNs) and, subsequently, muscle denervation and muscle atrophy. Nonetheless, certain subpopulations of MNs are more resistant to degeneration than others. Among somatic MNs, oculomotor neurons (OMNs), which control eye movement, persist throughout the disease. Generating systems that allow us to understand cell-intrinsic properties that promote resilience of OMNs in disease would help us to find therapeutic targets to treat ALS patients. We have previously generated OMNs through temporal overexpression of PHOX2A in mESC-derived neuronal progenitors. Identity of these in vitro-generated OMNs was confirmed with electrophysiology, immunocytochemistry and RNA sequencing experiments. In addition, OMNs were more resilient to excitotoxicity in comparison to mESC-derived spinal MNs, which could be explained by their higher levels of calcium buffering proteins and elevated Akt signaling. We have now generated OMNs from hiPSCs with the exclusive use of morphogens. We differentiated hiPSCs from healthy donors and isogenic lines with genome engineered ALS-causing mutations into OMNs, which showed co-expression of ISL1, PHOX2A and TUJ1 at 20DIV. Absence of HB9 expression in PHOX2A-positive cells indicated generation of OMNs, and their anterior A-P identity was suggested by the absence of HOXB4 expression. Finally, OMNs and spinal MNs derived from hiPSC, from both healthy donors and with genome engineered ALS-causing mutations, were exposed to ALS-like toxicity to investigate the cell-intrinsic resilience properties of OMNs in comparison to spinal MNs in health and disease. Further understanding will be achieved by comparing the transcriptome of in vitro-generated OMNs with spinal MNs in health and disease. In conclusion, we generated OMNs from hiPSC exclusively using morphogens. This approach establishes a biologically relevant model, closely mirroring their developmental origin, without the potential risk of off-target genomic integration associated with overexpression systems. This model will contribute to generating more translatable and reliable results, offering valuable insights into the resilience of OMNs, and consequently, identifying potential gene targets for treating ALS.

Keywords: Amyotrophic lateral sclerosis, oculomotor neurons, resilience

3:45 PM – 4:45 PM

165

GENERATION OF PATIENT-DERIVED AD BRAIN ORGANOID AND THE THERAPEUTIC EFFECT OF NGO TREATMENT

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Alzheimer's disease (AD) is known for one of the most renowned neurodegenerative disease with substantial amount of progress in the field of neuroscience and AD, yet there has been no significant improvement in AD treatment. AD patient derived iPSC was employed to generate cerebral brain organoid and was cultured until they expressed AD phenotypes; accumulation of amyloid-beta (Aβ) plaque and hyperphosphorylated tau (pTau)-derived neurofibrillary tangles (NFTs). We investigated whether treatment of nano graphene oxide (NGO) could decrease the expression of AD's major hallmarks. Even though NGO treatment did not have any effect towards the expression of pTau, however, accumulated Aβ plaques were diminished in NGO treated AD organoids. NGO activates autophagy pathway, targeting AMPK activation, and also decrease IFITM3 expression by decreasing pro inflammatory cytokine levels. Overall, development of AD brain organoid successfully mimics AD phenotype expressions, thus it could be used as a screening platform for novel AD treatment assessments.

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Keywords: Alzheimer's disease, brain organoid, nano graphene oxide

3:45 PM – 4:45 PM

167

INVESTIGATING THE EFFECTS OF CELL CULTURE COATINGS ON THE MATURATION AND ELECTROPHYSIOLOGY OF HIPSC-DERIVED NEURONS

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Neural cells derived from human induced pluripotent stem cells (hiPSC) hold immense potential to advance in vitro disease modeling and drug screening, as they provide a biologically relevant alternative to traditional animal-based approaches. While previous research has predominantly focused on the cell-cell interactions in both 2D and 3D models, the interaction of cells with their environment is often neglected. However, it is known that the composition and characteristics of the matrix significantly impact the cellular behavior by modulating mechanosensing and -transduction. Therefore, in the present study, we aim to investigate the influence of cultivation substrates on the maturation and spontaneous electrical activity of hiPSC-derived neurons in a 2D culture. We generated hiPSC-derived neurons through Neurogenin-2 overexpression (NGN2-neurons) and explored the impact of standard neuronal coatings like Laminin, Poly-L-Ornithine (PLO) and Matrigel on their maturation and functionality using microelectrode array (MEA) technology, gene expression profiling and neurite network assessment. In addition, we investigated the expression of key proteins involved in the mechanotransduction process. Although we observed no significant differences in the gene expression of mature neuronal markers such as TUBB3 and MAPT, differences in the density of the neurite networks were determined. Interestingly, despite having a less dense neuritic network, the cells cultured under certain conditions showed higher network bursting and synchronous firing. The differences in electrical activity depending on the culture substrate underline the importance of substrate selection in the establishment of in vitro

models. This emphasizes the need for increased consideration of the cellular environment to improve the reliability of in vitro disease models and drug screening platforms.

Keywords: hiPSC-derived neurons, mechanotransduction, micro-electrode array (MEA)

3:45 PM – 4:45 PM

169

IPSC-BASED BRAIN METASTASIS MODELS FOR DRUG DISCOVERY

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About 25% of cancer patients are affected by brain metastasis with severe neurological symptoms but face limited therapeutic options. Consequently, there is an unmet clinical need to explore therapeutic vulnerabilities of disseminated cancer cells (DCCs) that eventually spread from a primary tumor to secondary organs like the brain. For the identification of prospective pharmacological strategies, human preclinical models predicting the interplay between DCCs and the brain niche microenvironment are crucial. Therefore, we have successfully established a production workflow of human induced pluripotent stem cell (iPSC)-derived cortical organoids in a screening-compatible 96-well format. In order to mimic brain metastasis, cortical organoids were exposed to cancer cells of different primary tumor origin. Metastatic colonization of fluorescent-labeled melanoma cells has been visualized by advanced microscopy technologies and the model workflow validated using additional breast cancer cell lines and patient-derived DCCs. Comprehensive characterization of metastasis signatures has thoroughly been conducted on mRNA expression and protein levels. For a robust screening assay, we target these metastasis models with rationally selected blood-brain permeable tool compounds, tested in an iPSC-derived brain capillary endothelial cell model. With our metastasis platform, we aim to validate and profile drug candidates with specific on-target efficacy on metastatic cancer cells and minimal off-target cytotoxicity on the niche forming brain tissue.

Funding Source: SFB/TRR 305 funded by Deutsche Forschungsgemeinschaft (DFG)

Keywords: cortical organoids, brain metastasis, drug discovery

3:45 PM – 4:45 PM

171

NEURONS FROM PATIENTS WITH FRONTOTEMPORAL DEMENTIA RELEASE OSTEOPONTIN AND ELICIT AN INFLAMMATORY RESPONSE

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Frontotemporal dementia (FTD) is a group of neurodegenerative diseases and is one of the most common forms of early-onset dementia. FTD can be caused by widespread deposition of hyperphosphorylated tau in neurons and glial cells in patient brains, but the mechanisms leading to neurodegeneration remain largely unknown and curative therapeutic options do not exist. Here, we combined single-cell analyses of FTD patient brains with a stem cell culture and transplantation model of FTD. We identified disease phenotypes in FTD induced pluripotent stem cell-derived neurons related to oxidative stress, oxidative phosphorylation and neuroinflammation with an upregulation of the inflammation-associated protein osteopontin (OPN). Human FTD neurons showed impaired survival and elicited an increased microglial response after transplantation into the mouse forebrain, that we further characterized by single-nucleus RNA-sequencing of microdissected grafts. Notably, downregulation of OPN in engrafted FTD neurons resulted in improved engraftment and reduced microglial infiltration, indicating an immune-modulatory role of OPN in patient neurons, which may represent a potential therapeutic target in FTD.

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Keywords: frontotemporal dementia, transplantation, disease modeling

3:45 PM – 4:45 PM

173

REPROGRAMMING-DRIVEN DRUG DISCOVERY REVEALS SILDENAFIL EFFICACY IN MITOCHONDRIAL DISEASE

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Mitochondrial disease is a group of rare inherited conditions affecting mitochondrial function. One of the most severe forms of mitochondrial disease is represented by Leigh syndrome (LS) characterized by psychomotor regression and early mortality. Currently, there are no treatments for LS. To accelerate drug discovery of LS, we leveraged on drug repurposing, as this approach may bypass the challenges associated with developing new molecular entities for rare diseases. We made use of induced pluripotent stem cell (iPSC)-derived neuronal precursor cells (NPCs) from LS patients and screened a high-quality and well-annotated drug repurposing library consisting of 5,632 compounds. We identified phosphodiesterase 5 inhibitors (PDE5i) as leads with the ability to normalize mitochondrial polarization without causing toxicity. Among PDE5i, we focused on sildenafil, given its established safety profile in children. We confirmed the effectiveness of sildenafil in multiple NPCs derived from nine LS patients. Multi-omics analysis in NPCs and brain organoids from LS iPSCs revealed an amelioration of the disease signature by sildenafil through modulation of pathways related to nervous system development and neuron projection. Accordingly, treatment with sildenafil enhanced neuronal outgrowth capacity in patient-derived dopaminergic neurons. Sildenafil also prevented abnormal calcium response in patient-derived cultured brain organoid slices exposed to metabolic stress. Next, we treated a mouse of model of LS with sildenafil, and observed significant extension of life span and restoration of metabolic and encephalopathy phenotypes. We then confirmed acceptable permeability of sildenafil in a patient-derived blood-brain barrier model. Lastly, we initiated compassionate off-label chronic treatments with sildenafil in five LS patients that are showing encouraging results, as seen by improved neuromuscular function and lack of metabolic decompensation. These findings led the European Medicines Agency (EMA) to recognize sildenafil as an orphan medicine for LS. Taken together, our iPSC-driven drug discovery approach enabled the identification of sildenafil as a promising drug to be repurposed for the treatment of children affected by Leigh syndrome.

Funding Source: We acknowledge support from the European Joint Programme for Rare Diseases (EJPRD) (CureMILS Project), and the European Union (SIMPATHIC Project).

Keywords: drug screening, mitochondrial diseases, brain organoids

3:45 PM – 4:45 PM

175

PHARMACOLOGICAL MODULATION OF NEURITE OUTGROWTH IN STEM CELL-DERIVED PHOTORECEPTORS AND DIFFERENCES IN ROD AND CONE PHOTORECEPTOR NEURITOGENESIS IN VITRO

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Stem cell-derived retinal cell replacement therapies offer promise for the treatment of millions worldwide who suffer from sight loss resulting from retinal degeneration. Restoration of vision will depend on the efficacy of new synapse formation between transplanted photoreceptor cells and recipient inner retinal circuitry. Identification of factors that influence human photoreceptor neuritogenesis and synaptogenesis may be gained by analysis of cell behaviour in vitro. In this study, we investigated the effect of pharmacologic agents on neurite outgrowth of human photoreceptor cells. Embryonic stem cell lines (ESC) were differentiated into 3D retinal organoids to mimic retinal development and generate photoreceptors and inner retinal neurons. Retinal organoids were dissociated, replated in 2D cultures and photoreceptor behaviour analysed after treatment with 19 small molecules shown previously to enhance cortical neuron neurite outgrowth. Retinal cell cultures were immunolabelled against the photoreceptor marker Recoverin

and neurite outgrowth was quantified via high-content image analysis. Significantly enhanced neurite outgrowth was observed following treatment with Rho-kinase inhibitor Y-27632 or myosin II ATPase inhibitor blebbistatin, at micromolar concentrations. Differences in neuritogenesis between rod and cone photoreceptor cells were investigated using timelapse microscopy. A CRISPR-edited dual fluorescent ESC line was used to distinguish dissociated GFP+ rods and mScarlet+ cones after replating in vitro. Timelapse imaging revealed distinct neurite outgrowth behaviours between rods and cones. In contrast to NRL. GFP+ rods, ARR3.mScarlet+ cones autonomously sent neurites to their surroundings. This study uncovers mechanisms controlling human photoreceptor neuritogenesis showing that the processes of ESC-derived photoreceptors can be lengthened with agents that influence actomyosin. These data indicate that small molecule addition can enhance neurite outgrowth and suggest such pharmacological agents could be used to support formation of new synapses between transplanted photoreceptor cells and host neurons.

Funding Source: The Macular Society, National Institute of Health Research, Great Ormond Street Hospital Children's Charity.

Keywords: photoreceptor, neuritogenesis, pharmacological screen

3:45 PM – 4:45 PM

177

INVESTIGATING THE EFFECTS OF MICROGRAVITY ON IPSC-DERIVED NEURAL ORGANOIDS VIA STUDIES CONDUCTED ON THE INTERNATIONAL SPACE STATION

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Research conducted on the International Space Station (ISS) in low-Earth orbit (LEO) has shown that microgravity affects multiple organ systems. To investigate the effects of microgravity on the central nervous system (CNS), we developed a unique organoid strategy using induced pluripotent stem cells (iPSCs) derived from four individuals: those affected by Primary Progressive Multiple Sclerosis (PPMS), Parkinson's disease (PD), and two non-symptomatic controls. These iPSCs were differentiated into cortical and dopaminergic neural progenitor cells (NPCs) to model the specific regions of the brain that are affected in PPMS and PD, respectively. The NPCs were then combined with isogenic iPSC-derived microglia to generate organoids. These organoids were cultured for one month using a novel proprietary sealed cryovial culture method on board the International Space Station (ISS) and live samples were returned to Earth. Post-flight analyses of individual organoids included immunohistochemistry and transcriptome and secretome profiling. Our preliminary findings (1) confirm the expression of both neuron- and microglia-specific genes in organoids and (2) demonstrate lower levels of cell proliferation and higher levels of cell maturity in LEO-cultured organoids when compared to parallel cultures on Earth. These initial experiments have laid the groundwork for further investigation of the effects of microgravity on the brain. We are performing ongoing analyses of neural organoids from two additional flights to validate these findings. Our more recent studies include an expansion of this work in partnership with BioServe Space Technologies, using validated organoid flight hardware that allows medium exchange and drug challenge to be conducted on the organoids while aboard the ISS. Exploring the effects of microgravity on the CNS may provide insights into neurodegenerative diseases on Earth and aid in identification of potential novel therapeutics for patients.

Keywords: organoid, neurodegeneration, space

3:45 PM – 4:45 PM

179

EXPLORING DISEASE-ASSOCIATED FEATURES IN STEM CELL-DERIVED NEURAL GRAFTS FROM PATIENTS WITH GENETIC AND SPORADIC PARKINSON'S DISEASE

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Parkinson's disease (PD) is due to the loss of the dopaminergic (DA) neurons composing the nigrostriatal pathway and its focal degeneration makes it a suitable target for cell-based repair. The first clinical trials during the 80s and the 90s demonstrated that grafts derived from fetal ventral midbrain tissue can mature, integrate, and mediate motor recovery in patients with PD. Post-mortem analysis of these brains revealed the presence of Lewy bodies in the grafts, suggesting that the DA neurons slowly accumulated alpha-synuclein (aSyn) pathology over time. The ongoing clinical trials are based on transplantation of DA

neurons derived from embryonic stem cells (ESC) or healthy pluripotent stem cells (PSC). The potential development of PD pathology in these donor cells is also accepted to occur over a long period of time like for healthy fetal tissue, only affecting a small proportion of neurons without affecting graft functionality. However, for autologous grafting, the vulnerability of patients' cells to develop aSyn pathology in a PD environment needs to be further investigated. To evaluate this, we took advantage of a new, disease-mimicking xenograft model of PD. In this model, co-injection of AAV overexpressing the human aSyn gene and human aSyn pre-formed fibrils, in the substantia nigra of rats, results in extensive synucleinopathy and inflammation resembling human PD. We transplanted DA progenitors either from a patient-derived line carrying the triplication of the aSyn gene (AST18) or a patient-derived line with idiopathic PD (PD16) and compared them to grafts derived from the ESC line (RC17) used in STEM-PD. Grafts were characterized for up to 24-week post-transplantation by quantifying the number and density of DA cells as well as the development of aSyn aggregates. By assessing DA neurons with pathology as well as microglia activation, we showed that both AST18- and PD16-derived DA neurons developed significantly higher levels of aSyn aggregates compared to the RC17 grafts, indicating that cells derived from both monogenetic and idiopathic PD are prone to develop aSyn pathology after grafting into a PD-like brain. These results suggest that autologous transplantation without modifications to protect the cells from pathology may not be suitable in patient-derived stem cell therapy for PD.

Keywords: Parkinson's disease, alpha-Synuclein pathology, autologous transplantation

3:45 PM – 4:45 PM

181

INVESTIGATING DOPAMINERGIC-STRIATAL NEURON INTERACTION IN A REGIONALIZED MULTI-ORGANOID MODEL

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Understanding the development, function, and degeneration of human midbrain dopaminergic (DA) neurons is crucial for elucidating the pathophysiology of Parkinson's Disease (PD) and DA-related disorders. Due to the inaccessibility of the human brain, pluripotent stem cell-based models, such as brain organoids, represent an interesting approach to investigate the complexity of neuronal tissue in a dish. However, existing in vitro models still struggle to fully recapitulate human DA circuitries such as the nigrostriatal pathway, which connects DA neurons in the substantia nigra and their striatal targets. To address this issue, we developed a connectoid system, an in vitro model designed to recapitulate DA long-range projections and functional connections in the nigrostriatal pathway. This model integrates regionalized ventral midbrain (VM) and striatal (STR) organoids within a polydimethylsiloxane (PDMS) bioengineered device to spatially confine and guide axonal projections on a chip. We generated VM- and STR-patterned organoids, ensuring the presence of region-specific neurons through adaptation of established differentiation protocols. We showed that VM and STR organoids in the connectoid keep their regional identity, and follow defined neurodevelopmental trajectories also while in co-development. Within this setup, we observed long-range functional projections from VM to STR organoids and assessed the dynamics of axon growth using engineered cell lines and confocal live imaging. DA release measurements upon external stimuli further validated the functionality of midbrain DA neurons within our connectoids. Connectoids represent a novel approach in modeling the human nigrostriatal pathway in a more physiologically relevant context and can be easily adapted to recapitulate other circuitries of the human brain. Therefore, connectoids are an accessible, powerful, and robust platform for exploring DA neuron innervation dynamics, drug screening, and developmental studies, with direct applications in developing novel cell-based treatment strategies for PD.

Keywords: dopaminergic neurons, nigrostriatal pathway, human brain organoids

3:45 PM – 4:45 PM

183

MODELLING RETT SYNDROME USING IPSC-DERIVED CORTICAL ORGANOIDS

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Rett syndrome (RTT) is a rare neurological disorder caused by mutations in the methyl-CpG binding protein 2 (MECP2) gene, which plays an essential role in normal brain development. The onset of RTT typically occurs around 6-18 months after birth and is characterised by rapid deterioration of learnt behaviours with cognitive and physical disabilities. The molecular and cellular processes in the prenatal and post-natal period before symptom onset need to be better understood

as research is limited by the lack of brain tissue and human models. Cortical brain organoids are an emerging human 3D model that can mimic various developmental features at the cellular and molecular levels, which provides a powerful model for studying neurodevelopmental disorders. This project aims to characterise patient iPSC-derived cortical organoids to provide greater insight into the molecular complexity of Rett syndrome and identify novel clinical biomarkers and drug targets by performing a multi-omics characterisation. Neuroectoderm progenitor cells, neurons and astrocytes are present in the hCOs, which undergo further maturation when cultured long-term. Reduced neural activity was observed in the RTT-cortical organoids compared to their congenic controls at 150 and 180 days in vitro through live cell imaging and calcium signalling. Furthermore, the RTT hCOs resulted in a lower basal oxygen consumption rate than the controls indicating a pathological impairment in mitochondrial function. Preliminary studies validate the model's ability to recapitulate a RTT phenotype, which provides a novel model to understand the cellular and molecular pathology of the brain in the early developmental stages of neurodevelopmental disorders.

Funding Source: MRFF Grant and Luminesce Alliane

Keywords: neurodevelopmental disorder, disease modelling, cortical organoid (neural)

3:45 PM – 4:45 PM

185

FUNCTIONAL NETWORK LEVEL ABNORMALITIES OF NEURAL CIRCUITS IN A HUMAN IPSC MODEL OF DRAVET SYNDROME

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Dravet syndrome (DS), is a severe childhood epilepsy with limited response to current antiseizure medications (ASMs). New therapeutic drugs are therefore needed, as most ASM tested on animal models, have partially failed in developing efficacious treatment, and species translatability is often debated. Therefore, human predictable pre-clinical models are needed. Over 80% of DS cases are caused by de novo mutation in the SCN1A gene, encoding the alpha subunit of the voltage-gated sodium channel. Experimental mouse models and in vitro-based human induced pluripotent stem cell (hiPSCs) models have reported complex findings related to the underlying pathogenic mechanism. While earlier in vivo studies showed that DS pathology is a result of the disinhibition of GABAergic neurons, more recent works have alluded alterations in sodium function to also be present in excitatory neurons. However, the interplay between both cell types is essential in understanding how the SCN1A mutation affects their functional properties. Here, we investigated the functional phenotype of DS patient-specific hiPSC-derived neuronal networks using microelectrode arrays (MEAs). We generated two disease-relevant cell-type cultures: i) GABAergic enriched neurons and ii) mixed excitatory (glutamatergic) and inhibitory (GABAergic) neurons, from DS patient hiPSCs. We report that DS patient neurons displayed differential functional activity patterns in the GABAergic-enriched cultures. In the mixed cultures, a distinctive neuronal activity pattern of DS patients was detected in contrast to control neurons. Alterations at network-level activity were patient-specific which corresponded to the severity of their clinical diagnosis. Furthermore, multiparametric analysis with principal component analysis (PCA) that combined single-channel and network-level MEA features revealed distinct segregation between DS patients and control networks in the mixed cultures. This study highlights the competence of human-based stem cells in disease modeling to advance future studies related to epilepsy.

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Keywords: Dravet syndrome, human IPSCs, microelectrode arrays

3:45 PM – 4:45 PM

187

REPRODUCTION OF CELLULAR SENESCENCE IN IPS CELL-DERIVED NEURONS FOR MODELING OF NEURODEGENERATIVE DISEASES

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A critical limitation in the current modeling of neurological diseases is the difficulty of recapitulating cellular senescence in vitro. In this context, it has been suggested that hiPSC-derived neurons are immature compared to those in the adult brain and may not accurately recapitulate the pathological phenotypes of late-onset neurodegenerative diseases. To address this issue, some groups have proposed artificial senescence techniques such as the introduction of progerin, knockout of SATB1, treatment with telomerase catalytic activity inhibitors and knockdown of RanBP17. These techniques may mimic cellular senescence in hiPSC-derived neurons, but they are technically complicated and challenging to replicate in terms of throughput, efficiency, and uniformity. Recently, it was reported that a cocktail of three inhibitors, SBI-0206965 (an autophagy kinase ULK1 inhibitor), lopinavir (an HIV protease inhibitor), and O151 (a DNA glycosylase inhibitor), accelerated the senescence and reproduction of disease-specific phenotypes in an

amyotrophic lateral sclerosis hiPSC model. In this study, we screened compounds that may promote the maturation of hiPSC-derived neurons and identified JA1, an ATM kinase inhibitor that promotes maturation of hiPSC-derived neurons. The JA1-treated neurons also exhibited multiple cellular senescence phenotypes, including SA-beta-Gal positivity, abnormal autophagy, and decreased NAD/NADH ratio. This JA-induced cellular senescence was caused by DNA repair dysfunction, DNA replication stress, and cell cycle abnormalities via the HSP90 pathway. Furthermore, JA1 facilitated the reproduction of pathological phenotypes in the hiPSC model of late-onset neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. JA1 can easily reproduce cellular senescence in iPS cell-derived neurons, will contribute to neurological disease and aging research.

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Keywords: cellular senescence, ATM kinase, neurodegeneration

3:45 PM – 4:45 PM

189

PHENOTYPIC DRUG SCREEN USING A FUNCTIONAL IN VITRO MODEL OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and most common cause of dementia. It is characterized by severe memory loss and cognitive decline resulting from a progressive dysfunction and degeneration of specific neuronal populations including basal forebrain cholinergic neurons (BFCNs). This process is associated with pathological changes hallmarked by the accumulation of aggregated proteins and loss of synapses, as well as neuroinflammation involving activation of microglia, the primary immune cell of the brain. The etiology remains unclear, and while treatment with acetylcholinesterase inhibitors can ameliorate symptoms temporarily in some patients, there is a great need for development of disease-modifying therapies. In this study, we are developing a human pluripotent stem cell (hPSC)-based in vitro model of AD which we will use for phenotypic screening of a library of FDA-approved small molecules aiming to identify compounds that can enhance neuronal function and protect against degeneration. Our model is based on the generation and co-culture of BFCNs, astrocytes, and microglia from hiPSC lines carrying genetic variants associated with increased AD risk, specifically combining mutations in APP with a homozygous APOEe4 genotype. The primary readout for the screen is neuronal functionality in the form of acetylcholine release which is measured using acetylcholine-sensitive sniffer cells expressing a genetically encoded fluorescent sensor, allowing high-throughput imaging-based evaluation of compounds. With this agnostic drug discovery approach, we aim to identify novel neuroprotective targets, thus moving closer to the development of a treatment that can halt the progression of AD.

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Keywords: neurodegenerative disease, disease modeling, drug screen

3:45 PM – 4:45 PM

191

DEVELOPMENT OF IN VITRO ASSAYS TO EVALUATE THE IMPACT OF SGSH ENZYME REPLACEMENT THERAPY FOR SANFILIPPO SYNDROME TYPE A IN DIFFERENT BRAIN CELL TYPES

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Sanfilippo Syndrome type A (MPS IIIA) is a monogenetic disease caused by ~150 different loss-of-function mutations in the lysosomal enzyme N-Sulfoglucosamine Sulfohydrolase (SGSH), resulting in accumulation of heparan sulfate (HS) in lysosomes. Unfortunately, peripherally administered enzyme fails to cross the blood-brain-barrier. Therefore, to develop an efficient enzyme replacement therapy (ERT) approach for MPS IIIA, it is key to understand cellular and intracellular distribution of HS accumulation and SGSH activity. To that end, we have generated SGSH KO HEK cell lines to study intracellular accumulation of HS. As HS quantification by immunocytochemistry is challenging, due to its similarity to other glycosaminoglycans and poor antibody specificity, we have established a high-throughput enzymatic digestion assay coupled with liquid chromatography-tandem mass spectrometry (LC-MS) that allows accurate quantification of HS disaccharides. Using this method, we confirmed HS accumulation in KO cells compared to wildtype, and that HS can be degraded in vitro with recombinant SGSH treatment. To study SGSH function in brain relevant cell types, we differentiated induced pluripotent stem cells (iPSCs) into neurons, microglia and astrocytes to assess SGSH expression and activity by RNAseq and enzyme activity assay, respectively. We found that SGSH expression and activity is enhanced in microglia and astrocytes compared to neurons, suggesting their major contribution to MPS IIIA neuropathology. To characterize HS accumulation in brain relevant cell types in a disease relevant setting, we generated SGSH KO clones from the parental iPSCs and differentiated these to neurons, microglia and astrocytes with the purpose to evaluate cell specific HS accumulation and degradation after in vitro treatment with recombinant SGSH. Thus, we have developed in vitro assays that can be used to evaluate the efficacy of various SGSH ERTs in different brain cells paving the way for efficient drug development for patients living with MPS IIIA.

Keywords: MPS IIIA, drug discovery, stem cell-based model system

3:45 PM – 4:45 PM

193

DISEASE MODELLING WITH COMBINATIONS OF IPSC-DERIVED NEURAL CELL TYPES IN CO-CULTURE

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iPSC-derived neural cell types have emerged as valuable tools for modeling central nervous system (CNS) diseases, primarily due to their ability to faithfully represent the human genetic background. Commonly employed protocols either entail the undirected differentiation of iPSC into a heterogeneous mix of neural cell types or the generation of specific cell types through the controlled expression of transcription factors. While the latter approach, focusing on defined cell types, presents advantages over the former, it falls short in capturing the intricate complexity of the nervous system in single-cell type cultures. In our pursuit of more accurate disease models, we developed a series of co-culture models utilizing iPSC-derived neural cells to represent different endophenotypes of schizophrenia. These models comprised co-cultures (1) of microglia with neurons for the analysis of neuroinflammation, (2) of glutamatergic and GABAergic neurons to mimic disturbed microcircuitry, and (3) of glutamatergic and dopaminergic neurons to study dysfunctions of mesocortical connections. Our comprehensive findings revealed intriguing insights, such as heightened inflammasome activation in microglia and synaptic damage in neuron-microglia co-cultures. Moreover, our co-cultures featuring glutamatergic/GABAergic neurons uncovered phenotypes linked to synaptic connectivity and transmission not accessible in these neuronal subtypes cultivated separately. Lastly, the co-culture of glutamatergic/dopaminergic neurons exhibited increased neuronal activity, a phenomenon not observed in cultures featuring either glutamatergic or dopaminergic neurons alone. This study convincingly demonstrates the advantages of employing more complex co-culture models over simplistic cultures, offering an improved representation of schizophrenia and its multifaceted aspects.

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Keywords: iPSC-derived neural cells, schizophrenia phenotypes, co-culture models

3:45 PM – 4:45 PM

195

SMALL-MOLECULE MODULATION OF CAG RNA INTERACTORS AS POTENTIAL DRUG TARGETS FOR HUNTINGTON'S DISEASE

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Research on neurodegenerative diseases focuses on the disruption of RNA metabolism due to loss of function in RNA binding proteins (RBPs). RBPs play crucial roles in various cellular processes, including gene expression, splicing, RNA transport, and translation. In neurodegenerative conditions, RBPs experience decreased expression, mislocalization, and aggregation. In diseases characterized by CAG repeat expansions, such as Huntington's disease (HD), RNA containing CAG repeats forms nuclear RNA foci, which can sequester RBPs, disrupting RNA metabolism. In our study, we employ a novel approach utilizing the RNA-targeting Cas protein dCas13d fused with the proximity labeling protein APEX2 in a HEK293T model of HD to identify proteins bound to CAG RNA. Our APEX experiment reveals that mutant HTT exon 1 mRNA preferentially binds proteins associated with translation and stress granules. By employing small molecules to inhibit stress granule formation and assembly, we have observed reduced levels of the mutant allele, decreased polyQ protein aggregation, and increased neuronal survival from HD adult-onset induced pluripotent stem cell (iPSC)-derived striatal neurons. Our study opens avenues for potential therapeutic interventions aimed at restoring RNA homeostasis and alleviating the progression of HD.

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Keywords: Huntington's disease, protein aggregation, patient derived, striatal neurons, APEX, G3BP1, PolyQ, therapeutics, allele specific, chronic, HTT protein, CAG protein, RNA

3:45 PM – 4:45 PM

197

PRECLINICAL CHARACTERIZATION OF NRTX-1001, A HUMAN INHIBITORY INTERNEURON CELL THERAPY CANDIDATE IN PHASE I/II CLINICAL INVESTIGATION FOR DRUG-RESISTANT EPILEPSY

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Epilepsy is one of the most common neurological disorders worldwide. About one-third of patients have drug-resistant seizures, presenting an unmet need for new therapeutic strategies. Epilepsy is characterized by recurrent seizures and network hyperexcitability. GABAergic interneurons are essential to maintaining excitatory/inhibitory homeostasis in the brain, and interneuron loss/dysfunction can lead to overexcitation and spontaneous recurrent seizures. Surgical lobectomy to remove or ablate the seizure-generating brain region can be an option for some patients with refractory focal epilepsy but is not indicated or effective for all and can cause cognitive impairment. NRTX-1001 is an investigational cellular therapeutic candidate comprising GABAergic, post-mitotic pallial interneurons derived from human pluripotent stem cells. Clinically-compliant processes were used to reliably manufacture and cryopreserve NRTX-1001. Persistence, distribution, fate, and composition of the graft were investigated pre-clinically in the mouse intrahippocampal (IH) kainate model of chronic drug-resistant mesial temporal lobe epilepsy (MTLE). Mice received a single IH transplantation of NRTX-1001 during the chronic phase of the model which resulted in pronounced suppression of focal electrographic seizures and reduced hippocampal pathology. Across several preclinical studies, approximately two-thirds of epileptic mice that received NRTX-1001 were stably seizure-free 8 months post-transplantation. Single-cell RNA sequencing analyses of the grafted cells enabled the bioinformatic identification of human interneuron subtypes based on global gene expression. NRTX-1001 grafts persisted long-term with stable subtype composition. Parallel histological findings supported the subtype composition data, grafted cell persistence, and reduced dentate granule cell dispersion. No ectopic tissues, tumors, or teratomas were observed following transplantation of NRTX-1001, and no deleterious effects were observed in post-transplantation behavioral testing. NRTX-1001 is being evaluated for safety and efficacy in an ongoing phase I/II clinical trial (NCT05135091) for drug-resistant MTLE.

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Keywords: epilepsy, cell therapy, hESC

3:45 PM – 4:45 PM

199

EXPLORING ADAR1 DEPENDENCIES IN BREAST CANCER STEM CELLS METASTASIS

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Adenosine deaminase acting on double-stranded RNA (ADAR1) is a recognized driver of cancer stem cell immune evasion and epitranscriptomic remodeling. Essential for preventing autoinflammation, ADAR1 (A to I) editing also plays a crucial role in tumor resistance to immune checkpoint blockade. This latter effect is mainly attributed to ADAR1 p150 isoform, which harbors a Z-alpha domain capable of binding to and suppressing double-stranded RNA (dsRNA) in Z-conformation, like those arising from human retroviral elements expression. High levels of ADAR1 have been associated with a worse prognosis and reduced survival in patients with high-grade breast cancer (BC), especially in the HER2+ and triple-negative BC (TNBC) subgroup, which are the most prone to developing brain metastasis (up to 30% of TNBC and HER2+ cases). However, the role of ADAR1 in malignant breast cancer progression is not fully understood. We hypothesized that ADAR1 primes BC stem cell niches, facilitating their migration to the brain, with ADAR1 p150 potentially driving stem cells mobilization. To investigate this, a humanized mouse model of breast cancer brain metastases (BC-BrM) was established by neonatal intracerebroventricular injection of MDA-MD-231 TNBC cells in Rag2-/-γc-/- mice. Furthermore, BC-BrM samples were obtained from patient surgeries at the UC-San Diego hospitals. Using a nanoluc-GFP reporter to trace ADAR1 kinetics, we observed upregulation of ADAR1 p150 in BC-BrM derived from TNBC implants, along with activation of the WNT/β-catenin pathway and an increase in the cancer stem cell marker CD47. Additionally, the ADAR1 reporter was activated in TNBC implants during the metastatic switch from the brain to the spine. Upon shRNA knockdown of ADAR1, downregulation of CD47 was confirmed in the TNBC cells, suggesting that ADAR1 influences innate immune signaling in BC. In BC-BrM derived from patient samples, ADAR1 p150 was found to be overexpressed in cancer cells exhibiting high levels of CD47 indicating that the Z-alpha domain, and its targets, are required to maintain cancer cell stemness. We are currently further exploring ADAR1 p150-driven pathways that mediate the metastatic transition in BC-BrM. Overall, this study will improve our understanding of ADAR1 function in establishing and maintaining breast metastatic niches.

Keywords: ADAR1, breast cancer, brain metastasis

3:45 PM – 4:45 PM

201

ORGANOID ASSEMBLOIDS MODELING THE ROLE OF SEROTONIN DURING HUMAN CORTICAL DEVELOPMENT

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The development of the human cortex involves the coordination of several processes including cell proliferation, migration, differentiation, and connectivity. Alterations in these events can lead to pathological conditions such as psychiatric disorders. The neurotransmitter serotonin (5-HT) has been shown to play a significant role in this context, although its molecular effects are poorly understood. In this project we aim to model the role of serotonin during forebrain development using induced pluripotent stem cell (iPSC)-derived progenitors and organoids. We first developed a protocol for the generation of raphe-type organoids (RO). At early time points, RO are composed of progenitor cells expressing NKX2.2 and FOXA2 which further differentiate into serotonergic neurons expressing markers such as TPH2, VMAT2 and 5-HT. When applying the serotonin sensor sDarken release of endogenous serotonin could be detected. We further generated human iPSC-derived cortical progenitors and cortical organoids (CO), and established cortical-raphe assembloids to investigate serotonin's role during early human corticogenesis. When exposing cortical progenitors and CO to 5-HT, we observed increased proliferation of progenitor populations, including apical (aRG) and basal radial glial cells (bRG). Agonist and antagonist experiments revealed that 5- HT signalling is mediated via cell type specific receptor subtypes in these progenitor populations, 5-HTR2C in aRGs, and 5-HTR2A in bRGs. To mimic the innervation of the developing cortex with 5-HT neurons from the raphe nuclei, we employed the CO-RO assembloids. We found a widespread invasion of serotonergic afferents in CO, which displayed an increased progenitor proliferation in proximity to serotonergic afferents. By that our data suggest that organoids provide a valid platform to explore serotonin's role in corticogenesis and to investigate related diseases.

Keywords: raphe organoids, serotonin, cortical progenitors

3:45 PM – 4:45 PM

203

GENERATION OF HUMAN EXCITATORY FOREBRAIN NEURONS BY COOPERATIVE BINDING OF PRONEURAL NGN2 AND HOMEOBOX FACTOR EMX1

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Generation of defined neuronal subtypes from human pluripotent stem cells remains a challenge. The proneural factor NGN2 has been shown to overcome experimental variability observed by morphogen-guided differentiation and directly converts pluripotent stem cells into neurons but their cellular heterogeneity has not been investigated yet. Here we found that NGN2 reproducibly produces three different kinds of excitatory neurons characterized by partial co-activation of other neurotransmitter programs. We explored two principal approaches to achieve more precise specification: pre-patterning the chromatin landscape that NGN2 is exposed to and combining NGN2 with region-specific transcription factors. Unexpectedly, the chromatin context of regionalized neural progenitors only mildly altered genomic NGN2 binding and its transcriptional response and did not affect neuronal specification. In contrast, co-expression of region-specific homeobox factors such as EMX1 resulted in drastic re-distribution of NGN2 including recruitment to homeobox targets and resulted in glutamatergic neurons with silenced non-glutamatergic programs. These results provide the molecular basis for a blueprint for improved strategies for generating a plethora of defined neuronal subpopulations from pluripotent stem cells for therapeutic or disease-modeling purposes.

Funding Source: Simons Foundation

Keywords: neuronal reprogramming, epigenetics, disease modeling

3:45 PM – 4:45 PM

205

EFFECTS OF PRENATAL GABAPENTINOIDS EXPOSURE ON HUMAN CORTICAL NEURONS

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Prenatal substance exposure is a major public health concern associated with many detrimental fetal consequences. Unfortunately, polysubstance use in pregnancy is common. Gabapentinoids are widely used as treatments in psychiatry and neurology; however, they have

been increasingly reported as having potential for misuse. Moreover, gabapentinoids can cross the placental barrier. Due to difficulties in accessing fetal brains exposed to gabapentinoids, we used the human embryonic stem cell (hESC) line H9 to generate early, intermediate cortical progenitors and cortical neurons to modulate prenatal gabapentinoid exposure in vitro. Since the cortex is responsible for cognition and behavior, we focused on cortical development. We analyzed treated (10uM) and untreated (control) cultures for gene expressions, neurogenesis, and morphogenesis. At the early patterning stage, there was a significant increase in Tbr2+ intermediate progenitors in pregabalin- and gabapentin-treated cultures. In addition, there was a significant increase in the expression of cortical related genes Pax6, Foxg1, and Tbr2 in pregabalin-treated cultures, whereas gabapentin significantly increased Tbr2 expression solely. At the maturation stage, the number of mature cortical neurons was unchanged in pregabalin-treated cultures. At early maturation, gabapentin significantly increased Tbr1+ neurons, but not Ctip2+ neurons. At the genetic level, we screened the effects of pregabalin on different cortical layer related genes. Pregabalin significantly increased expression of Brn2 without significant effects on other screened genes. Meanwhile, gabapentin did not alter any cortical layer related genes. Regarding morphogenical analysis, both pregabalin and gabapentin significantly decreased neurite length, branches, and neurites of human cortical neurons. Our data also shows that the effects of pregabalin and gabapentin on the morphogenesis of cortical neurons differ based on the presence of maturation factors, such as GDNF and BDNF, suggesting a possible interaction mechanism. Our study demonstrates that exposure to gabapentinoids during early brain development may interfere with the neurogenesis and morphogenesis of various neuronal subpopulations.

Keywords: gabapentinoids, cortical development, prenatal exposure

3:45 PM – 4:45 PM

207

EXPLORING THE IMPACT OF PSILOCIN ON MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF HUMAN IPSC-DERIVED CORTICAL NEURONS: IMPLICATIONS FOR ANTIDEPRESSANT MECHANISMS

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Human induced pluripotent stem cell (hiPSC)-derived neurons are of great interest for deepening our understanding of the human nervous system, modeling neuropsychiatric disease mechanisms, and developing potential therapies. Psilocybin and its active metabolite, psilocin, have garnered recent attention as potential rapid-acting therapies for treatment-resistant depression, but little is known about their mechanisms of action. Studies in mice have shown that a single dose of psilocin can increase dendritic spines and promote synapse formation. However, this has not been demonstrated in human neurons due to the limitations of accessing the human brain. hiPSC-derived neurons present a unique opportunity to bridge this gap. In this study, we used a high-resolution light microscope to collect images of hiPSC-derived cortical neurons and multiple electrode array (MEA) assays to explore the impact of psilocin on neuronal morphology and electrophysiology. Seven paired cultures of hiPSC-derived, cortical neurons at 9-weeks post-differentiation were treated for 1 week with either psilocin (5µm) or vehicle. Psilocin significantly increased neurite number and increased the average length of neurites, neurite path length, and neurite branch points. On the MEA, hiPSC-derived neurons at 5 weeks post-differentiation were treated with 5 µm psilocin, followed by measures of network activity at 2, 24, and 48 h post-treatment. Psilocin significantly increases the firing rate, mean spikes per burst, and other measures of neuronal network activity. These effects peaked 24 h after treatment and persisted for up to 2 days. These findings suggest that psilocin promotes both structural and functional changes in hiPSC-derived cortical neurons, leading to increased network activity. These in vitro effects may contribute to the putative antidepressant effects of psilocin.

Keywords: psilocin, MEA, neurons

3:45 PM – 4:45 PM

209

TRANSLATING PATIENT STEM CELLS INTO PERSONALISED SCREENS FOR AGE-RELATED MACULAR DEGENERATION

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Age-related macular degeneration (AMD) is a leading cause of irreversible vision loss in the Western world. AMD contributes to 50% of all legal blindness with an estimated 12% of Australians over the age of 50 affected (~1 million). AMD is characterised by the presence of extracellular deposits, known as drusen, that form between the retinal pigment epithelium (RPE) cells and the underlying Bruch's membrane. Yet, the exact pathogenic mechanisms at play are not well elucidated despite intense international research efforts. This is due to limited experimental models of AMD, complicating the task of identifying new treatments. Human induced pluripotent stem cells (iPSCs) are a powerful tool to elucidate the mechanisms that confer disease risk or contribute to AMD development and progression. iPSCs derived from individuals with a specific genetic background can be differentiated into RPE cells that recapitulate AMD phenotype in vitro, e.g., produce drusen-like deposits and changes in mitochondrial function. Our previous work identified mitochondrial remodelling in iPSC-derived RPE cells from AMD patients. In this project we screened a library of 160 FDA-approved

drugs targeting mitochondrial pathways focusing on neuroprotection, antioxidation, and anti-inflammatory activities. We used two iPSC lines derived from AMD patients, which were differentiated to RPE cells for 60 days before passaging them into 96-well plates. iPSC-derived RPE cells were treated with drugs on days 7 and 10 post-passage with three concentrations per compound. We used TMRE (tetramethylrhodamine, ethyl ester) to monitor mitochondrial health following the exposure to the screened drugs and the mitochondrial uncoupler, FCCP, on day 14 post-passage. We identified several compounds able to protect mitochondria from the decrease in the membrane potential following exposure to FCCP in these iPSC-derived RPE cells. Using this approach will allow us to identify compounds with a therapeutic potential, dissect the molecular mechanism of their action and assess their potential for clinical translation.

Funding Source: Medical Research Future Fund

Keywords: age-related macular degeneration, retinal pigment epithelial cells, drug screening

3:45 PM – 4:45 PM

211

IMPAIRED UPTAKE AND CLEARANCE CAPACITY OF ASTROCYTES IN AN IPSC DERIVED PARKINSON'S DISEASE MODEL FROM P.A53T ALPHA SYNUCLEIN PATIENTS

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Astrocytes play important roles in sustaining neuronal health and are emerging as critical players in neurodegeneration processes. Yet, their contribution to Parkinson's disease (PD) pathology is understudied. Here, we leveraged an induced pluripotent stem cell (iPSC)-based model from PD patients harboring the p.A53T mutation in α-synuclein (αSyn), which causes an early and severe PD form. We generated iPSC-derived ventral midbrain dopaminergic neurons and astrocytes to investigate their reciprocal communication in the context of PD. Our data showed that healthy neurons treated with PD astrocyte-conditioned medium displayed reduced neuronal viability and increased levels of αSyn. Interestingly, treatment of PD neurons with healthy astrocyte medium reduced the number of intraneuronal protein aggregates, a hallmark of PD, and ameliorated neuronal survival and network formation. Vice versa, treatment of healthy astrocytes with PD neuron-conditioned medium resulted in efficient uptake of neuron-derived αSyn, a process that was hampered in PD astrocytes, indicating their reduced internalization capacity. In line, PD astrocytes exposed to αSyn pre-formed fibrils (PFFs) for 2h showed significantly lower uptake capacity as compared to healthy astrocytes. However, after 16h exposure, internalized PFFs were significantly more abundant in PD vs healthy astrocytes, pointing towards a retarded degradation process. Of relevance, the activity of Cathepsin B lysosomal enzyme was reduced in PD astrocytes and their lysosomal properties were impaired. Further, in PFF-loaded neuronal cells co-cultured with healthy astrocytes, we observed significantly enhanced clearance of αSyn PFFs as opposed to co-cultures with PD astrocytes. The operation of contact-mediated mechanisms via tunneling nanotubes is being investigated, in addition to non-contact mediated mechanisms. Overall, our data suggest that PD astrocytes, unlike healthy astrocytes, are unable to efficiently uptake and clear neuronal αSyn aggregates thus contributing to PD pathology. These results highlight a critical role for astrocytes in PD that may lead to the development of novel treatments.

Funding Source: Pasteur Joint International Research Unit PIU Neurodegenerative Diseases DiseasePhenoTarget-HFRI Research Projects to support Faculty members & researchers & the procurement of high cost research equipment Bodossaki Foundation

Keywords: Parkinson's disease, iPSC-derived astrocytes, tunneling nanotubes

3:45 PM – 4:45 PM

213

THE EDINBURGH PROGENITOR CELL BANK: IPSC-DERIVED CELLS POISED FOR NEURAL DIFFERENTIATION FOR THE RESEARCH COMMUNITY

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Human induced pluripotent stem cells (hiPSCs) can be differentiated into diverse types of cells for disease modelling and cell replacement therapy. A common problem with hiPSC differentiation protocols is their reproducibility and transferability across different laboratories. This often restricts the use of hiPSC lines and associated differentiation protocols to selected laboratories. One solution to this problem is to differentiate hiPSCs into committed progenitor cells and cryopreserve them in a state poised for differentiation. Test-thaws and quality control of the cryopreserved cells are performed before sharing with the research community. Furthermore, an allelic series of isogenic CRISPR-engineered iPSC lines can be simultaneously differentiated and provided as sets of cryopreserved progenitor cells for direct use

in experiments. Quality-controlled progenitors, already committed to a particular cell type, are ready for experimental studies in any laboratory with cell culture facilities. We have focused efforts on producing midbrain dopaminergic (mDA) progenitor cells that are more than 80% double-positive for LMX1A/EN1 by immunostaining and greater than 90% CORIN-positive by flow cytometry. We have also engineered an isogenic collection of hiPSC lines with deletion of the SNCA gene for Parkinson's disease modelling. These have been differentiated into mDA progenitors and available for distribution. The cryopreserved mDA neural progenitors provide a source of cells for transplantation studies or of mature human dopaminergic neurons for disease modelling applications. The Edinburgh Progenitor Cell Bank endeavours to provide the best quality neural progenitors to the academic community and facilitate user-friendly access to differentiated neuronal cells to help accelerate basic and applied research.

Funding Source: Funded by NC3Rs

Keywords: human induced pluripotent stem cells, neural differentiation, progenitors cell bank

3:45 PM – 4:45 PM

215

THE RHO/ROCK SIGNALING PATHWAY IS SENSITIVE TO UBE2A GENE DOSAGE ABNORMALITIES IN NEURAL CELLS DIFFERENTIATED FROM IPSCS

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X-linked intellectual disability syndrome type Nascimento was first described by Rafaella Nascimento in 2006. This syndrome is caused by loss-of-function mutations in UBE2A gene encoding ubiquitin-conjugated enzyme E2A. Databases have also reported cases of mental retardation associated with duplication of the locus containing the UBE2A gene. The UBE2A protein participates in transcription elongation, mitophagy, and translesion DNA synthesis. However, UBE2A functions in neurogenesis are still unknown. To identify cellular processes affected by the UBE2A dosage abnormalities in neural cells, we developed a cell model based on human iPSCs, including iPSCs with knockout and overexpression of UBE2A gene. We complemented this cell model with iPSCs derived from a patient with Nascimento's syndrome caused by the deletion of UBE2A gene. We differentiated iPSCs into neuronal precursors and glial cells for transcriptomic and proteomic analyses. The transcriptomic data analysis revealed above 1500 up-regulated and above 2000 down-regulated differentially expressed genes (DEGs) across the samples under the study. We identified 8 DEG clusters differentially dependent on the UBE2A gene dosage. The clusters included genes responsible for such biological processes as replication, post-replicate DNA repair, cell cycle regulation, actin cytoskeleton organization and neuronal migration. The last two processes are essential in neurodevelopment and are under the control of the Rho/ROCK signaling pathway. We observed a decreased expression of genes involved in the biological processes regulated by ROCK kinase: Rho GTPase activity (GO:0005096), the non-muscle myosin II complex (GO:0016459), as well as genes responsible for the actin filaments organization (GO:0030036), focal adhesions (GO:0005925). Interestingly, this effect was observed in neural cells, which had knockout or deletion of the UBE2A gene and, on the contrary, overexpressed UBE2A gene. The proteomic data correlated well with transcriptomic data. Thus, the Rho/ROCK signaling pathway demonstrated paradoxical sensitivity to the UBE2A gene dosage abnormalities in neural cells differentiated from iPSCs. Namely, knockout, deletion, or overexpression of UBE2A gene led to a similar decrease in expression of the Rho/ROCK signaling pathway genes.

Funding Source: The project is supported by RSF grant #21-65-00017

Keywords: UBE2A, X-linked intellectual developmental disorder type Nascimento, Rho/ROCK signaling pathway

3:45 PM – 4:45 PM

217

IDENTIFYING NEW TARGETS THAT PROTECT AGAINST THE CELL-TO-CELL PROPAGATION OF TAUOPATHY

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Tauopathies are a group of neurodegenerative disorders associated with cognitive decline that have in common the abnormal aggregation of tau protein in the brain. The cell-to-cell propagation of tau pathology between neurons is an important event in the clinical progression of these disorders. In collaboration with the Lead Discovery Center GmbH, we performed a high-throughput screening campaign to identify small molecules that can reduce tau spreading. For this purpose, we used tau biosensor cells, a genetically engineered cell line that generates intracellular aggregates in response to tau conformations with seeding potential. We identified a novel key regulator of tau internalization, and we validated this target using additional small molecule inhibitors as well as siRNA-mediated knockdown. We recently established a model of tau pathologic spreading using induced pluripotent stem cell-derived

neurons, which we are using to assess how tau spreading causes axonal degeneration and to validate the target's role in tau pathology. In addition, we are testing antisense oligonucleotides (ASOs) for specific reduction of the target as a potential therapeutic approach. Unlike small molecules, directly injecting ASOs into the cerebrospinal fluid should allow us to control the level and localization of the target. Through this approach, we hope to lower the putative side effects stemming from systemic drug administration. Results from this study will enable development of therapies to protect patients against tau spreading and tau-related cognitive decline and dementia.

Funding Source: BMBF, Technische Universität Dresden, Lead Discovery Center GmbH

Keywords: tauopathy, tau spreading, neurodegeneration

3:45 PM – 4:45 PM

219

OPEN SCIENCE INTEGRATIVE PORTAL COMBINES PATIENT-DERIVED IPSCS AND EXPLORATORY SINGLE CELL RNA SEQUENCING TRANSCRIPTOMICS DATA

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Patient-derived induced pluripotent stem cell (iPSC) technology holds promise for generating human brain cells in vitro, advancing research on neurological diseases. Through collaboration between the Clinical, Biospecimen, Imaging, and Genetic Repository (C-BIG) and the Early Drug Discovery Unit (EDDU) at the Montreal Neurological Institute, a biobank of over 150 patient-derived iPSCs was established to facilitate translational research in an open science framework. iPSCs undergo rigorous QC processing, ensuring the expression of OCT3/4, Nanog, Tra160-1, and SSEA4 by immunofluorescence as well as genetic stability through in-house cytogenetic analysis. While imaging and genomic data are accessible through a global portal, an additional module is being developed to include single cell RNA sequencing (scRNA) data, providing comprehensive multi-omic information to researchers. The most recent scRNA data set explores the transcriptional differences between healthy control and sporadic Parkinson's disease (PD) in iPSC-derived midbrain organoids at two different time points (100 and 200 days in culture). Organoids were grown and dissociated as previously described. Using the workflow from Parse Biosciences, samples were fixed until library prep was completed. Samples were then run through the Parse pipeline for demultiplexing of the combinatorial barcodes and then Seurat to visualize results on a umap and explore differentially expressed genes between disease and control. Different cell populations were identified within the organoids, including oligodendrocyte precursors, astrocytes, and a large population of dopaminergic progenitors and neurons. Gene Ontology (GO) analysis revealed differentially expressed genes involved in neural development, axonogenesis, response to misfolded proteins, protein localization, and microtubule organization. Genes such as NR2F1, previously reported to be downregulated by LRRK2 G2019S mutation in PD were identified and will be further explored through methods such as qPCR. Once this data is finalized, it will be deposited into the C-BIG repository, where other researchers can explore the data in the hopes of using it to better understand Parkinson's and other neurological diseases to help identify novel therapeutics.

Keywords: iPSCs, human midbrain organoids, single cell RNA sequencing

3:45 PM – 4:45 PM

221

TOWARDS THE DEVELOPMENT OF 3D MODELLING SYSTEM FOR GLIOBLASTOMA RESEARCH AND DRUG TESTING

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Glioblastoma multiforme (GBM), a highly aggressive brain tumour, requires innovative therapies and advanced in vitro models resembling the tumour microenvironment (TME). Over the past decade, GBM modelling has shifted from monolayer cultures to three-dimensional (3D) environments. However, the tumour's complexity necessitates optimising the modelling system. Moreover, there are still knowledge gaps

in the peculiarities of the cell behaviour and response to treatment in a 3D environment. In this study, we compared the response of 2D and 3D cultured GBM cells to several types of treatment and attempted to optimise the 3D model system to improve the TME resemblance. To reach the aim of this study, we established 3D spheroid cultures of GBM cells (U87MG line) and studied the cell behaviour in response to different microenvironmental factors and treatment conditions. Compared to the monolayer cultures, we detected a decreased sensitivity to the cytostatic treatment in the 3D-cultured GBM cells. Similarly, a distinct cell response was observed in 2D and 3D cultures after treatment with integrin-binding nanoparticles (cRGD-NPs). In monolayer cultures, the addition of cRGD-NPs led to the detachment of cells, while no visible effect of the cRGD-NPs was detected in 3D cultures. To improve the 3D culture conditions regarding the in vivo resemblance, we established a perfusion system that allowed us to study the hydrogel-based 3D invasion of cells in dynamic conditions under various treatments, as well as paracrine interaction with other cell types. The co-culture of GBM spheroids with multipotent mesenchymal stromal cells or astrocytes altered the invasive behaviour pattern. In conclusion, our work underscores the importance of 3D culture for understanding GBM therapy responses and provides an adaptable system for diverse experimental needs. Such a platform holds promise for preclinical research, offering new detailed insights into tumour microenvironments.

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Keywords: glioblastoma, disease modelling, 3D culture

3:45 PM – 4:45 PM

223

LOSS OF BRAT1 DISRUPTS NEUROGENESIS IN AN ORGANOID MODEL OF RIGIDITY AND MULTIFOCAL SEIZURE SYNDROME

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The Integrator Complex (INT) is a 17-subunit machinery that associates with RNA polymerase II (RNAPII) and functions as a critical transcription regulator. INT is essential for the 3'-end formation of a variety of non-coding RNAs and is a broad negative regulator of promoter-proximally paused RNAPII. Integrator subunit 11 (INTS11) houses the RNA endonuclease domain vital for Integrator to cleave nascent transcripts at all RNAPII loci, which is also an important activity for transcriptional repression. Consistent with a fundamental role in Integrator function, mutations within INT subunits cause neuronal dysfunction, including INTS11 genetic variants that give rise to a complex neurological syndrome marked by cerebellar ataxia, intellectual defects, and seizures. Similarly, mutations in BRAT1, a factor which has recently been shown to associate with INST11, also impede neurodevelopment causing a lethal neonatal multi-focus seizure syndrome (Rigidity and Multifocal Seizure Syndrome, Lethal Neonatal, RMSFL). We hypothesize that disruption in INTS9/11 association with BRAT1 negatively impacts neurogenesis. To that end, we have engineered human embryonic stem cells (hESCs) to be null for BRAT1. These syngeneic hESCs have been differentiated into neuronal brain organoids and we examine the phenotypic outcomes and altered gene expression that are disruptive to cell fate determination in neuronal cell types. Our data demonstrate a significant disruption in ventral neural cell fates and neural differentiation.

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Keywords: integrator, infantile seizures, neural differentiation

3:45 PM – 4:45 PM

225

STUDYING THE ORIGIN OF VASCULAR LEPTOMENINGEAL CELLS FROM HUMAN PLURIPOTENT STEM CELL CULTURES

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Human pluripotent stem cell (hPSC)-derived products for the treatment of Parkinson's disease are rapidly moving into clinical trials, with the first trials already ongoing. The aim of these treatments is to promote long-term amelioration of motor symptoms in Parkinson's patients, as has been seen in previous clinical studies using human fetal tissue for

transplantation. The hPSC-derived cell products are manufactured on the basis of well-defined protocols for the generation of authentic ventral midbrain dopaminergic (vmDA) progenitors, which have proven safe and efficacious in preclinical animal transplantation studies. Single cell RNA sequencing (scRNAseq) of such transplanted vmDA progenitor cells surprisingly revealed the presence of vascular leptomeningeal cells (VLMC) of human origin in the grafts. In this study, we investigate the developmental origin of VLMCs and we uncover the signaling pathways governing their differentiation. To decipher the origin of VLMCs in the ventral midbrain we used a clinical grade protocol for generating authentic ventral midbrain progenitors and sampled the cultures at different maturation stages for scRNAseq. This data revealed several developmental signaling pathways as possible drivers of a VLMC fate and clonal culturing analysis revealed the presence of common progenitors capable of giving rise to both neurons and VLMCs. We tested agonists and antagonists of the scRNAseq-identified pathways and found that activation of TGFb and FGF pathways during the neuronal maturation phase (i.e. after day 16) increased VLMC presence, while inhibition of either pathway had no effect. Furthermore, we found that increased seeding density during culture maturation could significantly reduce the amount of VLMCs. To further investigate the regional origin of VLMCs we screened hPSC cultures patterned towards different regions of the neural tube and found that VLMCs were mainly arising from cultures patterned towards ventral regions. This was consistent with the finding, that VLMCs could be generated from FOXA2+-sorted floor plate progenitor cells. With this study we hope to refine the molecular understanding of VLMCs in the ventral midbrain to ultimately improve the current differentiation protocols by minimizing non-therapeutic cells arising from the cell product.

Funding Source: Novo Nordisk foundation grant NNF21CC0073729

Keywords: VLMC, dopamine neurons, lineage tracing

3:45 PM – 4:45 PM

227

LABEL-FREE FUNCTIONAL ANALYSIS FOR THE CHARACTERIZATION OF IPSC-DERIVED NEURAL ORGANOID DEVELOPMENT AND MATURATION

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The flexibility and accessibility of induced pluripotent stem cell (iPSC) technology has enabled high-throughput reproduction of complex human biology in vitro. Indeed, rapid advances in stem cell technology have led to widespread adoption for the development of in vitro neuron electrophysiology models for drug discovery and safety screening. Furthermore, advanced cell preparations, such as spheroids or organoids, are intensively studied with aims to establish mature human phenotypes in vitro. The objective of this work is to develop and validate a live-cell analysis workflow for the characterization of neural organoids in vitro. First, whole-vessel live-cell imaging with the Omni was used to monitor iPSC colony formation and expansion in real-time. The Omni iPSC module was used to determine iPSC colony size and coverage to ensure consistent iPSC passaging. Furthermore, iPSC stemness was confirmed by fluorescence staining of the pluripotency markers TRA-1-60 and SSEA4-1. Lastly, the embryoid bodies were differentiated towards neural organoids. Real-time live-cell imaging was used to track the size and shape of embryoid body formation and the induction of neural differentiation. At day 50+, organoids were transferred to a multiwell microelectrode array (MEA) plate and allowed to attach. Impedance measurements were used to quantify the attachment of the organoids to the substrate and microelectrodes, as a measure of cell viability and electrode coverage. Broadband (1 – 5000 Hz) electrophysiological data was acquired and then separately processed for action potential detection (200 – 5000 Hz) and low frequency oscillations (1 – 50 Hz). The power spectral density was computed from the low frequency signal sampled after network burst events, and then absolute power was computed in the delta (1-4 Hz), theta (4-8 Hz), alpha (8-14 Hz), beta (14-30 Hz), and gamma (30-50 Hz) bands. The emergence and maturation of neural organoid electrophysiological activity was tracked via these measurements of spiking activity and low frequency oscillations, coupled with the long-term monitoring of size via live-cell imaging. This new workflow combining live-cell imaging and MEA measurements supports the continued development of in vitro 3D models of neural function.

Keywords: iPSC-derived neural ortanoids, multiwell microelectrode array, live-cell imaging

3:45 PM – 4:45 PM

229

GENERATION OF REGIONALISED HYPOTHALAMIC NEURONS FROM HUMAN EMBRYONIC STEM CELLS

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Essential behaviours such as appetite, sleep and thermoregulation are controlled by the hypothalamus, a forebrain-derived structure comprised of nuclei with high neuronal diversity and a complex developmental origin. The lineage trajectories of each of these nuclei is not fully understood, and this has hampered the development of protocols for generating subregional cultures of the hypothalamus from pluripotent stem cells. By combining previous literature with single-cell RNA sequencing (scRNA-Seq) datasets from mouse, chick and human fetal hypothalamus, we identified subtype-specific progenitor markers that can predict cell fates of the paraventricular nucleus (PVN), the arcuate nucleus (ARC) and the lateral hypothalamic area (LHA). Based on this information, we developed three hESC differentiation protocols

to produced subregionalised cultures of PVN, ARC and LHA neurons, respectively. Our PVN protocol produced cultures of OTP+/SIM1+ progenitors and gave rise to corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), oxytocin and growth hormone-releasing hormone (GHRH) neurons. These finding were validated via RT-qPCR, RNAscope and immunocytochemistry. Secondly, the ARC protocol was enriched for posterior tuberal progenitors and reproducibly generated appetite-regulating neurons of pro-opiomelanocortin (POMC) and agouti-related peptide (AGRP) subtypes. These neurons have been linked to metabolic diseases such as obesity and type 2 diabetes, making them a valuable in vitro model for drug screening and testing of obesity-related drugs like GLP1R agonists. Thirdly, our LHA protocol gave rise to neurons expressing melanin-concentrating hormone (MCH) and dynorphin (PDYN), as well as to the sleep-regulating hypocretin (HCRT) neurons. Upon transplantation into rodent brains, these LHA progenitors survived and matured into HCRT-expressing neurons in vivo. As the loss of HCRT is the underlying cause of the chronic sleep disorder Narcolepsy type 1, a protocol to generate HCRT neurons is an important step in testing a potential HCRT cell replacement therapy in preclinical studies. In summary, these novel protocols allow us to study the cellular and molecular properties of important subtype-specific human hypothalamic neurons.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) is supported by a Novo Nordisk Foundation grant number NNF21CC0073729.

Keywords: hypothalamus, brain development, single-cell RNA sequencing

3:45 PM – 4:45 PM

231

HUMAN INDUCED PLURIPOTENT STEM CELLS AS A MODEL TO EXPLORE PRADER WILLI SYNDROME

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Prader-Willi syndrome (PWS) is a neurodevelopmental condition generally caused by genomic imprinting defects. Currently, there are no curative treatments available, and patients can rely only on palliative interventions. The PWS locus covers approximately 4 megabases and encompasses about 15 genes, some of which undergo maternal imprinting. PWS results from the absence of gene expression on the paternal allele due to deletions, maternal uniparental disomy (UPD), or genomic defects, ultimately leading to the complete absence of expression of those genes. However, the noncoding RNA Snord116, whose function remains unclear, is absent in most of the patients and thus it is believed to be the primary factor contributing to the syndrome. We started from the peripheral blood mononuclear cells from patients who harbor the primary disease-causing mutations. From these cells, we derived induced pluripotent stem cells to investigate the pathomolecular mechanisms driving the pathology. We optimized a protocol to generate cortical excitatory neurons using a cocktail of small molecules that induce differentiation. Additionally, we observed that the PWS locus assumes a mature conformation, as evidenced by the imprinting of the adjacent maternally expressed UBE3A gene. We performed RNA sequencing on neurons at six weeks of differentiation to identify genes showing varied expression levels between patients and controls, as well as among patients with diverse mutations. We are currently validating some of the genes identified through this analysis. Next, considering the haploinsufficiency of GABAergic receptor genes in one of our patients, we conducted electrophysiological studies to assess potential hyperexcitability in our neurons. In summary, we refined a protocol to establish a useful model for investigating Prader Willi syndrome. We are currently assessing candidate differentially expressed genes identified through transcriptome analysis, aiming to elucidate the disease's pathophysiology. Additionally, considering the psychiatric phenotype observed in patients with maternal uniparental disomy (UPD), we are currently investigating a potential correlation between UBE3A, a maternally expressed gene, and these clinical manifestations, aiming to elucidate this phenotypic trait.

Keywords: iPSC disease modeling, neurodevelopmental disorder, imprinting defects

3:45 PM – 4:45 PM

233

HUMAN PLURIPOTENT STEM CELL-DERIVED MICROGLIA REGULATE NEURAL PRECURSOR ABUNDANCE BY SECRETING NEUROTROPHIC GROWTH FACTORS IN 2D CO-CULTURES AND 3D BRAIN ORGANOIDS

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Development of the human cerebral cortex requires the establishment of an appropriately sized pool of neural precursors (NPs) through tight regulation of their proliferation and differentiation into neurons and glia via intrinsic and extrinsic cues. Many neurodevelopmental disorders stem from convergent dysregulation of these early NP processes. Emerging evidence has delineated microglia, the resident macrophages of the brain, as critical modulators of brain development and function. However, fundamental questions regarding their diverse and potentially unique roles within the human NP niche remain largely unexplored. To address this gap, we used human pluripotent stem cell-derived cultures to model interactions between isogenic microglia-like cells (MLCs) and NPs in 2D co-cultures. Complementarily, we developed a protocol to

incorporate fluorescently tagged MLCs into 3D cortical organoids at a density comparable to the human fetal cortex. Using both models, we identified a density-dependent effect of MLCs in promoting proliferation and abundance of SOX2+ NPs. This effect was phenocopied by the addition of MLC-conditioned medium alone, highlighting a secreted growth factor-mediated mechanism. Furthermore, we sought to study interactions between MLCs and outer radial glia (oRG), a unique class of NPs proposed to drive human brain expansion, and observed a similar effect of increased oRG abundance upon MLC co-culture. To dissect the molecular mechanism, we conducted receptor-ligand interaction analyses on MLC and NP transcriptomic datasets. Among the top ligands, Insulin-like Growth Factor-1 (IGF1) stood out as a key proliferative factor almost exclusively expressed by microglia during cortical development, with its cognate receptor, IGF1-Receptor (IGF1R), being abundantly expressed by NPs. Perturbing the IGF1-IGF1R axis through genetic ablation of IGF1 production in MLCs, or via pharmacological inhibition of IGF1R signaling in NPs, partially reversed the trophic effect of MLCs on NPs. Together, our 2D and 3D co-culture models allow crucial experimental access to studying neuroimmune crosstalk during human brain development in health and disease. Using this platform, our data support a function of microglia in regulating the human NP pool size by secreting neurotrophic factors like IGF1.

Funding Source: This work was supported by the Simons Foundation, the Natural Sciences and Engineering Research Council of Canada, Medicine by Design, Stem Cell Network, Brain Canada, Brain and Behavior Research Foundation, and University of Toronto

Keywords: microglia, brain organoid, neuroimmune interactions

3:45 PM – 4:45 PM

235

AN IPSC MODEL OF AUTHENTIC STRIATAL NEURON DEVELOPMENT IDENTIFIES ALTERED DIFFERENTIATION KINETICS AND DIRECT/ INDIRECT MSN SUBTYPE FATE IMBALANCE DRIVEN BY CNVS IN THE AUTISM-ASSOCIATED 16P11.2 LOCUS

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During striatal development, medium spiny neurons (MSNs) differentiate and mature while integrating into subtype-specific direct and indirect basal ganglia pathways (dMSNs and iMSNs, respectively), which are crucial for cognitive, motor, and social behaviours. A growing body of evidence identifies genetic mutations disrupting striatal circuits as risk factors in a range of neurodevelopmental disorders (NDDs). This is the case with copy number variations (CNVs) in the 16p11.2 region, which is associated with schizophrenia, autism spectrum disorders, and intellectual disability. A study on 16p11.2 deletion mice found behavioural abnormalities, such as hyperactivity, circling, and deficits in movement control, potentially caused by an altered dMSN/iMSN ratio and associated synaptic defects in the striatum. Thus, there is an urgent need to develop reliable in vitro methods to generate human MSNs from PSCs to understand striatal development, study a role for these neurons in disease pathogenesis, as well as to develop potential therapies in regenerative medicine. In this study, we applied a protocol modified from our previously characterised Activin A-based striatal neuron differentiation and performed single-cell RNA sequencing of differentiating MSNs. We compared our data against published single-cell datasets from the human developing and adult striatum and confirm that our protocol recapitulates the cell populations found in the human striatum with significant accuracy and at a higher rate than previously published methods. Finally, using iPSC-derived MSNs as a model we demonstrate a role for 16p11.2 CNVs in striatal neurogenesis and MSN subtype differentiation. Our study reveals significant deficits in cell cycle kinetics in striatal progenitors that result in opposing temporal shifts in MSN production from iPSCs carrying either duplication or deletion of the 16p11.2 locus. Furthermore, we demonstrate an imbalance between MSN subtypes with a preferential bias towards dMSN generation in both 16p11.2 genotypes, which may explain the motor and cognitive behavioural changes found in human CNV carriers and 16p11.2 mouse models. We therefore prioritise promising targets within the 16p11.2 locus for further mechanistic investigations and development of new therapeutic interventions.

Keywords: medium spiny neuron, neurodevelopmental disorder, scRNAseq

3:45 PM – 4:45 PM

239

STUDYING DISEASE MECHANISMS OF DOMINANT OPTIC ATROPHY USING HUMAN PLURIPOTENT STEM CELL-BASED MODELS

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Dominant optic atrophy (DOA) is the most common inherited optic neuropathy worldwide. The majority of DOA is caused by mutations in the OPA1 gene that encodes a mitochondrial targeting protein playing

important roles in mitochondrial dynamics, membrane integrity, and cristae structure. Despite its expression in most somatic cell types, OPA1 mutations predominantly affect the retinal projection neurons, the retinal ganglion cells (RGCs). However, due to the tissue scarcity, mechanisms of DOA pathogenesis have not been extensively studied using human RGCs. In the study, we have used pluripotent stem cell (PSC)-derived retinal organoids and neurons to establish DOA disease models in vitro. The peripheral blood cells of DOA patients with distinct OPA1 mutations were reprogrammed to obtain iPSC lines. CRISPR-Cas mediated homologous directed repair (HDR) was subsequently performed to correct the mutation of a DOA patient's iPSCs to establish an isogenic control iPSC line. In addition, CRISPR-Cas9-mediated gene editing was used to create homozygous and heterozygous OPA1 mutant ESCs from a control line with a normal OPA1 gene. Using these isogenic OPA1 mutant and control PSC lines, we show that all heterozygous OPA1 mutant PSC lines can give rise to retinal organoids and produce RGCs. But OPA1 mutants show reduced OPA1 protein expression, altered mitochondrial morphology, and decreased cell respiration and mitochondrial ATP production rate. Furthermore, electrophysiological analysis by whole cell patch clamp recordings has revealed that OPA1 mutant neurons have altered ion channel properties and firing patterns compared to control PSC-derived neurons. Together, our results demonstrate that isogenic OPA1 mutant and control PSC lines are useful tools for establishing OPA1-DOA disease models using 3D-retinal organoid-derived RGCs and induced neurons. The identified OPA1 mutant phenotypes in vitro can thus serve as the basis to develop treatments or therapies for DOA.

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Keywords: OPA1 mutation caused dominant optic atrophy, PSC-derived human RGC, mitochondrial disease model

3:45 PM – 4:45 PM

241

EXPOSING IPSC-DERIVED BRAIN PERICYTE-LIKE CELLS TO ITACONATE SUPPORTS METABOLIC TREATMENT APPROACH FOR REPERFUSION INJURY

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While surmounting the high-risk state of limited supply of oxygen and nutrients to brain tissue, post-ischemia reperfusion triggers inflammatory responses, oxidative stress, and metabolic reprogramming, aggravating tissue damage. We have previously shown that the myeloid "immunometabolite" itaconate can mitigate these adverse effects of reoxygenation in mouse models by preventing a sudden increase in cellular respiration. To date, however, the roles of pericytes in the critical metabolic cascades at the neurovascular interface remain opaque. Here, we analyzed how human iPSC-derived brain pericyte-like cells alter their metabolic state in response to exogenous itaconate. Differentiation of iPSC via neural crest induction resulted in cells staining positive for PDGFRβ and showing upregulation of FOXF2 and NG2 (i.e., brain pericyte markers). For our metabolic study, we exposed them to 10 mM itaconate in [U-13C]glucose-supplemented media, and after 24 hours subjected supernatants and cell lysates to metabolomic analysis (via GC-MS). In particular, we found levels of succinate elevated over 10-fold compared to negative controls. This is in line with our previously-described results from rat astrocyte/neuron co-cultures and indicative of an impairment of succinate dehydrogenase (SDH) activity in the TCA cycle. Our metabolic flux studies also revealed that glucose utilization for TCA cycle metabolism was not significantly affected. Cell proliferation and PDGFRβ expression, meanwhile, remained unaffected. These results suggest that a potential treatment approach with itaconate would be further supported by metabolic adaptations in brain pericytes, which tightly interact with other cell types of the neurovascular unit (NVU). Considering the unmet medical need, therapeutically targeting metabolism to alleviate reperfusion injury effects can be a promising approach and our first insights into the reaction of human pericytes to itaconate further reinforces this idea.

Keywords: human iPSC-derived brain pericyte-like cells, metabolic treatment of reperfusion injury, itaconate metabolic regulation

3:45 PM – 4:45 PM

243

METFORMIN PROMOTES NEURAL STEM CELL ACTIVATION IN MOUSE AND HUMAN MODELS OF BRAIN INJURY

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Activating endogenous neural stem cells (NSCs) with therapeutic interventions holds great promise for regenerating the injured brain. An important non-invasive strategy to activate these cells is through the use of small molecules and drugs. In particular, administration of the type II diabetes medication metformin, has been shown to be sufficient for sensorimotor and cognitive recovery in pre-clinical mouse models

of stroke. Understanding the cell-based mechanisms underlying this recovery and the potential application to human cells has important implications for developing novel therapeutics. Importantly, it is known that metformin has pleiotropic effects in the mammalian brain, including NSC activation. Herein, we have examined the effects of metformin on distinct NSC populations in the mouse brain and investigated the same effects of metformin on human NSCs from human cerebral organoids (hCOs). Neural stem cells are comprised of two distinct populations: definitive NSCs (dNSCs) and primitive NSCs (pNSCs). dNSCs are the most abundant and well-characterized NSCs in the mammalian brain while pNSCs are a rare, quiescent population of NSCs that lie upstream of dNSCs. We have shown that dNSCs are responsive to metformin treatment but are not necessary for functional recovery. Thus, we hypothesized that the upstream pNSCs play an important role in metformin-mediated functional recovery. Herein, we demonstrate that pNSCs respond to injury and metformin treatment by expanding in number and potentially migrating to the site of injury in the early post-natal mouse brain. Relevant for human application, we have isolated both pNSC and dNSC populations from hCOs at various stages of development. Human pNSCs, a previously undescribed human cell population, and dNSCs are multipotent and self-renewing cells that are responsive to metformin. Using these NSC-containing hCOs, we have shown that inducing a stroke-like injury followed by metformin treatment increases expression of neuronal markers, which may be attributed to increased NSC activity, similar to what is observed in mouse studies. These findings have implications for understanding the fundamental biology of human NSCs and their potential to contribute to neural repair following brain injury.

Keywords: human neural stem cells, human cerebral organoids, neural repair

3:45 PM – 4:45 PM

245

MUTANT HUNTINGTIN IMPAIRS NEURODEVELOPMENT IN HUMAN BRAIN ORGANOIDS THROUGH CHCHD2-MEDIATED NEUROMETABOLIC FAILURE

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Expansion of the glutamine tract (poly-Q) in the protein Huntingtin (HTT) causes the neurodegenerative disorder Huntington's disease (HD). Emerging evidence suggests that mutant HTT (mHTT) disrupts brain development. To gain mechanistic insights into the neurodevelopmental impact of mHTT, we introduced a biallelic expansion of 70Q into human induced pluripotent stem cells using CRISPR/Cas9. 70Q introduction caused aberrant development of cerebral organoids with loss of neural progenitor organization. Single-cell transcriptomics of regionalized midbrain organoids further highlighted the impact of mHTT on progenitor development. Multi-omics along the neural lineage highlighted the dysregulation of the protein coiled-coil-helix-coiled-coil-helix domain containing 2 (CHCHD2), a transcription factor involved in mitochondrial integrated stress response and mitochondrial homeostasis. CHCHD2 repression was associated with abnormal mitochondrial morpho-dynamics and elevated resting energy expenditure in neural progenitors. Elimination of the poly-Q tract of HTT normalized CHCHD2 expression and specific mitochondrial defects. Hence, mHTT-mediated disruption of human neurodevelopment is paralleled by aberrant neurometabolic programming mediated by dysregulation of CHCHD2, which could then serve as an early intervention target for HD.

Funding Source: DFG (PR1527/5-1, PR1527/6-1 to AP; RTG2155 ProMoAge to HO & LK; SFB167 B07 to JP) BIH (to SD, JP, RK & AP) BMBF (AZ. 031L0211, 01GM2002A to AP) Medical Faculty, HHU (FoKo grant to AP & SC) NCN (2016/22/M/NZ2/00548, 2017/27/B/NZ1/02401 to PL)

Keywords: Huntington's disease, cerebral organoids, mitochondria

3:45 PM – 4:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

247

PRECLINICAL STUDY OF NEURAL REGENERATION PROMOTING CELLS DIFFERENTIATED FROM TONSIL-DERIVED MESENCHYMAL STEM CELLS FOR PERIPHERAL NERVE INJURY

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Although the peripheral nervous system normally regenerates after a certain period when the axon is physically damaged, spontaneous nerve regeneration becomes difficult when the peripheral nerve is severely injured, such as during tissue extraction for trauma or tumor treatment. Therefore, supportive treatment for nerve regeneration using Schwann cells or mesenchymal stem cells or microsurgery is needed to connect the severed nerves directly or insert a nerve conduit to connect them. Human tonsil-derived mesenchymal stem cells (TMSCs) can differentiate into Schwann cell-like cells (TMSC-SCs). We have been investigating the potential of TMSC-SCs for peripheral nerve and muscle regeneration in animal models of several peripheral neuropathies. The TMSC-SCs were manufactured in a good manufacturing practice facility and named neuronal regeneration promoting cells (NRPCs) for development. In this study, we developed peripheral nerve injury (PNI) rat models with sciatic nerve transection and treated them with the implantation of a mixture of NRPCs and fibrin glue (NRPC-fibrin glue) into the damaged sites of the nerves. Sciatic nerve and muscle morphology were observed, and motor functions were tested in treated rats with PNI. As a result of treatment, sciatic nerve regeneration was induced, and the histological findings of the muscle remained normal after NRPC-fibrin glue treatment. In addition, the neuromuscular junction was spared in NRPC-fibrin glue-treated rats. This study indicates that a mixture of NRPC-fibrin glue can be a therapeutic agent that enables peripheral nerve and muscle regeneration in patients with PNI.

Funding Source: Korean Fund for Regenerative Medicine (KFRM) grant, funded by the Korea government (the Ministry of Science and ICT, the Ministry of Health & Welfare) (22C0627L1-11)

Keywords: peripheral nerve injury, tonsil-derived mesenchymal stem cells, Schwann cell-like cells

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

249

EVALUATION OF BENZALKONIUM CHLORIDE INFLUENCE ON HUMAN LIMBAL EPITHELIAL STEM CELLS

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Self-renewing properties of corneal epithelium relies on limbal stem cells (LESCs). Preservatives such as benzalkonium chloride (BAC) are considered risk factors for iatrogenic dry eye disease. Eye drops containing BAC can damage not only the corneal epithelium, but also the limbal area. Cytokines regulate cell functions such as growth, differentiation or apoptosis. Early detection of their expression at molecular level can be useful in understanding the influence of BAC on LESCs and may help developing an in vitro toxicity model for drug discovery. This study aimed to assess the impact of BAC on the expression of genes related to proliferation, apoptosis and inflammation in LESCs. The study utilized LESCs isolated from human tissue. After treating LESCs with the dose of 0.0002% BAC for 48 h, cells were tested for migration and proliferation rate by scratch and colony forming assays, and processed towards total RNA extraction. The apoptosis (Bax, BCl2, tp53, Casp3, Casp7), inflammation (IL-1β, IL6, IL4, IL10), and cell cycle (CCND1, CCND2, E2F2, CDKN2C, CCNE1, CCNE2, Rb1) related genes expression was determined by RT-qPCR. Flow cytometry was used to differentiate cell populations of G0/G1, S, and G2/M cell cycle phases. Results suggest that BAC impairs proliferation, alters cell cycle progression and causes apoptosis. Under the influence of BAC, the expression of RB1 ($p< 0.001$), CDKN2C ($p< 0.001$), CCNE1 ($p< 0.01$), CCNE2 ($p< 0.01$), BAX ($p< 0.01$), CASP3 ($p< 0.05$), CASP7 ($p< 0.05$), IL1β ($p< 0.001$) was upregulated. The expression of E2F2 ($p< 0.001$), BCL2 ($p< 0.05$) and IL6 ($p< 0.001$) was downregulated. The expression of CCND1 and CCND2 genes was not changed and the expression of IL4, IL10 and CDKN2A was not detected. Flow cytometry revealed an increase ($p< 0.01$) of LESCs in S phase. Concluding, BAC is toxic to LESCs, induces overexpression of pro-apoptotic genes and significantly affects the cell cycle. BAC-treated LESCs have a lower proliferative potential and self-renewal capacity.

Funding Source: This research was funded in part by National Science Centre #2022/06/X/NZ3/00715. Authors kindly acknowledge the funding from Medical University of Silesia in Katowice grants: #PCN-2-047/K/2/I and #BNW-2-035/N/3/0, #RW/31/2024.

Keywords: benzalkonium chloride, dry eye disease, corneal epithelium regeneration

3:45 PM – 4:45 PM

251

COMPARISON OF LOW SHEAR STRESS COMMERCIAL AND SELF-DEVELOPED BIOREACTORS FOR IPSC EXPANSION

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The expansion of human induced pluripotent stem cells (hiPSCs) to high cell amount for drug screening or cell therapy requires the use of bioreactors, as the 2D condition is limited in its growing surface capacity. For this purpose, various types of 3D bioreactors are available on the market, such as suspension bioreactors with impeller, vertical-wheel bioreactors, wavebreaker bioreactors, etc. The bioreactor setups are mostly complex and the protocols difficult to transfer or adapt to different cells/labs. Given the sensitivity of hiPSCs to physical forces, maintaining low shear stress environment is crucial to preserve their pluripotency and viability during large-scale expansion. However, optimal conditions are challenging but necessary for successful cultivation and expansion in 3D. At Fraunhofer ISC, a self-built bioreactor called SUSI (SUSpension Incubator) with a low positioned impeller has been developed and optimized for low shear stress cultivation of hiPSCs using computational modeling in 2019. All designed parts were implemented as a scalable system and the setup and software were compiled for easy handling and transferability (only few parameters are adjustable). As a next step, we cultured together with Fraunhofer IBMT two hiPSC lines over ten days and 3 passages in the SUSI bioreactor and compared the output in parallel with a 2D and a commercially available impeller-free bioreactor, already successfully evaluated at Fraunhofer IBMT. Different media and protocols were also tested. The expanded cells were characterized in terms of morphology, aggregate diameter as well as yield and their stemness and quality were determined with FACS, qPCR and viability. As a result, we observed that the aggregates grown in the SUSI bioreactor were slightly larger than the other aggregates, while the yield was comparable in the different conditions and the pluripotency was maintained over the 10 days. In summary, we show the cell output and quality from the self-developed bioreactor and compare it with an impeller-free system, confirming its potential as an alternative device towards commercial upscaling solutions.

Keywords: self-developed bioreactor, iPSC expansion, low shear stress cultivation

3:45 PM – 4:45 PM

253

MITOCHONDRIAL DNA ANALYSIS USING MITOPORE

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Although mitochondrial DNA (mtDNA) mutations have been reported to impair stability, functionality, and differentiation potential of human induced pluripotent stem cells (iPSCs), it is not regularly included in iPSC quality control. Besides a lack of awareness for the matter, this is in part applicable to the lack of simple, cost-effective and user-friendly pipelines. To overcome this issue, we developed a workflow tailored to entry-level long-read nanopore sequencing and a corresponding webserver termed Mitopore. We demonstrate the applicability of Mitopore to provide fast and reliable information on single nucleotide variants (SNVs), haplogroups, and large deletions by validating the workflow with different patient-derived and iPSC samples harboring variable levels of mtDNA SNVs. To demonstrate the feasibility for quality control of iPSCs, we characterized the mtDNA integrity of the recently published male reference iPSC line KOLF2.1J by employing whole genome sequencing using state-of-the-art short- and long-read sequencing as well as targeted sequencing using our Mitopore workflow. We further show that mtDNA haplogroup may be used for cell line authentication in specific circumstances. In summary, we suggest to include assessment of mtDNA integrity into iPSC quality control workflows, either by analyzing whole genome sequencing-derived data or by employing targeted cost-effective long-read nanopore sequencing.

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Keywords: mitochondrial DNA, long-read sequencing, iPSC quality control

3:45 PM – 4:45 PM

255

REPROGRAMMING OF HUMAN FIBROBLASTS INTO RETINAL PROGENITOR CELLS TO RESCUE BLINDNESS

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In clinical trials, retinal progenitor cells (RPCs) are anticipated to rescue vision in late-stage photoreceptor degeneration, but the availability of primary human RPCs is limited to fetal tissue. We developed a method to directly reprogram human fibroblasts (HFs) into RPCs with small molecules, and tested its safety and therapeutic efficacy in a rodent model of photoreceptor loss. HFs were transduced with Vsx2–eGFP reporter, to determine the conversion of induced retinal progenitor cells (iRPCs). By fluorescence activated cell sorting (FACS), we sorted Vsx2–eGFP+ cells, and checked the gene expression profile utilizing quantitative reverse transcription PCR (qRT-PCR), and bulk RNA sequencing. To test in vitro potency, we used fura-2-based imaging to study glutamate receptor-mediated [Ca2+] i changes in fibroblasts and iRPCs. To test in vivo safety and therapeutic efficacy, one eye of Royal College of Surgeons (RCS) rat at P21 were subretinally transplanted with 5 µL of 2*105 cell suspension containing PBS (N=4), HFs (N=4), or iRPCs (N=4). The rats were then analyzed with electroretinogram (ERG) and light aversion tests monthly. At 4 month age, the eyes were cryo-fixed and sectioned for immunohistochemistry. After chemical reprogramming, there were 42.8% Vsx2–eGFP+ cells on FACS. In qRT-PCR, significant increase of Vsx2 expression was found in iRPCs.. In fura-2-based imaging, intracellular calcium concentrations were found to elevate in iRPCs upon glutamate stimulation (1mM), whereas HFs showed no response. In RCS rats, there was an improvement of ERG in the eyes transplanted with iRPCs at P28 and P56 ($p < 0.05$). In light aversion test at P112, RCS rats transplanted with iRPCs were found to spend significantly more time in the dark space, indicating vision rescue in late-stage photoreceptor loss. In immunohistochemistry, we found survival and integration of iRPCs in layer of rat photoreceptors, with layer-by-layer retinal morphology well preserved. By chemical reprogramming of human fibroblasts into induced retinal progenitor cells, we demonstrated its therapeutic potential in photoreceptor degeneration.

Keywords: cell therapy, retinal progenitor cells, photoreceptor degeneration

3:45 PM – 4:45 PM

257

HIGH QUALITY CONTROL HUMAN INDUCED PLURIPOTENT STEM CELL LINES AVAILABLE FOR RESEARCH

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Human induced pluripotent stem cells (hiPSCs) from healthy donors ('controls') can be generated from multiple somatic cell types from male or female donors of various age groups and ethnicities and with a variety of reprogramming vectors which all potentially could influence downstream applications. When choosing a control hiPSC line matching one or more parameters with those of other lines might therefore be desirable. A proper informed consent and quality control assessment are important prerequisites for the successful usage in general. Here we generated hiPSCs from male and female blood, skin or urine derived cells with either Sendai virus, self-replicating RNA, episomal vectors or lentivirus. hiPSC lines were subjected to extensive QC: >80% expressed pluripotency markers Nanog, OCT3/4 and SSEA4 by FACS, differentiated in vitro into derivatives of the three germ layers as assessed by immunofluorescent staining, displayed a normal karyotype (G-banding and/or Karyostat), tested negative for residual reprogramming vectors (qPCR), had a matched identity with the original primary cells as assessed by STR analysis and tested negative for various pathogens like CMV, HIV and mycoplasma. In addition we performed HLA profiling and the X chromosome status in female lines was determined. A list of publications using these lines for various research projects including genetic modifications is available. For two lines whole genome sequencing and transcriptome analysis were performed. Importantly for all lines a detailed informed consent form specifying the use in common areas of hiPSC research as well as limitations is available. Finally hiPSC lines have been registered at the hPSCreg.

Funding Source: The LUMC hiPSC hotel is part of The Novo Nordisk Foundation Center for Stem Cell Medicine which is supported by Novo Nordisk Foundation grants (NNF21CC0073729)

Keywords: hiPSC Reprogramming, high quality hiPSC lines, control research-grade hiPSC lines

3:45 PM – 4:45 PM

259

IN VITRO RECONSTITUTION OF THE FEMALE GERM CELL DEVELOPMENT USING HUMAN IPSCS AND FETAL GERM CELLS

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The development of ovarian reserve is a complex process occurring prenatally, involving intricate specification and maturation events spanning the first and second trimesters. Female primordial germ cells (PGCs) are specified in the peri-implantation embryo, after which they migrate through the embryo to the developing gonads, where they further mature to DDX4-positive fetal germ cells and enter meiosis. Female in vitro gametogenesis (IVG) aims to generate eggs using human induced pluripotent stem cells (hiPSCs), offering hope for reproduction in patients lacking functional ovaries. Presently, the field has achieved the generation of germ cell precursor, PGC-like cells (PGCLCs) from hiPSCs, equivalent to week 3 of development. To gain further understanding of oocyte development, we recapitulated female germ cell development in vitro using iPSCs and fetal ovary tissue. First, we set up a 3D culture system by reaggregating single cell suspension of human fetal ovaries and encapsulating them in hydrogel, which preserved the somatic microenvironment necessary for fetal germ cells to proliferate and mature. Following a 10-day culture period, DDX4+ fetal germ cell numbers increased up to five-fold, with over 25% progressing into meiosis I, indicated by the presence of SYCP3 and pH2AX(Ser139). Supplementing the culture medium with BMP2 and retinoic acid enhanced the yield of meiotic germ cells. Using this system, we co-cultured isolated PGCLCs derived from hiPSCs harboring endogenous fluorescent reporters (POU5F1::GFP and TUBA1B::GFP) with fetal ovary cells in 3D hydrogel for 3-4 weeks. Live cell imaging and immunofluorescence revealed proliferating PGCLCs within the aggregate, with some upregulating DDX4, which is expressed by post-migratory fetal germ cells. By contrast, PGCLCs aggregated and cultured in absence of fetal ovary cells show upregulation of migratory-stage PGC markers, such as CD38 and SUSD2, but not of post-migratory marker DDX4, confirming the necessity of the gonadal somatic niche for germ cell maturation. In conclusion, our in vitro model recapitulated the maturation of nascent PGCLCs to migratory PGCLCs, DDX4+ gonadal PGCLCs and the meiotic entry of female fetal germ cells, which provides a platform to unravel mechanisms of germ cell development and to advance the field of IVG in females.

Funding Source: Dutch Research Council (VICI-2018-9181964) Novo Nordisk Foundation (reNEW NNF21CC0073729)

Keywords: in vitro gametogenesis, oocyte development, primordial germ cells

3:45 PM – 4:45 PM

261

SINGLE CELL BIOPRINTING AS A PLATFORM TO STANDARDIZE CULTURE AND MAINTENANCE OF IPSC

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Human induced pluripotent stem cells (hiPSC) have emerged as promising cell source for the creation of tools in the fields of disease modelling, drug screening, and tissue engineering (Yamanaka, 2020). Despite the significant advancements, the reliance on animal-derived substrates and the lack of standardize methods in the stem cell culture hinders their integration into precision medicine (Ghasemi-Dehkordi et al., 2015). In response to these limitations, this study explores the innovative approach of bioprinting hiPSCs using a state-of-the-art, support-free single-cell bioprinter (Biopixlar, Fluicell). This technology allows for the precise patterning of individual cells to create complex tissue models (Jeffries et al., 2020). By bioprinting hiPSCs, we aim to overcome traditional hurdles by enabling the controlled delivery and culture of printed colonies over a chemically defined substrate. Therefore, we screened different positively charged molecules, needed for the bioprinting process, that would be used as adhesive coating to attach and culture cells. Among them, we selected PEI, PDL, and PLL for their chemical characteristics and the established use in the stem cell culture. Initial findings reveal that PEI effectively supports the growth and maintenance of hiPSC pluripotency across all tested concentrations. In contrast, PDL was associated with the dedifferentiation of hiPSCs, indicating its unsuitability for maintaining pluripotent stem cell characteristics. Interestingly, only low concentrations of PLL could sustaining hiPSC growth and pluripotency, highlighting the fine balance required between adhesive strength and cell pluripotentcy maintenance. Consequently, the study focused on PLL as the adhesive of choice for further bioprinting experiments, given its unique ability to support cell survival and attachment. Preliminary bioprinting tests demonstrated promising results, with iPSCs forming well-defined colonies while maintaining key pluripotency markers expression. This research represents a significant step towards the standardization of hiPSC culture techniques, offering new avenues for the application of hiPSCs in diverse fields.

Funding Source: This project has received funding from the European Union's Horizon 2020 FET Open programme under grant agreement No 964452

Keywords: hiPSC culture and maintenance, bioprinting, stem cell standardization

3:45 PM – 4:45 PM

263

RESCUE AND SEED & GO - ADDRESSING IPSC CULTURE AND ASSAY REPRODUCIBILITY CHALLENGES

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Induced pluripotent stem cells (iPSCs) have been crucial to advancing novel cell-based therapeutics, drug discovery and a number of other market areas. Despite significant advances in the culture of iPSCs, there remain many challenges with reproducibility, reliability and consistency of cell cultures, assays and data. Reproducibility issues also add significant costs and time to projects. We present data demonstrating the development of 2 novel platforms for addressing some of the key reproducibility challenges: 1. RESCUE recovers normal cells from a population which has acquired a mosaic genomic abnormality. Culture acquired genomic abnormalities (e.g 20q11.21 copy number gains and TP53 mutations) are present in as many as 30% of iPSC cell lines and can provide cells with significant growth advantages. We present data on the RESCUE of a cell line with a mosaic abnormality. Utilising a combination of our advanced single cell cloning methods and rapid genomic screening, high numbers of clones were derived, screened and triaged. Normal cell lines were rapidly identified, QC'd and expanded for cryopreservation. 2. SEED & GO is an advanced cryopreservation method that improves reproducibility of processes requiring single cell dissociated iPSCs whilst significantly reducing the time to assay by 10-14 days. Clump passaging and cryopreservation methods are a significant source of data inconsistency. SEED & GO single cell cryopreserved vials were differentiation ready within days of thaw. Our data demonstrates high viability (>90%), genomic stability and high uniformity from this cryopreservation method which can create 10's to 100's of vials. Together these two novel platforms offer researchers a cost-effective solution to significantly improve iPSC assay reproducibility and quality.

Keywords: iPSC, reproducibility, QC, genomic anomaly

3:45 PM – 4:45 PM

265

HIGH-THROUGHPUT SCREENING FOR CELL DEATH IN FUCHS ENDOTHELIAL CORNEAL DYSTROPHY

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Fuchs endothelial corneal dystrophy (FECD) is one of the leading causes of corneal transplantation caused by progressive loss of corneal endothelial cells. Corneal endothelial cells in FECD are vulnerable to oxidative stress leading to mitochondrial dysfunction, cell degeneration and death. While cell death is an essential aspect of FECD, previous reports have often analyzed immortalized cell lines, thus making cell death evaluation difficult. We, therefore, established a new in vitro FECD model to perform cell death assays precisely. Induced pluripotent stem cells (iPSCs) were established from peripheral blood mononuclear cells of FECD patients, which were then induced into corneal endothelial cells. Corneal endothelial cells were treated with H2O2 to mimic the pathophysiology of FECD and we investigated the responses to oxidative stress. Oxidative stress level of FECD was higher than control. Cell death ratio with H2O2 was upregulated in FECD than control. Additionally, induced cells were more prone to cell death than immortalized cells. ATP assay revealed that ATP level was significantly decreased with H2O2 in FECD. The Z factor, an indicator of the validity of high-throughput drug screening, was calculated. Z factor of the ATP assay met the requirements for an "excellent assay". We then conducted high-throughput drug screening and identified several hit compounds. In conclusion, this in vitro model of FECD may be suitable for assessing cell death phenotype in FECD. Hit compounds will be further assessed with other assays such as a mitochondrial transmembrane potential assay.

Funding Source: This work was supported by JSPS KAKENHI Grant Numbers JP19K09978 and JP20J40146, and grants from Japan Cornea Society (Novartis Pharma Grants 2020). The funders had no role in study design, data collection and analysis.

Keywords: Fuchs endothelial corneal dystrophy, high-throughput screening, cell death

3:45 PM – 4:45 PM

267

QUALITY CONTROL IN HUMAN ORGANOID MODELS

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Elegant studies in stem cells culture have facilitated the generation of 3D in vitro biomodels known as organoids, these have the capability to replicate the structures of tissues and organs. The organoid has proven to be valuable tools for modeling organ development and diseases, thereby offering a broad spectrum of applications in basic research, drug discovery, and regenerative medicine. However, it is imperative to acknowledge that during the generation of organoids, alterations may manifest, leading to a deviation from the authentic characteristics observed in the original tissue. Therefore, the characterization of these organoids is essential to ensure their suitability as biomodels. The Andalusian Public Health System Biobank (BBSSPA) has developed a minimal characterization protocol that includes morphological, cytogenetic, and molecular characterization to guarantee that the organoids maintain the characteristics of the tissue, traceability, and chromosomal status. G-Karyotiping: The organoids were treated with colcemid to stop mitosis, then exposed to a hypotonic solution. After treated with fixative solution the sample, it is spread onto slides, G-banding was performed on chromosomes, which were visualized under a microscope and mitotic images and analyzed to identify chromosomal abnormalities. Traceability control: Short Tamdem Repeats (STRs) analysis was performed following the ASN-0002-2022 standard by the ATCC Standards Development Organization recommendation. Morphological characterization. Organoids were characterized through haematoxylin and eosin-stained slides. Organoids were fixed, cryoprotected and embedded in OCT. Finally, 20 um sections were stained with haematoxylin and eosin to assess organoid morphology. Data obtained through optimization and adaptation of three techniques. The characterization protocols have been adapted to obtain the results that have enabled the analyses. The validation of organoid characterization methodologies allows to the BBSSPA to carry out exhaustive quality control of the organoids transferred to different research projects. It should be mandatory these assays for the deposit of organoids in banks and the publication of results in scientific journals.

Funding Source: This research was funded by Consejería de Salud y Consumo, Junta de Andalucía; and ISCIII Platform Biobanks and Biomodels co-financed by FEDER.

Keywords: organoid, quality control, characterization protocols

3:45 PM – 4:45 PM

269

GENE EDITING CELLS USING CRISPR-CAS9 ON BIOLAMININ 521 IMPROVES SINGLE CELL SURVIVAL AND CLONING, ENABLING CONSECUTIVE DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS ON BIORELEVANT MATRICES

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CRISPR/Cas9 gene editing technology is revolutionary in the field of genome editing. So far, obtaining single-cell clones of edited hiPS cells has been a major problem. Several researchers have demonstrated how Biolaminin® substrates can improve single-cell survival and pluripotency of CRISPR-Cas9-edited hPSCs and support high throughput hPSC clonal selection and expansion. Laminins are an extracellular matrix (ECM) protein family of around 16 different isoforms and a major component of the basement membrane, thus vital for maintenance and survival of tissues. The laminin-521 isoform is the major ECM protein expressed in the inner cell mass of the pre-implanted embryo and, therefore the most biologically relevant substrate for hESCs and iPSCs cultured in vitro and critical for regulating survival and self-renewal. In hPSC feeder-free cultures, Biolaminin 521 increased the single-cell cloning efficiency, compared to other ECMs, e.g. Matrigel or Geltrex. For CRISPR/Cas9 protocols in hPSC cultures, Biolaminin 521 coating supported the single cells seeding at a very low density. In a recent protocol, Biolaminin 521 and 111 substrates supported the effective differentiation of CRISPR/Cas9-edited hiPSC into cortical neurons and validation over 80 days. Moreover, Biolaminin 521 and -111 were efficient substrates for CRISPRi-mediated transcriptional silencing in hiPSCs and the differentiation of hiPSC into forebrain neural progenitor cells for loss-of-function studies in brain development research. The CRISPRi cells differentiated in cerebral organoids with steady transcriptional silencing after 4 months. In conclusion, Biolaminin substrates are highly suitable matrixes for CRISPR/Cas9 gene editing of hiPSC in a variety of different protocols including maintenance and differentiation. Biolaminin substrates improve single-cell survival and pluripotency and help achieve high transduction and silencing efficiency.

Keywords: cell culture substrate, gene editing incl. Crispr-Cas, single cells survival and cloning efficiency

3:45 PM – 4:45 PM

271

INDUCTION OF DNA METHYLATION BY TRANSCRIPTION IN AN IN VITRO IPS CELL MODEL

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Imprinted genes are controlled by differentially methylated imprinting centers resulting in allele specific gene expression. The imprinted Prader-Willi / Angelman syndrome locus (PWS/AS) on human chromosome 15 has a bipartite imprinting center, consisting of two functional elements: the PWS-SRO, which becomes methylated on the maternal allele and the AS-SRO promoter, located 35kb upstream to the PWS-SRO (SRO: shortest region of overlap). To test the assumption that transcription initiating at the AS-SRO promoter causes DNA methylation at the PWS-SRO, an iPSC-based human in vitro system was developed. By replacing the AS-SRO with an inducible promotor that is regulated by a doxycycline-responsive transactivator protein (rtTA), transcription initiation at the AS-SRO can be manipulated. To study if cellular differentiation could play a role in induction DNA methylation the experiments were performed in undifferentiated iPS cells, during embryoid body differentiation and after targeted differentiation into derivatives of the three germ layers. Transcription across the PWS-SRO was successfully induced by addition of doxycycline in all three experimental settings. Transcripts spliced onto exon 2 of the SNRPN gene, as observed for known oocyte-specific transcripts. Acquisition of DNA methylation at the PWS-SRO was analyzed by targeted deep bisulfite sequencing and could be, so far, observed to some extent only in the endoderm lineage. This might indicate that differentiation is needed as additional stimulus for induction of de novo DNA methylation. Further differentiation experiments are under way and results will be presented and discussed.

Funding Source: DFG - Deutsche Forschungsgemeinschaft

Keywords: genomic imprinting, DNA methylation, Prader-Willi/ Angelman syndrome locus

3:45 PM – 4:45 PM

273

MITOCHONDRIA-MEDIATED INTERCELLULAR COMMUNICATION: UNVEILING THE IMMUNOMODULATORY PROPERTIES OF AMNIOTIC EPITHELIAL CELLS

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Intercellular mitochondrial transfer has attracted great attention in various biomedical fields, including stem cell biology. Understanding the role of this phenomenon in the dialogue between stem and immune cells is essential to grasp the mechanisms leading to immune cell reprogramming, tissue damage and repair, and tumor development. Amniotic Epithelial Cells (AEC) represent an attractive stem cell source, exerting a notable pro-regenerative effect on injured tendons, primarily through the modulation of the early inflammatory phase by controlling different sets of immune cells. While most of the efforts have focused on the molecular mechanisms behind AEC paracrine immune-suppressive action, this study aims to explore their mitochondrial transfer to immune cells after the first evidence of hAEC mitochondria donation to H2O2-damaged cells. The obtained results reveal, for the first time, the presence of functional mitochondria on AEC-derived conditioned media (CM). In detail, ultracentrifugation of CM allowed to fraction EVs containing mitochondria as characterized by WB and FACS. The AEC-EVs, compared with the soluble fraction, showed an enhanced immunosuppressive effect by dumping the activation of PBMC and NFAT pathway in CD3/CD28-stimulated Jurkat cells. Moreover, confocal microscope analysis revealed the transfer of mitochondria to PBMCs exposed to AEC-EVs. Of note, this process was blocked by an anti-CD44-neutralising antibody, which also significantly inhibited the immune-suppressive action of AEC-EVs. Overall, these findings elucidate the mitochondria-mediated intercellular communication between AEC and immune cells as one of the key processes involved in their immunomodulatory properties, opening new insights for targeted therapeutics aimed at enhancing tendon regeneration.

Funding Source: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955685 (www.p4fit.eu).

Keywords: mitochondria, amniotic epithelial cells, immunomodulation

3:45 PM – 4:45 PM

TOPIC: PANCREAS

275

INDUCED PLURIPOTENT STEM CELL-BASED REPORTER SYSTEM TO MODEL POORLY UNDERSTOOD CANCERS: KIDNEY, PANCREAS

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Pancreatic, colorectal, and renal cancers are responsible of approximately 20% of new cancer cases in Europe. However, their incidence rates vary across Europe with eastern European countries showing the highest rates and this epidemiological variability is not accounted for by the known risk factors. Therefore, we set out to research the causes of the three cancers through the evaluation of the impact of endogenous factors found to be involved in chronic conditions such as obesity, diabetes, and chronic inflammation. For this, we used endogenous EGFP-tagged induced pluripotent stem cells (iPSCs) reporter cell lines, generated in the lab, signaling diverse cellular stress responses; P21 for DNA-damage, ICAM1, upregulated in inflammatory environment and MYC which is tumor/metabolic marker. These iPSCs were differentiated into pancreatic acinar and duct-like organoids and renal proximal tubule like cells. Subsequently, the cells were exposed to known factors involved in sustained inflammatory and metabolic signals such as TNFa, IL6, IL1b, Leptin, insulin, IGF1, free fatty acids, high glucose, and to their combination. The long-term effects were then analyzed after 7-day exposure through high-throughput imaging and researching the expression of early mitotic and metaplastic precursor signals. MYC and P21 showed a clear upregulation at Day7, within the 3D acinar structures when treated with IL6, IGF1 and leptin. This effect was scarce in presence of insulin and IL1b. This suggested that metabolic/mitotic and plastic impact happens in this model with specific factors as early as after 7-day exposure. This also indicates that iPSCs-based model can be used to model early events observed in chronic inflammatory and metabolic conditions that can be conducive or enhancing to cancer in a healthy tissue.

Funding Source: DISCERN project funded by the European Commission in the framework of the Horizon Europe Mission on Cancer

Keywords: induced pluripotent stem cells reporters, pancreatic and renal cancer, organoids

3:45 PM – 4:45 PM

277

SINGLE-CELL PROFILING UNLEASHES KRAS-DRIVEN REDRAFTING OF THE TUMOR MICROENVIRONMENT BEFORE CANCER ONSET

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Pancreatic cancer is a highly lethal disease, commonly characterized by aggressiveness and a distinct tumor microenvironment, where mutant KRAS signaling stands as a hallmark event in its pathophysiology. To deepen our understanding of KRAS-dependent signaling, stem cell-derived pancreatic ductal-like organoids (PDLOs) were induced by oncogenic KRAS expression, followed by time-resolved single-cell RNA- and bulk ATAC-sequencing. This unique precancerous state model facilitated a comprehensive characterization of early oncogenic events at cellular resolution. Specifically, mutated KRAS segregated two ductal subpopulations with distinct gene expression signatures, regardless of KRAS expression levels. While KRAShigh cluster 1 exhibited overexpression of genes associated with extracellular matrix remodeling, KRAShigh cluster 2 is predominantly characterized by enhanced cytokine-related signaling. Correlation with published data sets underscored the relevance of these distinct gene signatures in advanced cancer states within human patients. Various co-culture systems showed that KRASG12D-expressing PDLOs could induce cancer-associated fibroblast (CAF) activation while simultaneously shielding KRAS-activated, yet non-cancerous, organoids from T cell infiltration in vitro. Furthermore, in silico approaches were employed to reconstruct a virtual pancreatic cancer space, unveiling the KRAS-driven pathways mediating CAF reprogramming and T cell shielding preceding cancer onset. In summary, our human PDLO model allows us to explore the transition from normal to cancerous states, bridging the gaps in our understanding of tumor development and tumor niche preparation.

Keywords: pancreatic cancer, KRAS, pancreatic ductal-like organoids

3:45 PM – 4:45 PM

279

MODELLING JOHANSON-BLIZZARD-SYNDROME IN A DISH - MOLECULAR INSIGHTS INTO UBR1 DEFICIENCY USING PLURIPOTENT STEM CELL-DERIVED PANCREATIC ORGANOIDS

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Johanson-Blizzard syndrome (JBS) is a rare autosomal recessive genetic disorder characterized by congenital exocrine pancreatic insufficiency, nasal wing aplasia, and various symptoms of varying severity. Although we have previously identified bi-allelic loss-of-function mutations in the UBR1 gene as the genetic cause of JBS, the precise molecular pathomechanism remains unknown. Since loss of UBR1 can be partly compensated in mice, no model system has existed to fully recapitulate the phenotype of JBS patients. We have employed in vitro differentiation of human pluripotent stem cells (PSCs) to unravel the molecular mechanism of pancreatic insufficiency in JBS. Our collection includes JBS-specific PSCs-derived from patient's iPSCs, isogenic controls, and CRISPR/Cas9-engineered hESCs, enabling advanced disease modeling. UBR1-deficient and proficient PSCs exhibited comparable growth and differentiation patterns until the pancreatic progenitor (PP) stage, consistent with a normal pancreatic primordium in JBS patients. To explore later stages of pancreatic development, we employed xenograft and ex vivo culture models. Remarkably, grafts from UBR1-deficient PPs showed a significant absence of acinar pancreatic tissue after 8 weeks. Our immune cell-free model highlights a potential cell-intrinsic cause of pancreatic tissue degeneration in JBS. Currently, we are conducting time-resolved ex vivo and xenograft analyses alongside our in vitro differentiation into ductal, acinar, and endocrine organoids, to intricately dissect the degeneration processes. UBR1 functions as an E3 ubiquitin ligase in the N-end rule pathway, playing a crucial role in regulating protein degradation. Consistent with its function, global quantitative proteomics revealed a stage-specific upregulation of multiple proteins in UBR1-ablated cells starting from the PSC and PP stages. To pinpoint potential disease-causing substrates of JBS, we are currently establishing an E3-ligase substrate trapping strategy. In summary, our sophisticated JBS model enables us to recapitulate the disease mechanism of this rare and severe syndrome not only in the pancreas but also beyond, offering valuable insights into its pathophysiology.

Keywords: stem cell derived pancreatic organoids, exocrine pancreas, disease modelling

3:45 PM – 4:45 PM

283

DECIPHERING COLLECTIVE ONCOGENIC GNAS AND KRAS SIGNALING IN AN ORGANOID MODEL FOR CYSTIC PANCREATIC NEOPLASMS

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Intraductal Papillary Mucinous Neoplasm (IPMN) are cystic neoplasm of the pancreatic ducts that typically manifest at an advanced age and can progress to invasive adenocarcinoma. While certain morphological and spatial features can be indicative of progression, a mutation pattern defining this risk remains to be identified. IPMNs frequently harbor activating mutations in either GNAS or KRAS, or both; however, interaction and crosstalk between these oncogenes are poorly understood. Here, we engineered iPSCs derived from a McCune-Albright syndrome patient harboring either a wildtype or mutant GNAS^R201C along with an inducible oncogenic KRAS^G12D cassette. iPSCs were differentiated towards the pancreatic lineage, resulting in the formation of pancreatic duct-like organoids (PDLO). Subsequently, these organoids were induced with doxycycline for seven days to activate KRAS^G12D, after which they underwent molecular characterization through bulk RNA-sequencing and proteomics analysis. Additionally, pancreatic progenitor cells derived from the iPSCs were transplanted onto an ex vivo scaffold known as the porcine urinary bladder (PUB) model for non-disruptive long-term culture and observation. The engraftments on the PUB indicated that GNAS^R201C drives cyst formation, dysplasia progression and increased proliferation, while also reducing oncogene-induced senescence. Furthermore, both dysplasia progression and senescence were boosted by additional KRAS induction. At RNA and protein levels, the effects were classified as either additive, combinatorial or synergistic. This classification facilitated the identification and validation of potential pathways through which GNAS affects oncogene-induced senescence and early dysplasia formation. Using our iPSC-derived PDLO model, we can closely observe and identify key targets and players in early IPMN formation and progression. This model system is particularly valuable given the challenge associated with culturing patient-derived IPMN organoids and the elusive nature of risk factors for progression from IPMNs to invasive PDAC. In our study, we detected a reduction in oncogene-induced senescence driven by GNAS^R201C, along with an increased inflammatory and reduced genomic stability signature.

Keywords: pancreatic cancer, iPSC-derived pancreatic duct-like organoids, IPMN model

3:45 PM – 4:45 PM

285

VASCULARIZING STEM CELL DERIVED-ISLET ORGANOIDS WITH BLOOD VESSEL ORGANOIDS IMPROVE BETA CELLS PROPERTY AND TRANSPLANT SURVIVAL

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Islet endocrine cells generated from stem cells have been new sources of beta cells used for transplantation as type I diabetes (T1D) therapy strategy. Vessels bring nutrition and are also crucial niches for pancreatic islets. However, lack of vascularization in islet organoids attenuates beta cells property and impairs survival after transplantation. In this study, we vascularized stem cell derived-islet organoids in vitro by simply co-culture islet organoids with blood vessel organoids. Vascularization increased the percentage of beta cells in islet organoids and also increased mitochondrial size in individual beta cell. Functionally, vascularized islet organoids secrete more insulin upon glucose stimulation, indicating beta cells in vascularized islet organoids maybe more mature. More importantly, after transplanting these vascularized islet organoids into diabetes mice induced by STZ administration, it can quicken macrovascularization of graft in vivo, improve its survival and cure diabetes better compared with controls. Our approach thus provides a strategy for islet organoids vascularization in vitro that promote beta cells maturation and survival after transplantation.

Keywords: blood vessel organoids, stem cell derived islet organoids, vascularization

3:45 PM – 4:45 PM

287

GENOME WIDE CRISPR SCREENING TO STUDY CELL PLASTICITY IN IPSC DERIVED ISLETS

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Understanding the genetic mechanisms controlling beta cell de-differentiation or alpha-to-beta cell trans-differentiation holds a great potential for gene therapies of beta cell regeneration in diabetic patients. Pancreatic iPSC differentiation could provide with the cell types necessary to study those processes in the pancreatic islet. In this study, we have performed pooled CRISPR screens in stem cell derived islets to discover genetic programs that control cell identity at genome wide scale. For the multifactorial target validation, we are developing an arrayed screening platform. This study provides us with understanding of the pancreatic endocrine development and serves bases for approaches to restore minimal functional beta cell mass necessary for glycemic control in diabetic patients.

Keywords: directed pancreatic iPSC differentiation, genome wide CRISPR screen, dedifferentiation trans differentiation

3:45 PM – 4:45 PM

289

NON-CODING SNP RS6048205 CONTRIBUTES TO DIABETES SUSCEPTIBILITY VIA RXRA-MEDIATED FOXA2 OVEREXPRESSION DURING PANCREATIC DIFFERENTIATION

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Diabetes mellitus is a multifactorial disease with complex genetic underpinnings and environmental factors. Human genetics analysis has identified numerous single-nucleotide polymorphism (SNP) variants associated with diabetic traits. However, approximately 90% disease-associated SNPs are located in noncoding regions. Whether and how these variants contribute to diabetes are largely unknown. Here, we focus on a noncoding SNP rs6048205 (NC_000020.11:22578962:A>G), located in the downstream of FOXA2 gene locus with 2 kilobase-pairs away, which is associated with the symptoms of fasting-glucose (FG) and β cells function. To address these function of this variant in pancreatic differentiation and its potential pathological role, we first construct the isogenic human pluripotent stem cell lines carrying non-risk or risk allele (A vs. G) and the stepwise pancreatic differentiation assay indicates that the risk allele G impairs the generation of PDX1+/NKX6-1+ progenitor cells during pancreatic induction, and further results in the decrease of bona fide mono-hormonal β cells and abnormal increase of polyhormonal cells. Mechanistically, this risk variant G abnormally activates the expression of FOXA2 by introducing a new motif and specifically recruiting transcriptional factor RXRA in pancreatic progenitor stage. Consistent with this notion, inducible overexpression of FOXA2 could phenocopy the differentiation defect caused by the risk allele. Furthermore, the dysregulated FOXA2 binds to NKX6-1 gene locus and represses the transcription of NKX6-1, causing the abnormal differentiation of pancreatic lineage. More importantly, mice carrying the risk allele G exhibit abnormal pancreatic islet architecture, mild hyperglycemia and more sensitive to streptozotocin or high fat diet to eventually develop into diabetes. Our clinical observation further supports the important role of rs6048205-G in plasma glucose homeostasis. This study not only identifies a causal noncoding variant in diabetes susceptibility but also dissects the underlying gain-of-function mechanism by recruiting stage-specific factor.

Funding Source: National Natural Science Foundation of China (No. 31970608)

Keywords: noncoding variant, diabetes susceptibility, gain-of-function mutation

TRACK: EQUITY, DIVERSITY, AND INCLUSION (EDI)

3:45 PM – 4:45 PM

291

GETTING TO THE HEART OF IT: INTERACTIVE ENGAGEMENT ENABLES STUDENTS TO LEARN ABOUT STEM CELLS

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Getting to the heart of it: using stem cells to understand heart health and disease (UniStem 2023) was designed to provide students with a deeper understanding of the 'real science' of stem cell research and what it holds for the future, with a focus on heart regeneration and disease research. The event was run as part of UniStem - the largest educational outreach initiative on stem cell research across the globe, and in partnership with the Gene Technology Access Centre. Method and results. A total of 180 high school students and their teachers from 15 Victorian schools participated across two half-day sessions in Getting to the heart of it. Schools from lower socio-economic areas of Victoria were given priority to attend. Each session consisted of interactive activities, that enabled curiosity-lead learning where students analysed results to gain an understanding of the science. Students were asked to give feedback on the overall half-day session they attended. From the 180 students in attendance, 131 gave their feedback. • 92% of students rated their enjoyment of the program as high to very high • 98% of students agreed or strongly agreed that they learnt more about how stem cells are used in research • 94% of students agreed or strongly agreed that they learnt more about what it is like to work in a stem cell lab • 97% of students agreed or strongly agreed that they understood more about how researchers use stem cells to understand heart disease and try to treat it. Conclusion Quantitative and qualitative feedback from participating students demonstrated that attendance at Getting to the heart of it provided valuable experience of how laboratory research is conducted, with a focus on stem cell research. Students learnt about the different pathways into a career in science, and gained an understanding of how iPSC are used to both understand heart diseases but also as a tool for exploring potential therapies.

Keywords: engagement, outreach, stem cell

3:45 PM – 4:45 PM

293

ENGAGING WITH POTENTIAL BENEFICIARIES OF STEM CELL RESEARCH: THE CASE OF IN VITRO GAMETOGENESIS

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Gametes are essential for fertility. Recent advances in the field of in vitro gametogenesis (IVG) have raised hopes for the application of regenerative medicine to reproduction. However, as underlined by the ISSCR guidelines, the use of IVG to create embryos for implantation is ethically highly problematic and requires prior and in-depth ethical reflection and public engagement. Here, we report the results of the first empirical study of stakeholder perspectives on IVG to include a broad range of potential beneficiaries, namely people with infertility and LGBTQ+ people seeking to have a child in the US. These individuals could uniquely benefit from IVG to have a biological child. They also represent potential tissue donors for research and human subjects for clinical trials. We explored their views on IVG and its ethical and social implications through focus groups and individual interviews. We found that potential users were concerned not only that IVG technology produce functional and safe gametes, but also that it be accessible and equitable. Their main concerns were financial accessibility, inclusion of minorities, regulation of the industry and its uses, and scientific transparency. Our data point to areas where it is important for scientists to engage in order to promote the development of a stem cell technology that meets reproductive needs and does not increase reproductive health disparities. Our participants brought to the discussion of IVG the knowledge they gained from their experiences of living with infertility and using assisted reproductive technology. Our study illustrates public engagement as a two-way process that can serve not only to educate the public about cutting-edge research, but also to provide useful perspectives that ultimately support ethical research and clinical translation goals.

Funding Source: This research was supported by the National Human Genome Research Institute of the National Institute of Health, grant #R21HG012248.

Keywords: public engagement, bioethics, in vitro gametogenesis

TRACK: ETHICS, POLICY, AND STANDARDS (EPS)

3:45 PM – 4:45 PM

TOPIC: ETHICS

295

NAVIGATING THE FRONTIER: G-STEP PROJECT'S EXAMINATION OF THE REGULATORY LANDSCAPE OF IN VITRO GAMETOGENESIS RESEARCH AND POTENTIAL CLINICAL APPLICATION IN JAPAN

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Recent progress of stem cell research has enabled researchers to better understand mechanisms of early embryonic development, presenting opportunities for translating basic research into clinical applications, notably in assisted reproductive technology (ART). However, there are multiple research areas where ethical and regulatory issues need to be addressed. One such area is in vitro gametogenesis (IVG), which aims to generate gametes in vitro. Researchers have succeeded to produce sperms and eggs from mouse pluripotent stem cells (PSCs) such as ES cells and iPS cells, resulting in the birth of healthy offspring. Experiments with human cells are also progressing, and primordial germ cell-like cells (PGCLCs) have been produced from human PSCs. Our newly started project, G-STEP (Gametogenesis from Stem Cells and ELSI/Governance by Patient and public involvement), supported by the Japanese government, addresses the ethical considerations surrounding IVG and aims at proposing policy recommendations for the regulation of research. As the first step, we are analyzing regulatory landscapes related to basic research and potential clinical application of IVG in Japan. Our current results can be summarized in three points. First, regulations in Japan, specifically ethical guidelines from the government, are notably strict compared to international guidelines such as the ISSCR guidelines in that they allow the derivation of germ cells but prohibit fertilization. Second, a multitude of governmental guidelines regulating certain areas of human embryo and germ cell research has created confusion among researchers. Thirdly, the absence of a comprehensive law for ART, which presents challenges for clinicians and patients alike, could potentially pose further obstacles for the proper regulation of IVG research. This presentation aims to discuss the policy implications of our findings, providing insights for both Japanese and global research and clinical practices in the field of in vitro gametogenesis.

Funding Source: This research is supported by the funding Program: Responsible Innovation with Conscience and Agility (RInCA) by the Research Institute of Science and Technology for Society (RISTEX), Japan Science and Technology Agency (JST).

Keywords: in vitro gametogenesis, ethical research guidelines, assisted reproductive technology

3:45 PM – 4:45 PM

297

PRENATAL GENE EDITING: WHICH VALUES SHOULD GOVERN CLINICAL TRANSLATION?

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It has been anticipated that prenatal gene editing (PGE) would soon be introduced in the research setting. With these expectations, we need to proactively consider approaches to policy and values-based governance as PGE moves from research to clinical translation. This becomes more urgent when considering that reproductive technologies are one of the most variably governed areas of medicine, with substantial uptake in reproductive tourism in regions with lax regulatory oversight. Developing values-based governance for PGE requires considering the society in which policy is developed and where governance is implemented, and importantly, the socio-cultural and moral values underpinning them. Yet, up to now germline GE policies have adopted a technocratic approach with major emphasis on moratoria or other forms of control, while somatic GE have focused on safety issues. In all instances, they have left the underlying socio-ethical normative foundations vague and considerations for collective deliberation as secondary goals. To evaluate how a values-based governance framework for PGE could be developed, we conducted a comparative policy study (e.g. soft and hard laws) in ~40 jurisdictions to identify the rationale and values supporting policy and governance approaches to PGE. Our analysis showed that the principles of autonomy and dignity were predominant

across policies. Justice concerns were also prominent, although this notion was subject to different conceptualizations. In contrast, calls for assessing the socio-ethical implications of GE, or for fostering public engagement or other forms of collective participation appeared to be primarily rhetorical. In this presentation, we outline a values-based governance framework for PGE based on meaningful stakeholder inclusion and the incorporation of translational justice considerations.

Funding Source: Patient Supported Approaches to Gene Editing – PASAGE, National Human Genome Research Institute (grant R01HG011461)

Keywords: gene editing, ethics, policy

3:45 PM – 4:45 PM

299

LINEAGE-SPECIFIC CANCER-RELATED MUTATIONS IN PLURIPOTENT AND ADULT STEM CELLS

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Human pluripotent stem cells (hPSCs) are known to acquire genetic aberrations in culture, affecting their growth as well as their tumorigenic potential. To survey cancer-related mutations in hPSCs and their differentiated derivatives, we analyzed over 2,200 transcriptomes from about 150 independent lines. About 25% of the samples had at least one cancer-related mutation in either undifferentiated cells or in their ectodermal, mesodermal, and endodermal progeny cells. We have identified mutations in over 25 tier-1 tumor-related genes, and the majority of affected samples had mutations in the TP53 gene. Cells with TP53 mutations showed a pronounced selective advantage, perturbed target gene expression and altered cellular differentiation. To compare the rate and the type of mutations in pluripotent versus adult stem cells, we have analyzed several hundreds of samples from mesenchymal stem cells (MSCs), derived from adult tissues. We found that about 10% of the samples had cancer-related mutations, and some genes recurrently appear in different studies. All tumor-related genes identified in MSCs are interconnected, and show dramatically different prevalence from those identified in hPSCs. We thus demonstrate a lineage-specific mutation profile in pluripotent versus adult stem cells, attributed to their origin and growth conditions. These results may enable us to better understand the genetic stability of stem cells, and the forces that direct their aberrations in culture. These findings should have significant implications on the need for surveillance of cancer-related mutations in stem cells, especially in clinical applications. The initial part of this work was recently published in Nature Biotechnology (2024).

Keywords: human pluripotent and adult stem cells, cancer-related mutations, lineage specificity

3:45 PM – 4:45 PM

301

THE PROSPECT OF DIRECT BENEFIT IN FIRST-IN-HUMAN GENE THERAPY STUDIES IN MINORS - AN ETHICAL ANALYSIS

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Recent developments in gene transfer and gene editing hugely facilitated therapeutic advances in the treatment of monogenetic pediatric diseases. First-in-human (FIH) trials are a crucial step towards market authorization. Such trials involve notable uncertainties and risks, and for diseases that manifest in early childhood adult data are often lacking. In research involving minors, an important requirement to allow exposure to more than minimal risks is that there must be a prospect of direct benefit for the minors involved. However, in FIH pediatric gene therapy trials it can be questioned whether a certain therapy meets this requirement for a prospect of direct benefit and when such trials should be allowed, due to the uncertainty that accompanies them. To aid the review of these trials, we investigated definitions of the prospect of direct benefit in the ethical literature and important regulatory documents, such as the European Clinical Trial Regulation, FDA Common Rule and CIOMS guidelines. Additionally, we explored if and how preclinical research can substantiate evidence for the prospect of direct benefit. We found that “benefit” is inconsistently defined in regulatory documents, allowing different types of benefit to influence risk-benefit analysis. In some regulatory documents, direct benefit solely refers to clinical benefits that result from an intervention, whereas other regulations also include other, indirect, benefits in their definition of direct benefit. In addition, we found that what is meant with “prospect” is ill-defined in many regulatory documents. This raises questions on how this requirement for a “prospect” of direct benefit needs to be interpreted. We provide recommendations how this prospect could be understood. To do so, we look at different types of evidence - mechanical and statistical - that are used to substantiate claims of effectiveness, and thus potential direct benefit. Clearly defining and accurately interpreting the requirement for a prospect of direct benefit is crucial for effectively reviewing FIH pediatric gene therapy trials. A failure to do so could lead to mislabeling trials as providing a prospect of direct benefit, fostering therapeutic misconception and accepting trials with an unfavourable risk-benefit ratio.

Funding Source: Funded by the Novo Nordisk Foundation Center for Stem Cell Medicine, reNEW (NNF21CC0073729).

Keywords: prospect of direct benefit, first in human trials, ethics

3:45 PM – 4:45 PM

303

ATTITUDES TOWARDS STEM CELL LINE REGISTRATION

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The recently issued ISSCR guidelines for Standards in Stem Cell Research provide recommendations that human pluripotent stem cells lines should be registered in an internationally recognised platform such as hPSCreg.eu. These recommendations support best digital practises to unambiguously link stem cell provenance to data generated using those lines. Australia is relatively well connected with the international stem cell community but doesn't have the mandates for registration that exist in other jurisdictions, so is a useful testbed to assess attitudes and behaviours about data sharing generally, and stem cell registration specifically. In this project, we sought to understand what motivated Australian researchers to register their lines. We conducted interviews with 48 Australian stem cell stakeholders, including early career researchers, group leaders, facility leads, clinicians, and industry professionals, recruited from seven Australian cities. We found that most Australian stem cell researchers do not routinely register their lines, and consequently, most Australian lines are not registered. Journal requirements for a registered cell identifier was the main reason provided for those who had registered a stem cell line. When registration is perceived to be a hurdle to publication, researchers reported little incentive to explore other aspects of the registry, even when they expressed support for the open science values that registries facilitate. Although we have drawn on a relatively small sample size, these findings do suggest that journals play an important role in supporting registration practices. Our study also suggests that there is a lack of awareness of registries generally, and little understanding of how registration supports scientific integrity and data provenance when there is no established community of practise to support registration. We show that superficial engagement with registration can lead to negative perceptions about the platforms that support registration of human pluripotent stem cell lines.

Funding Source: The Australian Medical Research Future Fund, via the Stem Cell Therapies Mission, Grant ID 2008807, to RA, JL, DN, CW.

Keywords: standards, digital registration, cell line provenance

3:45 PM – 4:45 PM

305

ORGANOIDS TO 'CURE' DEAFNESS? A SCOPING REVIEW ON THE ETHICS OF USING ORGANOID TECHNOLOGY TO TREAT DEAFNESS

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Hearing loss impacts millions of people worldwide, yet effective treatments remain unavailable, leaving patients reliant on technological aids such as hearing aids or cochlear implants. Organoid technology is a promising new biomedical tool in the search for the cause and solution of deafness. Organoids are human tissues grown from stem cells that mimic an organ. Patient-specific organoids provide a means to investigate hereditary deafness, assess drug efficacy, and tailor treatments to individual patients. At the same time, a future in which organoids are used to investigate into and treat deafness also faces ethical concerns, especially regarding clinical application and its impact on deaf identity. While existing literature discusses the ethics of organoids or the ethics of deafness separately, an ethical analysis of using organoids for treating deafness is missing. To address this gap, we conducted a scoping review and created an overview of the ethical questions at stake. Literature was found in bioethics and biomedical databases using search strategies with the terms 'organoids and ethics' and 'deafness and ethics'. From these articles, we identified ethical challenges that are specific to using organoids to address deafness. The ethical concerns echo broader concerns surrounding medicalization, disability, and identity. Concurrently, the distinctive nature of organoids, also introduces novel ethical dimensions to the debate. These amongst others include

considerations related to moving from exploratory science to clinical practice, and to justice and equal access to medicine. After outlining the ethical analysis of organoids as a potential solution for addressing deafness, we discuss how the identified challenges should be navigated, and set an agenda for further deliberation on this topic.

Funding Source: This research is funded by the Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW), which is supported by a Novo Nordisk Foundation grant, number NNF21CC0073729.

Keywords: ethics, deafness, organoids

3:45 PM – 4:45 PM

307

WHAT IS A STEM CELL? THE EXPERT VIEW(S)

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What is the defining property of a stem cell? “Self-renewal. That’s it.” “Self-renewal and differentiation. As I say in my first-year lectures.” “Differentiation and proliferation. But I’m sure someone else will give you a different answer.” These are some answers pioneering researchers in the stem cell field gave when discussing what makes a cell a stem cell. But how were these definitions formed and does it matter? How is consensus formed and does that matter? Here we present an interview study of how 16 leading scientists in the stem cell area understand the foundational concepts within stem cell research – namely, stem cells and stemness. In characterising current schools of thought, we identify key parameters that govern the continuous construction of these definitions and their subsequent effects on research practice. We explore how personal ideas of ‘stem cells’ are consciously negotiated against each other, as well as the kinds of consensus that are sought after and/or idealised. We argue that how scientists make meaning around the question ‘what is a stem cell?’ shapes how the field evolves, with important implications for stem cell research in its entirety and especially its policy-making, standard-setting and public engagement efforts.

Funding Source: The Novo Nordisk Foundation (NNF) Center for Stem Cell Medicine is supported by a NNF grant (NNF21CC0073729). I.T. is the recipient of a fellowship from the NNF as part of the Copenhagen Bioscience Ph.D. Program (NNF20SA0035584).

Keywords: definitions, consensus, stem cells/stemness

TRACK: NEW TECHNOLOGIES (NT)

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

309

A INNOVATIVE APPROACH FOR CONDUCTING 3D ELECTROPHYSIOLOGICAL RECORDINGS WITHIN INTACT ORGANOIDS

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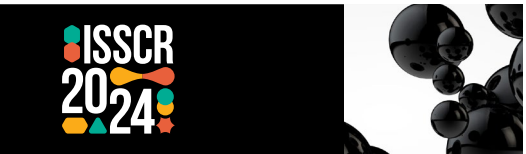
Organoid technology is a pivotal tool for exploring human physiology and diseases. Despite its potential, current readout capabilities constrain organoid electrophysiological research. Classical microelectrode arrays (MEA) fall short in capturing data from intact organoids, which may flatten in the 2D-MEA surface, jeopardizing physiological responses and data validity. To overcome this, we pioneered a mesh MEA, preventing morphological deformations, fostering 3D growth, and facilitating electrical activity recording within intact organoids over an extended period. Electrophysiological recordings of human brain organoids were performed in an MEA-2100 head stage from Multi-Channel Systems, accommodating classical MEA (c-MEA) and mesh MEA (m-MEA) chips. Extracellular neural activity, sampled at 25 kHz and filtered at 400 Hz for spike detection, accurately reflected action potential events on the membrane. Neuronal migration around the mesh was monitored using light microscopy. The mesh MEA integrates 60 titanium nitride electrodes (30 µm diameter), connected via a 200 µm-long polyamide filament pitch, forming a flexible mesh. The average noise level of c-MEA and m-MEA systems was comparable (± 10 µV). Spike time analysis revealed heightened activity after seven days on the m-MEA compared to acute recordings on c-MEA, with a mean firing rate of 5 Hz in classical MEA and 34 Hz in the mesh MEA. Microscopy images illustrated neuronal migration, dendritic growth, and axon development around the mesh structure and electrodes. These findings suggest that the m-MEA holds great promise for comprehensive, long-term organoid electrophysiological studies, providing deeper insights into human functions and disorders.

Keywords: organoid, electrophysiology, brain disorder



3:45 PM – 4:45 PM

TOPIC: CARDIAC

311

DATA-DRIVEN MULTI-GENE CONTROL FOR PRECISION CELL FATE PROGRAMMING

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Stem cell-based applications benefit from an ability to generate specific cell types. However, current differentiation protocols are often both imprecise and inefficient and require labor-intensive trial-and-error experimentation. There is thus a major need for methods that improve and standardize the cell differentiation workflow. Becuase cell identity is controlled by multi-gene networks, we therefore developed new CRISPR-based tools that perform simultaneous multi-gene activation and repression using combinations of dCas12a, Cas13d and dCas9. To learn which gene combinations to perturb, we developed a computational tool that uses scRNA-seq data to predict which genes to simultaneously activate and repress to convert a desired starting cell into a target cell. We combine these technologies into a unified workflow for data-driven cell fate engineering and demonstrate its use by generating mesoderm progenitors while actively repressing undesired cell types. This foundational technology for data-driven cell-fate control will enable new stem cell-based applications.

Funding Source: California Institute for Regenerative Medicine (CIRM) Human Frontier Science Program (HFSP)

Keywords: data-driven cell fate engineering, multi-gene control, precision cell programming

3:45 PM – 4:45 PM

313

LIGHT-RECONFIGURABLE SURFACE TOPOGRAPHY GUIDES HUMAN STEM CELL DERIVED CARDIOMYOCYTE ALIGNMENT AND ORIENTATION OF CONTRACTION

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Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are a powerful platform for drug development and cardiovascular research. However, their electrophysiology and structure are less developed than adult cardiomyocytes. Micropatterning and mechanical stimulation are known to induce hallmarks of adult-like cardiomyocytes, creating more advanced cell models. Here, we present a novel cell culture platform with a reconfigurable surface topography, with the unique capability for surface erasure and mechanical stimulation with light. Biomimetic micropatterns of distinct sizes (1 µm, 1.5 µm and 2.5 µm) were obtained by light-responsive Disperse Red1 glass on glass coverslips, subsequently coated with Parylene C. The surface relief gratings were inscribed on the substrates by interference lithography in the Lloyd's mirror configuration with a 488 nm continuous-wave laser with circular polarization at 500 mW/cm2 intensity over an area of 0.50 cm2. Commercially available topography was used as positive control for alignment. All topographies were imaged by atomic force microscopy. Magnetic-activated cell sorting was performed for hiPSC-CMs on day 20 of differentiation to improve cardiomyocyte purity. After that, cardiomyocyte monolayers were plated over the micropatterns. One-week post-plating, brightfield video recording, topography reconfiguration with uniform irradiation by blue LED, and immunostaining were performed. Contractility was quantified from the videos using digital image correlation and the contraction vector orientation distribution was determined. The cell alignment and vector orientation distributions were similar in the three pattern sizes, and comparable to commercial sample topographies. Thus, the micropattern size had little effect on cardiomyocyte alignment. The cells remained viable after the light-controlled topography reconfiguration, inducing morphological changes in the cells. To conclude, the system enables a unique opportunity to study cell-specific response to custom-shaped surface topographies. The micropatterns were capable of aligning cardiomyocytes comparably to commercially available ones. The setup combines alignment, erasure and stimulation, enabling novel drug development assays and cardiovascular research.

Keywords: cardiomyocyte, alignment, micropatterns

3:45 PM – 4:45 PM

315

TISSUE MICROENVIRONMENT DICTATES THE STATE OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED ENDOTHELIAL CELLS OF DISTINCT DEVELOPMENTAL ORIGIN IN 3D CARDIAC MICROTISSUES

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Endothelial cells (ECs), forming the inner lining of blood and lymphatic vessels, are known to originate from multiple developmental lineages, adapting to local cues to support organ function. Here we demonstrate that organotypic vasculature for the heart can be recreated in a three-dimensional cardiac microtissue (MT) model composed of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (CMs), cardiac fibroblasts (CFs) and endothelial cells (ECs). ECs in cardiac MTs upregulated expression of markers enriched in human intramyocardial ECs (iECs), such as CD36, CLDN5, APLNR, NOTCH4, IGFBP3, ARHGAP18, which were previously identified in the single-cell RNA-seq dataset from the human fetal heart (6.5-7 weeks post coitum). We further show that the local microenvironment largely dictates the organ-specific identity of hiPSC-derived ECs: we compared ECs of different developmental origins derived from two distinct mesoderm subtypes (cardiac and paraxial mesoderm) and found that independent of whether the ECs were cardiac or paraxial mesoderm derived, they acquired similar identities upon integration into cardiac microtissues. This was confirmed by single-cell RNA-seq. Overall, the results indicated that whilst the initial gene profile of ECs was dictated by developmental origin, this could be modified by the local tissue environment such that the original identity was lost and the organotypic identity acquired through local environmental signals. This developmental "plasticity" in ECs has implications for multiple pathological and disease states.

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Keywords: hiPSC-derived endothelial cells (hiPSC-ECs), hiPSC-derived 3D cardiac microtissues, organ specific ECs

3:45 PM – 4:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

317

MODELLING EMBRYO IMPLANTATION IN VITRO USING 3D ENDOMETRIAL ASSEMBLOIDS AND HUMAN BLASTOCYSTS

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Human pregnancy involves implantation of chromosomally diverse embryos in a uterine mucosa – the endometrium – subjected to cyclical breakdown and regeneration. Upon implantation of high-fitness embryos, the endometrium transforms into the decidua of pregnancy, which anchors and supports the placenta throughout gestation. Perturbations in these processes are believed to underpin prevalent reproductive disorders while, the mechanisms that account for persistent reproductive failure remain poorly understood. There is a vital need for a model involving the endometrium and human embryos since ethical reasons limit our ability to study human embryo implantation in vitro. For this, endometrial assembloids were established which are physiologically similar to endometrial tissue in morphology and function. Assembloids consist of epithelial compartments including gland-like organoids and a luminal epithelium which is the embryo attachment side, and a stromal compartment rich in fibroblasts, immune and endothelial cells. Hormonal stimulation results in the induction of decidual stromal (PRL, SCARA5 and DIO2) and epithelial (PAEP and SPP1) marker genes. Aiming to mimic implantation, assembloids underwent a 3-day long differentiation in a chemically defined medium, 8-br-cAMP, estradiol, and a progestin. Subsequently, assembloids were overlaid with single epithelial cells from the matching patient. Following a 24-hour long incubation period, human hatched day 6 blastocysts were positioned on the surface epithelium. Co-cultures were fixed on day 8 and immunofluorescent antibody labelling using markers for the epiblast (OCT4), primitive endoderm (GATA4) and trophectoderm (GATA3) were used to visualise the co-culture. Secretion of βhCG was detected on day 9 while by day 10, βhCG detection was enhanced and Cytokeratin7 was also detected around the embryo marking the syncytiotrophoblast and cytotrophoblast populations, respectively. The proposed system is an innovative method to recapitulate apposition, adhesion, and invasion during implantation. While many technical hurdles remain, the ability to establish patient-specific endometrial models is poised to lead to advances in our understanding of mechanisms of reproductive failure and the development of personalised therapeutic interventions.

Funding Source: EUTOPIA – Institutional Partnership University of Warwick (UK) – Vrije Universiteit Brussel (BE) Co-tutelle PhD Scholarship

Keywords: endometrium, assembloids, embryo

3:45 PM – 4:45 PM

319

HUMAN GASTROIDS RECAPITULATE DEVELOPMENTAL SYMMETRY BREAKING IN EARLY STOMACH ORGANOGENESIS

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Symmetry breaking is universal and instrumental for shaping the architectural and functional complexity in developing organs. As a highly structured organ, stomach features multi-lineage composition as well as regionalized functionality allocated along the fundic-antral axis. The emergence of such fundic-antral asymmetry is the first developmental milestone that defines the organ plan in early stomach organogenesis. However, the developmental principle of such gastric symmetry breaking in humans is poorly understood due to limited accessibility to early embryonic specimens and a lack of in vitro models. Here we report human gastroids, a self-organized multi-lineage gastric organoid derived from human pluripotent stem cells, to recapitulate gastric symmetry breaking in vitro. Through biomimetic multi-germ-layer co-development, we generate gastroids that feature an epithelial chamber with bipolar fundic-antral patterning, annexed with neural populations near the fundic domain while enveloped by mesenchymal cells, thus showing molecular, cellular, structural, and anatomical similarity to stomach development in vivo. Micro-surgery experiments further revealed the indispensability of non-endodermal cells and inter-tissue signaling crosstalk to instruct gastric symmetry breaking in a bi-potential environment. Our single cell transcriptomic profiling reveals spatiotemporal lineage trajectories, regulatory networks, and dynamic niche remodeling during human gastroid development. Combining single-cell analysis and perturbative experiments, we further identify neural tissues as a signaling center to drive fundic-antral symmetry breaking in gastric epithelium through WNT-mediated crosstalk. Together, this study established a high-fidelity organoid model to recapitulate the formation of fundic-antral asymmetry in human stomach organogenesis, and revealed a previously unappreciated paradigm of inter-tissue crosstalk in dictating axial patterning in digestive organs. This work thus opens new avenues to decipher and reconstruct developmental symmetry breaking in stomach organogenesis and help advance basic human developmental biology and organoid engineering.

Funding Source: This work is supported by the National Natural Science Foundation of China (U21A20203).

Keywords: synthetic development, stomach organ patterning, human pluripotent stem cells

3:45 PM – 4:45 PM

321

VISUALIZATION AND ANALYSIS OF LIVE ORGANOIDS DYNAMICS VIA LABEL-FREE 3D HIGH-RESOLUTION IMAGING WITH LOW-COHERENCE HOLOTOMOGRAPHY

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Three-dimensional (3D) and temporal visualization of live organoids is essential for understanding their cellular heterogeneity and physiological functions. Conventional 3D imaging techniques often involve invasive, detrimental labeling, which can potentially alter the biological status of the organoids. Holotomography offers a non-invasive approach to 3D imaging by utilizing the refractive index (RI) of the specimen. This RI-based methodology enables the translation of RI data into physical quantities that reflect the biological status of the organoids, thereby providing insightful and undisturbed observations. In this study, we employed low-coherence holotomography, an ideal technique for observing multicellular specimens, to investigate the morphological dynamics of murine small intestine organoids (sIOs) under cytotoxic conditions. The low-coherence holotomography system allowed us to monitor undisguised live organoids in their physiological state. We acquired 3D RI tomograms of live organoids embedded in Matrigel, following treatment with the cytotoxic agent cisplatin or a control vehicle. 3D visualization revealed the shrinkage of crypt due to the cell death, and the translocation of a single apoptotic cell. Through the volumetric analysis and RI-based quantitative measurements, we tracked the decrease in organoid volume, protein concentration, and mass, correlating these changes with the observed crypt shrinkage. In conclusion, low-coherence holotomography provides unique capabilities for determining the biological status of organoids, making it a valuable tool for basic research and therapeutic applications. By enabling non-invasive, label-free, and quantitative observation of live organoids, this technology has the potential to revolutionize the field of organoid research.

Keywords: live organoid imaging, organoid quantification, organoid drug response

ABSTRACT WITHDRAWN

3:45 PM – 4:45 PM

325

IDENTIFICATION OF LABEL-FREE RAMAN BIOMARKERS IN HUMAN SKIN STEM CELLS

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The transplantation of epidermal autografts has revolutionized skin tissue engineering. Recent studies unveiled two distinct stem cell populations—slow and fast cycling—in the interfollicular epidermis of mouse skin. However, effective biomarkers for distinguishing these heterogeneous stem cell populations remain elusive. Raman microspectroscopy, a powerful technique for analyzing molecular-based structures, has emerged as a promising avenue. Recent research, coupled with multivariate data analysis (MVA), successfully identified disease-related Raman spectra in aortic diseases in both mouse and human samples. This discovery underscores the potential of Raman imaging as a novel tool for biomarker identification, with profound implications for disease diagnostics and other research fields. Our study aims to assess the molecular components of human skin at the single-cell level using Raman microspectroscopy and imaging. By pinpointing Raman spectra that accurately represent the biochemical profiles and spatial information of heterogeneous stem cells and the undulating skin structure, we aim to discover specific biomarkers for customized transplantation treatments. Ultimately, our research seeks to establish a label-free evaluation system for skin models, with potential applications in transplantation, drug screening, and disease modeling for various organs beyond the skin.

Keywords: skin, imaging, biomarker

3:45 PM – 4:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

327

HARNESSING NEAREST-NEIGHBOUR GRAPHS TO BETTER UNDERSTAND SINGLE-CELL TOPOLOGIES AND CLUSTERING

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Single-cell RNA sequencing (scRNA-seq) provides high-resolution biological insights but yields complex and high-dimensional datasets. Uniform manifold approximate and projection (UMAP) is routinely used for dimensionality reduction in scRNA-seq data for cell clustering and visualization. However, low-dimensionality representations of scRNA-seq data in general are raising concerns about the capacity to preserve

underlying data complexities. Therefore, we propose a methodology to assess the robustness of dimensionality reduction topologies and cluster-cluster interactions. The overall principle is to investigate the nearest-neighbour graphs used in the UMAP and clustering algorithms to assess which cells or clusters are more robust and which ones are more malleable. We propose three techniques: 1. Visualization of Cell Neighbourhoods: For individual cells or cell groups, we visualize their cell neighbourhoods as either dots or density contours on a dimensionality reduction map, uncovering tightly interwoven or widely dispersed regions. This allows us to learn which clusters might need merging if they have largely overlapped neighbourhoods, and infer trajectory relationships of cells obscured by the reduction. 2. Quantification of Cell Neighbourhoods: For each cell, we quantify the distribution of its neighbouring cells by the variance in their coordinates, providing insights into the cell positioning in the reduction map. Large variance indicates diminished confidence in a cell's positioning. We found it reveals the reduction patterns of more loosely and tightly positioned cells, that can be leveraged to interpret the cell clustering better. 3. Complementary reductions: The cell neighbourhoods can be explored across various dimensionality reductions for insights. For example, diffusion map and force-directed layout can provide clues about cell development by preserving the continuous cell transitioning trajectories. We demonstrate their application on an atlas of early human development blastocyst data to uncover timing relationships and gastrulation stage trajectories. Overall, this neighbourhood analysis helps to understand and interpret the representations of scRNA-seq data in low-dimensional spaces.

Keywords: bioinformatics, computational biology, dimensionality reduction

3:45 PM – 4:45 PM

329

STEM CELL ASSOCIATED TECHNIQUES TO RESCUE THE NORTHERN WHITE RHINO FROM EXTINCTION

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With only two surviving females, the northern white rhino (NWR) is functionally extinct. To save the charismatic animals from extinction, the BioRescue consortium combines advanced assisted reproductive technologies and stem cell associated techniques. Natural oocytes can be derived from Fatu, the younger NWR individual, and subsequently in vitro fertilized by intracytoplasmic sperm injection (ICSI) with cryopreserved sperm of already deceased NWR bulls. Thus, the BioRescue consortium generated already more than 30 NWR embryos, which are awaiting transfer into surrogate mothers of the closely related, less threatened southern white rhino (SWR). Recently, we demonstrated the first successful embryo transfer in rhinos, which paved the way for NWR calves to be born from SWR mothers in the near future. However, one genetic mother is not sufficient to found a healthy, self-sustaining NWR population. To increase the genetic variation, we aim to differentiate gametes from induced pluripotent stem cells (iPSCs). Towards this aim, we reprogrammed fibroblasts from several deceased NWR individuals, and developed a primordial germ cell-like cell (PGCLC) induction protocol. Within the scope of our comprehensive quality control, we perform single-cell genome sequencing by STRAND-seq to analyze both the genetic integrity and the heterogeneity of source fibroblasts and generated iPSCs. Additionally, the data is used to assemble a state-of-the-art NWR reference genome. Current focus includes optimization of the PGCLC induction protocol and generation of PGCLCs from different NWR individuals. As maturation of PGCLCs into mature oocytes requires co-culture with somatic gonadal tissue, we are developing a protocol to differentiate fetal ovarian somatic cell-like cells (FOSLCs) from rhino stem cells. The two approaches for producing NWR embryos are accompanied by an ethical risk analysis, which adequately evaluates potential risks of the new species protection strategy, the well-being of individual animals, the prosperity of the entire subspecies, and complex social-ecological questions.

Funding Source: German government BMBF 01LC1902B BioRescue

Keywords: conservation, stem cell associated technique (SCAT), primordial-germ cells

3:45 PM – 4:45 PM

331

CELL SIZE REGULATES HUMAN ENDODERM SPECIFICATION THROUGH ACTOMYOSIN-DEPENDENT AMOT-YAP SIGNALING

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Cell size is a crucial physical property that significantly impacts cellular physiology and function. However, the influence of cell size on stem cell specification remains largely unknown. Here, we investigated the dynamic changes in cell size during the differentiation of human pluripotent stem cells into definitive endoderm (DE). Interestingly, cell size exhibited a gradual decrease as DE differentiation progressed with higher stiffness. Furthermore, the application of hypertonic pressure to accelerate the reduction in cell size significantly enhanced DE differentiation. By functionally intervening in mechanosensitive elements, we have identified actomyosin activity as a crucial mediator of both DE differentiation and cell size reduction. Mechanistically, the reduction in cell size induces actomyosin-dependent AMOT nuclear translocation, which suppresses YAP activity and thus facilitates DE differentiation. Together, our study has established a novel connection between cell size diminution and DE differentiation, which is mediated by myosin-dependent AMOT nuclear translocation. Additionally, our findings suggest that the application of osmotic pressure can effectively promote human endoderm lineage differentiation.

Keywords: osmotic pressure, cell size, human endoderm differentiation

3:45 PM – 4:45 PM

333

INSIGHTS INTO THE ENHANCING INMUNOMODULATORY EFFECTS OF HUMAN PLACENTAL MESENCHYMAL STEM CELLS ON INMUNE CELLS FROM TYPE 2 DIABETES PATIENTS IN CONTACT WITH COVID-19

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Mesenchymal stem cells (MSCs) can be obtained from different human tissues demostrating to have a great potential to enchance wound healing through multipotenciality and inmunomodulatory properties, presenting new opportunities for the advance of regenerative medicine. MSCs have shown to regulate the production of cytokines due to their immunomodulatory capacity, which could be used as a therapeutic alternative for inflammatory alterations induced by SARS-CoV-2 and diabetes mellitus type 2 (DMT2). Recent studies have shown that COVID-19 infection can produce a cytokine storm that release IL-6 and TNF-α causing insulin resistance, endothelial dysfunction, and damage of the pancreatic islets in DMT2, showing that chronic hyperglycemic patients are more vulnerable to have a negative impact in there inmune system. For this reason the aim of this study is to understand the inmunomodulatory effect of placenta-derived mesenchymal stem cells (PDMSCs) on peripheral blood mononuclear cells (PBMCs) of patients with DMT2 that have had infection with COVID-19. We were able to observe that PDMSCs showed a three-fold increase in the production of proinlfamatory cytokines IL-6, IDO and COX-2, when co-cultured with PBMCs from DMT2-COVID-19 patient;levels that were decreased when stimulated with LPS/INF- γ. There was a two-fold decrease in the expression of CD3, CD45, and CD16 phenotypes, and TNF-α proinflamatory cytokine in non-stimulated PBMCs from DMT2-COVID-19 patients. PDMSCs atenuated the expresión of IL-6, IL-12 proinflamatory cytokines and CD56 in both sitimulated PBMCs control and DMT2-COVID-19. PDMSCs increased the expression of IL-10 antiflamatory cytokine in stimulated PBMCs from DMT2-COVID-19 patient. Results showed that PDMSCs have the capacity of attenuating the production of proinflamatory cytokines in PBMCs from DMT2-COVID-19 patients after stimulated. Our results help us to elucidate the inmunomodulatory potential of PDMSCs for future studies regarding the remarkable abilitiy of MSCs in innovative regenerative medicine treatments.

Keywords: inmunomodulation, placental mesenchymal stem cells, cytokines

3:45 PM – 4:45 PM

335

ILLUMINATING DYNAMIC STRUCTURAL STATES IN HIPSCS THROUGH ENDOGENOUS GENE TAGGING

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The Allen Institute for Cell Science aims to understand the principles by which human induced pluripotent stem cells (hiPSCs) establish and maintain robust dynamic localization of cellular structures, and how they transition between states during differentiation and disease. We used CRISPR/Cas9 to create a collection of endogenously tagged hiPSC lines to illuminate organization in various cell states, including stem cells, migratory cells, cardiomyocytes and endothelial cells. The tagged proteins include notable membrane-bound and membrane-less cellular organelles, signaling complexes, phase transition markers, transcription factors and differentiation-specific structural and cell identity markers. To date, the Allen Cell Collection consists of 56 single- or compound-edited lines that have undergone extensive quality control testing to ensure genomic, cell biological, and stem cell integrity. Here, we highlight our gene-editing and quality control workflow for mono- and biallelic editing of expressed or silent genes that are either expressed in stem cells or specifically during differentiation. Furthermore, we demonstrate our efforts to increase tagging efficiency by utilizing Adeno-Associated Virus (AAV) to deliver donor DNAs and multiplexing transfection strategies for gene tagging at multiple loci, including those not expressed in stem cells. Our most recently developed lines are VIM-mEGFP (Vimentin) for visualizing intermediate filaments, the cadherins CDH2-mTagRFPt (N-cadherin) to label junctions in migratory cells, and CDH5-mEGFP (Vascular E-cadherin) for analysis of cell

adhesions in endothelial cells. We have also generated TBR2-mEGFP (EOMES) and TBXT-Halo (Brachyury) which contains tags on transcription factors specifying the stage of commitment when differentiation is triggered during epithelial-mesenchymal transition. These lines not only facilitate live imaging to study organizational principles of cell state transitions but also serve as valuable tools that we continue to make openly available to the biomedical research community. Our cell lines, donor plasmids, segmented 3D images of our cell lines, image analysis and visualization tools, integrated cell models and biological findings can be found at (www.allencell.org).

Keywords: EMT, gene tagging, iPSCs

3:45 PM – 4:45 PM

337

ENGINEERING FIRE: ILLUMINATING IRON REGULATION OF STEM CELL STATE AND FATE

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Cellular iron regulation is essential for the proper execution of early mammalian development. Iron regulatory proteins (IRP) are RNA-binding proteins that regulate cellular iron and are required for erythropoiesis, neurogenesis, and gut development, as well as the development of organs including the brain, heart, and liver. To achieve these complex mechanisms and beyond, cellular iron homeostasis is carefully controlled by the IRP-targeted network from pluripotency, yet the roles of cellular iron in the earliest cell fate decisions are not well known. We've developed a genetically-encoded dual biosensor for IRP-binding and cellular iron called FIRE (Fe-IRE) and are using it to determine the contributions of cellular iron homeostasis to pluripotency and cell state transitions at single cell resolution. We use our FIRE sensor as a new lens to understand the role of iron in the transcriptomic networks that parallel and indeed underlie early mammalian development. Using FIRE, our group couples imaging, cytometry, sequencing, and genetic perturbations with quantitative functional analysis to illuminate the contributions of cellular iron and IRP-binding to early mammalian stem cell state(s) and fate(s).

Funding Source: This work was supported by NIH/Eunice Kennedy Shriver National Institute of Child Health and Human Development Grant K08HD105017 and the Burroughs Wellcome Fund Career Award for Medical Scientists (both to CS).

Keywords: iron, sensor, RNA-binding

3:45 PM – 4:45 PM

339

DATA-DRIVEN ASSESSMENT OF BLASTOID MODELS USING AN INTEGRATED ATLAS OF EARLY DEVELOPMENT

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In vitro models of early human development, such as blastoids, hold immense potential for advancing our understanding of embryogenesis. However, the lack of comprehensive in vivo reference data hinders the evaluation and comparison of these models. Existing single-cell datasets on early development are heterogeneous in terms of timepoints and technologies utilised, posing challenges for integration. Thus, we conducted a full benchmark of single-cell integration methods to address this gap. Our approach resulted in the creation of an atlas of early embryos spanning embryonic days 3 to 19, combining data from plate-based and droplet-based single cell technologies. We then projected single-cell data from different blastoid models onto our atlas to identify the cell types present and assess the celltype composition of these in vitro systems. For pre- and post-implantation blastoids, we predicted the maturity of cells in the epiblast, hypoblast and trophoblast, determining if these lineages are developmentally synced. We further validated our findings orthogonally by gene expression signatures that are unaffected by data integration. Importantly, the strength of these gene expression signatures quantifies the confidence associated with data integration and annotation, providing insights into the reliability of our findings. Through our analysis, we drew conclusions about the molecular composition and developmental progression of different blastoid models, shedding light on their developmental trajectories and transcriptional resemblance to in vivo counterparts.

Funding Source: Singapore National Medical Research Council (NMRC) Open Fund Young Investigator Research Grant (OF-YIRG)

Keywords: early embryos, blastoid, single-cell atlas

3:45 PM – 4:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

341

GENERATION OF ALLOGENEIC CAR-NKT CELLS FOR CANCER IMMUNOTHERAPY THROUGH CD34+ HSPC GENE-ENGINEERING AND XENOGENEIC FEEDER-FREE CULTURE

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Autologous chimeric antigen receptor (CAR) T cell therapy faces challenges in manufacturing, cost, and patient selection that could be avoided by using 'off-the-shelf' products, such as allogeneic CAR natural killer T (AlloCAR-NKT) cells. Previously we reported a culture system for differentiating human CD34+ hematopoietic stem and progenitor cells (HSPCs) into AlloCAR-NKT cells, but the use of 3D xenogeneic feeder-dependent culture limits its clinical potential. Here we describe a xenogeneic feeder-free culture to generate IL-15-enhanced Allo-CAR-NKT cells targeting various cancers including multiple myeloma with high yield and purity. In vivo, the cells exhibit potent antitumor efficacy, expansion, and persistence. They selectively deplete immunosuppressive cells in the tumor microenviroment and antagonize tumor immune evasion via triple targeting of CAR, TCR and NK receptors. They exhibit a stable hypoimmunogenic phenotype that we trace to epigenetic and signaling regulation. These properties of AlloCAR-NKT cells, together with their low risk of graft vs host disease and cytokine release syndrome, support their potential for clinical translation.

Funding Source: Partnering Opportunity for Discovery Stage Research Projects Award, Partnering Opportunity for Translational Research Projects Awards from the California Institute for Regenerative Medicine (DISC2-11157 and TRAN1-12250)

Keywords: hematopoietic stem and progenitor cells, natural killer T cell, cancer immunotherapy

3:45 PM – 4:45 PM

343

EXHAUSTED IN SPACE HEMATOPOIETIC STEM CELLS RECOVER THEIR SELF-RENEWAL CAPACITY BY ADDITIONAL STROMAL CO-CULTURE

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Microgravity and higher inflammogenic conditions in space may potentially change the normal steady-state of HSC-microenvironment interaction status. We compared the self-renewal capacities of HSCs returning from culture in Space and paired parallel Ground cultures and investigated the possible recovery of HSC's lost replating ability by re-culturing the cells in a more supportive niche and recreating the beneficial microenvironment. To observe the cell cycle changes CD34 positive cells from aged normal BM (ABM) were transduced with Fucci Bicistronic Vector. Cells were cultured in 3D nanobioreactor (BR) for 42 d spaceflight mission and terrestrially as a Ground control. This BR was designed for supporting primary human HSC culture on orbit aboard the International Space Station. Post-mission cells were plated on primary autologous stroma. established from the their CD34 negative fraction of ABM, or Hs27a cell line monolayer, and then subjected to clonogenic assays. Some of these cultures were used for RNA Nanostring analysis with the Human Myeloid Innate Immunity 750 gene panel. Post flight the ABM cells had a significantly reduced ability to replate and create multilineage colonies. To determine if the impairment was permanent, we co-cultured the non-functional Space samples with autologous or HS27a stroma. In 12 days there was a strong expansion of cell numbers and colony formation in survival and self-renewal assays. Both the autologous stroma and HS27a cells were able to improve replating capacity comparable to the level of original naïve HSCs. The ability of Space BR HSCs to self-renew was significantly lower compared to Ground controls. Cell cycle analysis revealed that cells were in both proliferating and dormant stage after 54 d of total culture. We conducted NanoString RNA analysis on the replated colonies to evaluate changes indicative of functional recovery. Results showed differential expression of key stem cell genes including, WNT5A, IRF4, GATA2, and NGF supporting that impaired replating abilities could be partially prevented. Damage to the cells in Space or Ground BR long culture was not permanent, and could be reversed by replating them onto a supportive, nourishing niche. Nanostring RNA analysis confirmed that cellular damage appeared to be transient and could be reversed.

Keywords: nano-bioreactor, space, HSC

3:45 PM – 4:45 PM

345

A MICROFLUIDIC PLATFORM INTEGRATING FUNCTIONAL VASCULARIZED ORGANOIDS-ON-CHIP

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Despite rapid advancement in organoid and microvascular network systems, establishing a functional vasculature within organoids remains a formidable challenge in tissue engineering. Typically, organoids require transplantation into host animals to establish functional vascular circulation, an expensive process that lacks scalability for larger scale toxicity or drug screening. Our aim was to develop an in vitro system to convincingly achieve intravascular perfusion within organoids through functional anastomosis with neighbouring endothelial networks. Our solution leverages a synergistic blend of microfluidics and stem cell technology, resulting in a novel microfluidic serpentine device designed for organoid vascularization on-chip. We validated our method using human iPSC-derived Blood Vessel Organoids (BVOs), which comprise fine, dense capillary networks. These BVOs were intricately integrated into a HUVEC vascular bed. Cultured on-chip under flow conditions, the cells self-organized into interconnected networks, reflecting the physiological hierarchical organization of the in vivo vascular tree. The regions upstream and downstream with arteriole-venule sized vessels exhibited strong expression of the smooth muscle cell marker SM22, while the narrow capillaries in the BVOs maintained authentic hollow lumen structures with pericyte and basal membrane coverage. Importantly, the organoids' inner vasculature was perfusable, as demonstrated by live microbeads perfusion. Variable culture conditions (with and without flow / HUVEC network), revealed through RNA sequencing that vascularization and flow are vital for organoids' growth, function and maturation. To further showcase the utility of our microfluidic platform, we report enhanced functionality of pre-vascularized insulin-secreting pancreatic islet spheroids, when cultured using our approach. Altogether, our work provides a new inroad into exploring diverse topics, such as organoid lifespan enhancement through vascularization, exposure to drugs, nucleic acids or metabolic stress.

Keywords: organoid-on-chip, vascularization, microfluidics

3:45 PM – 4:45 PM

347

LONG-TERM EX VIVO HEMATOPOIETIC STEM CELL EXPANSION AS A TOOL TO IMPROVE THE FEASIBILITY OF GENE CORRECTION

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Autosomal recessive osteopetrosis (ARO) is a rare genetic disease, that affects osteoclast function causing bone marrow (BM) failure. The standard treatment is the hematopoietic stem cell transplantation (HSCT), but its success is limited by several constraints. Conversely, gene therapy (GT) could minimize the immune-related complications of allogeneic HSCT. Hematopoietic stem cells (HSCs) are very rare cells within the BM and are virtually absent from ARO BM. Ex vivo HSC expansion has been developed to improve the hematopoietic reconstitution in low-cell dose grafts. We are optimizing the combination of HSC expansion and gene therapy in order and to improve the feasibility of gene correction to treat osteopetrosis and other genetic diseases. We sorted HSCs (LSK CD34- CD150+) from BM and spleens of adult mice and pups. Cells were cultured in two different conditions: high O2 and low O2. We transduced cells using PGK.GFP lentiviral vector and we performed FACS analysis to examine the growth and phenotype of the expanded HSC cultures. Expanded cells were transplanted in vivo at different doses of HSC to test the long-term engrafting capacity. We checked for engraftment by FACS analysis in peripheral blood, BM, spleen and thymus. Flow cytometry analysis of culture showed that cells cultured with high O2 are characterized by a higher number of differentiated cells. Instead, HSC expanded cells derived from the low O2 culture maintain the expression of primitive LSK markers. After transduction, all cells express the GFP marker, with higher vector copy number (VCN) in cells cultured in low O2. Mice transplanted with untransduced low O2 cells showed higher engraftment than littermates transplanted with untransduced high O2 cells. In contrast, mice transplanted with transduced high O2 cells showed a higher engraftment than low O2 condition. In tissues, the percentage of GFP+ donor derived cells were comparable between the two conditions, showing that the transduction does not impact on the fitness to engraft long-term. We demonstrated that HSC expanded cultures can be efficiently transduced in vitro without impacting their engraftment potential. The long-term ex vivo HSC expansion protocol may improve the feasibility of gene correction to treat those diseases, like Osteopetrosis, in which limited HSC numbers are available.

Funding Source: Fondazione Telethon Core Grant E2 (TTAVC0522TT)

Keywords: gene therapy, osteopetrosis, ex vivo HSC expansion

3:45 PM – 4:45 PM

349

TRANSFER OF METABOLICALLY ENHANCED MSC-DERIVED MITOCHONDRIA IMPROVES THE ENGRAFTMENT POTENTIAL OF HUMAN UMBILICAL CORD BLOOD (UCB) CD34+ HEMATOPOIETIC STEM/PROGENITOR CELLS (HSPCS)

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UCB contains HSPCs with high expansion and low immunogenicity. Nonetheless, their use is limited due to low cell counts, resulting in delayed immune reconstitution. The role of mitochondria (MT) in hematopoietic cells' fate and function has been described as a regulator of self-renewal, differentiation, and engraftment. Reports of mitochondrial transfer (MitoT) from UC-MSC have resulted in better disease prognosis and boost of metabolic and functional parameters on target cells. Hence, we theorised that MitoT is one of the mechanisms by which the bone marrow (BM) niche exerts its function on HSPCs. The occurrence of MitoT was studied through cell culture and validated in a tamoxifen-induced (TAM) stroma-specific MT-labelled transgenic model (Dendra+ MT). Dendra+ MT originated from BM Prx1+ cells, which are mostly of mesenchymal origin. In addition, we studied how metabolically reprogrammed UC-MSCs-derived MT regulate HSPCs fate and function using a repopulating immunodeficient mice model. We report that adult mouse BM HSPCs can acquire Dendra+ MT upon TAM treatment under physiological conditions. The observed physiological transfer of MT was successfully reproduced in vitro, where HSPCs acquired MitoTracker+ MT in co-culture conditions with UC-MSCs dependent on the dose and hematopoietic recipient. Next, we studied the impact of MitoT on different hematopoietic subpopulations. However, to avoid the interference of other paracrine factors secreted by MSCs, we isolated MT from UC-MSCs under two different metabolic statuses (glycolytic and OXPHOS). We showed that OXPHOS UC-MSCs-derived MT-treated HSCs (HSPCsMToxp) led to high MT mass, glycolytic program, low Δκm, low ROS content and differentiated into CFU-GEMM. Furthermore, when replating single colonies, HSPCsMToxp led to the production of new colonies, suggesting the presence of self-renewing HSCs. Finally, we examined the engrafting and repopulation capacity of HSPCs. We demonstrated that HSPCsMToxp led to faster and higher short-term engraftment of total hematopoietic (2.96-fold) and lymphocytic (3.80-fold) lineages in the recipient. The effects of MitoT on increasing the expansion of HSPCs while preserving their stem cell potential suggest that MitoT could represent a therapeutic strategy to improve transplant efficiency in patients.

Funding Source: ANID-FONDECYT #1211749. ANID-Basal funding for Scientific and Technological Center of Excellence, IMPACT, #FB210024.

Keywords: hematopoietic stem/progenitor cells (hspcs), mitochondria, mitochondrial transfer/transplantation

3:45 PM – 4:45 PM

351

ENHANCING CRISPR GENE CORRECTION IN HEMATOPOIETIC STEM/PROGENITOR CELLS

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CRISPR/Cas9 is a powerful tool for precise gene correction of monogenic blood diseases, such as deficiency of adenosine deaminase 2 (DADA2), a rare disorder that causes recurrent inflammation and vascular damage. However, the efficiency of homology-directed repair (HDR) in CD34+ hematopoietic stem/progenitor cells (HSPCs) is low and limits the therapeutic potential of this approach. In this study, we used a Finnish founder mutation (R169Q) in ADA2 as a model locus for gene correction. We introduced four silent single nucleotide polymorphisms (SNPs) by electroporation using RNPs (ribonucleoproteins) and single-stranded oligodeoxynucleotide (ssODN) repair template in wild-type CD34+ cells isolated from umbilical cord blood. We evaluated the effect of Compound X, a small molecule inhibitor selected from a chemical compounds screen in T cells, on enhancing HDR in CD34+ cells. We also compared two methods for measuring the colony forming unit (CFU) output of edited cells: a solid assay based on morphology and pigment of cells, and a liquid assay based on cell surface markers (CD14, CD15, CD235), enabling a higher-throughput setup. Using droplet digital PCR (ddPCR) as a method to quantify edited cells, we found that Compound X increased HDR up to 80% in CD34+ cells, but reduced CFU output by 2-3 folds. Our results suggest that Compound X is a promising HDR modulator for gene correction in HSPCs, and could lead to the development of more effective gene therapies for monogenic blood diseases in the near future; however, its side effects need to be further investigated. We are currently performing in vivo experiments with Compound X and exploring novel editing strategies, while also working to align our protocols with current good manufacturing practice (cGMP) standards. Furthermore, we aim to assess and optimize the efficacy of Compound X, as well as similar compounds, in long-term repopulating hematopoietic stem cells (LT-HSCs), given their significant clinical importance and the crucial need to enhance editing efficiency in these cells.

Keywords: hematopoietic stem cells, genome editing, homology-directed repair (HDR) enhancement

3:45 PM – 4:45 PM

353

BASE EDITING ENABLES SINGLE-STEP, MULTIPLEX GENOME EDITING IN HUMAN IPSCS WITH NEGLIGIBLE GENOTOXICITY

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Next generation cancer therapies using genome edited immune cells is a rapidly evolving yet promising avenue of personalized medicine. Human induced pluripotent stem cells (iPSCs) are a genetically tractable platform for scalable in vitro production of engineered immune cells; however, multiplex editing using previous generation Cas9 nucleases to enhance effector function and overcome the immunosuppressive tumor microenvironment (TME) poses significant risks for genotoxicity and chromosomal translocations. To mitigate risks associated with double stranded breaks (DSB) induced by CRISPR/Cas9 nuclease, we utilized base editing (BE) to install precise nucleotide edits without DSB induction, achieving highly efficient, single step multiplexed genome editing in human iPSCs with minimal genotoxic side effects. Comparing Cas9 nuclease and adenosine BE (ABE8e) delivered as synthetic mRNA, we simultaneously edited 9 genes previously linked to enhanced function in immune cells derived from human iPSC. In singleplex, both Cas9 and ABE8e achieved >90% editing efficiencies at a single target (B2M). Conversely, in the multiplex setting, ABE8e achieved high editing rates (70% +/- 30%) compared to only 9% (+/- 8%) for Cas9. Cell toxicity was nearly undetectable with ABE8e, but was notably higher with Cas9 as evidenced by lower cell recovery and a (5-35x fold) induction in levels of P21 transcription, a protein involved in DNA damage response and cell cycle arrest. This result confirmed substantial impacts to genomic stability, stress, and DNA damage with Cas9 that is absent with ABE8e. Furthermore, cytogenetic analysis of Cas9-edited populations revealed chromosomal abnormalities, inversions, and random loss events linked to targeted loci that were notably absent in cells edited with ABE8e. These findings confirm that base editing allows single-step highly multiplexed editing of human iPSCs without the genotoxicity associated with nuclease-induced DSBs. We are currently conducting whole genome sequencing to further characterize chromosomal aberrations in these experiments. Our platform supports rapid production of highly multiplex-engineered iPSCs using ABE8e, ideal for in vitro characterization of diverse engineered immune cells with enhanced function for cancer immunotherapy and beyond.

Keywords: iPS engineering/genome engineering, base editing, adoptive cell transfer/immunotherapy

3:45 PM – 4:45 PM

355

DEVELOPMENT OF A NEXT-GENERATION EX VIVO DELIVERY PLATFORM USING SILICON NANONEEDLES FOR PHYSICAL CARGO DELIVERY INTO DIFFICULT-TO-TRANSFECT STEM CELLS

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The use and development of genetically modified stem cells as therapies is limited by a lack of efficient and safe delivery methods. Both viral vectors and electroporation-based biomolecule delivery have numerous challenges, including patient safety concerns, cell viability issues, cargo size limitations, cell type restrictions, a lack of dose control, and a lack of precision. These limitations compound and can cause cell functionality compromises that are observed either in the initial production of a therapy or downstream in the patient. Mekonos is developing an advanced delivery platform that will enable new innovative approaches across diverse cell types and payloads by overcoming many of the existing ex vivo delivery challenges – offering unprecedented viability, efficiency, and consistency in molecular payload delivery. Our novel cell engineering platform utilizes a controllable array of solid silicon nanoneedles to enable direct intracellular access of multiplexed biomolecular payload. Silicon nanoneedles are made using an advanced semiconductor process to develop unique chips that are scalable and precise, with nanoneedles of specific heights and widths. Cells are trapped using a microfluidic system that enables precise localization of each nanoneedle tip to a single cell (one needle per cell"), enabling a controlled, consistent delivery that is not possible with conventional delivery methods. The biomolecular payloads are adsorbed onto the surface of each nanoneedle using proprietary surface chemistry for controlled release inside the cell. Our nanoneedle delivery method can deliver nucleic acids, proteins, and RNP complexes into a wide variety of stem cells with higher viability and efficiency than current industry standard methods. We will present data showing - manipulation of multiple fragile cells (e.g., HSCs, iPSCs) with high efficiency (70%+) and high viability (95%+); nuclear delivery of DNA + RNP; large plasmid delivery (15kb+); and multiplexed delivery (simultaneous delivery of three plasmids). We believe that the combination of our semiconductor, microfluidic, and surface chemistry technologies will drive the future of stem cell therapy development and manufacturing through hyper-
precise cell engineering.

Keywords: ex-vivo delivery, cell therapy, gene editing

3:45 PM – 4:45 PM

357

IMPEDANCE-BASED SINGLE-CELL DISPENSING AND SCREENING FOR MONOCLONALITY VERIFICATION OF HUMAN PLURIPOTENT STEM CELLS

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Human induced pluripotent stem cells (hiPSCs) hold immense potential in regenerative medicine and disease modelling, yet obtaining monoclonal colonies remains challenging. This study presents a novel approach utilising an impedance-based single-cell dispenser (DISPENCELL™) and a high-throughput fluorescence-based imager (CloneSelect® Imager FL) for efficient isolation and screening of hiPSCs. The workflow involves dispensing single hiPSCs into 96- and 384-well plates followed by imaging for monoclonality verification. The DISPENCELL's gentle handling minimises cell stress, ensuring high viability and deposition efficiency. Automated imaging allows for rapid colony counting and monitoring of growth. Analysis features enable precise identification of monoclonal colonies based on size and shape criteria. The integration of DISPENCELL and CSI FL into an automated platform enhances throughput and reliability, facilitating gene editing and cell line development. Our results demonstrate the feasibility and effectiveness of this approach in obtaining monoclonal hiPSC colonies with minimal manual intervention. The Monoclonality Report feature provides objective evidence of clonality, streamlining the cell line development process. Overall, this innovative workflow offers a robust solution for single-cell dispensing and screening of hiPSCs, paving the way for advancements in stem cell research and therapeutic applications.

Keywords: iPSC, single-cell isolation, cell line development

3:45 PM – 4:45 PM

359

AUTOLOGOUS HUMANIZED MESENCHYMAL STEM CELL NICHES FOR SUCCESSFUL ENGRAFTMENT OF AML WITH DIVERSE RISK AND CYTOGENETIC BACKGROUND

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Acute myeloid leukemia (AML) is a heterogeneous group of myeloid malignancies in the human bone marrow (BM) characterized by excessive blast production and complex cytogenetics. Humanized mouse models represent the gold standard in assessing human malignancies, including AML, but they lack reproducibility and are prone to fail, especially in intermediate and low risk patient samples. This has significantly hindered in-depth analyses of the human AML development and treatment. We aimed at addressing these long-standing issues by employing an autologous humanized ossicle model. This human mini-bone encompasses AML niches that contain ectopic human bone, a mesenchymal stem/stroma cell (MSC) compartment and the AML hematopoietic cells, all from the same patient. To achieve this, we collected fresh BM aspirates from 7 AML patients and isolated the CD3-CD45+/CD34+ hematopoietic cells, which we cryopreserved, and expanded the rest for MSCs. MSCs, labelled with TdTomato, were then mixed with OssiGel, a cell-free extracellular matrix, and implanted subcutaneously in NSG mice. At 4 weeks, microtomography verified bone formation, followed by intra-ossicle transplantation of CD45+/CD34+ cells from the same patient. We monitored human engraftment in the periphery/mini-bones, and analyzed femurs and mini-bones for the presence of MSCs and hematopoietic cells by FACS, confocal microscopy and single cell omics. We successfully generated mature human mini-bones for all tested AML. Microtomography revealed leukemia donor-dependent differences in bone density. Successful long-term establishment of a human MSC and malignant cells' niche (16-33 weeks) was possible even with favorable (i.e. CBF1 mutated) and intermediate risk patient samples. Leukemia cells were detected in corresponding mouse femurs, albeit at low frequencies. Interestingly, single cell analysis evidenced marked differences in the AML populations developing in mouse femurs versus human mini-bones. These data provide the basis for a robust personalized modeling of human AML irrespective of the patient's risk classification, further allowing the interrogation of leukemia-niche cells' interactions. Further validation of the model may facilitate the development of patient-tailored therapies.

Funding Source: ERC Starting grant, Cancerfonden, Knut and Alice Wallenberg Stiftelsen

Keywords: humanized ossicles, mesenchymal niche, acute myeloid leukemia

3:45 PM – 4:45 PM

361

ENHANCED ANTI-TUMOR EFFECTS OF ENGINEERED MACROPHAGES DERIVED FROM PLURIPOTENT STEM CELL-DERIVED MYELOID CELL LINES

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Even after chimeric antigen receptor (CAR)-based immunotherapy has dramatically changed therapeutic approaches for malignancies, balancing therapeutic efficacy with labor and financial cost remains a major problem for immunotherapy. Current study developed the more powerful and less labor intensive approach to chimeric antigen receptor (CAR)-macrophage therapy for cancer and demonstrated its therapeutic effects of anti-HER2 CAR macrophages generated from human pluripotent stem cell (PSC)-derived immortalized myeloid cell

lines (ML). These ML-derived CAR macrophages (CAR-ML-MPs) established from two different PSC strains (KhES1 and 409 B2 strains) exhibit potent antigen-specific killing activity against HER2-expressing tumor cells by phagocytosis in vitro (p=0.0481 and p=0.0072 against Luc-HER2-K562 and p=0.0200 and p=0.0288 against OCUB-F in KhES1 and 409 B2 strains, respectively) and effectively inhibit tumor progression in vivo, resulting in a more than 1.5-fold increase in the longest survival of the tumor-bearing mice. CAR-ML-MPs provide a promising off-the-shelf cellular resource for tumor adoptive cell immunotherapy, solving the cost and time problems associated with conventional CAR-based immunotherapy.

Keywords: chimeric antigen receptor (CAR), pluripotent stem cell-derived macrophage, cell therapy

3:45 PM – 4:45 PM

367

CHIMERIC ANTIGEN RECEPTOR (CAR) - EXPRESSING M1 MACROPHAGES TARGET EFFICIENTLY EGFRVIII-MUTATED GLIOBLASTOMA CELLS

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Autologous or induced pluripotent stem cell (iPSC)-derived chimeric antigen receptor (CAR-T)-cells represent major promising cell therapy approaches. CAR-T cells have been efficiently used in hematological malignancies but their efficacy in solid tumors remain limited due to the poor infiltration, expansion and low persistence of the CAR-expressing T-cells in solid tumors. CAR therapies via the use of macrophages, offer a promising avenue due to their unique ability to infiltrate tumors and to initiate phagocytosis. To generate a model of M1 CAR-macrophage for cancer therapy, we have designed a novel CAR monocyte-construct to target EGFRvIII-expressing glioblastoma cells (DK-MGϫEGFRvIII) using THP-1 monocytic cell line able to differentiate towards macrophages. As it is necessary to use M1 macrophages to target solid tumors, in preliminary experiments we have established the efficient generation of M1 from THP-1 cells. The CAR structure comprises a ScFv recognizing specifically EGFRvIII and MEGF10 intracellular domain which enhances tethering and phagocytosis activity. CAR and Mock THP-1 monocytes were generated by using lentiviral transduction. CAR-Macs were sorted in order to obtain a 100% CAR-expressing cells. Both transgene expressing cells are GFP positive for tracking the cells during the assays. M1 macrophages were successfully generated by differentiating THP-1 monocytes using PMA (10ng/ ml) for 24h then LPS+IFNκ (100ng/ml and 20ng/ml respectively) for 48h. These cells have been characterized by the expression macrophage markers CD45, CD14, CD11b as well as the M1 marker CD80 (~70-80%). To evaluate their ability of phagocytosis, we co-cultured THP-1 derived mock or CAR-expressing macrophages with the target DK-MGϫEGFRvIII cells. Confocal microscopy experiments revealed highly efficient phagocytosis of DK-MGϫEGFRvIII cells by CAR-Macs (~60%) as compared to Mock-Macs (~15%). The evaluation of the pro-inflammatory and killing potential of the anti-EGFRvIII CAR-Mac- using ELISA as well as transcriptome experiments are in progress. This model is now being translated to iPSC-derived macrophages to generate a clinically applicable future cell therapy approach in all solid tumors expressing mutated EGFRvIII.

Funding Source: INSERM, ANR, PEPR

Keywords: IPSC-derived macrophages, cancer, chimeric antigen receptors

3:45 PM – 4:45 PM

TOPIC: KIDNEY

369

CHARACTERISING THE VARIABLE OCCURRENCE OF NON-NEPHRON FORMING CELL TYPES DURING STEM CELL-DERIVED KIDNEY ORGANOID DIFFERENTIATION

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Human iPSC-derived kidney tissue has the potential to provide renal replacement therapy for patients with kidney disease whose only current treatment options are kidney transplantation and dialysis. In our approach, stem cells are differentiated to kidney cell types in a single protocol, resulting in a heterogeneous population that includes critical populations that form functional nephrons, as well as a proportion of non-nephron forming cells. While some non-nephron forming cells are appropriately patterned to renal stroma, others represent off-target populations. The identity and proportion of non-nephron forming cells

varies by protocol, cell line and batch. Such technical variability is a major barrier to manufacturing quality control, necessitating methods to accurately identify and quantify these populations. In this study, we have used high-resolution single cell profiling to precisely identify all cell populations present and define these as on-target or off-target. We then correlate population gene markers to variable genes identified in a dataset of over 200 low-resolution bulk transcriptomes sampled throughout routine differentiation experiments performed in our laboratory over three years. By comparing this variability data to organoid outcomes we can gain insights into batch-level population changes and identify a set of quality markers that will support the development of stem cell-derived kidney therapies.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) is supported by a Novo Nordisk Foundation grant number NNF21CC0073729

Keywords: transcriptomics, kidney organoid, quality control

3:45 PM – 4:45 PM

371

IDENTIFICATION OF IMMUNE-MODULATING LIGANDS AND CYTOKINE EXPRESSION PATTERNS IN IPSC-DERIVED KIDNEY ORGANOIDS UPON INTERFERON STIMULATION

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Regenerative therapies, such as iPSC-derived kidney organoids, show a unique potential to be used as off-the-shelf therapies to regenerate malfunctioning tissues. However, allogeneic transplantation poses rejection risks due to human leukocyte antigen (HLA) mismatches and pro-inflammatory signaling. To date, the immunogenicity of organoids remains poorly understood, leaving uncertainty regarding immune-mediated rejection. The aim of our study is to elucidate immunomodulatory mechanisms to allow in vitro risk profiling of iPSC-derived kidney organoids. To this end, an immune response was simulated by exposing kidney organoids to interferon gamma in five independent cultures. Immunomodulatory capacity was assessed via quantification of pro- and anti-inflammatory cytokines, as well as HLA and co-stimulatory or inhibitory ligands, using Luminex and multiplex flow cytometry. Pro-inflammatory cytokines were significantly enhanced in stimulated (ST) compared to control (C) organoids; of which five cytokines had particular biological relevance. IL-5 (C: 34,25 vs ST: 584,35 pg/mL) in ST-organoids was comparable to serum levels in patients diagnosed with impaired transplant function. IL-15 (C: 0 vs ST: 378,79 pg/mL), MCP-1 (C: 703,66 vs ST: 1032,15 pg/mL) and IP-10 (C: 113,66 vs ST: 20293,26 pg/mL) in ST-organoids were resembling serum concentrations observed in patients experiencing acute kidney transplant rejection. In line with this HLAs (-A, Bw6, -C, -DR) were significantly upregulated upon stimulation, allowing allorecognition by T- and natural killer (NK) cells. The expression of co-stimulatory ligands together with HLA mismatches is essential for T- and NK cell activation. Consequently, the significant upregulation of stimulatory MICB and CD54 in ST-organoids shows an increased risk for T- and NK cell-mediated rejection. In contrast, reduced MICA activating ligand expression and an elevated IL-1ra/IL-1b ratio may elicit potential anti-inflammatory signaling. Overall, our study reveals a potential risk of immune cell activation. Our findings contribute to mechanistic insights, enabling targeted pathway modifications to mitigate rejection. In the continuation of this study, co-cultures of kidney organoids with immune cells will facilitate the formulation of an in vitro risk profile.

Keywords: kidney organoids, immunogenicity, HLA

3:45 PM – 4:45 PM

TOPIC: LIVER

373

LIGHT REGULATES LIVER METABOLISM THROUGH MELANOCYTES

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Numerous studies confirm the link between circadian rhythm disruption and metabolic disease development. Light, as the “switch” of the Earth, is the most important zeitgeber in the circadian rhythm system, but its metabolic impact has been oversimplified: light sensed by ipRGCs regulates the SCN, affecting lipid and glucose levels via neuroendocrine routes. The liver, as the metabolic center of the body, plays a central role in the process of circadian regulation of metabolic homeostasis. Our research found that melanocytes, originating from the neural crest, express opsins, indicating a photoreceptive function. Additionally, at the gene expression level, it has been proven that melanocytes express key enzymes for hormone synthesis, confirming that melanocytes also possess certain neuroendocrine functions. Since melanocytes, like the ipRGC-SCN pathway, have photoreceptive and neuroendocrine functions, could light also influence the circadian rhythm of the liver through melanocytes, leading to changes in body metabolism? Our laboratory has successfully constructed hiPSC-derived liver organoids and hiPSC-NASH models composed of hepatocytes, hepatic stellate cells, and liver sinusoidal endothelial cells. By co-culturing human melanocytes with hiPSC-derived hepatocytes in three dimensions, we found that melanocytes can affect the fat synthesis function of hiPSC-derived hepatocytes, but the molecular mechanisms involved require further exploration. Therefore, using CRISPR-Cas9, we constructed a fluorescent reporter hepatocyte line for NR1D1—a core clock gene known to be related to endocrinology and metabolism. We then exposed the melanocyte-liver organoid co-culture model to light of different

wavelengths, and by tracking NR1D1 in hepatocyte. Furthermore, to capture the effects of light on the liver's biological clock and metabolism through melanocytes. It breaks through the traditional "light-ipRGCs-SCN-liver" pathway, which sees light regulating body metabolism through the central biological clock. It proposes a new photoreceptive pathway, "light-melanocytes-liver," and studies the molecular mechanisms by which this pathway affects metabolism, thereby understanding the connection between circadian rhythm and human metabolism on multiple scales, dimensions, and levels.

Keywords: liver metabolism, circadian rhythm, light

3:45 PM – 4:45 PM

375

OPTIMIZATION OF INTRA-HEPATIC CHOLANGIOCYTE ORGANOID TRANSFECTION

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Intra-hepatic Cholangiocytes line the epithelium of the biliary tree located within the liver. This network of conduits drains the liver from toxins produced by the hepatocytes. Disease targeting this cell type are life threatening and there is currently no cure against end stage cholangiopathies. More recently, cholangiocytes have also been shown to be involved in regenerative processes occurring during chronic liver disease progression. Indeed, they seem to be able to transdifferentiate into hepatocytes. However, the molecular mechanisms involved remain to be fully investigated. To address this knowledge gap, we want to perform gain and loss of function experiments in intra-hepatic cholangiocytes organoids (ICOs). Indeed, cholangiocytes can be grown in vitro while maintaining their key characteristics including the expression of specific markers but also the capacity to generate hepatocyte like cells. Therefore, ICOs provide an important platform to study regenerative mechanisms occurring during liver diseases. Here, we described the development of methods to overexpress and knock down transgene in ICOs. For that, we have compared the efficiency of different transfection methods for the stable expression of reporter genes in ICOs. The cells were transfected using different methods including electroporation-based methods like nucleofection or transfection with the NEPA21 system and Lipofection. The efficacy of each method was analyses on different ICOs lines by immunofluorescence and FACs analyses. In addition, the resulting organoids were further characterized to demonstrate that transfection did not affect their basic characteristics. These experiments did establish the optimal transfection method for genomic engineering in ICOs. Such protocol pave the way for a broad range of applications including genetic screens and functional studies.

Keywords: intra-hepatic cholangiocyte organoids, transfection, liver

3:45 PM – 4:45 PM

377

SPATIO-TEMPORAL LIVER DYNAMICS SHAPES HEPATOCYTE HETEROGENEITY AND IMPACTS IN VIVO GENE THERAPY

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The liver is a central organ for metabolism and hepatocytes are the cells responsible for most of its functions. For this reason, the liver is an attractive target organ for in vivo gene therapy. Lentiviral vectors (LV) integrate into the target cell chromatin and are maintained as cells divide, thus potentially allowing life-long genetic correction even after administration to young individuals. Several previous studies investigated the cell types involved in tissue homeostasis and regeneration, however the mechanisms underlying post-natal liver growth and establishment of the mature hepatocyte phenotypes remain to be fully understood. Here we investigated liver tissue dynamics in mice and its impact on in vivo LV gene transfer. We evaluated clonal proliferation of hepatocytes in Alb-Cre/Rosa26-Confetti mice during growth and showed that approximately 20% of the cells generates continuously growing clusters covering more than 90% of the adult liver, indicating that only a fraction of newborn hepatocytes contributes to growth. We administered LV intravenously to newborn mice and showed a 4-fold higher LV-positive liver area compared to adults, with preferential transduction of the peri-central compared to the peri-portal area in adult-treated mice. We performed longitudinal spatial transcriptomics in the liver and observed that, in young mice, hepatocytes displayed a transcriptional profile similar to that of adult peri-portal hepatocytes, regardless of their position in the lobule. Interestingly, in adults, we identified high expression of the proteasome genes in peri-central hepatocytes, which were less permissive to LV gene transfer. Thus, we administered the proteasome inhibitor Bortezomib to adult mice before LV and achieved 4-fold higher liver gene transfer compared to LV-only treated mice, and a switch from peri-portal to peri-central transduction bias. These data suggests that proteasome activity acts as a restriction factor of LV mediated hepatocyte gene transfer, which can be overcome by its transient inhibition. Overall, our findings provide new

insights into the spatio-temporal dynamics of the liver during post-natal growth and hepatocyte heterogeneity, which extends our understanding of liver biology and have important implications for therapeutic applications.

Keywords: gene therapy, liver, hepatocyte heterogeneity

3:45 PM – 4:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

379

TRACING GENOMIC INSTABILITY IN INDUCED MESENCHYMAL STROMAL CELL MANUFACTURE: AN INTEGRATION-FREE TRANSFECTION APPROACH

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In this comprehensive study, we systematically explored the genomic alterations occurring from the initiation of induced pluripotent stem cell (iPSC) generation to the induced differentiation of mesenchymal stromal cells (iMSC). We observed ten copy number variations (CNVs) and four single nucleotide variations (SNVs) during the reprogramming, differentiation, and passaging phases. All four Sendai virus-induced iPSC (SV-iPSC) lines and two of the five episomal vector-induced iPSC (Epi-iPSC) lines exhibited CNVs during reprogramming. In contrast, differentiation-induced CNVs were observed in two Epi-iMSC lines. CNVs involved in duplication significantly affected genes associated with fundamental cellular functions, particularly those involved in replication and loss of genes essential for regulating cell division. SNVs have been identified exclusively in SV-derived cells during cell passaging and differentiation. A distinctive highlight of our study is the identification of TP53 mutations, a recognized indicator of genomic instability. Our research underscores the imperative need for a comprehensive genomic evaluation combining various genomic analyses to confirm the safety and efficiency of iMSCs for therapeutic use.

Funding Source: This research was supported by a grant (22202MFDS127) from Ministry of Food and Drug Safety in 2022-2024, the Ministry of Science, ICT (MSIT) (2022M3A9H1014126) and the MSIT/ the Ministry of Health & Welfare (23A0106L1).

Keywords: genomic instability, induced pluripotent stem cells (iPSCs), mesenchymal stromal cells (MSCs)

Clinical Trial ID number: IRB (P01-201910-31-005)

3:45 PM – 4:45 PM

381

HARNESSING THE POWER OF BIO ONE 3D BIOPRINTER FOR HIGH-THROUGHPUT STEM CELL APPLICATIONS

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Bioprinting, a transformative technique in stem cell research, enables 3D deposition of cells encapsulated in hydrogels, mimicking biological tissues. Stem cells, embedded in bioinks like type 1 collagen, can be printed into 3D constructs for in vitro culture, implantation, disease modelling, and drug testing. However, the scalability of natural matrices in 3D bioprinted models for high-throughput applications remains a challenge. CELLINK's BIO ONE biodispenser addresses this by integrating temperature control, bioink crosslinking and high-precision extrusion. To demonstrate the applicability of the BIO ONE in stem cell research, we used it to print neutralized type 1 collagen, TeloCol®-10 mixed with mesenchymal stem cells (MSCs), achieving the collagen concentration of 6 mg/mL. The MSCs embedded in TeloCol® droplets were dispensed into 96-well plates, thermally crosslinked for 20 minutes, and incubated after the addition of cell medium. Cell viability, cell number, and morphology were analysed using the ECHO REVOLVE™ microscope and the LIVE/DEAD fluorescent kit at 1-, 3-, and 7-days post-dispensing of 1 µL droplets. For cell homogeneity, 80 µL/ well of MSCs embedded in neutralized TeloCol® were incubated with PrestoBlueTM HS and fluorescence reading was performed by a multimode plate reader (VICTOR® Nivo™), 1-day post-dispensing. Hand casted droplets were used as control. The cell viability was approximately 90%, and the cell count consistently increased over the 7-day culture period. The captured images also showed comparable cell stretching in the MSCs used for BIO ONE dispensing, mirroring the behaviour observed in the control group. The cell homogeneity was also similar to the control group. These results confirm that the bioprinting process using BIO ONE does not interfere with the normal physiology of MSCs. Therefore, it can be effectively utilized in various stem cell research approaches, including personalized medicine, regenerative therapies, and disease modelling.

Keywords: bioprinting, collagen, mesenchymal stem cells

3:45 PM – 4:45 PM

383

EXPLORATION OF THE AMELOBLASTIC POTENTIAL OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED): IMPLICATIONS FOR BIO-TOOTH ENGINEERING

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Stem cell-based advances in bio-tooth engineering hold significant potential for addressing tooth repair or loss. The limited availability of ameloblast-generating dental stem cells markedly impedes the advancement of tooth bioengineering. The current study investigated the potential for stem cells from human exfoliated deciduous teeth (SHED) to regenerate teeth and showed odontogenic features, including amelogenesis, when co-cultured with human gingival epithelial cells (GECs) to replicate the complex interaction between mesenchymal and epithelial cells. In the current study, we assessed the odontogenic potential of SHED (n=10) by co-culture with human gingival epithelial cells (GEC). The cultures were maintained for different time intervals, viz. days 1, 7, and 12. Next, the ameloblast and odontoblast differentiation of SHED and GECs was investigated by examining cellular morphological alterations, immunocytochemistry (ENAM, AMLX), quantitative gene expression analysis (MMP-20, AMBN, AMGN, TFT, KRT-14, and DMP), and mineralization estimation using von Kossa and alkaline phosphatase assays. Our investigation suggested that SHED cellular morphology was altered and exhibited ameloblast-like and odontoblast-like morphologies. Compared to SHED and GEC-Alone control cultures, direct co-culture on days 7 and 12 showed a statistically significant increase in the expression of ameloblast and enamelin-related genes. AMGN expression increased 15-fold from days 1 to 12 ($p < 0.0001$). The co-culture of SHED with GKC induced differentiation of SHED and enhanced the expression of amelogenin-X and enamelin protein, as confirmed by specific staining. Co-culturing epithelial cells with SHED initiated mineralization, as shown by increased alkaline phosphatase activity and black-colored mineralized nodules in differentiated cells following von Kossa staining. Our work concludes that heterotypic recombination between SHED and epithelial cells induces odontogenic development and differentiates into ameloblast-like cells. These results advance our knowledge of the functions of SHED in regenerative dentistry and biotooth engineering.

Keywords: human exfoliated deciduous teeth stem cells, amelogenesis, biotooth engineering

3:45 PM – 4:45 PM

385

DEVELOPMENT OF AN IMAGE-BASED EVALUATION METHOD FOR GMP-GRADE MSC EXPANSION AND CRYOPRESERVATION MEDIA USING THE CELIGO IMAGE CYTOMETRY

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Mesenchymal stem cells (MSCs) are multipotent, self-restorative, can differentiate into multiple cell types, and are increasingly being considered for use in clinical therapy. However, they require specific medium for optimal culture conditions and long-term storage. Therefore, development of a GMP-grade medium manufacture with qualified traceable components under consistent and controlled workflow is critical for improving the efficiency, safety, and cost of cell therapy treatments. Media development requires assessment of viability, expansion efficacy, phenotypic expression, and morphology. However, traditional methods such as microscopy and flow cytometry are time-consuming and may compromise morphology and surface marker expression. In this work, we developed an image-based evaluation method using the Celigo® Image Cytometer for assessing media development metrics for a human MSC GMP, serum-free growth medium and a DMSO-free cryoprotection solution. Image cytometry has been employed to obtain high-speed, high-throughput data acquisition and analysis while minimizing perturbation to cell cultures by direct cell analysis in plates. We investigated the effects of medium composition including basal medium, growth factors, protein, mineral, AND lipid concentrations on MSC expansion and cryopreservation. Cell proliferation assays using Hoechst and propidium iodide were performed to simultaneously determine viability and population expansion directly in 24-well plates after storage in LN2 in the cryoprotectant solution or directly with primary cells. In addition, we performed visual assessment of morphology and phenotype as a quick QC process prior to flow cytometric analysis. Finally, we examined the differentiation potential of MSCs into adipocytes and osteocytes. The results demonstrated comparable cell growth, viability, morphology, phenotype, and differentiation potential against other industry available MSC cryoprotectant solutions with DMSO and culture media. The Celigo Image Cytometer was able to perform rapid, improved throughput screening of multiple conditions in DOE paradigms compared to traditional microscopy methods. The method enabled a more streamlined process for data acquisition and analysis, which may increase the effectiveness of media development.

Keywords: mesenchymal stem cell, cell culture and cryopreservation, cell image cytometer

3:45 PM – 4:45 PM

387

SAMPLING AND GERMLINE DIFFERENTIATION OF AVIAN MESENCHYMAL STEM CELLS: TOWARDS A RELEVANT APPROACH FOR GENETIC RESOURCES PRESERVATION?

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The preservation of avian genetic resources uses methods that remain unsatisfying. Indeed, cryopreservation of sperm is poorly effective and physically impossible for oocytes. Regarding primordial germ cells, collection, culture and cryopreservation require high level technical skills as well as the sacrifice of embryos, impeding effective availability. Contrarywise, mesenchymal stem cells (MSCs) are abundant and can be isolated from various tissues (bone marrow, fat, skin, etc.). Several teams report their transformation into gametes by induced transdifferentiation in vivo and in vitro. Recently, our laboratory has succeeded in isolating MSCs from feather follicles from living animals, and from post-mortem chicken tendons. These cells were cultured, amplified and we were able to induce differentiation towards a germline phenotype. This promising proof of concept raises questions of (i) its generalization to other avian species, (ii) confirmation of the germline competence of differentiated MSCs, (iii) maintenance of this competence after cryopreservation, and (iv) the determination of key mechanisms involved in germline-oriented transdifferentiation. We therefore plan to answer these questions, by implementing the technological suite developed in chickens to other species, by confirming the germline competence of differentiated MSCs and by identifying in vitro key players in this differentiation. Our research should thus contribute to define the best options for safeguarding the avian genomes of healthy, but also sterile, debilitated or even deceased animals and could have immediate practical consequences on biobanking policies in these species.

Keywords: mesenchymal stem cells, avian genetic resources, germline differentiation

3:45 PM – 4:45 PM

389

ENHANCED TISSUE REGENERATION THROUGH THE SYNERGISTIC COMPLEX OF PLACENTAL STEM CELL-DERIVED CONDITIONED MEDIA AND THERMOGEL

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Placenta-derived stem cells are notably free from ethical concerns due to their non-invasive acquisition at birth. Characterized by their high proliferative capacity and low immunogenicity, these cells have emerged as a significant focus of therapeutic research. Despite their potential, challenges in in vivo delivery and surface attachment, especially to skin, limit their application. This study explores the therapeutic potential of a hydrogel complex of placental stem cell-derived conditioned medium (CM) and hexanoyl glycol chitosan (HGC), which demonstrates a temperature-responsive sol-gel transition attributed to hexanoylation of glycol chitosan. Our goal was to overcome the limitations of stem cell delivery and surface adhesion, thereby enhancing the therapeutic applications of HGC-CM hydrogels. The CM, derived from mesenchymal stem cells of placental origin, was characterized through analysis of cell proliferation, differentiation, and expression of stem cell markers. The HGC-CM hydrogel, formulated through the physical amalgamation of HGC and CM, was optimized to ascertain its physical characteristics. This HGC-CM hydrogel demonstrated no cytotoxic effects and promoted cell proliferation in co-culture environments. Its capacity for regeneration was evidenced by positive outcomes in in vitro scratch assays and angiogenesis evaluations. Furthermore, the HGC-CM hydrogel showed no signs of immune rejection, evidenced by increased expression of the M2 marker associated with anti-inflammation. These findings collectively suggest the effectiveness of HGC-CM in wound healing, highlighting its potential as an innovative therapeutic approach that leverages stem cell benefits while minimizing immunogenicity concerns.

Funding Source: This work was supported by KOITA (Korea Industrial Technology Association) grant funded by MSIT (Korea Ministry of Environment). [R&DCENTER Capability Enhancement Project, grant number: 1711199718]

Keywords: placental stem cell therapy, conditioned media, thermogel

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3:45 PM – 4:45 PM

391

ASSESSMENT OF MESENCHYMAL STROMAL CELLS WITH A CLEAR DISTINCTION FROM STEM CELLS

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Currently widely used criteria for quality control of mesenchymal stromal cells (MSCs) are confounded with that of stem cells, presenting an urgent challenge for the understanding and translation of cell-based therapy. Stem cells are active in self-renewal and differentiation, but MSCs are silenced in this capacity. We thus develop an assessment specifically for MSCs based on the distinction in self-renewal and differentiation between MSCs and stem cells. Temporal trajectory analysis revealed a clear developmental cliff between MSCs and stem cells. There are 8 hub self-renewal genes DPPA4, LEF1, MYCN, NR6A1, SALL4, SFRP2, SOX2, and WNT11, are expressed in stem cells, but none of these expressed in MSCs. There are 5 functional genes of MSCs, TMEM119, FBLN5, KCNK2, CLDN11 and DKK1, are not expressed in stem cells. Therefore, these 5 “positive” and 8 “negative” genes can be defined as identification markers for MSCs, clearly distinguishing from stem cells. Furthermore, we found AD-MSCs have farther developmental distance from stem cells, and exhibit higher activities in extracellular matrix and secretory function regulation relative to MSCs from other tissues.

Keywords: mesenchymal stromal cell, stem cell, identification markers

3:45 PM – 4:45 PM

393

CULTIVATING DENTAL PULP STEM CELL EXTRACELLULAR VESICLES: IMPACT OF HOLLOW FIBER BIOREACTOR PORE SIZE

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Dental pulp stem cells (DPSC) are highly proliferative cells known for their remarkable ability to alleviate acute inflammation and facilitate long-term tissue healing and angiogenesis. These therapeutic effects are largely attributed to their paracrine factors, where extracellular vesicles (DPSC-EVs) playing a crucial role. However, the clinical validation of DPSC-EVs has been delayed by their limited production yields. In our research, we aimed to address this challenge by optimizing DPSC-EV production using a hollow fiber bioreactor (HFB). We hypothesized that employing HFBs with varying pore sizes could impact both the quantity and functionality of DPSC-EVs. To test this hypothesis, bioreactors with 5 kDa and 20 kDa pore sizes were used, and DPSC-EV production over 26 days was monitored. Our findings revealed that DPSCs cultured in the HFBs exhibited high EV production during the initial two weeks, yielding approximately 1010 particles per harvest. This production rate gradually declined with successive harvests. Analysis of the DPSC-EVs from both conditions via Western Blotting confirmed the presence of key markers such as Flotilin-1, CD63, and CD9, while transmission electron microscopy (TEM) images showcased their characteristic cup-shaped morphology. Moreover, we evaluated the pro-angiogenic properties of DPSC-EVs using scratch assay, demonstrating their ability to promote endothelial cell migration significantly regardless of pore size. Additionally, in an LPS-induced inflammation model investigated by flow cytometry, both DPSC-EVs exhibited notable anti-inflammatory effects. In conclusion, our study sheds light on the cultivation of DPSCs in HFBs and highlights that pore size variations do not significantly impact the production or functionality of DPSC-EVs. These findings hold promise for advancing the clinical application of DPSC and their extracellular vesicles in the field of regenerative medicine.

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Keywords: dental stem cells, bioreactor, extracellular vesicles

3:45 PM – 4:45 PM

395

ENHANCING CLINICAL SAFETY: VIRUS-INACTIVATED HUMAN PLATELET LYSATE AS SUPERIOR ALTERNATIVE TO FBS

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Clinical-grade Fetal Bovine Serum (FBS) has been used as standard cell culture supplement in cell therapy studies for several decades. However, there are strong concerns about the need to find an alternative for FBS, including its inconsistency in price and quality, the lack of traceability of its manufacturing, the risk of contamination from animal-derived pathogens and viruses, and the cruelty of serum extraction from living bovine fetuses (prohibited in the EU). The fast-growing field of cell-based therapy requires the highest quality and safety standards. Human platelet lysate (HPL) has the potential to meet these requirements. In general, HPL is produced from expired human thrombocyte concentrates, which are clinically tested transfusion products manufactured by certified blood donation centres. Repeated freezing and thawing of thrombocytes result in a highly enriched cocktail of essential growth factors and chemokines. Our innovative ELAREM™ platform consists of patented HPL cell culture media that promote growth of many different animal- and human-derived cell types, including stem cells. We guarantee high reproducibility and consistency in quality due to large batch pools consisting of hundreds of platelet donations, along

with the benefits of a GMP-compliant, xeno-free cell culture supplement. Thus, our ELAREM™ technology aims to bridge the gap between academic research, pre-clinical research and cell therapy. To guarantee highest safety for clinical use, we are enhancing our ELAREM™ products using state-of-the-art viral clearance technology to follow the latest guidelines for viral safety of biotechnology products (ICH Q5A(R2)). To this end, we have tested ionizing gamma irradiation to inactivate potential virus contaminants in HPL. At a low (>10 kGy) and medium dose (>20 kGy), gamma irradiation led to a mild reduction in cell growth of MSCs of 15% and 25%, respectively. In contrast, high doses >30 kGy led to a significantly higher reduction. Moreover, gamma irradiation did not affect total protein concentration, pH and osmolality of HPL, and only had an insignificantly low impact on cell growth-associated growth factors. Taken together, we are developing an ELAREM™ product that provides the highest clinical safety as well as efficient expansion potential for cell therapy preparations.

Funding Source: All activities were executed in the scope of the Eurostars' EXCELL project E!113731 and with support from EUREKA.

Keywords: human platelet lysate, mesenchymal stem cells (MSC), clinical safety

3:45 PM – 4:45 PM

397

ZERO-VALENT IRON PARTICLES PROMOTE GROWTH AND STEMNESS OF MESENCHYMAL STEM CELLS IN A XENO-FREE CULTURE

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Cell therapy is gaining prominence in clinical therapeutics, particularly in cancer immunotherapy, inflammatory diseases, and regenerative medicine. Mesenchymal stem cells (MSCs) are a key component of stem cell-based regenerative medicine, offering a promising approach for tissue reconstruction while addressing ethical concerns, histocompatibility issues, and the risk of teratoma formation. The versatility of adult MSCs positions them as a favored cell source in contemporary regenerative medicine. Ongoing research focuses on overcoming challenges related to maintaining stemness and efficiently enriching MSCs to meet clinical requirements. We synthesized zero-valent iron nanoparticles with a carboxyl-methyl cellulose (ZVI@CMC) shell. Transmission electron microscopy (TEM) observation showed the nondispersive particles have mean physical diameter of 70.17 ± 14.4 nm. Dynamic light scattering (DLS) measurement presented a mean hydrodynamic size of 78.8 ± 19.8 nm. Surface coating of CMC was confirmed by FTIR analysis and the change in zeta potential was observed indicating a shift to -31.51. We further discovered that a xeno-free medium containing the ZVI@CMC nanoparticles effectively promote MSC proliferation in a dose dependent manner between 0.2-1 μg/mL range while higher dose started to compromise their growth. ZVI@CMC induced reactive oxygen species (ROS) production after 6 hours of treatment. Flow cytometry analysis showed an increase in the S phase proportion and well maintenance of the stemness state using an array of biomarkers after continuous cultivation with the particles. Functional analysis underscored that MSCs continuously cultured with ZVI@CMC exhibited improved cell mobility, delayed aging, and were able to confer adipocytes and chondrocytes differentiation successfully by chemokine induction. These results highlight the beneficial impact of incorporating ZVI@CMC in MSC culture system, showcasing its potential not only in promoting growth and delaying aging but also in enhancing cell mobility and the capacity for induced differentiation in a xeno-free culture environment.

Funding Source: National Health Research Institutes grant NHRI-EX113-11103EI

Keywords: zero-valent iron nanoparticles (ZVI), mesenchymal stem cells (MSCs), xeno-free culture environment

3:45 PM – 4:45 PM

399

ROOM AIR OXYGEN IS A SIGNALING MOLECULE THAT TRIGGERS MSC SENESCENCE

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Traditional room air conditions are supraphysioxic for human bone marrow Mesenchymal Stromal Cells (MSC) used for in vitro testing or cellular therapies. Many researchers will use tri-gas incubators to maintain physioxic conditions around MSC during incubation, but then expose them to room air oxygen during handling in a traditional BSC. We previously showed that constant physiologic oxygen (5% O2), even for cell handling, improves human MSC yields and keeps them growing longer before senescence. We also showed that pericellular oxygen levels in cultures handled in room air took more than 80 minutes to equilibrate to 3% O2. Intracellular oxygen took even longer to recover, over 150 minutes. Here we show that breaking physioxia even for a quick room air medium change, triggers Hypoxia Induced Factor 1a (HIF-1a) modulation, and makes individual MSC more likely to stop dividing. We cultured human bone marrow MSC in a closed cell incubation and processing chamber (Xvivo System) for full-time control of oxygen, temperature, and CO2. MSC cultures were handled either in oxygen conditions matching incubation conditions (unbroken conditions) or under physioxia for incubation and room air conditions for routine cell culture (broken conditions). All cell culture media were pre-equilibrated to cell handling conditions. We found higher yields in each passage, and more total cell passages in cultures maintained full-time in physioxic conditions than in cultures handled under broken conditions (two-tailed T test, unequal variances). HIF-1a was down-regulated in broken conditions, but not in unbroken conditions. Long-term imaging data was used to assay cell division rates. Tracing individual cell lineages showed that once cells were exposed to room air supraphysioxia, they were far less likely to ever divide again. We concluded that maintaining constant physioxia around MSC during cell handling operations is critical to maximizing MSC yields. It also makes sense to use cell handling conditions that are controlled and reproducible from site-to-site to generate reproducible MSC data and performance.

Keywords: MSC, hypoxia, oxygen

3:45 PM – 4:45 PM

401

DESIGN, CREATION AND VALIDATION OF A SEMI-AUTOMATED WORKFLOW FOR THERAPEUTICS CELLS EXPANSION, HARVEST AND FINAL FILL

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Genetically modified organisms and cells are increasingly deployed at large scales and in clinical environments. Robust cells separation strategies are needed to prevent unintended contamination of therapeutics payloads in disposable systems during manufacturing operations. In this presentation, the design, building and validation of a closed-system MSC expansion process transferable to any BSL-2 lab, on-field or clinical settings will be presented. This work demonstrates the strategic integration of off-the-shelf consumables and standard lab equipment in a semi-automated closed modular workflow as a cost-effective approach when compared to “all-in-one” proprietary commercially available systems. The resulting assemblies exhibit unprecedented resistance/high-performance to cells processing operations and critical fluid handling control including storage. We will also discuss how precision controlled biomanufacturing strategy impacts product quality, process efficiency and reliability, speed across scalability and overall economics. Our latest results obtained on a set of carefully selected particles and cells lines (e.g. mesenchymal stem cells) will be exposed. This work provides a foundation for new and safer bioprocessing solutions that are re-designed to provide lower cost and more benefit to patients.

Keywords: microcarrier-cell separation, precision biomanufacturing, high resolution particles technology

3:45 PM – 4:45 PM

403

MAGNETIC NANOMATERIALS AS TOOLS FOR INFLUENCING STEM CELL FATE

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Magnetic nanoparticles (MNPs) and magnetic nanowires (NW) are increasingly used for various biomedical application in tracking sorting and drug delivery. Remote controlled actuation of stem cell incorporating MNP can be employed to deliver micro-mechanical stimulation during stem cell differentiation potentially increasing osteogenesis and chondrogenesis. Nickel magnetic responsive NW can be used as substrate for the delivery of magneto mechanical stimulation to differentiating MSCs. Human adipose derived stem cells (ADSCs) and Wharton jelly derived MSCs (WJMSC) loaded with proprietary MNPs were tested for viability, proliferative capabilities, culture induced senescence. In vitro osteogenesis adipogenesis of ADSCs-MNP as well as chondrogenic potential of ADSC and WJMSC –MNP exposed to magnetic field (MF) was tested. ADSC cultured on NW substrates with or without MF exposure were tested for viability. Osteogenic and chondrogenic differentiation was quantified. Stem cells retain viability and proliferative capabilities compared to non-loaded and become remote controllable within MF. MNP presence decrease stem cells culture induced senescence in ADSC but not WJMSCs. ADSCs–MNP display increased osteogenic and decreased adipogenesis when exposed to NF in a time, modality of exposure and intensity manner. ADSC-MNP increased chondrogenesis within MF. NW substrate supports attachment and viability of ADSC. Osteogenic conversion but not adipogenesis of ADSC cultured on NW substrates was found to increased by intermittent alternating MF exposure. Moreover, osteogenesis in ADSCs cultured on NW substrate could be detected even without specific differentiation media and enhanced by MF exposure. ADSCs-MNP display increased osteogenic and decreased adipogenic potential under MF dependently on exposure protocol. ADSC-MNP but not WJMSC display increased chondrogenesis increased by MF exposure. NW substrate and MF exposure can be used to obtain osteogenic conversion of ADSC even in the absence of osteogenic media, process increased by MF exposure. This can be used to design innovative modalities for engineering of implantable bone or for cell preconditioning in the case of cellular therapies for treating osteoporosis or other bone related diseases.

Funding Source: Financial support was received from Ministry of Research, Innovation and Digitization, CNCS/CCCDI -UEFISCDI, project number ERANET-EURONANOMED-3-OASIs, within PNCDI III

Keywords: magnetic nanomaterials, tissue derived stem cell differentiation, osteogenesis chondrogenesis

3:45 PM – 4:45 PM

405

BERBERINE IMPROVED THE THERAPEUTIC EFFICACY OF UC-MSCS FOR DSS-INDUCED COLITIS VIA AEROBIC GLYCOLYSIS

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The global prevalence of Inflammatory bowel disease (IBD) continues increasing, but effec-tive targeted treatments remain elusive. Mesenchymal stem cells (MSCs) possess self-renewal, multi-directional differentiation and distinctive immunoregulatory effects, and emerge as a promising candidate for IBD treatment. Accumulating evidence suggested that energy metabolism played a critical role in the therapeutic effects of MSCs. Berberine (BBR), a natural extract from plants of the Berberidaceae family, has been shown to modu-late various cell functions by reprogramming metabolism in the context of immune or meta-bolic-related diseases. In this study, we investigated the influence of BBR on the therapeutic effectiveness of MSCs in colitis. Our results revealed that BBR activated AMPK signaling pathway, upregulated the expression of glucose transporter 1 (GLUT1) and promoted aerobic glycolysis in MSCs. These alterations increased the production of tumor necrosis fac-tor-stimulated gene-6 (TSG-6), enhanced the immunomodulatory function and improved the therapeutic efficacy of MSCs. Inhibition of glycolysis or TSG-6 impaired the enhanced therapeutic effect of MSCs induced by berberine. Moreover, overexpression of GLUT1 ob-viously improved the therapeutic efficacy of MSCs. Taken together, our data indicated that BBR treatment improved the therapeutic effect of MSCs in colitis by inducing GLUT1-dependent aerobic glycolysis, providing evidence that BBR may serve as a potential glycolysis inducer for optimizing MSCs therapy in IBD. Our findings highlighted the im-portance of reprogramming the energy metabolism in MSCs for developing new therapeutic strategies for IBD and suggested that natural products like berberine may provide a safe and effective approach for optimizing MSCs-based therapy.

Keywords: berberine, mesenchymal stem cells, glycolysis

3:45 PM – 4:45 PM

TOPIC: MUSCULOSKELETAL

407

AN INTEGRATIVE MULTIOMICS APPROACH TO DECONSTRUCT BOVINE MYOGENESIS IN VITRO FOR CULTIVATED MEAT APPLICATIONS

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Traditional livestock farming for meat production significantly contrib-utes to greenhouse gas emissions, deforestation, zoonotic diseases, water pollution, and adverse animal welfare. Cultivated meat represents an emerging food technology aiming to offer a sustainable alternative to conventional meat production, typically through in vitro differentiation of myogenic progenitor cells (MPCs) into muscle fibers, the primary component of meat. However, little is known about the molecular determinants governing how MPCs give rise to muscle fibers in vitro, and whether manipulation of signaling pathways can enhance this procedure. To address these challenges, we employed multiomics tools to dissect the molecular landscape controlling bovine myogenic differentiation in vitro, further exploring whether this process can be augmented by small molecules. This effort unveiled enhanced medium formulation containing compounds that can rapidly and robustly increase MPC differentiation compared to a widely used conventional differentiation medium. By utilizing bulk and single-cell transcriptomics and proteomics, we compared the effect of conventional and enhanced media on MPCs alongside their differentiated progenies. We documented that the enhanced medium elicited de-differentiation into unique cell populations, in conjunction with formation of highly contractile fibers that expressed a diverse array of muscle differentiation markers. Notably, these in vitro-derived muscle cells more closely resembled in vivo bovine skeletal muscle cells, both molecularly and functionally. In summary, we developed an optimized method for the in vitro generation of bovine muscle cells, further exploring their molecular and functional characteristics. The reported medium and the accompanied molecular roadmap may assist efforts for in vitro meat production.

Keywords: cultivated meat, multi-omics integration, myogenesis

3:45 PM – 4:45 PM

409

MAGNETICALLY INDUCED SKELETAL MUSCLE SECRETOME EXHIBITS ANTICANCER POTENCY: SECRETOME PRECONDITIONING PARADIGM FOR LIQUID BIOMARKER DISCOVERY AND CHARACTERIZATION

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The goal of this work is to investigate the anticancer potency of magnetically induced muscle secretome on breast cancer in vitro, in vivo and ex vitro. The work also examines the function of magnetically mediated preconditioning paradigm to enhance muscle secretome release and production. Briefly (10 min) exposing C2C12 myotubes to low amplitude (1.5 milliTesla) pulsed electromagnetic fields (PEMFs) generated a conditioned media (pCM) that mitigated breast cancer cell growth, migration and invasiveness in vitro, whereas the conditioned media harvested from unexposed myotubes, representing constitutively released secretome (cCM), was less effective. Ex vivo, administering pCM to breast cancer microtumors engrafted onto the chorioallantoic membrane of the chicken egg reduced tumor volume and vascularity. In vivo, blood serum collected from PEMF-exposed or exercised mice allayed breast cancer cell growth, migration and invasiveness. Based on PEMF-induction of the muscle secretome, a preconditioning methodology was developed to enhance secretome production and release. HTRA1 (High-Temperature Requirement A Serine Peptidase 1) was upregulated in the pCM and was shown necessary for myogenic progression and anticancer potency. HTRA1 expression hence embodied key adaptive features of the pCM preconditioning paradigm and emulating an exercise-like effect. Brief and non-invasive PEMF stimulation may represent a method to commandeer the secretome response of muscle for clinical exploitation in cancer. In summary, brief and non-invasive pulsed electromagnetic field (PEMF) stimulation was shown to induce the secretome response of muscles akin to exercise to impact cancer viability and epithelial-mesenchymal transition (EMT). Moreover, the preconditioning of cells to PEMF may represent a method to induce the secretome response of muscles for clinical exploitation in cancer.

Funding Source: National University of Singapore

Keywords: musculoskeletal secretome, pulsed electromagnetic fields (PEMF), cancer

3:45 PM – 4:45 PM

411

STEM CELL DIFFERENTIATION THROUGH CRISPR-BASED MULTIMODAL TUNING OF GENE EXPRESSION

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Human synthetic biology aspires to mimic the sophistication of naturally occurring gene regulation. Emerging methods based on programmable genome modifiers are optimized for immortalized cell lines, which cannot model the breadth of human pathophysiology. Implementation of these approaches in human induced pluripotent stem cells (hiPSCs) and their derivatives is highly desirable, but several challenges remain. These include high sensitivity to genotoxic nucleases, toxicity and/or unstable expression of large transgenes — particularly during differentiation, and limited susceptibility to transfection or transduction of hPSC-derived cells. To address this technological gap, we developed CIRI (Combinatorial Inducible cRispr in Ipscs), a multimodal CRISPR/dCas9-based method to conditionally manipulate complex genetic circuits in hPSCs and their differentiated derivatives. CIRI leverages tetracycline-inducible single guide RNAs (sgRNAs) engineered to include modular RNA aptamers able to recruit effectors at target genomic sites. This bypasses the need for big dCas9 fusion proteins. Moreover, by combining different aptamers, RNA binding proteins, and effectors, CIRI allows the simultaneous execution of distinct regulatory activities at multiple loci; i.e., activating a set of genes (CRISPRa) while others are being repressed (CRISPRi). sgRNAs also contain distinct capture sequences that facilitate their identification in the context of pooled screens via single-cell RNA sequencing (scRNA-seq). Finally, all genetic instructions are inserted in genomic safe harbors to ensure stable expression. We first tested variations in sgRNA structures and effectors, and identified strategies that allow strong CRISPRa (i.e., MYOD1 expression in ranges achieved during muscle development) and CRISPRi (i.e., almost complete silencing of TFRC). We then deployed CIRI to forward program hPSCs into skeletal myocytes through the combined repression of OCT4, SOX2, and NANOG and the activation of MYOD1. Lastly, we performed a scRNA-seq screening to identify factors that improve myogenic differentiation and maturation. We anticipate that CIRI will enable both basic and translational applications from disease modeling to generation of cells precision-programmed for regenerative medicine.

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Keywords: multimodal CRISPR/dCAS9, forward programming, gene expression tuning

3:45 PM – 4:45 PM

TOPIC: NEURAL

415

LONG-TERM CONTINUOUS LINEAGE DECODING IN HUMAN BRAIN ORGANOIDS AND GRAFTS

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Human embryonic stem cell (hESC)-derived organoids mimic human organ development, providing an accessible and ethically justified model for studying human organogenesis in vitro. The continuous landscape of the long-term developmental lineage in human nervous system remains to be elucidated. Here, we develop a genetically defined progressive barcoding system, termed single-cell CRISPR lineage tracing (scCITING), to allow long-term lineage decoding in the hESC-derived organoids. Applying scCITING, we revealed an early regional patterning of human brain organoids. We further combined scCITING with the clonal splitting strategy and tracked the continuous maturation trajectories from progenitors to their terminal fates under different conditions by maturation in organoids or in grafts. Taking advantage of the long-term editing feature of scCITING, we unprecedently reconstructed the phylogenic fate maps for single progenitors by building a single-cell level tree rooting from each individual transcriptome-defined progenitor cell. We found the progenitor fates were mostly conserved cross the environments, despite of the distinctive cell type compositions in vivo and in vitro. Specifically, we identified multiple origins for midbrain dopaminergic neurons in both environments. Furthermore, we incorporated spatial transcriptome and uncovered different lineages and distributions of two transcriptionally distinct astrocyte subtypes in the transplanted grafts. Our approach is a stable system for studying the continuous single-cell lineage in human organoids, offering insights for understanding the organ development in human.

Keywords: lineage tracing, brain organoid, cell transplantation

3:45 PM – 4:45 PM

417

A LAYERED APPROACH TO SECURE THE SAFETY AND GRAFT-HOST INTEGRATION OF HIPSCS-DERIVED NEURAL NETWORK TISSUE FOR FUNCTIONAL REPAIR OF RATS COMPLETE SPINAL CORD INJURY

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The successful integration of human induced pluripotent stem cells (hiPSCs)-derived neurons with the host neural circuits is critical for functional repair of spinal cord injury (SCI). However, the desired donor fate and proper function in the injured spinal cord remains to be ensured. In this study, we have adopted gene engineering and tissue engineering technique to construct a human iPSC-derived neural network-like tissue with transfected thymidine kinase (TK) gene as proliferative-cell-depletion approach to increase security. After culturing in a 3-dimensional gelatin sponge scaffold for 14 days, a hiPSCs-derived neural network-like tissue (hiPSC-NN)with high rate of hiPSCs-derived neurons and synaptic transmission potential were constructed in vitro. With ganciclovir (GCV) application, proliferative cells were significantly depleted, leaving non-mitotic, mature neural cells for transplantation. Remarkably, the SCI rat receiving the transplantation of the hiPSC-NN demonstrated a gradual restoration of paralyzed hindlimb motor function, along with improved electrophysiological presentation when compared with the control groups, with no teratoma formation encountered in the non-TK iPSC group. Histological analysis showed that in the hiPSC-NN group, a large number of neurons had survived in the injury/graft site for 8 weeks, significantly surpassing the neuronal population in hiPSC-derived NPC implantation group. Furthermore, the survival donor neurons integrated with the regenerating CGRP-positive nerve fibers and host neurons by formation of synapses. A closer look suggested that a pro-regenerative microenvironment with“inflammatory privilege-like”was formed in the injury/graft site after transplantation of hiPSC-NN with altered macrophage/microglia profiles. The results suggest that the layer approach conferred a safety hiPSC-NN with a function of “neuronal relayers” to restore themotor function in the rats with complete SCI.

Funding Source: National Natural Science Foundation of China (82371395), Guangdong Provincial Hospital of Chinese Medicine (GXB202401, 2022KT1032), Guangzhou Basic and Applied Basic Research Foundation (2022A03J01464)

Keywords: spinal cord injury, tissue engineering, neural network-like tissue

3:45 PM – 4:45 PM

419

DE NOVO DESIGN OF A NOVEL TRKA AGONIST TO PROMOTE NERVE REGENERATION AND REDUCE NOCICEPTIVE SIGNALS

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Since its discovery as a potent molecule to promote axonal growth and nerve regeneration in adults, NGF was thought to be the key to prevent or reverse peripheral neuropathy. However, clinical trials failed due to myalgia and hyperalgesia observed as a response. Since then, the molecular mechanisms for NGF-mediated nociception at the cellular level have started to be deciphered, involving its two receptors: TrkA and P75NTR. For example, while the TrkA-NGF signaling promotes axon sprouting, the involvement of the P75NTR receptor has been linked to hyperexcitability and nociceptor gene expression, as shown by NGF mutants unable to bind P75NTR that fail to trigger deep pain sensations in patients. So far, the contributions of each receptor in the establishment of acute or chronic pain are yet to be fully elucidated. In this context, we took advantage of recent advances in de novo protein design to generate a potent TrkA-specific binder acting as an antagonist. Using novel deep-learning tools we designed rigid fusions of those binding domains to specifically cluster TrkA receptors to activate downstream signaling and induce endocytosis. In a model of fibroblast-to-neuron direct conversion through ASCL1 and NGN2 overexpression, we show that while both NGF and the TrkA-only binder promotes axon sprouting, TrkA specific activation does not induce the transcription of genes involved in nociception like TRPV1, CEBPB or TMEM160, as does NGF. Using this system, we aim to dissect the contribution of each receptor and downstream pathways such as PLCκ activation in the establishment of various pain signatures in neurons. This AI-designed TrkA agonist could have a broad impact in the field of pain management and nerve regeneration after injury.

Funding Source: D. Detraux is a recipient of an ISCRM postdoctoral fellowship

Keywords: nerve regeneration, pain signaling, AI-designed proteins

3:45 PM – 4:45 PM

421

EXPLORING THE IMPACT OF DRUG DIFFUSION IN THE DYNAMICS OF NEURONAL NETWORKS DERIVED FROM HUMAN IPSCS

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In vitro models combined with human induced pluripotent stem cells (hiPSCs) are an extensively used tool to study the complex mechanism of the human brain. Indeed, the use of hiPSCs-derived neuronal networks allow to create in vitro patient-specific models that most closely resembles the processes of the human system, providing a reliable model to test neuroactive properties of different compounds. This study explores how different drugs affected the electrophysiological activity of in vitro systems composed by excitatory (E) and inhibitory (I) neurons derived from hiPSCs. Our experimental model included three different culture configurations: 100% excitatory neurons (100E), 75% excitatory and 25% inhibitory neurons (75E25I), and 100% inhibitory neurons (100I). To these neuronal networks, 2-amino-5-phosphonovaleric (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and bicuculline (BIC) were administrated. The recordings of the spontaneous and chemically stimulated electrophysiological activity were performed by exploiting Micro-Electrodes Arrays (MEAs). The administration of APV and CNQX resulted in an unaffected firing activity, while led to a decrease of the bursting and network bursting activity of both 100E and 75E25I configurations. On the other hand, BIC led to a significant increase of the firing activity of the 100I and 75E25I, and of the bursting and network bursting activity of the 75E25I configuration. This comprehensive investigation sheds light on the different effects of APV, CNQX and BIC on the electrophysiological dynamics of neuronal networks with different excitatory-inhibitory configurations. These findings contribute to our understanding of drug-induced modulation in neuronal circuits and provide valuable insights for potential therapeutic investigations in neurological disorders.

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Keywords: in vitro model, chemical stimulation, electrophysiology

3:45 PM – 4:45 PM

423

HOMOPHILIC WIRING PRINCIPLES UNDERPIN NEURONAL NETWORK TOPOLOGY IN VITRO

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Economic efficiency has been a popular explanation for how networks self-organize within the developing nervous system. However, the precise nature of the economic negotiations governing this putative organizational principle remains unclear. Here, we address this question further by combining large-scale electrophysiological recordings, to characterize the functional connectivity of developing neuronal networks in vitro, with a generative modeling approach capable of simulating network formation. We find that the best fitting model uses a homophilic generative wiring principle in which neurons form connections to other neurons which are spatially proximal and have similar connectivity patterns to themselves. Homophilic generative models outperform more canonical models in which neurons wire depending upon their spatial proximity either alone or in combination with the extent of their local connectivity. This homophily-based mechanism for neuronal network emergence accounts for a wide range of observations that are described, but not sufficiently explained, by traditional analyses of network topology. Using rodent and human monolayer and organoid cultures, we show that homophilic generative mechanisms can accurately recapitulate the topology of emerging cellular functional connectivity, representing an important wiring principle and determining factor of neuronal network formation in vitro.

Keywords: neuronal network development, generative network model, high-density microelectrode arrays

3:45 PM – 4:45 PM

425

NEXT-GENERATION ELECTROPHYSIOLOGY FOR FUNCTIONAL CHARACTERIZATION OF HUMAN NEURAL ORGANOIDS

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Human induced pluripotent stem cell (hiPSC)-derived neural models have emerged as invaluable tools for studying neurological disorders, such as epilepsy, Alzheimer's, and Parkinson's disease.Real-time, label-free measurement of electrical activity in self-organizing in vitro cellular models provides critical insight into the complexity of their neuronal networks. High-density microelectrode arrays (HD-MEAs) enable non-invasive electrophysiological recordings from various electrogenic samples, including iPSC-derived neurons, retinal explants, brain slices, and neural organoids. In this study, we used MaxOne and MaxTwo high-density MEA platforms (MaxWell Biosystems AG, Switzerland), with 26,400 electrodes per well to record extracellular action potentials in neural organoids at different scales, ranging from cell population networks to single-cell resolution and subcellular levels. We showcased the flexible selection of electrodes for recording neural activity, increasing the reproducibility and statistical power of the data collected. Key metrics such as firing rate, spike amplitude, and network burst profile were extrapolated in a parallelized manner to capture even the smallest neuronal signals. Furthermore, we characterized axonal function and structure using the AxonTracking Assay, which allows measurement of action potential conduction velocity, latency, axonal length, and branching. This automated assay facilitates high-throughput characterization of disease models targeting axon initial segments, axonal branching, development, and conduction. MaxWell Biosystems' HD-MEA platforms, along with automatically generated plots and extracted metrics, provide a unique, user-friendly approach to identifying and isolating functionally active regions in 3D cultures. These powerful platforms enable long-term in vitro disease modeling and compound testing in acute recordings and/or longitudinal studies.

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Keywords: in vitro electrophysiology, HD-MEA, neural organoids

3:45 PM – 4:45 PM

427

A NOVEL ORGANOID-BASED PLATFORM TO DECODE REGIONAL DIVERSITY OF HUMAN MICROGLIA PHENOTYPES

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Microglia, the key tissue-resident immune cells of the brain, are actively involved in orchestrating various developmental processes. Previous studies have shown that microglia assume highly heterogenic molecular states that successively emerge over the course of brain development. However, the manifestation of this phenomenon in the human context and its functional implication remain unexplored due to the lack of suitable model systems. To overcome these limitations, we developed a modular forebrain assembloid model that allowed us to investigate the developmental diversification of human microglia (hMG) within a human brain-like environment. Using this model, we observed that forebrain-resident hMG are capable to shift phenotypes according to their spatial positioning within the developing assembloid. Most notably, we identified a population of hMG that appeared to be closely associated with developing axon tracts and defined by a specific cellular phenotype that was distinct from hMG populations observed in other regions of the assembloid. Correlative light electron microscopy further indicated that this population may indeed be adjusting to the specific functional demands dictated by the local microenvironment. Finally, single-cell RNA sequencing (scRNA-seq) performed in our models confirmed the presence of molecularly defined microglia states and suggested an environment-specific induction of these distinct functional phenotypes. Taken together, we describe the development and characterization of a modular organoid-based platform that enables the functional dissection of human microglia phenotypes during human brain development, a critical next step for deciphering their contribution to both normal neurodevelopmental processes and pathological conditions.

Keywords: microglia, brain organoids, neurodevelopment

3:45 PM – 4:45 PM

429

PULP FICTION: DIRECT REPROGRAMMING OF DPSCS INTO NEURONS

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Dental pulp is a connective tissue rich in nerves and blood vessels that houses progenitor cells, known as dental pulp stem cells (DPSCs). Due to their neural crest origin, DPSCs can be differentiated into neuronal cells under the right in vitro conditions. Our aim is to develop a novel stable, reproducible method to utilize the developmental potential of DPSCs using transdifferentiation, direct neuronal reprogramming. Our first experiments indicated that forced expression of the neuronal transcription factors Ascl1, Brn2, and Myt1l successfully transdifferentiated DPSCs into induced neurons (iNs). Moreover, optimized DPSC-iN conversion quantifiably met the efficiency and purity of standard fibroblast iNs, measured by automated high-content microscopy after immunostaining with mature neural markers (MAP2, TAU). Next, we reprogrammed and cultured DPSCs into transplantable 3D neurospheroid-like structures, with the administration of different culture conditions but with the same described direct reprogramming protocol. After 14 days of culturing, the spheroids showed mostly neuronal immunolabeling while some cells were positive for neural crest markers (HNK1, NF). We are currently characterizing and carefully phenotyping our DPSC-iNs derived from 4 different donors by using immunostaining followed by automated high-content microscopy, mass spectrometry, qRT-PCR, bulk RNA-seq, patch-clamp electrophysiology and DNA methylation array. We are also planning to use sc-RNA-sequencing on DPSC-iNs after barcoding native DPSCs to identify and sort the dental pulp progenitor cells from the mesenchymal elements, using a novel lineage tracing methodology in combination with previously acquired multiomics data. These experiments will define neural crest-related cell subpopulations within the pulp and ensure a way to identify the ones with neurogenic potential. In summary, DPSC-iNs represent a novel methodology to study donor-derived neurons from an ethical, easily accessible cellular source. In combination with the unique developmental origin, DPSC-iNs provide a novel model for developmental diseases mainly affecting children and adolescents.

Keywords: direct reprogramming, mesenchymal stromal cells, induced neurons

3:45 PM – 4:45 PM

431

GENERATION OF A NOVEL 3D MICROFLUIDIC IN VITRO HIPSC-DERIVED NEUROVASCULAR UNIT MODEL

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The blood-brain barrier (BBB) is formed by brain microvascular endothelial cells (BMECs) through interactions with other cells of the neurovascular unit (NVU), namely astrocytes (ACs) and pericytes (PCs). In vitro modeling of the human BBB is limited by the lack of robust protocols to generate BMECs from human iPSCs (hiPSCs) with high molecular fidelity and barrier functionality. Here, we report the generation of reprogrammed BMECs (rBMECs) through a combination of directed differentiation of hiPSCs into BBB-primed endothelial cells (bpECs) and reprogramming with two BBB-critical transcription factors, FOXF2 and ZIC3. rBMECs express a subset of the BBB gene repertoire, including tight junctions (TJs) and ECM components, and exhibit higher paracellular barrier properties and lower transcytosis compared to primary hBMECs. rBMECs were used to establish a 3D NVU model through co-culture with iPSC-derived ACs and PCs within the MIMETAS platform, which applies passive flow to enable endothelial tubule formation and interaction with ACs and PCs across an ECM channel. We report that rBMECs form 3D tubules, which stain for endothelial markers CD31 and CDH5 and TJ markers CLDN-5 and ZO-1. iPSC-derived ACs and PCs migrate through the ECM channel to colocalize with rBMEC tubules. Our iPSC-derived NVUs exhibit lower permeability to a 70kDa dextran and Biocytin tracer compared to primary cell-derived NVUs, suggesting low transcellular and paracellular transport, respectively. Furthermore, we report elevated trans-endothelial electrical resistance (TEER) in iPSC-derived NVUs compared to primary cell-derived NVUs. Together, these data suggest that our iPSC-derived NVUs can effectively model the human BBB for studies of healthy and pathological states.

Keywords: blood-brain barrier, NVU, transcriptional reprogramming

3:45 PM – 4:45 PM

433

GENERATION OF FUNCTIONAL 3D SPINAL CORD ORGANOIDS FROM HUMAN PLURIPOTENT STEM CELLS

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There is no cure for motor neuron (MN) diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy. Access to a reliable human MN model would be invaluable to help uncover disease mechanisms. Advanced culture models such as spinal cord organoids (SCO) contain a variety of tissue-specific cell types including MNs, glial cells, and interneurons, improving their physiological relevance. Here, we describe the STEMdiff™ Spinal Cord Organoid Differentiation Kit which generates SCOs from human pluripotent stem cells (hPSCs) at a high efficiency. A single-cell suspension of hPSCs was cultured for 6 days in AggreWell™800 plates containing organoid formation medium. The resulting organoids were replated and cultured in expansion medium from days 6 - 19, followed by differentiation medium from days 19 - 43, and STEMdiff™ Neural Organoid Basal 2 with supplement from day 43 onwards. We examined the expression of HOX genes by qPCR to determine the rostral-caudal patterning at day 19. Then, we evaluated the cell identity by qPCR and immunocytochemistry at specific days: day 19 for OLIG2+ and NKX6.1+ neural progenitor cells, day 30 for MNX1+, ISL1+, and/or FOXP1+ post-mitotic MNs, CHX10+ for interneurons, and finally day 75 for GFAP+ or MBP+ glial cells. We also measured spontaneous electrophysiological currents using the Axion microelectrode array system throughout the culture. The SCOs expressed HOXB4 and OLIG2 on day 19 and MNX1 and ISL1 on day 30 at significantly higher levels than hPSCs (fold changes relative to hPSCs of 29103, 1568, 1105, and 3059, respectively, $p < 0.0001$, $n = 11$). Moreover, we observed a large number of MNX1+, ISL1+, and/or CHAT+ MNs and CHX10+ interneurons at day 30 and GFAP+ or MBP+ glial cells at day 75. The SCOs displayed spontaneous firing as early as 4 weeks in culture (0.7154 ± 0.1982 Hz, weighted mean firing rate mean ± SEM), which became more synchronous and active with maturation (average synchrony index of 0.0672 and 0.3252 from weeks 4 and 9, respectively). Additionally, the SCOs had more bursts when matured in BrainPhys™ than Neural Organoid Basal 2 (13.52 and 0.40 bursts per minute respectively, $p = 0.0016$, $n = 5 - 8$). Taken together, STEMdiff™ Spinal Cord Organoid Differentiation Kit provides a powerful tool to generate functional hPSC-derived SCOs for in vitro studies of human MN diseases.

Keywords: spinal cord organoid, motor neuron disease, in vitro model

3:45 PM – 4:45 PM

435

MOLECULAR LINEAGE TRACING OF STEM CELL DERIVED DOPAMINE GRAFTS IN VIVO REVEALS A SHARED ORIGIN OF ALL GRAFT-DERIVED CELLS

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Human pluripotent stem cells (PSCs) differentiated into dopamine (DA) neurons provide an unlimited cell source for cell replacement therapy for Parkinson's disease (PD). However, histology and scRNA-seq profiling of resulting grafts in preclinical models have revealed that DA progenitors that are seemingly homogenous at the time of transplantation give rise to heterogenous grafts composed of different mature cell types. We have used single nuclei RNA-seq combined with molecular barcodes to determine origin and shared lineage of the mature cell types forming in grafts of dopaminergic progenitors currently explored in cell therapy for Parkinson's Disease. Single-cell chromatin accessibility and RNA expression paired maps identified only subtle chromatin accessibility differences among progenitors supporting their homogeneity, and together with the tracing data shows that the PSC-derived floor plate cells used for transplantation are tri-potent, giving rise to dopamine neurons, astrocytes and VLMCs. In parallel, we have also generated new barcode libraries designs for more efficient capture of the barcode transcript taking advantage of feature barcoding. This will allow for more detailed fate and state mapping in stem cell based regenerative approaches.

Keywords: lineage tracing, cell therapy for Parkinson's disease, multiome snRNA-seq

3:45 PM – 4:45 PM

437

EXPLORING THE IMPACT OF DIMENSIONALITY ON ELECTROPHYSIOLOGICAL ACTIVITY OF HIPSCS-DERIVED NEUROSPHEROIDS

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In vitro models are universally accepted as systems that reduce the complexity of the human brain and support the understanding of the pathophysiology of neuronal diseases by partly mimicking the in vivo conditions. Indeed, the human brain is characterized by several complex features such as three-dimensionality, heterogeneity, modularity, segregation, and integration. Reproducing these characteristics in experimental models in vitro is essential to obtain reliable systems and to conduct consistent studies. In this work, we present an experimental brain-on-a-chip model based on neuronal networks derived from human-induced pluripotent stem cells by focalising on three-dimensionality and heterogeneity. The aim of our study is to evaluate different sizes of neurospheroids coupled to high-density electronic devices to understand how their dimension affect the spontaneous electrophysiological activity. To achieve this goal, we realized neurospheroids composed (nominally) by 10^4, 2·10^4, and 3·10^4 cells. To implement the minimal neuronal heterogeneity, we generated 3D cultures composed by glutamatergic (75%) and GABAergic (25%) neurons as it is estimated in the cortex. We recorded the spontaneous electrophysiological activity from high-density devices with 4096 electrodes for 10 minutes in stable conditions after 60 days in vitro. For the analysis of the spontaneous electrophysiological activity, we focused on spiking patterns. Moreover, we performed a morphological characterization by computing the area of the neurospheroids. The 3D cultures showed significant differences with respect to their sizes. Furthermore, these differences were consistent with what emerged from the analysis of the spontaneous electrophysiological activity, which showed lower firing values when the number of cells into the neurospheroids decreased. In conclusion, our results suggest that neurospheroids composed by a number of cells lower than 3·10^4 do not show sustained electrophysiological activity. Our model and the obtained preliminary results represent the first step toward the construction of complex three-dimensional and heterogeneous brain-on-a-chip systems.

Funding Source: Work supported by #NEXTGENERATIONEU (NGEU) and funded by the Ministry of University and Research (MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE0000006) – (DN. 1553 11.10.2022)

Keywords: in vitro model, high density arrays, three dimensionality

3:45 PM – 4:45 PM

439

STRUCTURE-FUNCTION RELATIONSHIP IN HIPSC-DERIVED NEURONAL NETWORKS

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Brain function is substantially linked to the highly organized structure of neuronal networks. The structures of in vitro assembled neuronal circuits often exhibit variability across samples and undergo dynamic changes over time, posing challenges in the reliable recording of network functional output and its correlation with network structure. Employing the high-density multi-electrode array (HD-MEA) electrophysiology system, we demonstrated that neuronal networks derived from human induced pluripotent stem cells (hiPSCs) undergo

substantial structural transformations over months. Microscopy data revealed the progressive evolution of randomly formed network morphology from homogeneously distributed neurons in the initial month to the formation of smaller neuronal clusters in the second month, culminating in larger clusters by the third month. These substantial changes were accompanied by the movement of neuronal cell bodies and connecting axonal bundles. The displacement of neuronal cells and network clusters on the sensing substrate (>200µm in two months) correspondingly led to a shift in the spatial distribution of network activity maps, which we could correlate. Therefore, tracking the long-term activity profile during network development becomes exceedingly challenging, as the activity of the same neuron or network cluster can be recorded by different set of electrodes on different days. To address these challenges, we engineered neuronal circuits within a microfluidic-MEA coupled device to preserve the overall structure of the network and maintain stable neuronal cell positions over an extended period. Functional readouts from these engineered circuits demonstrated that spatial distribution of the network activity remain consistent over an extended duration. Furthermore, we observed significantly enhanced consistency of functional data between samples in engineered networks compared to the randomly formed networks. The physical confinements in these circuits facilitated the study of the impact of network structure on its function. These preliminary findings lay the foundation for developing human stem cell-derived neuronal systems with reproducible functional phenotypes, holding significant promise for in vitro functional phenotyping of neurological diseases.

Funding Source: Authors acknowledges funding from the Pro Retina Foundation, and the Volkswagen Foundation (Freigeist–A110720).

Keywords: microfluidics, microchannel device, brain-on-chip, neuonal networks, neuronal cluster, in vitro network development, human induced pluripotent stem cells, hiPSC

3:45 PM – 4:45 PM

441

EVALUATING THE EFFECT OF NERVE GROWTH FACTOR ON CATECHOLAMINERGIC NEURON DEVELOPMENT IN SYMPATHETIC NEURONAL ORGANOIDS

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Nerve growth factor (NGF) is a neurotrophin which is essential for cardiac innervation. Animal experiments suggest that dysregulation of NGF levels upon myocardial infarction greatly contribute to neuronal remodeling, norepinephrine level imbalance and lethal arrhythmias. In this study we investigate the role of exogenous NGF in human autonomic neurons in a 3D autonomic neuronal organoid enriched in sympathetic neurons (sympathetic neuronal organoid, SNO). NGF exerts its action mainly by binding to neurotrophic tyrosine kinase receptor (NTRK1). Temporal analysis of NTRK1 transcript levels during SNO development (day 15-60) revealed an increase of NTRK1 from day 30 onwards. In line with this data, early treatment (day 15-30) with exogenous NGF 30 ng/ml (day 15-30) neither enhanced the expression of noradrenergic markers as dopamine beta hydroxylase (DBH) and tyrosine hydroxylase (TH) nor affected noradrenaline levels (N=1 independent experiment, n=4 tissues). In contrast, SNO treated with NGF 30 ng/ml at a later stage (day 45-52) resulted in significantly higher transcript levels of noradrenergic markers (TH, DBH) vs cholinergic marker choline acetyltransferase (CHAT; N=3, n=12 tissues / group). Wholemount immunofluorescence further supported that NGF treatment increased the amount of DBH neurons in the SNO. Finally, catecholamine quantification in SNO by liquid chromatography mass spectrometry showed significantly higher levels of noradrenaline and lower levels of acetylcholine in tissues treated with NGF (N=3, n=12 tissues / group). This data support that exogenous NGF treatment between d45-52 can further enhance noradrenergic neuron (sympathetic) content in SNO vs catecholaminergic (parasympathetic).

Keywords: neurons, organoid, NGF

3:45 PM – 4:45 PM

443

ICG-MEDIATED PHOTOPORATION OF THE INNER LIMITING MEMBRANE TO ENHANCE RETINAL GANGLION CELL DELIVERY

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Glaucoma is a leading cause of irreversible blindness worldwide. Its primary hallmark is the progressive loss of retinal ganglion cells (RGCs) which inevitably leads to vision loss. A revolutionary cure for glaucoma could be the replacement of these lost neurons with stem-cell-derived RGCs. A major challenge to RGC transplantation is the limited migration of the transplanted cells into the retina following intravitreal injection due to the presence of the inner limiting membrane (ILM). The ILM represents a double-edged sword for RGC engraftment: on the one hand it greatly hinders cell migration into the retina, whereas on the other hand its presence is necessary for correct development of the transplanted RGCs. ILM photoporation offers a controllable method, allowing us to tune the integrity of the ILM to the needs of the iPSC-derived-RGCs. To photoporate the ILM, the photosensitizer indocyanine green (ICG) (0.1-1 mg/ml) was applied at the ILM side of bovine retinal explants followed by laser treatment (800 nm, 7 ns pulses, 1.65 J/cm2) with varying scanning patterns. Afterwards, iPSC-derived-RGCs expressing TdTomato (20.000 per 5 mm explant) were co-cultured for 7 days on untreated and photoporated flatmounts. On day 7, samples were fixed and stained, allowing us to quantify the number of RGCs per explant using confocal microscopy. Remarkably, RGC transplantation in ILM

perforated explants was found to be enhanced, seeing that the mean survival of the donor RGCs in perforated explants (0.25 mg/ml ICG) was 10.1±2.9% compared to 5.4±3.3% for untreated explants. Furthermore, several topographic spatial metrics imply that photoporation reduces donor RGC clustering: the nearest neighbor index increases from 0.38±0.05 to 0.57±0.07 in photoporated explants along with the regularity index which rises from 0,73±0.18 to 1,14±0.07. Our findings demonstrate that ICG-mediated ILM photoporation is a promising method to disrupt the ILM in a highly tunable and controlled manner. Preliminary experiments indicate that ILM photoporation can increase donor RGC survival and decrease clustering. Future studies will involve exploration of the impact of different laser scanning patterns on RGC transplantation and RGC development. In addition, we will compare photoporation with enzymatic digestion of the ILM using collagenase.

Funding Source: Emma De Coster is a doctoral fellow of the Research Foundation-Flanders, Belgium (FWO Vlaanderen, grant 1S19723N).

Keywords: cell delivery, iPSC-derived retinal ganglion cells, photoporation

3:45 PM – 4:45 PM

445

FUNCTIONAL RECONSTRUCTION OF DAMAGED BRAIN USING STEM CELLS, A TWO-WAY ROAD

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Stem cell therapy using human skin-derived neural progenitors holds much promise for the treatment of neurological disorders. One of the biggest challenges of this strategy is the confirmation of cell replacement leading to neuronal reconstruction of the damaged circuitry. Recently developed sophisticated research tools like optogenetic control of neuronal activity and rabies virus monosynaptic tracing, among others, have made it possible to provide solid evidence about the functional integration of grafted cells and its contribution to improved recovery in animal models of brain damage. Moreover, clinical trials in patients with Parkinson's Disease represent a proof of principle that stem cell-based neuronal replacement could work in humans. Our studies with transplantation of human skin-derived cells neurons in animal model of stroke showed bi-directional functional connectivity from host neurons to grafted cells and from the later to specific brain areas of the host brain. In addition, we demonstrated the contribution of graft-originated neurons to the functional recovery of the animals, supporting the hypothesis that human somatic cells reprogrammed into neurons can get integrated in the lesioned neuronal circuitry and opening new avenues for development of the future clinical applications.

Funding Source: This work is supported by the Ministry of Science and Innovation, Spanish Government.

Keywords: brain damage, pluripotent stem cells, functional integration

3:45 PM – 4:45 PM

447

MIGRATION AND DIFFERENTIATION OF DENTATE GYRUS (DG) STEM CELLS IN 3D HIPPOCAMPAL NEUROGENESIS MODELING PLATFORM

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Adult hippocampal neurogenesis including migration and differentiation of dentate gyrus (DG) niche stem cells are important to produce neurons and astrocytes as well as form more neural circuits in the adult hippocampal region. However, the lack of adequate in vitro 3D models leads to the cellular mechanism responsible for in vivo adult hippocampus not fully understood yet. Here, we introduced a 3D hippocampal morphogenic spheroid model in a microfluidic culture platform that enables to mimic DG stem cell migration and differentiation aspects of adult hippocampal neurogenesis. We investigated DG migration under the Rapgef2 knockdown condition and DG differentication in the DG-hippocampal integrated spheroid which was exhibited the gene expression changes related to neurogenesis, glial cell development and myelination, and neurotransmission, during DG migration procession. We suggest that this DG-hippocampal integrated spheroid as a promising in vitro model for recapitulating in vivo adult hippocampal neurogenesis.

Funding Source: This study was supported by the Institute for Basic Science of Korea (IBS-R011-D1) and by the Institute of Quantum Biophysics.

Keywords: adult hippocampal neurogenesis, dentate gyrus cell niche development, hippocampal morphogenic microfluidic chip

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

449

GENE-EXPRESSION MEMORY-BASED LINEAGE INFERENCE IN SC-RNASEQ DATASETS

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Assigning single cell transcriptomes to cellular lineage trees by lineage tracing has transformed our understanding of differentiation during development, regeneration, and disease. However, lineage tracing is technically demanding, and often limited in time-resolution, leaving

most scRNA-seq datasets devoid of lineage information. Especially the analysis of close lineage relationships to study cell fate decisions in in vivo settings and in human samples remains challenging. To address this gap, we developed Gene Expression Memory-based Lineage Inference (GEMLI), a computational tool allowing to robustly determine closely-related cell lineages solely from scRNA-seq datasets. This is possible by using the stability of subtle gene expression differences, instead of genetic marks, for lineage tracing. By identifying cells related over several cell divisions in scRNA-seq data, GEMLI allows to study heritable gene expression, to discriminate symmetric and asymmetric cell fate decisions, and to reconstruct small individual multicellular structures from pooled scRNA-seq datasets. Importantly, as GEMLI does not require any experimental engineering procedures and allows to track closely-related cells, it enables to study cell differentiation in vivo at unprecedented resolution. We showed GEMLIs broad applicability to analyze the differentiation landscape of hematopoietic stem cells, and to study the heterogeneity of individual intestinal crypts, and organoids, as well as individual pancreatic cancer metastases. The application of GEMLI to datasets of human breast cancer biopsies, a setting in which no other lineage tracing technique can be applied, identified cell lineages and previously unknown gene expression changes at the onset of cancer invasiveness in vivo. We expect GEMLI to greatly ease and expand the study of cell lineages in a wide range of physiological and pathological contexts.

Keywords: lineage tracing, scRNA-seq, cell fate decisions

3:45 PM – 4:45 PM

451

CONTROLLING STEM CELL ENERGY METABOLISM USING SURFACE TOPOGRAPHIES

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In vitro culture of mesenchymal stem cells (MSCs) is closely associated with rapid differentiation and loss of multi-lineage potential, impeding their large-scale expansion for use in regenerative therapies. Energy metabolism, and the fine regulation of glycolysis and oxidative phosphorylation (OXPHOS) fluxes, has emerged as a key factor in improving essential stem cell processes involving proliferation, self-renewal and cell fate. Whereas quiescent stem cells are primarily glycolytic, characterized by low levels of OXPHOS-generated reactive oxygen species to maintain stemness, rapid stem cell proliferation and differentiation is accompanied by a shift in bioenergetic metabolism towards OXPHOS and a loss of multipotency respectively. Distinct material properties have been shown to affect cell metabolism and support stem cell expansion while maintaining their multipotent phenotype. A better understanding of how MSC metabolism is affected by surface structures may be crucial in the development of a consistent MSC expansion platform. This study aims to characterize the surface topography-induced metabolic switch in MSCs. Bone-marrow derived MSCs were cultured on both flat and micro-topographically enhanced polystyrene growth substrates, fabricated by soft lithography and hot embossing techniques, in order to characterize the topography-induced metabolic switch in these cells. A resazurin-based metabolic assay indicates a reduced mitochondrial activity MSCs cultured on topography-enhanced substrates relative to flat controls after 7 days of culture. In addition, both a topography-induced increase and decrease in glycolytic flux (i.e. glucose consumption, lactate secretion, and differential expression of glycolytic genes) can be observed, for which directionality of the effect was dependent on topographical feature design. Further exploration of the topography-induced metabolic switch in MSCs and identifying the core signaling pathways involved allows for the development of a topography-enhanced cell culture platform that enables the control of stem cell energy metabolism and fate respectively.

Keywords: stem cell metabolism, surface topography, metabolic switch

3:45 PM – 4:45 PM

453

ENHANCING GENE EDITING EFFICIENCIES IN IPSCS: HIGH-THROUGHPUT SGRNA SCREENING AND NGS ANALYSIS ACROSS GENOMIC SAFE HARBOR SITES

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This project aimed to identify ideal genomic safe harbor (GSH) sites (and specific sgRNA sequences) that generate high gene editing knock-in efficiencies in induced pluripotent stem cells (iPSCs) using synthetic biology and machine learning. We utilized EditCo's Discovery Platform and its Optimal Design of Editing Strategies (ODES) to perform high-throughput screening of 276 sgRNAs across 25 GSH sites, utilizing electroporation of Cas9/sgRNA ribonucleoprotein complexes in an iPSC cell line together with single-stranded oligos as HDR templates. Optimization of the editing conditions was performed to be able to detect different KI efficiencies. Editing levels were assessed through Next Generation Sequencing (NGS). Library preparation from genomic DNA was performed using an automated platform that allowed generating eight data points from each electroporation reaction: having two technical replicates, testing two primer pairs per site, and performing each PCR per duplicate. In total, 4,608 samples were sequenced, obtaining a high reproducibility between technical replicates and primer pairs. Success rates from sequencing runs across plates ranged from 88.3% to 98.0%, with specific challenges noted, such as complete amplicon dropouts for certain guides indicating some sequencing challenges across different GSH sites. The integration of automated processes in the editing and sequencing phases underscores EditCo's innovative approach to the high throughput screening of guide RNA for optimal activity.

Keywords: CRISPR, high throughput, guide RNA screening

3:45 PM – 4:45 PM

455

EFFICIENT AND RELIABLE MEASLES REPROGRAMMING PLATFORM FOR THE GENERATION OF HUMAN IPSC

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Previous studies from our lab have established the Measles virus (MeV) vector as an efficient and safe method for reprogramming somatic cells into induced pluripotent stem cells (iPSC). However, the efficiency was limited to 0.2%. Here, we present the next generation of the MeV reprogramming vector. The MV(O)(SK)(M) previously established by Rallabandi et al. was modified by substituting the P gene of the wild strain, MVPwt(O)(SK)(M). After validation of the four reprogramming factors OCT4, SOX2, KLF4, and c-MYC expression, and the propagation of the MVPwt(O)(SK)(M) vector, the reprogramming ability was analyzed in 6 adult human fibroblasts (AHF). Reprogramming was achieved at low multiplicity of transduction in 5 out of the 6 AHFs with average efficiency between 1.25-2.25%. Only one AHF was reprogrammed with a low efficiency (0.23%). At day 25 post-transduction, we isolated iPSC clones from each reprogramming. Following iPSC amplification, immunostaining was performed to confirm the induction of endogenous pluripotency-associated markers, SSEA-4, TRA-1-81, TRA-1-60, and NANOG. The iPSC pluripotency propensity was further confirmed by spontaneous embryoid body (EB) formation and guided differentiation into three germ layers using immunostaining and confocal analysis. The rapid elimination of the vector from the iPSC at or before passage five was confirmed using qRT-PCR. Finally, we show the ability of the MeV-derived iPSC to differentiate into hematopoietic stem cells, pancreatic progenitor cells, neuronal progenitor cells, and terminal neurons. In conclusion, our study shows that the latest generation of MeV reprogramming vector is highly efficient and reproducibly reprograms adult human fibroblasts into iPSCs. Compared to other non-integrating reprogramming technologies, such as the Sendai system, MeV has a comparable to higher efficiency using a single vector, a lower multiplicity of transduction, and exhibits a faster vector elimination from iPSCs.

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Keywords: iPSC Reprogramming, viral vector, iPSC differentiation

3:45 PM – 4:45 PM

457

CATCHER: A BIOINFORMATIC TOOLBOX FOR CLONALITY AND TREATMENT-CONTROLLED SINGLE CELL GENOMICS SCREENS IN PLURIPOTENT STEM CELL MODELS

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Pooled functional genomics screens in pluripotent stem cell (PSC) models offer great potential to uncover disease-associated genes and molecular pathways. We recently established iPS2-seq (iPS-optimized inducible Postranscriptional Silencing in pool deconvoluted by single cell sequencing), which allows clonally controlled, single cell aware, isogenic engineered, and stage specific loss-of-function perturbations. iPS2-seq opens new possibilities thanks to the comparative analysis of single cell gene expression in cells with induced knockdown (KD) of a gene of interest and paired controls of the same clone but without LoF, reducing confounding factors due to genetic and epigenetic clonal variability. The new experimental design calls for a tailored computational and statistical framework capable of exploiting its strengths. We developed catcheR (clonality and treatment controlled shRNA effect findeR), a bioinformatic workflow written in R which exploits Docker containers to guarantee reproducibility. CatcheR allows great analysis flexibility while retaining good accessibility to researchers lacking strong computational expertise. catcheR first quantifies barcodes associated with KD perturbations, applying dynamic thresholds in order to robustly identify single cell transcriptomes associated with a single KD perturbation. Cells are grouped by clones characterized by an additional randomized barcode. Clones are quality controlled through orthogonal NGS analyses of the shRNA libraries, which allows filtering and denoising of molecular cloning errors. Subsequent steps include KD and control integration for batch correction, dimensionality reduction, clustering, and statistical analysis of the distributions of KD and controls in specific subpopulation(s). Additionally, biologically driven dimensionality reduction via autoencoders identifies pathways affected by the perturbations. KD effects can also be evaluated via enrichment/depletion and differential expression analysis of individual KD clones versus matched controls. In all, catcheR provides a comprehensive package to analyze iPS2-seq experiments, and provides a framework that could be applied to other methods designed to robustly control clonal and treatment variability in single cell genomics pooled screens.

Funding Source: Giovanni Armenise-Harvard Foundation Career Development Award 2021 (A.B.); Additional Ventures Single Ventricle Research Fund 2021 (A.B.); ERC Starting Grant (TRANS-3; 101076026 (A.B.)

Keywords: bioinformatics, genomic screen, single cell analysis

3:45 PM – 4:45 PM

459

EPIBINDERS: DE NOVO DESIGNED PROTEINS TO SELECTIVELY ALTER EPIGENETIC MODIFICATIONS

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It is well known that epigenetic modifications of histones and DNA govern gene accessibility and transcription. As such, selective recruitment of epigenetic modifying enzymes to particular gene loci has long been an interest of genetic engineers: the ability to precisely control DNA state would allow for manipulation of things like cell fate, stemness, and senescence by awakening or silencing genes necessary for those states. Meanwhile, de novo protein design has gone through a veritable revolution with the release of deep-learning methods such as RFDiffusion and ProteinMPNN. I will describe my efforts to create a designer epigenetic modifying molecule that utilizes dead Cas9 as a locator and a small de novo protein called a minibinder as an enzyme recruiter. Our binder design pipeline has produced 4 robust minibinders to DNMT3a, a de novo methyltransferase, and we are currently screening several thousand for Tet1 and Tet2, both demethylators. These minibinders bind to DNMT3a with affinities between 35 to 850 nM and I will describe our initial experiments in iPSCs to validate target protein binding and editing activity. I will also detail our future plans to effect precise epigenetic editing to induce differentiation and treat disease. This project represents an intrinsically novel approach to not only epigenetic specificity, but also stem cell research as a whole. Ultimately, our goal is to produce a fully de novo designed molecule that would allow precise control over cell state and fate change both for basic research and individualized treatments.

Keywords: epigenetics, protein design, epigenome engineering

3:45 PM – 4:45 PM

461

REVOLUTIONIZING STEM CELL SELECTION: A LABEL-FREE APPROACH USING THE VERLO IMAGING CELL SORTER

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In the evolving landscape of stem cell research, the ability to distinguish between undifferentiated and differentiated cells is paramount for advancing both basic science and therapeutic applications. The traditional reliance on fluorescent markers for cell sorting, although effective, introduces complexity and may affect cell viability and functionality. Our recent study leverages the VERLO™ Imaging Cell Sorter's capabilities to address this challenge in a label-free manner, revolutionizing the process of stem cell selection. Utilizing high-resolution imaging and advanced analytics, the VERLO sorter enables the precise identification and separation of undifferentiated from differentiated human pluripotent stem cells without the need for external markers. This approach preserves cell integrity and eliminates the potential impacts of labeling on cell physiology. Our results demonstrate a significant advancement in stem cell research methodologies, offering a more efficient, reliable, and non-invasive alternative for cell sorting. We successfully applied this label-free sorting technique to isolate pure populations of undifferentiated stem cells, achieving high efficiency and viability. This method not only enhances the potential for scalable stem cell applications but also opens new avenues for research into cellular development, disease modeling, and regenerative medicine. The utility of the VERLO Imaging Cell Sorter in this context underscores its value as a critical tool for the next generation of stem cell research and application.

Keywords: imaging cell sorter, cell differentiation, pluripotent stem cells

3:45 PM – 4:45 PM

463

SURFACE PROTEOMICS OF C-MANNOSYLTRANSFERASE-DEFICIENT HUMAN INDUCED PLURIPOTENT STEM CELLS USING A CLEAVABLE AMINOOXY-BIOTIN-REAGENT

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The attachment of single mannose molecules via covalent C-C-bonds to specific protein´s tryptophan residues within the ER (C-mannosylation) can be crucial for protein folding, secretion or plasma membrane (PM) localization. This unique type of protein glycosylation is catalyzed by enzymes designated as C-mannosyltransferases (C-Mans) for which four isoforms exist in Mammalia (DPY19L1-DPY19L4) whose substrate specificity remains poorly understood. We previously established DPY19L1- and DPY19L3-knockout (KO) human induced pluripotent stem cell (hiPSC) lines and by quantitative analysis of secreted proteins (secretomics) novel target proteins for C-Mans could be uncovered. However, for DPY19L4, identification of target proteins remains elusive. Therefore, applying CRISPR-Cas9, we additionally generated DPY19L4-KO hiPSCs. Proteins with impaired trafficking through the secretory pathway in DPY19L4-KO hiPSCs are candidates to be C-mannosylated by DPY19L4. Therefore, we intend to comparatively assess the plasma membrane proteome of wildtype (WT) and C-Man-deficient hiPSCs (C-Man-KOs). WT and C-Man-KOs are cultured as spheroids in Matrigel-free suspension culture to avoid contamination with foreign proteins. Cell-surface glycoproteins are specifically biotinylated upon oxidation with an aminooxy-biotin reagent containing a linker with a disulfide bridge that is cleavable by chemical reduction. Thereby glycosylated membrane proteins can be specifically captured based on the stringent avidin-biotin interaction, washed and then easily liberated by reductive cleavage of the linker. Initial proteomic analyses show strong enrichment of plasma membrane proteins and significantly reduced contamination with cytosolic proteins by this approach paving the way

for further studies intended to quantitatively compare the PM proteomes of WT and C-Man-KOs.

Funding Source: FOR2509, DFG, project number: 289991887

Keywords: surface proteomics, cell surface trafficking, C-mannosylation

3:45 PM – 4:45 PM

465

USE OF A MACHINE LEARNING PLATFORM AS A METHOD FOR IPSC CHARACTERIZATION & EXPANSION

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Machine learning tools offer opportunities to characterize cells and understand patterns that may not be clear to the human eye. Additionally, large numbers of images can be analyzed pixel-by-pixel and provide insights into how characteristics shared by colonies across images may be connected, helping to advance discoveries related to stem cell phenotype and function. This allows for the characterization of stem cells in culture and reduces the need for staining that is typically used throughout the iPSC generation and expansion process. Innovative tools like Landing Lens make the process of generating machine learning algorithms simple, by allowing users to easily label and classify images based on detection, segmentation, or classification. In relation to the characterization of stem cells, the segmentation tool provides the ability to label images pixel-by-pixel to train the model on what features in an image belong in different classes as defined by the user. The labels created are then fed into a deep-learning algorithm to generate a model, that can then be applied to new images to test for accuracy and precision. In this study, we utilized Landing Lens machine learning software to generate an algorithm that can be used to identify iPSC colonies containing differentiation, and those without. Images of iPSC colonies from the early stages of reprogramming, all the way through expansion were used to train the model on characteristics of iPSC cultures with varying levels of differentiation. This model used pixel-by-pixel labeling that was verified by multiple operators to ensure that the model training was as close to human observation as possible. Results indicate high precision and recall for the model created, and ideal colony identification was accurate when compared to operator labeling. This algorithm can also be applied as a platform through which to train operators in a manufacturing setting. Culturing iPSCs and being able to recognize differentiation as it appears and select pluripotent colonies requires a large amount of training. Using an AI-based model as a baseline through which to train operators may reduce training timelines and allow for more successful culture of iPSCs to generate cell therapies.

Keywords: iPSC, machine learning, characterization

3:45 PM – 4:45 PM

467

CHARACTERISTICS OF JUSTIFIABLE LABORATORY-BASED RESEARCH INVOLVING HUMAN PLURIPOTENT STEM CELL, HUMAN EMBRYO, AND RELATED RESEARCH

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The 2021 International Society for Stem Cell Research Guidelines for Stem Cell Research and Translation state that laboratory research involving human pluripotent stem cells, human embryos, and human gametes should be carefully scrutinized for scientific rigor and adequate justification before the research goes forward. However, the Guidelines do not specifically address how the assessment for scientific and ethical justification be made. Leaving out specific criteria for scientific and ethical justification is generally reasonable, considering that professional society guidelines need to be broadly applicable to many avenues of research, cannot be too directive as to supersede (or appear to supersede) policies and regulations of specific countries and jurisdictions, and cannot address advances that are at the time of writing unknown. Moreover, without knowing the details and implications of any particular research program, it is difficult to derive fair and unbiased criteria to assess it. Nonetheless, with recent advances in extended in vitro culture of embryos, in vitro gametogenesis, organoid derivation (including of neural origin), and embryo model development, it has become evident that some detailed review guidance would be beneficial to oversight bodies and researchers alike. Indeed, considering the ethical and scientific complexity and novelty of pluripotent stem cell research, the speed at which laboratory research capabilities are advancing, and public interest and concern regarding these avenues of research, the need to develop specific criteria for assessing “scientific rigor” and “appropriate scientific justification” has become urgent. Therefore, the ISSCR Ethics Committee conducted several internal workshops with invited experts in human embryo, embryo model, and other pluripotent stem cell research, to better understand the directions this research is going, as well as its implications and capabilities. Using this information, and examining criteria used by various jurisdictions and federal agencies to assess research proposals, we have arrived at a number of criteria that may be used by oversight and review committees, as well as by researchers, to address scientific rigor, and “appropriate” scientific and ethical justification in this rapidly evolving area of research.

Keywords: ethics, oversight, embryo

3:45 PM – 4:45 PM

469

BIOINK AND BEYOND: BIOPRINTING STRATEGIES FOR STEM CELL-BASED TISSUE ENGINEERING

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While traditional cell culture methods afford limited control over shape, 3D bioprinting provides extensive regulation of tissue morphology. Nevertheless, bioprinting encounters challenges due to elevated shear stresses and suboptimal shape fidelity. Consequently, hydrogels are frequently incorporated into the bioink; however, these hydrogel components may impede cellular self-assembly. To address these constraints, we developed a simple and inexpensive xanthan gum hydrogel based printing method. Upon depositing a bioink into this hydrogel embedding bath, the liquid phase of the ink diffuses into the xanthan matrix, facilitating the convergence of particles until they achieve contact. This process has been designated as 'diffusion packing'. We utilized this method for bioprinting of a variety of bioinks composed of different cells into dense fibers. The morphology of these tissues varied based on the fusion potential of the cells used. HPSCs were successfully printed and diffusion packed forming linear compacted tissues. The morphology of the fibers changed over the course of the first few hours. The stiffness of the support matrix had a drastic effect on the behavior of the tissue fibers. The hPSC fibers expanded during the first 12 hours and then compact into a denser construct in the stiff bath, while in the soft bath the fibers compacted without significant expansion. In addition, we 3D printed hPSC-derived cardiomyocytes and epicardial cells into a compacted connected fiber. The cells were randomly dispersed immediately after printing, but gradually migrated within the fiber to form a two-layered tissue consisting of a body of cardiomyocytes covered by a layer of epicardial cells, which resembles the organization in human heart tissue. Homogeneous distribution of alpha-actinin within the fiber and spontaneous beating was observed. After electrical pacing, cardiac fibers started beating synchronously and could follow a pacing frequency up to 4 Hz. In conclusion, we developed a novel bioprinting technique that enables the generation of complex tissues without lowering their potential for self-organization.

Keywords: tissue engineering, bioprinting, self-organizing hPSC-based tissue

3:45 PM – 4:45 PM

471

OPEN TOOLS AND RESOURCES FOR QUANTITATIVE IMAGE ANALYSIS OF CELLULAR ORGANIZATION

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The Allen Institute for Cell Science aims to understand the principles by which human induced pluripotent stem cells (hiPSCs) establish and maintain robust dynamic localization of cellular structures, and how they transition between states during differentiation and disease. To understand and predict cellular organization, we have created the Allen Cell Collection of fluorescently-tagged hiPSC lines and a suite of openly available image-based computational tools for quantitation and analyses. Each hiPSC line expresses a fluorescently tagged protein that represents a major cellular structure or organelle. The Allen Cell Collection now contains more than 56 high quality lines that target more than 40 key cellular structures and substructures, including a new set of cardiac, skeletal, and nuclear disease lines, and are available to academic and commercial researchers. Following tagging, we perform live 3D imaging via our microscopy pipeline and generate hundreds of thousands of high-resolution images. To increase throughput, we developed an automated hiPSC culture procedure using the Hamilton STAR liquid handling system compatible with high-resolution 3D microscopy. To meet the challenge of segmenting, analyzing, and visualizing this massive volume of imaging data, we have developed several tools which we make available to the public for free. This includes the Allen Cell and Structure Segmenter, which we have updated and made more accessible by developing napari plugins for each the Classic Image Segmentation workflow (already released) and recently the Iterative Deep Learning Workflow (coming soon). The latter is powered by a comprehensive deep learning tool for streamlining microscopy image transformations called CytoDL which we recently released for python users. In addition, we have developed a volume viewer tool with path-trace rendering called AGAVE, a visual analysis tool to interrogate biological simulations called Simularium, and an online tool to visualize and plot features of segmented images called the Cell Feature Explorer, among others. Finally, we provide educational resources, software, and analysis code developed at the Institute and our datasets are openly available to the community for access and download. These tools, resources, and data can be found at allencell.org.

Keywords: hiPSC, deep learning, cell culture

3:45 PM – 4:45 PM

475

HLA-BASED BANKING OF INDUCED PLURIPOTENT STEM CELLS IN SAUDI ARABIA

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Human iPSCs' derivation and use in clinical studies are transforming medicine. Yet, there is a high cost and long waiting time associated with autologous iPS-based cellular therapy. The genetic engineering of hypo-immunogenic iPS cell lines is hampered with numerous hurdles. Therefore, it is increasingly interesting to create cell stocks based on HLA haplotype distribution in a given population and assess the potential of HLA- based iPS banking for the Saudi population. By interrogated the HLA database of the Saudi Stem Cell Donor Registry (SSCDR), containing high-resolution HLA genotype data from 64,315 registered Saudi donors at the time of analysis, the most frequent HLA haplotypes in the Saudi population was determined. Furthermore, an in-house developed iterative algorithm was used to identify HLA matching percentages in the SSCDR database and cumulative coverage. These analysis revealed that the establishment of only 13 iPSC lines would match 30% of the Saudi population, 39 lines would attain 50% coverage, and 596 lines would be necessary for over 90% coverage. Subsequently, to develop a clinically relevant protocol for iPSCs generation, and to illustrate the applicability of the concept of HLA-based banking for cell therapy purposes, the first HLA-based iPS cell line in Saudi Arabia was generated. Clinically relevant methods were employed to generate the two iPS clones from a homozygous donor for the most prevalent HLA haplotype in the Saudi population. The proof-of-concept HLA-iPSCs, which cover 6.1% of the Saudi population, successfully demonstrated pluripotency and the ability to differentiate into various cell types including beating cardiomyocytes and neuronal progenitors. The comprehensive genetic analysis corroborated that all identified variants in the derived iPSCs were inherently present in the original donor sample and were classified as benign according to the standards set by the ACMG. The successful generation and validation of iPSC lines based on the most prevalent HLA haplotype in the Saudi population signify a promising step toward broadening the accessibility and applicability of stem cell therapies and regenerative medicine. Thus, our study sets a road map for introducing iPS-based cell therapy in the Kingdom of Saudi Arabia.

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Keywords: induced pluripotent stem cells, HLA-based banking, IPS-based therapies

3:45 PM – 4:45 PM

477

A PLATFORM FOR DIRECT CYTOSOLIC DELIVERY VIA MECHANOPORATION: OPENING OPPORTUNITIES FOR MULTIPLEXED AND SEQUENTIAL DELIVERIES ACCELERATING CELL REPROGRAMMING AND DIFFERENTIATION

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The discovery of human induced pluripotent stem cells (hiPSCs) has opened opportunities to treat previously intractable diseases across areas of unmet clinical needs including degenerative and metabolic diseases, genetic disorders and tissue regeneration. Protocols have been devised based on fundamental discoveries in developmental biology to reprogram somatic tissues into hiPSCs and to differentiate hiPSCs into cellular lineages of high therapeutic value. However, these protocols are often lengthy and costly, and reprogramming efficiency remains a key challenge. Many cell engineering strategies focus on permanent genome modification of cells which has long-term safety concerns including off-target genetic effects and permanent

expression or suppression of target genes. Further, current technologies for engineering human primary cells, such as electroporation and lipofection, present limitations in delivery of cargo without off-target effects. At Portal, we have developed a silicon membrane-based intracellular delivery technology that facilitates mechanoporation of cells. In this process, cells are temporarily rendered permeable as they pass through the membrane, allowing for delivery of cargos (e.g. RNAs, peptides, proteins) into the cytosol. Portal's technology is compatible with delivering multiplexed cargo mixtures across multiple types of cells and materials, and can be used for multiple sequential deliveries with minimal effect on gene expression. Our results in human iPSCs show high delivery efficiency and significant potential for our technology to overcome challenges to the directed differentiation of PSCs. In addition to PSCs, human hematopoietic stem cells (HSCs) are used to treat hematologic conditions; however, current clinical approaches still face major challenges. Portal technology shows promise to overcome challenges of the clinical application of HSCs through the use of RNA to modulate cell function. With continued progress in simplifying stem cell engineering through RNA-based technologies, we aim to unlock biological potential in the field of cell therapy while simultaneously reducing safety concerns associated with other delivery modalities.

Keywords: mRNA, reprogramming, differentiation

3:45 PM – 4:45 PM

479

DEVELOPMENT OF A HIGH-THROUGHPUT APPROACH TO IMAGING AND SORTING OF 3D CELL CULTURE

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Hindering the adoption of 3D cell culture models for high-throughput screening is a lack of control over 3D cell culture formation and a reliable non-invasive readout for these 3D cell culture models. Here, we were able to analyze, select and sort organoids and spheroids using a large object imaging flow cytometer, the COPAS VISION. As models, we used iPSC-derived retinal epithelial organoids and tumor spheroids. We were able to discriminate between stages of organoid/spheroid formation and expression of fluorescent markers. In contrast to traditional methods, where organoids are sorted out by hand under a microscope, our approach offers a high-throughput manner to assess quality and sort uniform organoids/spheroids to be used for testing, transplantation and disease modeling.

Keywords: 3D-Cell-Culture, imaging, sorting

3:45 PM – 4:45 PM

481

RECONFIGURING THE ACTIONS OF THE YAMANAKA FACTORS (OSKM) CAN SUPPORT DIRECT LINEAGE CONVERSIONS

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Reprogramming somatic cells to primed iPSCs reflects aspects of post-implantation human development, while naïve iPSCs capture the earlier, pre-implantation stages. However, the molecular principles underlying the emergence of these pluripotency states remain poorly understood due to the varied responses exhibited by individual cells when exposed to the Yamanaka factors (OSKM). To elucidate OSKM actions, we generated a comprehensive single-cell atlas capturing jointly both gene expression and chromatin accessibility in over 90,000 male and female human fibroblast cells as they reprogram into pluripotent stem cells. This provides a unique, time-resolved view of the regulatory events that guide cells towards either prime or naïve pluripotency. We find that conflicting gene expression programs resolve in a sequential manner, with somatic program decommissioning preceding the activation of prime and naïve pluripotency genes. At the epigenetic level, chromatin accessibility at lineage specific and pluripotency-related genes precedes gene expression and is concomitant with somatic enhancer silencing within the same cell, suggesting that dual changes in chromatin accessibility may prime cells for lineage commitment. To uncover novel regulators of somatic-to-hiPS reprogramming we devised a novel method that facilitates the overexpression of all annotated human transcription factor (TF) splice isoforms (>3500) in combination with OSKM, capturing 15000 reprogramming (SSEA4+) and non-reprogramming (SSEA4-) cells, and their corresponding transcriptome, epigenome, and TF genomic sites of impact. We identify novel facilitators and impediments of the reprogramming process and uncover epistatic effects of lineage-specific TFs on OSKM that result in the generation of endoderm and mesoderm progenitors by direct lineage conversion bypassing the iPSC state. Mechanistically these events lead to reconfiguration of OSKM binding. Our results provide proof of principle that OSKM induction can support direct conversion into alternate lineages through direct and indirect interactions with lineage-specific TFs.

Keywords: naive and primed hiPSC reprogramming, single cell multiomics, epigenetics and chromatin

3:45 PM – 4:45 PM

483

CRACKING THE CODE: 7-DAY DIRECTED DIFFERENTIATION OF HIGH PURITY MELANOCYTES FROM ANY HIPSC LINE

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Melanocytes are the cells responsible for pigment production within the epidermis and hair follicles. Many pathologies are associated with the dysfunction of melanocytes, including hyperpigmentation, hypopigmentation, vitiligo, and melanoma. A rapid, scalable, and reproducible protocol to produce melanocytes from multiple human induced pluripotent stem cell (hiPSC) lines would aid in the ability to study these associated disorders in a patient-relevant manner without the need to undergo the invasive procedure to obtain primary melanocytes. In this study, three different hiPSC lines – including a vitiligo patient line – were differentiated using small molecules and growth factors that modulated different signaling pathways through daily media exchanges. The different developmental stages within the melanocyte cell lineage were recapitulated over a seven day-long protocol until MITF+/SOX10+ melanocytes developed at a purity of greater than 90%. Moreover, these cells can subsequently be cryopreserved in order to establish a large bank of melanocytes ready to be used for downstream assays. Melanocytes were thawed and matured for one week before being characterized via qPCR and immunocytochemical staining for specific genes and proteins MITF, TYRP1, TYR, PAX3, KIT, DCT, SOX10, S100b, and PMEL. Rapid melanogenesis abilities were shown via microscopic and macroscopic pigment production in vitro after only one week of maturation. To demonstrate their utility as a simple assay for screening purposes, large scale batches of melanocytes were manufactured and banked. Melanocytes were thawed into 96-well plates and after a week of maturation were used to screen and identify pigment regulating agents. We show that 4-butylresorcinol, an inhibitor of tyrosinase and tyrosinase-related protein-1, can macroscopically decrease pigmentation in only three days. In summation, these data showcase the ability of multiple healthy and diseased iPSC lines to be rapidly and efficiently differentiated into functional melanocytes. This process allows for the investigation into different diseases implicated by melanocyte dysfunction.

Keywords: melanocytes, vitiligo, pigmentation

3:45 PM – 4:45 PM

485

NEW HIGH-THROUGHPUT INSTRUMENT FOR HIGH-RESOLUTION, GENOME-WIDE STRUCTURAL VARIATION DETECTION AT LOW VARIANT ALLELE FRACTION FOR CELL AND GENE THERAPY APPLICATIONS.

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In vitro culture and genome engineering in cell and gene therapy cause stress to the genome and can result in unintended changes, especially structural variations and chromosomal abnormalities. To ensure cell quality for downstream applications, appropriate genomic assessment methods are critical. Historically, karyotyping has been employed, but it is limited by its very low resolution and tedious laboratory workflow. Other methods such as PCR and targeted-sequencing can characterize only small genomic variants at specific loci. High resolution, genome wide structural variation (SV) detection remains an elusive undertaking. Especially when low variant allele fraction (VAF) SV detection is particularly important in cell and gene therapy applications. Optical genome mapping (OGM) is a technique that is revolutionizing cytogenomics. Here, we demonstrate the performance of a new high throughput system for generation of OGM data at the extraordinarily high coverage depth needed to interrogate SVs at ultra-low (≥1%) VAF. We show that with this instrument, 12 cartridges, each containing a single flow cell, can be loaded onto the carousal at once, and the instrument can collect at least 400× genome coverage for each of these flowcells in less than 24 hours. Data from the instrument can be processed by Bionano Access and VIA software to call structural variants, copy number variants (CNVs), regions of loss of heterozygosity (LOH), genome wide, and to assess based on reference gene annotations and likely functional impact. This new high throughput system with advanced bioinformatic data analysis has the potential to transform structural variation analysis in cell QC and bioprocessing. Stratys offers many advantages over the previous (Saphyr) generation of optical genome mapping instruments by increasing throughput up to 4× and enabling random access to loading and unloading samples at any time during a run. Optical genome mapping can help labs modernize and automate by providing an alternative to methods like karyotype, fluorescence in situ hybridization, and chromosomal microarray in genome integrity assessment applications for cell and gene therapy by offering sample to answer at very high resolution within 2 days.

Funding Source: Internal funding

Keywords: genome integrity, off target effects, cytogenetics

3:45 PM – 4:45 PM

487

THE TERRESTRIAL BENEFITS TO BIOMANUFACTURING IN LOW EARTH ORBIT

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Redwire's BioFabrication Facility (BFF) is the first 3D bioprinter on the International Space Station (ISS) and was designed to harness microgravity to advance 3D bioprinting. The ISS microgravity environment allows for 3D printing without sedimentation of cells and nutrients, instead printed components stay where they are printed. However, a significant number and variety of cells are required to 3D bioprint tissues and organs, representing a major roadblock to realizing the potential of biomanufacturing in microgravity. Research suggests that stemness and proliferative capacity are enhanced in stem cells grown in microgravity. To harness this effect, we have developed the Redwire Cell Factory, a suite of automated hardware committed to large scale production of biologics in all their forms. One part of the Cell Factory suite is the automated cell culture system for reprogramming cells into induced Pluripotent Stem Cells (iPSCs) on the ISS. The microgravity grown iPSCs may then be used aboard the ISS for fundamental research, the preparation of bioinks for 3D bioprinting, and direct cell therapy applications on Earth. The ability to manufacture cells, tissues, and whole organs in space for a patient on Earth, from that patient's own cells, could be regarded as one of the most significant advancements in healthcare in this century. The first element of the Redwire Cell Factory can maintain environmental conditions for cell growth, microscopy using phase, brightfield, or fluorescent imaging, as well as automate media changes on an individual well basis. The double locker payload has demonstrated successful transfection, maintenance, and imaging of primary cells while also maintaining a contamination free culture, and in a highly automated fashion with minimal human intervention. The transfection efficiency and cell viability were comparable between the Cell Factory and standard laboratory approaches. The combination of the Cell Factory technology with the BFF will enhance our ability to leverage microgravity for breakthroughs involving stem cells and 3D bioprinting. This combination of cell production and 3D bioprinting will be regarded as one of the most important and tangible returns on America's investment during the 30-year operation of the ISS.

Funding Source: NASA NRA NASA CCRPP

Keywords: biomanufacturing, reprogramming, microgravity

3:45 PM – 4:45 PM

489

CHARACTERIZATION OF NOVEL DNA-TARGETING CRISPR FAMILY PROTEINS FROM THE MICROBES

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The CRISPR/Cas system is a widely prevalent natural immune system in bacteria and archaea, primarily responsible for identifying and eradicating foreign genetic material. This system has evolved into a versatile and essential tool, playing crucial roles in genome editing, gene expression regulation, and in vivo therapies. In recent years, researchers have developed advanced gene-editing systems, such as base editors and prime editing systems, to address the imprecise repair consequences stemming from DNA double-strand breaks (DSBs) generated by conventional DNA base-editing tools. These innovative systems circumvent direct DSB induction and enable precise DSB repair. However, the size of classical gene-editing tools poses challenges for packaging and delivery when combined with base editing or prime editing systems using adeno-associated viruses. To address this issue, researchers have been exploring more compact gene-editing effectors in recent years. In the process, they have discovered the potential evolutionary relationship between the CRISPR/Cas system and the IS200/605 transposon family. In this study, we utilized bioinformatics approaches to identify 77 novel, compact Cas12 effectors originating from bacterial, archaeal genomes, and metagenomes, classified into seven distinct families. Six of these putative Cas12 effectors demonstrated gRNA-guided DNA double-strand cleavage activity. Future research will explore the biochemical properties of these six effectors through in vitro cleavage assays and evaluate their editing efficiency in mammalian cells. Additionally, we identified two putative Cas12 proteins homologous to the Cas12k family (with DNA-target binding activity but lacking DNA cleavage activity) that exhibit gRNA-guided DNA double-strand cleavage activity. This finding could potentially provide insights into the evolutionary development of the CRISPR/Cas12 system as a gRNA-guided DNA cleavage mechanism. While we have discovered several novel protein families and observed proteolytic activity in a subset of effectors, the specific cleavage mechanisms remain unclear. Future investigations will integrate in vitro cleavage assays and structural biology to further examine their function and underlying mechanisms.

Keywords: gene editing, CRISPR-Cas nucleases, base editing

3:45 PM – 4:45 PM

491

CROSS-SPECIES CRISPRI IN PRIMATE IPSCS TO STUDY GENE REGULATORY NETWORKS

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Perturbation screens in stem cells based on the CRISPR/Cas technology have emerged as important tools in developmental biology, enabling an efficient way to investigate gene regulatory networks (GRNs). CRISPR interference (CRISPRi) screens stand out as a powerful variant, employing a catalytically inactive (dead) dCas9 protein fused with a repressor domain such as KRAB, to selectively suppress specific target genes. Moreover, to distinguish primary from secondary perturbation effects, it is useful to implement an inducible expression of dCas9. Perturbation screens typically focus on a single species, but here we present the method in a cross-species setting that enables us to directly compare knockdown effects. Thus, we can leverage unique information on species-specificity and GRN conservation to study GRN evolution. We generated and validated stable KRAB-dCas9 iPS cell lines from three different primate species: Human (Homo sapiens), Gorilla (Gorilla gorilla) and Cynomolgus macaque (Macaca fascicularis). We confirmed dox-inducible expression of the KRAB-dCas9 construct that was integrated at the AAVS1-locus. Then we proved an efficient SOX2 knockdown on the RNA and protein level and moreover, identified subsequent effects on cell fate changes in the iPSCs. While association with a pluripotency profile was clearly reduced upon SOX2 knockdown, the cells were more likely to be associated with a mesodermal identity. Notably, these findings were consistent across species. Next, we performed a cross-species single-cell CRISPRi screen to compare the transcriptomic effects of knockdown of ~100 distinct transcription factors (TFs) between human and cynomolgus iPSCs. We found the target gene knockdown to be comparable across species, even though KRAB-dCas9 levels were varying. Furthermore, we could identify downstream targets of the perturbed TFs, as well as influences on the cell fate of the iPSCs upon specific TF-knockdown. Using this comprehensive data, we aim to study the regulatory networks of the perturbed TFs further and compare these between human and cynomolgus to unravel the evolution of gene regulation in primate iPSCs.

Funding Source: This project is funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) - project number 458888224.

Keywords: primate KRAB-dCas9 iPSCs, single-cell CRISPR screen, gene regulation

3:45 PM – 4:45 PM

493

UNRAVELING THE INTERPLAY BETWEEN SPATIAL A-TO-I EDITING AND CANCER STEM CELLS IN TUMOR MICROENVIRONMENTS

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Understanding the intricate relationships between A-to-I RNA editing and cancer stem cells (CSCs) holds immense promise in deciphering pivotal mechanisms of tumor and stem cell biology. A-to-I RNA editing, an epitranscriptomic process catalyzed by ADAR enzymes, has potential capabilities to contribute to the diversity of characteristics of CSCs through its influence on gene expressions and alternative splicing. Insights into the role of A-to-I editing on CSCs would be expected to not only provide a key to unveil significant interactions between cancer stem cells and tumors, but also offer potential avenues in therapeutic approaches. To decipher and map A-to-I editing events at molecular resolution across CSC microniches, we stained two CSC-related proteins, CD44 and ALDH1, across triple negative breast cancer (TNBC) tumor sections to identify regions of interest (ROI); these were then isolated using Spatially-resolved Laser Activated Sorting (SLACS) technology. REDITools analysis of RNA molecules from these laser-sorted ROI unveiled characteristic transcriptional A-to-I editing signatures in CD44low/-/ALDH1high microniches. These editing events were aligned with representation of non-synonymous editing of the GPX4 gene, a gene established as an important regulator in ferroptotic processes. In addition to heightened GPX4 expression, we noted a parallel enrichment of the ferroptosis gene set in the same CSC-like, A-to-I edited tumor microniches. Further analysis indicated a negative correlation between the frequency of A-to-I editing events of GPX4 in CSC-like microniches and clinical outcomes of TNBC patients. In this research, we discuss the impact of A-to-I editing on CSC properties, highlighting specific genes affected and the downstream implications of their altered expression. Accordingly, the relationship between spatial editing patterns with clinical outcomes and epitranscriptomic data of CSC-like microniches suggests that spatial A-to-I RNA editing could pave the way for innovative therapeutic strategies and facing consequent challenges of immune surveillance. This research emphasizes the value of understanding spatial RNA editing patterns for precision medicine approaches, ultimately honing in on targeting diseases within their specific contexts

Keywords: spatially-resolved laser activated cell sort, A-to-I editing, cancer stem cell microniches

3:45 PM – 4:45 PM

495

REVOLUTIONIZING TRANSFUSION MEDICINE: IPSC-DERIVED O-NEGATIVE RBCS

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We present a novel approach to address the shortage of universal donor blood, particularly for sickle cell disease patients. By reprogramming O-negative donor somatic cells into induced pluripotent stem cells (iPSCs), we can differentiate them into hematopoietic stem cells and eventually functional red blood cells (RBCs) free from the sickle cell mutation. This method could supply endless universal donor RBCs, reducing immune rejection risks. Challenges like optimizing differentiation protocols and confirming therapeutic safety are addressed using machine learning for protocol enhancement and computer vision for cell quality control. These advances in iPSC technology signify a transformative stride in transfusion medicine and patient care.

Keywords: universal, donor, blood

3:45 PM – 4:45 PM

497

A MACHINE LEARNING ENABLED IPSC PLATFORM FOR DISEASE MODELING, TARGET, AND DRUG DISCOVERY

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In this presentation, we unveil Insitro's cutting-edge platform that marries machine learning (ML) with induced pluripotent stem cell (iPSC) technology, heralding a new era in disease modeling and drug discovery. Our unique methodology synergizes high-content imaging—both fixed and live, RNA sequencing, and deep learning algorithms to accurately predict disease markers and unravel complex disease mechanisms. Central to our approach is the generation of a 'disease axis', a novel concept that maps the trajectory from diseased to healthy cellular states. In target discovery, we leverage both imaging and single-cell RNA sequencing data in pooled CRISPR screening, guided by self-supervised deep learning. This strategy effectively identifies genes that can shift cells along the disease axis from pathological to normal states. Our platform transcends traditional limitations by obviating the need for predefined biomarkers, thus facilitating the discovery of new therapeutic targets and drugs. The integration of AI in our biological research not only accelerates target identification and drug development but also sets a new benchmark in translational medicine. Through our work, we demonstrate the profound potential of AI to transform our understanding and treatment of complex diseases.

Funding Source: insitro/private

Keywords: machine learning, functional genomics, iPSC-disease modeling

3:45 PM – 4:45 PM

501

FULLY INTEGRATED PIPELINE FOR HPSC QUALITY CONTROL USING SNP ARRAY COPY NUMBER CALLING

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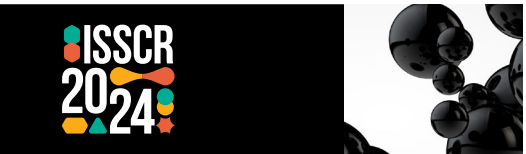
Human pluripotent stem cells (hPSC) or other cells growing continuously in culture tend to acquire mutations and genomic aberrations over time, even when specifically controlled cell banks are used. Therefore, occurrence of unwanted genomic abnormalities needs to be excluded to ensure high quality and reproducibility of experiments. One widely accessible method that combines low cost suitable for routine screening with a reasonable high genome wide resolution is the usage of single nucleotide polymorphism (SNP) arrays, which allow determination of copy number states on top of SNP genotyping. Despite the longstanding existence of SNP-array based copy number calling no easily accessible software package exists that allows a comprehensive analysis workflow. Existing tools either allow only individual analysis steps while requiring specialist bioinformatics knowledge or require commercial licenses without offering features specifically relevant to cell line quality control. To address this need, we have developed an easily accessible analysis pipeline for SNP array data-based copy number calling for cell line quality control. We compared the results of our implementation with current standards and developed strategies to improve copy number reliability based on manual CNV evaluation. The current pipeline allows users to easily run everything, from raw data processing to generation of an easily readable report. Additional features specifically tailored to cell line analysis include comparison of samples to a reference and cell line identity check using SNP profiles. For further development, we plan to include automatic annotation and scoring of copy number regions based on public databases. Additionally, we will extract hPSC specific features using an extensive data set collected as a collaborative effort among stem cell core facilities worldwide.

Keywords: quality control, copy number variation, automated analysis



3:45 PM – 4:45 PM

507

SPATIALLY RESOLVED LASER-ACTIVATED CELL SORTING (SLACS) AS A TRANSFORMATIVE TOOL IN STEM CELL RESEARCH

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In the field of stem cell research, where precision and specificity are crucial, the introduction of innovative tools can significantly advance our understanding of complex biological systems. This study introduces a novel spatial cell sorting technology, Spatially Resolved Laser-Activated Cell Sorting (SLACS), which is tailored to meet the intricate demands of stem cell research. SLACS is not just a tool for cell sorting; it represents a paradigm shift in how researchers can selectively target and isolate cells from specific regions of interest within heterogeneous cellular landscapes. The unique capability of SLACS lies in its precision and adaptability, allowing researchers to dissect complex tissue structures and sort cells with unprecedented accuracy. This is particularly beneficial in stem cell research, where understanding the microenvironment and cellular interactions is key to unraveling the mysteries of cell differentiation, development, and disease processes. Moreover, the integration of SLACS with various omics technologies, such as genomics, transcriptomics, and proteomics, amplifies its potential. This synergy has been instrumental in deepening our understanding of stem cell biology. By combining the spatial resolution of SLACS with the comprehensive analysis provided by omics technologies, researchers can now explore the molecular signatures and pathways in specific cell populations with a level of detail that was previously unattainable. This study not only highlights the technical advancements of the SLACS technology but also showcases its application in various complex aspects of stem cell research. The implications of this technology are far-reaching, opening new avenues for exploration and discovery in the realm of biological sciences. Through this integration, SLACS stands as a beacon of innovation, guiding researchers to new insights and understanding in the ever-evolving field of stem cell research.

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Keywords: spatial omics, cell sorting, novel technology

3:45 PM – 4:45 PM

509

NOVEL POLYVALENT IONIZABLE LIPIDS ENABLE TARGETED DELIVERY OF MRNA TO IMMUNE CELLS AND IPSC-DERIVED MSCS

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Since the introduction of the mRNA COVID-19 vaccines, ionizable lipid-based mRNA delivery systems have gained attention for their potential applications in immunotherapy and tissue engineering. However, current chemical transfection reagents suffer from low transfection efficiency when transfecting mRNA into certain primary cells and stem cells. To address this, we designed a library of 4,200 lipids of which a panel of 82 potential candidates were synthesized and screened in iPSC-derived MSCs (iMSCs) and human peripheral blood mononuclear cells (PBMCs). Lipids were designed with biodegradable branched tails and a spermine-derived backbone, a structural characteristic of the ToRNAdo™ Nucleic-Acid Delivery System. Cells were transfected with GFP-encoding RNA complexed with each of the candidate lipids. High content imaging analysis showed peak GFP expression 18-24 hours after transfection. Additionally, flow cytometric phenotyping was conducted 24 hours after transfection to assess GFP expression. 45 lipids induced GFP expression in CD14+ monocytes and CD3+ T cells. Over 60 of the novel lipids transfected iMSCs with 70-99% efficiency and 83-95% viability. Our data demonstrate novel polyvalent, ionizable lipids that enable delivery of mRNA into hard-to-transfect cells, including human CD14+ and CD3+ cells. These novel lipids may prove useful in the development of mRNA therapeutics targeting hard-to-transfect cell types.

Keywords: ionizable lipids, RNA delivery, transfection

3:45 PM – 4:45 PM

511

CANDIDATE STEM CELL ISOLATION AND TRANSPLANTATION IN HEXACORALLIA

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Stem cells are the foundation for cell therapy due to their ability to self-renew, differentiate into other cell types, and persist throughout the life of an organism. Stem cell isolation and transplantation have not yet been established in Hexacorallia – a cnidarian subclass containing stony corals and sea anemones. Here we demonstrate that candidate stem cells, in the hexacorallian Nematostella vectensis, can be transplanted into adult animals. These cells exhibited the hallmarks of stem cell functional properties; they integrated into recipients' tissues and rescued them from lethal doses of chemotherapy; additionally, these cells proliferated and survived serial transplantations. Notably, we showed that this cellular subpopulation can be enriched by sorting using species non-specific cell markers and that similar subpopulations of cells can be isolated from other hexacorallians, including stony corals. Marine heat waves and other anthropogenic stressors affect corals. Coral resilience can be linked to their genetics. Therefore, stem cell therapy, in which heat-resistant stem cells are transferred to heat-sensitive corals, may be possible. This research establishes the basis for studying stem cell biology on a functional level in Hexacorallia and opens the possibility of cell-based therapy in stony corals.

Funding Source: The European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 948476).

Keywords: stem cell transplantation, hexacorallia, nematostella vectensis

3:45 PM – 4:45 PM

TOPIC: PANCREAS

515

DEVELOPMENT OF A 2.5-DIMENSIONAL PANCREATIC DIFFERENTIATION PROTOCOL: INTEGRATING METABOLIC REGULATION WITH AN INNOVATIVE PATTERNING PLATE

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Optimal scaffolds and culture media are necessary to efficiently differentiate target cells from pluripotent stem cells. We previously established methods for generating stem cell-derived beta cells (SC-β) using two-dimensional cultures with supporting cells, extracellular matrices, and three-dimensional cultures using rotating cultures. Additionally, focusing on nutritional factors, we have developed efficient methods for generating SC-β by depriving methionine and zinc from the medium (Cell Rep 2022, STAR protocols 2023). In this study, we developed a 2.5-dimensional pancreatic differentiation protocol by combining a novel patterning plate (2.5D Plate) with methionine deprival, which enhances differentiation. 2.5D Plate possesses hundreds to thousands of circular cell adhesion areas with a diameter of 200μm on its bottom surface, enabling temperature-responsive cell detachment and forming

numerous uniform hemispheres. The combination of 2.5D Plate and methionine deprival was expected to allow the development of a uniform and efficient method for differentiating induced pluripotent stem cells (iPSCs) into SC-β cells. We differentiated iPSCs into SC-β cells using 2.5D Plate. Methionine deprival treatment was performed before the start of the differentiation culture. After 32 days of culture, the cells easily detached from the adhesive surface of 2.5D Plate by changing the temperature. The collected cells were evaluated by immunocytochemistry and static glucose-stimulated insulin secretion (GSIS) assay. Immunocytochemistry identified that SC-β cells expressed C-peptide and NKX6.1, indicating that 2.5D Plate combined with methionine deprivation was suitable for the generation of PSC into SC-β cells. GSIS assay confirmed that SC-β cells obtained on 2.5D Plate could secret C-peptide in response to glucose challenge. These findings suggest that using 2.5D Plate and methionine deprivation in differentiation culture might be advantageous in inducing SC-β cells more uniformly and efficiently.

Funding Source: JSPS Kakenhi to SK & NS

Keywords: 2.5D culture, methionine deprivation, stem cell-derived pancreatic beta cells

3:45 PM – 4:45 PM

517

INNOVATIVE DEVELOPMENT OF EFFICIENT CELL FATE CONVERSION THERAPY FOR INSULIN REPLACEMENT

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Cell fate reprogramming of stem cells and somatic cells into pancreatic beta cells is a promising approach to type 1 diabetes as cell replacement therapy and gene therapy. Regenerative medicine for diabetes is expected to be implemented in the First in Human study in which human iPS-derived SC-islets (VX-808) in combination with immunosuppressive drugs were transplanted into the liver via the portal vein (Markman et al. ADA 2022). However, it still remains to be solved the challenges of immune rejection, tumorigenicity to allografts, central necrosis in immunosuppressant devices and massive cost in allogeneic stem cell transplantation. Here we report the development of a method for producing artificial pancreatic islets with glucose-responsive insulin secretory capacity (GSIS) by cell fate transformation using somatic cells with an ultra-high efficiency that surpasses that of conventional methods. This technology can produce artificial pancreatic islets in about half the time of the process of producing iPSC-derived β-cells with a differentiation induction efficiency of about 80% or more as a result of the introduction of four transcriptional factor (TFs) genes (OKAP). As a result of transplantation of artificial pancreatic islets created from mouse somatic cells into the renal cortex of a type 1 diabetes model mouse, improvement of hyperglycemia and elevation of blood glucose levels after nephrectomy were observed. This technology is highly reproducible because it can induce expression of endogenous insulin from all somatic cells in more than 30 types of primary cultured cells and cell lines. This technology is advantageous in that it shows ultra-high efficiency of differentiation and reproducibility by 4 TFs gene transfer, is highly safe and applicable to autologous transplantation because it can generate artificial islets from the patient's own somatic cells. Taken together, our technology we developed could be a game changer as a booster toward near future clinical trial in the field for insulin replacement.

Funding Source: This work was supported by JSPS KAKENHI (JP20245946), JP22H04922 (AdAMS), AMED (23ym0126110h0001), JST START (JPMJSF2307), Kawano Masanori & Kose Foundations, President's Research Program Seed A (GP22A-004) Juntendo University.

Keywords: direct conversion, insulin replacement, diabetes

TRACK: PLURIPOTENCY AND DEVELOPMENT (PD)

3:45 PM – 4:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

519

HUMAN PCOS EMBRYOS EXPERIENCE DISORDERED EPIGENETIC REPROGRAMMING DURING PRE-IMPLANTATION DEVELOPMENT

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Polycystic Ovary Syndrome (PCOS) affects 20% of reproductive-aged women and is characterized by anovulation, hyperandrogenism, obesity, and hyperinsulinemia. PCOS also exhibits a significant intergenerational hereditary tendency. Epigenomic abnormalities have a significant impact on the pathogenesis and progression of PCOS. However, how epigenetic factors contribute to the specific causes of PCOS and the mechanisms of PCOS inheritance remain unclear. In this study, we collected oocytes and pre-implantation embryos from PCOS patients, performing transcriptomic and epigenomic analyses. The findings unveiled a suppressed gene expression pattern in PCOS-derived early embryos before the 8-cell stage, transitioning to an activated state by the blastocyst stage, in contrast to non-PCOS patients. Notably, defects were identified in the establishment of de novo H3K4me3 and the removal of H3K27me3 during zygotic genome activation (ZGA), signifying significant abnormalities during the epigenetic reprogramming of PCOS-derived early embryos. The observed aberrations in H3K4me3 and H3K27me3 modifications were correlated with dysregulated expression levels of the corresponding enzymes. Through the manipulation of enzyme expression levels in early mouse embryos, we generated histone modifications remodeled (HMR) mice. HMR mice displayed metabolic irregularities, including increased body weight, erratic estrous cycles, impaired glucose metabolism, and elevated testosterone levels, luteinizing hormone, and insulin. These findings indicate a strong predisposition of HMR mice to PCOS-like conditions. Additionally, early embryos derived from HMR mice exhibited metabolic and epigenetic abnormalities akin to those observed in embryos from

PCOS patients. In summary, our study comprehensively elucidates the epigenetic-mediated pathogenesis of PCOS and the molecular mechanisms underlying intergenerational inheritance. The findings are expected to provide novel targets for diagnosing, preventing, and treating PCOS and infertility by targeting and disrupting epigenetic abnormalities induced by environmental factors, hormones, lifestyle, and other factors. This research aims to enhance the success rates of assisted reproductive technologies and improve the health of offspring.

Keywords: human pre-implantation embryos, epigenetic modifications, PCOS

3:45 PM – 4:45 PM

TOPIC: CARDIAC

521

COHESIN DYNAMICS DURING CARDIOMYOCYTE DIFFERENTIATION: REGULATION OF TRANSCRIPTIONAL PROGRAMS

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Stem cell self-renewal and differentiation are orchestrated both by genetic and epigenetic cues that determine cell fate, and deregulation of these processes is often connected with developmental defects. One of the most frequent developmental abnormalities are congenital heart defects (CHDs) - structural abnormalities of the heart present at birth, often attributed to genetic mutations. Recent studies have identified mutations in genes associated with cohesin as CHD drivers. Cohesin is a protein complex that mediates sister chromatid cohesion, homologous recombination, and DNA looping. It has broad impacts on transcription, which likely underlies its role in cell differentiation. Our goal is to explore the role of cohesin dynamics in cell differentiation and shed light on how cohesion is regulated during that process. We are focusing on two key cohesin regulators – ESCO1 and WAPL, that play role in cohesion stabilization and release from chromatin, respectively. To investigate their roles in cell differentiation, we use a directed cardiogenesis protocol and genetically engineered mouse embryonic stem cells (mESCs) lines in which knocked-in biallelic degron tags can inducibly degrade ESCO1 or WAPL. We confirmed efficient protein degradation in both mESCs and cardiomyocyte cultures upon adding the dTAGV-1 molecule. We found that ESCO1 and WAPL protein levels changed dramatically during cardiac differentiation, in contrast to the cohesin core subunits, which remained stably expressed. This may suggest that cohesin dynamics are greatly reduced during the differentiation process. Our long-term goal is to understand how cohesin dynamics are entrained during differentiation and how this in turn impacts gene expression. Our ongoing research endeavors aim to bridge existing knowledge gaps concerning the role of the cohesin complex in the processes of cardiogenesis, offering valuable insights into the molecular mechanisms underlying differentiation and potential impacts on congenital heart defects.

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Keywords: differentiation, cohesin dynamics, regulation of transcription

3:45 PM – 4:45 PM

523

DISTINCT DISTRIBUTION OF OIP5OS1 COORDINATES EMBRYONIC STEM CELL DIFFERENTIATION

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The congenital heart disease is closely linked to cardiomyocyte differentiation in early stage of cardiac development. However, how non-coding RNAs regulate the process is largely unknown. Here, we identified a lncRNA, Oip5os1 (Rik), which expressed with dynamical alternation during differentiation, and underwent a distribution shift from cytoplasm in mesodermal period to nucleus in cardiac progenitor cell period. Overexpression of Rik significantly promoted mesodermal differentiation but inhibited the process in the cardiac progenitor cell period. When Rik was knockout, mesodermal differentiation was blocked, whereas in favor of the differentiation of cardiac progenitor cells. In mechanism， we found that the rich Rik in cytoplasm could bind with miR-26b, which resulted in reduction of inhibition to miR-26b-target gene Wnt5a, ultimately leading to upregulation of the β-CATENIN for promoting mesodermal differentiation. On the other hand, the nuclear-rich Rik in the cardiac progenitor cell period involved in alternative splicing with the highest scoring protein, A2BP1, a splicing protein. We first confirmed the Rik directly targeted to A2BP1, and then identified two A2BP1-targeted splicing sites at downstream of the third exon of Mef2c, a cardiac progenitor cell period-specific transcription factor. Subsequently, we found that forced expression of Rik really promoted

A2BP1 increase in the nucleus in cardiac progenitor cells. Rik overexpression promoted the upregulation of the Mef2c α2 isoform, whereas Rik knockdown tended to Mef2c α1 splicing. Bioinformatics analysis also indicated that cardiac progenitor cells are more inclined to be spliced to Mef2c α1 during the cardiac progenitor cell period, whereas interestingly in skeletal muscle cells, more Mef2c α2 were spliced. Therefore, our results suggest that nucleus rich Rik promotes the upregulation of A2BP1, which makes the cardiac progenitor cell-specific transcription factor Mef2c more inclined to be spliced to Mef2c α2, finally resulting in a limitation on cardiac progenitor cell differentiation. Taken together, our study demonstrates that Oip5os1 temporally regulates mesodermal differentiation by undergoing a distribution shift in nuclear, resulting in deciding the fate of embryonic stem cell differentiation.

Funding Source: National Natural Science Foundation of China (NSFC) :32071109

Keywords: embryonic stem cell differentiation, Wnt/β-catenin signaling pathway, alternative splicing

3:45 PM – 4:45 PM

525

CV4 MEDIA: THE DEVELOPMENT OF A NOVEL MEDIA FOR MATURATION OF HUMAN IPSC DERIVED MULTICELLULAR CARDIOVASCULAR ORGANOIDS

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Key limitations in the development of translational cardiovascular organoids are the relatively limited maturation and cellular complexity of current models and technologies. Specifically, the lack of a maturation media tailored to support organoid development and maturation of all 4 main cellular components of a cardiovascular tissue (cardiomyocytes, endothelial cells, cardiac fibroblasts, and smooth muscle cells). In the current study, we aimed at developing a novel media tailored to promote the maturation and self-assembly of all cardiovascular lines within a cardiac organoid. A CV4 media was established using a step-wise approach to assess the effect on the various cardiovascular cell types aiming at improving cardiomyocyte maturation and vessel network complexity as well as cell-viability, ATP content and functionality. Organoid maturation was assessed using functional assays for contraction force and relaxation dynamics, gene expression and proteomic profiling and ultrastructural maturation. CV4 medium induces maturation by using factors affecting cellular energetics, mitochondrial function, pro-hypertrophic mediators. Functional assessment by tailor-made high-throughput robotic system exhibited functional improvements following exposure to the CV4 media with 36% augmentation of contractile force ($p < 0.001$). Immunostaining studies revealed high degree sarcomeric maturation with longer sarcomere length. Organoid exposure to CV4 media resulted in the formation of vessel-like structures. The vessel network formed demonstrated a functional interplay between the endothelial calls and supporting cells. Metabolic assays revealed improved oxygen consumption rate indicative of enhanced maturation. Additionally, gene expression profiling and proteomics, revealed alterations in the expression of maturation-associated genes such as TNNI3 and angiogenic markers in the organoids exposed to the CV4 media. These findings underscore the effectiveness of the developed maturation media in promoting the maturation and self-assembly of the cardiovascular organoids. This study contributes to the advancement of methodologies for improving the fidelity of hiPSC based models, thus holding significant implications for cardiac tissue engineering and disease modeling applications.

Keywords: cardiovascular organoid, cardiovascular maturation, regenerative medicine

3:45 PM – 4:45 PM

527

MULTI-CHAMBER CARDIOIDS UNRAVEL HUMAN HEART DEVELOPMENT AND CARDIAC DEFECTS

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Defects in heart development are a leading cause of fetal mortality in humans. However, studying the impacts of mutations and drugs on various heart compartments during early development is challenging due to the inaccessibility of the human embryonic heart and its complex functional demands. To address this challenge, we have developed a human cardiac organoid (cardioid) platform capable of faithfully modeling the development of major embryonic heart compartments, including the left ventricle, right ventricle, atria, outflow tract, and atrioventricular canal. Our methodology efficiently generates progenitor subsets representing distinct identities from the first, anterior, and posterior second heart fields, enabling the reproducible high throughput creation of cardioids exhibiting compartment-specific gene expression profiles, morphologies, and functionality. By integrating these compartments to form multi-chambered cardioids, we can investigate communication processes underlying coordinated cardiac electrical activity. Leveraging our multi-chamber cardioid platform, we explore the etiology of congenital heart diseases by examining the effects of genetic knockouts (ISL1, TBX5, and FOXF1) on the development of different cardiac chambers. Additionally, we assess the varying impacts of different teratogens (Thalidomide, Acitretin, and Transretinal) on chamber-specific heart development. Finally, we demonstrate chamber-specific arrhythmogenic responses to established ion channel modulators (Ivabradine, Isoprenaline, and Bay K 8644), highlighting the nuanced interactions among different cardiac chambers. Overall, our findings underscore the utility of the multi-chambered cardioid platform as a valuable model for elucidating cardiac development mechanisms and screening for mutagenic or teratogenic effects contributing to congenital heart diseases.

Funding Source: Austrian Academy of Sciences (OEAW) and the Austrian Research Promotion Agency (FFG) (to the Mendjan lab), FWF Special Research Program SFB-F78, F 7811-B

Keywords: cardiac organoids (cardioids), congenital heart disease, human cardiac development

3:45 PM – 4:45 PM

529

UNRAVELING THE ROLE OF RNA-BINDING PROTEINS IN CARDIOMYOCYTE MATURATION

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Adult hearts cannot be repaired after injury, making cardiovascular disease a leading cause of death worldwide. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) offer a promising model for studying cardiac development, disease modeling, and regenerative therapy. However, their immature and fetal-like characteristics restrict their use in adult disease modeling and regenerative medicine. Therefore, it is crucial to identify strategies to improve cardiomyocyte maturation. To overcome this hurdle, various strategies have been employed to enhance the maturation of hPSC-CMs. However, molecular mechanisms underlying the maturation remain elusive. Our previous studies demonstrated that RNA-binding protein RBM24 mediates alternative splicing of core myofibrillogenesis genes during sarcomere assembly in a stage-specific manner. Moreover, we now reveal that RBM24 deficiency affects cardiomyocyte maturation, including the failure of myosin isoform switching, metabolism, immature calcium handling, and reduced mitochondria contents. Our mechanistic study further reveals that RBM24 regulates multiple types of non-coding RNAs, including long non-coding RNAs (LncRNAs), micro RNAs (miRNAs), and circular RNAs (CircRNA). Intriguingly, we identify a long non-coding RNA as a critical factor in modulating sarcomere myosin switching—a hallmark of cardiomyocyte maturation. In conclusion, our research reveals that RBM24-mediated cardiomyocyte maturation, involving critical processes like myosin switching, metabolism, and calcium handling, relies on long non-coding RNAs. These findings illuminate potential therapeutic targets for enhancing hPSC-CM maturation and advancing efforts in myocardial regeneration.

Keywords: cardiomyocyte maturation, RNA-binding protein, human pluripotent stem cell

3:45 PM – 4:45 PM

531

CARDIAC AND SKELETAL MUSCLE SPECIFICATION IN MES-DERIVED GASTRULOID MODELS

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The cardiopharyngeal mesoderm plays a crucial role in the development of both heart and head muscles, yet the precise mechanisms guiding its specification remain elusive. Currently, there is a lack of in vitro models capable of replicating the differentiation process of both heart and head muscles, hindering the study of these mechanisms. Our refined culture protocol has enabled gastruloids to develop beating regions and express markers indicative of both cardiac and skeletal myogenic profiles. Comparative analysis of gastruloids with mouse embryos has revealed strikingly similar spatio-temporal gene expression patterns between the two models. Single-cell RNA sequencing (scRNA-seq) analysis identified distinct cell populations corresponding to various developmental stages, including specific subpopulations within the cardiopharyngeal mesoderm. From this scRNA-seq data, we have delineated trajectories leading from cardiopharyngeal mesoderm to cardiomyocytes and myoblasts. Further analysis of scRNAseq cell clusters reveals the existence of different subpopulations of cardiomyocytes and subpopulations of myoblasts with intermediate differentiation status with “head-like” and “trunk-like” skeletal myoblasts. Lineage tracing experiments confirm distinct subpopulations of cardiomyocytes derived from different cardiac lineages. Collectively, these findings underscore the potential utility of gastruloids as an in vitro model for investigating the development of cardiac and head skeletal muscles.

Keywords: gastruloids, cardiopharyngeal mesoderm, cardiac specification

3:45 PM – 4:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

533

CONTRADICTORY ROLE OF MITF IN MELANOCYTE STEM CELLS FROM HUMAN PLURIPOTENT STEM CELLS IPSCS

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There are a lot of depigmentation diseases derived from the loss of melanocytes stimulated by stress or autoimmune disorders, the treatment principle is the pigment replenish. But traditional treatments, like surgical skin grafting, phototherapy, microneedling, include faults but not limited to severe scar formation, long treatment cycle, low efficiency, and endless recurrence. People have put forward isolating and manipulating melanocyte stem cells for tissue engineering as a source of abundant pure dependable human pigment for many years.

However, separation and purification of primary melanocyte stem cells still a complexity which is hindered by the lack of definite and specific surface marker. MITF is a key transcription factor that determines melanocyte fate and maintains the expansion or differentiation of melanocyte stem cells into melanocytes. Using CRISPR/Cas9 gene editing, we were able to visualize MITF gene expression using EGFP in real time during the differentiation of hiPSC into melanocytes. Through differentiation, there is no significant difference between the knock-in iPSCs and the control group. By visualizing MITF in induced pluripotent stem cells (iPSCs), our study attempts to facilitating the characterization of human melanocyte stem cells. In our study, we evaluate the self-renewal capability of stem cells with one of the character, sphere formation capability. We analyzed and compared EGFP+ single cells sorted by flow cytometry, we observed that EGFP-positive cells had strong spherical ability at 21 days of differentiation, which confirmed that MITF-expressing induced melanocytes have the stem cell properties of self-renewing. Interestingly, for passaged melanocytes induced from iPSCs, the cells with high MITF expression were not the cells with the strongest sphere-forming ability. On the contrary, the cells with relatively low MITF expression showed stronger stemness. As to whether MITF is low or high expressed, and its relationship with the fate of melanocyte cell lines has important research significance, further studies are needed to elucidate. In conclusion, our study confirmed the stemness of MITF positive melanocytes, and revealed the contradictory role of MITF, which provide inspiration for disease with a defection of melanocyte stem cells.

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Keywords: hiPSCs, melanocyte stem cells, stemness

3:45 PM – 4:45 PM

535

AGE SPECIFIC INTESTINAL STEM CELLS SHAPE CD4+ T CELL IMMUNITY IN THE HUMAN INTESTINE

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Early life immune responses need to be calibrated to induce responses against pathogens while maintaining tolerance against commensals. Previous studies from our group have investigated this critical time window in human development and have shown changes in intestinal immune cell populations (NK cells, CD8+ T cells, CD4+ T cells and ILCs) with increasing age; however, the underlying mechanisms regulating immune development in the human intestine are incompletely understood. Epithelial cells are regulators of adaptive immune cells and produce cytokines and chemokines. Here, we examined whether intestinal stem cells produce cytokines or chemokines that affect CD4+ T cell recruitment. Therefore, we generated human intestinal organoids (HIOs) from infant and adult tissues to mimic the age-specific intestinal epithelium. Because TLR5 has been shown to play a role in the development of immune homeostasis in the postnatal period, infant and adult HIOs were stimulated with the TLR5 ligand flagellin for 24 hours or long-term (8 weeks). Short-term flagellin stimulations induced mRNA and protein expression of the TH17 cell recruiting chemokine CCL20 to similar levels in infant and adult HIOs. In contrast, long-term flagellin stimulation resulted in significantly higher CCL20 mRNA and protein expression in infant HIOs as compared to adult HIOs. In line, scRNA sequencing and flow cytometric analyses of intra-epithelial lymphocytes demonstrated increased numbers of phenotypic TH17/22 cells in infant compared to adult intestines. IL-22, one of the hallmark cytokines of TH17/22 cells, is known to aid epithelial regeneration via STAT3, and indeed IL-22 enhanced intestinal stem cells (ISCs) proliferation in HIOs. Concordantly, an increased STAT3 signature in infant ISCs as compared to adult ISCs was detected in scRNAseq analyses of infant and adult epithelial cells. In summary, we demonstrate an age-specific intestinal immune network that favors TH17/22 responses where ISCs provide local signals to promote Th17/22 cell recruitment. In turn, IL-22 produced by intestinal TH17/22 cells enhanced ISC outgrowth generating a positive feedback loop after encountering flagellated bacteria to promote intestinal growth in young infants.

Funding Source: Daisy Huet Roell Foundation; The Leibniz Institute of Virology is supported by the 'Bundesministerium für Gesundheit' and the 'Behörde für Wissenschaft, Forschung, Gleichstellung und Bezirke' der Stadt Hamburg

Keywords: CD4+ T cells, CCL20, CCR6

3:45 PM – 4:45 PM

537

MULTIPOTENCY OF PITUITARY STEM/ PROGENITOR CELLS POSITIVE FOR PRRX1 IN MICE

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The anterior lobe (AL) of the pituitary gland is an endocrine organ composed of five types of hormone-producing cells and non-hormone-producing cells, such as stem/progenitor cells, vascular endothelial cells, and pericytes. Recently, we isolated the adult pituitary stem/progenitor cell (APSC) that are positive for the transcription factor paired related homeobox 1 (PRRX1) and revealed that these cells had the ability to differentiate into AL hormone-producing cells. However, it is not clear which type of terminally-differentiated cells is supplied by APSCs. In

this study, we evaluated the multipotency of APSCs. First, APSCs, which were isolated from the AL as reported previously, were subjected to three-dimensional culture for 1–4 days to form aggregates. Next, we analyzed the genetic characteristics of the aggregates using quantitative RT-PCR. The expression levels of the stem/progenitor cell markers Prrx1 and Sox2 did not change significantly during cultivation. On the other hand, the pituitary progenitor cell marker Prop1 increased from the beginning of culture, peaked on day 2, and then returned to basal levels. The anterior pituitary hormone genes, except for Tshb, increased gradually from day 0 to day 4 of culture and eventually reached approximately 22 to 58 times the basal level. In addition, the vascular endothelial cell marker Pecam1 and pericyte marker Cspg4 were also increased in culture. Immunofluorescence analysis showed a gradual increase in the percentage of anterior pituitary hormone-positive cells, except for the thyroid-stimulating hormone beta subunit, in aggregates (approximately 30 times from day 1 to day 4). Cells positive for the vascular endothelial cell marker CD31 and pericyte marker NG2 also appeared after culture. Thus, we found that the APSC has the capacity to differentiate into terminally-differentiated cells, including anterior pituitary hormone-producing cells, vascular endothelial cells, and pericytes. Interestingly, Gnrhr and Ghrhr, the receptor genes of hypothalamic hormones that regulate anterior pituitary hormone secretion, also increased in parallel with an increase in the number of hormone-producing cells. These results suggest that APSC-derived aggregates have the function of secreting hormones; they are functional pituitary organoids.

Funding Source: This work was supported by JSPS KAKENHI Grant Number 23KJ1597.

Keywords: pituitary stem/progenitor cell, three-dimensional culture, multipotency

3:45 PM – 4:45 PM

539

AKT REGULATES NUCLEUS-CYTOPLASMIC TRANSPORT OF PLURIPOTENCY FACTORS IN HUMAN AMNIOTIC EPITHELIAL CELLS

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Human amniotic epithelial cells (hAEC) derive from the post-implantation epiblast around embryonic day seven, displaying pluripotent stem cell features owing to their origin in the early embryo. Previous findings from our group have shown that hAEC express markers associated with an undifferentiated state, including OCT-4, SOX2 and NANOG. However, these transcription factors are observed in both the nucleus and cytoplasm of hAEC, whether in situ or cultured in vitro. Additionally, their cellular localization may be regulated through post-translational modifications. Notably, Akt-mediated phosphorylation at threonine 235 of OCT4 promotes its nuclear presence, facilitating the transcription of target genes such as SOX2 and NANOG. Here, we aimed to assess Akt-mediated nucleus-cytoplasmic transport of OCT4, SOX2, and NANOG in cultured hAEC in vitro over a 30-minute period. Immunocytochemistry was employed to determine the cellular localization of these transcription factors. Our observations indicate that in AKT-treated hAEC, OCT4 and SOX2 tended to localize exclusively in the nucleus, while NANOG was found in both compartments. These results suggest that Akt induces the nuclear localization of OCT4 and SOX2 in cultured hAEC. Overall, these findings contribute to our understanding of hAEC biology and may have implications for stem cell-based therapies and regenerative medicine.

Funding Source: This work received funding from the Instituto Nacional de Pererinatología, registry 2018-1-150 and 2021-1-24; Hospital Angeles Pedregal, registry HAP 2685.

Keywords: human amniotic epithelial cells, Akt, nucleus-cytoplasmic transport

3:45 PM – 4:45 PM

543

INDUCING LUNG DIFFERENTIATION IN HUMAN HEART-FORMING ORGANOIDS TO FORM COMPLEX LUNG-HFOS

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Heart-forming organoids (HFOs) derived from human pluripotent stem cells (hPSCs) represent a complex, highly structured in vitro model for heart, foregut and vasculature development. Specifically, HFOs are composed of a myocardial layer lined by endocardial-like cells and surrounded by septum-transversum-like anlagen; they further contain spatially and molecularly distinct anterior versus posterior foregut endoderm (AFE versus PFE) tissues and a vascular network. The architecture of HFOs closely resembles aspects of early native heart anlagen prior to heart tube formation, which is known to require an interplay with foregut endoderm development. In the embryo, the AFE

gives rise to different organs such as the lung, esophagus and thymus. In HFOs, the AFE is located in the inner core and represents immature tissue expressing progenitor markers of AFE-derived organs as revealed by single-cell RNA sequencing. To further advance the current HFO model towards embryo-like multi-tissue complexity, we here combine our HFO differentiation protocol with directed lung differentiation to induce lung epithelium formation. We show via flow cytometry and immunofluorescence staining that cells expressing the lung progenitor marker NKX2.1 form in the inner core, specifically within the epithelium lining the endodermal cavities. Following maturation, spheres expressing lung epithelium markers emerge from the HFOs. Notably, while both proximal and distal lung epithelial cells form over time, the overall 3D structure and established pattern of HFOs remain intact, despite the increase in tissue complexity. Thus, the first step towards a combined, self-organized heart-lung-vasculature model has been achieved, opening new perspectives for investigating human diseases in vitro, as well as advanced teratogenicity assessment and drug discovery approaches to replace animal experiments efficiently.

Keywords: organoid, heart, lung

3:45 PM – 4:45 PM

545

IDENTIFICATION OF STEM/PROGENITOR CELLS IN ANTERIOR LOBE OF BOVINE PITUITARY GLAND

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Hormone-producing cells in the anterior lobe (AL) of the pituitary gland secrete hormones that regulate growth, lactation and reproduction. This ability is an important factor for determining the industrial value of cattle. The stem/progenitor cell marker sex-determining region Y-box 2 (SOX2)-positive cells, which are putative pituitary stem/progenitor cells, are thought to contribute to the secretion of hormones by supplying hormone-producing cells. However, the differentiation potential of SOX2-expressing cells has not been elucidated because of the lack of techniques for isolating and culturing pituitary cells from bovines. In this study, we isolated the SOX2-positive cells from the AL of the bovine pituitary gland and investigated their differentiation potential. Immunohistochemical analysis revealed that SOX2-positive cells were localized in the cell layer bordering the Rathke's cleft and the parenchyma of the AL in calves (0 day) and adult cows (12 months). Next, a single-cell suspension of primary AL cells was prepared and subjected to two-dimensional culture. As a result, some cells adhered to a culture dish and proliferated. qRT-PCR showed that the expression levels of Sox2 and the stem cell niche marker Cdh1 were higher in proliferated cells than in tissues. Immunocytochemical analysis revealed that most of the proliferated cells were positive for PRRX1+2 (another pituitary stem/progenitor cell marker) and E-cadherin, in addition to SOX2, and were negative for hormones. Finally, the isolated SOX2-positive cells were subjected to three-dimensional culture. The results showed that SOX2-positive cells isolated from calves and adult cows formed floating aggregates, and hormone-positive cells were detected in some of them. Thus, the present study indicates that SOX2-positive cells in the AL of the bovine pituitary gland are stem/progenitor cells with the ability to differentiate into hormone-producing cells. Our results provide important insights into the mechanisms of hormone-producing cell supply in the pituitary gland and may lead to the development of technology to control growth and reproduction in cattle in the future.

Funding Source: This work was supported by JSPS KAKENHI Grant Number JP20K21371.

Keywords: bovine, pituitary gland, stem/progenitor cell

3:45 PM – 4:45 PM

547

A WORKFLOW TO QUANTIFY SINGLE-CELL MORPHOGENETIC FEATURES OF DEVELOPING MUCOCILIARY EPIDERMIS

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As the developing embryo forms, the simple mass of cells becomes more and more complex throughout the morphogenetic shaping of tissues. Morphogenetic processes display an astounding level of tissue self-organization, where an initially unorganized mass of cells rearranges to form a functional tissue. One example of such process is the formation of regurlarly patterned, multilayered tissue, such as mucociliary epithelia (MCE); however, the formation of the tissue from initially pluripotent cells remains uncharacterized. The morphogenetic shaping and cell fate choices in the mucociliary epithelium takes place both collectively and individually. Cells exhibit collective movement, but single cells in in the deep cell layer can also migrate individually and remodel their environment. Similarly, with fate choices, neighboring cells end up in differing cell types as intercalating cell types become evenly spaced out, but at the same time cells also signal to each other to regulate their differentiation. To resolve the morphogenetic behaviors across time and across the scale of individualism-collectivity, we quantify morphological and kinetic phenotypes of single cells in the embryonic Xenopus mucociliary epithelium. Using explanted prospective epidermis, aim to can image and quantify developmental dynamics in single-cell resolution. To achieve this, we have developed a state of the art quantitative imaging pipeline to track cell dynamics in the bottom layer of developing Xenopus epidermal explants. Simultaneously, we trace the positioning of different cell types throughout the development by visualizing the cell type specific marker genes in developmentally critical stages of the explant in order to link the cell trajectories to cell types. The detailed backtracking of the cell's histories allows us to connect individual cell behaviors to cell fate. By assaying the embryonic epidermis, we aim to provide an unprecedented detailed view of the developmental dynamics of a mucociliary epithelium. Understanding these fundamentals of mucociliary differentiation could provide a better understanding of pathological conditions arising from defective development of airway epithelia.

Funding Source: The Novo Nordisk Foundation for Stem Cell Medicine

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Keywords: mucociliary epithelium, machine learning, quantitative imaging

3:45 PM – 4:45 PM

549

PLURIPOTENT STEM CELL-DERIVED EXPANDABLE LUNG EPITHELIAL PROGENITORS AS A TOOL FOR INVESTIGATION OF ER-ASSOCIATED LUNG STRESS AND REGENERATION

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Lungs are constantly exposed to intrinsic and extrinsic factors that can deteriorate the fine pulmonary architecture. Modelling the lung stress response and regeneration can be addressed by two principal approaches. First, by direct differentiation of pluripotent stem cells to lung cells and second, by isolation and expansion of lung stem cells and progenitors, such as pneumocytes type II or airway basal cells. However, these models are limited by time and economic costs or small yields and suffer from significant phenotype shifts such as epithelial-to-mesenchymal plasticity (EMP) or senescence in long-term cultures. Here we demonstrate that Expandable Lung Epithelial Progenitors (ELEPs) differentiated from pluripotent stem cells maintain a high rate of proliferation and in parallel markers of immature alveolar or airway cells, e.g., lamellar bodies or expression of prosurfactant proteins, aquaporins, or podoplanin. The ELEPs expressing high levels of transcription factor NK2 Homeobox 1 (NKX2.1) can be propagated for a long time in in vitro culture and differentiated to terminal lung cell phenotypes using specifically formulated culture media (containing activators and inhibitors of Wnt/β-catenin and general TGF-β signalling, respectively) and culture setup (submerged 2D/air-liquid interface/3D low adhesion culture). Endoplasmic reticulum (ER) represents besides its proteosynthetic function a key cellular signalling hub, integrating metabolic cues and external factors to cell prosurvival and proapoptotic cell response. In addition, deregulation of ER homeostasis leads to alterations in membrane and secreted molecules and contributes to changes in functional cell morphology, such as EMP. The ELEPs upregulate ER-associated canonical signalling through IRE1a, PERK, and ATF6 during differentiation to terminal lung phenotypes. In addition, the ELEPs are highly sensitive to proteosynthetic stress that affects their differentiation trajectory and the establishment of functional phenotypes through altered expression/function of major cell adhesion molecules and downstream signalling. In summary, we demonstrate the ELEPs as the pulmonary cell model for investigation of cell stress integrating at ER during differentiation of lung cell types and lung regeneration.

Funding Source: This work was supported by the Czech Science Foundation (grant no. GA23-06675S) and by the Masaryk University (grant no. MUNI/A/1598/2023).

Keywords: lung cell differentiation, ER stress, epithelial-mesenchymal plasticity

3:45 PM – 4:45 PM

551

HOW MUCH DO WE KNOW ABOUT THE MOUSE RESPIRATORY EPITHELIAL STEM CELLS?

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Tissue-specific stem cell lines are useful tools for cell biology studies. Information on respiratory tissue cell lines is limited. A doxycycline-regulated epithelial precursor cell line was established from the lung tissue of a tTAxSV40 Tag double transgenic mouse. This cell line was characterized and found to mimic a rare subpopulation of club- and pneumocyte type II- dual cells. Cell growth was doxycycline-regulated and observed only when doxycycline was omitted from the medium or present at concentrations up to 1 mg/ml, higher concentrations were inhibitory. ACT+ ciliated cells were found upon implantation into immune-deficient mice. Cell growth was doxycycline-regulated in vitro. When transplanted subcutaneously into immune-deficient mice, these cells migrated to the lung to form organized chimeric structures of donor and host origins, with club cells in the terminal bronchioles, ACT+ ciliated cells along the epithelial lining, and pneumocyte type II-cells in the alveolar interstices. No such homing of donor cells to the lung was observed when the implanted mice were fed doxycycline-containing water. This lung stem cell line might be able to provide us with an

insight into the differentiation pathway of lung epithelial cells and with some understanding of the nature of air trophic-pulmonary epithelial cells. The results underline the possibility of a future application for somatic (stem / precursor) cells in tissue engineering of the damaged lung. Its ability to secrete and deliver soluble protein, might be a potential novel way for drug delivery. Stem cells are thought to proliferate and differentiate in response to a deficiency or as a result of injury. Successful migration to the target organ and subsequent maturation of these precursors could be attributed to a requirement of lung stem cells to search for an aerated environment. Our findings challenge some current concepts of stem cell biology. This lung stem cell line may become a rich source of cells for tissue engineering and cell-based therapy for lung injury. The route and protocol established for cell introduction into the lung may provide a novel alternative to delivery of soluble protein substances through the airways. This lung stem cell line might also be modified to provide models for screening drugs against respiratory infection.

Funding Source: Supported by DFG (BE793/8-1), INSERM, EC (BIO4-CT95-0284).

Keywords: Tet-off expression system in lung stem cells, lung disease and lung viral infection, drug screening and delivery

3:45 PM – 4:45 PM

553

A CULTURE PLATFORM WITH ALVEOLAR GEOMETRY FOR INDUCED PLURIPOTENT STEM CELL-DERIVED DISTAL LUNG ORGANOIDS

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Current human induced pluripotent stem cell (hiPSC)-derived distal lung organoids (DLOs) resemble fetal maturity and lack key cell types. They do not adequately resemble the adult lung which limits their utility for drug testing, cell therapy, or as an in vitro model. We must improve existing DLOs to better resemble the human lung in terms of their cell composition, functionality, and maturity. Traditionally, DLOs are cultured as self-organizing spheroids embedded in soft gel. This culture system does not mimic the native microenvironment, which is increasingly being recognized for its importance in directing cell behaviour. We hypothesize that culturing DLOs on a biomimetic platform will influence key signalling pathways that affect cell fate. We designed a culture platform with tunable geometry, stiffness, and extracellular matrix (ECM) composition to investigate the roles of these parameters on DLO development. A mold was fabricated using two-photon lithography 3D printing. Subsequent replica molding generated a hydrogel or PDMS cell culture substrate with dimples resembling 200 µm-diameter alveoli. The collagen type I hydrogel was optimized to achieve the same stiffness as human lung (2 kPa). We screened 36 ECM compositions to identify those that best support hiPSC-derived lung progenitor cell attachment over six hours. Mixtures including collagen type I and either vitronectin, fibronectin, collagen type III, tropoelastin, or laminin enhanced lung progenitor cell attachment. Next, lung progenitor differentiation to alveolar epithelial cells will be compared among the dimpled hydrogel, flat hydrogel, and traditional 3D spheroids. Differentiation will be compared at day 30, as this endpoint yielded the greatest proportion of alveolar epithelial cells in spheroids. Mechanical signalling pathways and epigenomic state modulate alveolar epithelial cell fate choice. Therefore, single cell RNA sequencing and epigenomics will be combined to identify active signalling pathways in each cell type within the heterogeneous DLO cell population. Overall, this project will elucidate microenvironmental cues that guide distal lung development, ultimately providing insight into which biophysical parameters must be leveraged to improve the fidelity of DLOs as an in vitro model of the adult lung.

Keywords: induced pluripotent stem cells, mechanotransduction, distal lung

3:45 PM – 4:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

555

HARNESSING GERMLINE STEM CELLS AS TOOLS FOR MARSUPIAL CONSERVATION

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Marsupial research and conservation are limited by the absence of advanced technologies such as genome engineering. This work aims to fill this gap by developing the first ever methods to culture and genetically modify marsupial spermatogonial stem cells (SSCs), and to generate induced pluripotent stem cells (iPSCs) for differentiation into germ cell lineages. These tools are necessary to achieve in-vitro gametogenesis as an avenue to generate genetically modified marsupials. Using fat-tailed dunnarts, a close living relative of several endangered/extinct marsupials, this study generated iPSC lines using transposase- or virus-based delivery of reprogramming factors. These iPSCs were assessed for hallmarks of pluripotency, including expression of pluripotency genes, germ layer gene expression under defined culture conditions, and the ability to form embryoid bodies. Further, iPSCs were assessed for their ability to differentiate into primordial germ cell-like cells (PGCLCs) under culture conditions developed for mouse and human iPSCs. Second, testis tissue digestion and culture protocols were developed for the dunnart, to enable maintenance and growth of SSCs in vitro. Stable dunnart iPSC lines were generated and expressed core pluripotency genes, and genes associated with germ lineages in trilineage differentiation assays. Further, iPSCs exhibited characteristic differentiation and organisation in embryoid body assays.

Exposure of dunnart iPSCs to PGCLC differentiation conditions induced expression of PRDM1, PRDM14, and ITGA6 that are hallmarks of PGCLC differentiation. Digestion and culture of primary dunnart testis tissue demonstrated that medium containing bFGF and GDNF is optimal for survival and expansion of dunnart testicular germ cells in vitro. These germ cells have been maintained in continuous culture for >14 weeks and survived multiple passages; a first for marsupial SSCs. Together with the successful generation of dunnart iPSCs and their germline differentiation, this study provides critical foundations for the successful generation of genetically modified Australian marsupials for use in "next generation" conservation efforts.

Funding Source: The Australian Research Council Colossal Biosciences Wilson Family Trust

Keywords: pluripotency, conservation, de-extinction, marsupials

3:45 PM – 4:45 PM

557

THE FIRST TWO BLASTOMERES CONTRIBUTE UNEQUALLY TO THE HUMAN EMBRYO

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Retrospective lineage reconstruction of humans predicts that dramatic clonal imbalances in the composition of the body can be traced to the 2-cell stage embryo. However, whether and how such clonal asymmetries arise in the pre-implantation embryo are unclear. Exploration of these questions in human embryos remained challenging due to extremely limited human zygote access and lack of appropriate techniques. We have performed the first prospective lineage tracing of human embryos, from the zygote to the blastocyst stage, using live imaging, non-invasive cell labelling and computational predictions to determine the contribution of each 2-cell blastomere to the epiblast (body), hypoblast (yolk sac) and trophectoderm (placenta). We discover that most epiblast cells originate from only one 2-cell stage blastomere in most embryos. Mechanistically, we observe that the first wave of inner cell allocation by asymmetric cell division at the 8- to 16-cell stage transition underpins this clonal imbalance, as only one to three cells become internalized at this stage unbalancing the inner cell founding population. Moreover, these internalized cells are more frequently derived from the first blastomere to divide at the 2-cell stage. We propose that cell division dynamics and a cell internalization bottleneck in the early embryo establish asymmetry in the clonal composition of the pre-implantation epiblast, the source of the future human body.

Funding Source: This work was supported by Human Science Frontiers Program, NOMIS Foundation, Wellcome Trust, Open Philanthropy Project, and the Curci and Weston Heavens Foundations.

Keywords: human embryo, lineage tracing, clonal imbalances

3:45 PM – 4:45 PM

559

XENO/FEEDER-FREE CULTURE USING LAMININ SUPPORTS RESETTING TOWARDS NAIVE PLURIPOTENCY IN HUMAN EMBRYONIC STEM CELLS

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Human pluripotent stem cells (hPSC) exist in a naïve or primed state of pluripotency, mimicking the pre- or post-implantation human epiblast cells in blastocysts, respectively, and are a valuable resource to study human early development. The field of naive hPSC has seen a surge in research activity over the years, particularly the use of naive hPSC for the generation of human blastoids, a powerful 3D model to study human embryogenesis in vitro in a way that was previously impossible. A major hurdle today is the lack of a standardized protocol for deriving naive hPSC without the use of mouse embryonic fibroblasts (MEF) or transgene overexpression. Therefore, we aimed to mimic the natural environment by culturing cells on laminin 521 (Biolamina LN521), which is one of the first cell adhesion proteins present in the inner cell mass of human blastocysts. We established this novel approach of culturing cells on LN521 during epigenetic resetting through histone deacetylation inhibition. We used several in-house primed hPSC lines to generate feeder-free naive hPSC. After the resetting, the cells were cultured in PXGL conditions on LN521 and passaged regularly into a single cell suspension without the addition of ROCK inhibitors. The naive cells produced using the LN521 culture method showed expression of naive pluripotency markers KLF17, NANOG and SUSD2 through RT-qPCR and presence of SUSD2 (96%) and CD75 (79.1%) using flow cytometry at passage 20. We hereby report a novel and standardized protocol for the derivation of naive cells and, most importantly, a model that supports naive pluripotency in culture without the need for MEF. Moreover, we can use an additional gelatin clean-up step during the cellular expansion to achieve a purer naive population without the need for cell sorting. This protocol is of a significant importance in the field of naive hPSC, by mimicking the natural niche of pluripotency and implementing

a xeno-free culture, our in vitro model is more similar to the in vivo situation. Moreover, generating naive hPSC using a stable LN521 feeder-free based model will lead to a more consistent and less labour-intensive culture resulting in a reduced risk of contamination, lower batch-to-batch variability, and better reproducibility of experiments.

Funding Source: FWO PhD fellowship fundamental research

Keywords: naive pluripotency, feeder-free culture, human embryonic stem cells

3:45 PM – 4:45 PM

561

IDENTIFYING REGULATORS OF HUMAN GENOMIC IMPRINTING UTILIZING UNIPARENTAL EMBRYONIC STEM CELLS

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Maternal and paternal genomes are essential for normal embryonic development, as the two genome copies are not functionally equivalent due to genomic imprinting, a process by which specific genes expression are determined by the parent of origin (paternal or maternal). Epigenetic variations, DNA methylation and histone modification are the main processes through which the cell maintains this expression pattern. Biparental cells (with both genome copies) offer a balanced genetic landscape for studying the interplay between maternal and paternal genetic contributions and imprinting effects on gene expression. We suggest utilizing our parthenogenic or androgenetic human embryonic stem cells, containing either the maternal or the paternal genome respectively, as a unique platform to identify regulators of parental imprinting in humans. Although this field has been a significant area of research for several decades, the central regulators and factors involved in this phenomenon, in human, are still poorly understood. We aim to identify imprinting regulators by designing a CRISPR-Cas9 knockout library of about 40 genes as candidates for imprinting regulators. All the candidate genes have been shown to be expressed in human embryonic stem cells functioning as DNA methyltransferase and/or histone modifiers. For two of these genes we have already demonstrated a significant effect on the imprinting locus in human chromosome 14. The identification of specific imprinting regulators would enable new insights on imprinting loci and pathways, allowing for novel understandings of the regulation of imprinting in human, along with potential therapeutic modes ways to affect imprinting disorders.

Keywords: parental imprinting, genetic screening, epigenetic regulators

3:45 PM – 4:45 PM

563

DIVERGENCES IN BIOELECTRIC STATE AND CALCIUM SIGNALING DURING EARLY EMBRYONIC STEM CELL DIFFERENTIATION

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Resting membrane potential (Vmem) and calcium signaling are spatio-temporally-specific, as a function of developmental stage and location. Changes in Vmem and increases in intracellular calcium ([Ca2+]i) regulate proliferation and differentiation of embryonic cells, with prior evidence suggesting calcium signaling is downstream of Vmem. However, it remains unclear how changes in the bioelectric and calcium signaling states of precursor cells connect with their differentiation trajectories. In this study, we measured Vmem and [Ca2+]i signals during differentiation of pluripotent embryonic stem cells (ESCs) towards neuroectoderm (NE) and mesendoderm (MZ) cells. We hypothesized that Vmem and calcium signals diverge from ESCs prior to acquisition of NE and MZ-like states. Mouse ESCs were differentiated in monolayer towards NE- or MZ-like states for 4 days. Cells were measured cells every 24 hours using DiBAC4(3) (for Vmem) and OGB1 (for [Ca2+]i). Cultures featured morphologies characteristic of early NE (SOX1+) and MZ (T+)-like states, with distinct regions of either flattened or dense three-dimensional ('cluster') morphologies. NE-directed cells were hyperpolarized relative to MZ-directed cells, with lower [Ca2+]i levels prior to germ layer marker acquisition. Colonies with clustered morphologies maintained relatively depolarized Vmem and higher calcium signals, closer to those of ESCs, despite their distinct cluster appearance. Within NE cultures, SOX1+ cells were hyperpolarized and had lower [Ca2+]i relative to OTX2+ (epiblast-like) cells. T+ cells are depolarized within MZ-like cell populations, with spatial variation in [Ca2+]i correlating with T expression. Our results demonstrate that divergences in Vmem and [Ca2+]i occur prior to germ layer marker protein expression, suggesting that a cell's bioelectric state may be connected with differentiation trajectory from ESCs. Identification of regional differences in both Vmem and [Ca2+]i suggests that even within in-vitro systems that lack the complexity of in vivo microenvironments, stem cells maintain regulated resting membrane potentials and calcium signals during germ layer differentiation. Our findings offer new insight complementing prior work in the field, which has largely been isolated to non-mammalian in vivo models.

Funding Source: Supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany´s Excellence Strategy – EXC-2068 – 390729961 and a Regent's Junior Faculty Fellowship (AD).

Keywords: bioelectricity, calcium signaling, germ layer differentiation

3:45 PM – 4:45 PM

565

CANNABINOID 2 RECEPTOR AGONISM INDUCES PROLIFERATION AND COMMITMENT TO SPERMATOGONIAL STEM CELL DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS

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Male factor infertility (MFI) reasons for 50% of infertility cases, 15% have no sperm due to germ cell aplasia. Although human induced pluripotent stem cell (hiPSC) derived spermatogonial stem cells (hSSC) present an autologous germ cell source, the yield is not adequate for clinical use. Some endocannabinoid system (ECS) components are distributed in human testes and promote proliferation of mouse embryonic stem cells. However, potential of ECS on hiPSCs and hiPSC derived hSSCs remains unknown. We hypothesize that stimulating ECS in hiPSCs can enhance their proliferation and improve the differentiation yield of hiPSC derived hSSCs. This could lead to an autologous cell based solution for MFI. For this aim, we reprogrammed iPSCs from commercial human dermal fibroblasts (hDF) by Sendai virus kit and differentiated them into hSSCs. qPCR assessed ECS receptors of hiPSCs and hiPSCs derived hSSCs. We revealed proliferative dose range for specific agonists on hiPSCs by real time cell analysis. Finally, highly expressed CB2R specific agonist CB65 was tested for potency on differentiation of hSSCs from hiPSCs. hiPSCs express OCT4, SOX2, and NANOG and are immunolabeled with OCT4-SSEA4 (92.09-92.98%), SOX2-TRA-1-60 (88.50-91.59%), NANOG (93.53-95.12%). High levels of ID4 (96.56-97.47%), PLZF (98.97-99.17%) expression and immune labeling confirmed hSSCs differentiation from hiPSCs. hiPSCs and hiPSCs derived hSSCs carry CB1R, CB2R, TRPV1, GPR55. Both hiPSCs and hiPSCs derived hSSCs express CB2R at higher levels compared to CB1R, TRPV1,GPR55 ($p< 0.005$ for all). CB65, a specific synthetic agonist for CB2R, enhances hiPSCs proliferation at 78-174 hours with an EC50 value of $2.092x10\text{-}8$ M ($p< 0.005$) compared to medium and DMSO controls. CB65 induced differentiation of hSSCs by increasing ID4+ and PLZF+ cells compared to control and the CBR2 antagonist groups and EC50 provided a homogeneous sized hSSCs population on day 10. The results indicate first time ECS receptor distribution and a dominant specific CB2R agonistic effect of CB65 on hiPSCs proliferation and differentiation to hSSCs. Here we set an effective dose range for CB65 to ameliorate the yield of hSSCs by restoring spermatogenesis when supplemented into culture medium. This could help enrich SSCs in germ cell aplasia for autotransplantation in MFI patients.

Funding Source: The Scientific and Technological Research Council of Turkey (#120S254, #22AG008) and Hacettepe University Scientific Research Projects Coordination Unit (# TSA-2021-19204) funded this study.

Keywords: human induced pluripotent stem cells, human spermatogonial stem cells, in vitro spermatogenesis

3:45 PM – 4:45 PM

567

A MICROFLUIDIC MODEL FOR HUMAN EMBRYONIC DISC AND AMNION FORMATION

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The first morphological milestone upon implantation of the human embryo is the emergence of the amniotic cavity. Amniogenesis is initiated in the epiblast upon rosette formation and continues throughout early development. Lumen expansion in the epiblast rosette results in an asymmetrical epithelial cyst, where tall columnar cells of the epiblast adjacent to the visceral endoderm are continuous with the squamous amniotic epithelium cells positioned near the trophoblast. Early postimplantation development is still a challenge to investigate due to ethical regulations around in vitro human embryo cultures, limited accessibility of relevant in vivo embryo samples and the significant evolutionary divergence to other model organisms. Stem cell-based embryo models represent a powerful tool to overcome these challenges. Here, we have established a microfluidic-based human pluripotent stem cell (hPSC) model to elucidate the molecular drivers of amnion formation. Using flow focusing technology, we generated primed hPSC-laden agarose microgels in a high-throughput fashion. Agarose microgels represent a biomimetic 3D environment that allowed hPSC to self-organize into homogenous squamous spheroids that resemble the molecular signature of the amniotic epithelium. Furthermore, we investigated the influence of various morphogens on amniogenesis, particularly focusing on the role of BMP, NODAL/TGFβ and FGF/MAPK signalling. This microfluidic-based system provides a scalable and controllable model to unravel the factors required for healthy embryonic disc and amnion development in humans. Ultimately, our model may aid in evaluating the effects of pharmaceuticals during pregnancy.

Keywords: amnion, microfluidic platform, agarose microgel suspension culture

3:45 PM – 4:45 PM

569

COORDINATION BETWEEN ENDODERM PROGRESSION AND GASTRULOID ELONGATION CONTROLS ENDODERMAL MORPHOTYPE CHOICE

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Embryonic development is highly robust. Morphogenetic variability between embryos, at least under ideal conditions, is largely quantitative. This robustness stands in contrast to in-vitro embryo-like models, which, like most organoids, can display a high degree of tissue morphogenetic variability. The source of this difference is not fully understood. We use the gastruloid model to study the morphogenetic progression of definitive endoderm (DE) and its divergence. We first catalog the different morphologies and characterize their statistics. We then learn predictive models for the lineage morphotype based on earlier expression and morphology measurements. Finally, we analyze these models to identify key drivers of morphotype variability, and devise personalized (gastruloid-specific) as well as global interventions that will lower this variability and steer morphotype choice. In the process we identify two types of coordination that are lacking in the in-vitro model but are required for robust gut tube formation. We expect the insights obtained here will improve the quality and usability of 3D embryo-like models, chart a methodology extendable to other organoids for controlling variability, and will also shed light on the factors that provide the embryo it's morphogenetic robustness.

Keywords: gastruloids, endoderm, embryo-like models

3:45 PM – 4:45 PM

571

BASE EDITING-MEDIATED MULTIPLEXED GENES INACTIVATION REVEALS ESSENTIAL ROLES OF DNMTS IN MOUSE GASTRULATION

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Dynamic regulation of DNA methylation by DNA methyltransferases (DNMTs) is critical for mammalian embryonic development. The function of individual DNMTs has been investigated through conventional knock-out strategies; however, the developmental effects of DNA methylation remain unclear partially due to the lack of effective tools to generate DNMTs-null embryos. Here, we first establish an IMGZ (Inactivation of Multiple Genes in Zygotes) system to simultaneously efficiently inactivate endogenous genes involved in DNA methylation and demethylation in zygotes through the introduction of a stop codon by hA3A-eBE-Y130F-mediated base editing. MGIZ-derived DNMTs-null embryos display embryonic lethal due to gastrulation failure at E7.5. Moreover, mutation combinations between DNMT and TET families showed DNMT1 or DNMT3A/3B is indispensable for mouse gastrulation and it's function independent of TET proteins. DNA methylome analysis reveals that DNMT1 and DNMT3A/3B jointly maintained DNA methylation of some crucial regions, including retrotransposons, enhancers and promoters which were hypermethylation in pre-implantation stage and may be critical for gastrulation. Promoter analysis showed that short genes, especially miRNAs, were more likely to maintain hypermethylation. CAS-seq revealed many miRNAs overexpression in DNMTs-null embryos, such as Let-7b, Mir127 and Mir541, which down-regulated some gastrulation-related genes, causing transcriptional instability. The introduction of a single mutant allele of six miRNAs and paternal IG-DMR partially restores primitive streak elongation in Dnmt-null embryos. Thus, our results unveil a novel epigenetic correlation between promoter methylation and suppression of miRNA expression for gastrulation and demonstrate that IMGZ can accelerate deciphering the functions of multiple genes in vivo.

Keywords: DNMT family genes, mouse embryo gastrulation, base editing mediated gene inactivation

3:45 PM – 4:45 PM

573

AN ESSENTIAL ROLE OF NUCLEOLAR RNA HELICASE DDX21 IN THE SELF-RENEWAL OF EMBRYONIC STEM CELL AND NEURAL STEM CELL

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Embryonic stem cells (ESCs) and neural stem cells (NSCs) are highly proliferating cells which display self-renewal property through epigenetic regulation. To maintain an active proliferating state, rapid protein synthesis is required and supported by active rDNA transcription, rRNA processing and ribosome biogenesis in the nucleolus. DDX21 is an RNA helicase primarily localized to the nucleolus for rRNA processing and ribosome biogenesis. To test whether DDX21 is required for the self-renewal of ESC and NSC, we depleted DDX21 in human ESC and NSC. Both stem cell types lost their ability to proliferate and thus compromised differentiation. DDX21 was found to interact with polycomb repressive complex 2 (PRC2), a chromatin modifier which generally suppresses the transcription of developmental genes through establishing H3K27me3. DDX21 loss was associated with downregulation of H3K27me3 mark. Using CUT&TAG-seq, we profiled genes disturbed when DDX21 was depleted. Additionally, nucleolar organization was changed. These results suggested that DDX21 is important for stem cell self-renewal through maintaining an epigenetic signature which might be linked to its helicase function in the nucleolus.

Funding Source: Hong Kong Research Grants Council (RGC) GRF Project No. 14104321; Health@InnoHK funding support from the Innovation Technology Commission of the Hong Kong SAR.

Keywords: DDX21, stem cell self-renewal, polycomb repressive complex

3:45 PM – 4:45 PM

575

A MESSAGE OF SELFLESS DEATH - DEVELOPMENTAL CELL COMPETITION IN GASTRULOIDS

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Cell competition (CC) is a comparison of fitness among neighboring cells in a tissue. In this process, viable but suboptimal cells undergo apoptosis when faced with 'fitter' wild-type (WT) cells. These same suboptimal cells may survive and grow when surrounded by equals, but even healthy WT cells may initiate apoptosis when surrounded by fitter 'supercompetitor' cells created by e.g. loss of p53. Developmental cell competition has been described in the early mammalian embryo, where it results in the death of up to 35% of cells within the epiblast. This may reflect a quality control checkpoint to eliminate suboptimal cells prior to gastrulation, the start of embryo construction. This situation has been recapitulated in vitro in 2D cultures of embryonic stem cells (ESC). These studies have shown that CC is not explained by differences in proliferation or nutrient scarcity and that it only occurs in differentiating but not pluripotent cells. Here, I use gastruloids, an ESC-based model of early mammalian development, to study CC in real time in a 3D context and with reproducible high-throughput capacity. Mosaic gastruloids consisting of two fluorescently distinct WT populations develop normal with equal contribution. However, mosaics of WT and p53KO cells undergo drastic competition, where even few supercompetitors suffice to outcompete WT cells almost entirely. In gastruloids, CC takes place during 48-96h after aggregation, which developmentally corresponds to E5.5-E7.5 in the mouse and mirrors the developmental timing of CC in the embryo. pH3 staining and gastruloid size titrations confirm that CC is not due to differences in proliferation or nutrient availability. Inhibiting apoptosis by overexpressing Bcl2 (specifically during 48-96h) entirely abolishes CC, confirming that it is executed through apoptosis. Neither pluripotent mosaic 3D aggregates, nor 3D Epi-gastruloids, which model more advanced developmental processes, demonstrate any competition, confirming that developmental CC is specific to pre-gastrulation. Competition gastruloids are thus a useful system to decipher the mechanisms mediating CC in mammalian embryos.

Funding Source: JF has been funded by "la Caixa" Foundation (ID 100010434 The fellowship code is "LCF/BQ/PI23/11970017") and an Advanced ERC award (MiniEmbryoBlueprint 834580) as well as from an EMBO postdoctoral fellowship (ALTF 605-2022).

Keywords: gastruloid, cell competition, apoptosis

3:45 PM – 4:45 PM

577

DECIPHERING HUMAN EMBRYO IMPLANTATION THROUGH EMBRYO–ENDOMETRIAL INTERFACE MODELING

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The implantation process represents the initial phase of physical interaction between the developing embryo and the maternal endometrium. A blastocyst hatches from the zona pellucida, exposing the trophectoderm–the main point of contact with the receptive endometrium, thus giving rise to the placenta and facilitating the exchange of nutrients, gas, and waste. Human endometrial receptivity is cyclic, peaking during the "implantation window" in the mid-luteal phase, governed by hormonal dynamics. The orchestration between the blastocyst's readiness for implantation and the endometrium's receptivity is pivotal for successful implantation. Yet, the detailed process underlying the embryo–endometrial interface during human implantation remains largely unknown, hindered by the ethical and technical challenges in procuring early-stage human biological samples. To bridge this knowledge gap, we harnessed in vitro models simulating the implantation process. We developed an apical-out endometrial organoid (AO-EMO), which exposes the apical surface of the epithelium and contains a dense endometrial stromal cells and an endothelial network, addresses the limitations of traditional organoids, including issues of apical accessibility, stromal representation, and vascularization. Through co-culture with human embryonic stem cell (ESC)-derived blastoids, we created feto–maternal assembloids that accurately mirror the implantation's initial phases—apposition, adhesion, and invasion. This model facilitated unprecedented observations of the direct interplay between fetal and maternal cells, uncovering the disruption of the endometrial epithelial barrier by syncytia that subsequently invade and integrate with human endometrial stromal cells. Validation with human blastocysts further corroborates these findings. The feto–maternal assembloids developed in this investigation offer a robust platform for elucidating the mechanisms of implantation, thereby enhancing our understanding and potentially informing new fertility treatment strategies.

Keywords: implantation, endometrial organoid, blastoid

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

579

DECIPHERING THE MECHANISMS BEHIND SPECIES-SPECIFIC DEVELOPMENTAL TIMESCALES

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We asked what mechanisms regulate species-specific timing of mammalian embryonic development. Using pluripotent stem cells (PSCs) and neural differentiation from multiple species under identical culture conditions we examined gene expression and chromatin accessibility in single cell resolution. The time-series analysis of human, cynomolgus monkey and mouse cells showed significant differences in the rates of up- and down-regulation of developmental genes in ascending order from slowest to fastest. Intriguingly, by analyzing G1-M phase genes and inhibition of mTOR pathway we noted that the rates of species-specific neural differentiation rates are uncoupled with cell cycle length. However, we observed differential usage of cohorts of genes associated with chromatin and metabolic regulation between species. Importantly, we identified an association of the glycogen synthesis gene UGP2 with the slower differentiation rate of human and cynomolgus monkey cells. Functional studies of UGP2 showed that it is governs the rate of neural differentiation. This study uncovers metabolic regulation of species-specific developmental rates.

Keywords: developmental speed, single cell sequencing, glycogen metabolism

TRACK: DISEASE MODELING AND DRUG DISCOVERY (DMDD)

Session II: Even

4:45 PM – 5:45 PM

TOPIC: NEURAL

102

HIPSC-DERIVED CORTICAL NEURONS IMPLICATE DYSFUNCTIONAL GLUTAMATERGIC DEVELOPMENT IN ADHD PATHOGENESIS

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Attention-deficit/hyperactivity disorder (ADHD) is a chronic neuropsychiatric disorder, significantly impacting life quality and increasing risk of premature death. Due to the complex and poorly understood aetiology of ADHD, current medication can only treat ~40% adult patients, and novel drug development has stalled. Previous research has primarily focused on the dopaminergic system, however changes in prefrontal cortex (PFC) function have also been reported in patients, implicating a potential role for glutamatergic neurons. Additionally, there has been a large reliance on animal models to date, which cannot successfully recapitulate clinical ADHD pathology. We therefore sought to develop a human-based, disease-relevant model to investigate ADHD aetiology, via the use of human induced pluripotent stem cell (hiPSC)-derived cortical neurons. hiPSCs generated from healthy controls and adult ADHD patients were differentiated into cortical neuron cultures consisting predominantly of glutamatergic neurons, as demonstrated by expression of the pan-neuronal marker TUBB3 and vesicular glutamate transporter vGLUT2. The presence of glutamatergic synapses was confirmed by colocalisation of the pre- and post-synaptic markers vGLUT1 and PSD95, and calcium imaging indicated spontaneous synaptic signalling, confirming the successful generation of mature cortical neurons. Case-control analysis revealed decreased expression of vGLUT2 and PSD95 in cortical neurons derived from ADHD patients, along with a reduction in the number of glutamatergic synapses formed. Furthermore, changes in extracellular glutamate were observed, and a significantly altered glutamate/glutamine balance identified. Calcium signalling analysis additionally revealed decreased peak amplitude in cortical neurons derived from ADHD patients, along with a significant reduction in firing frequency. We successfully generated hiPSC-derived functional cortical neurons from adult ADHD patients for the first time, which revealed inhibited glutamatergic synapse formation and synaptic signalling. Our results suggest dysfunctional glutamatergic development plays a key role in ADHD pathogenesis, providing a new avenue of investigation for further studies and potentially novel targets for future drug development.

Funding Source: Supported by a NARSAD Young Investigator Grant from the Brain & Behaviour Research Foundation

Keywords: hiPSCs, ADHD, psychiatry

4:45 PM – 5:45 PM

104

HUMAN SPINAL CORD ORGANOIDS AS A MODEL OF FETAL NEURAL TUBE MORPHOGENESIS IN MYELOMENINGOCELE

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Myelomeningocele (MMC) is a severe form of spina bifida associated with substantial neurologic morbidity. In vitro modeling systems of human spinal cord development may help to elucidate the underlying pathophysiology of the MMC spinal cord. We developed spinal cord organoids (SCO), defined as self-organized, three-dimensional clusters of spinal tissue, that were derived from human amniotic fluid induced pluripotent stem cells. Here, we used a variety of analyses, including immunofluorescent and single-cell transcriptomic approaches, to characterize and compare SCOs from healthy and MMC fetuses. Organoids possessed unique dorsal-ventral and rostro-caudal patterning. scRNAseq data identified five main cell type groups: neurons, glia/glia progenitors, neural progenitors, mesoderm, and dividing cells (Figure). Among the neuronal cell population, we found cells corresponding to each of the dorsal dI2-dI5 domains, and ventral V2a, V2b, V3, pMN and FP domains, as well as neural crest neurons. Oligodendrocytes and astrocytes, as well as mesodermal cells, including chondrocytes, osteoblast and osteoclasts, and smooth muscle, were also recognized when cultured for up to 130 days in vitro. Multielectrode arrays revealed functional electric activity by SCOs with evidence of emerging neuronal networks. Fetal spina bifida modeling in a clinically relevant microenvironment was successfully established by culturing MMC SCOs in second- and third-trimester amniotic fluid for 3 weeks. Taken together, these studies show that functional SCOs can recapitulate the cellular identity of the fetal spinal cord and represent a novel research platform to study the interplay between cellular, biochemical, and mechanical cues during human MMC neural tube morphogenesis.

Keywords: spinal cord organoids, induced pluripotent stem cells, myelomeningocele

4:45 PM – 5:45 PM

106

THE EFFECTS OF PMP22 OVEREXPRESSION ON CELLULAR STRESS IN CHARCOT-MARIE-TOOTH DISEASE TYPE 1A

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Charcot-Marie-Tooth (CMT) disease is a peripheral neuropathy occurring in 1 in 2,500 people worldwide. The most common form of this hereditary disease, CMT1A, is predominantly demyelinating and is caused by the Peripheral Myelin Protein 22 (PMP22) gene duplication. PMP22 is an aggregation-prone intrinsic membrane protein of the myelin sheath mainly produced by Schwann cells (SC), the myelinating cells of the peripheral nervous system. It is unknown how the overexpression of PMP22 contributes to the abnormal myelin sheath structure and SC dysfunction observed in CMT1A. Hence, no cure is available to date. PMP22 aggregates have been colocalized with lysosomes, suggesting autophagy pathway activation. Moreover, previous research has shown that myelinating cells are especially susceptible to lysosomal stress, suggesting that the overexpression of PMP22 may disrupt lysosomal function. This study investigates the poorly understood role of lysosomal dysfunction in CMT1A Schwann cells using Schwann cell precursors (SCP) derived from CMT1A patient iPSC. We used a human patient-in-a-dish model of Schwann cell precursors (SCP) derived from CMT1A patient iPSC and their isogenic controls. Protein ubiquitination and aggregation were observed via immunostainings in CMT1A iPSC and SCP. Furthermore, CMT1A SCP showed significantly higher protein levels of the lysosomal marker LAMP1 and lysosomal enzymes cathepsin B and D (CtB and CtD) compared to their isogenic controls, using immunocytochemistry. This indicated a significant increase in lysosomal amount and lysosomal enzymes. Additionally, CtB activity was significantly elevated in CMT1A SCP compared to their isogenic controls. Transmission electron microscopy images revealed an increased lysosomal amount in CMT1A SCP. We observed a significant increase in lysosomal amount and lysosomal enzymes in CMT1A SCP, indicating alterations in the lysosomal pathway in CMT1A. Nevertheless, further research is necessary to explore the mechanism and consequences of these lysosomal changes and their potential therapeutic implication, providing important insights into CMT1A and other neurodegenerative, demyelinating, and PMP22-related diseases.

Funding Source: FWO Vlaanderen

Keywords: Charcot-Marie-Tooth disease type 1A, protein aggregation, lysosomes

4:45 PM – 5:45 PM

108

INTEGRATION OF ELECTRIC CIRCUITS INTO TAILOR-MADE 3D NANOPRINTED MICROSCAFFOLDS FOR HUMAN IPSC-DERIVED NEURONAL NETWORK CULTIVATION

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The path to Brain-on-a-Chip (BoC) devices comprises three crucial cornerstones: (i) utilizing human induced pluripotent stem cell (hiPSC)-derived neuronal networks for mimicking human physiology, (ii) incorporating guiding cues to achieve precise three-dimensional brain-like assembly, and (iii) establishing an electrical interface for analyzing and interacting with these networks. Traditionally, cultivation platforms of hiPSC-derived neuronal networks are augmented either by 3D guiding cues or 2D microelectrode arrays (MEAs). Here, we propose a method to integrate circuitry into 3D microscaffolds that are connected to planar MEAs. This approaches and enables the fabrication of cultivation platforms for simultaneous single-cell 3D guiding and electrical readout of neuronal networks. Recently, we presented non-functional microscaffolds prepared by 3D nanoprinting (3DN) for hiPSC-derived guided neuronal networks. Tower-like structures with cavities on the top determined locations for neuron settling while interconnecting tunnels confined axonal outgrowth for neuronal network formation. Hence, alterations in the scaffold's layout would impact the formation of the respective networks. Yet, the electrophysiological readout was limited to invasive patch clamp recordings of selected cells within the network. To conduct collective long-term experiments, integrated electrodes inside the scaffolds are prepared by electrodeposition (ED) of gold. By implementing vertical tunnels at each tower cavity during 3DN, the gold ED was precisely guided toward the settling areas. The 3DN was conducted directly on custom-made or commercially available MEAs until the deposited gold filled the tunnels. Note, the electrode's surface topography can be modified by changing the deposition rate. Such an electrode morphology tuning allows for the optimization of the cell-electrode coupling. The versatile nature of the MEA fabrication in combination with 3DN and ED enables the production of microscaffolds as cultivation platforms with addressable electrodes of almost any shape. Our results take a significant step toward application in analyzing information transmission within hiPSC-derived neuronal networks and understanding the underlying mechanisms orchestrating information processing in 3D.

Keywords: brain-on-a-chip, 3D nanoprinting, microelectrode arrays

4:45 PM – 5:45 PM

110

INVESTIGATING NEUROIMMUNE/TUMOR INTERACTIONS WITH HUMAN BRAIN ORGANOIDS

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Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive pediatric brain tumor with a poor prognosis (< 12 months survival). Currently, there is no effective treatment for DIPG. Due to its invasive growth pattern within the pontine area, complete surgical resection of DIPG is difficult. Up to a quarter of the cells in a DIPG tumor originate from the immune lineage. In order to understand the interactions between tumor cells and microglia - the brain's primary immune cells - in a human 3D tissue context, we developed a neuroimmune-competent brain organoid/DIPG fusion model system. The microglia-containing brain organoids (MiCBOs) were generated through self-assembly and co-development of human embryonic stem cell-derived neuronal progenitor and GFP-labeled myeloid precursor cells. Extensive testing of various growth conditions established two optimal settings that result in substantial amounts of GFP+ microglia in organoids over two months. We applied confocal and two-photon live microscopy to assess the distribution and motility of the microglia in the MiCBOs and found viable, highly motile, and widely distributed microglia that show a high resemblance to those found in the human brain. Tumor cell invasion and microglia migration were evaluated by confocal live cell imaging as well as immunofluorescence stainings. Different primary tumor cell lines show distinguishable invasion patterns and grades of diffusion into the brain organoids. We further utilized single-cell RNA-Sequencing (scRNA-Seq) to reveal distinct clustering of microglia cells as a result of tumor confrontation and the emergence of a new molecular subgroup of tumor cells as a result of microglia exposure. This suggests signaling interactions between these two cell types in the organoids. In summary, our controllable, tractable, and modular MiCBO/DIPG fusion model system can be utilized to gain insights into the invasion process of DIPG cells and their interaction with brain cells, including microglia, in the tumor microenvironment. The MiCBO model is easily expandable for screening clinically relevant drugs or gene manipulations that induce an immune response to possibly suppress tumor progression.

Funding Source: Boston Children's Hospital Kirby Center Innovation Award Elsa U. Pardee Foundation

Keywords: microglia, immunocompetent, tumor cell invasion

4:45 PM – 5:45 PM

112

GENERATION OF HIGHLY PURE HUMAN INDUCED PLURIPOTENT STEM CELL HIPSC-DERIVED MOTOR NEURONS FOR MODELING MOVEMENT DISORDERS

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The limited access to patient brain tissues greatly impedes progress in neurological disease research. Human induced pluripotent stem cells (hiPSCs)-derived neurons provide unprecedented approaches for disease modelling and drug discovery. Using lentiviral delivery of transcription factors, we have established a protocol for the generation of highly pure motor neurons (MNs) based on hiPSC-induction and differentiation. Recently, we developed an indirect co-culture system using cell culture inserts, which physically separate iPSC-MNs and co-cultured astrocytes, enabling us to obtain highly pure MNs at late mature stages for biochemical studies. We used this system to model DYT1 dystonia, a movement disorder caused by the loss-of-function mutation in the TOR1A gene (ΔE). DYT1 iPSC-derived MNs successfully replicated disease-dependent cellular deficits, including deformed nuclei, impaired neurodevelopment, disrupted nuclear transport, and the unexpected finding of nuclear Lamin B1 mislocalization. Transmission electron microscopy (TEM) and immunogold labeling indicated that the dysregulated nuclear Lamin B1 led to the thickening of the nuclear lamina and deformation of the nucleus in DYT1 MNs. Moreover, using the co-culture system, we prepared a large amount of highly pure MNs from DYT1 iPSCs and isogenic controls and performed transcriptomic and proteomic studies. We found that the dysregulated genes in DYT1 MNs are enriched in neurodevelopment, synaptogenesis, synaptic transformation, neurotransmitter release, and nuclear transport. Through proteomic studies, we found that the mutant protein (ΔE) disrupted critical factors for various biological processes and neuron functions. Meanwhile, we also identified mislocalized nuclear Lamin B1-interacting proteins that are involved in signaling pathways for neuronal development and functions. Our study demonstrates the high value of hiPSC-derived MNs in modeling movement disorders and the reliability of the co-culture system in obtaining high quality MNs for identification of pathogenic factors using biochemical approaches. The dysregulated genes and proteins identified in DYT1 dystonia provide novel insights into the disease pathogenesis and offer molecular targets for potential development of therapeutic interventions.

Funding Source: NIH National Institute of Neurological Disorders and Stroke (NINDS) (NS112910 and NS133252 to B.D.), Department of Defense (DoD) (W81XWH2010186 to B.D), and LSU Health Shreveport Center for Brain Health (CBH) (to B.D.).

Keywords: human induced pluripotent stem cells (hiPSCs), motor neurons, movement disorder DYT1 dystonia

4:45 PM – 5:45 PM

114

IPSC-DERIVED NEURONAL MODEL FOR STUDYING MITOCHONDRIAL DEFECTS IN LEIGH'S SYNDROME

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Leigh's Syndrome (LS) is progressive neurodegenerative disorder linked to mitochondrial dysfunction. Pathogenesis of LS is not fully understood due to limitations of current disease models. Development of induced pluripotent stem cells (iPSCs) has opened the possibility of elucidating disease mechanisms. Here, we aimed to generate iPSC-derived neurons from fibroblasts obtained from a LS patient carrying ND6-mutation and healthy individuals and analyse the mitochondrial function and morphology. We reprogrammed fibroblasts from a patient with mt-ND6 mutation by lentiviral transduction. iPSC clones were expanded and characterized by immunostaining and real-time quantitative polymerase chain reaction (qRT-PCT) for pluripotency and genetic stability. iPSCs were differentiated into NSCs and neurons by defined medium. iPSC-derived neurons were analysed by high content image analysis for analysis of mitochondrial membrane potential and neuronal morphology. iPSCs from LS patient (LS-iPSCs) exhibited characteristic morphology and pluripotency. LS- iPSCs showed no chromosome aberrations and formed embryoid bodies that spontaneously differentiated and expressed markers of all three germ layers. LS-iPSCs successfully differentiated into NSCs expressing SOX2 and Nestin, and neurons expressing BIII-tubulin and MAP2. LS-neurons showed reduced mitochondrial membrane potential without significant differences in mitochondrial mass compared to controls. LS-neurons exhibited less roots per cell compared to controls. We successfully obtained iPSC-derived neurons from an LS patient exhibiting mitochondrial dysfunction and altered neuron morphology. These LS-neurons further assist the understanding of LS pathogenesis in the brain and develop new strategies for clinical diagnosis and therapeutic drug targets.

Keywords: neurons, Leigh's Syndrome (LS), induced pluripotent stem cells

4:45 PM – 5:45 PM

116

DISEASE MODELING OF HUMAN MYOCILIN MUTATION-ASSOCIATED GLAUCOMA: DEVELOPMENTAL ABNORMALITIES IN RETINAL GANGLION CELLS

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Glaucoma represents a group of diseases where the unifying theme is the progressive degeneration of retinal ganglion cells (RGCs), the main output neurons of the retina, leading to permanent vision loss. Mutation in the ubiquitous myocilin (MYOC) gene represents one of the most common genetic factors associated with primary open angle glaucoma (POAG). Here, we have tested the hypothesis that RGCs are MYOC mutations cellular target in addition to the generally accepted trabecular meshwork (TM), the linchpin of TM/ocular hypertension hypothesis of glaucomatous degeneration. We reprogrammed blood cells from a MYOCALA445VAL POAG patient into iPSCs and obtained isogenic iPSC lines by correcting the mutation through gene editing. We generated hRGCs from the mutant and isogenic iPSC lines by recapitulation of developmental mechanism through stage-specific 2D directed differentiation method. Temporal analysis of transcripts corresponding eye field transcription field gene (EFTF) expression during neural induction revealed their levels decreased in mutant RGCs with corresponding decrease in the number of RX+Pax6+ retinal progenitor cells (RPCs), compared to isogenic controls. Starting with the equal number of RPCs, the MYOC mutants generated significantly low number of hRGCs, compared to isogenic controls. The deficient generation of hRGCs by MYOC mutant RPCs was accompanied by decrease in the expression of RGC regulatory genes (e.g., ATOH7, BRN3b, ISL1) versus isogenic controls, suggesting adverse effects of the mutation on RGC developmental mechanism. Morphological abnormalities were also detected in MYOC mutant RGCs; both the complexity and length of the neurites of these cells were simpler and shorter in comparison to isogenic controls. The MYOC mutant RGCs were functionally immature in basal physiology and networking, as revealed by the whole cell and multiple electrodes array (MEA) recordings. Furthermore, when subjected to axotomy in a microfluidic model of axon regeneration the MYOC mutant RGCs were found deficient in axon regeneration relative to controls. Thus, the disease modeling revealed that the MYOC mutation in POAG may confer developmental susceptibility on RGCs, which in adult may be unmasked in the presence of risk factors, leading to degenerative changes.

Funding Source: NIHEY029778, NIHEY022051

Keywords: glaucoma disease modeling, retinal ganglion cells, myocilin

4:45 PM – 5:45 PM

118

MATURE BRAIN ORGANOID PLATFORM FOR THERAPEUTIC SCREENING FOR ALS/FTD

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Synapticure's mission is to provide access to help patients to the best care and treatments access the best treatment possible for their specific type of neurodegenerative disease regardless of where they live. We currently help >1600 patients across Dementia, Motor and Movement Disorders by tailoring today's best possible care to each person. Currently, effective diagnostics and treatments for Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are lacking, and newer therapies have translated poorly to humans, despite showing great promise in vitro and in vivo preclinical studies. TAR-DNA binding protein 43 (TDP-43) proteinopathy is a neuropathological feature observed in ~97% of ALS, up to 57% of Alzheimer's and ~45% of FTD cases. Synapticure is developing a novel patient-derived 3D platform for high-fidelity modeling and screening of TDP-43 proteinopathy and associated biomarkers. Through an engineering-like approach, Synapticure can form mature brain organoids (mbOrgs) from induced pluripotent stem cell (iPSC) derived mature human astrocytes (iA) and homogeneous cortical-like neurons (iN) in defined numbers and ratios, providing a 3D environment similar to those in the healthy human brain and recapitulating key features of ALS such as aging-related disease pathology never before reported in vitro. Synapticure's 3D models have been validated by assaying TDP-43 proteinopathy, which includes the evaluation of TDP-43 localization and phosphorylation, as well as the mis-splicing of stathmin-2 (STMN2), a recently characterized biomarker of TDP-43 proteinopathy. Here we show a 2D to 3D therapeutic screening pipeline for identifying compounds that rescue TDP-43 proteinopathy-associated phenotypes. We established and validated therapeutic screening in 2D, showing rescue of TDP-43 proteinopathy-associated cryptic splicing of STMN2. Additionally, we continue therapeutic screens in 3D mbOrgs to evaluate the potential of therapeutic candidates to rescue spontaneous TDP-43 proteinopathy. Synapticure is expanding the platform to patient-derived cells and to include other relevant cell types, confirming the ability to screen compounds against a broad genetic background as a step toward personalized treatment for ALS/FTD.

Funding Source: NIH SBIR - 1 R43 NS134458-01

Keywords: organoid, neurodegeneration, platform

4:45 PM – 5:45 PM

120

HUMAN IPSC DERIVED NOCICEPTORS AND ASSAY TO STUDY PAIN MECHANISMS

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Chronic pain disorders represent a major burden on society, inflicting individual suffering, disability and high healthcare costs. Analgesic treatments, particularly opiates can be harmful and for pain conditions such as neuropathic pain, there are no effective treatments for many patients. Drug discovery in pain remains challenging – in part due to the limitations of current models and historical data which has largely come from rodent studies. We present our standardised workflow for generating nociceptor subtypes from human induced pluripotent stem cells (iPSC). We have successfully generated non-peptidergic and peptidergic nociceptors that are functionally competent. We can assay responses using multi-well multielectrode arrays, calcium imaging and molecular analysis. We highlight key nociceptor biology that can be revealed using our methods. Human iPSC nociceptor models thus represent a key tool for investigating pain mechanisms, for target discovery and validation and for investigating pharmacodynamics.

Keywords: Nociceptor, chronic pain, peptidergic

4:45 PM – 5:45 PM

122

MODELLING SCHIZOPHRENIA NETWORKS IN HESC-DERIVED FOREBRAIN NEURONS

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Schizophrenia (SZ) is a debilitating hereditary neuropsychiatric disorder with a ± 1% prevalence. However, despite decades of intensive research, a thorough understanding of its etiology is currently lacking, hampering the development of much needed novel treatment strategies. Over the past years, research efforts have shifted towards elucidating the collective influence of genetic risk factors to uncover overarching disease mechanisms. This has resulted in the discovery of cell-type-specific protein-protein interaction networks centered around SZ risk genes, which themselves also exhibit enrichment for SZ risk. In this study, we investigate the roles of two genes which have recently been associated with SZ risk and are connected to such networks: the potassium channel HCN1 and the nuclear exportin protein XPO7. We generated heterozygous and homozygous KO lines for HCN1 and XPO7 in human embryonic stem cells and subsequently differentiated these into medial ganglionic derived inhibitory neurons and cortical excitatory neurons, two cell types heavily linked to the disease. Upon maturation in vitro, we found that HCN1-knockout excitatory neurons displayed electrophysiological hyperactivity as detected by multi-electrode array analysis. In contrast, we identified a significant reduction in the number of synaptic densities expressing synapsin 1 (SYN1) for the XPO7 KO lines, suggesting potential implications for synaptic function and neurotransmission. We are currently performing proteomic as well as single-cell transcriptomic analysis to elucidate the molecular mechanisms involved in these SZ-related neuronal dysregulations, aiming to provide a comprehensive understanding of the role of these genes in human neurons.

Keywords: schizophrenia, pluripotent stem cells, disease modelling

4:45 PM – 5:45 PM

124

USING HIPSC-DERIVED SORL1 E270K ASTROCYTES TO ELUCIDATE DIFFERENCES IN ALZHEIMERS DISEASE RISK DEPENDING ON THE GENETIC BACKGROUND

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SORL1, initially identified as an APOE receptor and regulator of cellular protein trafficking, is an important risk gene for Alzheimer's disease (AD). We found that the SORL1 E270K variant seems to have differential effects on AD risk depending on the genetic background, which we will attempt to better understand using iAstrocytes derived from our novel hiPSC differentiation protocol. This protocol combines low-density propagation of neural progenitors with lentiviral overexpression of NFIA, a key transcription factor for glial cell fate. iAstrocytes express a consistent repertoire of astrocyte markers at the transcript and protein levels and bulk RNAseq analysis shows robust expression of these markers as compared to two other commonly used astrocyte differentiation protocols. iAstrocytes also display robust calcium transient activity using a calcium-sensitive dye in response to mechanical stimulation or ATP. SNPs in SORL1 have been identified as risk factors for late-onset AD (LOAD), including the E270K variant in a Caribbean Hispanic population. This mutation maps to the cargo-carrying domain of the SORL1 protein and results in increased amyloidogenic processing of APP and enlarged early endosomes. We analyzed the effects of the SORL1 E270K variant on AD risk in the primarily Caucasian NIA-LOAD

cohort stratified by APOE genotype. Surprisingly, we have found that the E270K variant is protective (rather than deleterious) for LOAD risk specifically in APOE4 carriers in this population. We hypothesize that the SORL1 E270K mutation causes differential SORL1-substrate cargo binding interactions which has differential consequences depending on the genetic background. To test this hypothesis, we used CRISPR-mediated genome editing to generate SORL1 E270K knock-in hiPSCs. We then differentiated SORL1 E270K/+, SORL1 E270K/E270K, and isogenic control hiPSCs into iAstrocytes. Preliminary data shows that SORL1 E270K/E270K astrocytes have reduced phagocytosis of monomeric FITC-Aβ42, providing initial validation that E270K has some negative consequences in this cell type. We are now using this platform to identify the molecular mechanisms by which the genetic background of SORL1 E270K carriers confers differential LOAD risk.

Keywords: Alzheimer's disease, SORL1, astrocyte

4:45 PM – 5:45 PM

126

INVESTIGATING ALCOHOL-INDUCED BRAIN DAMAGE USING HUMAN-DERIVED INDUCED PLURIPOTENT STEM CELLS: A FOCUS ON NEUROTRANSMITTER RECEPTOR MECHANISMS

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Alcohol-related brain damage is a significant public health concern with far-reaching consequences exerting considerable societal burden. Understanding the mechanisms underlying alcohol-induced neurotoxicity is crucial for developing effective prevention and treatment strategies. However, the mechanism of alcohol is still unclear and has many limitations. In this study, we aimed to focus on alcohol's effects on neuronal function, especially in NMDA receptor activation and dopamine secretion. The effect of alcohol was confirmed in human derived induced pluripotent stem cells (hiPSCs) derived cortical neuron and cerebral organoids. Chronic ethanol exposure was induced by treating the cells or organoids with varying concentrations of alcohol (0mM, 25mM, 100mM) for 7 days. Ethanol exposure decreased the expression of mature neuronal markers (i.e. MAP2, TUJ1, and TBR1) and neurotrophic markers (i.e. BDNF, GDNF, and NGF) in a dose dependent manner. Moreover, we also confirmed neurotransmitter receptor mechanism related markers, hypothesizing that alcohol enhances NMDA receptor activity and dopamine secretion. Our finding robustly support this hypothesis, demonstrating dose-dependent upregulation of NMDA receptor subunits NR1 and NR2A expression alongside concurrent increases in dopamine levels. Our study provides insights into the mechanisms of alcohol induced brain damage, particularly regarding neurotransmitter receptor modulation using hiPSCs. By elucidating the mechanisms underlying alcohol-induced neurotoxicity, we pave the way for the development of targeted therapeutic interventions aimed at protecting and restoring brain function. Furthermore, our approach highlights the potential of hiPSCs as a valuable tool for studying alcohol-related brain damage and advancing translational research efforts.

Keywords: human induced pluripotent stem cell, alcohol related brain damage, NMDA receptor

4:45 PM – 5:45 PM

128

HUMAN NEUROBIOLOGY AT SCALE: STANDARDIZATION OF THE CORTICAL BRAIN ORGANOID PIPELINE TO MODEL NEURODEVELOPMENTAL DISORDERS

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Neurodevelopmental disorders (NDDs) are a broad group of conditions affecting nervous system formation and maturation, leading to a variety of cognitive and/or behavioral symptoms. Despite their high prevalence, the uniqueness of human brain development, the inherent limitations in primary brain tissue accessibility and the sheer complexity of NDD genetic architectures make their in vitro modelling challenging. Technologies based on induced pluripotent stem cells (iPSCs) have advanced rapidly over the past decades and patient-derived in vitro models, such as three-dimensional cortical brain organoids (CBOs), have grown into a transformative resource thanks to the recapitulation of key aspects of human brain cytoarchitecture, gene expression, and cellular function. Scaling such approaches to tackle NDD diversity remains however a fundamental unmet challenge, starting from the need of standardized protocols that would enable modelling NDD diversity and the neurodiversity of the human condition more broadly. Here we describe a pipeline we implemented to improve reproducibility and scalability of CBOs generation and profiling. To enhance the robustness of this pipeline, we included more than 40 stem cell lines from NDD

case-control matched cohorts, as well as iPSCs and embryonic stem cell isogenic lines carrying highly penetrant mutations of NDD-related genes. First, we adapted all lines to a novel, highly scalable, precoating-free culturing method, eliminating time-consuming steps and optimizing cell culture propagation techniques. After synchronizing the lines growth, we generated CBOs and profiled them at multiple time points through an integrated optimization of two complementary approaches: single-cell enzymatic dissociation of CBOs for fixed RNA sequencing, obtaining a multiplexed and scalable measurement of gene expression in single cells, and an adaptation of high-throughput tissue microarrays platforms to scale up immunofluorescence analysis of NDD-related biomarkers. Together, this pipeline overcomes critical challenges of iPSC and organoid-based NDD models, paving the way to the standardization of scaled culturing and profiling protocols, thereby domesticating experimental variability to improve our study of NDDs in the authenticity of the neurodiverse human condition.

Keywords: NDDs, organoids, standardization

4:45 PM – 5:45 PM

130

SLITRK DYSREGULATION IN PRE-CLINICAL MOUSE AND HIPSC-BASED MODELS OF ALPHA-SYNUCLEIN MEDIATED SYNAPTOPATHY

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Alpha-synuclein (αSyn) is a highly expressed and conserved presynaptic protein, which is considered a critical factor in the development of Parkinson's disease (PD). Synaptic dysfunction is presumed to be an early yet progressive pathological feature in PD, with the triggering mechanisms remaining undefined. An analysis of human-derived neurons from patients harboring the p.A53T mutation of αSyn has revealed deficiencies in synapse formation and function, alongside dysregulation of genes involved in synaptic signaling. Notably, among these genes, members of the SLITRK family of postsynaptic adhesion proteins (SLITRK1/2/4) have been identified to be significantly downregulated in mutant neurons. These proteins form trans-synaptic complexes with presynaptic LAR-RPTPs molecules, thereby governing neurite outgrowth, neuron survival, and synapse formation. To date SLITRKs have been linked to neurodevelopmental disorders, while their possible role in Parkinson's disease has not yet been determined. In this study, we use two protocols of hiPSC-derived dopaminergic neuronal differentiation, with the first resulting a culture system that contains glutamatergic, GABAergic and DA neurons and a second that yields highly enriched DA neurons and lacks the other two main subpopulations. This approach allows to dissect the neuronal networks that are negatively affected by αSyn-mediated trans-synaptic adhesion disturbances and define the synaptic components affected by SLITRK downregulation. Apart from the p.A53T-αSyn mutation, we apply our experimental approach in neurons derived from triplication αSyn patients to determine whether SLITRK dysregulation is also due to excessive production of αSyn. In parallel, we extend our studies in a transgenic mouse model that expresses the human p.A53T-αSyn and shows early signs of synaptopathy. A significant reduction in SLITRKs expression is already apparent from 4-months and is further enhanced at 12-months of age. Finally, subcellular localization of SLITRKs in A53T-αSyn hiPSC-derived and primary mouse hippocampal neurons reveals impairment of SLITRKs trafficking. Overall, our study identifies three novel mediators of αSyn-induced synaptopathy, underscoring a potential link between pathogenic αSyn expression and trans-synaptic adhesion mechanisms.

Funding Source: General Secretariat for Research & Innovation under the action "TAA TAEDR-0535850 - BrainPrecision"; Bodossakis Foundation Scholarship for Research Visitors (to E.A.); H.P.I. Excellence Scholarship -Nostos Foundation (to E.A).

Keywords: αSynuclein, synaptopathy, SLITRKs

4:45 PM – 5:45 PM

132

TGF-BETA AGONIST ENHANCED GLUTAMATERGIC DIFFERENTIATION OF PLURIPOTENT STEM CELLS

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Glutamatergic neurons are the predominant excitatory neurons producing glutamate neurotransmitter, which plays essential roles in cognition, learning, memory, and emotional control, in various parts of the brain, including hippocampus, limbic system, and neocortex. Successful differentiation of glutamatergic neural precursors from embryonic stem cells (ESCs) or human induced pluripotent stem cells (hiPSCs) can provide critical research tools for the study of neurodegenerative diseases including Alzheimer's disease (AD). Transforming growth factor-beta (TGFβ) is known to be a potential physiological regulator of neural stem cell proliferation and neuronal maturation. In this study, we hypothesized that the TGFβ signaling agonist, SRI can induce the differentiation of the glutamatergic precursor cells from ESCs or hiPSCs. We used H9 cells and healthy iPSCs using the glutamatergic differentiation protocol for 18 days. We detected the level of glutamatergic precursor cells and other neuronal populations by immunocytochemistry or real-time

PCR for the specific markers including TUBB3, VGLUT2, GAD1, etc. We found that SRI treatment increased the number of glutamatergic neurons, cell survivals, neuronal activities, mitochondrial activities, and oxygen consumption rates, compared to the vehicle group. These data suggest that TGFβ agonists can be used for the efficient glutamatergic differentiation of pluripotent stem cells and this protocol can be applied for the disease modeling to identify future therapeutic targets of AD, and cell replacement therapy.

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Keywords: human pluripotent stem cells, glutamatergic differentiation, TGFβ signaling

4:45 PM – 5:45 PM

134

STUDYING THE EFFECTS OF ENTEROVIRUS D68 INFECTION ON THE NEUROMUSCULAR JUNCTION AND MOTOR NEURON TOXICITY USING NOVEL CO-CULTURE METHODOLOGIES

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Acute flaccid myelitis (AFM) is a polio-like pediatric neuromuscular condition characterized by muscle weakness and atrophy and is most often caused by enterovirus D68 (EV-D68). EV-D68 remains a relatively understudied virus, and we currently do not understand the mechanisms behind how EV-D68 causes neurotoxicity in motor neurons, which causes AFM. Clinical observations, such as spontaneous recovery and similarity to enterovirus A71 (which rarely causes AFM), suggest that neuronal death is preceded by a period of reversible axonal dysfunction as opposed to longer nascent recovery periods following peripheral nerve Wallerian degeneration. This period of reversible dysfunction also suggests that studying the neuromuscular junction would provide vital insights into the physiological and structural alterations in the early stages of EV-D68-infected motor neurons. Using induced pluripotent stem cell (iPSCs) derived motor neurons and skeletal muscle, microfluidic co-culture devices, and microelectrode arrays, we have developed novel techniques to study the effects of EV-D68 infection on the neuromuscular junction to better mimic in vivo disease. We will further discuss how this model can be utilized to evaluate the function and morphology of axons, the neuromuscular junction, and muscle in the immediate phases after EV-D68 infection.

Keywords: acute flaccid myelitis, enterovirus D68 (EV-D68), neuromuscular junction and motor neurons

4:45 PM – 5:45 PM

136

ELUCIDATING THE IMPACT OF ALPHA SYNUCLEIN ON ASTROCYTIC DYSFUNCTION IN PARKINSON'S DISEASE

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Despite extensive progress, the mechanisms of Parkinson's disease (PD) remain poorly understood, and we lack disease-modifying treatments. Genetic risk factors are increasingly understood to act not only in disease-vulnerable ventral midbrain dopaminergic (mDA) neurons but also in other brain cell types like microglia and astrocytes. Here, we aim to characterize cell-autonomous and non-autonomous disease phenotypes and mechanisms in human induced pluripotent stem cell (iPSC)-derived astrocytes carrying PD-relevant genetic variants. We used a previously described astrocyte PD model (LRRK2 G2019S) as a positive control and chose a genetically simple patient-derived alpha-synuclein (SNCA) triplication gene-dosage model with isogenic controls (S3W series: 4-copy, 2-copy, 0-copy SNCA), and differentiated these into mDa neurons and astrocytes. We recorded calcium dynamics (gCamp8s) in neurons and astrocytes, analyzed protein expression, cell morphology, and SNCA localization, and performed alpha-syn fibril uptake experiments. Additionally, we performed astrocyte-neuron co-culture assays to measure SNCA transfer and astrocyte-induced neurotoxicity. We found that astrocytes derived from S3W iPSC lines had comparable expression of canonical markers of mature astrocytes and response to inflammatory stimuli. We then looked at calcium activity, a functional aspect of astrocyte biology, and found that 4-copy SNCA astrocytes had significantly diminished calcium release from ER stores after physiological activation. In addition, SNCA triplication astrocytes displayed increased number and size of lysosomes and increased colocalization of early endosome marker (Rab7) and SNCA. Finally, co-culture experiments revealed that PD astrocytes are toxic to neurons, reducing their numbers and neurite complexity. We conclude that elevated SNCA expression alone is sufficient to perturb human astrocytes' cell-intrinsic cell biology and function, ultimately resulting in neurotoxicity. Our ongoing work further characterizes these perturbations and the dialog between astrocytes and neurons. Our findings thus far demonstrate the value of the S3W allelic series iPSC-astrocytes as a model for elucidating the role of SCNA in PD and brain development and function.

Funding Source: Funded by Aligning Science Across Parkinson's (ASAP) initiative (ASAP-000472)

Keywords: alpha-synuclein, Parkinson's disease, iPSC-derived brain cells

4:45 PM – 5:45 PM

138

STRATIFICATION OF IDIOPATHIC PARKINSON'S DISEASE USING PHENOTYPIC CHARACTERISTICS FROM PATIENT IPS CELL-DERIVED NEURONS

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Parkinson's disease (PD) is a neurodegenerative disease caused by progressive loss of midbrain dopaminergic neurons. Patients with PD exhibit a highly heterogeneous clinical presentation, with differences in response to medication and underlying pathogenic mechanisms. Therefore, identification of individual pathological features and prediction of treatment response may significantly improve patient outcomes. We previously developed a semi-automated system to detect phenotypic differences in iPS cell-derived neurons associated with familial PD (PARK2 and PARK6) and identified bromocriptine as a promising therapeutic candidate (Yamaguchi et al., Stem Cell Rep. 2020). In this study, we investigated the potential of patient-specific iPSCs to inform personalized therapeutic strategies for idiopathic PD (iPD). We analyzed iPSCs derived from 70 iPD patients with diverse clinical characteristics, including age, sex, disease duration, and symptom severity. Cellular phenotypes such as apoptosis levels, α-synuclein accumulation, and mitochondrial clearance levels were assessed using our previously developed semi-automated system. While no significant group-wide differences were observed compared to controls, we identified remarkable variability in cellular phenotypes among iPD patients. To explore the potential for personalized therapy, we focused on the variability in the efficacy of bromocriptine. We performed a subclassification based on clinical parameters and cellular phenotypes. Interestingly, the neuroprotective effect of bromocriptine was found to correlate with specific serological markers. This finding highlights the potential of integrating patient-specific iPSC-based cellular phenotypes with clinical data to subclassify iPD patients and predict their response to specific therapeutic agents. This approach could pave the way for the development of personalized disease-modifying therapies for PD.

Keywords: Parkinson's disease, disease modeling, patient stratification

4:45 PM – 5:45 PM

140

DEVELOPMENT OF A 3D RETINA ORGAN MODEL FOR DRUG DISCOVERY TO BE SUPPORTED BY IPSC-DERIVED RPE CELLS - ELECTRORETINOGRAM (ERG) UP TO 13 HOURS POST-MORTEM

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Neuroretinal degeneration as Age-related Macular Degeneration (AMD) and Diabetes Retinopathy (DR) are major causes of blindness worldwide and though treatments are available, no regenerative, curative approaches exist. Moreover, diseases rely on costly and frequent intraocular injections. There is thus a high unmet need for innovative regenerative cell therapies to respond to increasing numbers of patients suffering from neuroretinal degeneration. The development of novel therapeutics necessitates, however, drug discovery tools of highest quality and transferability to patients. Cell culture models lack complexity of the multilayered retina and are thus, only limitedly transferable. On the other hand, animal testing lacks transferability due to species differences and raises ethical concerns. Our purpose is the development of a 3D retina model co-cultured with iPSC-derived RPE cells from healthy and diseased donors offering new ways in drug development. Here, we present ERGs of pig retina measured up to 13h post-mortem. Pig eyes were collected from a slaughterhouse ca. 3h postmortem in a cooled, opaque box for immediate dark-adaptation. After retina isolation, samples were illuminated with flashlights from 30 to 30K mcd.s/m2 from 4-10 flashes of 2-10s. ERGs were recorded using the Ocuscience ERG ex vivo adapter at different time points up to 13h postmortem. After ⩾4h of dark adaptation, 16 retina samples derived from 3 eyes were isolated under dim red light and used for ERG recording. Normal A and B waves could be recorded in all 16 retina from 6h up to 13h postmortem without significant differences between time points. At highest intensity, the mean measured A wave amplitude was -68±115mV (-14 and -350mV) and 678±1482mV (32 and 716mV) for the B wave. The mean implicit time for the A wave was 0.47±0.30ms and for the B wave 5.7±0.39ms. Shown ERG measurements prove activity not only of photoreceptor but also of bipolar cells for 0.5d offering functional testing in the development of personalized regenerative cell therapies. Next, neuronal survival will be preserved by co-culture

of the retina with iPSC-derived RPE cells to enable functional analyses for multiple days. Moreover, the use of iPSC-derived RPE cells from patients will significantly advance midterm drug testing in a highly transferable disease model.

Keywords: 3D retina organ model - electroretinogram post-mortem, neuroretinal degeneration diseases, disease modeling - drug discovery tool

4:45 PM – 5:45 PM

144

WHAT CAUSES DIFFERENCES IN THE ONSET OF ALEXANDER DISEASE DEPENDING ON THE SITE OF GFAP MUTATION?

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Alexander disease (AxD) is often caused by single nucleotide mutations in the glial fibrillary acidic protein (GFAP) gene, which is mainly expressed by astrocytes in the central nervous system (CNS). Accumulation of Rosenthal fibers (RFs) in astrocytes is a pathological hallmark of AxD. The disease has been classified into two subtypes based on neurological symptoms and lesion locations in the CNS, with type 1 and type 2 affecting the forebrain and the hindbrain, respectively. The most severe form of AxD occurs in early childhood, and most patients die within a few months. Currently, the only treatment for AxD is symptomatic therapy, so the development of new therapies is urgently needed. In addition, AxD affects a demyelinating disease involving oligodendrocytes, another type of glial cell in the CNS, and is classified as a leukodystrophy. The pathophysiology of AxD is largely unclear. In this study, we attempted to create a pathological model of AxD by inducing astrocytes in symptomatic brain regions, forebrain and hindbrain, using AxD-derived induced pluripotent stem (iPS) cells and isogenic iPS cells by correcting the GFAP mutation site using the CRISPR/Cas9 nickase to determine whether the formation of RFs is region-specific. We used iPS cells from two different AxD patients, each with different GFAP gene mutation sites. As a result, a higher percentage of GFAP-expressing astrocytes derived from AxD patients formed RFs than that of corrected GFAP-expressing cells. Moreover, differences in cell morphology were observed depending on the differentiated brain regions. In the future, we would like to investigate these differences in detail by examining changes in the time course of gene expression during differentiation. Furthermore, to analyze three-dimensional structural abnormalities that are difficult to analyze in vitro and investigate the relationship between astrocytes and perivascular structures, we plan to generate chimeric mice with human glial cells to reproduce and analyze the pathophysiology of AxD in vivo. According to these basic data, we would like to understand the pathogenesis of AxD and develop therapeutic methods.

Keywords: Alexander's disease, astrocyte, rosenthal fibers

4:45 PM – 5:45 PM

146

FAST AND EFFICIENT GENERATION OF HUMAN CORTICAL NGN2 - INDUCIBLE NEURONS IN A 3D SUSPENSION BIOREACTOR

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Human induced pluripotent stem cells (hiPSCs) have emerged as a valuable and versatile tool for research and drug development. To meet the growing demand for high-quality hiPSCs and well-characterised derivatives, the European Bank for Induced Pluripotent Stem Cells (EBiSC) was established in 2014 to provide a centralised platform for standardised cells and workflows. We present a fast and efficient protocol for the differentiation of genetically engineered, doxycycline-inducible neurogenin 2 (iNGN2)-expressing hiPSCs into cortical excitatory neurons using a benchtop 3D cell culture incubator. Briefly, single cell suspensions of hiPSCs were allowed to form aggregates under continuous agitation, followed by the addition of doxycycline to drive neuronal lineage commitment. After two days of transgene induction, the aggregates were enzymatically dissociated and the cells were either cryopreserved or directly replated for terminal maturation. iNGN2 neurons are generated in a 3D environment in high yield and are also available as the hiPSC-derived product EBiSC-NEUR1 in the EBiSC catalogue. In addition to the early expression of classical neuronal markers such as beta-III-tubulin and microtubule-associated protein 2, iNGN2 neurons display forebrain identity and form complex and dense neuritic networks upon replating. As cultures mature, iNGN2 neurons express vesicular glutamate transporters and show robust spontaneous spiking with high synchrony when co-cultured with human primary astrocytes, exhibiting functional properties similar to mature excitatory neurons. In conclusion, we demonstrate the fast and efficient generation of iNGN2 neurons in a 3D environment with promising applications in disease modelling, high-throughput phenotypic drug screening and large-scale toxicity testing.

Funding Source: This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement No 821362. The JU receives support from the European Union's Horizon 2020 RIA programme and EFPIA.

Keywords: NGN2 neurons, 3D environment, EBiSC

4:45 PM – 5:45 PM

148

SULF1 IS REQUIRED FOR SPECIFICATION OF HUMAN MIDBRAIN DOPAMINERGIC CELLS

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Midbrain dopaminergic (mDA) progenitors are central to cell replacement therapy strategies for Parkinson's disease. Like all cells, mDA cells require specific morphogens at the right concentration and time but precisely how the combination of morphogens leads to this cell type is not well understood. Here, we used in vitro human induced pluripotent stem cell (iPSC) induction techniques to control morphogen dose and timing. We conducted an RNA sequencing (RNA-seq) experiment to identify genes that are highly upregulated when iPSCs are induced into mDA progenitors. Our data led to the identification of SULF1, a heparan sulfate 6-O-endosulfatase, as a remarkably accurate marker of mDA progenitor cells, which we confirmed by single-cell RNA-seq analyses from mouse and human. To assess SULF1's function in mDA cells, we generated heterozygous and homozygous SULF1 knockout lines using CRISPR-Cas9 (SULF1+/-; SULF1-/-, respectively) and show that engineered loss of SULF1 leads to a SULF1 dose-dependent loss of FOXA2 and LMX1A, two important mDA progenitor markers. Impairments associated with SULF1-/- could be rescued with 6-O desulfated heparin treatment, suggesting a direct connection between sulfatase activity and the development of human mDA cells. These results demonstrate a requirement for SULF1 in mDA development and suggest it may affect concentration gradients of morphogens by sculpting extracellular receptors that respond to those morphogens.

Keywords: human midbrain dopaminergic progenitors, morphogens, sulfatase

4:45 PM – 5:45 PM

150

MODELING NEURONAL SENESCENCE AND ALZHEIMER'S DISEASE USING NEURONS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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As the human lifespan increases, age-related disorders, including Alzheimer's disease (AD), are emerging as significant public health challenges. Cellular senescence, often considered aging at the cellular level, may play a direct role in age-related disorders. Recent studies have identified senescence-like characteristics in post-mitotic differentiated cells, including neurons, posing a need to decipher the underlying interplay of neuronal senescence and AD. Here, we first employed human induced pluripotent stem cells-derived neurons (hiPSC-iNs) with extended culture time to obtain senescent neurons. We demonstrated that a prolonged culture of hiPSC-iNs period of up to 4 months could give rise to in vitro neuronal senescence characterized by various cellular senescence phenotypes, including elevated levels of P21, senescence-associated β-galactosidase, and senescence-associated secretory phenotypes (SASP). Moreover, global transcriptome profiling showed that these prolonged cultured hiPSC-iNs exhibited gene expression patterns similar to the aging human brain. To further determine whether these senescent hiPSC-iNs presented a more pronounced AD phenotype, we applied hiPSC-iNs with AD risk mutations. We found that prolonged culture duration, as well as familial AD mutations, can give rise to increased beta-amyloid 42/40 ratio and elevated phosphorylated Tau levels. Lastly, treatment of senolytic drugs on these senescent neurons demonstrated reduced senescence and AD phenotypes. Overall, our research provides a model to understand neuronal senescence and its effect on AD development. Moreover, this model can serve as a platform for drug screening targeting AD treatment and discovering beneficial or harmful substances for healthy brains in future studies.

Keywords: iPSC-derived neurons, Alzheimer's disease, neuronal senescence

4:45 PM – 5:45 PM

152

OPTIMIZATION OF THE CRYOPRESERVING LOW-IMMUNOGENIC HUMAN IPSCS-DERIVED DOPAMINERGIC PROGENITORS

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Parkinson's disease stems from the death of dopaminergic neurons in the substantia nigra of the brain, leading to cognitive and motor impairments. While there are medications available, their efficacy is limited, prompting a growing interest in long-term symptom improvement through cellular therapies. In previous literature and research, transplantation of dopamine neuron precursor cells derived from autologous hiPSCs has been proposed as having therapeutic potential. However, whether iPSCs from patient sources remain healthy and function normally after differentiation into dopamine neuron precursor cells post-transplantation is another critical therapeutic consideration. Using low-immunogenicity hiPSCs derived from healthy human sources and differentiated into dopamine neuron precursor cells can mitigate rejection reactions and provide a healthier cell source. Our research and application focus on Li-iPSCs (low-immunogenicity induced pluripotent stem cells), which have undergone genetic editing to eliminate the expression of Human Leukocyte Antigen class I (HLA-I) and β2 microglobulin (B2M), thereby reducing immunogenicity. To meet the future demand for large-scale allogeneic cell transplantation, this study aims to differentiate low-immunogenicity iPSCs into dopamine neuron precursor cells (Li-iPSC-DAP) and develop suitable cryopreservation

methods for these cells, aiming to reduce costs through batch cell production. We freeze and thaw dopamine neuron precursor cells after several weeks to test cell viability and characteristics under different storage times, confirming and optimizing the quality of Li-iPSC-DAP differentiation processes and cell cryopreservation stability. Experimental results demonstrate that our Li-iPSC-DAP differentiation technique produces high-quality dopamine precursor cell phenotypes, expressing biomarkers such as FOXA2, LMX1A, and NESTIN, with more stable results achieved using controlled cell cryopreservation methods compared to traditional freezing methods while maintaining biomarker expression. This study provides a process to assist in bridging quality systems and preclinical trials, ultimately developing into a mass-produced cellular therapy product for clinical treatment of Parkinson's disease.

Keywords: induced pluripotent stem cells, dopaminergic progenitor cells, cryopreservation

4:45 PM – 5:45 PM

154

MACHINE LEARNING REGRESSION MODELS PERFORMANCE EVALUATION FOR PREDICTING CELL VIABILITY IN BIOMATERIALS APPLIED IN 3D NEURAL MODELS MANUFACTURE

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Three-dimensional (3D) models facilitate the pathophysiological elucidation of disorders such as neurodegenerative diseases by mimicking in vitro processes that occur in vivo. However, their production is expensive and time-consuming, and ensuring biomaterial cytocompatibility, among other factors, is crucial. In this context, machine learning can be a valuable tool for predicting these properties and optimizing the overall process. Therefore, this study aims to assess the performance metrics of regression algorithms in predicting cell viability in biomaterials used for manufacturing 3D neural models. The dataset was compiled from indexed research articles employing stem cells and neural phenotypes in the 3D bioprinting process. Three categorical variables (biomaterial type, cell type, and viability analysis method) and five numerical variables (printing temperature and speed, culture time, cell density, and viability). RStudio software was employed to conduct machine learning and implement supervised regression models, including support vector regression (SVR), multilinear regression (MLR), random forest (RF), and gradient boosting (GB). A regression model was selected for prediction based on the highest R-squared and lowest Mean Squared Error (MSE) performance metrics values over k-fold cross-validation training evaluations (k = 2, 5, and 10) compared to other models. Compared to alternative algorithms, MLR exhibited superior performance (MSE = 0.02924359 and R-squared = 0.7692261), especially when contrasted with RF (p-value = 0.5948662). Biomaterial and cell types emerged as variables with the highest correlation values of importance in predicting viability (0.56975328). Experimental validation of the predictive values determined by MLR combining biomaterials and human iPSCs will be conducted. Subsequently, a graphical interface will be developed for users of 3D bioprinting technology to accelerate the development of new 3D in vitro disease models.

Funding Source: São Paulo Research Foundation: 2022/15909-3 and 2018/12605-8; Brazilian National Council for Scientific and Technological Development: 406258/2022-8; Coordination of Superior Level Staff Improvement: Financial Code 001.

Keywords: 3D bioprinting, machine learning, biomaterials

4:45 PM – 5:45 PM

156

SAME CHALLENGE, DIFFERENT SOLUTION: HOW DIFFERENT NEURONS RESPOND TO AUTOPHAGY INHIBITION MAY PREDICT DISEASE VULNERABILITY

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Autophagy is one of the two major protein degradation systems in the cell and basal autophagy levels are important for protein homeostasis (proteostasis). A decline in autophagy may contribute to aging, and aging is the highest risk factor for neurodegenerative diseases. Here we set out to probe the hypothesis that inhibition of autophagy in neurons triggers distinct, neuron subtype-specific - possibly neurodegenerative - phenotypes that reflect the particular vulnerability of those neurons to disease. We first derived excitatory cortical (CTX) and spinal motor neurons (SMNs) from human pluripotent stem cells (hPSCs). Proteomic and transcriptomic analyses were performed upon autophagy inhibition in CTX and SMNs in an engineered hPSC model where autophagy is solely under external control through doxycycline. Strikingly, CTX and SMNs showed distinct responses to autophagy inhibition. SMNs rapidly acquired a senescence-associated degenerative phenotype. In contrast, CTX proved more resilient to autophagy inhibition by selective upregulation of an ER stress-related response. Those differences were reflected in both proteomic and transcriptomic data with SMNs showing a strong enrichment under OFF vs ON condition for proteins associated with neurodegeneration (Alzheimer's, Parkinson's and ALS) and activation of cell death pathways (senescence, p53). The SMN senescence profile was validated by immunoblot of p53 and by b-Gal staining. CTX but not SMNs showed ER-related alterations including a decline in ER tracker red signal, while elevated oxidative stress by MitoSOX was detected in both neuronal subtypes. Finally, we observed that neuronal differentiation was remarkably sensitive to autophagy levels, questioning the view of autophagy as a mere bystander and homeostatic actor in cellular physiology and neural development. In

addition, degradation of the covalently bound ATG5-ATG12 complex was highly protracted under OFF conditions without a complete loss even after months of doxycycline withdrawal in neurons. Therefore, we engineered and validated several independent degron systems for the rapid and complete inhibition of autophagy in human neurons to interrogate the physiological role of autophagy during neuronal development and to abolish residual autophagy activity in mature neurons.

Funding Source: Research Fellowship of the German Research Foundation (DFG) (401380638); NIH Exploratory/Developmental Research Grant Award from the National Institute Of Neurological Disorders And Stroke (R21NS116545)

Keywords: neurodegenerative disorders, autophagy, aging

4:45 PM – 5:45 PM

158

UNRAVELING MITOCHONDRIAL DYNAMICS IN ADULT ADHD: EXPLORING THE EFFECT OF PARK2 COPY NUMBER VARIANTS USING HIPSC-DERIVED CORTICAL NEURONS

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Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent neurodevelopmental disorder, persisting into adulthood in more than 50 % of cases. Inadequate treatment response affects up to 60 % of adults, highlighting the urgent need for effective medications. Copy number variations (CNVs) in the PARK2 gene have been associated with ADHD development, potentially linked to its role in mitochondrial (Mt) quality control. This study aimed to explore the impact of PARK2 CNVs on ADHD pathophysiology and investigate the possibility of Mt function as a therapeutic target. We analyzed Mt function and energy metabolism in ADHD-relevant models derived from human induced pluripotent stem cells (hiPSCs), specifically neural progenitor cells (NPCs) and cortical neurons (CNs). NPCs were successfully generated from individuals with ADHD, including PARK2 CNV carriers and wildtype carriers (Wt), alongside healthy controls (HC). Immunofluorescence (IF) labeling for NPC markers PAX6 and SOX2 ensured high-quality NPC lines, selecting those with > 80 % positive labeling for subsequent Mt analyses and neuronal differentiation. Protein expression of the neuron markers TUBB3 and vGLUT2 validated differentiation into glutamatergic CN cultures, with calcium imaging and multielectrode array (MEA) analysis additionally confirming spontaneous synaptic signaling. Initial Mt analyses revealed increased reactive oxygen species abundance in PARK2 CNV models compared to Wt and HC, implying possible disease mechanisms. Additionally, IF labeling suggested alterations in Mt co-localization with PARK2 in PARK2 CNV carriers relative to Wt and HC, potentially indicating impaired recruitment. Ongoing investigations, including Seahorse flux analysis and continuous MEA measurements, aim to provide additional insights into the effect of PARK2 CNVs on neural activity and Mt alterations. These preliminary findings contribute to our understanding of the molecular processes underlying ADHD and highlight Mt dysfunction as a potential therapeutic target. This study also demonstrates the utility of hiPSC-derived neuronal model systems in unveiling the mechanisms of neurodevelopmental disorders.

Keywords: hiPSC-derived neuronal in vitro models, PARK2 CNVs in adult ADHD, mitochondrial dysfunction

4:45 PM – 5:45 PM

160

EXPLORING THE POTENTIAL OF (POLY)PHENOL METABOLITES IN AN INNOVATIVE BRAIN-ON-CHIP OF PARKINSON'S DISEASE

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(Poly)phenols, abundant in fruits and vegetables, have emerged as pleiotropic compounds against PD. We identified gut-derived polyphenol metabolites (PMs) as abundant in the bloodstream, capable of crossing the blood-brain barrier (BBB), being protective against neurodegeneration and neuroinflammation, particularly in a 3D neuronal system. However, PMs' mechanisms in a comprehensive/translatable model of the full brain for Parkinson's disease (PD) have never been explored. By taking advantage of a new microphysiological system (MPS) of a brain-on-chip, we will disclose PMs' molecular potential against PD. The new two-compartment model developed will employ a dopaminergic insult applied to human brain microvascular endothelial cells (HBMEC), at the blood site, in combination with dopaminergic neurons derived from human neuronal stem cells (LUHMES), human astrocytes (HASTR/ci35) and human microglia (HMC3), at the brain site. PMs will be infused from the blood site and alterations in the different cell types monitored. Optimal conditions regarding cell seeding, extracellular matrix, and shear stress, were tested and implemented, while full system differentiation status unveiled. The response of brain cells to the dopaminergic neurotoxicant in the MPS will be disclosed. Single PMs or PMs mixture will be infused from the blood side, mimicking the real-life nutritionally relevant concentrations observed in humans, and their role in tackling PD-related hallmarks will be uncovered, including benefits against BBB impairment, dopaminergic cell death, oxidative stress, neuroinflammation, cell crosstalk, and alpha-synuclein phosphorylation. All parameters optimized for the MPS will be compared with static conditions. In the end, we hope to provide physiologically relevant insights for the nutritional management of PD by unveiling PMs' physiologic mode of action in an avant-garde cell model of the pathology, bypassing the need for animal experimentation.

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Keywords: (poly)phenol metabolites, Parkinson's disease, microphysiological systems

4:45 PM – 5:45 PM

162

IMMUNOCOMPETENT HUMAN MIDBRAIN ORGANOIDS TO STUDY NEUROINFLAMMATION IN PARKINSON'S DISEASE

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Parkinson's Disease (PD) is a severe neurodegenerative disorder which is characterized by significant loss of dopaminergic neurons in the midbrain. Neuroinflammation is recognized as a major pathological feature underlying neurodegeneration in PD, highlighting possible disease mechanisms associated with microglial dysfunction. However, neuroinflammatory mechanisms involved in PD pathogenesis are not fully understood. There is a pressing need for a human-specific model system that contains both neural and immune components to investigate microglia-mediated neuroinflammation in PD. Human midbrain organoids (hMBO) are a pluripotent stem cell (PSC)-derived 3D cultures that largely mimics the midbrain in terms of cellular composition, organization, and function. Yet, current organoid systems lack immune cells, limiting their application to study neuroinflammation in neurodegenerative diseases. To address this challenge, we developed immunocompetent hMBOs by co-culturing PSC-derived microglia with hMBO under defined conditions. Our results reveal that microglia can efficiently incorporate into hMBOs, be maintained for extended period, express microglia-specific markers and exhibit maturation signatures. In summary, we report a novel approach for the generation of human midbrain organoids containing microglia that could be used as a platform for basic, translational and disease modeling research applications.

Keywords: brain organoids, microglia, neuroinflammation, midbrain

4:45 PM – 5:45 PM

164

ESTABLISHMENT OF HUMAN IPS CELL LINES DERIVED FROM PATIENTS WITH NEUROFIBROMATOSIS 1

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Neurofibromatosis type 1 (NF1) is known to develop malignant peripheral nerve sheath tumor (MPNST) when neurofibroma progresses to plexiform neurofibromas. Mutations in genes such as NF1 are thought to be involved in the malignant transformation, although the mechanism is not fully understood. Recently, we generated a neurofibroma model that progresses to plexiform neurofibromas by establishing and inducing differentiation of human iPS cells derived from NF1 patients. First, peripheral blood mononuclear cells (PBMCs) derived from NF1 patients were isolated and initialised using PBMCs and SRV-iPSC4-Vector, after which colonies were picked up and cultured as single colonies. At the same time, iPS cells were also established from healthy individuals without NF1 mutation as controls. The established iPS cells were confirmed to be pluripotent by fluorescence immunostaining using pluripotency markers (NANOG, OCT4) to confirm marker expression, and flow cytometry showed that almost 100% of the cells were SSEA4 positive. Targeted sequence analysis of the NF1 exon confirmed that the patient's pathological mutation was inherited by the iPS cells, and it was concluded that a disease-specific iPS cell line was successfully established from the patient's PBMCs. In addition, as the differentiation process from iPS cells to Schwann cell precursors called NF1-derived cells is said to be mediated by neural crest cells, we induced differentiation into neural crest cells from healthy and patient-derived iPS cell lines. As a result, increased mRNA expression was observed

by qRT-PCR using neural crest markers (SOX10, P75, TFAP2B) and protein-level expression was also confirmed by fluorescent immunostaining using neural crest markers (AP-2a, SOX10). Furthermore, flow cytometry confirmed that almost all cells were P75 positive. From these results, it was concluded that both healthy and patient-derived iPS cells were successfully induced to differentiate into neural crest cells. This study enables the generation of the new NF1 model that closely resembles the actual pathology by using iPS cells with patient genomic information, and brings us one step closer to understanding the mechanism of malignant transformation of neurofibroma in NF1 patients.

Funding Source: Japan Agency for Medical Research and Development (AMED)

Keywords: neurofibromatosis type 1 (NF1), disease-specific iPS cells, neural crest cells

4:45 PM – 5:45 PM

166

GENERATION OF THREE-DIMENSIONAL NEURAL ORGANOIDS USING DIRECT CELL REPROGRAMMING

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Three-dimensional (3D) neural organoids are valuable tools for disease modelling. However, current protocols are lengthy and have limited applicability to diseases of aging due to the cells undergoing DNA rejuvenation. Direct-to-induced neural precursor (iNP) cell reprogramming converts human fibroblasts into region-specific neural precursor cells and allows the maintenance of epigenetic and aging factors. We propose the use of directly reprogrammed iNPs to generate cortical and striatal region-specific neural organoids will provide an enhanced approach to modelling of neurological diseases of aging. iNPs were directly reprogrammed from human fibroblasts by transient expression of SOX2 and PAX6 using chemically modified mRNA. Organoids were generated by suspending iNPs in ultra-low attachment plates with gentle rotation and timed growth factor exposure to drive differentiation to a cortical or striatal neuronal fate. Differentiation was evaluated using immunocytochemistry (ICC) and quantitative polymerase chain reaction (qPCR) for cortical or striatal markers. Organoids progressively increased in size over 21 days, reaching a uniform size of approximately 1mm diameter. A plate shaker to distribute nutrients was critical to the success of the protocol. Striatal organoids were positive for the striatal lineage markers CTIP2, TUJ1 and DARPP32 at Days 7 and 14 of differentiation, as confirmed by qPCR and ICC. Cortical organoids were positive for the cortical lineage markers TUJ1 and vGLUT1, at Days 7 and 14 of differentiation. We next investigated the ability to generate striatal organoids from fibroblasts derived from donors with adult-onset Huntington's disease (HD). We produced HD-iNP-derived striatal organoids co-expressing DARPP32 with TUJ1. By Day 7 of differentiation, the HD organoids expressed mutant huntingtin protein. This study demonstrates for the first time the ability to generate organoids from directly reprogrammed iNPs from healthy and HD donors, with region specific neuronal differentiation by Day 7 of culture. The reduced time required to generate organoids from iNPs, and the ability for direct-to-iNP reprogramming to maintain epigenetic and aging signatures advances current organoid methods and will facilitate future modelling of neurological diseases of aging.

Funding Source: Neurological Foundation, Hudson-Nilon Trust

Keywords: direct cell reprogramming, organoids, Huntington's disease

4:45 PM – 5:45 PM

168

DISSECTING GLIOBLASTOMA INVASION USING 3D BRAIN ORGANOIDS

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Glioblastoma multiforme (GBM) is a highly aggressive brain tumor that has a poor prognosis due to tumor recurrence and lack of effective treatment options. In order to address this challenge, we developed a 3D in vitro model of GBM that incorporates a co-culture of human iPSC-derived brain organoids with patient-derived GBM cells and its tumor microenvironment components. To establish this model, we genetically engineered patient derived GBM cells with a secreted luciferase construct bound to an RFP reporter to monitor live cell survival following different therapeutic treatments. The cells were then co-cultured with iPSC-derived brain organoids, and over a period of 15-40 days, both cell lines invaded and grew extensively into the organoid. Subsequently, the 3D Glio-organoids were treated with the standard of care therapeutic agent for glioblastoma, TMZ at different dosages as well as with new therapeutic drugs targeting RNA modifying enzymes and compared to traditional 2D growth cell cultures. Our results demonstrate that our co-culture model is optimal for evaluating the survival rate of the glioblastoma cells after being exposure to different therapeutic agents as well as for genetically dissect the invasive mechanisms of the different glioblastoma patient cells within the healthy brain organoid. Overall, our 3D in vitro model of GBM provides an innovative approach to improve preclinical models for glioblastoma, giving in vitro human alternative platforms and thereby reducing the number of experimental animals.

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Keywords: brain organoids, glioblastoma, 3D co-culture system

4:45 PM – 5:45 PM

170

EXPLORING AUTISM RISK GENES THROUGH HIGH-CONTENT CRISPRI SCREENS IN HUMAN IPSC-DERIVED NEURAL CELL VILLAGES

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Autism spectrum disorder (ASD) is a complex genetic condition associated with over one hundred known genes linked to ASD risk. While some genes have defined roles, how most other genes drive disease risk remains elusive, necessitating novel approaches for risk variant assessment on a larger and more informative scale. In this work, we establish a high-content screen using CRISPR gene editing and cell villages of human induced pluripotent stem cell (iPSC)-derived neural cells to identify gene expression patterns in ASD. Cell lines from different donors are mixed into a 'cell village,' which allows for the screening of tens to hundreds of genetic backgrounds at the same time. To this extent, we created an iPSC village transduced with a pooled CRISPRi library targeting ASD risk genes, differentiated this village into neural progenitor cells, neurons, and astrocytes, and performed single-cell RNA-sequencing (scRNA-seq) for gene expression analysis. The results aim to uncover the cellular phenotypes affected by perturbing ASD risk genes (perturbed variants). To extend our risk variant analysis, we explore a subset of naturally occurring common and rare loss-of-function variants found in ASD patients (natural variants). A case-control cohort of 120 individuals, comprising 40 ASD case lines with rare variants, 40 ASD case lines with common variants, and 40 healthy control lines, were pooled into a cell village and characterized by scRNA-seq. This data will be used to determine which perturbed variant phenotypes persist in natural patient genetic backgrounds and will help distinguish gene-related effects from genetic background influences. These results will then be integrated with complementary morphological and functional datasets. Taken together, this work will provide insight into the neurobiological roles of ASD risk genes and allow for the clustering of variants into common mechanisms of action while considering human genetic variation.

Keywords: CRISPR screening, cell villages, autism spectrum disorder

4:45 PM – 5:45 PM

172

TARGETED AND PROGRAMMABLE RNA EDITING FOR THE CORRECTION OF LRRK2 G2019S MUTATION: A PRECISION MEDICINE APPROACH TO PARKINSONS DISEASE

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Parkinson's disease (PD) is a debilitating neurodegenerative disorder causing severe motor, and non-motor dysfunction, and cognitive impairments, affecting more than 10 million people worldwide. The LRRK2 p.G2019S (c.6055G>A) mutation, identified as the most common single-point genetic determinant of PD, results in the hyperactivation of leucine-rich repeat kinase-2 (LRRK2), leading to the clinical manifestations and neuropathology associated with the disease. Current treatments for PD are symptomatic, and to date, there are no approved therapies proven to cure, halt, or stop disease progression. In this study, we utilized a targeted RNA editing technique that harnesses endogenous adenosine- deaminase-acting-on-RNA (ADAR) enzymes to repair LRRK2 p.G2019S mutation at the mRNA level. ADAR enzymes, which are naturally abundant, edit adenosine to inosine in double-stranded RNA, a change that is interpreted as guanosine by cellular machinery. Unlike current kinase inhibitors that have severe side effects affecting the lungs and kidneys, ADAR-mediated correction increases the normal function of the LRRK2 kinase. In a high-throughput workflow, we screened a library of 10^5 gRNA sequences to target and edit the G-to-A point mutation and identified a leading ADAR-recruiting antisense guide RNA (ASOV6) that we tested both in human iPSC-derived neurons and a humanized LRRK2 mouse model. By incorporating ASOV6 into an adeno-associated virus 9 (AAV9) and transducing it in human iPSC-derived neuronal cultures from a patient homozygous for LRRK2 p.G2019S, we detected a more than 40% editing of the LRRK2 mutation in FACS-sorted neurons. Furthermore, we conducted bilateral stereotactic injections of this AAV9 into a humanized LRRK2 mouse model and harvested the brains at 1 and 3-month time points. We will measure the RNA editing level through sequencing and in situ hybridization (Duplex Basescope). We will also measure phosphorylated substrates of LRRK2 kinase activity (total LRRK2, pLRRK2, Rab10, pRab10) in both iPSC-derived neurons and in mouse brain tissue. The efficient and precise correction of the pathogenic LRRK2 p.G2019S mutation into wild-type LRRK2, thus regaining its normal function, achieved in this way promises to be a breakthrough in precision genetic medicine for Parkinson's disease.

Funding Source: Stanford Wu Tsai Neuroscience: Translate Award Program, The Michael J. Fox Foundation

Keywords: ADAR: RNA editing, LRRK2 in Parkinson's disease, in situ hybridization: duplex basescope

4:45 PM – 5:45 PM

174

INVESTIGATING THE FUNCTIONAL RESPONSE OF NOVEL HIPSC-DERIVED HYPOTHALAMIC NEURONS TO PHYSIOLOGICALLY RELEVANT STIMULI

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The Arcuate Nucleus of the Ventral Hypothalamus (ARC) is at the epicenter of CNS response to peripheral circulating levels of metabolic signals via two major neuronal subtypes: agouti-related protein (AgRP) and pro-opiomelanocortin (POMC) expressing neurons. These neurons work in opposition to inhibit or elicit satiety signals respectively. A greater understanding of how these neuronal subtypes in the ARC orchestrate satiety signals and energy expenditure could open new avenues for medically alleviating obesity and related diseases such as type 2 diabetes, hyperlipidemia, high blood pressure, and cardiovascular disease. Among the challenges in studying these neurons for drug development is the lack of translationally relevant models of the hypothalamus. Our team recently developed a novel, robust and scalable, differentiation approach to generate cultures of ARC neurons, with a predominant expression of pro-opiomelanocortin (POMC), tyrosine hydroxylase (TH) and somatostatin (SST). Here we report our recent findings, encompassing time-course transcriptomic data, mid-throughput-multi-electrode and patch-clamp electrophysiology analyses, as well as protein expression data. Our results demonstrate these cultures express ARC specific markers robustly across cell lines and dynamically respond to physiologically relevant hormonal stimuli, including insulin, circulating nutrients such as glucose, neuropeptides like kisspeptin, and FDA-approved anti-diabetic drugs known as incretin mimetics. These results also suggest plasticity in POMC and insulin receptor expression in the neurons of the ARC. Our findings demonstrate that this hiPSC-derived ARC model is a suitable and translationally relevant platform to investigate metabolic and reproductive disorders associated with the hypothalamus. Additionally, this model serves as a valuable resource for conducting safety and efficacy study of preclinical drug candidates. Together, our results suggest that our hiPSC-derived ARC model can be used to establish high-throughput assays for drug screening and therapeutic advancement.

Funding Source: This work was funded by the Regenerative Medicine Program (RMP) of the NIH Common Fund and in part by the Intramural/Extramural research program of the NCATS, NIH.

Keywords: hypothalamus, obesity, neuroendocrine

4:45 PM – 5:45 PM

176

DISSECTING THE NEUROTROPISM OF MPOX VIRUS USING HUMAN STEM CELL-BASED MODELS

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Mpox, formerly known as monkeypox, is a zoonotic illness of international concern that can lead to severe disease including neurological sequelae. In the majority of severe cases, patients present with neuropsychiatric complications including seizures, confusion, and encephalitis, indicating that MPXV can invade the central nervous system (CNS). However, it remains unclear what the neurotropism of monkeypox virus (MPXV) is and how MPXV infection leads to neurological deficits. Here, we determined the neurotropism and neurovirulence of MPXV using human pluripotent stem cell- (hPSC)-derived neural stem cells, astrocytes, cortical neurons, and microglia together with ex vivo human brain tissue. We found that MPXV infects and replicates more efficiently in astrocytes and microglia compared to cortical neurons, which unlike glial cells showed activation of distinct antiviral programs that may confer differential susceptibility to MPXV. Ex vivo infection of human brain tissue confirmed the susceptibility of astrocytes to MPXV infection, which also had the strongest disease-associated changes. Molecular pathway analyses revealed induction of cellular senescence and a senescence-associated secretory phenotype upon MPXV infection in astrocytes. Finally, we demonstrated that antiviral treatment using tecovirimat inhibits MPXV replication and prevents virus-induced senescence in hPSC-derived astrocytes. Altogether, leveraging hPSC-derived brain cells, we reveal MPXV-induced cell type-specific effects at

the molecular and cellular level, which provide important insights into the neuropathogenesis of MPXV infection. Furthermore, we provide evidence for the therapeutic potential of tecovirimat treatment to target both MPXV replication in the CNS and MPXV-induced neuropathology.

Keywords: virology, encephalitis, neuroimunnology

4:45 PM – 5:45 PM

178

ENVIRONMENTAL FACTORS IN GENETIC COMPENSATION - PHENOTYPIC VARIABILITY IN ACTB LOSS-OF-FUNCTION MUTATION ASSOCIATED DISEASES

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Beta-actin (ACTB) heterozygous loss-of-function (LOF) mutations are associated with pleiotropic developmental disorders (DD) entailing intellectual disability and frequent organ malformations in affected individuals. Mutations in the ACTB gene locus that lead to haploinsufficiency range from small- nonsense and frameshift to large-scale mutations and induce a characteristic phenotype with considerable variations in expressivity and disease severity between affected individuals. These phenotypic variations may be attributed to genetic compensation, which can be directly or indirectly influenced by environmental factors. We generated two CRISPR/Cas9-edited human induced pluripotent stem cell (iPSC) lines carrying heterozygous and homozygous indel mutations in ACTB exon 4 that exhibit characteristic stem-cell like features. Both mutants display an upregulation in modifier gene expression and respective decrease in ACTB transcript and protein levels. To unravel the tissue-specific impact of dominant genes, we generated human cortical brain organoids that recapitulate human fetal brain development. While heterozygous mutants exhibit microencephaly and a reduced capacity to generate ventricular zones, as well as fewer neuronal induction markers compared to wildtype counterparts, mutants with a homozygous indel experience severe impairments that lead to disintegration of spheroids after forty days. The developmental delay observed in ACTB heterozygous organoids is consistent with symptoms of cognitive impairments characteristic for patients suffering from ACTB haploinsufficiency. Notably, up to our knowledge, no patient case exists that expresses a full ACTB knockout, confirming the lethality of a homozygous LOF that is represented by the developmental termination observed in the brain organoids. These mutants provide a powerful model to examine the molecular and functional consequences of ACTB haploinsufficiency and LOF, develop effective treatments, and explore the complex interplay of genetic compensation and environmental factors on phenotypic variability observed in monogenic DDs. Finally, this exploration aids in elucidating mechanisms that alleviate and intervene with disease progression in the early developmental stages.

Keywords: developmental disorder, genetic compensation, phenotypic variability

4:45 PM – 5:45 PM

180

IPSC-BASED DISEASE MODELING FOR MONOGENIC AND POLYGENIC MACULOPATHIES LED TO THE DISCOVERY OF METFORMIN AS A POTENTIAL TREATMENT

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Retinal pigment epithelium (RPE) is a monolayer of pigmented cells supporting photoreceptor (PR) and choroidal capillaries in retina maintaining proper vision. Maculaopathies like Stargardt disease (STGD1, a mutation in ABCA4) and age-related macular degeneration (AMD, a polygenic) share RPE phenotypes, which include sub/intra cellular lipid-rich deposits, cellular dysfunction and eventual atrophy leading to PR death and vision loss. Recently we demonstrated hallmark cellular features of these diseases in vitro using patient-specific induced pluripotent stem cell-derived RPE (iRPE). The bulk RNA-seq revealed common pathways affected were lysosomal activity, autophagy and fatty acid oxidation indicating disrupted metabolism as a common factor. Therefore, we aim to test the efficacy of metformin, a senolytic drug known to improve autophagy, fatty acid oxidation and lysosomal pH in iRPE in vitro and Abca4-/- mice in vivo focusing on key disease feature like sub-RPE lipid deposits. AMD and STGD1 iRPE challenged with physiological stressors with or without metformin (3mM) were analyzed for functional readouts and transcriptomics analysis. Abca4-/- mice given oral metformin (400 mg/kg/day) for 8 months, comparable to the human dose were used for tracking lipofuscin accumulation by fundus autofluorescence imaging and lipid analysis in retina and RPE/choroid. Metformin reduced sub/intra cellular lipid deposits in AMD and STGD1 iRPE (BODIPY staining and LipidUNet analysis). Treated iRPE had higher junctional integrity and the ability to process PR outer segments. In mice, metformin reaches to the retina and RPE layers (MS) and treated eyes showed reduced lipofuscin accumulation by fundus imaging and significantly reduced retinal metabolites, A2E by LC-MS/MS. scRNA-seq of mice RPE and bulk RNAseq of iRPE indicated the mechanism of action primarily through fatty acid oxidation and lysosomal pathways. Our findings confirm the potentials of metformin as an effective treatment for STGD1 and AMD, working through RPE lysosomal and fatty acid oxidation pathways both in vitro and in vivo model. To develop therapeutic delivery form for human ADM and STGD1, we plan to test eyedrop formulation of metformin in rabbit model. This work underscores the utility of iPS cell technology for disease modeling and drug discovery.

Keywords: Stargardt disease, age-related macular degeneration, metformin

4:45 PM – 5:45 PM

182

GENERATION OF A FULLY HUMAN PLURIPOTENT STEM CELL-DERIVED MODEL OF BLOOD-BRAIN BARRIER FOR DISEASE MODELING

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The blood–brain barrier (BBB) is the highly selective interface between the systemic circulation and the neural parenchyma which is required for brain homeostasis. We established detailed procedures to differentiate human pluripotent-stem cells (hPSCs) into endothelial, mural and astroglial cells by combining an original combination of small molecule cocktails with the expression of lineage-specific transcription factors. To assemble the three cell types all together we decided to generate assembloids that recapitulate the spatial organization of the BBB either on transwell filters or as free-floating aggregates. We demonstrated that BBBOs display a tight and homogeneous endothelial cell layer with well-organized cell junctions. BBB functionality has been validated showing high values of trans-epithelial electric resistance and impermeability to dyes with high molecular mass. Furthermore, we tested the BBB integrity by testing the differential crossing of two types of viruses: adeno-associated viruses (AAVs) and lentivirus (LVs). The BBBOs effectively blocked the crossing of LVs and most AAVs. In contrast, the AAV-PHP.B was able to efficiently transverse the BBBOs when overexpressing its transcytosis receptor Ly6A. These results demonstrate that BBBOs have acquired a highly selective permeability producing a robust in vitro neurovascular model with long-lasting stability and functions. We used BBBOs to model autoimmune encephalitis caused by anti-NMDAR antibodies developed by patients. BBBOs are able to efficiently block the passage of antibodies confirming the selective impermeability of these structures. However, inflammation stimuli triggered by a combination of pro-inflammatory cytokines lead to a selective reduction in the BBBO impermeability promoting the pronounced crossing of anti-NMDAR antibodies. These data promise to reveal the biological causes leading to BBB disruption and unleashing the progression of autoimmune encephalitis.

Keywords: blood-brain-barrier, differentiation of human pluripotent-stem cells (hPSCs) into endothelial, mural and astroglial cells, autoimmune encephalitis

4:45 PM – 5:45 PM

184

SENSORY NEURONS DERIVED FROM ALS PATIENT DERIVED IPSCS CARRYING C9ORF72 MUTATION DISPLAY MOLECULAR PATHOLOGIES IN VITRO

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Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by motor neuron degeneration. While extensive research has focused on the central nervous system aspects of the disease, there is limited understanding of the involvement of other cell types within the motor neuron microenvironment. In recent years, clinical observations and animal studies have implied the degeneration of sensory neurons (SN) associated with ALS. However, understanding the role and contribution of SN to the disease pathology is lacking in human culture models. Our study aimed to fill this gap by exploring the molecular impacts of the ALS-causing C9ORF72 mutation on human induced pluripotent stem cell (iPSC)-derived SN. We characterized the SN derived from healthy, ALS-patient, and isogenic human iPSC lines at the molecular and functional levels by gene and protein expression of SN markers, Ca2+ imaging, and pharmacological stimulation. Next, we analyzed the hallmark pathologies of ALS, including endoplasmic reticulum (ER) stress, mitochondrial function, DNA damage response, TDP43 protein mislocalization, and oxidative stress in SN cultures by using qRT-PCR, immunostaining, and CellROX assays. SNs were characterized by gene and protein expression analysis of general neural and SN markers, Tuj1, Peripherin, and Vglut2. Functional characterization of SN cultures by Ca2+ imaging demonstrated similar pharmacological responses to different chemical stimulants, ATP, Menthol, and Capsaicin, independent of the genetic background. Gene expression analysis revealed that mRNA levels of ER stress-related CHOP1 and XBP1 genes were significantly increased, along with a decreased expression of mitochondrial function related ATP5A1 and SIRT3 genes in ALS-SNs compared to healthy controls. Moreover, the nuclear to cytoplasmic ratio of TDP43 protein showed mislocalization in ALS-SN by immunostaining. Also, a significant increase in DNA damage-associated pATM protein levels and oxidative stress levels were detected in ALS-SN compared to healthy and isogenic controls. Our findings revealed hallmark cellular pathologies associated with ALS in human SN carrying C9ORF72 mutations. We provide the first in vitro evidence for the possible contribution of SN defects in C9ORF72 associated ALS pathologies.

Funding Source: This study was supported by European Union Joint Programme Neurodegenerative Disease (JPND) Research grant

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Keywords: Amyotrophic Lateral Sclerosis (ALS), C9ORF72, iPSCs-derived sensory neurons

4:45 PM – 5:45 PM

186

SURFACE CONJUGATED EXTRACELLULAR VESICLES: APPLYING THE NEXT GENERATION THERAPEUTICS OF TRAUMATIC OPTIC NEUROPATHY

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Stem cell therapy has been widely proposed as an alternative treatment for refractory optic nerve disease. Although mesenchymal stem cells with various tissue regeneration and recovery capabilities are considered as potential cell therapy, they have limitations in the clinical setting. To overcome this problem, we investigated the applicability of extracellular vesicles (EVs), isolated from human placenta-derived mesenchymal stem cells (hPMSCs) as a new treatment. To improve nerve recovery function, we generated EVs had surface conjugated with peptide X. R28 cells, retinal precursor cells, were exposed to CoCl2, and then they were treated with EVs for 24 h. In order to examine the regulation of target protein and signal pathway during recovery process by EVs in optic nerve disease models, we performed immunoblot assay and single cell RNA sequencing using in vitro and in vivo optic nerve disease models. We observed that EVs could restore abnormal regulation of neuroregeration markers in damaged models. The surface-conjugated EVs (SC_EVs) have been more effective in moderating neuroregeneration and hypoxic-induced damages. Moreover, single cell RNA-seqeunce analysis using R28 cells revealed that transcription of genes involved in RGC regeneration, neuroregeration, angiogenesis were considerably regulated by SC_EVs. In addition, we have identified the signal pathway mediated during recovery process by SC_EVs. Taken together, we could suggest that SC_EVs were more effective than naïve EVs for optic nerve repair. Therefore, we expect that SC_EVs could be a viable solution as an alternative therapy for treatment of optic nerve injury.

Funding Source: These studies are supported by the National Research Foundation of Korea (NRF) (grant: 2021R1A2C2010523).

Keywords: traumatic optic neuropathy, extracellular vesicles, neuroregeration

4:45 PM – 5:45 PM

188

SEEING BEYOND THE EYE: UNRAVELING RETINOBLASTOMA'S SECRETS WITH RETINAL ORGANOIDS

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Retinoblastoma is a rare and aggressive tumor of the developing retina that initiates in utero with the biallelic inactivation of the tumor suppressor gene RB1. This disease poses unique challenges to researchers and clinicians due to its early onset and complexity. Unlike most solid tumors, retinoblastoma is not diagnosed via biopsy. Instead, tumor tissue is only available upon enucleation when advanced disease requires removal of the eye. This limitation has impeded research progress into the origins and therapeutic vulnerabilities of the disease. While significant strides have been made in understanding the molecular mechanisms underlying retinoblastoma using cell lines and animal models, there remains a critical need for more accurate and clinically relevant systems to study the disease. In this study, we leverage retinal organoids derived from human pluripotent stem cells to model retinoblastoma in a physiologically relevant context. Toward this goal, our lab generated retinal organoids from RB1-null stem cells. By introducing RB1 loss and other genetic alterations associated with retinoblastoma into retinal organoids, we can recapitulate key aspects of tumor initiation and progression observed in patients. Our lab was the first to generate spontaneous retinoblastoma from retinal organoids and demonstrate that they are indistinguishable from human retinoblastomas via molecular, cellular, and genomic analyses. Here we expand on these findings and characterize the development and tumorigenicity of RB1-null retinal organoids in vitro and in vivo. Early-stage RB1-null retinal organoids develop normally and efficiently form spontaneous tumors when dissociated and introduced into the vitreous of athymic nude mice. Furthermore, gene expression profiles suggest that RB1-null organoids undergo malignant transformation in vitro. Our findings demonstrate the ability to generate patient-specific retinal organoids that allow for personalized modeling of retinoblastoma, offering opportunities for precision medicine approaches. Overall, our study highlights the potential of retinal organoids as a powerful tool for modeling retinoblastoma and advancing our understanding of this devastating childhood cancer.

Keywords: pediatric cancer modeling, tumorigenesis, retinal organoid derived retinoblastoma

4:45 PM – 5:45 PM

190

MODELING OF SPINAL MUSCULAR ATROPHY WITH PATIENT-SPECIFIC NEUROMUSCULAR ORGANOIDS

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Spinal muscular atrophy (SMA) is a neuromuscular disease that affects the integrity of neuromuscular junctions (NMJs), leading to motor impairments, muscular atrophy, and death in the most severe cases. It is caused by a homozygous deletion of the Survival Motor Neuron 1 (SMN1) gene and retention of its paralogue SMN2 gene. The severity of the disease is inversely correlated with the copy number of SMN2 (type I, type II, and type III). Although three FDA-approved drugs have shown promising outcomes by restoring global levels of SMN proteins, they are not curative. These unmet needs highlight the importance of understanding the disease onset and progression in a patient-specific manner and identifying non-SMN-dependent therapeutics to enhance treatment efficacy. To address this issue, we have successfully generated neuromuscular organoids (NMOs) from types I, II, and III patients' urine-derived induced pluripotent stem cells for modeling neuromuscular defects. Healthy individuals' urine cells served as wild-type (WT) control for comparison. By day 10 of culture, NMOs generated from SMA patients are relatively smaller in size with irregular shape compared with the characteristic oval morphology of a WT organoid in which the neural and muscle regions can be easily distinguished. By day 30, skeletal muscle fibers expressing myosin skeletal fast (Fast MyHC) were localized in a well-defined region of the WT organoids, where TUJ1+ neurites innervated the muscle region to form NMJs marked by alpha-bungarotoxin (BTX). Type II and III NMOs expressed a small portion of MyHC+ muscle fibers innervated by a low amount of TUJ1+ axonal tracts and BTX, whereas expression of these markers was low in type I NMO. Consistently, we observed a more robust contractile activity of WT NMOs than SMA NMOs, indicating defective NMJs in SMA NMOs. At day 50, expression of MN marker ISL1/2, TUJ1, Schwann cell marker MBP, and astrocyte marker (GFAP) were reduced in type II SMA NMOs compared to WT NMOs, demonstrating defects in neural, skeletal muscle, and glial lineage development in correlation with disease severity. These results highlight the potential of patient-specific NMOs for personalized SMA disease modeling, outcome prediction, and therapeutic development to enhance treatment efficacy for SMA patients.

Funding Source: This work was supported by grants from the Research Grants Council of Hong Kong (GRF_17114619, GRF_17102420, R7018-23), URC Seed Fund for Collaborative Research, and Liu Po Shan/Dr Vincent Liu Endowment Fund for Motor Neurone Disease.

Keywords: neuromuscular organoids, spinal muscular atrophy, neuromuscular junction

4:45 PM – 5:45 PM

194

ESTABLISHMENT OF THE IN VITRO MODEL OF CHARCOT-MARIE-TOOTH DISEASE USING HIPSCS

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Charcot-Marie-Tooth disease (CMT) is a hereditary peripheral neuropathy affecting both motor and sensory neurons. Mutations of the PMP22 gene are considered as the cause of demyelination, yet the molecular mechanisms remain unclear. This study aims to establish an in vitro demyelination model using patient-derived iPSCs to elucidate the pathogenesis of CMT and develop therapeutic approaches. from The iPSCs were established from T cells from a patient with CMT harboring a duplication of the PMP22 gene (CMT1A) by transducing Yamanaka factors (Klf4, Oct3/4, Sox2, c-Myc) using Sendai virus vectors. The established iPSCs showed a normal karyotype and the PMP22 gene duplication was conserved. Pluripotency was verified through immunofluorescence and in vitro differentiation assays. To gain insight into the CMT pathophysiology, CMT1-iPSCs were differentiated into Schwann cells. Both Schwann cell precursors (SCPs) and Schwann cells (SCs) showed corresponding morphologies. qPCR and immunochemical staining confirm the expression of Schwann cell precursor markers (SOX10, NGFR, GAP43) and mature Schwann cell markers (MPZ, GAP43, S100β). Notably, an elevation of PMP22 expression in patient-derived SCPs and SCs was observed. In this study, we successfully established a stable and expandable source of CMT1A iPSCs, Schwann cell precursors, and Schwann cells, which is expected to support further study on the elucidation of pathology and drug screening.

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Keywords: Schwann cell, Charcot-Marie-Tooth disease, peripheral neuropathy

4:45 PM – 5:45 PM

196

HYPO-EXCITABILITY IN STEM CELL-DERIVED CORTICAL GABAERGIC INTERNEURONS IN SGCE MUTATION-POSITIVE MYOCLONUS DYSTONIA

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Myoclonus Dystonia is a dominantly inherited form of dystonia caused by mutations in SGCE gene, encoding the ε-sarcoglycan protein. Work to date suggests that dystonia is caused by the disruption to the basal ganglia-cerebello-thalamo-cortical neuronal networks, with deficits in striatal medium spiny neurons and cortical glutamatergic neurons having been described in human induced pluripotent stem cell (iPSC)-derived models. Although cortical GABAergic neuronal involvement has been implicated in human physiological studies, no studies to date have derived in vitro cellular models of cortical GABAergic interneurons to study Myoclonus Dystonia. Here, we have differentiated medial ganglionic eminence (MGE)-derived cortical GABAergic interneurons from an SGCE missense mutation (Exon 5 c.622G > A, p.Gly221Asp) positive patient-derived iPSCs and their matched wild-type SGCE control lines. We found that no significant differences in the expression of either early MGE progenitor (NKX2.1, FOXG1, OTX2) or late GABAergic interneuron (GAD67, SST, CB) markers. However, SGCE mutation-carrying interneurons exhibited less complex neurite branching than control neurons. Whole-cell patch clamp characterization identified that compared to control neurons, SGCE mutation-carrying interneurons exhibited significantly lower amplitude and maximum rise slope of evoked-action potential, less repetitive firing, and fewer spontaneous excitatory and inhibitory postsynaptic currents. Moreover, SGCE mutation-harboring interneurons were significantly less active in Axion Maestro Pro multielectrode array assay than control neurons and less responsive to L-glutamate in high-throughput intracellular Ca2+ assay. Finally, deep single-cell transcriptomic analysis identified a network of genes and gene ontology pathways potentially underlying the cellular deficits observed in mutant interneurons caused by SGCE missense mutation. Overall, our study demonstrates a decreased morphological complexity and excitability of MGE-derived cortical GABAergic interneurons in the context of SGCE mutation. These changes potentially represent key components of the neuronal network deficit underlying the hyperkinetic clinical phenotypes for both Myoclonus Dystonia and the wider spectrum of dystonic disorders.

Funding Source: Medical Research Council Clinician-Scientist Fellowship MR/V036084/1

Keywords: dystonia, cortical GABAergic interneurons, hypoexcitability

4:45 PM – 5:45 PM

198

UNRAVELING THE ROLE OF EYS GENE IN RETINITIS PIGMENTOSA TYPE 25 PROGRESSION USING RETINAL ORGANOIDS

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Retinitis pigmentosa type 25 (RP25), caused by mutations in the EYS gene, is one of the most common forms of autosomal recessive RP across various ethnicities. Despite its prevalence, multiple obstacles have hampered therapeutic developments, including the large size of the EYS gene, lack of understanding of its function, and absence of it in standard animal models (mouse, rat, guinea pig). To better understand EYS function, we developed an in vitro disease model for RP25 using patient-derived pluripotent stem cells (iPSC) and retinal organoids (RO). We used iPSCs from three controls and two patients diagnosed with RP25 carrying mutations in the EYS gene: i) Patient carrying c.3567delA, p.G1190Dfs*3 in exon 23 and c.4829-4832delCATT, p.S1610Ffs*7 in exon 26; and ii) patient carrying a homozygous mutation c.8897G>A, p.G2945E in exon 43. The hiPSC lines had normal karyotypes, expressed pluripotency surface markers and pluripotency genes, and successfully differentiated into the three germ layers. RO were produced and characterized at various maturation stages. At the early stages of retinal differentiation, RP25 lines had disorganized neuroectoderm and surface ectoderm, with lower expression of key ocular genes (PAX6, SIX6, CHX10, RAX) and higher expression of RPE genes (TYR). This disorganization persisted through RO formation and maturation. RP25-derived RO showed decreased retinal thickness and RO formation, disorganization of bipolar and photoreceptor cells, increased pigmentation, and immature photoreceptors with aberrant outer segment formation. Furthermore, different dynamics have been observed during calcium imaging of retinal neural cells in RP25 and control RO. An investigation is underway to examine and determine variations in their functional patterns. This RO model was shown to be effective in reproducing distinct differences between RP25 and controls at both structural and functional levels, and these differences were consistent across multiple lines carrying different EYS gene mutations. Our findings shed light on EYS' complex role in RP25 disease, providing valuable insights for potential therapeutic strategies

Keywords: retinitis pigmentosa, retinal organoids, EYS gene

4:45 PM – 5:45 PM

200

INTEGRATIVE TRANSCRIPTOMICS/ METABOLOMICS ANALYSES OF A SCHIZOPHRENIA IPSC MODEL REVEAL GABA DYSREGULATION

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Schizophrenia (SCZ) is a neuropsychiatric disorder, caused by a combination of genetic and environmental factors. The etiology behind the disorder remains elusive and is hypothesized to be associated with the dysregulated response to neurotransmitters, such as dopamine and glutamate. However, a putative link between dysregulated metabolites and distorted neurodevelopment has not been assessed yet. In this study, we aim to investigate a presumed correlation between the transcriptome and metabolome in a SCZ model, employing patient-derived induced pluripotent stem cells (iPSCs). For this purpose, iPSCs were differentiated towards cortical neurons and samples were collected longitudinally at defined developmental stages, such as neuroepithelium, radial glia, and young and mature neuronal stages. The samples were subsequently analyzed by bulk RNA-sequencing and targeted metabolomics and the two modalities were used to construct integrative networks in silico. The network analysis revealed a distortion of the gamma-aminobutyric acid (GABA) neurotransmitter levels in the SCZ lines during the rosette maturation stage. Further investigation unveiled a downregulation in the main GABA production pathway, through the decarboxylation of glutamate from GAD1/2. Furthermore, we observed an upregulation of the alternative GABA biosynthetic pathway through putrescine in SCZ, indicative of a compensatory mechanism. In summary, our study establishes a novel in silico approach of correlating in vitro metabolic and transcriptomic data and secondly reveals dysregulated GABA biosynthetic pathways in SCZ, indicating a disruption of the cortical excitatory/inhibitory balance. Our study provides a novel tool for gaining insights into the cellular and genetic mechanisms underlying the multifaceted pathophysiology of psychiatric disorders, as here exemplified by SCZ.

Keywords: schizophrenia, iPSC disease modeling, transcriptomics/ metabolomics

4:45 PM – 5:45 PM

202

MODELLING CORTICO-LIMBIC SYSTEM IN VITRO TO STUDY HUMAN NEURODEVELOPMENT AND DISEASE

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The limbic system is essential in emotion processing and memory formation and encompasses multiple brain regions, including the hippocampus and amygdala. Alterations in these brain structures and their interaction with the cerebral cortex have been highlighted as hallmarks of neurological disorders. However, the neuroanatomy and functionality of the cortico-limbic axis remain largely unknown. Stem cell-derived 3D organoids provide an excellent opportunity to expand our understanding of these complex cellular networks and how their alterations result in disease. Here, we describe novel methods to generate 3D hippocampus and amygdala models and to connect them to cortical organoids, providing a platform to study cortico-limbic system interactions and investigate the impact of neurological disorders on these regions. The cerebral cortex, hippocampus, and amygdala develop from the embryonic dorsal telencephalon in a process that is finely regulated by morphogens. By exposing iPSC aggregates to BMP-WNT or FGF-SFRP signalling and adapting the cultures to growth in spinner-flask bioreactors, we achieved the long-term development of an extensive compendium of hippocampal and amygdala cell types. ScRNA-seq and immunohistochemistry analysis at 9, 18, 30, 90, and 180 days revealed that while at early stages of in vitro development, organoids have the potential to generate multiple regions of the developing nervous system, in long-term cultures they show a specific hippocampus and amygdala identity. We investigated the functional activity of our models using a high-resolution multielectrode array system and detected 3D neural network activity at 90 and 180 days in both models. To recreate the interactions between the limbic system and the cerebral cortex, we used previously characterised cortical organoids and our novel

hippocampus and amygdala models. We investigated the connectivity among distinct brain regions with long-term live-imaging and functional assays by generating each organoid using iPSC lines engineered to express different fluorescent reporter genes. These newly established models provide an unprecedented platform to investigate how the cortico-limbic system is built and how abnormalities cause disease to identify the pathophysiological mechanisms of neurological disorders.

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Keywords: cortico-limbic system, stem cell-derived 3D organoids, neurological disorders

4:45 PM – 5:45 PM

204

MODELLING SETBP1-ASSOCIATED RARE NEURODEVELOPMENTAL DISORDERS USING IPSCS

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Heterozygous mutations in the gene SETBP1 (SET binding protein 1) cause 2 distinct rare autosomal dominant neurological disorders: Schinzel-Giedion Syndrome (SGS) and SETBP1 Haploinsufficiency Disorder (SHD). Missense gain-of-function mutations in a mutational hotspot in SETBP1 cause Schinzel-Giedion syndrome, a disorder characterized by severe intellectual disability, epilepsy, neurological degeneration, and bone and renal abnormalities. Conversely, loss-of-function mutations cause SETBP1 haploinsufficiency, a disease characterized by mild to severe intellectual disability, autism and expressive language impairment. Rare diseases like SGS and SHD are often understudied and their mechanism of disease is not well understood, which is a major limitation for their diagnosis, care and therapy development. We generated iPSC-derived forebrain cortical Neural Progenitor Cells (NPCs) from 2 SGS patients and 2 SHD patients and their respective sex-matched controls, in addition to genetically engineered isogenic KOs of SETBP1 for additional independent validation of disease effects. We demonstrate that SETBP1 protein dose is depleted by about 50% in SHD, consistent with haploinsufficiency, and is increased 10-20 fold in SGS due impaired protein degradation via the proteasome. Further characterization of SGS SETBP1 protein demonstrates that its half-life is also significantly increased. SETBP1 binds to the SET oncoprotein, which is a known inhibitor of PP2A amongst other effects. We found that SETBP1 dose in SGS and SHD reciprocally alters PP2A as measured by AKT activation. Increased activation of AKT observed in SGS NPCs was reversible with the PP2A activator FTY720, suggesting that AKT hyperactivation in SGS is caused by PP2A inhibition. These data suggest that SETBP1 has an important regulatory effect on the SET-PP2A-AKT axis. The mirror effects on this pathway observed in SGS and SHD may underline some features of both diseases. This work redefines the molecular spectrum of SGS and SHD and reveals a novel mechanism for both diseases.

Funding Source: CONACYT, FRQ, European Joint Programme on Rare Diseases, SETBP1 Society, AGBU.

Keywords: rare disease, PP2A signalling, dosage disorders

4:45 PM – 5:45 PM

206

HUMAN DISEASE MODEL OF THE AUTISM SPECTRUM DISORDER ASSOCIATED CAV1.3 L271H GAIN-OF-FUNCTION MUTATION EXHIBITS ACCELERATED NEURODEVELOPMENT AND REDUCED NEURONAL FUNCTIONALITY

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Voltage-gated calcium channels (VGCCs) are highly expressed throughout the human brain and involved in many physiological processes. Recent reports pinpoint VGCCs as key modulators of early neurodevelopment. Gain-of-function mutations in L-type VGCCs, such as those observed in the Cav1.3-encoding CACNA1D gene, have been

implicated in various neurological pathologies, including Autism Spectrum Disorders (ASD). However, our understanding of how CACNA1D gain-of-function mutations impact developmental processes or the physiology of human neurons remains limited. Here, we describe the generation of an induced pluripotent stem cell (iPSC)-line carrying the heterozygous Cav1.3 L271H mutation through reprogramming of peripheral blood mononuclear cells (PBMC) obtained from a patient diagnosed with a severe neurodevelopmental disorder. This novel iPSC line was used to establish a disease model that includes multiple stages of neurodevelopment, comprising neural progenitor cells (NPC), human (midbrain) neurons and cortical organoids. Functional analysis of the mutant cells revealed alterations in calcium signaling and electrical activity, affecting the resting membrane potential, action potential shapes and firing frequency of both mutant NPCs and neurons. Accelerated neurogenesis was observed in both two- and three-dimensional models of early human neurodevelopment upon neuronal differentiation of the mutant cells. Additionally, patient-derived organoids exhibited structural alterations indicative of deficits in neural rosette self-organization, a prerequisite for proper corticogenesis. Moreover, transcriptomic analysis of the patient-derived cell lines highlighted the dysregulation of genes that may act downstream of the Cav1.3 channel and regulate neurogenesis. While many of those genes have previously been associated with ASD and other neurodevelopmental disorders, novel disease-associated genes will be discussed. Overall, this study not only broadens our understanding of the role Cav1.3 channels play during neurodevelopment and how Cav1.3 gain-of-function mutations contribute to CACNA1D channelopathies but also provides a platform for drug screening, thereby paving the way for novel therapeutic strategies for affected individuals.

Keywords: voltage-gated calcium channels, disease model, induced pluripotent stem cells

4:45 PM – 5:45 PM

208

DOSE-DEPENDENT EFFECTS OF SUPERNUMERARY SEX CHROMOSOMES ON BRAIN ORGANOID ARCHITECTURE AND FUNCTION

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Klinefelter syndrome (KS) is the most prevalent aneuploidy in males and is characterized by a 47,XXY karyotype. Less frequently, higher-grade sex chromosome aneuploidies (HGAs) can also occur. KS and HGA patients display a broad spectrum of neuronal clinical manifestations, including cognitive deficits, seizures, autistic traits, and delays in motor, speech, and language skills. The severity of cognitive impairment directly correlates with the number of additional sex complements. Despite the significant incidence of sex chromosome disorders, there has been a sustained demand for cellular models that elucidate the transcriptional and epigenetic consequences of X chromosome aneuploidies and their implications for human health. We previously generated a paradigmatic cohort of KS and HGA iPSCs to investigate the transcriptional consequences of X chromosome overdosage in 49,XXXXY, 48,XXXY, and 47,XXY patients. Leveraging this cohort, we modeled the impact of sex-chromosome aneuploidies during early neurodevelopment using iPSCs-derived X aneuploid cortical brain organoids. Intriguingly, X aneuploid brain organoids retain the epigenetically determined inactivation status of supernumerary X chromosomes during extended differentiation periods thus serving as an ideal 3D neurodevelopmental model. Through a comprehensive analysis integrating morphological, transcriptomic, and functional assessments, we demonstrated that supernumerary sex chromosomes detrimentally affect neural patterning, cortical architecture, and electrophysiological properties of brain organoids in a dose-dependent fashion. Thus, our study underscores the use of brain organoids as a valuable platform for modeling the molecular and cellular consequences of X chromosome overdosage during early neurodevelopment.

Keywords: Klinefelter syndrome, X-chromosome aneuploidies, brain organoids

4:45 PM – 5:45 PM

210

MODELING DISTINCT IMPACTS OF EPILEPSY ON CORTICAL AND HIPPOCAMPAL NEURAL NETWORK ACTIVITIES USING INDUCED PLURIPOTENT STEM CELL-DERIVED ORGANOIDS AND ASSEMBLOIDS

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Many neurodevelopmental disorders are associated with impairments in multiple cognitive domains. Epilepsy, for example, is associated with recurrent seizures impacting the cerebral cortex, but it is also linked to memory impairment involving the hippocampus. How a single disorder affects these different brain regions remains unclear. Here, we developed a pipeline to create and compare cortical-ganglionic eminence (GE) and hippocampus-GE assembloids to investigate the impact of epilepsy on network-level activities including neural oscillations in each area. Using a patient-derived induced pluripotent stem cell model for developmental epileptic encephalopathy-13 (DEE-13) associated with a gain of function allele in the SCN8A sodium channel, we observed marked hyperexcitability and epileptiform discharges in cortical-GE assembloids. By contrast, hippocampus-GE assembloids remained normal in excitability but instead showed deficits in memory-linked neural activities due to a loss of a specific group of inhibitory interneurons. Optogenetic activation of inhibitory pathways partially rescued these hippocampal deficits. Together these findings illustrate differential impacts of a single pathogenic gene mutation across brain regions and establish hippocampal-GE assembloids as a platform to investigate the effects of neurodevelopmental disorders on hippocampal learning and memory.

Keywords: organoid, epilepsy, neural activities

4:45 PM – 5:45 PM

212

DIFFERENTIATION PATHWAYS DURING CORTICOGENESIS INVOLVING TRANSCRIPTIONAL FACTORS BCL11B AND SATB2 ARE BENEFICIAL AFTER ISCHEMIC BRAIN LESION IN THE ADULT MOUSE

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Therapeutic approaches of stroke need addressing neuroprotection and/or neurorestoration. We hypothesized that the cellular and molecular mechanisms active during fetal brain development and cortex differentiation could as well contribute to the brain repair after ischemic brain injury. As candidate developmental genes having the hypothetic potential for the brain repair, we have chosen two corticogenesis-related transcription factors, BCL11B (B-cell lymphoma/leukemia 11B, aka CTIP2—chicken ovalbumin upstream promoter transcription factor interacting protein 2) and SATB2 (special AT-rich sequence binding protein 2). As a model for the human ischemic stroke, transient middle cerebral artery occlusion for 30 minutes was performed in mice. Three and 7 days after ischemic brain injury the animals were analyzed by in vivo magnetic resonance imaging, they were assessed functionally by neurological deficit score and the isolated brains were analyzed by immunohistochemistry. BCL11B and SATB2 were indeed reactivated after ischemic brain injury and their expression was higher in the lesioned animals than in the control, sham operated animals. BCL11B co-localized with another transcription factor, ATF3, known to be beneficial after ischemic stroke. Moreover, the expression of corticogenesis-related transcription factors correlated positively with the recovery rates measured by both, structural parameters (lesion reduction visualized by magnetic resonance imaging), and functional parameters (functional recovery measured by a decrease of neurological deficit score). The performed study supported the initial hypothesis that after brain injury there is a reactivation of genes active during neuronal differentiation in the fetal brain. The functional importance of the corticogenesis-related transcription factors in the brain repair was claimed by the fact that they were associated with the parameters of structural and functional recovery. This opens an exciting perspective of the potential therapeutical interventions oriented to enhance the reactivation of developmentally relevant genes. The developmental mechanisms can be a basis for neurorestoration strategies contributing to the regenerative medicine applications in neurology.

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Keywords: stroke, brain repair, in vivo imaging

4:45 PM – 5:45 PM

214

TRANSCRIPTIONAL AND EPIGENETIC MODULATION INDUCED BY GBM DRIVER MUTATIONS ON NEURAL STEM CELLS

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Glioblastoma multiforme (GBM) is the most aggressive malignant brain cancer in adults, characterized by a median overall survival of only 15 months. This poor outcome is primarily a result of intra-tumoral genetic heterogeneity driven by multiple gene mutations. The mesenchymal GBM subtype is associated with the poorest survival rates and frequently presents with mutations in the tumour suppressors; NF1 and PTEN, along with amplification and mutation of EGFR. Despite the genetic diversity among GBM stem cells, they share a phenotypic similarity with neural stem cells (NSC). However, the molecular mechanisms orchestrating the shift from normal to cancer cells remain still poorly understood. To investigate the consequences on chromatin organization induced by these GBM driver mutations, we generated human neural stem cells with isogenic NF1 loss, PTEN loss or EGFRvIII overexpression, along with a triple mutant cell line termed NPE to model the mesenchymal subtype. Analysis of accessible chromatin regions revealed that the single hits, NF1 loss and PTEN loss, as well as the NPE

model, drive the cells to a different state with profound changes of the chromatin landscape compared to the parental NSCs. In contrast, cells overexpressing EGFRvIII alone maintain a chromatin state close to the original NSCs. The open chromatin regions in NF1 loss, PTEN loss and NPE show enrichment of key functional pathways relevant to GBM such as the epithelial-mesenchymal transition and inflammatory signalling, which positively correlates with increased expression of genes that are hallmarks of these pathways. Furthermore, motif enrichment analysis of open chromatin regions indicates that motifs for the SOX transcription factor family members were highly enriched within parental and EGFRvIII overexpressing cells, while motifs for the Fos/Jun and RUNX family were among the most highly enriched within the NF1 loss, PTEN loss and NPE cells, implying a potential shift in transcription factor binding induced by NF1 and PTEN mutations. These findings suggest that NF1 and PTEN loss may play a role in reshaping the chromatin landscape, establishing a pre-malignant chromatin organization status that 'primes' cells for the malignant transformation characteristic of the mesenchymal GBM subtype.

Keywords: glioblastoma, chromatin organization, neural stem cells

4:45 PM – 5:45 PM

216

MULTI-OMICS PROFILING OF DIFFERENT DISEASE SEVERITIES IN A HUMAN CEREBRAL ORGANOID MODEL OF LIS1-LISSENCEPHALY IDENTIFIED SHARED AND SEVERITY-DEPENDENT DISEASE MECHANISMS

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Lissencephaly is a developmental cortical malformation characterized by reduced to absent gyri and disorganized cortex. Heterozygous mutations in the LIS1 gene, encoding a regulator of the microtubule motor dynein, cause lissencephaly with different clinical severities. While the clinical disease spectrum correlates with the degree of lissencephaly, the location and type of mutation do not. Here, we leveraged forebrain-type organoids from LIS1 patients diagnosed with mild, moderate, or severe lissencephaly to identify severity grade-related phenotypic convergence and divergence. We found varying degrees of alterations of cytoarchitecture and neurogenesis, correlating with disease severity. Multi-Omic analyses depict a severity-related dysregulation of forebrain development and especially progenitor cell homeostasis. We identified shared disease mechanisms, including microtubule destabilization and N-cadherin binding, and severity grade-dependent perturbances in unfolded protein binding, alternative splicing, and WNT-signaling to be critical for the development of the in vitro phenotype. Rescue experiments and drug repurposing analyses suggest a combinatorial severity-dependent therapeutic strategy. Our data indicate a direct link between the genotype and phenotype in LIS1-lissencephaly, elucidate

disease-linked molecular mechanisms that might underly the development of the different clinical severity grades of LIS1-lissencephaly and show that the organoid-based system is sensitive enough to capture different disease severities in vitro.

Funding Source: Ministry of Innovation Science and Research of North Rhine-Westphalia (Junior Research Group, to J.L.), the ERA-NET NEURON, JTC 2015 Neurodevelopmental Disorders, STEM-MCD (to J.L., F.F. and N.B-B.) the Hector Stiftung II (to J.L.).

Keywords: disease modeling, forebrain organoids, multi omics

4:45 PM – 5:45 PM

218

UNLOCKING DISEASE INSIGHTS: HARNESSING IPSC TECHNOLOGY TO RECAPITULATE RP11 AND ADOA DISEASE PHENOTYPES IN-VITRO

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The emergence of human induced pluripotent stem cells (iPSCs) offers unprecedented avenues for disease modeling. Utilizing iPSC-derived cells in two and three-dimensional in-vitro models provide a straightforward yet powerful approach to investigate mechanisms underlying disease pathogenesis. Our studies focus on leveraging induced iPSC technology to recreate disease phenotypes of Retinitis Pigmentosa 11 (RP11) and Autosomal Dominant Optic Atrophy (ADOA) in-vitro. Ultimately, our work aims to accurately model human diseases to enhance our understanding of rare diseases and validate novel therapeutic approaches. We demonstrate long-term successful differentiation of retinal pigment epithelial (RPE) and retinal ganglion cells (RGC) exhibiting RP11 and ADOA pathology, respectively. Our internally validated protocols facilitate the generation of cultures with comparable quality control parameters between patient-derived and wildtype or gene-corrected cells as assessed by flow cytometric expression of phenotype-specific markers. Further, our models exhibit disease-associated deficiencies, demonstrated by target gene and protein expression coupled with functional and morphological studies. For example, RP11 patient-derived iPSC-RPE exhibit reduced expression of the PRPF31 gene, reflective of a haploinsufficient phenotype, in addition to alterations in cell perimeter and cell density as assessed by ZO-1 ICC and SEM. Similarly, ADOA patient-derived iPSC-RGC exhibit a 25-62% reduction in OPA1 protein levels, the primary driver of ADOA pathogenesis alongside disrupted mitochondrial morphometry including mitochondrial fragmentation. Our in-vitro models reliably and reproducibly recapitulate the disease phenotypes of RP11 and ADOA, shedding light on their underlying mechanisms and allowing preclinical validation of therapeutic molecules. This has enabled the de-risking of our lead therapeutic candidate, now in clinical stage, and paved the way for another candidate entering its Investigational New Drug stage, showcasing the translational potential and therapeutic relevance of our human disease models and our findings.

Keywords: induced pluripotent stem cells, disease modeling, Retinitis Pigmentosa 11

4:45 PM – 5:45 PM

220

IPSC DERIVED RPE CELLS AND PORCINE RETINA CO-CULTURE AS ADVANCED IN VITRO MODEL FOR DRUG DISCOVERY IN NEURORETINAL DEGENERATION

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With increased life expectancy, there is more than ever pressure on biomedical science to develop innovative therapies particularly for age-related degenerative diseases. Safety and efficacy of novel drugs are still tested in in-vivo models. Induced pluripotent stem cells (iPSC) offer a framework for personalised medicine & disease modelling while retina culture provides cost effective, high through put system with complex interactions, we aimed to merge both systems to develop an iPSC derived retinal pigment epithelium cells (iRPE) porcine retina (pRetina) co-culture system as advanced in vitro model for drug discovery while reducing the number of animals used. A Cas iPSC clone was differentiated to iRPE cells. iRPE were characterized by FACS (CD140b). 50K iRPE cells (P3) were transfected with the pFAR-SB100X transposase (0.03µg) & pFAR4-SB-Venus (0.47µg) plasmids & cultured for

1-week. Transfection efficiency, morphology & viability were determined with image-based cytometry, microscopy, and acridin-orange staining. iRPE P3 were co-cultured with pRetinas (5-8 hrs post-mortem, sourced from local slaughterhouse) in Ames' medium supplemented with 5% FBS, 1% N2 & B27 at 21°C for 7d. Viability was analysed by Propidium iodide (retina) & acridin-orange staining (iRPE). Morphology was evaluated by bright field microscopy. Oxidative stress was measured by Griess reagent. CD140b marker was detected by FACS. Functional iRPE were successfully produced as shown by upregulation of RPE markers. Transfection rate was 100% with a mean fluorescence of 3.7K; mean cell number was 35K and viability 86%. Co-culture of iRPE-pRetinae showed a protective effect as evidenced by 92% viability of iRPE cells, and low necrosis (D1: 21.15, D7: 38.17 mean grey value, $p< 0.05$). Retina & iRPE cells maintained their typical morphology. Oxidative stress remained low (fresh: ND, cultured: 0.0038 AU, $p=0.048$). CD140b marker expression was preserved in iRPE (n= 5-12). In conclusion, iRPE-pRetina culture shows mutual benefits maintaining morphology, high viability and minimal oxidative stress. Transfection of iRPE cells was very efficient. This finding suggests that the model can be used as a tool for development of personalised medicine as well as disease modelling improving transferability to humans while reducing animals.

Funding Source: La Fondation de Bienfaisance Pierre & Andrée HAAS

Keywords: personalized medicine and disease modelling, induced retinal pigment epithelial cells, porcine retina, age related degenerative disease

4:45 PM – 5:45 PM

222

RESCUE OF PROGRANULIN DEFICIENCY IN FTD-GRN AND CLN11 IN VITRO MODELS USING HIPSC-DERIVED MICROGLIA

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The GRN gene encodes for progranulin (PGRN), a secreted glycoprotein linked to a multitude of functions: lysosomal processing, inflammation, development, and wound repair. Gene dosage variation in GRN causes distinct disorders: homozygous mutations cause neuronal ceroid lipofuscinosis 11 (CLN11) while heterozygous mutations cause Frontotemporal Dementia (FTD-GRN). CLN11 is a lysosomal storage disorder with early adulthood onset while FTD-GRN is a rapid progressing neurodegenerative disease starting around the sixth decade of life and 100% penetrant by age 80. FTD-GRN causes a myriad of cognitive and behavioral symptoms. There is no consensus on standard of care for CLN11 and FTD-GRN, only a focus in minimizing symptoms. Experimental therapies, like AAVs or recombinant protein infusions, aim to restore PGRN levels but potential issues remain. Recombinant proteins do not provide a persistent supply and AAVs restore progranulin only in a subset of cells which may be insufficient or induce supraphysiological progranulin expression in individual cells that could be detrimental. Allogeneic off-the-shelf donor cells, capable of long-term survival in the CNS while secreting wildtype levels of progranulin, offer a potential treatment for FTD-GRN. Here we show that human iPSC-derived microglia secrete wildtype levels of progranulin and can restore deficiency in GRN mutant cells through metabolic cross-correction. We developed isogenic iPSC lines modelling CLN11 and FTD-GRN progranulin dosage and successfully differentiated them into cortical neurons and microglia. Utilizing a co-culture assay, we can demonstrate successful progranulin transfer from healthy microglia into deficient cells and modulation of downstream targets. To probe the changes brought by co-culture with healthy microglia, we performed a transcriptomic analysis of corrected cells to confirm rescue of progranulin targets and assess additional changes. We also functionally characterized PGRN-deficient microglia and neurons before and after co-culture. Our results highlight the potential of iPSC-derived microglia as an alternative therapy for FTD-GRN, providing long-lasting persistent supply of PGRN to a deficient CNS with possibility of additional benefits from supporting functions of healthy microglia to diseased CNS cells.

Keywords: FTD, cell therapy, progranulin

4:45 PM – 5:45 PM

224

GENERATION OF SNCA-TAGGED IPSC LINES FOR AGGREGATION MODELING OF LYSOSOMAL STORAGE DISORDERS

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Alpha-synuclein (a-syn) is an important protein in the neuronal synapse encoded by the SNCA gene, but its exact function has yet to be fully understood. The aggregation of a-syn is closely linked to neurodegeneration, particularly in Parkinson's disease and other synucleinopathies. When the proteins misfold and clump together, they form insoluble aggregates which interferes cell functions and can trigger oxidative stress and neuroinflammation. These aggregates can spread from one cell to another, propagating the pathology throughout the brain. While making up the primary aggregate in synucleinopathies, secondary aggregation of a-syn can also be found in many early onset neurodegenerative diseases such as lysosomal storage disorders (LSDs). LSDs make up a group of more than 70 different metabolic disorders, with shared feature of impaired lysosomal function leading to accumulation

of macromolecules. Most of the primary aggregates of LSD are metabolites that are not genetically encoded, which makes visualization of aggregation dynamics and their cell-to-cell spreading very challenging. Through CRISPR/Cas9-based genome editing, we have introduced tags either to the N- and C-terminal of SNCA, with HA-tags to the N- or C-terminal and mCherry to the N-terminal, which will allow us to visualize aggregation in real time through live imaging. This approach enables us to study a-syn aggregation, a-syn dynamics under physiological conditions, as well as understanding how it interacts with other proteins and molecules in the synapse and the cell. Through pharmacological intervention with GCase inhibitor CBE we can mimic the effect of GBA1 mutations to induce aggregation, which acts as proof-of-concept that these lines can be used for modeling of aggregation in LSDs. We believe that these tagged lines, in addition to their use for studies of lysosomal storage disorders, can be an important resource in research of Parkinson's Disease and other synucleinopathies as well as for basic research into the function of a-syn itself.

Keywords: alpha-synuclein, aggregation, reporter

4:45 PM – 5:45 PM

226

GENERATION OF DORSAL SPINAL GABAERGIC NEURONS FROM HUMAN URINE CELLS BY DIRECT REPROGRAMMING FOR THE TREATMENT OF CENTRAL NEUROPATHIC PAIN

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Central neuropathic pain (CNP) is a chronic condition following spinal cord injury (SCI), which results from the loss of GABAergic inhibitory interneurons (GINs) in the dorsal horn, causing spontaneous hyperalgesia and allodynia. Unfortunately, there are currently no effective therapies available to cure CNP, primarily due to the non-regenerative nature of the injured spinal cord. Stem cell-based transplantation has emerged as a potential therapy for compensating the loss of GINs. Despite promising pre-clinical studies have been conducted, aiming to mitigate SCI-induced neuropathic pain through transplanting forebrain GINs derived from human pluripotent stem cells (hPSCs) or fibroblasts into the lesion sites, further clinical applications are limited due to concerns such as tumorigenicity associated with hPSCs, heterogeneity of grafted cells, and unmatched graft with recipient. Therefore, it is essential to generate homogenous dorsal spinal GINs from patient somatic cells by direct lineage reprogramming. Through a systematic screening process using small molecules differentiation strategy from hPSCs, we have identified four transcription factors (TFs), namely PTF1A, LBX1, ASCL1, and BRN2, as key specifiers for dorsal spinal GABAergic neurons. We further demonstrated the sufficiency of these TFs in different combinations for direct conversion of hPSCs into dorsal spinal GINs, which is also confirmed by our single cell RNA-sequencing analysis. In parallel, we have developed an AAV-based GABA-reporter system that allows us to lively monitor the reprogramming efficacy and obtain gene expression profiling of GINs. Our observations show that these GINs reprogrammed from hPSCs via defined TFs exhibited better survival and integration with host, including expressing high potion of mature GABAergic markers, and improving somatosensory function and locomotor activity compared to neural stem cells at 3 and 6 months after transplantation into the lesion sites of SCI rats. Importantly, we have achieved the trans-differentiation of human urine cells into dorsal spinal neural progenitors using the identified TFs. In the future, we will further identify effective combinations of TFs for direct reprogramming of GINs from human urine cells and evaluate their therapeutic potential in SCI model.

Funding Source: RGC Ref No. 17110520

Keywords: spinal cord injury, neural stem cell, central neuropathic pain

4:45 PM – 5:45 PM

230

ESTABLISHMENT OF AXON-OMICS FOR MOTOR NEURON DISORDERS USING COMPARTMENTALIZED MICROFLUIDIC CHAMBERS

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Hereditary Spastic Paraplegia (HSP) is a type of motor neuron disorder (MND) characterized by progressive spasticity and weakness in the lower limbs due to the degeneration of upper motor neurons. HSP is a genetically heterogeneous disorder with over 80 genes implicated in its pathogenesis. There are no therapies that halt disease progression, highlighting the need for research into fundamental biology of HSP. The progressive degeneration of long axonal tracts makes HSP a unique genetically tractable model of axonal degeneration and dysfunction. Our group has generated a set of over >40 patient and genetically engineered (CRISPR/Cas9) iPSC lines with different genotypes of HSP. Using compartmentalized microfluidic chambers, we have established a pioneering platform that integrates imaging-based analysis of axonal phenotypes in iPSC-derived cortical neurons with axon-omics analyses. The imaging-based platform allows to analyze axonal phenotypes, including quantification of axonal outgrowth, swellings, and growth cone morphology. In addition, our workflow includes axon-seq and axon-proteome analyses in human iPSC-derived cortical neurons, allowing the exploration of molecular signatures underlying axonal dysfunction. Historically, axon proteomics has been challenging; the low protein yield from isolated axons has limited mass spectrometry (MS)

analysis to only the most abundant proteins. However, we have now leveraged breakthroughs in high sensitivity data-independent acquisition (DIA)-MS to quantify more than 5,000 proteins from axons isolated from a microfluidic device. By elucidating common axonal disease signatures and identifying novel therapeutic targets and biomarkers, our approach holds promises to advance the understanding and treatment of HSP and other MNDs.

Funding Source: Tom Wahlig Foundation (to SH and AD)

Keywords: motor neuron disorder, axon-omics, axonal phenotypes

4:45 PM – 5:45 PM

232

IMPACT OF 3q29 MICRODELETION AND DUPLICATION SYNDROMES ON HUMAN BRAIN NEURONAL DEVELOPMENT

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The 3q29 loci pose a significant risk for neuropsychiatric disorders throughout an individual's lifetime. These disorders encompass a wide range of developmental neuropsychiatric conditions, such as Schizophrenia, Attention Deficit Hyperactivity Disorder, Anxiety, Autism Spectrum Disorder, and Psychosis Spectrum Disorders. Given its strong correlation with neuropsychiatric phenotypes and its relatively low complexity (involving only 22 genes in the locus), the 3q29 deletion and duplication syndrome presents a compelling opportunity for molecular dissection. In-depth research into the neurodevelopmental phenotype resulting from dosage effects of this locus may unveil a fundamental disruption in neurodevelopmental processes that underlie conditions like schizophrenia, autism, and intellectual disability. To achieve this objective, we successfully derived induced pluripotent stem cells (iPSCs) from patients with 3q29 deletion and duplication and subsequently differentiated them into excitatory neurons. Our research findings reveal that mutations within the 3q29 locus affect gene expression during the stages of neuronal precursor and immature neuron development. By day 20 of differentiation, both deletion and duplication cultures exhibited increased expression of NESTIN and PAX6, neural progenitor markers, compared to controls. Furthermore, 3q29 mutations influenced neuronal morphology and the production of immature neurons. We also examined the expression of presynaptic, postsynaptic proteins, and mature neuronal markers to investigate synapse formation. Our results indicate elevated expression of MAP2, SYN, and PSD95 in deletion and duplication neurons compared to controls, with a more pronounced increase observed in duplication cultures. Further analysis of neural activity through calcium imaging and multielectrode arrays unveiled those neurons affected by 3q29 deletion and duplication exhibited heightened levels of hyperactivity compared to control neurons. Taken together, our findings demonstrate the profound influence of the 3q29 locus on neural development and shed light on potential electrophysiological mechanisms underlying the associated disorders.

Keywords: 3q29 microdeletion and duplication syndromes, excitatory neurons, psychosis

4:45 PM – 5:45 PM

234

MODELLING STARGARDT DISEASE USING THREE-DIMENSIONAL RETINAL ORGANOIDS

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Stargardt disease (STGD1) is an autosomal recessive genetic disorder affecting the gene coding for the ABCA4 protein, leading to a juvenile form of severe macular dystrophy. Accounting for nearly 7% of retinopathies, STGD1 currently lacks any treatment. To address the limitations of existing STGD1 models, such as the lack of macula in rodents, the utilization of human induced pluripotent stem cells (hiPSCs) carrying the disease-associated mutations represents a promising technology. With the differentiating capabilities of hiPSCs, it is now possible to generate multi-layered 3D structures resembling optical vesicles, referred to as retinal organoids (ROs), recapitulating human retinal structure and physiology in vitro. The objective of our project is to model the visual cycle, a critical process for vision that is deregulated in STGD1, using hiPSCs and ROs from patients and healthy donors. In our initial experiments, we successfully cultivated mature ROs that exhibited proper organization and discernible photoreceptors (PRs). Furthermore, we verified the expression of ABCA4 in ROs by digital PCR (dPCR). Additionally, mature RPE cells with correct polarization was obtained, as evidenced by RPE65 expression and ZO-1 localization in confocal imaging. Notably, our findings confirmed that RPE cells expressed low levels of ABCA4, using dPCR. Differentiated RPE cells were also able to metabolize retinol, a metabolite from the visual cycle, into retinal, as demonstrated by metabolite measurements using mass spectrometry. Finally, we captured detailed images of RPE cells through electron microscopy, demonstrating characteristic microvilli and melanosomes. Our next step involves recreating the visual cycle by coculturing ROs on a monolayer of RPE cells. For that, we are developing PDMS-based moulds to accommodate for RO conformation and maximise PR-RPE interaction. We confirmed that RPE cells were able to adhere and grow on these moulds. Comparative analysis between patient-derived and healthy control models will contribute to a more comprehensive understanding of the cellular and molecular mechanisms underlying the observed retinal degeneration in patients with STGD1. Our STGD1 models may also be used to assess and optimize gene therapy strategies targeting the visual cycle and developed by our lab.

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Keywords: retinal organoid, Stargardt, visual cycle

4:45 PM – 5:45 PM

236

USING HUMAN PLURIPOTENT STEM CELL-DERIVED NEURO-IMMUNE ORGANOIDS TO MODEL CENTRAL NERVOUS SYSTEM DEMYELINATION

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Demyelinating disorders are presently difficult to cure, as details of their etiology and disease course remain unclear. One of the most prevalent demyelinating diseases is multiple sclerosis, as thousands of individuals around the world live with the debilitating condition. I focus on the role of brain resident macrophages, the microglia, as I hypothesize that they are key culprits during demyelination and may promote regeneration. There are currently no humanized models of demyelination that include microglia. To better delve into the biology of these diseases and develop therapies, I generated a novel model of demyelination using human pluripotent stem cells (hPSCs) that are differentiated into neuro-immune organoids. Our demyelination model is integrated into our study of X-linked adrenoleukodystrophy (ALD), a monogenic demyelinating disease, and leads the way in the use of cellular and gene-based therapies, while remaining incurable. To generate the organoids, I differentiated hPSCs using patterning and growth factors. These organoids contain neurons, astrocytes, oligodendrocytes and microglia. In conjunction, I used CRISPR gene editing to generate ALD mutant cultures and their isogenic controls. I successfully demonstrated that acute treatment with lysophosphatidylcholine (LPC) results in significant MBP loss and fragmentation, and observed increased astrogliosis and elevation of NFL levels in the medium following 24 hours of treatment. Using multi-omics profiling, I will monitor microglial state changes upon LPC treatment, and the impact of their presence on the demyelination process. I hypothesize that ALD mutant organoids are more susceptible to acute demyelination than controls, owing to their dysfunctional microglia, which display aberrant lipid storage. This system will allow us to screen for therapeutic interventions modulating microglial reactivity, with the aim of limiting demyelination and mitigating metabolic anomalies in microglia. This platform also provides the exciting opportunity to assess cellular and chemical interventions that promote remyelination.

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Keywords: organoid, microglia, Leukodystrophy

4:45 PM – 5:45 PM

238

GLOBAL ORGANELLE PROFILING: COMBINING STEM CELL MODELS AND PROTEOMICS TO UNCOVER DISEASE MECHANISM IN NEURODEGENERATIVE DISEASES

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Over the past two decades, significant strides have been made in understanding the genetic basis of neurodegenerative diseases, revealing a complex array of disease-associated variants across cellular dysfunctions. Despite these advancements, pinpointing the specific cellular pathways and identifying the most impacted cell types have proven elusive. This challenge underscores the critical need for novel methodologies to decode the cellular consequences of genetic risk factors in neurodegenerative diseases. In response, we introduce a comprehensive global profiling strategy that leverages human induced pluripotent stem cells (iPSCs) and CRISPR technology to investigate the proteomic impact of genetic variants on cellular organelles. By developing fluorescent- and epitope-tagged organellar protein markers, we can examine the morphology, positioning, and dynamics of organelles in live cells, alongside profiling their protein content through immuno-precipitation and mass spectrometry. This methodology allows for the high-resolution mapping of protein subcellular localization across all cellular organelles. Our innovative approach has culminated in the generation of isogenic human iPSC lines, carrying key genetic risk factors for Alzheimer's disease, such as APOE4, APP, and PSEN1 mutations. Through the validation of these engineered lines' differentiation potential into iNeurons and iMicroglia, we unearthed early endo-lysosomal remodeling phenomena within Alzheimer's disease models. These insights not only shed light on alterations in cellular architecture and pathways but also establish a comparative framework for evaluating the impacts of various cell types and mutations. Our study provides a novel framework for understanding the complex cellular mechanisms

implicated in neurodegenerative diseases through global organelle profiling. By combining stem cell models with advanced CRISPR and proteomic techniques, we elucidate the role of genetic risk factors in altering organelle function, offering a foundation for future therapeutic strategies aimed at mitigating these disruptions.

Keywords: human induced pluripotent stem cells (iPSCs), Alzheimer's disease, CRISPR technology, organelle profiling

4:45 PM – 5:45 PM

240

HUMAN IPSC-BASED CELLULAR SYSTEMS TO MODEL AUTOSOMAL DOMINANT LEUKODYSTROPHY

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Autosomal dominant leukodystrophy (ADLD) is a slowly, progressive, genetic, and fatal neurological disorder. The genetic cause of ADLD is Lamin B1 (LMNB1) overexpression due to coding duplications or non-coding deletions at the LMNB1 locus. Lamin B1 is a component of the inner nuclear membrane of cells and although LMNB1 is ubiquitously expressed, it appears that neurons and glial cells are particularly sensible to its dosage. Currently, only symptomatic and palliative treatments are available for this fatal disease. Since its discovery, human induced pluripotent stem cell (hiPSC) technology has open to the generation of novel and pathological-relevant in vitro models for Central Nervous System human diseases lacking appropriate model systems. In this work we describe the reprogramming of peripheral blood mononuclear cells and fibroblasts from ADLD patients carrying distinct genetic mutations into hiPSCs by Sendai Virus-based method. These hiPSC lines were characterized to assess their pluripotency state by means of qRT-PCR and immunofluorescence assay. Additionally, embryoid bodies formation assay was employed to evaluate their functional pluripotency. In parallel we set up a procedure for the differentiation of hiPSCs into neuronal and glial 2D and 3D cell models including regionalized brain organoids. These cultures were characterized to assess the expression of stage-specific markers and to uncover potential ADLD-relevant phenotypic and functional region-specific alterations. In conclusion, patient-derived ADLD hiPSC lines alongside hiPSCs based technologies might represent valuable tools for studies aiming to investigate ADLD-specific molecular and cellular alterations. These models hold promise for the development of targeted therapies aimed at ameliorating ADLD pathology.

Keywords: ADLD, LAMIN B1, hiPSCs

4:45 PM – 5:45 PM

242

FELODIPINE EFFICIENCY ANALYSIS ON INDUCED NEURONS DERIVED FROM HUNTINGTON'S DISEASE FELL-HD CLINICAL TRIAL PATIENTS

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, caused by CAG expansions in the huntingtin gene, which results in the aggregation of the mutated huntingtin protein (mHtt). HD is uncurable, and after disease onset around 30-40 years of age patients die within the next 10-20 years. Autophagy, a lysosomal degradation pathway ensuring cytoplasmic homeostasis is dysfunctional in HD, contributing to insufficient mHTT protein removal. The Fell-HD clinical trial is based on repurposing Felodipine, an already licensed L-type calcium channel blocker and antihypertensive drug with a low chance of side effects. Felodipine significantly increases autophagy in animal models of HD and subsequently reduces the level of mHTT, neurodegeneration, and disease symptoms, like tremors and loss of motor coordination. In this current project, in parallel with the FELL-HD trial, we are testing Felodipine drug efficacy in induced neurons (iN) directly reprogrammed from the FELL-HD cohort's skin fibroblasts. Transdifferentiated iNs keep the genetic and aging signatures of the donor bypassing any stem cell or neuroprogenitor phase during conversion. We converted 7 control and 18 HD patients' fibroblasts with mild symptoms to iNs with the same conversion efficiency and purity. In the current cohort, we are using the Horvath epigenetic clock to investigate the presence of accelerated aging. HD-iNs showed a less elaborated neuronal morphology. We used 0.1 uM and 1 uM felodipine treatment for 24h and 72h. After 28 days of conversion followed by Felodipine treatment iNs were fixed and counterstained using neuronal (TAU, MAP2) and autophagy markers (p62, LC3, LAMP1) to determine neuronal morphology and subcellular autophagy changes using high-content automated screening microscopy. Our preliminary results showed that Felodipine treatment enhances autophagy in a subset of patients while having no adverse effect on other HD-iNs. These preclinical results will be directly compared and correlated with FELL-HD trial outcomes and the patient's cognitive and motor scores. This project using an in vitro preclinical iN model can provide predictive information about drug effectiveness, opening a new dimension in clinical trial optimization and personalized medicine.

Keywords: Huntington disease, induced neurons, Felodipine

Clinical Trial ID number: ISRCTN56240656

4:45 PM – 5:45 PM

244

DEVELOPING ISOGENIC HUMAN INDUCED PLURIPOTENT CELL LINES TO MODEL CELL-CELL INTERACTIONS IN INHERITED RETINAL DEGENERATIVE DISEASE

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Developing cell-based therapy for inherited retinal diseases (IRDs) has been challenging due to the lack of suitable pre-clinical models. Retinal organoids have been developed in our lab as a way of studying the interaction between USH2A-diseased retinal cells and allogenic healthy retinal cells. However, allogenic cells are likely to harbor uncharacterized genetic variability that makes associating the impact of specific USH2A variants and their interactions impossible. Thus, we aim to generate isogenic USH2A-diseased/healthy induced pluripotent stem cell lines, which will allow us to investigate healthy/diseased cell interactions in specific rare IRDs. Sanger sequencing for USH2A-iPSCs confirmed the USH2A c.2299delG;c.1256G>T compound heterozygous mutations and were used for CRISPR construct design. After construct delivery by electroporation, Sanger sequencing showed the addition of guanine at the c.2299delG site in bulk iPSCs. Corrected alleles were detected by ddPCR, with the highest corrected proportion reaching 88.9%. To introduce the c.2299delG mutation in healthy iPSCs, a similar CRISPR design was applied. ddPCR has shown an 83.5% introduced USH2A-diseased allele in the subclone after transfection. Further subcloning is required to reach a 100% (1) isogenic USH2A-corrected healthy iPSC and (2) engineered USH2A-diseased iPSC population. To allow tracing of healthy cell progeny in the co-cultures, mScarlet fluorescent protein was knocked-in to the histone 2B locus in healthy iPSCs by CRISPR with a 1.76% transfection efficiency. Puromycin was used to purify the transfected cells. Single-cell colonies were then isolated and expanded. A 100% mScarlet expression in the RFP knocked-in healthy line was confirmed by nuclear Hoechst co-staining. With the establishment of isogenic cell lines, we can generate isogenic co-cultures of retinal cells to help us understand how a diseased host retinal environment in specific IRDs may influence the ability of donor cells to interact and restore retinal function in stem cell therapy.

Keywords: inherited retinal diseases, iPSC disease modelling, stem cell therapy

4:45 PM – 5:45 PM

246

MAPPING MICROGLIAL DIVERSITY THROUGH IMAGE-BASED PHENOTYPIC SUBGROUPING

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Microglia, the primary immune cells of the central nervous system, exhibit a spectrum of morphologies and phenotypes in response to environmental cues. Current methodologies, such as single-cell RNA sequencing, provide valuable insights into microglial biology but often overlook subtle, yet critical, phenotypic variations. Here, we present a novel, automated approach that leverages imaging, deep learning, and data clustering to subgroup microglia based on morphological and phenotypic data extracted from images. Our methodology employs human iPSC-derived microglia, enabling us to study microglial responses to various chemical compounds. We utilize both general staining approaches, such as cell painting, and specific staining techniques, including direct labeling of microglia-specific proteins (e.g., CD45, NFkB, ASC). The resulting images are analyzed using deep learning-based feature extraction or expert-defined segmentation-based feature extraction. Phenotypic feature-based community detection enables us to screen for compounds that impact specific microglial phenotypic properties and subpopulations in this highly plastic cell type. This makes our approach an attractive strategy for identifying chemical compounds that selectively impact phenotypic features of choice, thereby providing a more targeted and efficient approach towards compound screening. Our approach offers several advantages to commonly used strategies. Firstly, it allows for the detection of subtle phenotypic effects induced by chemical compounds, which may be overlooked by sequencing-based methods. Secondly, our assay is fully automated, enhancing reproducibility and scalability. Finally, by focusing on phenotypic data, our approach provides a more holistic view of microglial behavior, complementing the gene expression data obtained from bulk and single cell RNA sequencing.

Funding Source: Zentrales Innovationsprogramm Mittelstand (ZIM) des Bundesministeriums für Wirtschaft und Klimaschutz

Keywords: microglia, phenotypic screening, artificial intelligence

4:45 PM – 5:45 PM

TOPIC: NO TISSUE SPECIFICITY

248

IN VITRO AND IN VIVO EVALUATION OF GENETICALLY CORRECTED RPE CELLS FOR AUTOLOGOUS CELL THERAPY OF HEREDITARY RETINAL DYSTROPHIES

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The generation of genetically corrected RPE cells from patient-derived human induced pluripotent stem cells (hiPSCs) may provide a source for autologous therapeutically-relevant treatment for this rare retinal dystrophy as well to create the human cellular disease models. Recently, we generated a faithful RPE model from patient bearing mutation in the MERTK gene, and genetically corrected the RPEs using CRISPR/Cas9 system. For further application in humans, it is necessary to do in vivo validation for cell survival. The RPE cells from the genetically corrected patient-derived hiPSCs were characterized using immunocytological methods using differentiation and cell polarization markers, electron microscopy, RT-PCR, TEER measurement, ELISA test, Western Blot and phagocytic assay. RPE-scaffolds were implanted into minipig eyes. Due to the xenogeneic transplantation of human cells into minipig eyes, tacrolimus immunosuppression therapy was used. At 2-week follow-up from transplantation, non-invasive OCT and fundus camera examinations of the implanted RPE-scaffolds were performed. Consequently, euthanasia, histological and immunohistochemical investigations of the implanted retina were performed. All generated RPE cells exhibit typical features characteristic of this cell line. We demonstrated the reestablishment of the expression of full-length MERTK protein as well as the reversion of lost phagocytic function of hiPSC-RPEs in vitro, which represents the first example of its kind in this field. Histological and immunohistochemical investigation of the RPE implants and adjacent retina showed structurally healthy RPE-scaffold implant and neuroretinal cells above the implant. The generated hiPSC-derived RPEs in clinical grade conditions display the typical characteristics of mature RPEs re-establishing the phagocytic function in the genetically corrected patient-derived RPE cells in vitro. Functional in vivo studies in minipigs confirmed the RPE survival as well as survival of adjacent photoreceptors, which implies potential application for treatment of patients.

Keywords: retina, retinal pigment epithelium, gene correction, cell therapy

4:45 PM – 5:45 PM

250

GENERATION OF THE IMBA IPSC BIOBANK: A RESOURCE OF HIGH-QUALITY REFERENCE CONTROL PANELS OF HUMAN INDUCED PLURIPOTENT STEM CELL LINES

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High-quality reference control panels are indispensable for development and disease-modeling studies. To meet this demand, we have generated thirty induced pluripotent stem cell lines (iPSC) from PBMCs and fibroblasts of eleven healthy donors using a non-integrating reprogramming technology. All derived clones have undergone extensive quality control according to the prevailing recommendations of the International Society for Stem Cell Research. They are free of microbial and viral contamination, display genetic stability and demonstrate high levels of undifferentiated marker expression and trilineage

differentiation potential. The resource is available to all scientists and commercial institutions via the IMBA iPSC Biobank (https://shop.vbc.ac.at/ipsc_biobank/), an initiative to strengthen and support research into stem cells and tissue engineering.

Funding Source: Work in the IMBA Stem Cell Core facility is supported by the Austrian Academy of Sciences.

Keywords: open resource, iPSC Biobank, reference iPSCs

4:45 PM – 5:45 PM

252

ENHANCED EARLY QUALITY CONTROL OF HUMAN INDUCED PLURIPOTENT STEM CELL USING HIPSCORE

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Due to their tremendous potential, e.g. for individualized regenerative medicine, the number of studies involving human induced pluripotent stem cells (iPSCs) have increased dramatically. Simultaneously, there is a lack of standardization in the context of iPSC quality control. Early quality control includes the functional assessment of pluripotency, i.e. the ability of iPSCs to differentiate into the three primary germ layers endoderm, ectoderm, and mesoderm. Here, we employed directed differentiation, long-read transcriptome sequencing, and qPCR to develop a machine learning-based workflow for the robust and reliable assessment of iPSC pluripotency. By using fifteen different iPSC lines differentiated by two commercially available directed differentiation protocols, we identified and validated a set of marker genes for the use in a qPCR-based gene expression panel. To enable automated, reproducible, and robust classification we integrated these data into a machine learning-based scoring system termed hiPSCore which provides reliable assessment of iPSC pluripotency. The hiPSCore performs with high precision, sensitivity, and accuracy on previously unknown data and can be used to assess the capacity of iPSCs to differentiate into more complex cell types and structures, e.g. from definitive endoderm into branching lung organoids. By putting the ISSCR marker recommendations to practical use, this study accelerates standardization of early iPSC quality control and contributes to increased reproducibility of iPSC-based research.

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Keywords: human induced pluripotent stem cells, pluripotency quality control, machine learning

4:45 PM – 5:45 PM

254

ORGAN-SPECIFIC EXPRESSION DATA STEER CHOICE OF LAMININ PROTEINS FOR IN VITRO STUDIES, FOR BIOLOGICALLY RELEVANT DRUG DISCOVERY AND DEVELOPMENT PLATFORMS

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The Drug Discovery and Development (DDD) process incorporates multiple cell culture platforms to assess the safety and efficacy of lead compounds against a target for an indication. In recent years, with the advancement of stem cell research protocols, there has been shift in focus from 2D cell culture techniques to more complex patient-derived models. Human pluripotent stem cells (hPSCs) require an extracellular matrix (ECM) for maintenance, expansion, and differentiation; laminins, a large ECM protein family enriched within basement membranes of epithelial- and endothelial tissues, are an essential part of stem cell niches. While laminins are increasingly implemented in hPSC research, standardised protocols using biologically relevant ECM in DDD, across the board, have not been developed. Thus, we assessed protein and gene expression patterns of laminin isoforms and their associated integrin binding partners in vivo in four distinct tissues commonly used in DDD: the heart, liver, kidney and central nervous system, before translation to proliferation and expression experiments in specified cell-types on laminin substrates. We found the expression pattern is largely tissue- and cell type specific, for example, in the kidney. We also show that providing the relevant laminin isoform in vitro can lead to increased standardization and functionality. For example, 43-times more dopaminergic neurons, and >80% functional cardiovascular progenitor cells, were observed when culturing with relevant laminin isoforms. On the contrary, mutations to laminin genes and truncations result in laminopathies, such as Pierson's syndrome, cardiomyopathy and neuromuscular junction disorder. Taken together, we demonstrate cell-specific expression of full-length laminin isoforms and putative integrin binding partners. While abnormalities to LAM genes are responsible for established laminopathies, in vivo expression data and in vitro studies can determine the correct laminin for optimal cell culture conditions by initiating biologically relevant processes in vitro. By doing so, differentiation, reproducibility and cell maturity issues can be addressed in more sophisticated DDD applications, by laying the foundations for standardized protocols across different disease areas.

Keywords: biological relevant cell models and assays, laminins are tissue-specific in expression, importance of ECM for good prediction

4:45 PM – 5:45 PM

256

FUNDAMENTAL APPLICATIONS OF FETAL TISSUE IN BIOMEDICAL RESEARCH

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Human fetal tissue, defined as tissue or cells obtained from a human embryo or fetus after a spontaneous or induced abortion, is an important resource for stem cell research and regenerative medicine. Despite its fundamental importance in these areas, substantial misinformation persists in public and political discourse surrounding its uses. Here, we highlight four primary, concrete applications for human fetal tissue and cells derived from human fetal tissue. First, human fetal tissue is the gold standard comparator for cell culture and tissue model systems. Second, it is widely used for studies on both normal development and disease states. Third, many humanized mouse models, which are important in understanding development, tissue homeostasis, and infectious disease, rely on human fetal tissue. Finally, human fetal tissue is routinely used for vaccine development and manufacture. These applications reflect special characteristics exhibited by human fetal tissue, including increased proliferative capacity and developmental potential relative to adult tissues. Overall, these points underscore the value of human fetal tissue research in biomedical science.

Keywords: fetal tissue research, tissue models, vaccine development

4:45 PM – 5:45 PM

258

DISEASE-IN-A-DISH: USING STEM CELLS TO CHARACTERIZE ELECTRONIC CIGARETTE HARM TO HUMAN EMBRYOS

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Exposure of pregnant women to flavor chemicals in electronic cigarettes (ECs) may influence human prenatal development. Human embryonic stem cells (hESCs), which model the epiblast, were used to study the effects of vanillin on early postimplantation development. Vanillin, which is used at high concentrations in ECs, can activate TRP (transient-receptor-potential) channels, which may alter calcium homeostasis in embryos. We tested the hypothesis that vanillin, at concentrations likely to reach the embryo in pregnant users, adversely affects human embryonic development through activation of TRPV channels. A Ca2+ influx assay using Fluo-8 dye was performed to determine if vanillin activated TRPV channels shown to be present in hESCs. Vanillin (1-100 µM) increased [Ca2+]i which was blocked by a specific TRPV4 inhibitor (HC067047). µM vanillin also inhibited mitochondrial reductase activity in MTT assay, and this effect was reversed by the TRPV4 channel inhibitor. To determine if activation of TRPV4 channels by vanillin affected hESCs growth, time-lapse data were collected in a BioStation CT, and growth features were extracted using StemCellQC software. Vanillin (45-450 µM) inhibited the increase in colony area during 96 hours of in-vitro incubation. TRPV4 inhibitor reversed this effect and restored colony growth to that of the untreated controls. hESCs treated with vanillin were then evaluated for cell death using brightness/total area ratio in StemCellQC. This ratio was elevated in vanillin-treated colonies, indicating an increase in cell death, an effect that was blocked by TRPV4 inhibitor. Vanillin also affected hESC colony phenotype via TRPV4 channels. Major/minor axis ratio extracted from time-lapse videos using StemCellQC indicated that treated colonies had an elongated rather than circular shape. Given the high concentrations of vanillin in ECs and the low concentrations that perturb calcium homeostasis and influence critical processes in early embryonic development through TRPV channels, it is likely that human embryos would be affected by vanillin in pregnant women who vape. These data could help prevent alterations in normal development leading to birth defects. The use of ECs by pregnant women should be discouraged until the effects of flavor chemicals on their embryos are fully understood.

Keywords: human embryonic stem cells, prenatal development, electronic cigarettes (ECs)

4:45 PM – 5:45 PM

260

AN ALL-IN-ONE LONG-READ SEQUENCING ASSAY FOR THOROUGH GENOMIC ANALYSIS POST GENETIC MANIPULATION

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Large knockins (KIs), such as for gene humanization, cDNA overexpression, replacement, reporters and conditional KIs, are usually a challenging type of gene editing in cell line and animal model creation, compared to simple knockout (KO) and introduction of single-nucleotide polymorphism (SNPs) and small tags. Junction PCRs are the most commonly used method to confirm targeted integration, and it is increasingly difficult to obtain conclusive genotyping results with larger sizes and inserts that are more sophisticated. Here we report

the development of a universal genotyping assay to analyze on-target, random and off-target integration by combining target capture and long read sequencing. Using the method, we successfully analyzed various large KIs in mice and iPSCs, up to 10 kb, that were problematic by junction PCRs for on-target detection and captured integration events contributed by either random donor insertions and/or potential off targeting sites. As importantly, we further demonstrated the power of the assay and identified random integration sites of CAR expression cassette in CAR-T cells generated, a useful safety check to help reduce cancer risk from CAR-T immunotherapies. In summary, the all-in-one assay is cost effective and high throughput via multiplexing for screening founder animals and clonal cell lines for validation of the correct insertion in the genome. Additionally, the method may help shed light on mechanisms for homology-dependent repair as well as off targeting and potentially be a quality control measure for cell therapies involving random integration of DNA molecules in the genome.

Keywords: long-read sequencing, CRISPR-mediated gene editing, CAR-T

4:45 PM – 5:45 PM

262

A COMPLETE WORKFLOW FOR HIGH-EFFICIENCY CRISPR/CAS9-MEDIATED GENE EDITING IN PLURIPOTENT STEM CELLS

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Disease-specific human induced pluripotent stem cells (hiPSCs) can be generated directly from individuals with known disease characteristics. Alternatively, healthy hiPSCs can be modified using genome editing approaches to introduce disease-causing genetic mutations to study the biological response of those mutations. The emergence of CRISPR/Cas9 technology has made gene editing easier to achieve; yet, a vital step to get homogenous edited populations, producing single-cell clones of hiPSCs is still a major challenge in the field. In contrast to colony culture of hiPSCs, the specific composition of Cellartis® DEF-CS™ Culture System allows culture of hiPSCs in a non-colony, monolayer fashion, enabling very high rates of single-cell survival and clone expansion. Here we introduce a complete workflow that achieves a high number of edited and expandable hiPSC clones that maintain the hallmarks of pluripotency. Cas9/sgRNA ribonucleoprotein complex was delivered into hiPSCs either via electroporation or via gesicles, followed by successful single cell cloning of edited hiPSCs. Endogenous gene knockout efficiencies of up to 65% were achieved for the membrane protein CD81. Seeding of single edited hiPSCs into a 96-well plate either by limiting dilution or by fluorescence activated cell sorting, resulted in a survival rate >50% using both methods. Notably, edited hiPSC clones maintained their pluripotency markers even after expansion (>90% positive for Oct4, Tra-1-60 and SSEA-4). The described workflow implementing the DEF-CS™ culture system results in a high number of edited and expandable hiPSC clones, making it highly suitable for disease modelling.

Keywords: disease modelling, single-cell cloning, gene knockout

4:45 PM – 5:45 PM

264

PARP INHIBITION ELONGATES TELOMERE LENGTH IN MOUSE AND HUMAN PLURIPOTENT STEM CELLS

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Telomere is a tandem TTAGGG sequence located at the end of linear chromosomes. The length of the telomere can be extended through the telomerase dependent or the telomerase independent homologous recombination (HR) mediated alternative lengthening (ALT) pathway. Mutations in telomerase-associated genes, such as TERT, TERC, or DKC, often lead to abnormally short telomere lengths at the cellular level and so-called “telomere syndromes” manifested as dyskeratosis congenita, aplastic anemia, or idiopathic pulmonary fibrosis at the individual level. PARP1 is a crucial player in DNA repair. In the clinic, PARP inhibitors have been used to induce synthetic lethality to tumor cells that are already deficient in HR repair. Here we hypothesize that in HR-competent, PARP inhibition will favor HR-mediated ALT repair in the cells to elongate their telomere lengths. In the present work, we show that PARP inhibition elongated the telomere lengths in both wildtype and Terc knockout mouse embryonic stem cells (ESCs). Similarly, PARP inhibition also elongated the telomere lengths in human induced pluripotent stem cells (iPSCs). The present work demonstrates that PARP inhibition is an effective means to elongate telomere lengths in HR-competent mammalian stem cells, including ones that are telomerase defective, and suggests that PARP inhibition drugs may be repurposed to benefit telomere syndrome patients.

Funding Source: National Science and Technology Council, Taipei, Taiwan, R.O.C. Grant number NSTC 112-2313-B-002-036

Keywords PARP inhibition, telomere length, pluripotent stem cells

4:45 PM – 5:45 PM

266

EROSION OF X-CHROMOSOME INACTIVATION IN FEMALE HIPSCS IS HETEROGENEOUS AND PERSISTS DURING DIFFERENTIATION

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The erosion of the inactive X chromosome (Xi) in female human pluripotent stem cell (hPSC) cultures disrupts normal XIST RNA expression and leads to reactivation of a subset of X-linked genes. Despite its common occurrence, this phenomenon is often overlooked by the stem cell community, with implications for both fundamental and translational applications of hPSCs remaining incompletely addressed. Investigating erosion dynamics in female induced pluripotent stem cells (hiPSCs), our study reveals that XCI erosion is a frequent yet heterogeneous phenomenon, resulting in reactivation of several X-linked genes. The likelihood of a gene to erode increases for escapees, those located on the short arm of the X chromosome, and within H3K27me3-enriched domains. Importantly, increased X-linked gene expression upon erosion does not globally impact (hydroxy) methylation levels in hiPSCs or at imprinted regions. Our study further explores the consequences of erosion during trilineage commitment and cardiac differentiation, demonstrating that XCI erosion does not hinder specification to ectoderm, mesoderm, and endoderm or generation of functional cardiomyocytes. Importantly, the erosion pattern persists during differentiation, carrying implications for basic, translational, and clinical applications. We underscore the importance of raising awareness within the stem cell community regarding XCI erosion and advocate for its inclusion in comprehensive hiPSC quality control.

Funding Source: This work was funded by the X-HUMAN 2022.01532. PTDC Grant from Fundação para a Ciência e a Tecnologia (FCT). Simão T. da Rocha is supported by an assistant research contract 2021.00660. CEECIND from FCT.

Keywords: epigenetics, X-chromosome inactivation, human iPSCs

4:45 PM – 5:45 PM

268

THE EUROPEAN BANK FOR INDUCED PLURIPOTENT STEM CELLS: A SUSTAINABLE AND WORLDWIDE IPSC REPOSITORY

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Since their introduction in 2007, induced pluripotent stem cells (iPSCs) have transformed biomedical research by offering unprecedented opportunities for in vitro disease modelling, drug discovery, and personalised medicine using physiologically relevant human cell models. In response to the growing demand for standardised iPSC resources, the European Bank for Induced Pluripotent Stem Cells (EBiSC) was established in 2014. EBiSC serves as a centralized platform and infrastructure for the acquisition, storage, and dissemination of iPSCs and associated data, aiming to enhance reusability and reproducibility in iPSC research endeavours. The primary objective of EBiSC is to facilitate the sharing of iPSCs derived from donated samples across diverse disease contexts. By providing access to a comprehensive collection of iPSC lines along with associated protocols, training materials, and openly accessible datasets, EBiSC enables iPSC research within both non-profit and commercial organisations. This initiative was initiated under two funded project phases as public-private partnerships, receiving support from the Innovative Medicines Initiative and the European Federation of Pharmaceutical Industries and Associations (EFPIA). To enable long-term sustainability, it is now implementing a model that ensures continued availability and accessibility of iPSC resources. A cornerstone of EBiSC's approach is its collaborative biobanking model by being jointly supported by two not-for-profit research organisations: Fraunhofer UK and Fraunhofer IBMT. By sharing lessons learned and best practices in iPSC collection, characterisation, and distribution, EBiSC aims to enhance the usability and impact of iPSC resources while maintaining rigorous standards for quality control, ethical governance, and data management. In conclusion, EBiSC provides a vital infrastructure for the standardized acquisition and sharing of iPSC resources across Europe and beyond. Through its collaborative efforts, EBiSC seeks to advance scientific knowledge, accelerate understanding of disease, and ultimately improve patient outcomes, thereby benefiting researchers, patients, and the broader scientific community alike.

Funding Source: This work has received support from the EU / EFPIA / Innovative Medicines Initiative 2 Joint Undertaking (EBiSC grant n°115582 and EBiSC2 grant n°821362). This communication reflects the author's views.

Keywords: iPSC, quality control, disease

4:45 PM – 5:45 PM

270

CROSSTALK OF SYMPATHETIC NEURONS AND CARDIOMYOCYTES IN A PATIENT-SPECIFIC INDUCED PLURIPOTENT STEM CELL TAKOTSUBO-MODEL

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Takotsubo syndrome (TTS), also known as stress-induced cardiomyopathy, is characterized by an acute left ventricular dysfunction, usually in the absence of stenosis. Because a dysregulated central autonomic nervous system resulting in sympathetic overstimulation is suspected to be involved in developing the cardiac phenotype, TTS can be described as a brain-heart syndrome. Previous work identified a significantly higher catecholamine concentration in patients with acute TTS. Our previous work identified enhanced sensitivity to catecholamine-induced stress toxicity and a genetic predisposition as mechanisms associated with TTS by using a TTS patient-specific induced pluripotent cardiac stem cell (iPSC) model. Here, we aim to investigate whether iPSC-derived TTS sympathetic neurons (SN) impact iPSC-cardiomyocytes (CM) and whether they also contribute to the development of TTS. TTS-iPSC-lines harboring different genetic variants were used to establish a 3-step protocol, including neuronal progenitor aggregates and 2D monolayer stages. Immunohistochemical staining and qPCR data showed SN-specific marker expression (PHOX2B, TH, and DBH) increase with a characteristic ganglionic receptor profile (CHRNA3, CHRNB4) throughout the differentiation. Quantitative analyses by FLOW cytometry of 25-day-old SN indicated ~80% of the population's autonomic progenitors (PHOX2B+ cells). Finally, basal and KCl-stimulated noradrenaline secretion proved SN functionality. In the next step, we established a protocol for CM and SN co-culturing. We demonstrated crosstalk of both cell types by mimicking a stress-triggered response from the SNs using the specific nicotinic acetylcholine receptor agonist Cytisine. Enhanced beating frequency of co-cultured iPSC-CM was demonstrated using a multielectrode array. In a mix-and-match approach, the neuronal TTS mutagenic influence on the cardiac phenotype will be further analyzed. In conclusion, we established an effective protocol for the differentiation of functional iPSC-SNs that can be used in co-culture of TTS-iPSC-CM and -SN to confirm the functional interaction between both cell types. Investigating the impact of TTS genetic predisposition on sympathetic neurons next to cardiomyocytes is crucial to identifying potential drug targets to treat TTS.

Keywords: Takotsubo syndrome, induced pluripotent stem cells, sympathetic overstimulation

4:45 PM – 5:45 PM

272

ADVANCED 3D CELL MODELS FOR NEXT GENERATION THERAPEUTICS: STANDARDIZED ORGANOID CO-CULTURES AND ASSAYS IN MICROWELL PLATES

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Clinical trials for drug candidates have a high failure rate, often due to disparities between in vitro and in vivo efficacy. Addressing this challenge requires improved cell models that replicate organ-specific biology to identify relevant therapeutic targets. Organoids, capable of mimicking native tissue complexity, offer a promising solution that recapitulates in vivo physiology in an organ-specific context. However, they present numerous challenges regarding reproducibility, scalability, and analyses. Organoid cultures heavily rely on a solid extracellular matrix (ECM), leading to pronounced size and shape heterogeneity, and imaging difficulties. The organoids are trapped in the ECM and are not equally accessible to other cell types and molecules, hindering the establishment of co-cultures and robust assay development. Here we present a novel approach to standardize multicellular (co)-cultures: Gri3D®, a ready-to-use platform for high-throughput and reproducible organoid culture. Based on an array of microwells, the platform enables the high-throughput generation of single organoids of precise sizes in suspension-like conditions, and in a single focal plane. The system includes a pipetting port for safe media exchange. It facilitates the assessment of next-generation therapeutics, such as bispecific antibodies, immune modulators, or engineered immune cells, which are in direct contact with organoid models. We showcase two compelling applications of Gri3D® in assessing next-generation therapeutics. Firstly, its role to study receptor-mediated antibody transcytosis within blood-brain barrier organoid arrays. Secondly, its utility in evaluating the off-target toxicities of CAR T-cell therapy on adult stem cell-derived gastrointestinal organoids. The microwell system significantly enhances the efficiency and reproducibility of 3D organoid and co-culture workflows. It integrates with automation and supports a wide range of assays, including traditional whole-well luminescence and fluorescence-based approaches, as well as high-throughput image-based screening assays.

Keywords: organoid, immunotherapies, microwell

4:45 PM – 5:45 PM

TOPIC: PANCREAS

274

ADVANCING TYPE II DIABETES MODELING: FROM IMPROVED STEM CELL PROTOCOLS TO NOVEL USER-FRIENDLY MICROPHYSIOLOGICAL PLATFORM

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There is a critical need for improved in vitro systems to model type II diabetes to advance therapeutic drug candidates to preclinical studies. Significant advances have been made in this direction, with human pluripotent stem cells (PSCs) holding the highest potential as a limitless source of β-cells for disease modeling and cellular therapy for diabetes. While many protocols have been published describing the differentiation of insulin-expressing cells from PSCs, it is unclear which yield the most functional cells with reproducible glucose-stimulated insulin secretion (GSIS). In this study, we performed a side-by-side comparison between conventional and state-of-the-art protocols to determine which yields the most functionally mature cells. Our results unequivocally demonstrate that the newer protocols yield clusters with enhanced maturity, characterized by mono-hormonal insulin expression and robust, reproducible GSIS. Besides being glucose-responsive, these pancreatic aggregates exhibit responsiveness to incretin hormones, further underscoring their physiological relevance. Additionally, we demonstrated that cryopreserved pancreatic progenitors yield aggregates that are capable of GSIS. Lastly, our team has developed an easy-to-use, low-cost pillar/perfusion plate that does not require pumps and specialized equipment and allows for studies of inter-organ endocrine crosstalk. Overall, this platform has the potential to facilitate drug development by providing more predictive outcomes and human models for preclinical studies.

Keywords: functional SC-islets, pancreatic cryopreservation, inter-organ crosstalk platform

4:45 PM – 5:45 PM

276

VOLUMETRIC BIOPRINTING OF AN ENDOCRINE PANCREAS PLATFORM FOR DRUG SCREENING USING FUNCTIONAL HUMAN IPSC-DERIVED ISLETS

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Pancreatic islets of Langerhans regulate circulating glucose levels. Loss or dysfunction of beta cells in the islets results in diabetes mellitus, which affects over 500 million people worldwide. The current treatment for diabetes relies on the delivery of insulin, sometimes in combination with other insulin-sensitizing drugs, but does not recapitulate the innate glycemic control. Thus, novel therapies to address the limitations of the current treatments are needed. Bioprinting, biomaterials, and (stem) cell engineering provide a novel toolbox to design bioinspired systems that recapitulate salient organ functions. This study combines the generation of induced pluripotent stem cells (iPSC)-derived islets with light-based bioprinting to engineer an advanced in vitro model of the endocrine human pancreas. Human pancreatic islets were generated from iPSC following a seven-stage protocol. When reaching stage 7, iPSC-derived islets were collected and pancreatic constructs were generated via layerless and shear stress-free, light-based Volumetric Bioprinting (VBP). Concentrations ranging from 2%-7% (w/v%) of optically tuned bioresins, namely gelatin methacrylate (GelMa) and alginate methacrylate (AlgMa), were used. iPSC-derived islets were characterized via immunofluorescence and single cell transcriptomics. Insulin release on bioprinted constructs was assessed with a static and dynamic (flow of 100 µL/min for two hours) glucose stimulated insulin secretion (GSIS) assay. VBP allowed to produce 50-70 mm3 pancreatic constructs in 30 s and posed no mechanical or chemical stress on iPSC-derived islets, which were highly viable for 21 days after printing. Stiffer, higher polymer content GelMa hydrogels presented the highest islet viability. Islet metabolic activity remained constant over time. Bioprinted islets showed mature, insulin, glucagon and somatostatin, single-hormone producing cells after 21 days both characterized via immunofluorescence and single cell transcriptomics. Bioprinted islets are functional and possess a glucose-responsive insulin secretion profile, and the system is compatible with supplementation with anti-diabetic drugs. Overall, this technology opens up to new possibilities on developing a novel platform for the in vitro testing of islet functionality.

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Keywords: endocrine pancreas, diabetes, bioprinting

4:45 PM – 5:45 PM

278

GENERATION OF HUMAN STEM CELL DERIVED-ALPHA-LIKE CELLS THAT HAVE IMPAIRED REGULATION OF GLUCAGON SECRETION FOR TYPE 2 DIABETES RESEARCH

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Glucagon produced by pancreatic alpha cells is crucial in maintaining glucose homeostasis. However, individuals with type 2 diabetes (T2D) experience hyperglucagonemia both when fasting and after meals, leading to increased glucose production in the liver and exacerbating hyperglycemia of this condition. Despite the longstanding awareness of this phenomenon, scientific inquiry into the functioning of alpha cells, particularly in the context of T2D, has historically received less attention compared to research on beta cells and insulin. Here, we developed a protocol for generating alpha-like cells from human stem cells (SC-alpha) that have impaired regulation of glucagon secretion while maintaining insulin secretion function that might be useful for T2D modeling and drug development in vitro. In a 6-stage stepwise planar differentiation process, we obtained pancreatic progenitor cells (Stage 4), with over 50% positive for PDX1 and fewer than 10% positive for NKX6.1. The ARX protein that maintains alpha cell identity was activated and detectable at this stage. By targeting the cytoskeleton with latrunculin A at the first 24 hours of stage 5, we could generate >30% percent of Glucagon+ cells at day 7 of stage 5. Continuing differentiation until day 7 of Stage 6, and then suspending the cells in ultralow attachment cell culture plates, we obtained >55% of Glucagon+ cells, and >40% of cells were positive for C-peptide. Under 20 mM of glucose stimulation, these SC-alpha cell clusters showed a significant increase in insulin secretion (Insulin secretion index=1.14, $P< 0.01$), and a significant increase in glucagon secretion (Glucagon secretion index=2.10, $P< 0.05$). In contrast, the detected glucagon secretion from stem cell-derived beta-like cell clusters following the published protocol was negligible and nonsignificant (Glucagon secretion index=0.91, $P>0.05$). Overall, the resulting SC-alpha cell clusters represented impaired regulation of glucagon secretion ability while still retaining a normal but inefficient insulin secretion ability, which might play a role in T2D research.

Funding Source: Southern and Eastern Norway Regional Health Authority, project number 2023028. UiO: Life Science, Summer research projects for students.

Keywords: glucagon, stem cell differentiation, human stem cell-derived alpha-like cells

4:45 PM – 5:45 PM

280

STEM-CELL DERIVED BETA-LIKE CELLS FROM PATIENTS WITH CYSTIC FIBROSIS RELATED DIABETES PROTECT MICE FROM DIABETES

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Cystic fibrosis is a disease best known for its detrimental effects on lung function, but other organs are also affected. CFTR mutations affect the pancreas, and approximately 50% of adult patients with CF develop cystic fibrosis associated diabetes (CFRD). The management of one, let alone two serious diseases is exceedingly challenging. If normal glucose homeostasis in CF patients could be restored, it would greatly increase the quality of life, and likely decrease mortality. To reach this therapeutic goal, we first need to understand the mechanisms of beta cell dysfunction in CFRD. Here we show that stem cell derived beta cells from subjects with CFRD show normal differentiation, normal function including insulin synthesis and stimulated secretion. We also show that stem cell derived beta cells can regulate blood glucose levels in a mouse model of diabetes. Therefore, beta cell dysfunction in CFRD patients is not due to CFTR deficiency in the beta cell, but secondary to a compromised integrity of the organ within which the beta cells reside, the pancreas. Because CFRD is not caused by autoimmunity, grafting autologous stem cells may not require immune protection. Thus, beta cell replacement in CFRD subjects is an attainable goal.

Funding Source: This work was supported by the CF foundation, by the Leona and Harry Helmsley Charitable Trust for the collection cell lines 2015PG-T1D069, the NYSCF-Robertson award to D.E., and by the American Diabetes Association 1-16-ICTS-029.

Keywords: CFTR, beta cells, autologous cell therapy

4:45 PM – 5:45 PM

282

SCALABLE GENERATION OF 3D PANCREATIC ISLET ORGANOIDS FROM HUMAN PLURIPOTENT STEM CELLS IN SUSPENSION BIOREACTORS

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We describe a scalable method for the robust generation of 3D pancreatic islet-like organoids from human pluripotent stem cells entirely in suspension bioreactors. Our protocol involves a 6-stage, 20-day directed differentiation process, resulting in the production of 104-105 organoids. These organoids comprise α- and β-like cells that exhibit glucose-responsive insulin and glucagon secretion. We detail methods for culturing, passaging, and cryopreserving stem cells as suspended 3D clusters, and for differentiating them through specific growth media and exogenous factors added in a stepwise manner. Additionally, we address quality control measures, troubleshooting strategies, and functional assays. Finally, we illustrate research applications to 1) Generate bioelectronics-implanted, "cyborg" islets for multimodal charting of maturing α- and β-cell functional and gene expression states, 2) Reveal how circadian clock and splicing controllers shape islet functional maturity, 3) Study how functional maturity steers islet inflammation in autoimmune diabetes. These approaches and resulting data inform efforts to create fully functional human stem cell-derived islet organoids in the laboratory for basic research and regenerative medicine.

Keywords: directed stem cell differentiation, pancreatic islet beta cells, 3D organoid engineering

4:45 PM – 5:45 PM

284

DISRUPTION OF RFX6 IMPAIRS IPSC-DERIVED ISLET ORGANOID DEVELOPMENT AND VIABILITY, WHILE MAINTAINING PDX1+/NKX6.1+ PROGENITOR FORMATION

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Sequencing studies have revealed that RFX6 defects are linked to both monogenic and polygenic forms of diabetes. While it is established that homozygous mutations in the RFX6 gene result in neonatal diabetes with a hypoplastic pancreas, the precise mechanism driving this remains unclear. To fill this knowledge gap, we examined the expression patterns of RFX6 in distinct populations at various stages, utilizing single-cell analysis and hESCs with an HA tag knock-in to RFX6. Furthermore, we generated multiple iPSC lines with RFX6 knockout (RFX6 KO iPSCs) to evaluate its influence on different stages of pancreatic development. RFX6 expression was detected in PDX1+ cells in the hESC-derived posterior foregut (PF). However, in the pancreatic progenitors (PPs), RFX6 did not co-localize with PDX1 and NKX6.1, but instead with NEUROG3, NKX2.2, and islet hormones in the endocrine progenitor (EPs) and islets. Single-cell analysis revealed elevated RFX6 expression in endocrine clusters across various hESC-derived pancreatic differentiation stages. Upon differentiating iPSCs lacking RFX6 into pancreatic islets, a significant decrease in PDX1 expression at the PF stage was observed, although it did not affect PPs co-expressing PDX1 and NKX6.1. RNA sequencing uncovered the downregulation of essential genes involved in pancreatic endocrine differentiation, insulin secretion, and ion transport due to RFX6 deficiency. Furthermore, RFX6 deficiency results in the formation of smaller islet organoids due to increased cellular apoptosis, linked to reduced Catalase (CAT) expression, implying a protective role for RFX6. Overexpression of RFX6 reverses defective phenotypes in PPs and EPs. These findings suggest that pancreatic hypoplasia and reduced islet cell formation associated with RFX6 mutations are not due to alterations in PDX1+/NKX6.1+ PPs but instead result from cellular apoptosis and downregulation of pancreatic endocrine genes. Our study presents a valuable human islet model, offering a platform for the exploration and identification of novel therapeutic targets.

Keywords: transcription factors, endocrine specification, pancreatic hypoplasia, diabetes

4:45 PM – 5:45 PM

286

CELL-TYPE-SPECIFIC TRANSCRIPTIONAL AND CHROMATIN ANALYSIS OF ISLET STRESS RESPONSE

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Pancreatic beta cell death or dysfunction is a hallmark of type 1 diabetes (T1D). As diabetes develops and progresses, it triggers an increase in both endoplasmic reticulum (ER) and inflammatory stress. These stress mechanisms have been observed in primary human islets and stem cell-derived islets (SC-islets) after transplantation

into mouse models. However, previous studies have not examined cell-type-specific responses. Such responses are important due to the heterogeneous nature of the islet of Langerhans. Here, we utilized single-cell RNA and ATAC sequencing to investigate stress responses in primary human islets to thapsigargin, cytokines (IL1B, IFNG, TNFa), or Brefeldin A. Our results showed that beta, alpha, and ductal cells are the most susceptible to stress compared to other cell types. Many of the responses in our system mimic transcriptional differences found in T1D patients. From this data, we then leveraged SC-islets to analyze candidate genes that modulate stress responses. We found that CIB1, a calcium-binding protein, was upregulated under stress conditions and, when knocked out in SC-islets, showed disruption to function. In addition, using drug-gene interaction databases, we identified drugs that can reduce apoptosis under stress conditions in SC-islets. Our data provide a valuable resource to comprehend cell-type-specific responses to stress, which is critical in understanding why beta cells are preferentially attacked during diabetes, which is currently unknown, and provides candidate genes for further investigation.

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Keywords: endoplasmic reticulum and inflammation in pancreatic islets, single-cell RNA and ATAC sequencing, stem-cell derived islets

4:45 PM – 5:45 PM

288

DIABETES-ASSOCIATED REGULATORY SEQUENCES REVEAL NOVEL PATHWAYS IN PANCREATIC DEVELOPMENT AND DISEASE

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Rare early onset cases of diabetes stem from single gene mutations, whereas frequent gene variants can also contribute to multifactorial diabetes, indicating shared disease mechanisms. Our recent study identified ONECUT1 as a gene implicated in both monogenic and multifactorial diabetes, establishing it as crucial target for investigating key signaling pathways in diabetes pathology. However, many diabetic characteristics remain unexplained by typical genetic alterations, highlighting the underestimated significance of the non-coding genome. We identified a neonatal diabetes patient carrying a heterozygous ONECUT1 truncating variant. Remarkably, the patient exhibited a phenotype akin to individuals with homozygous ONECUT1 truncating mutations. We detected an additional heterozygous deletion in the non-coding genome, which led us to hypothesize that it could impact the regulation of the remaining ONECUT1 allele. Consequently, the dysregulation of the ONECUT1-dependent transcriptional machinery crucial for proper betacell development occurs, ultimately leading to the severe diabetic phenotype observed in the patient. Following deletion of specific genomic regions in human embryonic stem cells, we initiated their differentiation into the pancreatic endocrine lineage and meticulously characterized the developmental stages. Importantly, we replicated the patient's diabetic phenotype, elucidating deficiencies in both pancreatic development and betacell functionality. The differentiation of pancreatic progenitors was notably hindered by the loss of the regulatory region associated with nearly complete ablation of ONECUT1. Moreover, the stem cell-derived islets exhibited a reduced number of b-cells as well as impaired insulin secretion even in heterozygous knockouts. Altogether, our ongoing comprehensive mechanistic characterization will provide insights into the precise role of the identified regulatory elements in pancreatic betacell development and diabetes pathophysiology, emphasizing the significance of the non-coding genome. By unveiling unknown molecular pathomechanisms linked to alterations in regulatory sequences, we contribute to a deeper understanding of the pancreatic transcriptional network and establish the foundation for personalized diabetes treatment.

Keywords: non-coding genome, monogenic diabetes, stem cell-derived islets

TRACK: EQUITY, DIVERSITY AND INCLUSION (EDI)

4:45 PM – 5:45 PM

290

EXPANDING PRECISION MEDICINE TO ADDRESS TREATMENTS FOR PATIENTS WITH NEUROPSYCHIATRIC DISEASES

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Induced pluripotent stem cells (iPSCs) researches are developing more specific treatments for neuropsychiatric diseases. However, it is necessary to expand the access to this technology to increase the efficacy of precision medicine. The goal of this project is to study the mental health and the biology of individuals from Aracatu, a town located in the state of Bahia, in Brazil. There are approximately 14,000 people living in this town, the majority at rural areas. Aracatu's population has been declining and there are also reports of high incidence of consanguineous marriages. The demographic features of this town may allow to access biological information of individuals that carry very important genetic constructions with ethnic and ancestral traits poorly studied since far. To evaluate the potential of iPSCs in addressing treatments for neuropsychiatric diseases, some key words such as "mental health", "efficacy", "drug", and "stem cell" were searched in the PubMed database. The "AND" operator was used between each term. The search was also filtered for: "Clinical Trial", "Meta-Analysis", "Randomized Controlled Trial", and "Systematic Review". As an inclusion criterion, only papers addressing neuropsychiatric diseases were add to research. As an exclusion criterion, papers that did not use stem cell approaches were removed from research. The search found 8 papers and further applying the selection criterion, 2 papers were analyzed. In general, these approaches transform cells from skin biopsies into iPSCs. Next, the transformation of iPSCs into neurons allows to better understand what is happening with the brain cells of individuals with neuropsychiatric diseases. In addition, these iPSCs-derived neurons can also be used to evaluate which drug could be more suitable for each patient. Mental health care is still neglected and social, economic

and cultural backgrounds are barriers that limit access and adherence to care and necessary treatments. Financing initiatives that diversify the studied population potencialize our comprehension about the human health. It is also expected improvements on the self-perception of the culture and local values, which strength the regional identity and people well-being. In conclusion, this study may contribute to expand the access to precision medicine.

Keywords: precision medicine, access, metal health care

4:45 PM – 5:45 PM

292

CONTEMPORARY CELL AND GENE THERAPIES: INTEGRATION OF ACADEMIC, CLINICAL, AND INDUSTRY NEEDS AND PERSPECTIVES IN A NEW GRADUATE MASTERS SUBJECT

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Multiple cell and gene therapies have recently achieved clinical approval, and numerous others are in the process of regulatory approval globally. The Alliance for Regenerative Medicine's Workforce Report "Gap Analysis for the Cell and Gene Therapy Sector", among others, have identified workforce shortfalls as a significant challenge for the future of the sector internationally. At The University of Melbourne, we have developed a pipeline of stem cell education in undergraduate programs including the Bachelor of Science and Bachelor of Biomedicine. More recently, we have delivered a new graduate coursework subject in the Master of Biomedical Sciences, "Contemporary Cell and Gene Therapies". This subject was deliberately designed to address challenges facing the cell and gene therapy sector, by incorporating and embedding industry into the curriculum. This subject is a unique offering for a tertiary institution in Australia. This subject focussed on the gaps of knowledge and expertise associated with the successful development of cell and gene therapies including translation, regulation, manufacturing, and patient needs and perspectives. In addition, the subject consciously addressed career opportunities within academia, industry, and those that interface academia and industry. Students were exposed to National and International experts from varied backgrounds, and through 'purpose and product' completed assessments that developed skills and knowledge in the aforementioned areas. Students were anonymously surveyed on their experience at the beginning, middle and end of semester using Likert scale and open ended questions. Results indicate that students found the frontier nature of the subject intellectually engaging, appreciating opportunities to actively engage with guest speakers and academic co-ordinators. Group work and collaboration could be enhanced, and incorporation of more ethics and health economics were desired. Regardless of the student's research project and background, the majority of students gained confidence in the field of cell and gene therapies, and developed skills useful for their future careers. This subject is innovative in its approach and reach and reflects the current global need for an expansion of education in the field of cell and gene therapies.

Keywords: education, cell and gene therapy, industry

4:45 PM – 5:45 PM

294

REGULATORY FRAMEWORK FOR BIOETHICAL AND LEGAL USE OF STEM CELLS IN LATIN AMERICA: A CALL-TO-ACTION

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Human stem cell research in Latin America raises serious ethical and regulatory controversies with a recent increase in the establishment of private clinics offering unregulated therapies based on insufficient scientific rationale and clinical evidence. Latin America must restructure itself to face the challenges posed by stem cell research and provide an appropriate environment to halt stem cell tourism and gain intellectual sovereignty. Many jurisdictions have intended to approve legislations to fill the gaps governing the use of stem cells, exemplified by the Panamanian Executive Decree No. 179 of June 8, 2018. Five years since the N°179 decree approval, makes the appropriate timing for a critical SWOT analysis of the progress, setbacks, opportunities, strengths, and weaknesses that different regulations in Latin America have made to solve this issue, being the purpose of this talk. Regulations stating that stem cell clinical trials must be evaluated by the National Committee of Bioethics in Research with the advisory role of the National Transplant Committee regarding safety and protection; trials must be conducted in centers with licenses granted for the extraction and transplantation of anatomical component; and that related research must follow the regulatory process of an investigational new drug by the National Directorate of Pharmacy and Drugs of the Ministry of Health, among other policies, have diminished the licenses granted to clinics offering unapproved stem cell treatments. However, the unfortunate lack of sanctions applied to illegally established clinics has weakened regulatory actions and caused a recent increase in the proliferation of clandestine centers advertising stem cell tourism. The collective efforts in Panama to strengthen legislations regarding stem cell research and its clinical translation seeks to halt premature commercialization of unapproved stem cell treatments and exemplifies future actions that other countries can take in regulatory formulations. The pressure received in recent years from other sectors that seek to abolish current regulatory policies urges a call-to-action to reinforce international collaborations that may collectively strengthen stem cell-related legislations in Latin America and other regions.

Funding Source: Gorgas Memorial Institute for Health Studies
Comité Nacional de Bioética de la Investigación Sistema Nacional de Investigación (SNI), SENACYT

Keywords: regulatory policies, ethics, Latin America

TRACK: ETHICS, POLICY, AND STANDARDS (EPS)

4:45 PM – 5:45 PM

TOPIC: ETHICS

296

WHO SHOULD BE INCLUDED IN FIRST-IN-HUMAN TRIALS? A SYSTEMATIC REVIEW OF REASONS

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First-in-human (FIH) trials mark a significant turning point in translational research, with novel therapies being tested in humans for the first time. Who should be included in FIH trials is a topic of ongoing debate amongst researchers and clinicians and an emerging topic within bioethical literature. Yet, no comprehensive overview of the literature on this topic has been constructed before. Therefore, this systematic review of reasons provides the first comprehensive overview of the reasons for and against the inclusion of different participant groups for FIH trials. 170 reasons were identified amongst six potential participant categories: healthy volunteers, patients (general), patients with less advanced-stage diseases, patients with more advanced-stage diseases, vulnerable populations, and diverse participant groups. These reasons were then classified under seven broader themes: non-maleficence, beneficence, scientific value, efficiency, respect for persons, duty of care, and justice. This review highlights multiple challenges within the existing literature, including ambiguous or poorly defined reasons, a tendency to confuse FIH research with (medical) care, and the complexity of identifying and defining vulnerable populations. Finally, by drawing on the results, recommendations for in- and exclusion criteria for FIH trials are offered.

Funding Source: Funded by reNEW, the Novo Nordisk Foundation Center for Stem Cell Medicine (NNF21CC0073729).

Keywords: first-in-human, clinical trials, participant selection

4:45 PM – 5:45 PM

298

DYNAMIC GOVERNANCE: A NEW ERA FOR CONSENT FOR STEM CELL RESEARCH

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Given rapidly evolving stem cell (SC) science, participatory research platforms have been signaled as effective tools to promote longitudinal participant engagement and interactive decision-making. Governance infrastructures have streamlined scientific and ethical provenance verification of human pluripotent SC lines (hPSC). Yet other technological developments are changing the nature of research and governance on stored biospecimens, calling into question the validity of previously obtained consent models, particularly in the context of unforeseen downstream uses (e.g. SC-derived embryo models, organoids, human-animal chimeras, synthetic embryos, etc.). Moreover, the adoption of e-healthcare platforms is driving major transformations in how data are collected, stored, processed, and exchanged, prompting a reappraisal of how consent is obtained and managed, particularly with respect to clinical data. In addition, health data literacy and trust in governance structures are novel elements that need to be addressed to educate participants about technical advances driving both digital infrastructures and hPSC usage. Informed consent is not an "ethics-clearing" exercise. It provides the opportunity for partnership through which participants and scientists can use shared understanding to navigate scientific and ethical frontiers. The complexities of the SC field and the wide spectrum of participants' (and other stakeholders') interests, expectations, and values can best be captured by a system that enables to broaden the scope of governance. Building on existing governance strategies providing ongoing support for broad consent frameworks, and against the background conventional (one-time) consent format, this presentation will explore the benefits and challenges of implementing dynamic consent and governance models for SC, including data management issues. Learning from regional examples piloting dynamic consent models for biobanking and SC research in Europe (e.g. Norway, Italy, Human Pluripotent Stem Cell Registry – hPSCreg), we explore the linkage between dynamic consent and governance, arguing for the need to shift focus from mere future use authorization to more interactive and robust participatory oversight of SC research and clinical translation.

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Keywords: pluripotent stem cells, ethics, governance

4:45 PM – 5:45 PM

300

EXPLORING HEALTHCARE PROFESSIONAL-PATIENT COMMUNICATION ABOUT STEM CELL THERAPIES - A SCOPING REVIEW

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Communication about stem cells between healthcare professionals (HCP) and patients can be complex. This can include discussing conventional (proven or regulated) stem therapies that are potentially curative and addressing queries about experimental (unproven or unregulated) stem cell therapies. The aim of this scoping review is to investigate what is known about HCP-patient communication when discussing stem cell therapies. Four electronic databases were systematically searched for relevant studies dated year 2000 to present. All identified citations were imported to Covidence online software. Following an inclusion and exclusion criteria, titles and abstracts were screened by two reviewers. Conflicts discussed and finalised with the help of a third reviewer. This process was repeated for full text reviews. Twenty-six papers met the inclusion criteria. A data collection tool was created, and two reviewers extracted data from included papers. Mixed method descriptive analysis, and a thematic analysis guided by a functions of medical communication research framework were performed on extracted data. Majority of included studies addressed communication in context of conventional stem cell therapies, such as bone marrow transplants for blood cancers or disorders. Few (n=7) investigated communication about experimental stem cell therapies, with the majority set in Australia (n=3) and the United States (n=3), from the perspective of physicians (n=5), and in context of varied health conditions. Thematic analysis suggests HCP-patient discussion about stem cell therapies is realised between different communication styles that do not always align to patient's needs, hopes or expectations. Findings suggest there is a mismatch in how information is sought, given, and received about experimental stem cell therapies. This scoping review highlights the importance of context, and a framework acknowledging environmental and personal determinants to HCP-patient communication helps inform strategies to support HCP navigate these interactions. This includes, recognising context of health conditions and healthcare settings, reflecting on HCP intra-personal skills, and considering the role of HCPs other than physicians.

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Keywords: healthcare professional-patient communication, stem cell therapies, health condition and health setting context

4:45 PM – 5:45 PM

302

STEM CELL LITIGATION IN THE US

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Unproven stem cell interventions are recognised as a severe issue in several countries. Disturbingly, some patients who underwent these dubious treatments suffer adverse effects, including fatalities, after receiving these treatments. Previously, these therapies were only offered in the developing world. However, such procedures exist today even in the developed world, including the US and Australia. There is already litigation against such clinics in the US, some of which have led to settlements. Some of the unscrupulous businesses that offered those therapies are now liquidated. One of the cases is Moorer v. StemGenex Medical Group, Inc. (StemGenex). StemGenex made claims that its product could treat diabetes and a wide range of autoimmune and neurological disorders such as lupus, multiple sclerosis, Crohn's disease, Alzheimer's disease and Parkinson's disease. After inspecting their San Diego facility, the FDA issued a warning letter to StemGenex. "StemGenex™ product appeared to be generated using technology based on a now-expired patent owned by AdiStem Limited. The technology involved adipose-derived stem cells acquired via liposuction and processed with platelet-rich plasma and lights of various wavelengths. The so-called "activated" cell mixture was then administered back to patients (autologously) via intravenous infusion or other routes. This presentation argues the importance of the law's role in stemming the problem of untested stem cell interventions. It explores the current law and regulation in this area and analyses the cases that have come before the court, including cases that involve class action.

Keywords: law, ethics, court cases

4:45 PM – 5:45 PM

304

ADDING A RISK-BASED APPROACH TO FUTUREPROOF THE ADVANCED THERAPY CLASSIFICATION PROCEDURE AND TO FILL THE REGULATORY GAPS BETWEEN IN VIVO AND EX VIVO GENE THERAPIES IN EUROPE

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Cell and gene therapies are classified as Advanced Therapy Medicinal Products (ATMPs) by the European Medicine Agency (EMA). There are three main types: gene therapy medicines, which act by inserting recombinant genes into the body; somatic-cell therapy medicines, which contain substantially manipulated cells/tissues or cells/tissues intended for non-homologous use; and tissue-engineered medicines, which contain cells/tissues that have been modified for the purpose of regeneration or replacement of human tissue. An ATMP classification affects the full development cycle of a therapy and opens doors to specific regulatory advice services and expert committees. A Risk-Based Approach (RBA) leads ATMP development through tailored guidelines, rather than a 'one size fits all' approach of traditional medicines. Patient outcome monitoring and post-marketing surveillance for ATMPs also differs from non-ATMPs. Innovative approaches that move towards virus-free gene editing and in vivo delivery of gene therapy components have uncovered gaps in the ATMP classification system. Because of the strict definitions and the lack of an intermediate medicinal product classified as a genetically modified organism, the classification system not always recognizes in vivo gene therapy as an ATMP. In these cases, theoretically, a therapy targeting the same genetic sequence, using the same components and resulting in the same genetic modification in the patient can either be an ATMP or not, solely depending on its delivery method. We studied the verdicts from the ATMP classification procedure published by the EMA and collected examples of therapies currently being developed as demonstrator examples of 'borderline' ATMP products, which risk being excluded due to the rigidness of the current definitions. We propose a new ATMP classification system in which the RBA is leading for the classification outcome, which allows for flexibility and will be more futureproof for new emerging innovative approaches. This prevents products that are ATMPs in spirit to be condemned to a 'one size fits all' developmental path, reducing delays and increasing product safety. For gene therapies, this will ensure products that have the same effect and risk profile to be treated equally and provide more transparency to patients and general public.

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Keywords: Advanced Therapy Medicinal Product (ATMP) classification procedure, regulatory science and policy, European Medicine Agency (EMA)

4:45 PM – 5:45 PM

306

DEFINED CULTURE CONDITIONS ROBUSTLY MAINTAIN HUMAN STEM CELL PLURIPOTENCY VIA TIGHTLY CONTROLLED CA2+ SIGNALING

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Induced pluripotent stem cells (iPSCs) have significant potential for disease modeling and cell therapies. However, their wide-spread application has faced challenges, including significant batch-to-batch variabilities, and notable distinctions when compared to embryonic stem cells (ESCs). Although it is known that some of these disparities stem from using undefined culture conditions and the reprogramming procedure, the precise mechanisms remain understudied. Here, we compared gene expression data from over 100 iPSC and ESC lines cultivated under undefined and defined conditions. Defined conditions significantly reduced inter-PSC line variability, irrespective of PSC cell type, highlighting the importance of standardization to minimize PSC biases. This variability is concurrent with decreased expression of somatic cell marker and germ layer differentiation genes and increased expression of Ca2+-binding proteins. The significance of tightly controlled Ca2+ signaling in hPSC pluripotency in defined conditions was also confirmed. A deeper understanding of these processes may aid in standardizing and improving defined hPSC culture conditions.

Keywords: human induced pluripotent stem cells, defined culture conditions, calcium signaling

4:45 PM – 5:45 PM

308

COST-EFFECTIVENESS OF STEM CELL-DERIVED THERAPIES FOR DIABETES: A SYSTEMATIC REVIEW TO INFORM POTENTIAL FOR COMMERCIALIZATION AND REIMBURSEMENT

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Diabetes is a global health concern that incurs significant economic and societal costs. Research activities are currently being conducted to develop advanced stem cell-derived therapies for diabetes type 1 that have the potential to improve health outcomes. These therapies, however, are associated with high costs and uncertainty regarding its cost-effectiveness, which affect its potential for commercialization and reimbursement. This review aims to provide an overview of available evidence on cost-effectiveness of stem cell-derived therapies for diabetes. We conducted a systematic review in PubMed/MEDLINE, EMBASE, and HTA databases. Titles and abstracts were screened based on inclusion and exclusion criteria. Relevant publications were subjected to full text screening to assess eligibility. A total of 487 publications were initially identified, of which three studies were eligible. These three studies conducted early cost-effectiveness analyses for a potential stem cell-derived islet cell product and identified scenarios wherein the product would be considered cost-effective. Compared to intensive insulin therapy, all three studies estimated the stem cell-derived islet cell product will increase life-long costs, with costs-effectiveness ranging from a relatively favorable €50,000 per quality-adjusted life years (QALY) to an unfavorable €300,000 per QALY. The studies highlighted the role of manufacturing strategy and production scale as key considerations that significantly affect cost and cost-effectiveness. Cost-effectiveness was also seen to be largely affected by the reduction in the need for immunosuppression and a longer graft survival. A major limitation is that early cost-effectiveness analyses are associated with uncertainty surrounding estimates due to unknown long-term outcomes, as well as uncertainties with the effectiveness data, cost estimates, and modelling assumptions. Despite uncertainties, the results of this review indicate that stem cell-derived therapies for diabetes can potentially be cost-effective provided that strategies are implemented to mitigate costs.

Funding Source: This review is conducted under the reNEW project supported by Novo Nordisk Foundation grant number: NNF21CC0073729

Keywords: stem cell, diabetes, cost effectiveness

TRACK: NEW TECHNOLOGIES (NT)

4:45 PM – 5:45 PM

TOPIC: CARDIAC

310

BIO-ENGINEERING AND RECELLULARIZATION OF CARDIAC-DERIVED EXTRACELLULAR MATRIX

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Patients with univentricular circulation are at the most severe end of congenital heart disease. They currently undergo the Fontan procedure, utilizing a rigid synthetic Gore-Tex conduit, leading to long-term complications due to its rigidity. Addressing the limitations of the Fontan conduit, a potential solution involves using decellularized extracellular matrix scaffolds (dECMs) reseeded with cardiac cells, with recent advancements in 3D bioprinting allowing for customized grafts. For future applications innervation is still a missing link necessary for the graft to respond to physiological demands. We have previously shown that in vitro, the presence of cells derived from the outer layer of the heart, so-called epicardium-derived cells (EPDCs), stimulates neuronal outgrowth, likely by a paracrine effect. We aim to investigate the potential beneficial effect of EPDC-derived extracellular matrix (EPDC-ECM) compared to porcine heart extracellular matrix (pECM) and their combination (ECMs combination) on neuronal and myocardial cell survival, maturation and neurite outgrowth. Preliminary results suggest hiPSC-derived cardiomyocytes (hiPSC-CMs) exhibit a more organized and dense sarcomeric structure when cultured on EPDC-ECM and a tendency of increased expression of maturation genes when cultured on ECMs combination. Additionally, PC12 neuronal cells showed increased survival on EPDC-ECM, and increased neurite outgrowth on ECM combination, compared to collagen. Ongoing work involves utilizing PC12 and/or hiPSC-CM suspensions with EPDC-ECM, pECM or ECMs combination for bioink development, aiming for 3D bioprinting a biological graft. Further analysis focuses on cell survival, maturation, and neurite outgrowth. In conclusion, preliminary data shows that EPDC-ECM and cardiac pECM support recellularization with hiPSC-CMs or PC12 neuronal cells. Additionally, a combination of EPDC-ECM and cardiac pECM might be suitable as a bioink for producing a 3D-bioprinted graft, supporting cardiomyocyte maturation, neurite outgrowth, and innervation.

Keywords: fontan, ECM, bioprinting

4:45 PM – 5:45 PM

312

TEMPO: A HIPSC LINE WITH GENETICALLY ENGINEERED FLUORESCENT SENSORS TO TRACK STRUCTURE, FUNCTION, AND CELL CYCLE PROGRESSION IN DEVELOPMENT AND MORPHOGENESIS

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During morphogenesis, cells undergo structural and functional changes while progressing through the cell cycle. Yet, imaging cell cycle progression, structure, and function is challenging since most sensors use green/red fluorescent proteins (GFP/RFP). To tackle this problem, we created FUCCIplex: a cell cycle sensor that can be multiplexed with most GFP/RFP-based sensors since it uses cyan (mTurquoise2) and far-red (miRFP670) fluorescent proteins to mark various cell cycle phases. We introduced FUCCIplex and the actin sensor RFP-LifeAct in WTC-11 hiPSCs that already expressed the functional calcium sensor GCaMP6f. To stop tracking the cell cycle in mature cardiomyocytes, we placed FUCCIplex under the control of the EF1alpha promoter, which is reportedly downregulated in terminally differentiated cells. We called this line TEMPO since our sensors synergize to enable the Tracking of Early and Mature Phenotypes Optically. To validate TEMPO, we combined live cell confocal imaging and single-cell RNA sequencing of hiPSCs differentiated in 2D and 3D cardiac models. We confirmed that FUCCIplex is highly expressed in hiPSCs and the three germ layers but is reduced along the cardiomyogenic lineage and fully switched off in cardiomyocytes exhibiting a mature cytoskeletal architecture (LifeAct) and robust calcium cycling (GCaMP). Finally, to show the advantage of TEMPO hiPSCs in testing drugs, we performed a study with nocodazole, an anti-cancer drug with cardiac side effects. In cycling non-cardiomyocytes derived from TEMPO hiPSCs, we recapitulated the drug's known mechanism of action: accumulation of cells in the M-phase due to tubulin depolymerization, followed by cell death. Since TEMPO hiPSC-derived cardiomyocytes did not express FUCCIplex, we used a far-red fluorescent die (SPY650-tubulin) to image the microtubule, actin, and calcium dynamics before, during, and after treatment with nocodazole (18 hr total). We showed that tubulin assembly and calcium transients were drastically affected while the contractile machinery remained active. Our data suggest that the TEMPO hiPSC line is a powerful and versatile platform for in-vitro cardiac morphogenesis and drug testing studies.

Funding Source: This work was supported by the ERC Stg SYNBIO.ECM #852560

Keywords: cardiac morphogenesis, cell cycle, drug testing

4:45 PM – 5:45 PM

314

ANALYSIS OF HIPSC-CMS ELECTROPHYSIOLOGY ON BIOCOMPATIBLE ELECTROMECHANOACTIVE SCAFFOLD

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Heart-on-a-chip for drug testing seems attractive approach to evaluate the possible cardiotoxic effects on cardiomyocyte viability and electrophysiology. The need in recapitulation of the native cardiac microenvironment for the advancement of human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) maturation is becoming increasingly evident. Our aim was to create a 3D system with a biocompatible scaffold, coated with a conductive layer, suitable for culturing and maturation of hiPSC-CMs and compatible with electrophysiology parameter measurement techniques. Elastic scaffold was fabricated by electrospinning of gelatin-glucose microfibers followed by the vapor-phase and electrochemical deposition of conductive polymer polypyrrole (PPy) to create electroconductive layer. hiPSC-CMs at different maturation levels (at 6 vs 14 weeks) were used to test biocompatibility of the scaffold. To increase cell attachment, scaffold was treated with 0.1 M HCl and coated with Geltrex. Cells were stained with fluorescent dyes (Calcein AM, DAPI). Electrophysiological properties were analysed using intracellular calcium imaging (Cal520) and action potential recordings with sharp electrodes. HiPSC-CMs on coverslips were used as a 2D control. Calcein AM staining showed that high number of hiPSC-CMs attached to gelatine/glucose/PPy scaffold proving that the scaffold is biocompatible and cells remain viable even after long term incubation (> 2 weeks). HiPSC-CMs at lower maturity (6 weeks) had better attachment properties to it, indicating that more mature cardiomyocytes (14 weeks) gradually lose their capacity to attach to PPy surface. Intracellular calcium imaging showed that both types of cells generated synchronous spontaneous calcium oscillations on gelatine/glucose/PPy scaffolds. Action potential recordings in 3D were similar to those in 2D, and confirmed differences in hiPSC-CMs maturation levels – at lower maturation level hiPSC-CMs had lower upstroke velocity as compared to more matured hiPSC-CMs. To conclude, gelatine/glucose/

PPy scaffold is biocompatible for hiPSC-CMs cultivation, and suitable for evaluation of cardiomyocyte electrophysiology properties. Such electromechanoactive scaffold can be further employed for development of heart-on-a-chip model.

Funding Source: Horizon2020 program "ElectroMechanoActive Polymer-based Scaffolds for Heart-on-Chip (EMAPS-Cardio)", no. 953138 — EMAPS-Cardio.

Keywords: cardiomyocytes, polypyrrole, electrophysiology

4:45 PM – 5:45 PM

316

SINGLE CELL TRANSCIPTIONAL PERTURBOME IN PLURIPOTENT STEM CELL ORGANOIDS

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Functional genomics screens in pluripotent stem cell (PSC) models offer immense potential yet are plagued by several specific challenges. These include high sensitivity to genotoxic nucleases, genetic and epigenetic clonal variability, asynchronous and heterogeneous differentiation, unstable transgene expression, and limited susceptibility to transfection and transduction after differentiation. Screens in emerging PSC-based organoids compound these challenges with increased cell type diversity and morphological constraints. To bridge this technological gap we developed iPS2-seq: iPS-optimized inducible. Postranscriptional Silencing in pool deconvoluted by single cell sequencing. This method allows phenotype-agnostic screens in PSCs and their derivatives through mRNA-depleting, clonally controlled, single cell aware, isogenic engineered, and stage specific loss-of-function (LoF) perturbations. iPS2-seq is compatible with both commercial microfluidics and homebrew split-pool scRNA-seq protocols, enabling a variety of screen designs in terms of scale, cost, and input material. We demonstrate this technology by studying congenital heart disease-associated genes in both monolayer cardiomyocytes and cardiac organoids derived from human induced PSCs (hiPSCs). iPS2-seq robustly assigns unique perturbations to >75% of analyzed cells, and identifies and controls for confounding effects arising from molecular cloning inaccuracies and iPSC clonal variability. Pseudotime analyses allows ranking of LoF perturbations by their effect on cardiomyogenesis. Clone- and induction-matched enrichment analyses in organoid cell type clusters identify genes involved in developmental bifurcations and highlights 3D culture-specific functions. In all, the iPS2-seq platform promises to standardize, strengthen, and democratize access to functional single cell genomics in and beyond hPSC-derived organoids.

Funding Source: ERC Starting Grant (TRANS-3; 101076026 - A.B.); Giovanni Armenise-Harvard Foundation Career Development Award 2021 (A.B.); Additional Ventures Single Ventricle Research Fund 2021 (A.B. and S.M.)

Keywords: single cell genomics, organoids, screening

4:45 PM – 5:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

318

DECIPHERING THE HETEROGENEITY OF DIFFERENTIATING HPSC-DERIVED CORNEAL LIMBAL STEM CELLS THROUGH SINGLE-CELL RNA-SEQUENCING

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A comprehensive understanding of the human pluripotent stem cell (hPSC) differentiation process stands as a prerequisite for the development hPSC-based therapeutics. In this study, single-cell RNA-sequencing (scRNA-seq) was performed to decipher the heterogeneity during differentiation of three hPSC lines towards corneal limbal stem cells (LSCs). The scRNA-seq data revealed nine clusters encompassing the entire differentiation process, among which five followed the anticipated differentiation path of LSCs. The remaining four clusters were previously undescribed cell states that were annotated as either mesodermal-like or undifferentiated subpopulations, and their prevalence was hPSC line-dependent. Distinct cluster-specific marker genes identified in this study were confirmed by immunofluorescence analysis and employed to purify hPSC-derived LSCs, which effectively minimized the variation in the line-dependent differentiation efficiency. In summary, scRNA-seq offered molecular insights into the heterogeneity of hPSC-LSC differentiation, allowing a data-driven strategy for consistent and robust generation of LSCs, essential for future advancement toward clinical translation.

Funding Source: Research Council of Finland, Sigrid Jusélius Foundation, National Eye and Tissue Bank Foundation Filand, Dutch Reserach Council (NWO), European Joint Programme Rare Diseases, ZonMw Open, COST Action CA18116.

Keywords: corneal limba stem cells, pluripotent stem cells, single-cell RNA-seq

4:45 PM – 5:45 PM

320

PREFABRICATION OF EPIDERMAL GROWTH FACTOR (EGF) LOADED ALGINATE-HYALURONIC ACID (ALGHA) MICROBEADS SYSTEM FOR PUNCH-INDUCED WOUNDS IN RAT MODEL

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Alginate is a naturally derived polymer that is widely used for tissue engineering to treat acute and chronic injuries by maintaining wound integrity. Hyaluronic acid (HA) is an important part of the extracellular matrix (ECM), and HA along with alginate is used for the preparation of hydrogels in biomaterials research. EGF is one of the growth factors that help in wound healing by inducing cell proliferation. As our ALG-HA bead system exhibited significant results related to wound healing, we aimed to test the effect of the same system on EGF for wound healing purposes. We have used alginate (Alg) and hyaluronic acid (HA) composite (80:20) beads for epidermal growth factor (EGF) delivery, using heparin as a cross-linker. Beads were prepared in CaCl2 at different concentrations; 90:10, 80:20, 60:40, 40:60, and 20:80. Based on the degradation behavior, the beads were prepared using 80:20 (AlgHa) along with 2 µg of heparin using 23-gauge needles. An injector (at the speed of 10 ml/hour) was used for the drop-by-drop pouring into 5 molar CaCl2 solution with continuous gentle stirring Encapsulated EGF showed significant EGF entrapment in the AlgHaEGF100 and AlgHaEGF150 groups along with the sustained release of EGF within five days. For in vitro studies, encapsulated beads demonstrated biocompatibility in rat L929 cells and significant migration at the concentration of 50 and 10 ng/ml of EGF within 24 hours. AlgHaEGF100 and AlgHaEGF150 significantly improved the expression of Fetal Liver Kinase 1 (FLK-1) and Intercellular Adhesion Molecule-1 (ICAM-1) in rbMSCs. In vivo assessment showed 69.12 % to 76.9 % of wound closure in the AlgHaEGF100 and AlgHaEGF150 groups, respectively. Both the treatment groups exhibited significant epithelialization and wound closure gaps within two weeks. Immunohistochemistry of both groups shows markedly significant levels of ICAM-1, FLK-1, and FN. Clinically, frequent dressing changes are one of the major barriers to wound healing. Our bead system is proposed to maintain therapeutic levels of EGF at the wound site, thereby avoiding the need for daily or frequent dressing changes. It can be a promising candidate for wound healing by providing convenience to both patients and healthcare workers.

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) 2020R1A2C1100891. This research was also partially supported by the Soonchunhyang University research fund.

Keywords: epidermal growth factor, microbead, wound

4:45 PM – 5:45 PM

322

IN SILICO MODELING OF INDUCED PLURIPOTENT STEM CELL POPULATION DYNAMICS TOWARDS AIRWAY EPITHELIUM

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Differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into endoderm derivates and specifically towards airway lineages has wide implications for the modeling and treatment of lung disease. As such, significant research has been conducted on establishing protocols for producing iPSC-derived definitive endoderm (DE) and early lung progenitor (LP) populations. These endeavors have primarily relied on in vitro and ex vivo trial and error experiments, with limited attention to utilizing in silico modeling needed for accelerated discovery by reducing time, cost, and variance and improving cell yields. We utilize a model development approach outlined previously by the authors, here focusing on modeling multicellular populations undergoing differentiation. Our strategy proposes multiple biology-informed differential equation-based models considering varying cellular differentiation lineages and cell growth models. These assumptions encompass the various states stem cells transition through to reach a differentiated state, along with different growth models. We then calibrate these models using in vitro data from BU3 NGST iPSCs, which are NK2 Homeobox 1 green fluorescence protein (NKX2.1-GFP) and Surfactant Protein C tdTomato red fluorescent protein (SFTPC-tdTomato) tagged. These reporters, which track the differentiation to lung progenitor cells and Type 2 alveolar epithelial cells, respectively, allow for tracking and quantifying the differentiation process. Finally, we identify the most suitable model by subjecting these models to rigorous selection processes. Our analysis shows that models without transitory cellular states better predict cell population evolution than models with one or two transitory states, something that corresponds to prior experimental observations. Also, in the observed models, space-limited growth models, e.g., logistic and Gompertz growth, outperform exponential growth models. Also, we show that a combined error function best defines model variance. To our knowledge, this is the first mathematical model of the population dynamics of multicellular cultures of iPSCs differentiating into DE. This model will be a tool for furthering our understanding of the differentiation process and optimizing it for various applications.

Funding Source: This study is funded by the Canadian Institutes of Health Research (CIHR) in partnership with the Natural Sciences and Engineering Research Council of Canada (NSERC), and Canada First Research Excellence Fund (CFREF).

Keywords: induced pluripotent stem cells, definitive endoderm, in silico modeling

4:45 PM – 5:45 PM

324

BIOINSPIRED HYALURONAN-BASED HYDROGEL FUELS BIPOTENTIAL LUNG ORGANOID FORMATION VIA PIEZO1 MECHANOSENSATION

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Lung diseases are leading global causes of death, arising from genetic disorders, infections, or environmental factors. Recent advancements in induced pluripotent stem cell differentiation, facilitate bronchiolar and alveolar lung organoid derivation, enabling research on lung hypoplasia, pathogenesis and fibrosis. However, the generation of these organoids through conventional Matrigel encapsulation relies on stem cells' self-organization with minimal external intervention, leading to challenges such as low reproducibility and shape heterogeneity. The absence of hyaluronan (HA) in Matrigel, a major extracellular matrix component in the native lung interstitium and alveolar space, complicates and retardates the specification of bronchiolar and alveolar cell types, making organoid generation inefficient and time-consuming. To address this, integrating stem cell biology, mechanobiology, bioengineering and AI-assisted organoid live tracking becomes crucial. Here, inspired by the natural lung habitat, a novel hybrid hydrogel combining HA and 20 % v/v Matrigel was created. Besides the biochemical cues, the viscoelastic and mechanical performance of HA hydrogel was also enhanced. The AI-based Segment Anything Model facilitated precise non-invasive detection of the dynamic changes in organoid morphology when cultured within the matrices. Organoid development in HA hydrogel was highly expedited compared to traditional Matrigel culture, yielding bipotential organoids containing both SOX9+ SOX2+ distal, SOX2+ proximal conductive airway cells and SOX9- SFTPC+ mature alveolar epithelial type 2 cells within 8 days. High levels of active integrin beta 1, Piezo1 and an approximately 3.4-fold increase in myosin IIA indicate mechano-sensing and transduction signaling activation induced by altered viscoelasticity from this hydrogel. This bioinspired approach offers a robust model for biomedical research, facilitating rapid investigation into treatments for respiratory diseases and understanding of surfactant trafficking. Further, it enables exploration of the underlying biomechanical mechanisms to improve the controllability of organoid generation on demand.

Funding Source: Helmholtz Association of German Research Centers through program-oriented funding, Helmholtz Imaging Project "AIOrganoid", China Scholarship Council (202208320106), Helmholtz Association-Munich School for Data Science.

Keywords: lung organoid, hyaluronan hydrogel, biomechanics

4:45 PM – 5:45 PM

326

LIPID NANOPARTICLES FOR MUTATION AGNOSTIC CORRECTION OF CYSTIC FIBROSIS-CAUSING MUTATIONS IN AIRWAY STEM CELLS

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Monogenic disorders like cystic fibrosis (CF) are appealing targets for gene therapies. Effective mutation-agnostic correction of CF-causing errors in the cystic fibrosis transmembrane conductance regulator (CFTR) gene requires genomic integration of large double-stranded DNA (dsDNA) cassettes that are difficult to package and deliver reliably to airway basal stem cells (ABSCs). We report the design and testing of lipid nanoparticles (LNPs) configured to transport CRISPR/Cas9 payloads designed for site-specific insertion of CFTR in ABSCs. Nanoparticle formulations were systematically screened with varying ratios of Cas9-encoding mRNA, single guide RNAs (sgRNAs), and donor DNA templates to optimize gene editing in a human bronchial epithelial cell line (16HBE14o-). For these tests, we applied dsDNA donors designed to insert a mCitrine reporter within the endogenous 5' untranslated region of CFTR in 16HBE14o- cells via homology directed repair (HDR) to optimize LNP-based delivery of the gene editing cargoes. Encapsulation of dsDNA donors with Cas9-mRNA and sgRNA in a single LNP yielded ca. 14% integration of the mCitrine reporter measured by digital droplet PCR (ddPCR) compared to ca. 10% when the donor was packaged separately. The dsDNA:Cas9-mRNA and nitrogen-to-phosphate (N/P) ratios were then varied to improve the integration of a CFTR-encoding cassette in 16HBEge-G542x CF mutant cells. LNPs with 3:1 w/w dsDNA:Cas9-mRNA demonstrated up to 3.5% integration. Restoration of CFTR function in edited cells was evaluated via Ussing chamber analysis. Initial validation of codon optimized CFTR dsDNA donors electroporated in 16HBEge-G542x cells restored CFTR-dependent Cl- current to ca. 80% of wild-type. Altogether, this versatile

LNP platform adds new capabilities for transporting large gene editing machinery to airway epithelial cells, paving the way for therapeutic solutions that achieve site-specific correction of any CFTR mutation in ABSC populations.

Funding Source: NIH DP5D028181 CIRM DISC0-14458

Keywords: gene delivery, epithelial stem cells, cystic fibrosis

4:45 PM – 5:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

328

INTRACELLULAR MECHANICAL PROPERTY CHANGES DURING NON-CANONICAL CYTOKINESIS IN MOUSE ONE-CELL EMBRYOS

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In fertilization, the gametes (sperm and oocyte) combine to produce a totipotent one-cell embryo. This behavior is governed by mechanically active matter, but intracellular mechanics are difficult to study directly and are poorly understood. We are now harnessing the relatively large size (in cellular terms) of one-cell mouse embryos (~100 micrometer diameter) and recent biophysical advances to address this challenge and assess mammalian intracellular mechanobiology. Fully internalized nanodevices within one cell embryos revealed intracellular rheological changes and identified a program of forces and changes to cytoplasmic mechanical properties that are apparently required for mouse embryo development from fertilization to the first cell division. High resolution, single-cell RNA sequencing (scRNA-seq) following polyadenylation-independent library preparation revealed that the onset of embryonic gene activation (embryonic gene activation, EGA) occurred in human and mouse embryos at the one-cell stage. Predicted upstream regulators of these genes are not only shared between mouse and human but are oncogenes. Inhibiting the mouse ortholog of MYC, c-Myc, which was predicted to drive EGA gene expression within four hours of fertilization (immediate EGA, iEGA) caused acute cytokinetic collapse: embryos arrested before or soon after the first cell division. Consistent with observations from live imaging and the tolerance of one-cell embryonic cell division to canonical cytokinetic inhibitors, iEGA includes unique set of genes that we suggest modulating non-canonical first cytokinesis. Taken together, these and other data suggest that novel cytokinetic features are shared by one-cell embryos and certain types of cancer cells. This work sheds light on the fundamental involvement of programmed mechanobiological and transcriptional changes during the establishment of totipotency and opens new windows to understanding parallels with cell division in cancer.

Keywords: mammalian embryonic gene activation, mammalian intracellular mechanobiology, cytokinesis

4:45 PM – 5:45 PM

330

DECODING MOUSE SEX DETERMINATION: THE PATH TOWARDS AN IN VITRO SERTOLI CELL DIFFERENTIATION MODEL

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During testicular development, Sertoli cells emerge as pivotal regulators marking the onset of sex-specific differentiation in gonadal cells. Despite the well-established understanding of Sertoli cell differentiation from their immediate progenitors, uncertainties persist regarding factors guiding gonadal primordium specification before embryonic day 10.5 (E10.5) in the mouse. This study aimed to bridge this knowledge gap by employing the novel 'DCM time-machine' technique as an alternative for lineage tracing, that allows retrospective analyses of gene expression. In parallel, we optimized in vitro differentiation of XY mouse embryonic stem cells towards gonadal somatic cells and achieved expression of early Sertoli cell marker genes. Using the DCM time-machine we identified transcriptomic signatures of gonadal somatic progenitors between E7.5 and E10.5. Combined with embryonic single cell RNA sequencing data, our analyses pointed towards the (splanchnic) lateral plate mesoderm as a potential gonadal somatic cell primordium. Ongoing efforts involve validating transcriptomic signatures using immunofluorescence and RNA FISH. Subsequent analyses will investigate signaling pathways that influence gonadal primordium specification and explore male-female transcriptomic differences in gonadal precursors. The overarching aim is to refine the current differentiation protocol with insights gained from transcriptomic analyses, ultimately leading to the development of an in vitro model for sex determination and gametogenesis.

Keywords: mouse gonadal development, retrospective transcriptomics, cell-cell signaling

4:45 PM – 5:45 PM

332

REGULATORY NETWORK DYNAMICS OF SMALL NON-CODING RNA DURING IN VITRO GERM LAYER DIFFERENTIATON

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Small non-coding RNAs (sRNAs) have increasingly been found to have a wide range of functions in both healthy and pathophysiological conditions. However, little is known about their regulatory programs during early development and their role in establishing cell identity. To address this, we differentiated in vitro two isogenic induced-pluripotent stem cell lines towards mesoderm, endoderm, and ectoderm. We leveraged sequencing technologies to measure changes in the mRNA, sRNA, chromatin accessibility and methylation patterns at various time points during differentiation. This allowed us to identify novel sRNA, mRNA and enhancer regions playing a role during germ layer commitment. By integrating all the “omic” modalities together, we characterised dynamic microRNA-mRNA regulatory network motifs that guide pluripotency exit towards each germ layer. In addition, we found a potential regulatory role of tRNA during cell fate commitment, both through RNA modifications and tRNA fragmentation. This study provides a first high-throughput insight into the presence or absence of specific groups of sRNAs in iPSCs upon differentiation into each of the three germ-layers.

Funding Source: This work is supported by the European Union (ERC, i-SignalTrace, 101042634), a Novo Nordisk Foundation grant (NNF21CC0073729; reNEW), and a Marie Skłodowska-Curie grant (No. 813282).

Keywords: small non-coding RNA, cell fate commitment, multiomics

4:45 PM – 5:45 PM

334

FLEXIBLE AUTOMATION AND SCALABLE LIMS IN CELL CULTURE WORK: ENSURING EFFICIENCY AND FAIR DATA MANAGEMENT

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To promote the evolution of the rapidly advancing field of induced pluripotent stem cell culture research from bench to bedside, the arduous manual reprogramming process followed by the subsequent verification of iPSC pluripotency and its differentiation into defined lineages mandate at least some if not the complete automation of this complex process. Automation will alleviate the current limitations on the scale and reproducibility of this technology. With a goal to automate reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), the Center of Modeling Pediatric Diseases at St Jude in conjunction with Thermo CRS designed an automation system that consists of a Hamilton Vantage, a Hamilton Capper, Decapper, a Cytation V microscope, a HiG4 centrifuge, a 500 plate Cytomat Incubator, a KingFisher Flex, a Cell Celector, and an F7 arm that facilitates the transfer of plates from one instrument to another. All these instruments are housed inside a custom built BSCII enclosure designed by Enviro-Flo. Together these instruments have allowed us to automate the entire reprogramming process starting from a vial of somatic cells and ending with a quality-controlled iPSC line. In addition, the scheduling software Momentum has been configured to execute batches of work that can be reused or reorganized to generate a completely different process to address the inherent variability in biological systems. Furthermore, we have also designed an in-house Laboratory Information Management System (LIMS) to manage the vast and varied data generated through automated processes and to ensure that the data generated adheres to the FAIR principles—making it Findable, Accessible, Interoperable, and Reusable. Our LIMS architecture has also been designed to meet downstream scalability, configurability vital to a dynamic research environment, where workflows and data types are subject to changes thereby future proofing the cell culture workflow against evolving research demands. The synergy between a flexible automation and a scalable, configurable LIMS are crucial for the successful implementation of automated processes in stem cell culture research, ensuring not only the efficiency and accuracy of experiments but also the integrity and utility of the data generated.

Funding Source: St Jude Children’s Research Hospital

Keywords: reprogramming, flexible automation, scalable LIMS

4:45 PM – 5:45 PM

336

RIBOSWITCH-EQUIPPED RNA VIRAL VECTOR CONTROLS NANOG EXPRESSION FOR PLURIPOTENCY MAINTENANCE IN MOUSE EMBRYONIC STEM CELLS

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Controlling stem cell fate in large scale is a challenge in regenerative medicine. Replicable RNA viral vectors carrying transgenes in mammalian cells allow safe and low-cost cell regulation without genome integration. We have developed a vesicular stomatitis virus (VSV) vector equipped with riboswitches (HDVGuaM8) that can control transgene expression in mammalian cells using the small molecule guanine. We previously discovered a VSV vector variant with two novel mutations that allowed it to efficiently replicate in mouse embryonic stem cells (mESCs). In this study, we further improved the vector by incorporating a fluorescent reporter gene and a drug selection marker that enabled stable maintenance of the vector in propagating mESCs. By culturing mESCs in the presence of guanine and puromycin, the cells can be propagated while stably maintaining the VSV vector without expressing the transgene. Nanog, which is critical for pluripotency maintenance in ESCs, was incorporated as a transgene replacing the fluorescent reporter gene in the VSV vector (Nanog-VSV). Nanog-VSV-infected mESCs were stably propagated in the presence of guanine and puromycin in the culture for pluripotency maintenance. The cells were then transferred to a differentiation induction medium with or without guanine. After five days of culture, strong alkaline phosphatase activity was observed under the Nanog induction condition. Flow cytometric analysis also revealed expression of the pluripotency marker (SSEA-1) and a VSV protein (VSV-N) under the Nanog induction condition, whereas the cells under the Nanog suppression condition showed reduced expression levels. Next, to demonstrate the removal of the viral vector from mESCs, the Nanog-induced cells were transferred back to the pluripotency maintenance medium with guanine and ribavirin. Flow cytometric analysis detecting VSV-N protein in the cells indicated that the VSV vector was removed after culturing the mESCs in the presence of guanine and ribavirin for 15 days. Three additional days of culture without guanine and ribavirin did not restore the fraction of mESCs expressing VSV-N. Therefore, we demonstrated the use of our VSV vector to maintain pluripotency of mESCs and subsequent removal of the vector by chemical treatment.

Funding Source: The research was funded by Okinawa Institute of Science and Technology Graduate University (OIST) and JSPS KAKENHI grants (20K15669 and 22K15000) awarded to N. K.

Keywords: viral vector, riboswitch, embryonic stem cell

4:45 PM – 5:45 PM

338

PARP-DEPENDENT PROTEOMIC REPROGRAMMING OF THE UBIQUITINOME MEDIATES PLURIPOTENT STEM CELL REVERSION TO A FUNCTIONAL HUMAN 8-CELL (8C)-LIKE EMBRYONIC STEM CELL STATE

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Poly-ADP-ribose (PAR)ylation and ubiquitination of the proteome are both implicated in regulating balances between embryonic stem cell self-renewal and differentiation. However, the role of these post-translational modifications in human embryonic development is poorly understood. We have demonstrated that continuous culture of primed hPSC with non-specific Poly-ADP-ribose polymerase (PARP) inhibition chemically reprogrammed them into Tankyrase/PARP inhibitor-regulated naïve (TIRN) hPSC with significantly improved functionality. Here, we reveal that global suppression of both PARP1 and TNKS activities in TIRN-hPSC resulted in activation of expression of pioneer factors (TPRXL, HOX, GATA) that regulate human zygotic genome activation (ZGA) and 8C blastomere stages. Since TNKS1/2 and PARP1 both catalyze the PARylation of hundreds of protein substrates destined for degradation in the ubiquitin-proteasome system (UPS), we postulated that PARP-dependent ubiquitination may regulate the activity or stability of these pioneer factors. We first differentially evaluated protein targets potentially modified by mono- and poly-ribosylation and ubiquitination by Tandem Mass Tag mass spectrometry of the whole proteome; followed by ubiquitinome studies on isogenic primed vs. 8C-like TIRN-hPSC. Differential protein expression of 8C-like cells was analyzed by proteomic gene ontology (GO) and pathway analysis. PARP1/TNKS targets were validated by Western blotting using PAR-affinity resins, and the effects of UPS inhibitor MG132 on protein expression was studied. We observed an accumulation of ubiquitin-conjugated proteins in TIRN-hPSC of key transcriptional regulators (NANOG, GATA6) that regulate stemness. A multi-omics approach (that included ssRNA-Seq and ChiP) further revealed that TIRN reversion resulted in PARP-mediated proteogenomic reprogramming of the ubiquitin code; including a global ubiquitin-conjugated rewiring of histones, deubiquitinases, and E3 ligases. We propose that PARP-dependent ubiquitination may directly modulate the proteostasis of pioneer factors involved in maintaining an 8C state. Further chemical manipulation of PARP-dependent ubiquitination may be a gateway to deriving stable human 8C-stage totipotent stem cells for regenerative medicine.

Funding Source: This work was supported by grants from NIH/NEI (R01EY032113), The Maryland Stem Cell Research Fund (2023-MSCRFV-5995; 2023-MSCRFV-6248), and The Lisa Dean Moseley Foundation.

Keywords: 8-cell-like embryonic stem cells, ubiquitinome, TNKS/PARP inhibitor-regulated naïve cells

4:45 PM – 5:45 PM

340

INDUCING HYBRID E/M PHENOTYPE IN AMNIOTIC EPITHELIAL CELLS VIA CONTROLLED INHIBITION OF COMPLETE EMT, RESULTING IN ENHANCED STEM-LIKE AND REGENERATIVE CHARACTERISTICS

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Epithelial-mesenchymal plasticity (EMP) represents a novel biological concept that has evolved from the previously established epithelial-mesenchymal transition (EMT). EMP encompasses the dynamic trans-differentiation of epithelial (E) cells into mesenchymal (M) cells, involving the generation of an indeterminate number of cells exhibiting hybrid E/M phenotypes. These hybrid cells displaying a combination of (E) and (M) features, have recently been shown to be predominantly implicated in processes such as tumor metastasis, embryogenesis, and tissue regeneration. This becomes feasible as hybrid E/M cells, in comparison to their purely epithelial (E) or mesenchymal (M) counterparts, possess enhanced stem-like characteristics and increased plasticity resulting from extensive reprogramming. Nonetheless, these hybrid E/M cells manifest collective migration and foster the development of an immune-suppressive microenvironment, which are two critical facets of the metastatic process. Amniotic epithelial cells (AECs) have been extensively investigated for their ability to undergo EMT during pregnancy and regenerative processes. Moreover, the destiny of these cells can be easily redirected by using progesterone, which promotes the retention of (E) phenotype and inhibits AECs' complete trans-differentiation into the (M) cells. In this study, we presented evidence that inhibiting complete EMT induces the generation of AECs with hybrid E/M phenotypes. To this regard, we found an increased expression in stemness gene and an enhanced in vitro osteogenic differentiation. Furthermore, AECs in this state exhibit a collective migration, thus suggesting a switch toward a hybrid E/M form. In support to this hypothesis, we also found an increased in vivo regeneration when these cells are transplanted into a tendon lesion. Finally, the inhibition of full EMT demonstrated to also potentiate AECs immunomodulatory properties, inhibiting leukocyte proliferation and macrophage activation. This data establishes a link between the inhibition of complete EMT and the generation of hybrid E/M forms in AECs, which could be of great interest for cell therapy and regenerative medicine due to their distinctive biological properties.

Funding Source: 2022YXHEET - Hybrid E/M AEC States: Insights into the Relationship Between stemness and regenerative role

Keywords: amniotic epithelial cell, epithelial mesenchymal transition, hybrid E/M phenotype

4:45 PM – 5:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

342

BASE EDITING OF HUMAN HEMATOPOIETIC STEM AND PROGENITOR CELLS WITH THE PIN-POINT PLATFORM

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Hematopoietic stem and progenitor cells (HSPCs) are a foundational cell type for the development of engineered therapies. Given their susceptibility to DNA damage, it is crucial to employ gene editing technologies that minimize genotoxicity. Base editors offer an efficient strategy to mitigate the challenges posed by nuclease-induced double-strand breaks (DSBs), such as activation of DNA damage response and chromosomal aberrations. We have developed the Pin-pointTM platform, which enables the modular assembly of the base editor, including a DNA binding Cas and a DNA deaminase, via the interaction between an aptamer binding protein fused to the deaminase and an RNA aptamer located in the sequence-targeting guide RNA (gRNA). Allowing modifications of the DNA without relying on the introduction of DSBs, the Pin-point platform enables complex genetic modifications in a single intervention as demonstrated in primary human T cells and iPSCs, where we achieved efficient base editing at multiple sites and simultaneous targeted transgene knock-in without compromising genome integrity. The advanced safety profile of this technology makes it well suited to HSPCs. By optimising reagent design and delivery conditions of a Pin-point base editor composed of Rat APOBEC1 and SpCas9 nickase mRNAs, we achieve up to 80% C to T conversion at the B2M locus with high levels of editing purity and very low incidence of indels, an indirect measure of DSBs occurrence. Using the optimised conditions, we then targeted two separate loci known to reactivate γ-globin expression: the erythroid enhancer of the repressor BCL11A and the BCL11A binding site in the HBG promoter. We achieved a high level of base editing at both loci that corresponded with an increase in γ-globin mRNA and protein expression. Edited HSPCs retained viability, immunophenotype, and differentiation potential toward the erythroid lineage in vitro. The ability to base edit HSPCs efficiently and safely, while retaining high cell viability and differentiation capability, demonstrates the strength of the Pin-point™ platform as a tool for the generation of advanced cell therapies using sensitive cell types.

Keywords: base editing, HSPCs, genome engineering

4:45 PM – 5:45 PM

344

PROCESS DEVELOPMENT FOR PLURIPOTENT STEM CELL DIFFERENTIATION IN THE QUANTUM CELL EXPANSION SYSTEM

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Pluripotent stem cells, as an unlimited source of starting material that has potential for differentiation into any functional cell type, constitute a great promise for cell-based therapies. CiRA Foundation, in partnership with Terumo Blood and Cell Technologies, works on the development of an automated, scalable, and cost-effective process for high-quality iPS cells manufacturing and differentiation in the Quantum Cell Expansion System, scalable functionally closed platform for perfusion-based cell culture in hollow-fiber bioreactors. Last year we reported our progress in the development of large-scale pluripotent stem cell expansion protocol. Since then, we have further optimized the process on the Quantum Flex platform with variable bioreactor size and improved control software, and improved its cost-effectiveness with the use of customized culture medium, that utilizes the separation of intra- and extracapillary circulation by supplying costly medium containing recombinant proteins and growth factors to the intracapillary compartment only, with the extracapillary compartment feeding realized with basal medium supplemented with low molecular weight components. Since Quantum system supports expansion of both, adherent and suspension cells, we have decided to develop differentiation protocols for natural killer cells and cardiomyocytes, as a validation of the platform for various cell types. Here we are going to show our progress in the adaptation of the differentiation protocols for the Quantum system.

Funding Source: Japan Agency for Medical Research and Development, grant JP23bm1323001

Keywords: pluripotent stem cells, differentiation, closed culture system

4:45 PM – 5:45 PM

346

AN INTEGRATED HUMAN BONE MARROW ATLAS FOR LEUKAEMIC DISORDERS AND AGEING

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The bone marrow (BM), which is central to haematopoietic development, is implicated in various haematopoietic malignancies, including acute myeloid leukaemia (AML), chronic myeloid leukaemia, chronic myelomonocytic leukaemia, myelodysplastic syndromes, and multiple myeloma (MM). Understanding how these diseases emerge from their healthy counterparts may help identify potential intervention strategies to counter the transformation process. An atlas that incorporates both healthy and diseased data is required to identify common subpopulations and pathogenic trajectories across different malignancies. Currently, no such resource exists. Therefore, we constructed a one-million human BM cell atlas to investigate changes in gene expression (GE) profiles across cell types during disease progression and ageing. Since batch-effect correction can introduce spurious data transformation, we benchmarked various single-cell data integration methods, and extensively compared corrected and uncorrected GE profiles. Our atlas accurately recapitulates previously established hallmarks in each disease, including disease-specific cell type expansion and dysregulated pathways. The substantial cell count in our atlas facilitated the identification and characterisation of rare stem and progenitor populations that give rise to haematopoietic malignancies. We applied dissimilarity metrics to the single-cell transcriptomes and identified leukaemia-specific populations, uncovering lineage infidelity in blast-like cells from AML samples, and distinct haematopoietic stem cell (HSC) sub-populations in MM. Our atlas enables deep exploration of the interplay between the microenvironment and leukemic stem cell phenotypes, including dormancy, potency, and proliferation capacity, contributing valuable insights into identifying potential therapeutic targets. Furthermore, we aim to compare pre-leukemic stem cells to HSCs in ageing BM, shedding light on the impact of ageing on the emergence of haematopoietic malignancies.

Keywords: single-cell atlas, leukaemia, ageing bone marrow

4:45 PM – 5:45 PM

348

RNA-DRIVEN REPROGRAMMING TO INDUCED DENDRITIC CELLS MEDIATED BY TRANSCRIPTION FACTORS AND MIRNAS

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Direct reprogramming is a potent in vitro model of development and a source of therapeutically relevant cell types, but its utilization as a gene therapy remains constrained by low efficiencies in primary somatic cells and the use of viral vectors. Our group has previously described the generation of induced dendritic cells (iDCs) by lentiviral-mediated expression of PU.1, IRF8 and BATF3 (PIB), which resemble conventional type 1 dendritic cells (cDC1) crucial in driving anti-tumor immunity. Here, we hypothesized that direct reprogramming to iDCs can be mediated by a non-viral RNA cocktail. As microRNAs (miRNAs) were previously shown to promote reprogramming we screened 15 candidates linked to hematopoiesis or reprogramming by co-transduction of pri-miRNA sequences and PIB in Clec9a-tdTomato mouse embryonic fibroblasts. We identified miR-124 and miR-142 that improved phenotypic by upregulating expression of CD45, MHC-II and XCR1 in a dose-dependent manner, while maintaining iDC function. The action of the two miRNAs were conserved in human dermal fibroblasts, with miR-124 and miR-142 increasing reprogramming efficiency and fidelity, respectively. At the transcriptional level, both miRNAs but particularly miR-142 promoted expression of cDC1-restricted genes CLEC9A and XCR1 leading to increased transcriptional affiliation to human cDC1s. In contrast, miR-124 showed activity at the early stages of reprogramming, increasing chromatin accessibility sites for PU.1 binding. Co-transfection, but not pre-treatment with mature miR-124-3p RNA mimic recapitulated lentiviral pri-miR-124 expression, reinforcing the importance of PIB and miR-124 cooperation. Finally, delivering TFs as mRNAs and combining with miRNAs drove robust phenotypic reprogramming of human iDCs which was further enhanced upon JAK inhibition with ruxolitinib. Collectively, our work highlights the feasibility of non-viral iDC reprogramming by combining mRNA, miRNAs and small molecules and paves the way for safer and scalable immunotherapies.

Funding Source: This project is supported by the European Research Council and the Knut and Alice Wallenberg Foundation.

Keywords: reprogramming, dendritic cell, miRNA

4:45 PM – 5:45 PM

352

INVESTIGATING THE EXTRACELLULAR MICROENVIRONMENT AS A POTENTIAL TARGET TO MONITOR AND CONTROL LARGE-SCALE HAEMATOPOIETIC DIFFERENTIATION

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Allogeneic stem cell-derived therapies are being investigated for several indications. In-vitro haematopoietic differentiation of pluripotent stem cells (PSCs) holds great promise for the development of novel therapies, however in many cases requires large cell numbers per dose. To support reproducible and rapid scale-up of cell cultures to meet clinical needs, manufacturing must abandon planar flask technologies and adopt automated bioreactor systems. Despite the drive towards automation, cell therapies are inherently more prone to variability, with increased risk of batch failure and costs if processes are not sufficiently controlled. In-process analytics can help maintain control and reduce risks of failure. However, it is difficult to apply analytics that are used for monitoring expansion and differentiation in planar flasks to bioreactor systems. The extracellular microenvironment is composed of a myriad of molecules consumed and produced by cells during differentiation, potentially alluding to cell state and cell fate. Using, a model haematopoietic process to generate haematopoietic progenitor cells (HPCs), we have characterised he cell secretome with respect to metabolites, miRNAs, and secreted proteins. miRNA was isolated from the spent media and several screening methods have been applied to the material to perform multi-parametric analysis in conjunction with single-cell transcriptomics to determine potentially novel biomarkers for monitoring future large-scale differentiation processes. Our initial proof of concept work has yielded potentially novel miRNA markers in the haematopoietic differentiation process for spent media via Illumina sequencing and NanoString Technologies. These markers have shown consistent differential expression across all the platforms assessed. Furthermore, we have assessed additional timepoints for in-depth process characterisation and facilitate deeper process understanding and to assist in the prediction of run success. In collaboration with Earlham Institute, we have successfully characterised a process for generating hematopoietic progenitor cells from iPSC using multiomics analysis and therefore, identified the extracellular microenvironment for monitoring large-scale haematopoietic differentiation.

Keywords: haematopoietic progenitor cells, exosomes, miRNA, secretome, iPSCs, stem cells

4:45 PM – 5:45 PM

354

IMPACTS OF CATIONIC LIPID-DNA COMPLEXES ON HEMATOPOIETIC STEM CELLS IN VIVO

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The burgeoning field of nanomedicine has revolutionized the landscape of precision medicine, offering novel avenues in disease diagnosis, treatment, and vaccine development. Cationic lipids, a prominent class of nanocarriers, have emerged as leading non-viral vectors in gene therapy, demonstrating significant promise in drug delivery applications. The systemic administration of cationic nanoparticles has been a significant barrier to their clinical translation due to toxicity concerns. Understanding the in vivo behavior of cationic lipids is crucial, given their potential impact on critical biological components such as hematopoietic stem cells (HSC). These cells are essential for maintaining the body's homeostasis, and their interaction with cationic lipids is a key factor in determining the safety and efficacy of these nanoparticles. In this study, we evaluated the in vivo differences between intravenous cationic lipid nanoparticles (CLN) loaded with various nucleic acids, focusing on their potential harm to immune cells and HSC. We observed significant effects of CLN/siRNA and CLN/DNA treatments on these cells, particularly noting a marked increase in the amplification of long-term hematopoietic stem cells (LT-HSC). Additionally, we investigated the in vivo dynamics of CLN/DNA, finding that LT-HSC amplification in bone marrow (BM) and spleen (SP) peaked 24 h post-CLN/DNA treatment. Crucially, our competitive bone marrow transplantation experiments revealed a significant reduction in the ability to maintain hematopoiesis and reestablish blood-cell production in CLN/DNA-treated mice. Our findings indicate that CLN/DNA induces notable alterations in the immune and hematopoietic systems, adversely affecting the long-term regenerative capacity of HSC.

Keywords: hematopoietic cells, cationic lipid-DNA complexes, toxicity

4:45 PM – 5:45 PM

356

GENERATION OF OFF-THE-SHELF CAR-NKT CELLS FOR CANCER IMMUNOTHERAPY WITH GENETICALLY ENGINEERED HSPCS AND SCALABLE DIFFERENTIATION CULTURE

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Current CAR-T cell therapy's clinical potential is hampered by its autologous nature that poses significant challenges in manufacturing, costs, and patient selection. This spurs demand for off-the-shelf therapies. Here we introduce an ex vivo feeder-free culture to differentiate gene-engineered HSPCs into allogeneic NKT cells and their CAR-armed derivatives (AlloCAR-NKT cells). The AlloCAR-NKT cells are generated at high yield, purity, and robustness at the research scale used in our studies. These cells exhibit potent antitumor efficacy, showing effective tumor homing, expansion, and persistence in vivo. Impressively, AlloCAR-NKT cells can alter the tumor microenvironment by selectively depleting immunosuppressive TAMs and MDSCs, and can antagonize tumor immune evasion by deploying CAR/TCR/NKR triple-targeting mechanisms. AlloCAR-NKT cells also demonstrate an appealing safety profile with low GvHD and CRS risks, and exhibit a stable "hypoimmunogenic" phenotype attributed to epigenetic and signaling regulations. The reported technology presents a scalable strategy for diverse allogeneic CAR-NKT cell products, with potential for clinical translation and commercialization against various cancers.

Funding Source: CIRM (DISC2-11157, TRAN1-12250), DoD CDMRP PRCRP Impact Award (CA200456), UCLA BSCRC Innovation Award, Ablon Scholars Award, NIH T32 Award (CA009120).

Keywords: off-the-shelf CAR-NKT cells, cancer immunotherapy, scalable HSC-NKT differentiation culture

4:45 PM – 5:45 PM

358

A PLATFORM FOR RAPID AND EFFICIENT ENGINEERING OF MULTI-EDITED CLONAL IPSC LINES FOR ALLOGENEIC T CELL THERAPIES

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Induced pluripotent stem cells (iPSCs) have enormous potential to be used as a homogeneous, well-characterized starting material for the derivation of diverse immune cell subtypes for therapeutic applications. Through their ability to self-replicate genetically identical clones, master cell lines can be generated with increasingly complex genetic modifications. Yet, building multi-edited iPSC lines that show appropriate control of transgene expression throughout iPSC differentiation and in the final iPSC-derived cell product remains challenging. Here, we have built a MAD7-based gene editing platform that enables modification of iPSCs with more than 80% knock-out efficiency and targeted integration of non-viral transgenes within a range of 25-70% knock-in efficiency. In addition, we have created a method for achieving multiple edits in a single campaign and subsequent clonal selection and characterization. Using this platform, we were able to rapidly create multi-edited clonal iPSC lines with various combinations of promoters and integration sites for temporal and dose-dependent control of transgenes. After investigating transgene expression throughout T cell differentiation and in activated iPSC-derived CAR-T cells, we were able to identify a cell line design for a clinical iPSC-derived CAR-T product that contains up to five knock-outs and up to four different transgene knock-ins with desired expression profiles.

Keywords: clonal iPSC engineering, multiplex genome editing, transgene regulation

4:45 PM – 5:45 PM

360

ADVANCING DEVELOPMENT OF HEMATOPOIETIC STEM CELL THERAPY: ENHANCED HSC ISOLATION

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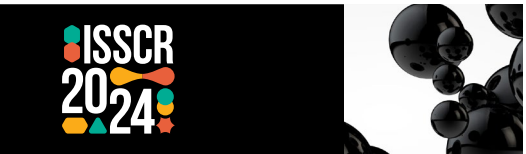
Hematopoietic stem cell (HSC) transplantation has become a critical treatment approach for a number of diseases since 1960s. With the innovation in gene editing technologies, now gene modified autologous HSCs have gain attractions being used as therapeutic options for certain diseases. Recently FDA approved gene therapies for sickle cell disease shine more lights on the value of genetically engineered HSCs. Since HSCs are present in peripheral blood at very low frequency, efficient isolation of HSCs is key for advancing cell therapy development, promising breakthroughs in regenerative medicine and cell-based cancer therapies. However, conventional methods for isolating CD34+ HSCs are time-consuming and often result in significant cell loss, negatively affecting efficiency and viability. Here we present an innovative approach aimed at bypassing these challenges, making a significant advancement in stem cell isolation techniques. Our methodology redefines HSC isolation by eliminating the need for magnetic columns, streamlining the process while maintaining high purity. With our MARS workflow, a purity exceeding 94% was achieved from sample with starting concentrations 0.2%, while significantly reducing experiment time by approximately 50% and number of steps from 8 to 4 in comparison to conventional methods (for 50 mL input sample). Automated serial cell enrichment ensures efficiency, as quick re-running of samples through the magnetic channel enhances the result. This workflow extends across various sample types, including cord blood, peripheral blood, PBMCs as well as mobilized apheresis products. The protocol, with fewer steps than traditional methods, ensures maximum cell viability throughout. Automated CD34+ enrichment enhances purity, reproducibility, recovery, and viability of isolated cells, and the isolation yields 5 to 8 times more CD34+ cells than the column method. This not only reduces the relative cost but also provides an increased volume of cells suitable for biobanking and ensures a richer stem cell base for successful therapy outcomes. Citations: Lee JY, Hong SH. Int J Stem Cells. 2020 Mar;13(1):1-12. Ivanovic Z. World J Stem Cells. 2010 Apr;2(2):18-23. Wang X, & Riviere I. Mol Ther Methods Clin Dev. 2017 Jun;5.

Keywords: hematopoietic stem cells, stem cell therapy, cell and gene therapy



4:45 PM – 5:45 PM

362

IPSC DERIVED CAR-MACROPHAGES FOR IMMUNE CELL THERAPIES

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Chimeric antigen receptor (CAR) T cell therapies have successfully treated hematological malignancies. Macrophages have also gained attention as an immunotherapy owing to their immunomodulatory capacity and ability to infiltrate solid tumors and phagocytize tumor cells. The first-generation CD3ζ-based CAR-macrophages could phagocytose tumor cells in an antigen-dependent manner (Zhang, JHO, 2020). Here we engineered induced pluripotent stem cell-derived macrophages (iMACs) with toll-like receptor 4 intracellular toll/IL-1R (TIR) domain-containing CARs resulting in a markedly enhanced antitumor effect over first-generation CAR-macrophages (Lei, Nature Immunology, 2023). Moreover, the design of a tandem CD3ζ-TIR dual signaling CAR endows iMACs with both target engulfment capacity and antigen-dependent M1 polarization and M2 resistance in a nuclear factor kappa B (NF-κB)-dependent manner, as well as the capacity to modulate the tumor microenvironment. We also outline a mechanism of tumor cell elimination by CAR-induced efferocytosis against tumor cell apoptotic bodies. Taken together, we provide a second-generation CAR-iMAC with an ability for orthogonal phagocytosis and polarization and superior antitumor functions in treating solid tumors relative to first-generation CAR-macrophages. In addition to engineering the CAR molecule, we also used CRISPR/Cas9 to engineer the iMACs to enhance their functions, including editing metabolism gene ACOD1/IRG1 to confer enhanced polarization(Wang, Nature Communications, 2023), and editing macrophage checkpoint molecules such as Siglic5/10 to repress their “don’t eat me” signals (Wu, PNAS, 2023). With these technology platforms, we are now moving toward manufacturing clinical grade CAR-iMACs and translating them for treating solid tumors.

Keywords: CAR-macrophage, solid tumor, iPSC differentiation

4:45 PM – 5:45 PM

364

HUMANIZED BONE MARROW MICROENVIRONMENT PROMOTES HEMATOPOIETIC STEM/PROGENITOR CELL EXPANSION AND REJUVENATION IN VIVO

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Immunodeficient mouse model provides the opportunity to study human hematopoietic stem cells (HSCs) in vivo through xenotransplantation. However, the fundamental differences between human and mouse bone marrow microenvironment (BME) necessitates myeloablative pre-conditioning before transplantation. Whether humanization of the BME in immunodeficient mice could promote expansion or rejuvenation of human HSCs in vivo remains unknown. SCF and CXCL12 are pivotal niche factors that promote HSC maintenance by bone marrow stromal cells (BMSCs). Whereas CXCL12 is highly conserved between human and mouse, the sequence homology of SCF is around 80%, which exhibits species-specific activity. In this study, we generated a human SCF knock-in immunodeficient mouse model (SCF-KI in NCG background) by CRISPR/Cas9, in which the mouse Scf gene was replaced by a transmembrane form of human SCF. Immunostaining and flow cytometry showed robust expression of human SCF in mouse BMSCs in the perivascular region. Homozygous SCF-KI mice were embryonic lethal while the heterozygous mice had normal hematopoiesis but significant less HSCs in the bone marrow and spleen. Remarkably, SCF-KI mice enable nonconditioned human cord blood CD34+ cell transplantation by retro-orbital injection, which gave significantly higher peripheral blood, bone marrow and spleen chimerism as compared to non-irradiated or sub-lethally irradiated NCG mice. Human red blood cells were also detected in the bone marrow and spleen of SCF-KI mice. Flow cytometry analyses revealed a 240~330-fold increase of human CD34+ cells in the bone marrow of SCF-KI mice, indicating dramatic expansion of hematopoietic stem/progenitor cells (HSPCs). In secondary transplantation, SCF-KI mice also gave significantly higher bone marrow chimerism as compared to sub-lethally irradiated NCG mice, suggesting that human HSPCs could be serially expanded in these mice. To test whether SCF-KI mice could be used to transplant human bone marrow HSPCs, we performed intra-femoral injection of aged human CD34+ cells, and found robust rejuvenation effects. Taken together, we demonstrated that humanization of the BME is a novel strategy to expand and rejuvenate human HSPCs in vivo, which could be exploited to treat hematological diseases or reverse hematopoietic aging.

Funding Source: The National Key R&D Program of China, National Natural Science Foundation of China, Fundamental Research Funds for the Central Universities, and Peak Disciplines (Type IV) of Institutions of Higher Learning in Shanghai.

Keywords: hematopoietic stem cell, humanized immunodeficient mice, rejuvenation

4:45 PM – 5:45 PM

366

THE REFINEMENT OF EARLY MOLECULAR STEPS IN HUMAN HEMATOPOIETIC STEM CELL FATE CONTROL REVEALED AN IMMUNOMODULATORY ROLE OF PD-L2

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Despite intense efforts over the last decades, the human hematopoietic stem cell (HSC) compartment remains heterogeneous and molecular changes regulating stemness and initial differentiation are still poorly understood. To resolve these early steps in HSC fate choices, we employed deep-targeted single-cell CITE-Seq on over 62,000 FACS-enriched hematopoietic stem and progenitor cells (HSPCs) isolated from 15 healthy donors of three age groups. Cluster and pseudotime diffusion analyses identified 9 immature cell subpopulations of HSPCs branching into four trajectories. TRADE-Seq analyses revealed continuous gene expression patterns along pseudotime from the most immature HSCs to early-lineage committed progenitors. While HSPCs from young donors showed an increased and balanced production of differentiated progenitors of all lineages, HSCs from old donors were increased in number and showed a decelerated differentiation kinetics in pseudotime. Several genes - so far not reported in human HSCs - showed differential expression during the early steps in lineage commitment. Importantly, the immune receptor Programmed death ligand 2 (PD-L2/CD273) was the only surface marker upregulated in the most immature HSCs. We confirmed these findings in mobilized samples by flow cytometry. RNA-Seq uncovered a unique molecular signature of PD-L2hi HSCs, with an upregulation of stem cell genes (HOPX, MLLT3, MPL), and a downregulation of the cell-cycle regulator CDK6. In vitro assays and video microscopy showed a delay in cell cycle entry and differentiation of PD-L2hi expressing HSCs. Since PD-L2 is a ligand for PD1 on activated T-cells, we investigated the immunomodulatory function of PD-L2hi HSPCs using an allogeneic lymphocyte reaction. Co-culturing T-cells with PD-L2hi HSPCs demonstrated diminished T-cell proliferation, elevated secretion of anti-inflammatory cytokines, and reduced activation of cytotoxic T-cells. Furthermore, HSPCs showed a preference for differentiating into myeloid progenitors after exposure to allogeneic T-cells. These findings suggest an immune modulatory function by the expression of PD-L2 in a subset of immature HSCs. Functional and molecular consequences on both, immune cells and HSCs, after cell-cell contact will be further investigated by single-cell technologies.

Keywords: hematopoietic stem cells, single-cell analysis, immune modulation

4:45 PM – 5:45 PM

368

NOVEL REGULATORY SEQUENCES DRIVE PERSISTENT TRANSGENE EXPRESSION DURING DIRECTED DIFFERENTIATION OF IPSCS TO LYMPHOCYTES AND MACROPHAGES

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Cell therapies derived from knock-in human induced pluripotent stem cells (iPSCs) hold potential for increased scalability, broader accessibility, and superior production of genetically identical immune cells that express therapeutic transgenes. Such transgenes are often paired with synthetic promoters, which can drive high levels of protein expression but are often silenced during differentiation. To address this issue, we designed novel sequences comprising a synthetic promoter (EF1α or SFC), an upstream ubiquitous chromatin opening element (UCOE), and a GFP reporter. These sequences (GFP under EF1α alone, EF1α with a UCOE (uEF1α), SFC alone, or SFC with a UCOE (uSFC)) were inserted into the AAVS1 safe harbor locus of mRNA-reprogrammed iPSCs, which were then differentiated via static culture into lymphocytes and macrophages. The gene-edited iPSCs expressed the pluripotency markers TRA-1-60 (wild type = 96.9%, SFC = 99.9%, uSFC = 99.9%, EF1α = 95.2%, uEF1α = 99.9%) and TRA-1-81 (wild type = 97.0%, SFC = 99.8%, uSFC = 99.9%, EF1α = 99.9%, uEF1α = 99.9%) and yielded comparable numbers of CD34+ hematopoietic progenitor cells upon differentiation (CD34+ cells as a percentage of iPSCs initially seeded: wild type = 4.4%, SFC = 5.0%, uSFC = 2.3%, EF1α = 3.8%, uEF1α = 4.1%). EF1α and uEF1α iPSC-derived lymphocytes were positive for CD7, and EF1α, uEF1α, and uSFC iPSC-derived macrophages were positive for CD13, CD14, CD33, and CD45. Furthermore, we found that uEF1α exhibited less differentiation-induced gene silencing than did EF1α. GFP expression in EF1α and uEF1α iPSCs was uniformly high (98.4% and 99.9%, respectively); however, in the iPSC-derived lymphocytes, the percentage of GFP+ cells was 36.5-fold higher under uEF1α (73%) than under EF1α (2%), and in the iPSC-derived macrophages, the percentage of GFP+ cells was 13-fold higher under uEF1α (78%) than under EF1α (6%). In summary, we present novel synthetic regulatory sequences that mitigate silencing during differentiation of iPSCs into lymphocytes or macrophages without interfering with differentiation or introducing unintended phenotypic changes. These sequence elements may prove useful in engineering cells with functional transgenes encoding therapeutic proteins, and could contribute to the development of effective engineered iPSC-based therapies.

Keywords: induced pluripotent stem cells (iPSCs), gene editing, macrophages

4:45 PM – 5:45 PM

TOPIC: KIDNEY

370

ROUTINE IMAGE-BASED QUALITY ASSESSMENT OF KIDNEY ORGANOID TISSUE THROUGH OPTIMISED DATA COLLECTION AND ANALYSIS PIPELINES

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Human stem cell-derived kidney organoids provide an exciting opportunity to develop new therapies and better understand human tissue formation. Our approach involves the directed differentiation of human induced pluripotent stem cells to kidney fate and aggregation of differentiated cells into tissue masses that form both nephrons and a surrounding stroma. We have developed methods to control and scale production, as well as mouse models of engraftment, providing the basis for future therapeutic development. A key barrier to translation is the quantitative assessment of nephron quality during tissue production and in subsequent pre-clinical models. Methods such as single-cell transcriptomics provide vital insight into cell identity yet are not amenable to routine batch assessment and do not capture the 3D morphological information necessary to assess nephron quality. Focussing on an organoid method previously developed by our group to produce scalable micro-organoids in suspension culture, we are developing image analytics to assess tissue growth at different stages of differentiation and in a mouse model of engraftment. We have optimised image collection and apply computational approaches, including the use of generalised AI models with minimal manual annotation to efficiently quantify and assess tissue morphology. This approach, when routinely applied to evaluating organoid morphology before and after transplantation, will enable advances in kidney tissue engineering.

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Keywords: kidney organoid, image analysis, AI

4:45 PM – 5:45 PM

TOPIC: LIVER

372

HYDROGEL-BASED ENCAPSULATION OF IPSC-DERIVED HEPATOCYTES RETAINS CELL VIABILITY AND FUNCTION OFFERING A NOVEL METHOD FOR THE TRANSPORT OF LIVE CELLS FOR LARGE-SCALE DRUG SCREENING AND EFFICACY STUDIES

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Cell cryopreservation allows the banking and shipping of large number of cells for use in drug discovery. Despite the benefits of the method, isolated human hepatocytes are poorly resistant to this process, and hepatocyte cryopreservation often results in cells with compromised quality due to significant alterations in morphology and function, post-thawing. Therefore, there is a need to overcome these limitations and identify alternative approaches that allow the efficient transport of fully-functional hepatocytes. We hypothesized that freezing-free, hydrogel-based encapsulation of DefiniGEN induced pluripotent stem cell (iPSC)-derived hepatocytes (Opti-HEP) in 2D format retains Opti-HEP viability and differentiation status, allowing their overseas transport for large-scale drug screening and efficacy studies. To investigate this, wild-type iPSCs were differentiated towards Opti-HEP for 4 weeks. Cells were subsequently seeded in 96-well plates and encapsulated using the Atelerix preservation platform for 72-168 hours at either room temperature or 37oC. Following completion of the encapsulation period, hydrogel was released, and cells were cultured in standard hepatocyte medium until the end of maturation, whereupon cell viability and function were measured by PrestoBlue cell viability assay and qPCR, respectively. Cell viability and morphology were comparable between non- (control) and encapsulated Opti-HEP across all timepoints when cells were cultured at 37oC; however, a significant decrease in cell viability was observed when both cultures were kept at room temperature, indicative of increased hepatocyte sensitivity in lower than 37oC environments. Supporting these data, no significant differences in the mRNA levels of the hepatic maturity markers albumin, alpha-1-antitrypsin, and hepatocyte nuclear factor 4A between 37oC-cultured control and encapsulated Opti-HEP were observed, suggestive of no adverse effects of cell encapsulation in Opti-HEP maturation. To conclude, we have demonstrated a novel method of hepatocyte preservation that removes the variability introduced by the cryopreservation/thawing processes, and introduces the capability to ship viable and fully-functional live cells for the screening and efficacy testing of novel treatments.

Keywords: liver, in vitro, cryopreservation

4:45 PM – 5:45 PM

374

THE ESTABLISHMENT OF A VASCULARIZED LIVER LOBULE-LIKE TISSUE

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The lack of perfusable vasculature limits the scale of organoids, making it difficult for them to grow further to the size of the native tissue. The liver lobule, which is at the millimeter-scale, serves as the basic functional unit of the liver. Constructing a perfusable vasculature is the initial challenge in creating a liver lobule-like tissue in vitro. However, traditional three-dimensional cultivation lacks the dynamic blood flow environment found in vivo, making it difficult to develop perfusable vascular networks. To address this issue, we combined microfluidic systems with the self-organization of hepatocytes, endothelial cells, and hepatic stellate cells (HSCs) to construct a vascularized liver lobule-like tissue ex vivo. The liver lobule-like tissue (LLB) demonstrated multicellular communications crucial for tissue formation. The endothelial cells in the LLB tissue exhibited some characteristics of liver sinusoid endothelial cells (LSECs), including marker gene expression and fenestration. The periportal-to-pericentral axis in liver lobules, also known as zonation, and contributes to zonal pathologies after liver injury. By developing zonal signals, we established partial metabolic zonation of hepatocytes ex vivo and successfully mimicked APAP-induced zonal injury. This human vascularized liver lobule-like tissue serves as a benchmark for reconstructing functional liver lobules in vitro, disease modeling, and drug screening.

Keywords: organ on chip, vasculature, liver lobule

4:45 PM – 5:45 PM

376

DEDICATED SAFEGUARD REPRESSORS CANALIZE CELL IDENTITY AND SUPPRESS TUMORIGENESIS

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Cell fate plasticity enables development, but destabilization of cell identity is a hallmark of diseases such as cancer. Cell fate induction and maintenance require the activation of lineage-specific programs and the silencing of alternative identities. In contrast to individual lineage-inducing master regulators that bind and activate cell-type-specific genes, little is known about how unwanted fates are suppressed by a limited number of available transcription factors. We previously found that the neuron-specific repressor MYT1L could suppress many non-neuronal gene programs to induce and safeguard neuronal cell fate and function. Here, we computationally predict additional transcriptional repressor candidates for 18 cell types that can suppress many alternative fates to safeguard the desired cell identity. We validated hepatocyte-specific candidates using cell fate reprogramming, revealing that PROX1 enhanced hepatocyte identity and suppressed several alternate cell identities. Mechanistically, direct repression of PROX1 target genes, including alternate fate master regulators Prrx1 and Pparg, promoted hepatocyte fate. Deleting Prox1 impaired hepatocyte formation and caused the induction of alternate fate markers. In line with cancer patient data, Prox1 depletion caused hepatocyte fate loss, while overexpression prevented neoplastic transformation during mouse liver tumorigenesis. Our findings demonstrate the utility of our safeguard repressor prediction and provide mechanistic evidence for PROX1 as a hepatocyte safeguard. This supports a model where individual cell type-specific safeguard repressors actively suppress many alternative fates to safeguard lineage choice and prevent cell fate plasticity and disease.

Keywords: cell fate plasticity, reprogramming, safeguard repressor

4:45 PM – 5:45 PM

378

LIVER AND PANCREATIC ENDODERM PROGENITORS SPECIFY THE STELLATE CELL PHENOTYPE IN IPSC-BASED SYSTEMS

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During embryo development mesenchymal cells promote the differentiation and maturation of the endoderm. However, to what extent the endoderm educates the mesenchyme is poorly understood. Stellate cells, are stromal mesenchymal cells present in the liver and the pancreas, responsible for the maintenance of the tissue architecture and the wound-healing response. The direct comparison of liver and pancreatic stellate cell revealed organ specific signatures in embryonic and adult human tissues at scRNA-seq level. Here, we investigate the role of the endoderm progenitors in the specification of stellate cell phenotype in the liver and the pancreas using an in vitro co-culture system derived from iPSCs. In this study, we showed that in vitro differentiation towards foregut posterior endoderm (FPE) was accompanied with the expression of progenitor markers of the pancreas (PDX1) and liver (AFP and HNF4A). Further, we committed the FPE to pancreatic (NKX6.1, PDX1) and hepatic (HNF4a, AFP, ALB) lineages when exposed to pancreatic cues (FGF7, Vit-C, SANT1, RA, LDN and TPB) or hepatic morphogens (BMP4 and bFGF). Conditioned media from liver and pancreatic progenitors induced the acquisition of organ-specific characteristics of iPSC-derived immature stellate cells, thus upregulating the expression of pancreatic BACH2, SLIT3, and IGF2 and hepatic RELN, ROBO2, and HGF markers. Remarkably, the direct contact in a 3D spheroid of stellate cells and endoderm progenitors further enhanced the overexpression of organ specific markers and promoted organ-specific morphogenesis, suggesting the need for cell-cell contact in modulating stellate cell-epithelial behavior. Overall, our findings show that stellate cells from iPSCs acquire organ-specific phenotypes and suggest that the direct co-culture coeducate both endoderm and mesenchymal populations improving in vitro model accuracy for organ and disease modelling.

Keywords: in vitro modelling, organ specification, epithelial-mesenchymal crosstalk

4:45 PM – 5:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

380

PURIFIED MESENCHYMAL STEM CELLS RESIST BETTER TO CRYOPRESERVATION THAN WHOLE STROMAL VASCULAR CELLS

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The standardization of cryopreservation protocols displays a necessary turning point to maximize viability and functionalities after long-lasting freezing. Adipose tissue-derived mesenchymal stem cells represent a powerful tool for regenerative application, through autologous re-injection; therefore, a correct cryopreservation as well as thawing of lipoaspirate-derived cellular components under GMP conditions is fundamental for future applications. Here, we characterized cellular component of lipoaspirate-derived stromal vascular fraction (SVF) through multicolour flow cytometry, by identifying CD45-CD34-CD146+ pericytes, CD45-CD34+CD146+ endothelial cells, as well as CD45-CD34+CD146- adipose stem cells (ASC). Then, we purified ASC by plating SVF for a week with specific culture medium and we stored at -196°C liquid nitrogen several cryovials of both SVF and ASC, for biobanking purpose. Afterwards, we thawed both SVF and ASC cryovials to investigate whether the long-lasting freezing processes may affect viability and recovery capabilities of heterogenous cell population as well as purified ASC. We found an increased recovery capability and viability in ASC, compared to SVF, assessed through NucleoCounter measurements. Moreover, we demonstrated the ability of ASC to maintain the same number of cells after thawing, whereas SVF displayed a significant decrease in cell number, compared to pre-freezing cells, pointing out the ability of ASC to resist to freezing-thawing processes. However, we found that SVF displayed a significant decreased capabilities in restoring both number and viability of cells after being thawed, following -196°C nitrogen cryopreservation. On the other hand, ASC showed the ability to completely restore in number and viability, following the same SVF cryo-preservative process, leading us to investigate the cellular mechanism involved in the resistance to freezing-thawing processes, then allowing a correct cryopreservation. The proper cryopreservation of ASC can be envisaged as a crucial target to enforce their potential for future clinical applications. Our results provided preliminary data to trigger the investigation on ASC potential to long-lasting cryopreservation, to highlight specific cryoprotective mechanisms, for future clinical applications.

Keywords: lipoaspirate, adipose stem cell, cryopreservation

4:45 PM – 5:45 PM

382

PANEL OPTIMIZATION FOR IMMUNOPHENOTYPING OF CANINE IMMUNE CELLS USING FULL SPECTRUM FLOW CYTOMETRY

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Advancements in fluorescence flow cytometry led to the development of full spectrum flow cytometry to accommodate a large panel of antibodies with multiple fluorochromes to better characterize various cell populations. In contrast to conventional flow cytometry, which primarily measures the peak emission of each fluorophore in a target detector, full spectrum flow cytometry uses a larger number of detectors with narrow band-pass filters. This allows the entire emission spectrum for every fluorophore to be captured across all lasers, creating a detailed signature of each fluorophore. This makes it possible to distinguish fluorophores with very similar emission maxima but unique overall spectrum. Despite these advances, a reliable antibody panel to reproducibly immunophenotype canine immune cells has not been reported. This is largely because of a lack of demonstration of antibody specificity and an optimal antibody concentration. Antibody titration is important to develop a high-dimensional flow cytometry panel. Using an incorrect antibody concentration can increase spread, decrease resolution, increase aggregation of reagents, and give rise to nonspecific binding. In addition, an optimized method to isolate canine peripheral blood mononuclear cells (PBMCs) has also not been reported. Here, we report an optimized procedure for gradient separation of canine PBMCs. We tested a variety of commercially available antibodies for canine immune cell markers and determined a set of antibodies that generate an optimal signal when used at a specific concentration. We present a 10-color panel able to resolve broad immune compartment populations in freshly prepared canine PBMCs that includes only surface markers to avoid the need for fixation and permeabilization steps. The panel presented here identifies CD4 T cells, CD8 T cells, B cells, and monocytes. Finally, our panel is optimized to detect cell populations in a small volume (~50 µls) of whole blood. Maximizing the amount of information that can be obtained from a single sample may help address the issue of limited sample availability. Disclaimer: This abstract reflects the views of the authors and should not be construed to represent FDA's views or policies

Funding Source: US FDA

Keywords: mesenchymal stem cells, pre-clinical animal model, veterinary medicine

4:45 PM – 5:45 PM

384

R9 ASSESSMENT: A NOVEL FLUORESCENT DYE TESTED ON STEM CELLS FROM APICAL PAPILLA

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In this research study, the focus was on assessing the cytotoxicity of a newly developed fluorescent dye, R9, on stem cells from the apical papilla. The aim was to test the effectiveness of the dye in connecting with proteins and RNA, which are crucial components of cellular processes. The researchers evaluated the nucleic acid binding properties of the dye, in particular its specificity towards RNA and DNA. To test the cytotoxicity of R9, a three-step protocol was followed. Mesenchymal stem cells were isolated from the apical papilla, and then the dye was administered to the cells in two different vessels - a 48 well plate and a 25 cm2 plastic flask. The plate was used for immediate observation using the InCell Analyzer 6000, while the flask was observed using flow cytometry after 24 hours of adding the dye. The researchers observed cellular penetration and recorded the results using the InCell Analyzer 6000 at intervals of 30 minutes for six hours. The differentiation between apoptotic and alive cells was made using the Anexine V kit for flow cytometry on Navios (BioRad). The apoptotic cells were further analyzed using FITC-conjugated anti-anexine V antibody, while the nuclei were counterstained with DAPI. The study's results indicate that a majority of the tested dye did not exhibit any cytotoxic effects on the cells. Furthermore, differences were observed between live and fixed cells. In live cells, the dye's spectrum was in the excitation peak at 559 nm and an emission peak at 583 nm. On the other hand, in fixed cells treated with 4% formalin, the spectrum shifted to 651 nm with an emission peak at 670 nm. The researchers emphasized the need for new and specific dyes that can aid in understanding cellular processes. Currently, there is only one RNA-specific binding dye available on the market. Developing new nontoxic dyes that can distinguish between RNA and DNA or bind to specific regions in proteins would provide valuable tools for understanding transmembrane transport, RNA translation, and protein folding and interactions

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Keywords: novel dyes, stem cells, mesenchymal stem cells

4:45 PM – 5:45 PM

386

UTILIZING ADIPOSE-DERIVED MESENCHYMAL STEM CELLS DIFFERENTIATED INTO ENDOTHELIAL CELLS FOR THE RE-ENDOTHELIALIZATION OF DECELLULARIZED OMENTUM FLAPS

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Extensive defects in soft tissue profoundly affect both patient quality of life and functional abilities, presenting a notable challenge in finding suitable repair strategies. The utilization of decellularization/recellularization techniques for tissues and organs has emerged as a promising avenue in tissue engineering. However, a key obstacle in achieving this objective resides in the process of angiogenesis and re-endothelialization of acellular vasculature. The capacity of adipose-derived mesenchymal stem cells (MSCs) to transform into various cell types, including endothelial cells, renders them appealing candidates for stimulating angiogenesis. This research aims to investigate the efficient differentiation of adipose-derived MSCs into ECs and explores the application of decellularization and re-endothelialization techniques on omentum flaps using endothelial-differentiated adipose-derived MSCs. MSCs were extracted from porcine adipose tissue through collagenase digestion and were characterized via flow cytometry. Subsequently, they were prompted to differentiate into endothelial cells and assessed for the expression of endothelial-specific markers and functions. Additionally, a low-concentration detergent protocol was employed for flap perfusion-decellularization. Re-endothelialization of preconditioned flaps with endothelial-differentiated adipose-derived MSCs was accomplished via perfusion-based seeding. Histological assessment, immunohistochemistry analysis, and cell viability testing were conducted post-seeding. MSCs demonstrated positivity for mesenchymal surface markers while exhibiting negativity for typical hematopoietic and endothelial markers. The findings illustrated the successful differentiation of expanded adipose-derived MSCs into ECs. Furthermore, our methodologies provided evidence of omentum flap decellularization, cellular attachment, and early signs of regenerated neo-endothelium. These results also shed light on the potential viability and effectiveness of employing decellularized omentum flaps as frameworks for adipose-derived MSCs differentiated into endothelial cells. This research contributes to the progress of tissue engineering and regenerative medicine.

Keywords: adipose-derived mesenchymal stem cells, endothelial differentiation, tissue-engineered flaps

4:45 PM – 5:45 PM

388

RESEARCH ON IMPROVING MESENCHYMAL STEM CELL (MSC) FUNCTION BY ULTRASOUND TREATMENT

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Mesenchymal stem cells (MSCs) are multipotent stem cells which are widely known for immunomodulatory properties and paracrine effects, thereby presenting a great value in various therapeutic applications. Some researchers have reported in recent years on modified MSC using gene modification techniques to enhance therapeutic effects and increase productivity. However, the modification of MSCs using gene modification techniques have challenges as it takes time and efforts. In this study, we aim to simply add new functions to MSCs. Some of methods have been used to these ends, but one new technique having significant interest is the use of ultrasound. It has already been reported that treating MSCs with ultrasound improves their migratory ability. We examined conditions to improve cell function with multiple parameters such as wavelength, intensity, and irradiation time. As a result of comparing gene expression variations of MSCs before and after ultrasound treatment by RNA sequencing, it was shown that expression variations occur in specific genes without affecting cell survival or MSC surface markers. Based on the results of gene expression, further research on functional modification of MSCs by ultrasound treatment is underway. This study suggests the possibility of enhancing the therapeutic effects by treating MSCs with ultrasound before administration to patients.

Keywords: mesenchymal stem cells, ultrasound, improving function

4:45 PM – 5:45 PM

390

MOVING TOWARDS REGULATORY-COMPLIANT CELL THERAPY: USING HUMAN PLATELET LYSATE FOR IPSC MAINTENANCE AND DIFFERENTIATION

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While the first transplants of blood stem cells date back to the 1950s, the field of cell therapy has only recently emerged due to the establishment of reliable methods for cultivating clinically relevant cell types. The majority of cell therapy companies is currently conducting clinical trials in phase I or II, with some reaching phase III of securing early market approvals. As more companies enter phase III, there will be a substantial demand for scale-up, process validation and quality assurance of critical raw materials. Induced pluripotent stem cells (iPSCs), derived from adult stem cells, hold great therapeutic potential, capable of differentiating into any somatic cell type without ethical concerns. As iPSCs approach clinical use for transplantation, the requirement for agents that complay with regulatory practices needs to be addressed as the availability of appropriate subtrates is very limited. Human platelet lysate (HPL), a xeno-free cell culture supplement, demonstrated efficacy in supporting iPSCs differentiation into clinically relevant cell types, particulary mesenchymal stromal cell-like cells (iMSCs). PL BioScience GmbH (PLBS) from expired human thrombocyte concentrates, which are clinically tested transfusion products manufactured by certified blood donation centres. Our innovative ELAREM™ platform consists of patented GMP-compliant human platelet lysate products that promote attachment and growth of various cells. We guarantee high reproducibility and consistency in quality. We have successfully shown that iPSCs can be grown and differentiated into iMSCs using ELAREM™. Taken together, with ELAREM™, we provide xeno-free cell culture solutions that are clinically safe and fit the needs of all fields of cell culture application.

Keywords: human platelet lysate, iPSCs, cellular therapy

4:45 PM – 5:45 PM

392

MANIPULATION OF CELLULAR PLASTICITY PREVENTS HETEROGENEITY OF PROPAGATED AD-MSCS FOR THERAPEUTIC APPLICATION

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Stem cell therapy holds an unprecedented promise for treating degenerative diseases. However, the batch-to-batch heterogeneity of propagated cells poses a significant challenge for clinical applications. Here, we elucidated how culture conditions manipulate the plasticity in cell fate decisions leading to heterogeneity of cell populations. Using a strategy of reverse enrichment of corresponding feature genes, we identified the central role of p38-MAPK in regulating the cell fate decisions. Further studies revealed that the AGE-RAGE-p38-MAPK axis of adipose-derived mesenchymal stromal cells (AD-MSCs) was responsible for the emergence of pro-embolic subpopulation of the propagated cells. Timely manipulation of the activity of the AGE-RAGE pathway shifted the fate of AD-MSCs and eliminated the heterogeneity in pre-embolic risk of these cells. This finding indicates that closely monitoring critical components in cultures can eliminate the batch-to-batch heterogeneity of propagated cells for therapeutic applications.

Keywords: AD-MSCs, heterogeneity, plasticity

4:45 PM – 5:45 PM

394

REPORTER-FREE GENERATION OF IPSC-DERIVED TISSUE-SPECIFIC CELLS ENGINEERED FOR THE STABLE EXPRESSION OF IMMUNOMODULATORY PROTEINS

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Use of pro- (e.g., IL7 and IL15) and anti-inflammatory cytokines (e.g., IL4 and IL10) are being explored in cell therapy for their roles in directing and enhancing immunomodulation and survival of immune cells to alleviate conditions such as cancer and inflammatory disease, respectively. Due to their natural capacity to target inflamed and cancerous tissues, alongside their ability to surmount the clonality, expandability, and engineering hurdles linked to tissue-derived mesenchymal stem cells (MSCs), iPSC-derived MSCs (iMSCs) offer an optimal engineering platform for localized delivery of transgenic cytokines. Transgene expression, however, can be unstable or silenced during

the differentiation of iPSCs to engineered iMSCs (EiMSCs). To ensure sustained transgene expression upon differentiation, we explored the use of ubiquitous chromatin-opening elements (UCOEs) in transgenic cassettes that express green fluorescent protein (GFP) under the EF1α promoter. Cassettes with and without a UCOE were inserted into the AAVS1 safe-harbor locus in iPSCs using mRNA encoding UltraSlice™ gene-editing endonucleases and single-stranded DNA (ssDNA) repair templates. Clonal iPSC lines were isolated by the single-cell deposition of GFP-positive cells, and biallelic insertion into AAVS1 was verified. Upon differentiation to EiMSCs, uniform GFP expression was only observed with a UCOE (>99% vs. ≤30% without) and persisted after 10 passages and 2 freeze-thaw cycles. We then applied this workflow to create pro- and anti-inflammatory EiMSC lines that are designed to overexpress the IL7-IL15 and IL4-IL10 fusion proteins, respectively. High knock-in efficiencies into iPSCs (~10%) enabled by the use of UltraSlice™ has allowed the establishment of clonal transgenic EiMSC lines without the use of a reporter system. EiMSCs stably express IL7-IL15 at 250 pg/mL, measured pre-passage in culture. These cell lines may prove promising in the development of therapies to treat a wide range of diseases.

Keywords: engineered iPSCs, overcome gene silencing, EiMSC express fusion inflammatory cytokines

4:45 PM – 5:45 PM

396

DEVELOPMENT OF MICROCARRIERS SUITABLE FOR MASS CULTURE OF THERAPEUTIC CELLS

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In the industrialization of regenerative medicine, there is an increasing need for mass culture of therapeutic cells, and microcarriers provide promising solution. We have developed new microcarriers for regenerative medicine applications. Our microcarriers are made of polyvinyl alcohol (PVA) and their surface are covalently coated with collagen. Due to the characteristics of the material, PVA microcarriers are highly elastic and unbreakable, and generate less debris during stirring. It's important that no debris is generated when agitating cells in a bioreactor, because debris smaller than about 100μm is difficult to separate from cells. Additionally, we also verified the safety of PVA microcarriers. Testing for leachables and extractables was performed according to guidelines applicable to polymeric single-use components in biopharmaceutical manufacturing, and safety evaluation was obtained according to medical device standards. We used this PVA microcarriers to expand human mesenchymal stem cells (hMSCs). As a result, PVA microcarriers supported a 2.6-fold expansion of hMSCs in a 30-mL single-use stirred bioreactor after 7 days culture period, comparable to that of commercially available microcarriers. We also attempted bead-to-bead transfer by adding fresh microcarrier on days 7 and days 14, and we succeeded in long term culture for 21 days. These results suggest that these PVA microcarriers can contribute to the large-scale culture of hMSCs for regenerative medicine and cell therapy.

Keywords: mass culture, microcarrier, mesenchymal stem cell

4:45 PM – 5:45 PM

398

STEM CELL EXPANSION FROM HUMAN UMBILICAL CORD: A SCALE-OUT STRATEGY

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In human stem cells are present in limited numbers, unable to fulfill the necessary demand for regeneration and repair. Organ or tissue regeneration requires 10 to 400 million mesenchymal stem cells (MSCs) per dose for transplantation. The ex-vivo expansion of MSCs are vital to fulfill the requisite cell number for therapeutic use. The current research was designed to optimize a novel protocol to achieve qualitative and quantitative scaling of MSCs. These recultured human umbilical cord (hUC) tissue derived MSC were evaluated for Immunophenotypic, immunocytochemical staining, trilineage differentiation, population doubling time and number, gene expression markers for proliferation, cell cycle progression, senescence-associated β-Galactosidase assay, human reverse transcriptase telomerase (hTERT) expression, mycoplasma, cytomegalovirus, and endotoxin detection. The results for the pluripotent gene expression markers Nanog, Sox-2, and Oct-4 for each recultured hUC-MSC revealed no statistically significant differences. The immunophenotypic markers CD90, CD73, CD105, CD44, Vimentin, CD29, Stro-1, and Lin28 were positively expressed by these recultured expanded MSCs, but CD34, CD11b, CD19, CD45, and HLA-DR were not. Up until passage 15, the recultured hUC-MSC population continued to multiply and double in size. Normalized proliferative gene expression of PAX6, BMP2, and TGF1 shows no difference between recultured hUC-MSC groups. Nevertheless, a significant (***$p < 0.001$) increase in the mitotic phase of the cell cycle analysed in recultured hUC-MSC groups. Delayed cellular senescence was observed (**$p < 0.01$) by increased expression of human telomerase reverse transcriptase (hTERT) at recultured numbers 8–10. The senescence-associated β-Galactosidase was not observed in repeatedly recultured hUC-MSCs. The quality of recultured hUC-MSCs was maintained and showed negative expression of mycoplasma, cytomegalovirus, and endotoxin. This study eventually results in the development of a cutting-edge protocol for scaling the stem cell population. It may rapidly increase the cell dose in vitro required for in vivo implantation. Since these MSCs were isolated from the same recultured hUC, they have persistent MSC stemness, as indicated by the International Society of Cellular Therapy (ISCT).

Keywords: human umbilical cord, expansion, mesenchymal stem cells

4:45 PM – 5:45 PM

400

POTENTIAL OF HUMENSCS AS A CLINICAL TREATMENT FOR HEART AND ENDOMETRIAL DISEASES

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The autologous stem cells with clinical significance were almost all invasively obtained. The menstrual blood as a stem cell source, was easier to obtain than other kinds of others, such as adipose or umbilical cords. We cultured huMenSCs (Human menstrual blood stem cells) in the serum-free media and passaged to 20+ passages in vitro. The fourth to sixth passage of the huMenSCs had successfully differentiated into the ectoderm, the endoderm and the adipose cells in vitro. The cells were free of the mycoplasma, chlamydia and endotoxin, and with a normal karyotype (46, XX). The huMenSCs EVs could reduce the rate of cell apoptosis, cardiac fibrosis and inflammation caused by oxidative stress in in vitro cultured heart muscle cells. The effects of huMenSCs and their extracelluar vesicles (EVs) on endometrial during the estrus cycle in mice is being undertaken.

Funding Source: The work is supported by Macau FDCT fund (0054/2022/A)

Keywords: huMenSCs, multipotential, oxidative stress

4:45 PM – 5:45 PM

402

MESENCHYMAL STEM CELLS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS UTILIZING AUTOMATED PRODUCTION POSSESS SUPERIOR IMMUNOMODULATORY POTENTIALS

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Induced pluripotent stem cells (iPSCs) can differentiate into any cell type, which makes them an attractive cell resource in regenerative medicine and drug discovery. However, the major barrier is unable to supply uniform quality of target cells from iPSCs due to the difficulties with handling iPSCs, which are highly sensitive to artificial factors and environmental alterations. Establishment of an automated iPSC cell culture system can overcome these difficulties. Although MSCs exhibit multiple differentiation potency and have shown promising therapeutic effects in a broad range of diseases, these applications can be hindered by their limited expansion ability and variation across donors. Here, programmable all-in-one Panasonic automated culture machine was utilized to derive iMSCs from human iPSCs. To further decode the transcriptomic information and uncover the cellular homogeneity in iMSCs derived either from automated or manual process, single cell RNA sequencing analysis using BD Rhapsody system were performed. Initial characterization revealed that automatic and manual iMSCs were positive for CD29, CD44, CD73, CD90, CD105, HLA-ABC, and negative for CD45, HLA-DR, as present by single cell RNA sequencing and flow cytometry data. To discover the potential signaling interactions between different cellular subpopulations, we interrogated our data with CellChat, which could predict the ligand and receptor interactions at single cell resolution. iMSCs derived from automatic production highly expressed WUCAM, PVR, and L-CAM signaling in comparison with manual iMSCs expressed DESMOSOME, SEMA4, and CD86 signaling pathways. To further validated the interaction between iMSCs and immune cells, FACS sorted PAPPA+ and HIST1H4C+ subpopulations were co-cultured with human primary NK cells which revealed iMSC subpopulations derived from automatic iMSCs primed NK cells for increased release of IFN-γ in response to IL-12 and IL-18. In contrast to cytotoxic CD56dim NK cells, CD56bright NK cells primed by automatic iMSC subpopulation expressed higher levels of CCR2 and then were more sensitive to CCL2. The current work elucidated the possibility of derivation of functional iMSCs with superior immunomodulatory properties utilizing automated production method.

Keywords: iMSCs, panasonic automatic cell culture system, BD rhapsody single cell analysis system

4:45 PM – 5:45 PM

404

TIMELY MODIFICATION OF MICROENVIRONMENT STIFFNESS IMPROVES CHONDROGENESIS OF SINGLE CELL HIMSCS

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Osteoarthritis (OA) is a widespread debilitating disease that currently has no effective treatments, and can lead to the need for total joint replacement surgery. Generation of de novo cartilage from cell types such as hiPSC-derived mesenchymal stromal cells (hiMSCs) is attractive for regenerative applications due to their sustainability as a stable cell source for OA therapies and disease modelling. However, the quality of neocartilage generated by MSCs is inferior due to increased hypertrophy compared to that of primary chondrocytes. To improve hiMSC chondrogenesis, deeper understanding of factors impacting cell fate during differentiation are needed. The study of microenvironment influence, specifically of stiffness, via single cell microgels (SCMs) on

MSC differentiation has previously been shown in adipogenesis and osteogenesis. We here explore the effect of three stiffnesses: soft (20kPa), stiff (80kPa), and post-cure (soft gels stiffened after seven days of differentiation) for chondrogenesis of hiMSCs in SCMs. We found that SCMs remained stable and supported hiMSC survival (>80%, cells non-proliferative) throughout 21 day chondrogenesis. Immunohistochemistry (IHC) showed expression of chondrogenic marker COL2 at day 21 in >80% of cells in soft and post-cure microgels, while it was only expressed in 35% of stiff microgels. IHC of bone marker SPP1 was expressed in 60% of soft but not in post-cure nor in stiff microgels, and hypertrophy marker COL10 was expressed in 85% of soft, 57% of post-cure, and 20% of stiff microgels. We demonstrate that, similar to bone marrow derived MSCs, hiMSCs differentiate and deposit neocartilage matrix in SCMs. Results also show that post-cure of SCMs enhances early commitment towards COL2 expression while preventing SPP1 expression and reducing COL10 expression. Therefore, we advocate that time-dependent control of the hiMSC microenvironment can be applied to reduce hypertrophy in neocartilage deposition. Further insight into key factors regulating chondrogenic cell fate decisions is ongoing through application of single cell multi-omics analysis to hiMSCs encapsulated in SCMs. In due time this will allow targeted improvement of hiMSC differentiation and neocartilage quality for treatment of OA.

Funding Source: Financial support was received from the Dutch Research Council (NWO, OCENW.GROOT.2019.079)

Keywords: single cell microgel, chondrogenesis, matrix stiffness, inical

4:45 PM – 5:45 PM

TOPIC: MUSCULOSKELETAL

406

MODULATION OF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM/MEDICINAL SIGNALING CELLS WITH ASCORBIC ACID AND SILK FIBROIN: EFFECTS ON PROLIFERATION, CHONDROGENIC DIFFERENTIATION AND ENCAPSULATION

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The key aspect of the therapeutic potential of MSCs for cartilage therapies is their proliferation and differentiation potential. Existing therapies utilize the immunomodulatory functions of undifferentiated cells, while MSCs differentiated into chondrocytes represent a possible alternative to true chondrocytes used in matrix-induced autologous chondrocyte implantation (MACI). In the first case, timely multiplication of MSCs to sufficient numbers is required, and in the second case, constitutive expression of hypertrophic markers associated with standard protocols for chondrogenic differentiation must be reduced or eliminated to ensure their use in MACI. As ascorbic acid (AA) is central to both MSC proliferation and chondrogenic differentiation, we investigated the effect of AA on the proliferation of caninen adipose-derived MSCs (cAMSCs) grown on a plastic surface and on silk fibroin (SF)-induced chondrogenesis of cAMSCs. A non-standardized procedure of SF-induced chondrogenesis, as shown in our previous research, could potentially lead to different outcomes in gene expression compared to commonly performed chondrogenesis. In this study, we have shown that AA significantly affects the proliferation potential of undifferentiated cAMSCs on plastic surfaces and is also a key factor of cell culture medium for determining the expression profile of chondrogenic and hypertrophic genes during SF-induced chondrogenesis. With the aim of developing a clinically relevant product with specifically modified cells suitable for either cartilage or bone regeneration depending on their genetic profile, we have optimized the protocol for the preparation of an injectable SF hydrogel for encapsulation of cAMSCs. Our study is of great importance to the scientific fields of regenerative medicine and tissue engineering as it improves the protocol for the expansion of cAMSCs with AA and utilizes the combination of SF and AA to modulate the chondrogenesis of cAMSCs. The results also have far-reaching applications in drug delivery. The SF hydrogel could be used as a vehicle for the slow and steady release of other therapeutic agents, such as extracellular vesicles from MSCs. The development of MSC-based cell-free off-the-shelf products will pave the way for the routine use of stem cell-based therapies in the future.

Funding Source: This work was supported by Slovenian Research and Innovation Agency (ARIS) research program P4-0053 and postdoctoral project Z4-4559.

Keywords: canine mesenchymal stem cells, proliferation and differentiation potential, effects of silk fibroin and ascorbic acid

4:45 PM – 5:45 PM

408

LIQUID CRYSTAL ELASTOMER AS SCAFFOLD FOR SUPPORTING OSTEOGENIC DIFFERENTIATION OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Liquid crystal elastomers (LCEs) are crosslinked liquid crystal polymers demonstrating the stimuli responsiveness of liquid crystals and elastic properties of conventional elastomers. The anisotropy and heterogeneity of LCE mimic load-bearing tissues such as bones, muscles, and connective tissues. In this study, collagen-based LCE was synthesized by the amine–acrylate click chemistry of aza-Michael addition and employed as a 3D scaffold for bone tissue engineering. Human bone marrow-derived mesenchymal stem cells (hBMSCs) were cultured on collagen-based LCE and stimulated to differentiate osteogenically. The morphology of hBMSCs was examined with a fluorescence microscope by staining with 4',6-diamidino-2-phenylindole and rhodamine phalloidin to visualize the nucleus and cytoskeleton, respectively. Our results demonstrate that no significant difference in cell shape and internal organization was observed when hBMSCs were cultured on collagen-based LCE. In addition, no significant cytotoxicity of collagen-based LCE, as determined by the WST-1 cell viability assay of hBMSCs, was detected after rinsing the biomaterial with water and equilibrated in phosphate-buffered saline, indicating that the cross-linking and polymerization process followed by the washing procedure

effectively eliminated potentially toxic components of the biomaterial. When the glass substrate was coated with the planar alignment reagent, polyvinyl alcohol, the planarly aligned liquid crystal mesogen in collagen-based LCE guided hBMSCs to grow and differentiate along the LCE network. By studying the change in optical properties of the stimuli-responsive collagen-based LCE caused by cell–matrix interactions, including LCE-induced alignment of cells and cell-induced reorganization of LCE, whose composition was further modified by extracellular matrix synthesis and mineral deposition during osteogenic differentiation, biosensing functionalities of collagen-based LCE correlated to osteogenic differentiation were established.

Funding Source: This study was financially supported by the National Science and Technology Council, Taiwan, under grant No. NSTC 112-2314-B-309-001.

Keywords: liquid crystal elastomer, bone tissue engineering, 3-dimensional culture

4:45 PM – 5:45 PM

410

ENHANCING CULTURED MEAT PRODUCTION: AUTOPHAGY REGULATION FOR STEMNESS AND AGE-RELATED EFFICIENCY

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This study focuses on enhancing cultured meat production by exploring the regulation of autophagy for maintaining stemness and optimizing age-related efficiency in muscle stem cell lines. Cultured meat offers a sustainable and ethical alternative to conventional animal meat by utilizing bovine-derived muscle satellite/stem cells for in vitro meat production. To overcome the mass production challenges caused by limited cell division in these stem cells, our goal is to develop an optimized stem cell line with enhanced proliferation and differentiation capabilities. We investigated the proliferation and differentiation capacities of bovine muscle-derived satellite/stem cells, comparing cells isolated from adult cattle aged 38 to 60 months and fetuses found during slaughter. Our findings reveal that fetal muscle cells exhibit higher growth capacity, attachment, and proliferation after primary culture, alongside a greater presence of muscle satellite cell-specific markers compared to adult muscle cells. Adult muscle cells showed lower expression levels of mesenchymal stem cell and myogenic markers, indicating a closer gene profile to a differentiated state compared to fetal cells. Through transcriptome analysis and the comparison of myogenic marker expression, we assessed the undifferentiation maintenance capability of muscle satellite cells. The significance of autophagy induction in maintaining the undifferentiation state, especially in adult muscle cells, which displayed impaired ability to form plurinucleate and contractile structures under differentiation conditions. Our study introduces a protocol that uses autophagy regulation to prevent muscle stem cells from differentiating prematurely, improving their ability to maintain stemness and proliferate. By determining the best conditions and cattle age for muscle cell differentiation, we aim to create optimized bovine muscle-derived stem cell lines for efficient and scalable cultured meat production, offering a sustainable and ethical meat alternative while enhancing production quality and efficiency.

Funding Source: This work was supported by Korea IPET through High Value-added Food Technology Development program, funded by MAFRA (321025-05) and by the KRIBB Research Initiative Program Grants (KGM4562323)

Keywords: cultured meat, muscle stem cell, transcriptome analysis

4:45 PM – 5:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

412

USING ARTIFICIAL INTELLIGENCE TO AID CURATION OF DATASETS IN THE STEMFORMATICS ATLAS

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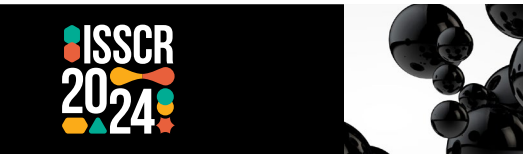
Stemformatics is a platform for sharing and visualising stem cell transcriptomic data. Stemformatics hosts an extensive data collection that offers an in-depth view of stem cell biology with a particular focus on haematopoiesis and myeloid subsets. We have created integrated atlases to compare primary and iPSC derived macrophages and dendritic cells. These atlases are proving useful for cross-dataset comparisons, enabling researchers to annotate myeloid subsets in external single cell data, and identify tissue-specific or activation properties of laboratory models or primary cells. While the atlas approach allows us to identify shared patterns of gene expression that define cell types, we are also interested in deeper exploration of the laboratory variables that contribute to myeloid differentiation and activation. Our long-term vision to develop Artificial Intelligence models to identify experimental or environmental drivers of complex gene expression phenotypes. Specifically, we are implementing Deep Neural Networks to study the influence of growth factor combinations, timing or dose on myeloid differentiation outcomes. This requires deep curation of the experimental metadata accompanying a transcriptional dataset. Here, we assessed the usefulness of Large Language Models for method extraction from academic papers to assist with rapid curation and integration of public data. These models are able to rapidly and accurately partition data from structured formats, like the NCBI GEO database, but require



human curation for extraction of information from published methods, especially where details are dispersed across figure legends and supplemental files. Stemformatics atlases, including curated metadata tables are freely available at www.stemformatics.org and updates are provided via the Git repository https://github.com/wellslab/s4m-api.

Funding Source: Australian National Health and Medical Research Council Synergy grant APP1186371 to CW.

Keywords: transcriptional atlas, artificial intelligence, computational stem cell biology

4:45 PM – 5:45 PM

TOPIC: NEURAL

414

PROFILING OF G-QUADRUPLEX LANDSCAPES BY SINGLE-CELL CUT&TAG IN MIXED CELL POPULATIONS

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Combination of CUT&Tag technology with droplet-based single-cell library preparation enables the high-quality single-cell mapping of chromatin modifications. Here, we apply single-cell CUT&Tag (scCUT&Tag) to quantify DNA G-quadruplexe (G4) structures at single-cell level in various cell lines. G4 scCUT&Tag allows us to distinguish different cell types of mixed cell populations based on G4 heterogeneity and quantify cluster specific co-quadruplexed sites over putative regulatory elements. Moreover, unpaired multi-omic integration of G4 scCUT&Tag with annotated scRNA-Seq reference data aid to describe the co-enrichments of G4 signals with oligodendrocyte marker gene transcription and subtype specific G4s following label transfer. We demonstrate the utility of G4 scCUT&Tag on low cell numbers and single cells. Additionally, we show that the applied single-cell computational pipelines on the G4 scCUT&Tag data enable the higher level characterization of G4s.

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Keywords: G-quadruplex, multi-omics, single-cell CUT&Tag

4:45 PM – 5:45 PM

416

USING ELECTROCHEMISTRY TO STUDY NEUROTRANSMITTER RELEASE FROM HUMAN STEM CELL-DERIVED 3D MODEL SYSTEMS IN REAL-TIME

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Validating the function of stem cell-derived somatic cells in vitro is crucial to substantiate these model systems for disease modelling as well as drug screening. Most cell types, including immune cells, secretory cells of the gut, and most prominently neurons, react to external stimuli by secreting signaling molecules, e.g. neurotransmitters like Serotonin (5HT) and Histamine (HA). Monitoring the dynamics of these molecules in real-time is still a challenge with researchers currently relying mostly on genetically engineered fluorescent sensors. In comparison, Fast-Scan Cyclic Voltammetry (FSCV) uses the intrinsic electrochemical properties of the secreted molecules without the need for the expression of exogenous sensors. A single carbon fiber-microelectrode is usually placed in the tissue of interest and used not only to identify the molecule released, but also to monitor dynamic changes of the extracellular concentration on a sub-second scale. Here, we optimized FSCV measurements for different cell populations derived from human stem cells, including neuronal spheroids, gut organoids, and immune cells. First, serotonergic neurons were differentiated from human induced pluripotent stem cells (iPSCs) and 3D neuronal spheroids were generated. To validate neuronal functionality and their value for pharmacological screening, the release and reuptake of 5HT was analyzed. Upon electrical stimulation, neurons released Serotonin and the kinetics of the subsequent reuptake was impacted by an acute treatment with selective-serotonin reuptake inhibitors (SSRIs). Secondly, gut organoids containing enterochromaffin cells derived from human primary stem cells were analyzed. These cells showed a spontaneous serotonergic tone, and both the frequency and amplitude of the releases were increase by chemical stimulation. Finally, iPSC-derived microglia, as the major immune cell type of the brain, were embedded into a droplet of extracellular matrix to obtain a 3D cellular structure. Lipopolysaccharide (LPS) was used to trigger an inflammatory reaction, and microglia were found to release HA, as a pro-inflammatory response. In summary, FSCV is a promising and powerful new tool to evaluate the secretory function of a wide range of 3D cellular models in vitro.

Keywords: neurotransmitter release and reuptake, electrochemistry, organoids and spheroids

4:45 PM – 5:45 PM

418

UNVEILING THE SPATIAL EPITRANSCRIPTOMIC AND EVOLUTIONARY DYNAMICS OF ZIKA VIRUS INFECTION IN HUMAN BRAIN ORGANOIDS

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Zika virus (ZIKV) poses a significant neurodevelopmental threat, evidenced by its association with microcephaly and other congenital abnormalities. Conventional models have failed to elucidate the complexity of ZIKV pathogenesis due to the lack of human pathophysiological context and spatial resolution. Recent advances in spatial epitranscriptomics hold promise for bridging this knowledge gap. This study aims to dissect the spatial epitranscriptomic landscape of ZIKV-infected brain organoids, focusing on the role of Adenosine-to-Inosine (A-to-I) RNA editing in modulating host-virus interactions and their implications for viral pathogenesis and potential therapeutic interventions. Utilizing induced pluripotent stem cell (iPSC) technology, we differentiated brain organoids containing distinct neural progenitor and mature neuron populations. Upon ZIKV infection, we applied spatial-histopathological examination-linked epitranscriptomics converged to transcriptomics with sequencing (Select-seq) to isolate and analyze discrete microniches. Our approach enabled full-length sequencing of host and viral transcriptomes, along with precise A-to-I RNA editing profiling. Our findings reveal a distinct spatial distribution of A-to-I RNA editing events within ZIKV-infected organoids, correlating with unique gene expression signatures and cellular phenotypes. Notably, A-to-I editing was found to be differentially regulated in ZIKV envelope protein-expressing cells, suggesting a potential mechanism of viral evasion or adaptation. Furthermore, we identified patterns of host-virus interaction that may contribute to the neuropathological outcomes associated with ZIKV. The integration of spatial epitranscriptomics into the study of ZIKV infection provides unprecedented insight into the virus-host interplay at a cellular level. Our pioneering approach underpins the significance of spatial context in understanding viral pathogenesis and opens avenues for the development of targeted antiviral therapies. The application of advanced technology in virology ushers in a new era of precision medicine, enabling the dissection of complex pathogenetic processes and fostering the development of personalized therapeutic strategies against ZIKV and other neuropathogenic viruses.

Funding Source: This research was supported by the Ministry of Science and ICT (MSIT) of the Republic of Korea and the National Research Foundation of Korea (RS-2023-00266110toA.C.L.).

Keywords: Zika virus infection, brain organoids, host-virus spatial epitranscriptomics

4:45 PM – 5:45 PM

420

CHARACTERIZATION OF A HUMAN MIDBRAIN ORGANOID CONTAINING DOPAMINERGIC NEURONS

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Parkinson's Disease (PD) is an incurable neurological disease affecting 1% of the population above 60 years of age. The main pathological hallmark of this disease is the degeneration of dopaminergic neurons in the substantia nigra in the midbrain, leading to lack of dopamine in the human forebrain. This may be triggered by insoluble aggregates of misfolded alpha-synuclein and have been shown to have prion-like transmission. Although in vivo experiments in rodent models have been instrumental for the identification of the pathological hallmarks of PD, the underlying cause remains unknown rendering treatment impossible. Human induced pluripotent stem cells (hiPSCs) derived organoids offer new opportunities to investigate and treat neurological diseases. To decipher the molecular mechanisms underlying the pathophysiology of PD, we aimed to generate a human organoid containing ventral midbrain dopaminergic (mDA) neurons, based on a previously established model, the bioengineered neuronal organoid (BENO) (Zafeiriou et al., 2020). The midbrain BENO (mBENO) is generated from hiPSCs embedded in collagen hydrogel, which are patterned into ventral midbrain by growth factors and small molecules over a time course of 30 days. On day 62 mBENOs were analysed for floor plate (FOXA2), midbrain (EN1) and mDA neuron (TH) markers. Real time PCR analysis shows a five to ten-fold higher expression of TH, a 30-60-fold higher expression of EN1 and a 6-9-fold higher expression for FOXA2 compared to undifferentiated hiPSCs (n=4). Wholemount immunofluorescence analysis validated the high abundance of midbrain dopaminergic neurons marked by FOXA2 and TH expression, as well as specialized TH+ cells by GIRK2 and CALB1 staining. Finally, we quantified the dopamine content of mBENO via liquid chromatography mass spectrometry. In conclusion, mBENO represents a novel human midbrain organoid model enriched in ventral dopamine producing neurons. In the future, we will explore the mBENO potential in modelling PD by patient and transgenic lines or as mDA source for cell replacement therapy.

Keywords: midbrain organoid, dopaminergic neurons, Parkinson

4:45 PM – 5:45 PM

422

MITOCHONDRIAL DNA EDITING IN IPSC MODELS OF MATERNALLY INHERITED LEIGH SYNDROME

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Until recently, the generation of disease models for mitochondrially encoded diseases has been challenging, due to the hurdles of mtDNA editing, resulting in a paucity of such models. Inefficient transport of nucleic acids into mitochondria and a lack of efficient double-strand break repair mechanisms impede the use of CRISPR/Cas as genome editing tool in mitochondria. Instead, a recently developed genome engineering tool termed DddA-derived cytosine base editor (DdCBE) enables the reliable introduction of precise modifications into mtDNA in form of C•G bp to T•A bp transitions. Here, we aim to optimize the use of this novel tool in induced pluripotent stem cells (iPSCs) to facilitate the study of mitochondrial disorders. We have identified two pathogenic mutations associated with Maternally inherited Leigh syndrome (MILS) that can be targeted by DdCBE. MILS is a form of Leigh syndrome caused by mutations in mitochondrial DNA (mtDNA) genes coding for components of the mitochondrial respiratory chain (RC) and is the most severe mitochondrial disorder affecting children. Already, we were able to design, clone, and identify the most efficient DdCBE constructs for each target site, affording maximal on-target and minimal bystander editing. On-target editing efficiencies detected in HEK293 cells during construct optimization experiments were in line with the literature. Further, we added red and green fluorescent reporters to the best performing construct pairs to facilitate transfection optimizations, as well as fluorescence activated cell sorting (FACS). A change of promoter markedly increased reporter fluorescence (indicating concomitant increase in DdCBE expression) in iPS cells. Currently, we are exploring different transfection strategies and sorting experiments are ongoing. Next, we will monitor the changes in mtDNA mutation level after DdCBE-based editing in iPSCs in terms of on- and off-target editing. Resulting edited iPSC lines can be differentiated into neural cells in order to address the impact of these changes on human brain development, furthering our understanding of this disorder with unmet medical need.

Keywords: mtDNA editing, DdCBE, disease modeling

4:45 PM – 5:45 PM

424

IMPROVING CELL PURITY WITH LINEAGE RESTRICTED UNDIFFERENTIATED STEM CELLS: A NOVEL APPROACH FOR GENERATING DOPAMINERGIC NEURONS

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The differentiation of human pluripotent stem cells (hPSCs) into mesencephalic dopaminergic (mesDA) neurons requires a precise combination of extrinsic factors that recapitulate the in vivo environment and timing. Current methods are capable of generating mesDA neurons after long-term culture in vitro; however, when mesDA progenitors are transplanted in vivo the resulting mesDA neurons are only minor components of the graft. In this study, we genetically modified pluripotent stem cells to generate an alternative type of stem cell that we named lineage-restricted undifferentiated stem cells (LR-USCs). LR-USCs are prevented from differentiating into a broad range of nondopaminergic cell types due to the knockout of genes critical for the specification of alternate lineages. Specifically, we targeted transcription factors involved in the production of hindbrain and spinal cord regions. When LR-USCs were differentiated under conditions known to promote midbrain specification, 69% of the cells they generated were bona fide caudal midbrain floor plate progenitors, i.e., were quadruple-positive for FOXA2/LMX1A/OTX2/EN1, whereas only 25% of the cells generated from human embryonic stem cells (hESCs) were caudal midbrain floor plate progenitors. Furthermore, LR-USCs generated 32% dopaminergic (DA) neurons, whereas hESCs from the same genetic background generated 9%. When differentiated under conditions known to promote hindbrain specification, 32% of LR-USCs maintained a midbrain identity (OTX2/EN1 double-positive), whereas 3% of hESCs did so. We show that when midbrain patterned LR-USC progenitors were transplanted into 6-hydroxydopamine lesioned rats they restored function in a clinically relevant non-pharmacological behavioral test, whilst midbrain

patterned hESC-derived progenitors did not. This strategy demonstrates how lineage restriction can prevent the development of undesirable lineages and enhance the conditions necessary for the generation of mesDA neurons.

Keywords: lineage restriction, dopaminergic neurons, gene editing

4:45 PM – 5:45 PM

426

EFFECT OF BHB ON IN VITRO DOPAMINERGIC NEURON DIFFERENTIATION FROM PLURIPOTENT STEM CELLS

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Neuronal differentiation is a highly regulated process, in which various morphogens are capable of giving specific identity to various cell lineages. These molecules have been used as tools for the study of highly regulated processes in embryonic development, with the aim to provide models for the study of fundamental events during neuronal differentiation. There is limited information on the role of metabolites derived from physiological cellular metabolism in development; some metabolites have been proposed to be protective factors for neurons in different neurodegenerative models. In this work, we explored the role of beta-hydroxybutyrate (BHB), a metabolite present throughout life, from conception to adulthood in the neuronal microenvironment. BHB has been proposed as an inflammatory, epigenetic, energetic and neuroprotective regulator. We exposed human embryonic stem cell (ESC)-derived neural progenitors, growing under conditions of dopaminergic neuronal differentiation to 3 concentrations of BHB (1, 3, and 5 mM). It was observed that exposure of neural progenitors to this metabolite significantly promotes higher expression of markers associated with dopaminergic specification, compared to a control group without BHB. To further explore the mechanism behind these gene-expression changes, we mapped the regions associated to transcriptional activation using CUT&RUN sequencing. One of the epigenetic modifications which have previously been associated to the changes in chromatin accessibility, in presence of BHB, is H3K27ac. In order to obtain global information of the influence of this metabolite in gene expression, we evaluated this mark in our differentiation model, with and without BHB. These results indicate that BHB can enhance dopaminergic differentiation of pluripotent stem cells.

Funding Source: Supported by PAPIIT-UNAM (IN219122) and Conacyt (CF2023-I-1668).

Keywords: dopaminergic neurons, beta-hydroxybutyrate, neurodevelopment

ABSTRACT WITHDRAWN

4:45 PM – 5:45 PM

430

EXPLORING 3D CULTURING METHODS FOR DIRECT NEURONAL REPROGRAMMING OF ADULT HUMAN FIBROBLASTS

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Direct neuronal reprogramming of adult human dermal fibroblasts (hDFs) can be used for several applications such as disease modeling, and cell therapy. Some challenges with current two-dimensional (2D) protocols for direct neuronal reprogramming are to achieve high efficiency and to keep the converted neurons in culture for extended periods. Moreover, directly reprogrammed neurons have low survival rate and limited integration after transplantation compared to stem cell differentiated neurons. In this study, we explore three-dimensional (3D) culturing methods to overcome these issues, using either 3D microculture arrays or fibroblast-mediated contraction of the extracellular matrix component collagen to form spheres. By adapting viral vector combinations, hDFs can be reprogrammed to different neuronal subtypes that express subtype-specific markers. After undergoing reprogramming toward dopaminergic fate, 3D cultures exhibit functional activity. Following in vitro studies, induced dopaminergic neurospheroids were transplanted into the striatum of a preclinical rat model for Parkinson's disease, with graft integration and functionality shown 8 weeks post-transplantation. With these 3D culturing methods, it is possible to overcome some major bottlenecks in direct reprogramming of fibroblasts. The protocols are easily adaptable and can be used to reprogram adult human fibroblasts to different neuronal subtypes, as well as be used for transplantation for therapeutic purposes.

Keywords: direct reprogramming, fibroblast to neuron reprogramming, cell transplantation

4:45 PM – 5:45 PM

432

TRANSFORMING DRUG DISCOVERY WITH LUMIRDT, ADVANCED HUMAN IPSC DISEASE MODEL AND 3D ORGANOIDS

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The utilization of iPSC (induced pluripotent stem cell)-derived models has garnered significant attention as a promising alternative to animal experimentation, aligning with the principles of the 3Rs (Replacement, Reduction, and Refinement). iPSC-derived cells have found widespread application in toxicity testing, disease modeling, and drug discovery due to their capacity to mimic crucial aspects of human biology. LumiSTAR Real-time Detection Technology (LumiRDT) represents a state-of-the-art innovation enabling the real-time quantification of phenotypic patterns, both spatially and temporally. This advancement facilitates the simultaneous assessment of multiple parameters, offering valuable insights into cellular responses over prolonged durations. A standout feature of the LumiRDT series is its capability to monitor cell responses for more than a month, rendering it suitable for investigating the long-term impacts of pharmaceuticals. Moreover, the LumiRDT series is compatible with 3D cell cultures, further broadening its utility. We have established a high-throughput phenotypic screening platform leveraging iPSC technology. Utilizing Alzheimer's disease (AD) as an illustrative example, we have demonstrated that iPSC-derived cortical neurons and astrocytes can replicate key disease characteristics in both 2D/3D models. These include morphological alterations, changes in reactive oxygen species (ROS) levels, aberrant calcium activity, and the accumulation of amyloid-beta (Aβ) peptides, notably the Aβ42/Aβ40 ratio implicated in AD pathology. Additionally, we showcase a non-invasive method for optically controlling and observing triggered cellular activity in iPSC-derived 3D cardiomyocytes, facilitating high-throughput drug screening and toxicity assessments. By harnessing the capabilities of LumiRDT, researchers can gain valuable insights into the cellular mechanisms underlying various diseases. This technology streamlines high-throughput screening of potential drugs and facilitates the identification of novel therapeutic targets. Ultimately, the combination of iPSC-based models with LumiRDT holds great promise for advancing drug screening and testing methodologies.

Keywords: iPSC derived neural cultures, 3D organoids, LumiRDT, bioimaging, disease modelling, high-content screening

4:45 PM – 5:45 PM

434

ENHANCING NEURAL STEM CELL COMMITMENT THROUGH ON-CHIP NEURAL INDUCTION: A STRATEGY FOR IPSC-BASED THERAPIES

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The potential for autologous therapeutics through the clinical application of induced pluripotent stem cells (iPSCs) is substantial. However, the current process for iPSC generation is costly, time-consuming, and demands standardization for therapeutic application. Our proposed approach introduces a streamlined microfluidic method for reprogramming fibroblasts into iPSCs, followed by their subsequent differentiation into neural stem cells (NSCs). Leveraging microphysiological technology, our method significantly reduces reagent usage for reprogramming by 100-fold and decreases the number of input cells by 9-fold. iPSCs generated through microfluidic reprogramming of fibroblasts exhibit upregulation of pluripotency markers and downregulation of fibroblast markers, comparable to those reprogrammed under standardized well conditions. The NSCs differentiated in microfluidic chips demonstrate an upregulation of neuroectodermal markers (ZIC1, PAX6, SOX1), underscoring their inclination towards nervous system development. A comparative analysis of cells obtained on conventional well plates and microfluidic chips for reprogramming and neural induction through bulk RNA sequencing was conducted. Pathway enrichment analysis of NSCs generated in the chip reveals enrichment in neural stem cell development and an enhanced commitment to the neural stem cell lineage at an earlier time point, attributed to the confined microfluidic chip environment. In summary, this microfluidic approach offers the potential for automation, establishing a closed system that minimizes batch-to-batch variations and eliminates the necessity for manual colony picking. Consequently, the method provides a cost-efficient pipeline for reprogramming and differentiating iPSCs in the realm of cellular therapeutics, potentially advancing efforts in making autologous therapies affordable on an industrial scale.

Keywords: on-chip somatic cell reprogramming and neural induction, cost-efficient pipeline, autologous therapies

4:45 PM – 5:45 PM

436

PHARMACOLOGICAL IMPACTS ON NEURONAL CONNECTIVITY: EXPLORING THE CHANGE OF CORRELATION IN HUMAN-DERIVED NEURONAL NETWORK

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The human brain's functioning depends on the interaction between about a thousand billion of neurons, which are linked among complex connectivity circuits and communicate to exchange information, adapt to internal or external stimuli, and synchronize their activity. Investigating changes in the connectivity after drugs' administration to in vitro neuronal networks aim to assess the role of different neural types (e.g., glutamatergic or GABAergic neurons) within the intricate connectivity circuits. This would provide a more comprehensive understanding of the role of both excitatory and inhibitory components. Our model includes the integration of human induced pluripotent stem cells (hiPSCs) with micro-electrode arrays (MEAs), providing a controlled in vitro environment. In this work, our experimental system included two culture configurations: the homogenous configuration, composed of exclusively glutamatergic neurons (100E), and the heterogeneous configuration, constituted by 75% of glutamatergic neurons and 25% of GABAergic neurons (75E25I). At the Day In Vitro 70, we recorded the spontaneous electrophysiological activity (10 minutes) and the chemically stimulated one (40 minutes). In particular, we tested three drugs, acting as antagonists of three of the most relevant ionotropic receptors of the human brain, i.e., 2-amino-5-phosphonovaleric (APV), acting as NMDA receptors antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), functioning as AMPA receptors antagonist, and bicuculline (BIC), working as GABA receptors antagonist. We analysed the electrophysiological activity to understand how the connectivity of the hiPSCs-derived neuronal networks was modified by the abovementioned drugs. Briefly, the administration of APV and CNQX led to a strong decrease in connectivity of both neuronal network configurations, whereas the BIC administration caused a significant increase in the correlation in the heterogeneous configuration. In conclusion, understanding the drug-induced modulation of neuronal connectivity provided a step forward for investigating targeted treatments to restore or regulate neural network functionality, thereby contributing to advancements in neuroscientific research and clinical applications.

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Keywords: in vitro model, connectivity, chemical stimulation

4:45 PM – 5:45 PM

438

DEVELOPMENT OF A SERUM-FREE CULTURE SYSTEM FOR RETINAL/IRIS PIGMENT EPITHELIAL CELLS IN STEM CELL AND GENE THERAPY TO TREAT NEURORETINAL DEGENERATION

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Ageing populations led to an increase of incurable degenerative age-related diseases. Stem cell approaches are in development to close this gap. To reach the clinic, the implementation of xeno-free production processes is crucial. Culture of stem cells in serum-free medium is well established, while the culture of stem cell-derived post-mitotic cells lack similar protocols. We aim to implement a xeno-free culture of retinal (RPE) and iris (IPE) pigment epithelial cells as cell products of highest safety. To allow RPE/IPE cell culture and testing of large panels of influencing factors, an incremental high-throughput approach has been developed (human platelet lysate [hPL], culture surface, glucose, linoleic & linolenic acids, growth factors, and hormones were identified to be optimized). First, 50k porcine RPE (pRPE, n=6-18) cells were cultured for 8d in 1% FBS to assure cell survival while testing hPL (0.5-20%) as serum replacement analyzing morphology (microscopy), cell number, and viability (acridine-orange staining). The best 3 concentrations were validated in a 14d culture and additionally analyzed for RPE65 expression. Validation was repeated in human IPE cells (hIPE, n=3). Cell numbers of the 12% (39'310±12'660 cells/well) and 8% (38'049±9'449 cells/well) hPL groups did not differ significantly to the 10% FBS group (44'569±7'495 cells/well). Viability was high in all groups except the negative control (88.10±1.16% [12% hPL] to 93.26±2.63% [8% hPL] and 94.23±3.62% [2% hPL]). Micrographs confirmed proliferation, viability, and normal morphology in 5-10% hPL. During validation, 5-10% hPL were compared to 10% FBS showing similar cell numbers (46'6267±6'227 to 59'600±17'486 cells/well, p>0.05) and viability (89.9±1.3 to 92.77±1.6%). RPE65 was highly expressed in hPL-supplemented medium cultures. Transfer to 25k hIPE cells in 8% hPL-supplemented medium demonstrated normal morphology, high pigmentation, cell number (15'660±1'508 cells/well after trypsinization) and viability (82.4±4.9%). Results of the ongoing project show efficient FBS-replacement by 5-10% hPL in post-mitotic pRPE/hIPE cells. Testing of further parameters will allow FBS-avoidance offering xeno-free manufacturing processes for emerging new approaches in cell therapy for regenerative, personalized medicine.

Keywords: xeno-free, retinal pigment epithelial cells, neuroretinal degeneration

4:45 PM – 5:45 PM

442

CORRECTING A COMMON CRB1 MUTATION BY RNA BASE EDITING USING IPSC-DERIVED CELL MODELS

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Retinitis pigmentosa (RP) is an inherited retinal dystrophy that leads to visual impairment and blindness. Many of the causative genes for RP are responsible for photoreceptor or retinal epithelial cell functionality such as phototransduction, the visual cycle or homeostasis. Amongst them, is the Crumbs complex protein CRB1, with over 150 identified mutations causing missense changes or null alleles. The most common mutation in the CRB1 gene is C948Y, caused by a single base pair exchange from guanine to adenine at position 2,843 of the longest CRB1 splice variant. Although several gene therapeutic approaches are being explored, there is no treatment option for retinopathies to this day. Furthermore, in case of CRB1, a variety of isoforms with different length and expression pattern complicate their development. Although classical gene therapy methods may enable the modification or replacement of the mutated CRB1 gene, the associated risks of the treatment are too high. A potential alternative to classical gene therapies involves the use of a novel class of oligonucleotides (ASO) which harness endogenous A-to-I RNA editing enzymes to correct G-to-A base pair exchanges as for instance the C948Y mutation in CRB1. Using a modified approach called RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing), a restoration of the disease-causing point mutation C948Y in CRB1 was achieved. As an initial in vitro model for the treatment, we used hiPSC-derived Müller glia cells, considering them as a potential target cell type for the therapy. Using transfection and electroporation of CRB1-targeting ASO, we achieved 10-40 % genotypical point mutation correction. As a next step, we explored the potential of ASOs to penetrate retinal tissue by using retinal organoids derived from RP affected patients and from healthy donors. To find the optimal transduction technique for 3D tissues, a variety of methods such as lipofection, electroporation, magnetofection and nanoparticles is explored. The correction efficiency of the point mutation in the 3D retinal organoids was lower compared to 2D, highlighting the importance of three dimensional cell models. This sets the ground for the development of further RESTORE ASOs to target inherited point mutations with unmet clinical needs.

Keywords: retinal organoids, retinitis pigmentosa, RNA editing

4:45 PM – 5:45 PM

444

TRANS-OMIC PROFILING UNCOVERS MOLECULAR CONTROLS OF EARLY HUMAN CEREBRAL ORGANOID FORMATION

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Defining molecular controls that orchestrate human brain development is essential for uncovering the complexity behind neurodevelopment and the pathogenesis of neurological disorders. Due to the difficulties in accessing embryonic and fetal brain tissues, the differentiation of human pluripotent stem cell (hPSC)-derived three-dimensional neural organoids has made it possible to recapitulate this developmental process in vitro and provide a unique opportunity to investigate human brain development and disease. To elucidate the molecular programs that drive this highly dynamic process, here, we generate a comprehensive trans-omic map of the phosphoproteome, proteome, and transcriptome of the initial stages of pluripotency and neural differentiation towards the formation of human cerebral organoids (hCOs). Our integrative analysis uncovers key phospho-signalling events underlying neural lineage differentiation, and their convergence on transcriptional (co-)factors and chromatin remodellers that govern downstream gene regulatory networks (GRNs). Comparative analysis with developing human and mouse embryos using these GRNs demonstrates the fidelity of our hCOs in modelling embryonic brain development. Finally, we demonstrate biochemical modulation of the AKT signalling as a key molecular switch for controlling hCO formation. Our data provides a comprehensive resource to gain insight into the molecular controls in human embryonic brain development and also provide a guide for future development of protocols for hCO differentiation.

Funding Source: National Health and Medical Research Council Investigator Grant, Metcalf Prize to P.Y.; a Luminesce Alliance support to A.G-C.; Research Training Program and Children's Medical Research Institute postgraduate scholarship to C.C.

Keywords: cerebral organoid, multi-omics, signalling

ABSTRACT WITHDRAWN

4:45 PM – 5:45 PM

TOPIC: NO TISSUE SPECIFICITY

448

EFFICIENT SINGLE-CELL CLONING AND MONOCLONAL CULTURE GENERATION OF GENE-EDITED HUMAN IPSCS IN LOW VOLUMES

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Genome-engineering of human iPSCs is often hampered by low single-cell cloning efficiency and high uncertainty of culture clonality, which can render the generation of high-quality cell lines laborious, expensive, and time-consuming. Here, we show how a novel microfluidic cell culture approach can be used to obtain clonal hiPSC lines with high efficiency as part of CRISPR-Cas9 genome-editing. By exploiting interfacial tension, small-scale cell culture chambers (GRIDs) can be fabricated on polystyrene dishes with an immiscible translucent fluorocarbon overlay. Each GRID-chamber operates with less than 1 µL of cell culture medium to cultivate single cells into clonal colonies. The chambers' unique optical properties allow for the identification of single cells directly after cell plating, ensuring upfront monoclonality assurance and downstream traceability. Using a defined workflow as

part of our automated Cloning Platform, we demonstrate high cloning efficiency across several genetically distinct hiPSC lines. Additionally, phenotyping of clonal hiPSC colonies revealed that cells maintained the expression of pluripotency markers and their genomic integrity.

Keywords: cloning, monoclonality, CRISPR

4:45 PM – 5:45 PM

450

WHATTHECELL: A COMPUTATIONAL TOOLKIT FOR DEMULTIPLEXING POOLED CULTURE DATA AND QUANTIFYING AMBIENT RNA EXTENT AND ORIGIN

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Pooled culture, in which cells from multiple individuals and/or species are processed together in a dish, is an increasingly popular strategy for droplet-based single cell sequencing studies. This design allows experimenters to efficiently scale cell culture experiments, isolate cell intrinsic differences, and mitigate both artifacts and false positive results due to batch effects in arrayed cell culture while improving ease of doublet identification to uncover the effects of inter- and intra-species genetic variation on gene expression and regulation. We present WhatTheCell, a new computational toolkit for processing data from pooled culture experiments. We demonstrate the ability of WhatTheCell to separate multi-species mixtures of cells without aligning to reference genomes, and to identify cells' individual of origin by clustering mitochondrial haplotypes without prior knowledge of genotypes or the number of individuals present. We also demonstrate a WhatTheCell program that uses genotype data to identify cells' individual of origin, which is fast and accurate even in the case of inter-species tetraploid composite cell lines. Finally, we present a new method for simultaneously quantifying ambient RNA contamination per droplet and modeling the individuals of origin in the ambient RNA pool, given sequencing data from a pooled experiment and a genotype panel. Using this tool, we explore the effects of ambient RNA contamination on expression of specific genes. We expect WhatTheCell to be widely applicable for pooled culture "cellular village" approaches as well as for pooled single cell capture strategies.

Keywords: bioinformatics, scRNA-seq, multiplexing

4:45 PM – 5:45 PM

452

OPTICAL GENOME MAPPING AS A TOOL FOR MONITORING FINE-STRUCTURAL GENOMIC STABILITY IN LONG-TERM CULTURED INDUCED PLURIPOTENT STEM CELLS

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Genetic integrity testing of induced pluripotent stem cells (iPSCs) is crucial for human disease and drug therapy studies. The conventional approach is by karyotyping and genome-wide SNP array, while submicroscopic genetic changes in iPSCs throughout passaging are poorly investigated. Here, we monitored genomic stability of the reference iPSC line IMBAi001-A in different stages of culturing by optical genome mapping (OGM), an emerging, fluorescent-label-based method for in-situ detection of structural rearrangements with high resolution. Independent triplicates of early, mid- and late iPSC cultures, corresponding to customary master bank- and expansion passages, were processed on the Bionano G165 Saphyr platform. Concurrently, cells were monitored by karyotyping and Nanopore long-read WGS (ONT). To maximize analysis efficiency of the data obtained from automatic calling of the Bionano software (Access v1.7.2, Solve v3.7), we applied a bioinformatic pipeline incorporating 200 in-house OGM-characterized genomes and public repositories. Following data curation, only SVs < 800 kB remained, 98% thereof sized < 20 kbp, supporting normal karyograms. Most SVs (94%) were conserved between all passages. A subset of 229 SVs were supported by OGM only in early or late passages. However, WGS data confirmed none of them as truly passage-specific, indicating a single-digit dropout rate of OGM, mainly in the detection range close to the system´s cutoff (500 bp). Additionally, we subjected the complete SV dataset to benchmarking against ONT, assessing specific strengths of OGM particularly regarding characterization of balanced SVs and copy-number gains. By OGM we were able to determine high genomic stability of IMBAi001-A across long-term culturing, while evidence for singular variants with low allele frequencies, possibly indicating passage variation through mosaicism, was also detected. Due to its ability to bridge blind spots in the current quality control of iPSCs, OGM may be highly useful to complement

karyotyping, when used in combination with additional strategies for data analysis. It further allows efficient, genome-wide query of SVs of potential clinical interest. Our study presents a provisional blueprint for efficient interpretation of iPSC monitoring by OGM.

Funding Source: Work in the IMBA Stem Cell Core facility is supported by the Austrian Academy of Sciences.

Keywords: optical genome mapping, iPSC quality control, genomic integrity

4:45 PM – 5:45 PM

454

AN OPTIMIZED AND VERSATILE SMALL MOLECULE-BASED SUPPLEMENT MITIGATES STRESS AND IMPROVES SURVIVAL OF PSCS AND PSC-DERIVED CELLS

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Advancements in cell culture media development have led to improved survival, expansion and maintenance of PSCs and PSC-derived cells; however, poor cell survival and recovery following stress-inducing culture applications remain a significant challenge. The use of ROCK inhibitors (RI) provides moderate improvement in recovery of PSCs in some routine applications (single-cell passaging and cryopreservation), but RIs show little improvement in survival of differentiated cells. Existing commercially available cell recovery supplements offer improved performance compared to RIs, yet are only recommended for a limited number of applications. Here we present GibcoTM CultureCEPTTM Supplement, a new and highly versatile cell recovery reagent. The CultureCEPT Supplement is offered at 1000x concentration and the formulation is based on advancements at the NIH's National Center for Advancing Translational Sciences (NCATS). This study demonstrates the versatility and superior performance of CultureCEPT compared to existing recovery reagents in: (1) Recovery of PSCs from single-cell passaging (monolayer and spheroid) and cryopreservation, (2) Recovery of PSC-derived cells (neurons and cardiomyocytes) from dissociation and cryopreservation, (3) Recovery of organoid and spheroid models (lung and neuron) from cryopreservation. For many of these applications CultureCEPT improves survival by 25-50% compared to the RI Y-27632. CultureCEPT Supplement reduces cellular stress and improves viability of stem cells, differentiated cells, and primary cells during a wide variety of handling and processing steps where cell damage and death can limit the success of a workflow.

Keywords: PSC, recovery, supplement

4:45 PM – 5:45 PM

456

SYSTEMATIC FIDELITY ASSESSMENT OF RETINAL ORGANOIDS AND BLASTOIDS FOR MODELLING HUMAN RETINA AND DEVELOPMENT

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Stem cell-derived organoid systems, such as retinal organoids and blastoids, have become increasingly used for modelling human organs and development. Assessing the fidelity of these stem cell-derived organoids is essential for their utility in recapitulating the human counterparts. In this work, we first introduce a computational framework to systematically evaluate the molecular and cellular fidelity of the retinal organoids derived from various culture protocols. Specifically, we perform an extensive meta-atlas characterization of cellular identities using single-cell transcriptome data generated from the human retina to curate a retinal-cell-identity map, and subsequently assess the fidelity of the retinal organoids in modelling their human counterpart. Next, we extend the computational framework to evaluate stem cell-derived blastoids for recapitulating the architecture and cellular constitution of the human blastocysts in early human development. This involves establishing a reference map of cell identity and lineage differentiation of the human blastocysts through the integration and curation of single-cell transcriptome data of ex vivo blastocysts profiled at a range of developmental timepoints in culture, and use this reference map to assess the coverage and the authenticity of cell lineages and the progression of lineage differentiation of various blastoid models generated with different cellular sources and protocols. In summary, this work provides the computational methodology and framework for systematic fidelity assessment of stem cell-derived organoids for modelling human organs and development.

Funding Source: A NHMRC Investigator Grant to Pengyi Yang and a Metcalf Prize for Stem Cell Research to Pengyi Yang

Keywords: organoid, blastoid, cell identity

4:45 PM – 5:45 PM

458

DEMOCRATIZING HUMAN PLURIPOTENT CELL GENOME EDITING, EXPANSION AND DIFFERENTIATION THROUGH CULTURE MEDIA OPTIMIZATION

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Cost effective, practical, and robust culture of human pluripotent stem cells (hPSCs) is required for both basic and translational applications. This is especially crucial for large scale expansion of hPSCs for cell therapy, which ought to be made accessible to many patients irrespective of their socioeconomical background. Basal 8 (B8) has emerged as a cost-effective solution for negligible-cost, weekend-free, and chemically-defined hPSC culture. However, homebrewing of some recombinant growth factors for B8 is a bottleneck towards both access and reproducibility of this technology. Moreover, we found the published B8 formulation to be suboptimal in normoxic hPSC culture, which is widely used particularly for scaling up hPSC culture in suspension. Lastly, the suitability of B8 for the development of genome-edited, 3D-cultured hPSC-derived cells remains to be assessed. To meet these challenges we formulated B8 with commercially available, animal-free growth factors, refined its composition to support the culture of the widely-used WTC11 hiPSC line, and thoroughly characterized its performance compared to alternatives. Our formulation of B8 stabilizes pluripotency through higher concentrations of TGFß-3, though this remains at 2-fold lower levels than TGFß-1 in Essential 8 (E8). Costs are further offset by using 8-fold less thermostable FGF2 (t-FGF2), which is produced without any tag and truncated to increase potency. We compared weekend-free protocols using B8 or t-FGF2-containing homemade E8 media to commercial E8 that was replaced daily. Despite morphological changes, cells in B8 exhibited the highest levels of NANOG and other primed pluripotency markers, as indicated by flow cytometry and RNA sequencing. B8 also matched or outperformed other media with regards to genome editing via homologous directed recombination, germ layer specification, terminal cardiac differentiation in monolayer, and generation of cardiac organoids in 3D cultures. Overall, we demonstrate that a modified B8 formulation promises to democratize the generation of engineered cells for a wide range of applications.

Funding Source: Giovanni Armenise-Harvard Foundation Career Development Award 2021; ERC Starting Grant (TRANS-3; 101076026)

Keywords: hPSC culture, medium optimization, growth factors

4:45 PM – 5:45 PM

460

ENHANCING HUMAN PLURIPOTENT STEM CELL MONOCLONALITY WITH IMPEDANCE-BASED DISPENSING AND HIGH-THROUGHPUT IMAGING

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This poster presents a novel method for isolating and verifying monoclonal human pluripotent stem cells using an impedance-based single-cell dispenser (DispenCell™) and an automated high-throughput imager (CloneSelect® Imager FL). Our integrated approach significantly improves the efficiency and reliability of monoclonal cell-line development, crucial for bioprocess and drug screening advancements. The process involves dispensing cells into multi-well plates, followed by detailed imaging and growth monitoring. Results demonstrate DispenCell's gentle cell handling with high viability and CloneSelect® Imager FL's effectiveness in confirming monoclonality. This innovative technique is a substantial step forward in stem cell research and biotechnological applications.

Keywords: monoclonality verification, cell line development, single cell dispensing

4:45 PM – 5:45 PM

462

APPLYING SPATIAL TRANSCRIPTOMICS TO DETERMINE THE ORGANISATION OF CELL POPULATIONS WITHIN MULTIPLE PLURIPOTENT STEM CELL DERIVED ORGANOIDS

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Spatial transcriptomics (ST) is a novel, disruptive technology that permits exploration of gene regulatory networks with spatial and temporal resolution. The insight provided by ST platforms promises to shed new light on developmental biology across an array of tissue types including organoids. Organoids offer the opportunity to model development and disease while overcoming many of the limitations of animal models or 2D cell cultures. As organoids can be cultured from genetically engineered stem cell and patient cell lines, they permit the detailed analysis of the transcriptional perturbations that underlie disease pathogenesis. Organoids display an inherent 3D organisation in vitro, making them prime candidates for exploration with ST. However, due to the limitations of commercially available ST technologies and the small size of organoids, there have been relatively few studies using ST to investigate organoids. Furthermore, they have been limited to exploration of a single type of organoid at a time. Here we present the use of STOmics, currently the only ST platform to offer sub-cellular resolution while capturing transcriptome-wide information, to profile an array of organoids including: brain, heart, kidney, lung, cartilage, blood and engineered heart valve tissue. Our data demonstrates the varied utility of the STOmics platform to determine the organisation of cell populations within this range of pluripotent stem cell derived organoids, with certain tissues requiring further optimisation. Furthermore, we provide examples of profiling multiple organoids from a single chip to maximise data capture. With current exploration of organoid structure and transcriptional signatures limited in their scope, a more thorough understanding of the localised gene expression driving organisation within each system is needed to generate organoids more closely resembling human organs. By providing a systematic overview of ST data across these varied tissue types, this study illustrates how ST can be harnessed improve our understanding of human organoids.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine is supported by Novo Nordisk Foundation grants (NNF21CC0073729). STOmics consumables and sequencing awarded under STOmics Grant Program.

Keywords: spatial transcriptomics, organoids, STOmics

4:45 PM – 5:45 PM

464

GENERATION OF AN ADVANCED STRAIGHT-IN ACCEPTOR HUMAN IPSC LINE WITH TWO LANDING PADS FOR INTEGRATING LARGE DNA PAYLOADS

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Integration of large DNA fragments (>10 kb) into the genome of human induced-pluripotent stem cells (hiPSCs) can be challenging. Recently, we developed the STRAIGHT-IN (Serine and Tyrosine Recombinase-Assisted Integration of Genes for High-Throughput INvestigation) platform allowing for seamless integration of large DNA payloads into acceptor lines carrying the STRAIGHT-IN landing pad (LP). Here, we further developed the system and generated a new acceptor hiPSC line harboring two orthogonal LPs within the AAVS1 genomic safe harbor loci of the hiPSC line LUMC0099iCTRL04 (hPSCreg LUMCi004-A). The LPs are equipped with two Bxb1 AttP sites using either a central "GT" or "GA" dinucleotide sequence allowing for the specific integration of two different donor plasmids without cross-over. Successful integration of the GT donor plasmid enables expression of the BleoR positive selection marker while the GA donor plasmid results in expression of PuroR, allowing antibiotic selection with zeocin and puromycin, respectively. Furthermore, the novel GA LP enables excision of all auxiliary elements using the Flp-FRT system, whereas the GT LP uses Cre-loxP. To generate the line, we electroporated our previously generated v2 acceptor hiPSC line (LUMCi004-A-1) with Cas9-gRNA RNP complex targeting the intact AAVS1 allele, along with a targeting construct (TC). This TC contains homology arms, flanked by FRT/FRT-F3 sites, a PGK promotor driving the expression of mScarlet, Bxb1-attP-GA site and a promoterless PuroR selection marker. Following recovery and expansion, mScarlet+ cells were clonally isolated and correct targeting of the LP was verified using PCR screening. The derived line was subjected to extensive quality control and has a normal karyotype, can differentiate in vitro into the three germ layers and >80% of the cells expressed markers of the undifferentiated state. In conclusion, this resulting hiPSC line containing the newest version of STRAIGHT-IN is versatile and can be used for various complex research applications including transgene overexpression and generation of reporter lines.

Funding Source: LUMC hiPSC Hotel is part of the Novo Nordisk Foundation Center for Stem Cell Medicine and is supported by Novo Nordisk Foundation grants (NNF21CC0073729).

Keywords: hiPSC, STRAIGHT-IN, integrating large DNA payloads

4:45 PM – 5:45 PM

466

TRANSLATIONAL FRAMEWORK FOR CELL COUNTING METHODOLOGY FOR IPSC FROM ASSAY ESTABLISHMENT TO GMP-MANUFACTURING

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Induced pluripotent stem cells (iPSCs) present novel challenges in transitioning from small- to large-scale manufacturing for cell therapy products. Allogeneic, stem cell-derived products have great potential to treat large numbers of patients, however, they require rigorous in-process controls to ensure product quality and improve the chance for success in the patient. One of the most important metrics in the measurement of cell product quality is cell viability and cell number. iPSCs are prone to a high degree of variability and are directly influenced by the methods, equipment, and operators. Automated cell counting platforms alleviate the variability that may arise between operators; however, the validity of the counting method needs to be verified for each cell type based on culture conditions. This is particularly relevant for iPSCs that go through various stages of passaging that are reliant on viable cell densities for decisions on split ratios and seeding densities. In this study, we evaluated cell counting methods for iPSCs at multiple stages of scale-up, including single-cell passaging in adherent culture, suspension culture in shaker flasks, and suspension culture in small-scale bioreactors. We utilized an ISO 20391-2 guided study design to develop a standardized framework for accurate cell counting for iPSCs in 2D and 3D culture systems. Utilizing 5 independent dilutions of cells, flow-based, and image-based methods were used to determine viable cell densities in the absence of reference material and based on the tools and guidance provided by the International Organization for Standards and the US National Institute of Standards and Technology. The developed methods were technology transferred to process development teams involved in cell therapy manufacturing to further operationalize in a GMP manufacturing setting. The resulting cell counting framework was specifically designed and tested to enable iPSC culture in various formats; enabling a smooth transition of methods to ensure accuracy in the quantification of viable cells while building a platform that allows for evaluation of method performance, operator training, and testing to ensure that measurements are consistent.

Keywords: iPSC, viability, manufacturing

4:45 PM – 5:45 PM

468

TRANSIENT NAIVE REPROGRAMMING CORRECTS HUMAN IPS CELLS FUNCTIONALLY AND EPIGENETICALLY

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Cells undergo significant epigenome reconfiguration during reprogramming to human induced pluripotent stem cells (hiPS cells). However, notable differences exist between the epigenomes of hiPS cells and human embryonic stem (hES) cells, impacting hiPS cell function and rendering them not equally capable. Differences include epigenetic

memory of the originating cell population and aberrations that arise during reprogramming itself. The mechanisms and dynamics underpinning these processes are not fully understood. We characterized the persistence and emergence of these differences by performing genome-wide DNA methylation profiling throughout primed and naive reprogramming of human somatic cells to hiPS cells. We find that reprogramming-induced epigenetic aberrations manifest midway through primed reprogramming, while DNA demethylation initiates early in naive reprogramming. Utilizing this knowledge, we devised a transient-naive-treatment (TNT) reprogramming strategy to emulate the epigenetic reset that occurs during early human embryogenesis. We show that the epigenetic memory in hiPS cells is concentrated in cell of origin-dependent repressive chromatin marked by H3K9me3, lamin-B1 and aberrant CpH methylation. TNT reprogramming effectively reconfigures such domains to a hES-cell-like state but does not disrupt genomic imprinting, thereby avoiding problems associated with long-term naive culturing. Using an isogenic system, we demonstrate that TNT reprogramming can correct transposable element overexpression and differential gene expression seen in conventional hiPS cells, and that TNT-reprogrammed hiPS and hES cells show similar differentiation efficiencies. Moreover, TNT reprogramming enhances the differentiation of hiPS cells derived from multiple cell types and different germ layers. Thus, TNT reprogramming corrects epigenetic memory and aberrations, producing hiPS cells that are molecularly and functionally more similar to hES cells than conventional hiPS cells. We foresee TNT reprogramming becoming a new standard for biomedical and therapeutic applications and providing a novel system for studying epigenetic memory.

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Keywords: epigenetic memory, human induced pluripotent stem cells reprogramming

4:45 PM – 5:45 PM

470

FROM MANUAL TO AUTOMATED: REVOLUTIONIZING HIPSC DIFFERENTIATION BY INCREASING EFFICIENCY AND FIDELITY FOR NPCS, HSCS, AND LPCS, WITH CELLRAFT TECHNOLOGY

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Differentiation of patient-derived and genetically modified hiPSCs provides unmatched potential in developing accurate disease models. Despite numerous available protocols and commercial kits for hiPSC differentiation, the process of differentiating hiPSCs is highly technical, often requiring manual manipulation, which can lead to inefficiency and lack of reproducibility. We hypothesized that CellRaft® technology could overcome technical challenges in differentiating hiPSCs into cell types representing each lineage. Using the CellRaft Array and the CellRaft AIR® System, protocols were developed to improve efficiency, providing an automated solution for these manual workflows. To optimize neural progenitor (NPC) differentiation, embryoid bodies were seeded on CellRaft Arrays in neural induction media to monitor formation of neural rosettes. Automated isolation of CellRafts containing neural rosettes was performed using the AIR System, eliminating the need for manual dissection or rosette selection reagents. Post-isolation staining revealed 100% of CellRafts containing neural rosettes were Pax6 positive, indicating high fidelity of NPC differentiation. For efficient hematopoietic progenitor (HSC) differentiation, manual selection of 100-200µm aggregates is a critical step requiring precise timing and collection methods. We utilized software tools selecting size-specific monoclonal hiPSC colonies for isolation into collections plates for HSC differentiation. This automated method increased colony survival by 100% compared to the standard protocol with >100,000 monoclonal CD34+ HSCs per selected colony within 2 weeks. During lung progenitor (LPC) differentiation, a high degree of heterogeneity exists within the population in the mesendoderm state necessitating purification after differentiation. Using the CellRaft Array, we stained for the endoderm-specific marker, CXCR4 and selected populations for isolation using the AIR System, increasing the accuracy of LPC differentiation. These data demonstrate how automating the manual steps of hiPSC differentiation, including image-based monitoring, phenotypic selection, and isolation, increases the efficiency and fidelity of hiPSC differentiation, which accelerates the development of clinically relevant models.

Keywords: iPSC, differentiation, automation

4:45 PM – 5:45 PM

472

ADVANCE BIOPROCESSING WITH CUSTOMISED MICROCARRIERS: ENHANCING ANCHORAGE DEPENDENT CELL YIELDS

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Aim Microcarriers, essential in bioprocessing since the 1960s, are microscale beads used in bioreactors for cell growth. Despite their widespread use, innovation in this sector has been limited, leading to challenges in industrial scaling. Primary issues include particulates and residuals from construction materials, non-biodegradability, and a lack of customizability. Customizability is crucial as different cell types require specific physical and chemical substrate properties; an aspect inadequately addressed in current microcarrier technology. Research has extensively shown that cell growth, and differentiation are significantly influenced by substrate properties. We have applied this knowledge to develop adaptable microcarriers. Results To replicate physiological conditions, Smart MCs utilized microfluidics technology to create 150-micron diameter microcarriers with adjustable physical and chemical properties, such as stiffness and surface charges. This study focuses on the X1 series of microcarriers in three stiffness levels: X1S (soft, 16.67 kPA), X1M (medium, 37.9 kPA), and X1H (hard, 77 kPA).

Experimental data showed a strong correlation between microcarrier stiffness and cell proliferation. The study involved multiple cell types, including C2C12 mouse myoblasts, L929 mouse fibroblasts, and MARC-145 monkey kidney cells, to assess the impact of microcarrier stiffness on cell behaviour. C2C12 and L929 were seeded at 4 cells/microcarrier and harvested on day 5, whereas MARC-145 was seeded at 16 cells/microcarrier and harvested on day 3. C2C12 preferred X1S microcarriers, while L929 and MARC-145 favoured X1M. C2C12 has 20.5 folds increase in 5 days, L929 has 20.3 folds increase in 5 days and MARC-145 has 6.5 folds increase in 3 days. The higher cell yields from X1 microcarriers compared to competitors were attributed to enhanced cell proliferation, their biodegradable nature enabling better cell harvesting, and differentiation capability to muscle cells. The bespoke microcarriers represent a significant breakthrough in bioprocessing technology. Aligning microcarrier characteristics with specific requirements improved cell growth. This innovation surmounts traditional microcarrier limitations, setting new standards for efficiency and versatility in the field.

Funding Source: Funded by SmartMCS and the Agency for Science Technology and Research (A*STAR)

Keywords: microcarriers, stem cells, tensile strength

4:45 PM – 5:45 PM

474

AI BASED ANALYSIS OF THE SPHEROID CULTIVATION PROCESS IN REAL TIME

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Spheroids and organoids are three-dimensional aggregates of cells, which are used in disease modeling, drug screening and tissue engineering. However, the demand for large quantities of spheroids with reproducible properties and high quality poses a challenge as the complex and labor-intensive cultivation process requires constant quality control. In particular, the reliability and quality of the organoids produced are critical for applications such as drug screening, where meaningful data is needed to evaluate new drug candidates. These requirements reduce the scalability of spheroid production and transfer to industry. This work describes a proof-of-concept study on the automatic transport of the spheroids from a bioreactor via a microfluidic system and their optical analysis. A microfluidic chip is placed under a microscope, which captures the spheroids during their transport through the channel. An edge computing device analyses the growth of the spheroids by segmenting and classifying the images and calculating the properties of the spheroids, e.g., area, shape and homogeneity. The analysis is performed with an artificial neuronal network model, trained to segment the spheroids presented in the frame and additionally classify them into four different classes: “broken”, “intact”, “double”, and “out-of-frame”. We trained three variations of the “You Only Look Once” model to evaluate their performance for our application. Further analysis of the spheroids is carried out using classical image processing algorithms. The system can be used to analyze spheroids during culture medium changes and help biologists to refine the cultivation processes. The algorithm needs less than 1 second to process a single frame, which allows the analysis of spheroids and the display of relevant statistics in real-time. This innovative approach holds promise for improving efficiency and scalability in spheroid-based applications within biomedical research and biotechnology.

Funding Source: This work was supported by the Fraunhofer Center for Sensor Intelligence (ZSI)

Keywords: spheroids, edge computing, microfluidic

4:45 PM – 5:45 PM

476

LNP-BASED MRNA DELIVERY OF AN ADVANCED PIGGYBAC TRANSPOSASE FOR IMPROVED RECOVERY OF CRISPR PRIME EDITED HUMAN IPSC

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The versatility and efficiency of CRISPR prime editors (PE) makes them precious tools for direct modification of human iPSC. We and others have shown that reversible genomic integration of piggyBac PE cassettes (PB-PE) is an effective method for editing of hiPSC, which allows for tuning of the genome edited fraction in bulk cell populations over time. However, removal of the PB-PE cassettes by excision and HSV thymidine kinase-based fialuridine selection can be tedious work due to inefficient transposon excision. In addition, we have observed that this can result in loss of the genome edited hiPSC fraction, likely due to loss of clonal diversity in the bulk. This problem is even more pronounced when available prime editors for a desired genomic modification have low efficiency. To this end, we first created an improved version of the excision optimized hyperactive piggyBac transposase (hyPBase_exo) by human codon optimization and N-terminal addition of a nucleolus-predominant (NP) signal peptide from the HIV-1 TAT protein (hco.NP-hyPBase_exo). Overexpression of this vector in prime edited hiPSC followed by fialuridine selection resulted in a 1.4-fold increase

in recovered clones when compared with standard hyPBase_exo. We then packaged in vitro transcribed mRNA of hco.NP-hyPBase_exo into specially formulated lipid nanoparticles (LNPs). Those were delivered into prime edited hiPSC, which resulted in another 7.2-fold enhancement when compared to plasmid-based methods. Together, we report a 10-fold improvement in recovery of prime edited hiPSC clones when compared to previous methods, which allowed for robust isolation of excised and genome edited bulk cell populations.

Keywords: CRISPR-PE, piggyBac, LNP

4:45 PM – 5:45 PM

478

A HIGHLY EFFICIENT DONOR SYSTEM FOR CRISPR KNOCK-IN EDITING IN MAMMALIAN CELLS

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CRISPR is a potent tool widely used for genome editing. While CRISPR knock-out, achieved through indel mutations via cellular repair mechanisms, has proven remarkably effective, the knock-in system for exogenous fragment insertion encounters is challenging due to limited specificity and efficiency. Two primary methods for exogenous fragment insertion, NHEJ (Non-Homologous End Joining) and HR (Homologous Recombination), exist. The HR allows for the construction of DNA insertions with precise junctions but is comparatively less efficient than NHEJ. To address these limitations, our system focuses on enhancing the efficiency of HR-based genome editing. Primarily, we have optimized the Lenti All-In-One CRISPR system to augment the delivery and targeting efficiency of the Cas9-gRNA complex. The system has been successfully employed to knock out Glutamine Synthetase (GLUL) in HEK293T cells and MAP kinases (MAP2K3) in MDA-MB-468 cells, exhibiting superior efficiency compared to commercially available tools. Secondly, we have implemented a pseudo-lenti system to amplify the entry capacity of donor DNA with homology arms into target cells. This technique has shown an improved HR effect in the experiment to tag a GFP at the C terminals of the CDC25A gene. Lastly, we leverage proximity effects by fusing Cas9 with a capture protein that can grasp a bait sequence attached to the donor DNA, thereby facilitating its transfer to the target sites. Our system has demonstrated exceptional efficacy as a CRISPR knock-out platform and a powerful tool for DNA tagging functionality on target genes in mammalian cells.

Keywords: genome editing, CRISPR, vector

4:45 PM – 5:45 PM

480

FACILITATING MODEL DEVELOPMENT FOR ORGAN-ON-CHIP RESEARCH AND IMPLEMENTATION

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Although organ-on-chip technology in combination with human stem cells could transform fundamental research as well as pre-clinical drug development pipelines, successful implementation of this technology hinges on the microfluidic devices being able to support stem cell derivatives in a healthy physiological state. This is still a challenge. To support the development of well-qualified, accessible in vitro models that can be readily used by the research community, the Leiden Organ-on-Chip Centre supports research by qualifying physiologically relevant, advanced human-based in vitro models that recapitulate the key aspects of human biology in health and disease. To this end, we make use of well-characterized induced pluripotent stem cell (hiPSCs) lines to generate a range of differentiated human cell types, such as cardiomyocytes and epi- or endothelial cell types, which are combined into advanced culture systems that enable modelling of their cell-cell and cell-device interplay to mimic the chosen organ in vitro. Key physiological features can be added including (3D) structure, natural and synthetic extracellular matrices, compartmentalization, flow, air/liquid interfaces and contractile forces. For each model, we select the optimal combinations of biology, such as gene-edited patient or control hiPSC lines and differentiation protocols, and technology, ranging from commercially available systems to in-house prototyped chip systems and tailor-made microfluidic set-ups. Recently, we developed and optimized in vitro models that allow to study the physiology of hiPSC-derived cardiomyocyte contractility in health and disease, as well as a vasculogenesis that could be used to resolve specific vascular defects associated with a genetic vascular disease. Our overarching goal is to help researchers select the optimal biological and technological elements for their research question, together develop and qualify human-based in vitro models that facilitate the study of human (patho-) physiology, and to enable the implementation of human-based models for pre-clinical testing.

Funding Source: This work is supported by Novo Nordisk Foundation grants (NNF21CC0073729).

Keywords: organ-on-chip, disease modeling, microfluidics

4:45 PM – 5:45 PM

482

VERSATILE HIGH-SPEED MICROFLUIDIC CELL SORTING OF HUMAN PLURIPOTENT STEM CELLS AND THEIR DERIVATIVES

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iPSCs with their potential to differentiate into countless different cell types hold great potential for allogenic or autologous cellular therapy. This innovative therapeutic approaches often entail the application of clearly defined cells types or subsets that need to be isolated and enriched from heterogeneous cell populations. Flow sorters are technically well capable of purifying cells but were predominantly designed for research applications. Open systems and harsh sorting conditions therefore often prevent the translation of respective research protocols into the clinic. We hereby show how a gentle microchip-based sorting technology can enable enrichment of pluripotent stem cells and their derivatives in the safety of a sterile cartridge during different steps along a standard PSC workflow. Human fibroblasts were reprogrammed via repeated mRNA transfections and resulted in a mixture of successfully generated iPSCs and non- or partly reprogrammed undefined cells. This heterogeneous cell culture was stained with Tra-1-60-PE and sorted immediately after reprogramming. The iPSCs could be successfully enriched from varying purities of 4-41% to 85-99%. Furthermore transgenic iPSC constitutively expressing GFP to a differing degree could be effectively enriched from 13-51% to a pure population of 72-98%. To obtain monoclonal transgenic iPSCs the sorted fraction was applied to a single-cell dispenser and seeded as single cells in 96-well plates. The cells showed high plating efficiency after the combination of sorting and subsequent single-cell dispension while maintaining typical iPSC morphology. Next, different iPSC-derived cell types were enriched after differentiation. Retinal pigmented epithelial cells could be enriched from a purity of 51-69% to 93-98% and showed a strongly pigmented phenotype after sorting. In other approaches cardiomyocytes were enriched from 59-82% to 85-94% and CD144-positive endothelial cells could be enriched from 48-49% to 90-96%. In all applications a high viability of over 85% was maintained after sorting. In summary, this gentle and sterile sorting technology allows for efficient and reliable enrichment of iPSCs and their derivatives and therefore facilitates working with clearly defined cell populations in a wide variety of PSC applications.

Keywords: cell sorting, microfluidics, flow cytometry

4:45 PM – 5:45 PM

484

YMETHX: DECIPHERING THE INTERPLAY OF DNA METHYLATION AND GENE EXPRESSION IN MALE SEX CHROMOSOME ANEUPLOIDIES

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Sex chromosome aneuploidies (SCAs) are characterized by a non-standard complement of sex chromosomes beyond the conventional diploid set (46,XX for females and 46,XY for males), leading to diverse clinical manifestations. The study of SCAs presents a unique challenge due to the variable penetrance of the phenotypical manifestations and the complex epigenetic mechanisms regulating X chromosome inactivation in X aneuploid patients. Despite the high prevalence of SCAs in the general population, the underliyng dysregulated epigenetic mechanisms, including DNA methylation, remain poorly understood. To address this gap in knowledge, our study introduces YmethX, an innovative web-based resource designed to elucidate the regulatory landscape and the complex interplay between DNA methylation and gene expression in SCAs. We integrated DNA methylation and transcriptomic profiles obtained from a paradigmatic cohort of SCA and non-affected male control iPSCs representative of 47,XXY, 47,XYY, 48,XXXY, and 49,XXXXY karyotypes and their differentiated derivatives. Our findings uncover significant differentially methylated regions, mostly located on the X chromosome, correlating with altered gene expression and mirroring the number of supernumerary sex chromosome, shedding light on potential epigenetic underpinnings of the phenotypic diversity in SCAs. YmethX advances our understanding of SCAs and offers a comprehensive platform for the broader research community to explore the epigenetic and transcriptomic landscape of SCAs. YmethX stands out for its user-friendly interface and robust analytical capabilities, making it an invaluable tool for advancing our knowledge of the impact of SCAs on human health.

Keywords: sex chromosome aneuploidies, Klinefelter syndrome, Jacob syndrome

4:45 PM – 5:45 PM

486

ANIMAL-FREE AND CHEMICALLY DEFINED COATING SOLUTION TO MAKE INDUCED PLURIPOTENT STEM CELLS FEEL AT HOME

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In vitro cell culture systems play a crucial role in biomedical research. In this context, induced pluripotent stem cells (iPSC) are becoming increasingly relevant for disease modelling, drug testing, and toxicological studies due to their ability to better replicate human physiological conditions compared to animal models and immortalized human cell lines. Moreover, they have the potential to revolutionize regenerative medicine and solve patients´ dependency from donors´ availability to repair injured or degenerated tissues. Currently, the gold standard coating solution for in vitro expansion and differentiation of iPSC is Matrigel®, a solubilized membrane preparation extracted from mouse sarcoma. High batch-to-batch variability, ethical and regulatory challenges related to animal welfare and the incompatibility with regenerative medicine applications due to animal components are the main reasons for the urgent need to replace this system. To address this need, we have developed a chemically defined and animal-free polymer-peptide conjugate that mimics the extracellular matrix (ECM) of the stem cell niche. The polymeric component of our solution can be easily applied to cell culture surfaces to create a robust and homogeneous coating. Being completely inert, this coating prevents unspecific binding of cells and proteins giving a considerable advantage in drug testing applications and toxicological studies. The functionalization of this polymer platform with peptide sequences from proteins of the ECM provides specific cell attachment and receptor triggering on a background of low non-specific interaction. In a benchmark study, our polymer-peptide conjugate supported iPSC adhesion, growth, and maintenance of pluripotency comparably to an animal-based product (GeltrexTM) and a xeno-free product (human recombinant Vitronectin). Currently, we are investigating the ability of our coating to support iPSC differentiation towards cardiomyocytes and cortical neurons. All in all, our new research product provides a xeno-free and fully synthetic yet biologically relevant coating solution for the expansion, and potentially the differentiation of human iPSC in an in vivo-like environment for pre-clinical applications and the implementation of these cells in regenerative medicine.

Keywords: ECM-mimicking polymeric platform, synthetic polymer-peptide conjugate, chemically defined coating solution

4:45 PM – 5:45 PM

488

REPROPLEX: A SCALABLE PLATFORM FOR IPSC REPROGRAMMING

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Induced pluripotent stem cells (iPSCs) represent a groundbreaking advancement in understanding complex diseases, drug discovery and offering personalised therapies. The reprogramming of somatic cells into iPSCs holds immense potential but is accompanied by numerous challenges that must be addressed to fully harness their research and therapeutic benefits. One of the primary challenges in iPSC reprogramming is the low efficiency and variability of the process. Furthermore, the cost, the time-consuming and labour-intensive nature of iPSC derivation and characterisation add complexity to their widespread use. To address these challenges we developed ReproPlexTM - miniaturised and parallelised platform that is scalable and allows the generation of 100s of iPSC lines rapidly and in parallel. Optimisation of culture conditions has enhanced the robustness and reproducibility and iPSC lines generated using our proprietary technology are high-quality and cost-effective. Our novel iPSC reprogramming platform offers an unprecedented opportunity for the establishment of large-scale iPSC banks for population-level cellular studies powering precision medicine and conducting in vitro clinical trials.

Keywords: reprogramming, precision medicine, in vitro clinical trial

4:45 PM – 5:45 PM

490

HALF-SERUM DMEM: REVOLUTIONIZING CELL CULTURE MEDIUM TO REDUCE FBS DEPENDENCY, COSTS, AND ENVIRONMENTAL IMPACT

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Fetal bovine serum (FBS) has been an essential element in long-term in vitro cell culture experiments due to it containing diverse nutrients for cell viability. However, recent movements to reduce livestock cultivation and frequent outbreaks of infectious diseases in cattle have posed challenges in the continuous production of FBS. Moreover, given the increased development of pharmaceuticals utilizing cell cultures, the price of FBS has risen. To address this issue, we developed a medium that only requires half the amount of FBS when culturing cells. We named this medium Half-Serum DMEM, and we demonstrate here that during a 20-days culture, its performance aligns with that of the existing medium in terms of cell cycle, morphology, isoform protein expression, lentivirus production, cell growth, cell death, and the regulation

of inflammation through major signaling pathways, even after cell culturing. Half-Serum DMEM will play a pivotal role in alleviating the financial burden on medical scientists. This will ultimately contribute to cost savings regarding patients' healthcare expenses. Additionally, the use of Half-Serum DMEM aligns with the international trend of minimizing livestock consumption to curb carbon emissions. Finally, these advancements are expected to have a positive impact on both economic and environmental aspects.

Funding Source: Pharmicell Co., Ltd.

Keywords: fetal bovine serum (FBS), cell culture, costs, half-serum DMEM, livestock consumption, carbon emissions, economic, environmental

4:45 PM – 5:45 PM

492

EXTRACELLULAR VESICLE SOURCE AND ISOLATION METHOD ARE SIGNIFICANTLY CONNECTED WITH CARGO AND FUNCTION

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Extracellular vesicles (EVs), formerly considered "cell dust", constitute a new universe of cell-to-cell contact-independent signaling particles. Over the past decade, EV-related stem cell research has increased from 162 articles in 2013 to 2,229 articles in 2022, representing a rise from 0.6% to 6.8% of all stem cell articles in PubMed, respectively. We performed a systematic review of all 20,364 original EV research articles selected from 40,684 EV-related records in PubMed 2013–2022 to display the global EV research landscape. We used machine-learning to categorize the high-dimensional data and further dissected significant associations between 36 parameters within the categories EV source, isolation method, cargo and function. We found unexpected correlations between multiple parameters within the four categories indicating prevalent experimental strategies based on significant cargo connectivity with function of interest being associated with certain EV sources or isolation strategies. EV source-function and source-cargo parameter correlation was most significant. Considering 1.32 x 10E155 possible combinations of the 36 search parameters, we are now able to display a comprehensive landscape of current global EV research. We propose that the conceptually relevant association of EV source and isolation method with cargo and function will guide strategic conclusions enhancing future EV research and product development. Based on this study, we built an open-source database, https://ev-zone.org, containing more than 21,000 EV publications. It enables researchers to navigate the vast landscape of EV discovery, identifying key trends and making complex targeted searches using regular expression patterns. By making this resource available, we aim to catalyze advancements in EV research, encouraging the integration of EV-based communication, as novel mechanistic category of cell-to-cell contact-independent signaling, into stem cell research.

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Keywords: extracellular vesicles (EVs), regenerative medicine, machine learning

4:45 PM – 5:45 PM

494

EFFICIENT AND GENTLE NON-VIRAL ENGINEERING OF IPSCS BY PHOTOPORATION

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Engineered induced pluripotent stem cells (iPSCs) are an excellent source for the production of cells for cancer immunotherapy, e.g. (off-the-shelf) iPSC-derived CAR-NK or CAR-T cells or for the treatment of genetic disorders in an autologous setting. Unfortunately, the many promising applications of gene-edited iPSCs have not yet been able to reach their full potential, among other due to suboptimal transfection technologies. Electroporation is a popular transfection technology, yet it is notorious for its detrimental effects on the viability of sensitive iPSCs. Photoporation is an up-and-coming alternative intracellular delivery method which has gained interest due to its gentleness to cells. The modality is based on the combination of laser light and photothermal nanoparticles to create transient pores in the plasma membrane, thus allowing uptake of external macromolecules. In its most traditional form, cells are first incubated with photothermal nanoparticles, e.g., gold nanoparticles (AuNPs), which can adsorb to the cell membrane. Local heating effects upon laser irradiation create pores in the membrane through which compounds in the surrounding cell medium can enter the cell. Here we explored if photoporation can be

applied to sensitive iPSCs, aiming at high cell viability while achieving high intracellular delivery efficiency. Using fluorescently labelled dextrans as model cargo molecules, a delivery efficiency of 69 ± 4 % was achieved while maintaining 95 ± 4% viable cells based on metabolic activity. This provides a first clear indication that AuNP-photoporation can reach high delivery percentages while maintaining high viability in iPSCs. Other cargo molecules, such as CRISPR/Cas9 ribonucleoproteins, will be tested next to further explore this promising technology for the transfection of iPSCs.

Funding Source: European Innovation Council

Keywords: non-viral transfection, engineered cell therapy, photoporation

4:45 PM – 5:45 PM

496

CEPT SMALL MOLECULE COCKTAIL SAFEGUARDS GENOME STABILITY IN HUMAN PLURIPOTENT STEM CELLS DURING LONG-TERM PASSAGING

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Human pluripotent stem cells (hPSCs) are characterized by extensive self-renewal capacity but can acquire genetic and karyotypic abnormalities during in vitro culture. Genomic instability in sensitive hPSCs remains poorly understood, posing a major obstacle for the safe therapeutic use of hPSCs for clinical applications. Recently, we established the four-part small mole culture cocktail termed CEPT (chroman 1, emricasan, polyamines, trans-ISRIB) as a new chemical platform for stress-free cell culture and utilization of hPSCs (i.e., routine cell passaging, cryopreservation/thawing, single cell cloning, cell aggregation for embryoid bodies and organoids). In the present study, we asked how the CEPT cocktail may affect the long-term culture of hPSCs. Using the CompacTSelecT robotic cell culture platform to minimize investigator bias and increase standardization, we cultured 7 different hPSC lines for 40 passages and serially passaged them in DMSO (control), Y-27632, and CEPT (24 h exposure at each passage). Whole genome sequencing and careful comparison of different cell lines and treatment groups revealed that CEPT is capable of protecting hPSCs against passaging-associated DNA damage as measured by the Comet assay, thereby preventing the accumulation of single nucleotide variants (SNPs) and other mutations in coding and non-coding genomic regions after long-term culture. These findings not only enhance our mechanistic understanding of the long-standing observation that hPSCs acquire non-random genetic abnormalities but also demonstrate that the use of CEPT can prevent the accumulation of such abnormalities and increase genome stability in hPSCs. Taken together, consistent application of CEPT at each passage can increase the quality and safety of hPSCs, representing an end-to-end solution for regenerative medicine and future cell therapies.

Funding Source: This work was funded by the Regenerative Medicine Program (RMP) of the NIH Common Fund and the intramural research program of the National Center for Advancing Translational Sciences (NCATS), NIH.

Keywords: genomic instability, CEPT, DNA damage

4:45 PM – 5:45 PM

498

AUTOMATION OF IPSC CULTURE, PASSAGING AND EXPANSION WITH CELLXPRESS.AI INSTRUMENT

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iPSC -derived cell models became a popular tool enabling to generate various cell types, organoids and tissues. Stem cells are also widely used to create disease-specific phenotypes using CRISPR and to discover new drug targets using patient derived samples. The process is often limited by labor-intensive and highly demanding steps for culture. To alleviate the limitations that come with labor-intensive protocols we developed a cell culture automation solution CellXpress.ai. The instrument enables automation of cell culture for iPSC. It contains automated imager, liquid handler, and incubator, connected by AI- powered software. Development of cell cultures was monitored by periodic imaging and analysis, which can trigger automatic decisions to initiate passaging, end-point assay, or troubleshooting steps. We present results from the automation of commonly used iPSC culture protocols, including cluster or single- cell passaging. iPSC culture was started from the source 6 well plate with iPSC (Coriell GM25256). We automated the basic iPSC protocol recommended by STEMCELL Technologies. The automated stem cell media exchange was done automatically every 24 hours using a liquid handler and plate tilting platform. Plates were monitored by imaging every 12h. Machine-learning based image analysis allowed to detect iPSC colonies and determine the cell area, confluency, also enabled detection of cell phenotype changes or differentiation. The software also allowed to visualize plots corresponding to cell growth. After 4-5 days cells were automatically harvested by pipetting after treatment with ReLeSR or TrypLE reagents, then re-suspended, and plated into additional Matrigel-coated 6-well plates. All steps were done via an automated process that included cell maintenance, passaging, expansion, also monitoring by imaging, image analysis, and image-based decision making. Passaging steps were triggered by a user-defined parameters, e.g. cell confluency. The presence of differentiated cells was also automatically detected during the process. The process described above allows to overcome challenges of manual protocol and enables automation of iPSC culture, also iPSC expansion. Cell culture automation has a great potential to reduce labor also to increase productivity, throughput and reproducibility.

Keywords: automation, iPSC, machine learning

4:45 PM – 5:45 PM

500

VASCULARIZATION OF THE ASSEMBLED SPHEROIDS

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Spheroids are frequently employed as a therapeutic approach and transplanted in vivo because they more accurately replicate the microphysiological environment. The successful engraftment and vascularization of transplants are critical for their sustained viability and integration into host tissue. Among the diverse methods for creating spheroids, the utilization of magnetic force expedites cell aggregation and spatial control. To promote vascularization, we employed magnetic bioprinting to construct vascular assemblies by coating magnetic spheroids with vascular endothelial cells containing internalized magnetic nanoparticles (MNPs). MNPs internalization did not compromise cell viability or the expression of genes associated with vascularization. Upon transplantation, these vascular assemblies swiftly sprouted and integrated into host tissue within three days. Furthermore, when intramuscularly transplanted into a hindlimb ischemic model, they notably reduced necrosis levels. Given their facile fabrication via MNPs-coated spheroids and magnetic control, these vascular assemblies hold promise for various applications involving spheroids with diverse functionalities.

Funding Source: This work was supported by Korea government grant funded by the Ministry of Science and ICT (MSIT, NRF-2021R1A2C2008821, NRF-2022M3A9B6082680, NRF-2023R1A2C2007283, and RS-2023-00254825).

Keywords: vascularization, magnetic nanoparticles, assembloid

4:45 PM – 5:45 PM

502

A PROTOCOL FOR LARGER INSERTIONS AND INVESTIGATIONS OF ALLELIC DROP OUT AT THE LUND UNIVERSITY CELL AND GENE THERAPY CORE

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CRISPR and human induced pluripotent stem cells (iPSCs) constitute a transformative duo which has nothing less than revolutionized medical research. In recent years, core facilities have emerged as pivotal players, harmonizing procedures and offering expertise in the swiftly advancing fields of iPSCs and gene editing. The Cell and Gene Therapy (CAGT) Core at Lund University and provides iPSC reprogramming as well as custom iPSC edits. In addition to the iPSC-related services the CAGT also offers custom viral vector and mRNA production. Here we present our protocol for insertion of plasmid-based repair templates into iPSCs, using a staggered two-day, two delivery method approach of the plasmid and the Cas9/sgRNA RNP. Our approach involves lipid-mediated transfection of iPSCs with a donor plasmid containing the fluorescent tag of interest, in this example case CopGFP, along with homology arms flanked by single guide RNA (gRNA) recognition sequence. Followed by nucleofection of cells with Cas9/sgRNA ribonucleoprotein complexes (RNPs) the following day. In this very successful example edit we observed about 40% of edited cells (as assessed via flow cytometry). Here we also present our investigation into the frequency of the recently described allelic drop out (loss of hemizygosity) at the edited locus. Here we collected DNA samples from our and European core facilities and performed a validated TaqMan copy number assay of the edited locus. In conclusion, we present a protocol for plasmid-based repair template insertion into iPSCs and an investigation into allelic drop out frequency. We also would like to underscore the important role of core facilities in advancing CRISPR and iPSC technology in medical research, emphasizing the importance of standardized procedures and expertise in gene editing and stem cell research.

Keywords: iPSC, core facilities, loss of homozygosity

4:45 PM – 5:45 PM

504

ENHANCING THE EFFICIENCY OF HUMAN PLURIPOTENT STEM CELL CLONING WITH THE WOLF G2 CELL SORTER: ACHIEVING HIGH PURITY AND CLONAL OUTGROWTH

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Human pluripotent stem cells, such as induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), hold significant promise for advancing both basic cell research and clinical applications. A major hurdle in harnessing their full potential is the efficient and accurate selection of clones that are both functional and pure. Traditional methods, such as manual colony picking and limiting dilution, are either low-throughput, labor-intensive or lack the precision required for selecting the most suitable clones. These methods also struggle to maintain the pluripotency of colonies, which is crucial for applications requiring cell differentiation, such as disease modeling. Identifying and selecting stem cells that remain undifferentiated is possible using fluorescence-activated cell sorting (FACS) to isolate cells based on the expression of specific markers, such as SSEA-4 and TRA-1-60-R. For efficient single-cell sorting and cultivation, technologies like the WOLF G2® Cell Sorter and the N1 Single-Cell Dispenser have shown promise. These tools can gently deposit a single cell into a well using low pressure (less than 2 psi), which supports the growth of undifferentiated colonies at a higher rate than traditional methods like limiting dilution. We have successfully utilized the WOLF G2 to sort GM2338 stem cells with over 90% efficiency. This process resulted in more than 40% clonal outgrowth, with the cells maintaining expression of key pluripotency markers SSEA-4 and TRA-1-60-R, indicating a significant improvement in the efficiency and reliability of stem cell cloning techniques.

Keywords: cell line development, clonal outgrowth, cell sorting

4:45 PM – 5:45 PM

506

NANOWIRE ARRAYS USED AS TOOLS TO MANIPULATE STEM CELLS: THE POTENTIAL OF NANONEEDLES FOR CARGO DELIVERY, STEM CELL GENERATION, AND DIRECTED CELL DIFFERENTIATION

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Nanowire arrays—arrays of vertically arranged high aspect ratio nanostructures—have been demonstrated to be potent tools for advanced applications such as sensing, stimulation, or biological cargo delivery. Their enormous potential in stem cell technologies, however, is rarely elaborated on. Here, we discuss nanowire arrays used as cell culture substrates and their versatile range of applications enabled by the unique interaction between the cells and the nanowires. The concept of nanoneedle-mediated cargo delivery to cells will be introduced, including the delivery of proteins, mRNA, and plasmid DNA to adherent and non-adherent cells. Immunocytochemistry imaging and scanning electron microscopy demonstrate that human induced pluripotent stem cells and their derivatives are compatible with the nanoscale topography of a wide range of nanowire array geometries. In addition, we imaged the interface between the cells and the individual nanowires, showing a tight interaction crucial for later applications. Viability studies confirm no profound impact on cell health in the case of the majority of employed nanowire substrates. However, particular nanowire arrangements showed the capability to control, for example, cell proliferation or neurite guiding. Concludingly, we believe that the versatility of nanowire arrays might pave the way for novel modalities to manipulate stem cells and thus facilitate regenerative medicine and stem cell research.

Funding Source: We thank the ARC Training Centre for Cell & Tissue Engineering Technologies (IC190100026). JH acknowledges the support from the Alexander von Humboldt Foundation through a Feodor Lynen research fellowship.

Keywords: nanoneedle arrays, non-viral cargo delivery, silicon

4:45 PM – 5:45 PM

508

SUSPENSION-INDUCED STEM CELL TRANSITION: A NON-TRANSGENIC METHOD TO GENERATE ADULT STEM CELLS FROM MOUSE AND HUMAN SOMATIC CELLS

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Adult stem cells (ASCs) can be cultured with difficulty from most tissues, often requiring chemical or transgenic modification to achieve adequate quantities. We show here that mouse primary fibroblasts, grown in suspension, change from the elongated and flattened morphology observed under standard adherent culture conditions of generating rounded cells with large nuclei and scant cytoplasm and expressing the mesenchymal stem cell (MSC) marker (Sca1; Ly6A) within 24 hrs. We describe here a suspension culture method that, irrespective of the lineage used, mouse fibroblast or primary human somatic cells (fibroblasts, hepatocytes and keratinocytes), is capable of generating a high yield of cells in spheroid form which display the expression of ASC surface markers, circumventing the anoikis which often occurs at this stage. Moreover, mouse fibroblastderived spheroids can be differentiated into adipogenic and osteogenic lineages. An analysis of singlecell RNA sequence data in mouse fibroblasts identified eight distinct cell clusters with one in particular comprising approximately 10% of the cells showing high levels of proliferative capacity expressing high levels of genes related to MSCs and self-renewal as well as the extracellular matrix (ECM). The rapid, high-yield generation of proliferative, multi-potent ASC-like cells via the process we term suspension-induced stem cell transition (SIST) could have significant implications for regenerative medicine.

Funding Source: 1. Canadian Institutes of Health Research 2. J.P. Bickell Foundation Medical Research Grant 3. CHEO Research Institute Research Growth Award

Keywords: adult stem cells, somatic cells, anoikis, reprogramming, single-cell RNA sequence

4:45 PM – 5:45 PM

510

DEVELOPING STEM CELL TRANSPLANTATION IN SEA ANEMONES AND CORALS; TOWARD CELL-THERAPY IN CORALS

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Reef corals are the foundation of ecosystems that host much of the ocean's biodiversity. They are under severe threat from anthropogenic stressors, particularly global warming. Efforts to mitigate the damage are informed by research on understanding and transferring naturally-occurring resilient genotypes. This has a direct parallel in medicine; cell- or gene-therapy, which is founded on an ability to isolate and then transplant stem cells. This ability/technology does not exist for any Hexacorallian species. We are developing robust tools for the isolation, characterization, and transplantation of Hexacorallian candidate stem cells. We have succeeded, using a Hexacorallian model of Nematostella vectensis utilizing its transgenic lines, to show candidate stem cell transplantation. Using this, we could follow in vivo cells in transplanted animals for up to 2 months. Using confocal microscopy and flow cytometry we showed the integration and proliferation of the transplanted cells into the tissues. We showed the cell integration and genetic chimerism using PCR and qPCR. Using serial transplantation, we showed the longevity of our candidate stem cells. We showed the presence of the transplanted cells in wound healing. Additionally, using machine learning analysis, we have shown cell differentiation to other cell types. Finally, we showed that the candidate stem cell transplantation can rescue animals from a lethal chemotherapy treatment. Moreover, since we are using species non-specific methods we show our capability of isolating those candidate stem cells from other Hexacorallian species, including stony corals. We have succeeded in transplantation in two species of stony corals. In addition, we do immunological analysis to understand the cell acceptance in Hexacoralians. In this work we utilize medical approaches toward ecological problems, opening new frontiers for stem cell-based therapy.

Funding Source: The project has received funding from the European Research Council (ERC) grant agreement No. 948476.

Keywords: stem cell therapy, corals, stem cell transplantation

4:45 PM – 5:45 PM

512

FLOW-IMAGING AS PAT-TOOL FOR CELL AGGREGATE MONITORING

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New and complex manufacturing and cultivation processes in the cell therapy field are a challenge to the well-established process analytical technologies (PAT) and monitoring solutions of today. As stem cells and other somatic cells cannot be cultivated in a solitary state, advanced analysis tools are required to overcome the associated intricacies. The efficient cultivation of induced pluripotent stem cells (iPSCs) in suspension is one of the many but essential tasks to tackle in the field, since they are a key intermediate product for various allogenic therapeutic approaches. One significant difference of iPSCs to carcinoma-based cell lines is their functioning pathway for programmed cell death (particularly anoikis), leading to growth in clusters or 'islands' in adherent cultures and cell aggregates or 'embryoid bodies' (EBs) in suspension cultures. To monitor and control the size of such objects, as well as ensuring a comparable degree of cell dissociation after harvest or before inoculation is of utmost importance for cultivation consistency and reproducibility. Current state of the art technologies for particle size and size distribution analysis are limited as they cannot provide more information of the object for deeper characterization, which is crucial if multiple subpopulations are present in the culture. In this work, an object classification system using image-morphological parameters, delivered by a flow-cell-based device has been developed to enhance the understanding of iPSC cultivations in two specific applications: the sizing of EBs and the assessment of cell dissociation efficacy in a semi-quantitative manner. The performance of this new methodology was evaluated against the traditional Coulter counter-based technology, which is the current standard for particle size analysis. Our new approach offers a significant advancement in the analysis of such complex cell cultures within the cell therapy domain. By providing detailed insights not just into the size but also into the morphology of cell aggregates, this method facilitates improved comparability and can herewith improve consistency in the cultivation of iPSCs, thereby supporting the development of various therapeutic strategies.

Keywords: Imaging, flow, morphology

4:45 PM – 5:45 PM

514

PROXIMITY PROTEIN PROFILING OF THE REORGANIZATION OF THE CYTOSKELETON DURING EARLY SOMATIC CELL REPROGRAMMING

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Reprogramming somatic cells towards induced pluripotent stem cells involves a multitude of sub-cellular changes inclusive of a global reorganization of the proteome. Though many studies have shown a step-wise resetting and time-defined expression changes during reprogramming, the dynamic protein-protein interaction remodeling that occurs during this dedifferentiation has been poorly characterized. Of particular importance are the changes in protein organization in one of the earliest hurdles known as the mesenchymal-to-epithelial transition (MET) during the initiation phase, which hosts a remodeling of cell morphology and notably the cellular cytoskeleton. In order to understand this remodeling, I have optimized and implemented a proximity dependent biotinylation (BioID) strategy through lentiviral delivery in reprogramming cells, detecting proximal proteins which in turn can define dynamic protein associations within a specific compartment of the cell. Using 27 cytoskeleton localized proteins (baits) profiled at 4 time points throughout the initiation phase, 3,488 high-confidence protein associations were identified against controls. These associations highlight distinct and uncharacterized changes in protein-protein associations during cytoskeleton remodeling. To delineate differential protein associations from natural expression changes, isobaric labeling (TMTpro16) was used to quantify the abundance changes of 7,060

proteins during the same time points. A systematic pairwise comparison of the differential profiles found across BioID and protein abundance levels will enable an unbiased selection of targets (e.g. members of the Pp6 phosphatase holoenzyme) for further validation. These prime candidates can be considered as key regulators of MET and the remodeling of early reprogramming cells as their localization, and thus function, do not correlate with protein expression changes. Current work aims to define the role of identified targets in this remodeling, a critical early step in somatic cell reprogramming. By combining my dynamic reprogramming cytoskeleton map with other –omics data, this data will serve at making new predictions in the order of events responsible for somatic cell reprogramming and the proteins that regulate network remodeling.

Keywords: proximity-dependent biotinylation, somatic cell reprogramming, proteomics

4:45 PM – 5:45 PM

TOPIC: PANCREAS

516

UNVEILING NOVEL TRANSCRIPTION FACTOR COMBINATIONS FOR EFFICIENT REPROGRAMMING OF PANCREATIC PROGENITORS INTO β-CELLS

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Diabetes, characterized by the pancreas's inability to regulate blood glucose levels, is a growing global health concern, affecting approximately 140 million people worldwide, with projections indicating an increase to 300 million by 2025. Islet transplantation stands out as a promising cell therapy approach, yet it faces issues like organ donor shortages and scalability hurdles. To address these obstacles, researchers are exploring scalable sources of β-cells, notably deriving them from stem cells such as hESCs and iPSCs. Despite these efforts, existing protocols encounter obstacles such as time-consuming procedures, high costs, lack of reproducibility, and variable efficiency (20-60%). Moreover, derived β-cells often exhibit immaturity, non-functionality characterized by inadequate glucose-stimulated insulin secretion, and deficiencies in key transcription factors (TFs), leading to polyhormonal phenotypes. Cellular reprogramming using TFs has emerged as a promising alternative to achieve faster, cost-effective, and efficient protocols. However, traditional trial-and-error approaches restrict the exploration of TF combinations. In 2008, Pdx1, Ngn3 and MafA were shown to convert adult mouse pancreatic exocrine cells into β-cells in vivo, and since that time this core set of TFs has been shown to reprogram cells from other tissues derived from endoderm. In this study, we employ a bead-based combinatorial screening technology, Combicult, to test over 10,000 different combinations of TFs from a set of 10 developmental regulators of pancreogenesis. Our findings reveal novel TF combinations surpassing existing protocols in efficiently reprogramming pancreatic progenitors into β-cells. These TFs can be administered sequentially or simultaneously through mRNA delivery using LNPs, eliminating genetic footprints in reprogrammed β-cells. In summary, our innovative screening platform has uncovered novel reprogramming protocols with the potential to scale up β-islet cell production. Moreover, these TFs hold promise for in vivo reprogramming, enabling the conversion of closely related cell types into β-cells. This breakthrough opens avenues for discovering reprogramming protocols for various other cell types, revolutionizing cell and gene therapy applications.

Funding Source: Ministry of Education, Singapore Start-Up Grant

Keywords: cellular reprogramming, beta cell therapy, diabetes

4:45 PM – 5:45 PM

518

3UTR GENETIC VARIANTS ALTER PANCREATIC RNA REGULATION IN A CELL TYPE SPECIFIC MANNER: IMPLICATIONS FOR STEM CELL-DERIVED ISLETS

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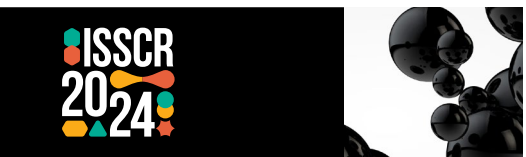
Stem cell-derived (SC-) islets offer the promise of cell replacement therapy in insulin-deficient diabetes mellitus, yet donor genetic variation can impact the differentiation and functionality of insulin-producing SC-beta cells, which is related to mRNA stability. Expression quantitative trait loci (eQTLs) are genetic variants in the 3'UTR that influence this stability. Understanding eQTL regulation in adult beta cells helps explaining functional variation of SC-beta cells, but past eQTL studies were limited by using bulk pancreas samples, overlooking cell-specific differences. To address this gap, we developed a pipeline to identify 3'UTR eQTLs from published scRNA-seq datasets of all major adult pancreatic cell types. Our dataset contains over 37000 eQTLs of which 60% act only in one cell type. 57% and 51% of eQTLs are found in alpha and acinar cells, respectively, and a small portion is present in the supporting endothelial (2%) and fibroblast (6%) cell types. 118 eQTL signals colocalize with T2D diabetes or obesity GWAS risk alleles, indicating potential functional effects. Notably, in beta cells, we observed that 1455 genes are regulated by 3827 3'UTR eQTLs, these genes include key beta cell functional genes, such as INS, PDX1, and SLC30A8. We found that eQTL variants directly alter the binding motifs of RNA binding proteins (RBPs) and microRNAs (miRNAs). One of such eQTLs,



rs701848, was present in the 3'UTR of PTEN that modifies the binding of the beta cell-specific miRNA mir-127-5p which is relevant for insulin secretory capacity and cell proliferation. In conclusion, our data sheds light on the 3'UTR regulation landscape in the mRNA of individual pancreatic cell types. Ultimately, understanding cell-specific eQTLs could explain variability in generation and function of SC-islets for replacement therapies.

Funding Source: Unite2CureType1 grant from Foundation DON and the Dutch Diabetes Research Foundation; The Novo Nordisk Foundation Center for Stem Cell Medicine is supported by Novo Nordisk Foundation grants (NNF21CC0073729).

Keywords: 3'UTR eQTL, beta cells, SC-islets

TRACK: PLURIPOTENCY AND DEVELOPMENT (PD)

4:45 PM – 5:45 PM

TOPIC: CARDIAC

520

PUSHING FORWARD THE MATURITY OF PLURIPOTENT STEM CELL-DERIVED LEFT VENTRICLE CARDIOMYOCYTES USING ADULT HEART TISSUE CUES

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Impaired left ventricle (LV) function often leads to debilitating and fatal cardiovascular disease making in-vitro models of LV cardiomyocytes extremely desirable. Human pluripotent stem cells are an endless resource of specialised human cell types, including cardiomyocytes. However, the most commonly used differentiation protocols generate heterogenous and immature cardiomyocytes, which greatly reduces their utility for cardiotoxicity studies, disease modelling or drug development. We developed a protocol that generates near-homogeneous populations of left ventricle-like cardiomyocytes (hPSC-LV-CMs) with increased maturity. Despite the faster pace of maturation achieved with our protocol, and the maturation improvement observed over time, our lab is working towards understanding and pushing forward the maturation of hPSC-LV-CMs. Gene set enrichment analysis comparing our cardiomyocytes at day 20 of differentiation with adult human LV cardiomyocytes confirmed they primarily lack metabolic maturation. Though fatty acid-enriched media is well described in the literature to mature hPSC-CMs, we observed that fatty acids only promoted increased metabolic and morphological maturation when delivered in DMEM basal media. We identified this is attributed to increased levels of calcium, magnesium and glutamine in DMEM. The extra magnesium appears to be particularly important for enabling maturity and we are now investigating its mode of action. These results contribute to a mechanistic understanding of the process of cardiomyocyte maturation and ultimately may help improve faithfulness of the hPSC LV-CM in vitro model for disease modelling and drug development.

Funding Source: This work was supported by grants from the Wellcome Trust (210987/Z/18/Z), and LifeArc (LifeArc-Crick Translation Fund).

Keywords: cardiomyocytes, maturation, fatty acid

4:45 PM – 5:45 PM

522

RNA-BINDING PROTEIN SAMD4A ASSOCIATES WITH FGF2 MOTIF TO REGULATE SELF-RENEWAL AND CARDIOMYOCYTES LINEAGE COMMITMENT IN HUMAN EMBRYONIC STEM CELLS

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RNA-binding proteins (RBPs) are involved in almost the entire process of cardiac development by regulation of hESC pluripotency and cardiomyocyte differentiation. However, many RBPs how regulate cardiomyocyte lineage commitment are still unclear. Here, we found that the RNA binding protein SAMD4A showed an accordingly expression pattern in cardiomyocyte differentiation from the ESCs of both human and mouse with the characteristics of key driver genes. SAMD4A showed a specific expression during heart development, and knockdown of SAMD4A caused hESC to exit from early pluripotency and fail to maintain the self-renewal state. However, the situation induced repression of cardiac mesoderm differentiation and impairment of hESC-derived cardiomyocyte function. We further confirmed that SAMD4A could bind to the FGF2 motif to modulate its translation, which thereby limited stem cell self-renewal and lineage specification through mTOR signaling. Therefore, our study demonstrates the indispensable role of SAMD4A in pluripotency, self-renewal, and cardiomyocyte fate determination in human embryonic stem cells, which provides experimental evidence for understanding cardiac development and congenital heart disease mechanism.

Funding Source: National Natural Science Foundation of China (NSFC) 474:32071109

Keywords: SAMD4A, RNA-binding protein, hESC

4:45 PM – 5:45 PM

524

A HETEROGENEITY OF TRANSCRIPTIONAL AND EPIGENETIC PROFILE IN HUMAN INDUCED PLURIPOTENT STEM CELL CLONES FROM THE SAME DONOR

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Human induced pluripotent stem cells (hiPSCs) offer immense potential for developmental studies, disease modeling, and clinical applications. Despite their self-renewal and pluripotency, the reproducibility of research is challenged by the inherent variability among hiPSC populations. While prior investigations have explored variations in iPSCs based on donor cell types and reprogramming methods, our research more focused on the clonal heterogeneity within hiPSCs lines derived from a single source. In this study, we identified distinct hiPSC clones demonstrating varying capacities to differentiate into cardiomyocytes, delineating them as productive clones (PC) and non-productive clones (NPC). Employing comprehensive analyses, including phospho-kinase array, RNA sequencing (RNA-seq), and Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), we aimed to discern differences in signal pathways, transcriptomic profiles, and chromatin states between PC and NPC. Integrative analysis of these datasets unveiled candidate genes positively correlated with cardiac differentiation propensity, enriching our comprehension of the intricate interplay among genetic, epigenetic, and signaling factors influencing hiPSC clonal heterogeneity. These findings offer valuable insights into predictive biomarkers for selecting optimal hiPSC clones for cardiac differentiation.

Funding Source: KFRM (Korean Fund for Regenerative Medicine) BK21 (Brain Korea 21) LIM Foundation

Keywords: hiPSC clonal heterogeneity, cardiac differentiation, transcriptome and epigenetic profile

4:45 PM – 5:45 PM

526

UNVEILING CARDIAC GASTRULOID MODEL: INVESTIGATING ENVIRONMENTAL AND INTRINSIC FACTORS FOR IMPROVED UNDERSTANDING OF EARLY ORGAN DEVELOPMENT

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The ability of gastruloids to efficiently form complex structures has improved our ability to study organ development in early embryonic models. The embryonic heart is inaccessible to basic manipulations, and current models fail to accurately represent the effects of mutations, medications, and environmental factors on the specialized functions of different cardiac compartments. In-vitro embryo-like models based on embryonic stem cells have improved greatly in recent years and can be used to study and manipulate early developmental processes. Here, we aim to establish the effect of environmental and intrinsic factors in cardiac gastruloid models. We employ different embryonic stem cells carrying fluorescent reporters to live-monitor the formation of the cardiac domain in gastruloids. We have introduced hypoxic conditions to understand the effect and need of hypoxia in cardiac gastruloids. We employ a supervised machine learning approach to characterize the factors affecting cardiac morphogenesis in these gastruloids. By analyzing the models, we devise interventions that will reduce the inter-organoid variability, to provide robust, reproducible cardiac organoids which will be useful in understanding the development more transparently.

Funding Source: SUMO, EIC

Keywords: cardiac gastruloid, environmental and intrinsic factors, hypoxia

4:45 PM – 5:45 PM

528

IRX3 ORCHESTRATES CARDIAC FATE IN HUMAN IPSC-DERIVED CARDIOMYOCYTES THROUGH INTERACTION WITH KEY TRANSCRIPTION FACTORS

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The regenerative capacity of the adult human heart is limited, highlighting the potential of human induced pluripotent stem cell (hiPSC)-based therapies for treating myocardial infarction. However, hiPSC-derived cardiomyocytes (hiPSC-CMs) often exhibit immature features in terms of structural, metabolic, and functional properties. A significant challenge lies in deciphering the precise mechanisms by which various cardiac transcription factors (cTFs) interact to determine cell fate. In this study, we demonstrate how depletion of the transcription factor IRX3 promotes early cardiac commitment and enhances the maturation of hiPSC-CMs by interacting with key cTFs. We first established the temporal expression of IRX3 during hiPSC-CM differentiation and subsequently investigated its role by generating two IRX3 mutant hiPSC lines (IRX3cl1-/- and IRX3cl2-/-). Depletion of IRX3 in cardiac progenitors resulted in elevated expression of cardiac development genes, including GATA4, NKX2-5, TBX5, HAND1/2, MEF2A/C, and Wnt-signaling genes, coupled with increased proliferation. Differentiation of IRX3-depleted cells yielded more cardiomyocytes with enhanced expression of TNNI1 and CX43. Notably, IRX3-depleted cardiomyocytes showed improved sarcomere alignment and enhanced cell-to-cell communication, indicative of structural maturation. These findings were accompanied by electrophysiological improvements, including shortened action potential and calcium decay durations, increased calcium peak and faster contraction-relaxation activity. Furthermore, our in silico analysis, validated by in vitro data, suggests that IRX3 regulates cardiomyocyte differentiation by targeting the transcription of TBX5, GATA4 and NKX2-5, along with their respective targets. Collectively, our findings underscore the potential of IRX3 depletion in facilitating the molecular and functional development of human cardiomyocytes, offering insights for advancing hiPSC-based cardiac therapies.

Funding Source: This project was supported by FAPESP [INCT-20214/50889-7 to JEK, 2013/17368-0 to JEK, 2015/50216-5 to JEK, 2021/14876-1 to NDF, 2023/05709-0 to NDF] and CNPq [INCT-465586/2014-7 to JEK, 309179/2013-0 to JEK, 442643/2020-9 to JEK].

Keywords: IRX3, cardiac commitment, hiPSC-derived cardiomyocytes maturation

4:45 PM – 5:45 PM

530

DISSECTING THE CHROMATIN TOPOLOGY UNDERPINNINGS OF CONGENITAL HEART DEFECTS USING ORGANOIDS

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Congenital heart disease (CHD) is the most prevalent severe birth defect, affecting ~1% of newborns. Mutations in transcription factors and chromatin remodelers are prevalent in CHD patients, but how they lead to disease is poorly understood. Dynamics in three-dimensional chromatin architecture play a pivotal role during normal cardiac development and may be dysregulated in CHD. We used TTN as a gene model to gather mechanistic insights into this process and how it relates to disease. TTN undergoes a profound topological rearrangement during embryonic development of cardiomyocytes, relocating from transcriptionally inactive chromatin proximal to the nuclear lamina to active chromatin in the nucleoplasm. We investigated the underlying mechanisms using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and chamber-specific multilineage cardiac organoids. We identified CTCF as a repressor of TTN topological activation, as well as of several other key cardiac genes undergoing a similar rearrangement. We hypothesized that gene repression is the result of CTCF-mediated intergenic looping, which is prominent in hPSCs and weakened after differentiation. To test this notion, we identified CTCF binding sites on the TTN locus and used CRISPR/Cas9 to mutate two sites predicted to be involved in CTCF-mediated loops formation. We validated the abrogation of CTCF binding with ChIP-qPCR, and investigated the resulting effects on TTN gene structure and expression using promoter-capture Hi-C and RT-qPCR. Release of CTCF-mediated repression coincides with recruitment of the pioneer transcription factor GATA4. We found that this gene is necessary for the topological activation of TTN, and its knockdown leads to an expanding cardiac progenitor population that drifts towards a stromal cell fate. Similar perturbations in CTCF have the opposite outcome. These results shed light on the mechanisms responsible for lineage specific gene activation during cardiogenesis. Since CTCF and GATA4 are mutated in patients with syndromic or isolated CHD, this mechanism may be disease-relevant and could be disrupted also in CHD due to environmental exposures. Chamber-specific cardioids offer a promising avenue to explore these future directions, as well as to test potential therapies.

Funding Source: Giovanni Armenise-Harvard Foundation Career Development Award 2021 (A.B.); Additional Ventures Single Ventricle Research Fund 2021 (A.B and S.M.); ERC Starting Grant (TRANS-3; 101076026 – A.B.)

Keywords: chromatin topology, cardiac development, congenital heart disease (CHD)

4:45 PM – 5:45 PM

532

UNVEILING RNA 5-METHYLCYTOSINE MODIFICATIONS IN PSC-DRIVEN CARDIOMYOCYTE DIFFERENTIATION

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Heart disease is a major global leading cause of death. The limitations of adult myocardial regeneration lead to the poor prognosis of heart disease. Therefore, promoting myocardium regeneration is a clinically unmet need. Using pluripotent stem cell-derived cardiomyocytes (PSC-CMs) for myocardial regeneration is a key focus in regenerative medicine research. However, the immaturity of PSC-CMs restricts their entry into clinical therapeutic applications, and there is still no method to induce PSC-CMs to complete maturation. Epigenetic regulations by DNA methylation, histone modifications and non-coding RNA have been shown to affect cardiac differentiation. However, whether RNA post-transcriptional modifications are involved in the process is still largely unknown. In this study, we aim to explore the regulation of PSC differentiation into cardiomyocytes and the key evidence for mediating the maturation of PSC-CMs through RNA 5-methylcytosine modifications. The RNA bisulfite sequencing (bsRNA-seq) analysis was utilized to characterize the alterations of m5C landscape during cellular differentiation in three unique types of derived-cardiomyocytes from their hPSCs—the human amniotic fluid-derived stem cells induced pluripotent stem cell (hAFSC-iPSC), human peripheral blood mononuclear cell (PBMC) induced pluripotent stem cell-SC81103 (hiPSC-1103), and human embryonic stem cells RUES2 (hESC). The result unveiled a hypomethylated RNA m5C landscape in hPSC-CMs, with 1335 consensus differentially methylated m5C in all these three types of hPSCs were enriched in the oxidative phosphorylation (OXPHOS) pathway and demonstrated the differences and uniqueness in the differentiation process of hPSCs from three different source. Our dataset of the RNA m5C epitranscriptome landscape of hPSCs and derived hPSC-CMs, offers valuable insights into RNA m5C regulatory mechanisms in cell-fate commitment and pluripotency during myocardial differentiation, and contributes to shedding light on potential applications in myocardial differentiation and maturation.

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Keywords: cardiomyocyte, pluripotent stem cells, 5-methylcytosine deposition

4:45 PM – 5:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

534

HOW MUCH DO WE KNOW ABOUT THE MOUSE RESPIRATORY EPITHELIAL STEM CELLS? PARTIAL CHARACTERIZATION OF A DOXYCYCLINE-REGULATED MULTI-LINEAGE MOUSE RESPIRATORY EPITHELIAL PRECURSOR CELL LINE IN VITRO

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Tissue-specific stem cell lines are useful tools for cell biology studies. Information on respiratory tissue cell lines is limited. A doxycycline-regulated epithelial precursor cell line was established from the lung tissue of a tTAxSV40 Tag double transgenic mouse. In this study, we have partially characterized this cell line in vitro. It was partially characterized using cell viability and death assays, H3-thymidine incorporation assay, chloride efflux assay, Western blotting of proteins secreted, RT-PCR assays for RNA isolated. It was found to mimic a rare subpopulation of club- and pneumocyte type II-dual cells with multiple phenotypes. Cell growth was doxycycline-regulated and observed only when doxycycline was omitted from the medium or present at concentrations up to 1 mg/ml, higher concentrations were inhibitory.his lung stem cell line might be able to provide us with an insight into the differentiation pathway of lung epithelial cells as well as with some understanding of the nature of air trophic-pulmonary epithelial cells. The results of this study underline the possibility of a future application for somatic (stem/precursor) cells in tissue replacement and tissue engineering of the damaged lung. Its ability to secrete and deliver soluble protein, might be a potential novel way for drug delivery.

Funding Source: DFG, EU Biotechnology

Keywords: Tet-on, tet-off, stem cell line, club- and pneumocyte type II-dual cells, doxycycline-regulated lung stem cell line

4:45 PM – 5:45 PM

536

DEVELOPMENT OF PLURIPOTENT STEM CELL-DERIVED EPIDERMAL ORGANOIDS THAT GENERATE EFFECTIVE EXTRACELLULAR VESICLES IN SKIN REGENERATION

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Extracellular vesicles (EVs) are promising cell-free delivery vehicles in regenerative medicine. Notably, EVs from three-dimensional (3D) models are being used for investigating its physiologic and pathological contents and functions. As 3D organoids closely recapitulate in vivo environment, this system provides more relevant micro-environment which influence cellular processes and therapeutic response. Although the roles of EVs derived from skin cells such as keratinocytes or fibroblasts in cutaneous tissue have been reported, the functions of 3D-EVs from epidermal tissue substitutes remain unknown because of the absence of a 3D epidermal model. Therefore, we developed a new method for differentiating epidermal organoids (EpiOs) from induced pluripotent stem cells (iPSCs). Compared to primary keratinocyte-derived epidermal organoids (kEpiOs), differentiated iPSC-derived epidermal organoids (iEpiOs) were also easily cultured and maintained throughout multiple organoid passages, while maintaining molecular and functional features resembling in vivo epidermis. Mature iEpiOs enhance epidermal stem cell populations and retain their ability to further differentiate into other skin compartment lineages, such as hair follicle stem cells. Further, 3D-cultured iEpiOs generate high-performance EVs in three-fold quantity compared with those from 2D-cultured iEpiOs. The 3D-iEpiO-EVs expressed high levels of the angiogenic factor VEGF and microRNAs like miR-31-5p and miR-146a-5p. The 3D-iEpiO-EVs contributed to cell proliferation, migration, and angiogenesis, providing a promising tool for wound healing. Overall, the newly developed iEpiOs offer a powerful model for basic and translational skin research as well as future therapeutic applications through iEpiO-secreted extracellular vesicles.

Funding Source: This work was supported by the National Research Foundation of Korea (2022R1A2C1008763, 2019R1C1C1010569 and 2021R1C1C2095616) and the KRIBB Research Initiative Program (KGM5322321).

Keywords: epidermal organoids, extracellular vesicle, wound healing

4:45 PM – 5:45 PM

538

DISSECTING CHROMATIN ACCESSIBILITY CHANGES DURING IN VITRO DIFFERENTIATION OF PLURIPOTENT STEM CELL-DERIVED LIMBAL EPITHELIAL STEM CELLS

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The cornea is the transparent tissue at the front of the eye that allows light through. Limbal epithelial stem cells (LESC), located in the limbus, at the edge of the cornea, are vital for maintaining cornea transparency and health by continuously replenishing the corneal epithelium. Because of this regenerative potential, understanding LESC lineage commitment and identity is essential for developing LESC-based therapies valuable for corneal transplantation or regenerative medicine. We have differentiated human ESC and iPSC lines into LESC using in house established protocols spanning a total of 25 days, and performed single cell Assay for Transposase-Accessible Chromatin with high-throughput sequencing (scATACseq) at 3 different stages of differentiation (days 0, 11 and 25) in order to analyse chromatin accessibility dynamics across the process. These data were then integrated with previously performed scRNAseq on cells at the same stages of differentiation. Results from both ESC- and iPSC- derived cells show 6 distinct cell populations based on global chromatin accessibility profiles, which correlate with the major cell clusters identified in scRNA-seq data. Gene regulatory network analysis using both scATACseq and scRNAseq data is currently being performed to identify the key transcription factors driving LESC fate but also to identify and understand the mechanisms underlying cell heterogeneity during the differentiation process. This work will uncover novel epigenetic mechanisms and key drivers potentially involved in the dynamic process of LESC lineage commitment, providing novel information for generating LESCs suitable for transplantation and regenerative medicine.

Keywords: cornea, pluripotent stem cells, regenerative medicine

4:45 PM – 5:45 PM

540

EARLY HUMAN FETAL LUNG ATLAS REVEALS THE TEMPORAL DYNAMICS OF EPITHELIAL CELL PLASTICITY

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Insights into human fetal lung development have largely been informed by animal models, however there are important developmental differences which suggests the presence of human-specific mechanisms that are not captured. Differentiation of human pluripotent stem cells (hPSC) towards lung model developmental stages can capture “fetal-like” phenotypes, but benchmarking these cells to the specific stage during human fetal lung development has largely been missed. Here, we used single cell RNA sequencing to create a transcriptomic cell atlas of 19 normal human fetal lung tissues from gestational weeks (GW) 10-19 capturing >150, 000 cells. Our key findings focused on the epithelial compartment where 19 epithelial cell types/states were identified. We identified novel temporal dynamics of epithelial development originating from progenitor cells that express abundantly high levels of CFTR. Lineage trajectory analysis show that NKX2-1+SOX9+CFTR+ cells give rise to SOX2lowCFTR+ and SOX2highCFTR+ cells, and both can generate SCGB3A2+SFTPB+CFTR+ cells. These latter progenitor cells are the immediate precursors of specialized cell types including pulmonary neuroendocrine early (< GW 14), and ciliated (> GW 14) and club cells (> GW 16). Ligand-receptor inference analysis using CellChat revealed differential stromal cell - SCGB3A2+SFTPB+CFTR+ epithelial cell interactions including the canonical WNT2/2B signaling pathway. Finally, we showed that integration of transcriptomic datasets of hPSC-derived “fetal lung cells” and “fetal lung organoids” (representing an earlier and later stage of fetal epithelial cell differentiation respectively), captured the CFTR+ progenitor cell lineage trajectory, as observed in our fetal lung cell atlas. Our study provides novel insights into human fetal lung epithelial plasticity and potentially how dysregulation of these developmental pathways contributes to congenital lung abnormalities. In addition, our work supports the use of hPSC-derived differentiation protocols to study important human-specific lung developmental mechanisms.

Funding Source: This work is supported by University of Toronto Data Science Institute, the Canadian Stem Cell Network Early Investigator Innovation Award, SickKids Foundation & CIHR-IHDCYH New Investigator Award.

Keywords: human pluripotent stem cell differentiation, lung development, single-cell RNA sequencing

4:45 PM – 5:45 PM

542

HOW TO BUILD LUNG EPITHELIA: INVOLVEMENT OF EXTRACELLULAR MATRIX IN SHAPING CILIATED TISSUES

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Every day, we expose our lungs to about 25000 breathing cycles and inhale around 11000 litres of air. This air enters our lungs unfiltered and contains many pollutants, such as viruses, bacteria, or dust particles. In a healthy lung, our airways are protected by a tissue called “mucociliary epithelium” (MCE). MCEs cover our respiratory tract with a layer of protective mucous that traps harmful particles and transports them out of the lung with help of beating ciliated cells. The cells majorly responsible for this function are goblet and multiciliated cells, respectively. In many lung diseases such as asthma or chronic obstructive pulmonary disease, this protective process is impaired. Altered mucous production, dysfunctional ciliary beating, but also varying cell type composition and their positioning within the tissue can result in improper protection and impede breathing in patients. In this project, we aim to understand how MCEs arise from a pool of multipotent stem cells in the first place. We propose that the extracellular matrix protein hyaluronic acid (HA) could play a dual role in determining cell fate and in shaping the tissue by exerting both mechanical and molecular cues. We explore HA’s implication in the development of Xenopus laevis MCE with high-resolution imaging techniques. When manipulating HA levels, we find changing cell type composition and patterning. We believe that our research provides important lessons on how to build lung tissue from stem cells and will pave the way for treating respiratory diseases in the future.

Funding Source: NNF21CC0073729

Keywords: epithelial stem cells, tissue mechanics, extracellular matrix

4:45 PM – 5:45 PM

544

HIPPO PATHWAY MARKERS IN BASAL KERATINOCYTES

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The aim of this research is to explore the significance of Hippo pathway for cell proliferation and differentiation in human keratinocytes. Keratinocytes were isolated, cultures and trypsinized (0.05 % trypsin-EDTA, Gibco) (5 min, 37 °C). Magnetic separation was performed with cell surface markers CD71 and integrin a6b4. The cultures than were seeded in black 96 well plate and cDNA was isolated with FastLane Cell cDNA kit (50), Cat. No: 215011, which directly isolates cDNA from cultured cells without RNA purification. Then the cDNA is transferred to RT2 Profiler PCR Array (384-Well [4x96] Format) for Human Hippo Signaling, Cat.No 330231 PAHS-172ZA and a PCR on qRTPCR was administered. The keratinocytes are separated in 3 groups, related to the cell surface markers. We defined the expression of ACTG1, AMOTL, CASP3, CRB,LAT,YWHA, TEAD, ACTB, B2M, etc. We characterized a panel of Hippo related genes in groups such as: Upstream Hippo Signaling Regulators, Hippo Core Kinase Complex, Downstream Mediators of Hippo Signaling, Hippo Signaling Target Genes, Contact Inhibition, Cell Polarity, Scaffolding Proteins. Keratinocytes stem cells are with a very specific regulation and cell growth. The proof of expression of this markers send us one step closer to understanding their regulation of expression and the proliferation and differentiation potential in the adult organism.

Funding Source: Project BG-RRP-2.004-0004-C01 financed by Bulgarian National Science Fund. The research is financed by the Bulgarian national plan for recovery and resilience.

Keywords: HIPPO pathway, keratinocytes, RT2 Profiler PCR array

4:45 PM – 5:45 PM

546

DIETARY ARACHIDONIC ACID EVOKES ENDOMETRIAL STEM CELL PLASTICITY

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Endometrial ailments such as endometriosis, infertility, and endometrial cancer impact 1 in 4 women in the US, accentuating the significant need to improve our understanding of endometrial biology. Endometrial stem cells regenerate the endometrium monthly, and their dysregulation is implicated in endometrial diseases. Recent studies have underscored the capacity for the metabolic reprogramming of stem cells, but little is known about the metabolic regulation of stem cell states in endometrial physiology and disease. Here, we explore how fatty acids affect endometrial stem cell regeneration. We performed a fatty acid (FA) screen on human and mouse endometrial organoids and discovered a subset of ω-6 fatty acids converging on arachidonic acid (AA) with stem cell-enhancing effects. Using bulk and single-cell RNA sequencing analysis, we found dietary AA evokes de novo stem cell states, dedifferentiation programs, and repair-associated stem cell reprogramming signatures in response to AA. Mechanistically, AA begets Prostaglandin E2 (PGE2) and activates the Ptger4 – cAMP - PKA signaling axis to promote stemness in mice and humans. These results demonstrate that dietary AA is a conserved promoter of endometrial regeneration that mimics the injury repair response through PGE2-Ptger4 signaling. These findings highlight the significance of dietary fatty acids in regulating endometrial tissue regeneration and the potential to treat degenerative endometrial ailments.

Keywords: diet, plasticity, regeneration

4:45 PM – 5:45 PM

548

MAPPING HUMAN INNER EAR DEVELOPMENT: INSIGHTS FROM SINGLE-CELL TRANSCRIPTOMICS

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The inner ear, essential for auditory perception and balance, relies on specialized cell types whose developmental mechanisms in humans remain incompletely understood. While animal models provide valuable insights, human inner ear development remains predominantly characterized by descriptive approaches. The advent of single-cell transcriptomics presents a promising avenue to elucidate the intricacies of human inner ear development. In this study, we present a comprehensive human single-nucleus RNA transcriptomic atlas encompassing the entire membranous inner ear, across nine time points from fetal weeks 7 to 15. Analyzing data from over 55,000 cells, we uncover the dynamic molecular landscape that drives the differentiation of epithelial, neuronal, and mesenchymal cells in both vestibular and cochlear regions. This comprehensive mapping illuminates key signaling pathways essential for the development of critical nonsensory epithelial cells, highlighting the roles of well-conserved signaling pathways in forming the vestibular system's nonsensory dark cells and the cochlea's stria vascularis - two specialized epithelia vital for inner ear function. Furthermore, we find a broad diversity of otic mesenchymal cells that has not previously been acknowledged. Our characterization not only advances understanding of human inner ear development but also holds significant promise to understand pathophysiological mechanisms leading to hearing loss and balance disorders. Furthermore, harnessing these insights to enhance culture models, such as human iPSC-derived inner ear organoids, would enhance their utility in addressing in vitro inquiries into developmental processes and their effectiveness as disease models.

Funding Source: This work was supported by the Novo Nordisk Foundation (NNF21CC0073729)

Keywords: human, inner ear development, single-nucleus RNA transcriptomics

4:45 PM – 5:45 PM

550

DIVERSE SURFACE ENVIRONMENTS INFLUENCE CELLULAR COMPOSITION IN HUMAN INTESTINAL ORGANOIDS

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Human intestinal organoids (hIOs), derived from human pluripotent stem cells (hPSCs), offer valuable insights into intestinal development. However, existing culture methods, predominantly Matrigel-based, struggle to fully replicate the diverse cellular spectrum found in the human intestine. Despite advancements in 3D cell culture extracellular matrices (ECMs), Matrigel or hydrogels remain standard. In our study, we employed a thin film for hIO cultivation, revealing distinct cellular compositions influenced by the polymer coating, as confirmed through genetic and histological analyses. In contrast to conventional Matrigel domes, we subjected hIO cultures to suspension conditions for 28 days under various thin films, including ULA or zero gravity systems, unveiling noticeable differences. Our investigation highlighted variations in cell type compositions within hIO cultures on thin films, with a clear correlation observed with surface hydrophobicity favoring conducive environments for epithelium or mesenchyme growth. Furthermore, we delineated distinct composition ratios of cell types, such as intestinal stem cells and enteroendocrine cells, within hIOs cultured on thin films. In summary, our results enable observing distinct cell type development in suspended hIOs based on surface or environmental factors. Furthermore, this opens up opportunities for the targeted cultivation of cells crucial for regenerative medicine, extending beyond the realms of cell differentiation and developmental studies.

Funding Source: This work was supported by the Korean Fund for Regenerative Medicine (KFRM) grant funded by the Ministry of Science and ICT, the Ministry of Health & Welfare (21A0404L1), and a grant from the Technology Innovation Program (No. 20008777).

Keywords: human intestinal organoid, extracellular matrix, suspension culture

4:45 PM – 5:45 PM

552

CRANIAL ECTOMESENCHYME AS A SOURCE OF EPIDERMAL STEM CELLS

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Pdgfrα (platelet-derived growth factor receptor alpha) is a general marker of mesenchymal cells such as fibroblasts. We observed that the Pdgfrα-lineage cells significantly contributed to the epidermal keratinocytes in the developing skin of the pdgfra::cre;flox-stop-flox-tdTomato mice. In the newborn mice, the Pdgfrα-lineage keratinocytes were observed in the skin of head, back, and abdomen as populations of 13.9%, 8.1%, and 8.4%, respectively. The population increased by aging, reaching to nearly 80% of the epidermal keratinocytes in the adult mice. To examine the detailed Pdgfrα-lineage keratinocyte composition in the

adult mouse skin, we performed scRNA-seq of the skin in the 8-week-old mice. We found that the Pdgfrα-lineage keratinocytes dominantly contributed to the interfollicular epidermis in the head and back skin, suggesting different developmental origins of the interfollicular and follicular keratinocytes. To search for developmental origin of the Pdgfrα-lineage keratinocytes, we used P0::cre;flox-stop-flox-tdTomato mice, in which we can trace the lineages of the ectodermal mesenchymal cells such as neural crest cells. We found that the P0-lineage cells accounted for more than 70% of the keratinocytes in the head and the back skin at the age of 8 weeks, indicating that the Pdgfrα-lineage keratinocytes in the skin were originated from the ectodermal mesenchymal cells (ectomesenchyme). The first wave of the ectomesenchyme are known to delaminate from the lateral non-neural surface ectoderm of the cranial neural fold in the early embryonic developmental stage. We found the cranial ectomesenchyme expressing Pdgfrα could be traced as the P0-lineages in the mouse embryo, suggesting that the Pdgfrα- and P0-lineage keratinocytes of the interfollicular epidermis possibly originated from the cranial ectomesenchyme. To validate our in vivo findings, we induced cranial ectomesenchyme from human induced pluripotent stem cells to interrogate their potency for raising epidermal keratinocytes. The induced cranial ectomesenchyme expressing Pdgfrα and p75 had a potency to differentiate into epidermal keratinocytes expressing epidermal stem cell markers p63 and K5/14. These results may provide a new insight to the cranial ectomesenchyme for developing interfollicular epidermal stem cells.

Keywords: skin development, ectodermal mesenchymal cells, human iPS cells

4:45 PM – 5:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

554

DEFINING EMBRYO DORMANCY AT SINGLE-CELL RESOLUTION REVEALS CRYPTIC TRANSCRIPTIONAL DYNAMICS APACE WITH YAP-MEDIATED MECHANOTRANSDUCTION

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The implantation of the blastocyst into the uterine wall is a key step of the reproductive cycle that mediates the connection of the embryo to the maternal tissues during the early stages of pregnancy. Interestingly, in some species, including the house mouse, this process can be put on hold, diverting embryonic development into a reversible state of dormancy, known as diapause. In contrast to the “normal” (transient) embryogenesis, which have been intensively studied, the embryonic diapause is still an extremely enigmatic state. Here, we characterised the transcriptome of murine embryos during entry, maintenance and exit of diapause at single-cell resolution, in parallel with the “normal” pre- to post-implantation transition, revealing the hidden transcriptional dynamics while the embryo seemingly resides in a state of suspended animation. Focusing on the pluripotent lineage, we uncovered the activation of a cryptic, diapause-specific transcriptional response. In particular, we found that integrin/Yap signalling is required for preserving the developmental capacity of the dormant embryo.

Funding Source: This work was supported by the European Research Council (ERC) Consolidator grant (MORPHEUS, 101043753) and the Collaborative Research Center 1348 ‘Dynamic Cellular Interfaces’ grant (1348/2, B09).

Keywords: embryo dormancy, embryonic diapause, single-cell RNA-seq

4:45 PM – 5:45 PM

556

METTL3 DEPENDENT M6A RNA METHYLATION REWIRES GENE REGULATORY NETWORK IN HUMAN EMBRYONIC STEM CELLS THROUGH TRANSPOSABLE ELEMENTS

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N6-methyladenosine (m6A) is the most prevalent RNA modification with diverse regulatory roles. While its functions in mouse embryonic stem cells (mESCs) are well-documented, its role in human embryonic stem cells (hESCs) remains largely unexplored. Here, using human expanded potential stem cells (hEPSCs) as a model, we uncovered the role of METTL3, the main enzyme responsible for m6A deposition, in gene regulatory network through regulating transposable elements. Utilizing CRISPR-Cas9 technology, we generated inducible METTL3 knockout (iKO) hEPSCs and noted significant upregulation of early epiblast naive pluripotent genes. Interesting, only a minority of these upregulated naïve genes are m6A-modified, suggesting an indirect mechanism of m6A regulation. Additionally, human trophoblast differentiation was impaired following METTL3 iKO. Considering the capacity of transposable elements (TEs) to remodel gene regulatory networks,

we examined TE expression and observed upregulated expression of two primate-specific retrotransposons, SVA_D and HERVK/LTR5_Hs, associated with increased enhancer activity after METTL3 iKO. Notably, we identified that METTL3 interacts with SVA_D loci and facilitate the deposition of repressive marker H3K9me3. On the other hand, increased binding of active transcriptional factors and reduced DNA methylation were observed on LTR5_Hs upon METTL3 iKO. Circular chromosome conformation capture sequencing (4C-seq) revealed direct interactions between these two retrotransposons and promoters of naïve genes, and excision of specific retrotransposon loci compromised the upregulation of naïve genes following METTL3 iKO. Through re-analyzing RNA-seq and epigenetic data of human embryo data, we hypothesized that METTL3 may affect zygote genome activation (ZGA) by repressing SVA_D. Our results propose that METTL3-dependent RNA methylation can significantly alter the gene regulatory network of human pluripotent stem cells and early embryo through TEs.

Keywords: METTL3 and m6A RNA methylation, pluripotency, transposable elements

4:45 PM – 5:45 PM

558

TRANSCRIPTION FACTOR NETWORK REGULATING THE FIRST LINEAGE CHOICE IN HUMAN EMBRYONIC STEM CELL MODEL

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The first lineage choice in human development delineates trophectoderm from the inner cell mass. Previous studies show that Polycomb repressive complex 2 (PRC2) is involved in maintaining naïve pluripotency and restricting human embryonic stem cell (hESC) ability to form trophectoderm (TE) and mesoderm (M) lineages. However, the lineage-specific transcription factors which are driving naïve hESC to form TE and M lineages in vitro are unclear. Elucidating the hierarchy of these transcription factors would enhance our understanding of the functional lineages involved in early human embryo development and could be used to inform state-of-art embryonic lineage models. Here, we aimed to validate candidate transcription factors using immunofluorescence in combination with epigenetic single-cell methods. By examining the fluctuation of TE and M markers in a hESC activation and capacitation model with perturbations to PRC2 levels on the previously selected transcription factor panel, we aim to evaluate a panel of transcription factors potentially involved in the first lineage choice. Inhibition of PRC2 under permissible culture conditions allows the naïve hESC model to form TE- and M- like lineages, which, in combination with functional perturbations, allows us to establish the degree of involvement, cooperativity, and possible hierarchy for the selected transcription factors and signaling pathways. Finally, our experiments establish how PRC2 shields naïve hESC from the activity of lineage-specific TFs.

Keywords: human embryonic stem cells, first lineage choice, naïve pluripotency

4:45 PM – 5:45 PM

560

SSCTF1 REGULATES CODING AND NONCODING TRANSCRIPTIONS IN MOUSE AND HUMAN SPERMATOGONIA STEM CELLS

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Spermatogenesis is a complex biological process orchestrated by a series of precisely regulated programs, encompassing spermatogonial stem cell (SSC) self-renewal and differentiation, meiosis, and spermiogenesis. In this study, using bioinformatic analysis and in vitro SSC differentiation, we identified SSCTF1 as a key transcriptional regulator in spermatogonia development. First, utilizing bioinformatic analysis and a germline-specific SSCTF1 knockout mouse model, we validated SSCTF1's indispensable role in determining mouse spermatogonia cell fate commitment. Depletion of SSCTF1 resulted in the depletion of pro-spermatogonia in the fetal testis, impaired spermatogenesis, and consequent male infertility. Furthermore, combining in vitro differentiation of human embryonic stem cells into SSCs, alongside various gene editing techniques and multiomic methodologies, we elucidated SSCTF1's regulatory role over protein-coding genes crucial for spermatogonia development (e.g., GFRA1, ID4, THY1), as well as a subset of long non-coding RNAs (e.g., LncKDM2A, LncKDM6B, LncARID1B) associated with histone remodeling. This regulation influenced the histone modification status of key gene loci downstream, thereby impacting spermatogonia development. Through protein co-immunoprecipitation experiments coupled with mass spectrometry analysis, we examined the potential pathogenic mechanism of SSCTF1 mutations newly identified in non-obstructive azoospermia patients. Our findings revealed that mutant SSCTF1 failed to efficiently recruit cofactors involved in transcription initiation and chromatin remodeling, thereby disrupting the transcriptional activation of downstream target genes and resulting in aberrant spermatogonia development. In summary, our study characterizes SSCTF1 as a prominent transcription factor governing SSCs in both mice and humans. We elucidated SSCTF1's ability to regulate not only protein-coding genes but also lncRNAs involved in chromatin remodeling, thus influencing spermatogonia cell fate commitment. Additionally, our investigation into the pathogenic mechanisms of SSCTF1 mutations associated with non-obstructive azoospermia offers novel insights into the diagnosis and treatment of related disorders.

Keywords: spermatogonial stem cell, transcriptional regulator, non-obstructive azoospermia

4:45 PM – 5:45 PM

562

THE EFFECTS OF Y CHROMOSOME GENES ON MOUSE ES CELLS

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The Y chromosome has a role not only in sex determination and spermatogenesis but also in embryogenesis, health, and diseases. In mice, four genes on the Y chromosome (Kdm5d, Ddx3y, Uty, and Eif2s3y) are expressed ubiquitously and thus may have an important role in non-reproductive organs. In ES cells, gene expression is different depending on the sex chromosome pair. However, there is no research to investigate the role of single Y chromosome genes. In this study, we established four single Y gene KO ES cells and conducted RNA sequencing of these cells, as well as XY and XO ES cells. In each single Y chromosome gene KO line, the whole gene body was deleted by the Crispr-Cas9 system, and the deletion was confirmed by DNA sequencing. In RNA-sequencing analysis, each single Y chromosome gene knockout affected the expression of other Y chromosome genes. Surprisingly, the number of differentially expressed genes between single Y gene KO lines and XY ES cells is larger than between XO and XY ES cells. Furthermore, although Kdm5d is not expressed in ES cells, the Kdm5d KO had the largest number of differentially expressed genes. These results suggest that Y chromosome genes form a collaborative network that affects autosomal gene expression. Our data also showed that a single Y gene KO could affect the transcriptome of the ES cells more drastically than the Y chromosome loss. Our results will provide a clue to elucidate the regulatory network of the Y chromosome genes. It will also help to find an essential gene of the Y chromosome in organs.

Funding Source: This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (CC2052), the UK Medical Research Council (CC2052), and the Wellcome Trust (CC2052) and JSPS Overseas Research Fellowships.

Keywords: Y chromosome genes, RNA-seq, ES cells

4:45 PM – 5:45 PM

564

AN M6A-IGF2BP1-OTX2-TFAP2C AXIS RESTRICTS HUMAN GERM CELL FATE DURING GERMLINE SPECIFICATION

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Germ cells are the carriers of genetic and epigenetic information from parents to offspring. Defects in the establishment of germline may lead to infertility, germ cell tumors, and birth defects. Primordial germ cells (PGCs), the precursors of germline, are specified early during embryogenesis and establish the germ cell lineage during development. In this study, we discovered that N6-methyladnosine (m6A)-mediated post-transcriptional regulation is crucial for the specification of human germline. Knockout of the key m6A writer METTL3 leads to increased percentage of human PGC-like cells (hPGCLCs), suggesting that m6A may play key roles in restricting germ cell fate. Consistently, over-expression of m6A erasers FTO or ALKBH5 also results in increased percentage of hPGCLCs. Furthermore, we discovered that knockout of the m6A reader IGF2BP1 leads to the same phenotype, suggesting that m6A machinery is essential for regulating germ cell fate. Through bioinformatic analysis and small-scale CRISPRi screen, we characterized OTX2 mRNA as the key downstream factor for the m6A regulation in germ cell lineage. Site-specific-removal of m6A modification in OTX2 mRNAs recapitulates the same phenotype as OTX2 knockout or IGF2BP1 knockout, which is the increased percentage of hPGCLCs. Moreover, we discovered that OTX2 suppresses TFAP2C during germ cell lineage specification. In summary, we characterized the m6A-IGF2BP1-OTX2-TFAP2C axis in restricting human germ cell fate specification.

Keywords: primordial germ cells, m6A modification, human embryonic stem cells

4:45 PM – 5:45 PM

566

CHARACTERIZING GERM CELL SPECIFICATION THROUGH SINGLE CELL MULTIOMICS

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The first step to ensure successful sexual reproduction and species survival is the correct differentiation of primordial germ cells (PGCs). Murine PGCs arise in the post-implantation epiblast in response to external signals including BMP4 and WNT from the adjacent

extraembryonic tissue. Cells exposed to this signalling environment can enter the germline and upregulate key PGC factors such as Blimp1, Prdm14 and Ap2γ. However what governs the first steps in the decision of cells to enter the germline remains unknown. We have carried out single cell omics (scRNA-seq and scATAC-seq) analysis at short time intervals during in vitro germline specification. Our results show that epiblast-like cells (EpiLCs) are homogenous at both gene expression and chromatin level indicating that there is not a subpopulation of EpiLCs that are predisposed for germline differentiation. Rather, two clear populations emerge after 48h of exposure to external signals: a small population of cells expressing early PGC markers (i.e Blimp1, Ap2y, Esrrb) and a larger group of cells expressing somatic markers (i.e T, Mixl1, Wnt3a). Commitment of cells to these fates is asynchronous, with cells entering the germline earlier than the somatic fate. In addition, mutually exclusive expression patterns suggest roles for novel potential regulators of germline versus somatic cell fate choice. These data presents the first steps of germline commitment on a single cell level and at high temporal resolution showing when and how cells commit to the germline and the soma.

Funding Source: Medical Research Council (MRC)

Keywords: development, single cell RNA-seq/ATAC-seq, germline commitment

4:45 PM – 5:45 PM

568

A GENETIC REPORTER FOR HUMAN NAÏVE PLURIPOTENCY BASED ON KLF17 EXPRESSION

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Pluripotent stem cells can exist in two primary cellular states referred to as naïve or primed. Naive pluripotent stem cells possess enhanced developmental potency compared to conventional primed states. However, generating and maintaining human naive pluripotent stem cells remains challenging. Developing tools to mark and isolate naive cells from heterogeneous stem cell cultures will facilitate the identification of genetic and epigenetic regulators of primed-to-naïve state conversion. In this work, we first analyzed expression data of human embryos collected at different stages of development to identify candidate genes that may be specific to naïve pluripotency. Based on this analysis, we selected KLF17 as a gene specifically expressed in naive pluripotent stem cells and engineered a KLF17-T2A-EGFP fluorescent reporter in human induced pluripotent stem cells (iPSCs) using CRISPR/Cas9-based genome editing. We validated this GFP-based reporter system during primed-to-naive conversion under several established culture conditions via immunofluorescence and flow cytometry and showed that KLF17 expression marks bona fide naïve stem cells. The reporter line allows for precise quantification of naïve cells in heterogenous cultures and will be useful in identifying novel regulators of primed-to-naïve stem cell conversion.

Funding Source: TUBITAK 2247A grant 121C316

Keywords: KLF17, naive stem cells, pluripotency

4:45 PM – 5:45 PM

570

AUTOCRINE MFGE-8 ENHANCES HUMAN PLURIPOTENT STEM CELL SELF-RENEWAL AND SURVIVAL BY DELIVERING GSTS WITHIN EXTRACELLULAR VESICLES

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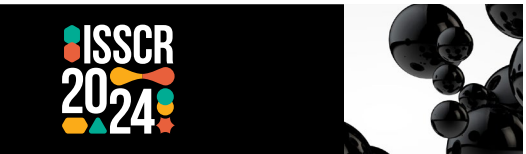
Autocrine and paracrine communication are mediated through the secretome, which can be broadly classified into two categories: extracellular vesicles (EVs) and soluble secretory factors (SFs). Although EVs and SFs are capable of playing their role independently, they can achieve a synergistic collaboration by generating a structure, referred to as a protein corona, which envelops the surface of EVs via interactive engagement of SFs with both the surface and the surrounding environment of EVs. Human pluripotent stem cells (hPSCs) also utilize EVs and SFs to communicate with neighboring cells. While it has been reported that EVs and SFs play crucial roles in preserving the self-renewal, pluripotency, and survival of hPSCs, there is a lack of studies exploring the cooperative association between EVs and SFs in modulating the stemness of hPSCs. We observed that SFs secreted from hPSCs augment the functions of autocrine EVs in promoting self-renewal and survival of hPSCs by creating the protein corona. Particularly, we found that a glycoprotein milk fat globule-EGF factor 8 (MFGE-8) secreted from hPSCs is incorporated onto the surface corona layer of hPSC-derived EVs, markedly amplifying the self-renewal and survival of hPSCs through the activation of the αvβ5/AKT axis. This signaling axis enables the simultaneous enhancement of EV uptake into hPSCs and the progression of the cell cycle. A pivotal element orchestrating this process following EV uptake is the existence of antioxidant enzymes located in the lumen of EVs, specifically those linked to glutathione (GSH) metabolism including glutathione S-transferases (GSTs). Upon internalization of EVs, these enzymes function to alleviate oxidative stress by facilitating



the regeneration of GSH in hPSCs and contribute to the maintenance of self-renewal and survival of hPSCs. In summary, our findings reveal that MFGE-8 present in the protein corona of hPSC-derived EVs plays a key role in delivering luminal antioxidant enzymes between hPSCs and this process is indispensable for the self-renewal and survival of hPSCs in vitro.

Funding Source: This work was funded by the National Research Foundation of Korea Grant (No. RS-2023-00280923) and the Korean Fund for Regenerative Medicine grant (No. 22A0204L1) from the Ministry of Science & ICT and the Ministry of Health & Welfare.

Keywords: human pluripotent stem cells, extracellular vesicles, milk fat globule-EGF factor 8

4:45 PM – 5:45 PM

572

ANALYSIS OF DEVELOPMENTAL ROLE OF RETINOIC ACID IN A MOUSE GASTRULOID MODEL

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In vivo studies have shown that retinoic acid strongly effects body plan formation and cell fate decisions in post-implantation stage embryos. However, due to the limitations of in vivo studies, the dynamic information of this signaling pathway on axial elongation and cell fate decisions is still unclear. Embryonic stem cells can self-organize into 3D embryo-like structures, termed gastruloids. With these gastruloid models, we can mimic post-implantation stage embryos in vitro, which allows us to analyze the dynamics of signaling gradients. To understand the role of retinoic acid signaling during early embryonic development, we cultured mouse gastruloids with different doses of retinol, a precursor of retinoic acid, and performed (time-lapse) imaging with germ layer-specific reporters. Also, we utilized single-cell RNA sequencing to investigate the impact of retinoic acid on cellular heterogeneity. Our (time-lapse) imaging data showed a critical role for retinoic acid in axial elongation as well as hox gene expression as observed in vivo. Additionally, single-cell RNA sequencing data demonstrates how retinoic acid maintains the balance between mesoderm and ectoderm commitment as well as mesoderm maturation within gastruloids. Together, these data demonstrate that retinoic acid is an important modulator of axial elongation, body plan formation, cell fate commitment and maturation.

Funding Source: This work is supported by a Novo Nordisk Foundation grant (NNF21CC0073729; reNEW)

Keywords: gastruloids, early embryonic development, retinoic acid signaling

4:45 PM – 5:45 PM

574

CHARACTERIZATION OF FATE TRAJECTORIES IN HUMAN POST-IMPLANTATION EMBRYOS

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Understanding human peri-implantation development is necessary to improve in vitro fertilisation. While tremendous progresses have recently shed light on human preimplantation development, little is known about fate transitions in human post-implantation embryos. Technical progress, such as scRNAseq and integrated stem cell models have dramatically improved our ability to understand human peri-implantation development. However, in-depth analysis and systematic validation in human embryos remains necessary to build a reliable reference. Here, we combine scRNAseq analysis and immuno-fluorescence of finely staged human embryos to decipher the sequences of molecular events characterizing post-implantation fate progression. We started by an in-depth integration of three datasets spanning the developmental period from 3dpf until 14dpf. Inference of gene regulatory networks led us to identify transcription factors linked with cell fate transition, within that developmental time-frame. Immunofluorescence in human embryos allowed validation of key transcription factors, as putative fate progression markers. Our results highlight the gradual acquisition of markers, such as ZIC3 for pluripotency, clarifying the pluripotency continuum, and RXRA for trophectoderm, shedding light on trophectoderm maturation and onset of syncytiotrophoblasts while cytotrophoblasts are preserved within the embryo. By systematically comparing results with stem cells models, such as naive PSC, primed PSC, TSC, TELC and blastoids, we refine stage matching of all the main cellular models with the human embryos, based on transcriptomics, immuno-fluorescence and proteomics datasets. Our analysis will be embedded in a fully browsable interface, as for our human pre-implantation embryo mapping (Meistermann et al 2021).

Funding Source: This work is funded by ANR.

Keywords: human embryo development, cell fate, omics

4:45 PM – 5:45 PM

576

COMPARATIVE PROTEOMIC LANDSCAPES PROVIDE INSIGHTS INTO MAMMALIAN PREIMPLANTATION DEVELOPMENT

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Preimplantation development of human embryos has been thoroughly investigated at transcriptomic, translatomic and epigenetic levels in the past decade. However, a deep and resourceful proteomic landscape of this process is a missing gap for our comprehensive understanding of human preimplantation development. Here, we utilized our recently developed Comprehensive Solution of Ultrasensitive Proteome Technology to measure the proteomic profiles of oocytes and early embryos, identifying nearly 8,000 proteins in humans, using only five oocytes or embryos per stage. Additionally, over 6,200 proteins were detected in various developmental stages of mice. We observed divergent proteomic dynamics before and around zygotic genome activation (ZGA) between the two species. Integrative analysis with translatome data revealed extensive and stage-specific protein turnover events. Multi-omics analysis indicated that ZGA transcripts contribute to protein accumulation in blastocysts. Additionally, several transcriptional regulators were found to be essential for early development in mice, thereby linking ZGA to the first lineage specification. Our study may reshape the framework of mammalian preimplantation development and lay a foundation for future investigations.

Keywords: preimplantation development, ultrasensitive proteomics, multi-omics analysis

4:45 PM – 5:45 PM

578

YAP ACTIVITY IMPACTS THE SURVIVAL OF NAÏVE AND PRIMED HUMAN EMBRYONIC STEM CELLS THROUGH CONTACT-MEDIATED MECHANISMS

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Human pluripotent stem cells (hPSCs) possess the remarkable ability to differentiate into any cell type of the body, serving as a powerful substrate to model human development in vitro. While these cells are seemingly equipotent, there is heterogeneity in the transcriptional state of hPSCs, creating a selection pressure during lineage commitment. Among the transcription factors regulating the balance between pluripotency and differentiation in hPSCs, yes-associated protein (YAP) emerges as a crucial player. Despite a similar growth rate when individually maintained, hPSCs with engineered YAP overexpression have been shown to exhibit a growth advantage in co-culture with wild-type (WT) hPSCs, particularly in the primed pluripotent state (corresponding to the post-implantation epiblast). Whether this growth advantage is conserved across pluripotency remains uncertain. To investigate this, we induced YAP- and WT-hPSCs into the naïve pluripotent state (corresponding to the pre-implantation blastocyst). Our findings reveal similar morphologies in both YAP- and WT- hPSCs following naïve induction, with higher SUSD2 (naïve marker) and lower CD90 (primed marker) expression compared to the primed state. When maintained in their separate cultures, WT- and YAP-hPSCs exhibit similar growth rates in both naïve and primed pluripotency. Notably, YAP-hPSCs progressively dominate WT in co-culture and the speed of overtake is amplified in naïve conditions. To monitor contact-mediated interactions between YAP- and WT-hPSCs, we genetically engineered the cells to express a “synthetic Notch”, inducing the robust expression of a fluorescent reporter upon cell-cell contact between YAP- and WT-hPSCs in co-culture. Using these lines, live imaging reveals a higher rate of WT cell loss upon interaction with YAP cells. Overall, our work unveils the conservative role of YAP transcription factor in affecting hPSC survival in a contact-mediated manner, allowing pluripotent cells to coordinate their fate outcomes with neighbours in naïve and primed pluripotency.

Funding Source: Canadian Institutes of Health Research (CIHR)

Keywords: naive hPSCs, primed hPSCs, YAP

4:45 PM – 5:45 PM

580

EVOLUTIONARY CONSERVED CELL-CELL ADHESION DYNAMICS DURING PLURIPOTENCY TRANSITIONS

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Pluripotency transitions and cell fate decisions are often approached from a transcription factor-centric perspective. However, cell-cell adhesions, influenced by the biophysical environment, play an additional crucial role in embryonic development and exhibit remarkably dynamic expression profiles. Understanding the interplay between biomechanics, cell-cell adhesion, and transcriptional gene regulatory networks is essential for unravelling the mechanisms underlying embryonic development. In this study, we exert microenvironmental control over embryonic stem cells, utilising microfluidic encapsulation in microgels to stabilise naive pluripotency. This approach upregulates the expression of Plakoglobin (Jup), a less-known vertebrate homolog of β-catenin, underlining the need for a deeper comprehension of the dynamics of cell-cell adhesion changes during pluripotency transitions. Furthermore, we find that in the epiblast, Plakoglobin is exclusively expressed at the blastocyst stage in human and mouse embryos, further fortifying the link between Plakoglobin and naive pluripotency in vivo. Overexpression of Plakoglobin effectively reinstates the gene regulatory network governing naive pluripotency under metastable conditions, validated through single-cell transcriptome profiling. This study illuminates the complex dynamics of cell-cell adhesion during pluripotency transitions, offering insights into the mechanisms governing embryonic development and cell differentiation.

Keywords: pluripotency, early development, cell-cell adhesions

FRIDAY, 12 JULY, 2024

TRACK: PLURIPOTENCY AND DEVELOPMENT (PD)

Session III: Odd

3:45 PM – 4:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

101

TRACKING OF EARLY CELL FATE DECISIONS OF IPSC BASED ON DNA METHYLATION

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Quality control of induced pluripotent stem cells (iPSC) remains a challenge. For validation of pluripotency, the trilineage differentiation potential towards endoderm, mesoderm, and ectoderm is crucial. Conventional quality control involves assessing morphological criteria, surface markers, gene expression changes, or teratoma formation in mouse models, but these are difficult to standardize and quantify. Recognizing the increasing need to accurately characterize iPSC and their derivates, we established PluripotencyScreen – an epigenetic biomarker to track early cell fate decisions through DNA methylation at specific CG dinucleotides (CpG). Using the computational framework CimpleG, we identified three characteristic CpG at pluripotent state and after differentiation into endoderm, mesoderm, and ectoderm based on own Illumina Methylation BeadChip data. From these signatures, we established a pluripotency score, that not only tracks reprogramming of iPSC but also indicates differentiation capacity, as well as lineage-specific scores to monitor both, the directed differentiation of iPSC and multilineage differentiation in embryoid bodies. Validation through targeted pyrosequencing assays of 58 iPSC lines and DNA samples confirmed the reliability of site-specific DNA methylation for characterization of iPSC. The assays revealed iPSC lines with inhibited ectodermal differentiation capacity in embryoid bodies. They correctly showed reduced ectodermal cell fractions in PRDM8 and YAP1 knockout lines. Furthermore, targeted pyrosequencing reflected the impact of culture media and differences between directed and undirected differentiation towards neuroectoderm. We will demonstrate ongoing optimization and

validation of the procedure. Taken together, PluripotencyScreen facilitates reliable and reproducible quality control of iPSC by quantifying pluripotency and early lineage-specific commitment.

Keywords: iPSC, pluripotency, DNA methylation

3:45 PM – 4:45 PM

103

UNCOVERING THE ROLE OF HDAC3 IN EARLY MOUSE DEVELOPMENT

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Histone acetylation has long been used as a proxy for the identification of active gene regulatory elements (GREs). However, whether and to what extent chromatin marks, in general, play an instructive role in gene regulation remains elusive. Intriguingly, recent evidence has shown that histone deacetylase 3 (HDAC3), a known transcriptional repressor, localizes to active GREs in mouse embryonic stem cells (mESCs), suggesting a role for their dynamic regulation. Since genetic ablation of HDAC3 results in embryonic lethality at the time of implantation, here we investigate the role of HDAC3 in the context of exit from naïve pluripotency. For this purpose, we have generated an auxin-inducible degron system for HDAC3 in mESCs. We used this system to test the immediate effects of HDAC3 loss on gene expression in cells differentiating from naïve to formative state, the in vitro counterpart of the implanting epiblast. Additionally, we performed FLASH-seq to generate single-embryo RNA sequencing data from E4.5 and E6.5 embryos. By integrating this data with ChIP-seq results for HDAC3 and different histone marks, we aim to decipher the extent to which HDAC3 is involved in enhancer utilization and get an insight on why epigenetic repressors localize to active GREs.

Funding Source: This work was supported by grants from Novo Nordisk Fonden (NNF) (NNF21CC0073729), Lundbeckfonden (Lundbeck Foundation) (R345-2020-1497) and Danmarks Frie Forskningsfond (DFF) (0134-00031B, 0169-00031B).

Keywords: HDAC3, pluripotency, acetylation

3:45 PM – 4:45 PM

105

MAINTENANCE OF NAIVE HUMAN PLURIPOTENT STEM CELLS IN FEEDER-FREE CONDITIONS

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Pluripotent stem cells (PSCs) can give rise to all adult cell types and self-renew indefinitely in vitro. Particularly, two pluripotency states have been determined both in mice and human, which differ in their differentiation potential, signalling pathways, and cytokines required for self-renewal and proliferation: naive, resembling the pre-implantation epiblast, and primed, mimicking a more developmentally advanced state. Naive mouse pluripotent stem cells (mPSCs) are cultured on gelatin-coated plates in an optimised and defined medium. However, given the recent derivation of naive human pluripotent stem cells (hPSCs), their culture media are still being optimised, and they expand robustly on a layer of inactivated mouse embryonic fibroblasts (MEFs). The presence of MEFs is challenging and introduces complexities and concerns for experimental set-up, downstream analysis, and applications of naive hPSCs. In this project on naive hPSCs, the effects on cell morphology, proliferation, and maintenance of pluripotency have been assessed by culturing hPSCs in a feeder-free condition, called serum coating. After a period of stabilisation, characterised by reduced proliferation compared to the standard condition on MEFs, naive hPSCs on serum coating exhibit growth rate, clonogenicity, pluripotency genes profile, and differentiation potential similar to naive hPSCs cultured on MEFs. RNA-Seq analysis at different time points during the transition from MEFs to serum coating will provide insights into the adaptive mechanisms involved. In sum, this work demonstrates that it is possible to culture naive hPSCs in feeder-free conditions while preserving their features, broadening the range of potential applications of these cells in developmental modelling, cell therapy, and regenerative medicine.

Funding Source: ERC Starting Grant (MetEpiStem) and Marie Curie Grant (PLURImet).

Keywords: human naive pluripotency, stem cell biology, feeder-free culture optimization

3:45 PM – 4:45 PM

107

DNMT3L SAFEGUARDS HUMAN NAÏVE PLURIPOTENCY THROUGH PRECISE REGULATION OF NAÏVE-SPECIFIC TRANSPOSABLE ELEMENTS AND DNA METHYLATION

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Naïve human pluripotent stem cells (hPSCs) serve as a critical tool for exploring early lineage specification and recapitulating pre- and post-implantation human embryo development in vitro. DNMT3L is the most highly enriched chromatin factor in naïve compared to primed hPSCs. However, the molecular function of DNMT3L in regulating naïve human pluripotency remains elusive. Here, we implemented CRISPR/Cas9 technology to generate DNMT3L-null naïve hPSCs which demonstrated abnormal morphological changes and slower self-renewal rate compared to wild-type cells Furthermore, transcriptomic analysis revealed a decrease and an increase in the expression of naïve- and primed-specific transposable elements; respectively. Chromatin enrichment for proteomics in DNMT3L-null naïve hPSCs revealed an increase in the enrichment of several zinc finger proteins associated with regulation of transposable elements. Interestingly, DNMT3L interactome analysis revealed associations with HDAC1 and TRIM28/KAP1, suggesting regulatory roles in chromatin repression and transposable element suppression. Additionally, whole genome DNA methylation analyses demonstrated reduced global DNA methylation upon DNMT3L depletion over poised and bivalent promoters. Moreover, DNMT3L depletion promoted induction of the naïve trophectoderm fate, while also impairing blastoid formation through reduced cavity size and perturbed lineage specification. In summary, our study highlights the pivotal role of DNMT3L in regulating early lineage commitment, shedding light on its multifaceted impact on transcriptional regulation, chromatin dynamics, DNA methylation, and lineage specification in naïve hPSCs.

Funding Source: This work is supported by the FWO, KU Leuven University.

Keywords: DNMT3L naive human, pluripotent stem cells, transposable elements

3:45 PM – 4:45 PM

109

HUMAN PLURIPOTENT STEM CELLS CULTURED IN THE MTESR FEEDER-FREE SYSTEM SHOW PROPERTIES MORE CLOSELY RELATED TO THE FORMATIVE RATHER THAN THE PRIMED STATE

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In the mammalian embryo, a formative pluripotent phase was proposed to exist at the early post-implantation period, during the transition from the pre-implantation naïve- to the post-implantation primed- epiblast. We previously reported that by recapitulating a laminin component of the extracellular matrix niche during embryonic formative transition and defined culture conditions, we established cultures of human pluripotent stem cells with properties of the formative epiblast (termed LN-hPSCs). LN-hPSCs exhibited a post-implantation-epiblast gene expression profile, inactive canonical Wnt signaling, lack of mesendodermal markers, and competence to initiate primordial germ cell (PGC) specification. Here we sought to characterize the pluripotency state of hPSCs cultured in the commonly used mTeSR system (mTeSR-hPSCs), in comparison with LN and primed hPSCs. Human PSCs in the three culture systems expressed POU5F1 and NANOG, and showed similar competence to initiate differentiation into the three germ layers in vitro. Similar to LN and primed hPSCs, mTeSR-hPSCs downregulated the expression of naïve pluripotency genes, and upregulated the expression of post-implantation epiblast markers. Notably, the expression of two early post-implantation epiblast markers, OTX2 and FOXD3, was downregulated in mTeSR, compared with LN-hPSCs. The FGF and TGFβ-dependent signaling pathways were required for the self-renewal of mTeSR, LN, and primed hPSCs. Wnt signaling was inactive in mTeSR and LN-hPSCs, as evidenced by the lack of expression of mesendodermal markers. In contrast, primed hPSCs exhibited heterogeneous Wnt signaling activity. Finally, comparison of the efficiency of PGC

specification showed that BMP4-induced EBs generated from LN-hPSCs exhibited significantly higher percentages of cells co-expressing AP2 gamma and SOX17 compared with EBs generated from mTeSR and primed hPSCs. Taken together, these results show that mTeSR-hPSCs exhibit properties of the formative epiblast, and are more closely related to LN-hPSCs than primed hPSCs. The higher efficiency of PGC specification of LN compared with mTeSR-hPSCs may suggest that despite the similarities between the two culture systems, LN and mTeSR-hPSCs represent a slightly different phase along the post-implantation pluripotency continuum.

Funding Source: Donation from Judy and Sidney Swartz

Keywords: pluripotent stem cells, formative pluripotency, primordial germ cell specification

3:45 PM – 4:45 PM

111

INDUCTION AND IN SILICO STAGING OF HUMAN GASTRULOIDS WITH NEURAL TUBE, SEGMENTED SOMITES & ADVANCED CELL TYPES

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Post-implantation embryogenesis is a critical period in human embryo development, albeit understudied because of limited availability of clinical samples from that early window. Embryonic organoids were recently developed to model post-implantation embryogenesis in vitro. For example, human gastruloids develop three germ layers and elongate to form anterior-posterior axis similar to post-implantation human embryos. However, conventional human gastruloids fail to robustly form a neural tube and segmented somites. We generated and analyzed single cell data on conventional human gastruloids to understand this limitation, and found that a progenitor population, neuromesodermal progenitors or NMPs, are biased towards mesodermal fates, resulting in underdevelopment of the neural lineage. We further found that a specific temporal regime of retinoic acid (RA) restored balance to NMP bipotency in human gastruloids. This regime, together with Matrigel supplementation, successfully induced human gastruloids that robustly formed both a neural tube and segmented somites, while also exhibiting the advancement of neural, mesodermal and endodermal lineages to more advanced cell types. We term this model human 'RA-gastruloids'. To contextualize human RA-gastruloids, we further developed a cross-species, somite-resolved computational staging approach to benchmark all available stembryo models against in vivo mouse, human and cynomolgus monkey development. We found that human RA-gastruloids developed to a more advanced stage than all other reported models to date, comparable to E9.5 mouse embryos and CS11 cynomolgus monkey embryos. Genetic and chemical perturbation of human RA-gastruloids further demonstrated its ability to model molecular mechanisms underlying human gastrulation. In summary, human RA-gastruloids extend the repertoire of embryonic organoid models. The robustness and low interindividual variability with which they form, together with the morphological structures and cell types represented, suggest they may be a powerful model in which to implement large-scale genetic screens aimed at better understanding the molecular and cellular biology of post-implantation human embryogenesis.

Funding Source: Paul G. Allen Frontiers Group (Allen Discovery Center for Cell Lineage Tracing), PI: Jay Shendure National Human Genome Research Institute (grant # UM1HG011586), PI: Jay Shendure Howard Hughes Medical Institute, PI: Jay Shendure

Keywords: gastruloids, post-implantation embryogenesis, single-cell sequencing

3:45 PM – 4:45 PM

113

REVEAL X-LINKED GENE DOSAGE EFFECTS ON THE SPECIFICATION OF HUMAN PRIMORDIAL GERM CELLS

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Human primordial germ cell-like cells (hPGCLCs) can be generated from pluripotent stem cells (PSCs) but the differentiation efficiency of female hPSCs is often lower than that of male hPSCs. Moreover, Klinefelter Syndrome (KS), a condition characterized by the presence of an extra X-chromosome in males often presents the failure of germline specification and infertility. In this study, we therefore investigate how X-linked gene dosage affects hPGCLC specification potential in both healthy and diseased conditions. We reveal that the X-chromosome plays a multifaceted inhibitory role in hPGCLC induction. The inhibitory effects of TGF-beta/activin A and BMP pathway by escape genes are demonstrated by the increased yield of hPGCLCs upon siRNA knockdown experiments. The elevated escapees-induced regulatory network has consequential effects on cellular metabolism, mitochondrial morphology, and ultimately progenitor competence, which subsequently inhibits hPGCLC induction. Importantly, failure to properly decrease X-linked gene expression at a time when SOX17 is induced by BMP signaling further causes cell death. These findings shed novel insights into the hPGC specification by linking the discrepant role of SOX17 between mice and humans with X-linked gene dosage effects. Moreover, our findings can be further applied to enhance the induction and survival efficiency of PGCLCs from hiPSCs for disease modeling and mechanistic studies.

Keywords: X-linked gene dosage, germ cells, mitochondrial function

3:45 PM – 4:45 PM

115

DERIVATION OF CYNOMOLGUS MONKEY INDUCED TROPHOBLAST STEM CELLS VIA NUCLEAR REPROGRAMMING

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The placenta is a unique organ developed during pregnancy that serves as a maternal-fetal interface to ensure proper growth and development of the fetus. Dysfunction of the placenta can lead to defective development of the fetus and pregnancy failure, therefore understanding placental development is crucial. Phylogenetically, non-human primates are very similar to humans, and this is especially true for placentation, making them an excellent model species for studying early primate placentation. Although studying placentation in non-human primates is possible, it is still extremely difficult due to logistical and ethical barriers. As an alternative, trophoblast stem cells (TS cells), can be derived from blastocysts or first trimester placentas, these TS cells have shown to be of great utility to model placenta biology and disease. However, they still require access to non-human blastocyst or early placenta. Here we report the generation of cynomolgus monkey induced trophoblast stem cells (iTS cells) via OCT4, SOX2, KLF4, c-MYC (OSKM)-mediated reprogramming of fibroblasts. The cynomolgus monkey iTS cells are capable of long-term proliferation and demonstrate key features of trophoblast lineage using whole genome transcriptional profiling in comparison to de novo TS cell counterparts. Functionally, the iTS cells can differentiate into the in vivo subtypes; syncytiotrophoblasts and extravillous cytotrophoblast cells. Furthermore, we have shown that cynomolgus monkey iTS cells can self-organise to form a trophoblast organoid as their human counterparts. As iTS cells are more easily accessible than TS cells derived from primary placental tissues or non-human blastocyst, iTS cells represent a valuable and tractable in vitro stem cell model that could be applied widely to assist in the understanding of primate placentation.

Funding Source: National Health and Medical Research Council (NHMRC) project grants APP1104560 and APP2004627; Synergy grant 2019251.

Keywords: induced trophoblast stem cells (iTSCs), nuclear reprogramming, cynomolgus monkey

3:45 PM – 4:45 PM

117

IN VITRO DIFFERENTIATION TOWARDS GRANULOSA LIKE CELLS: CREATING THE HUMAN OVARIAN NICHE

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All current stem cell-derived models of human embryo development inherently lack the very first steps in the process, starting from fertilization. To develop such a model, a prerequisite is to obtain also the gametes, eggs and sperm, from pluripotent stem cells (hiPSCs). For the development and maturation of oocytes, the support of a somatic niche, initially formed by granulosa cells (GCs) is essential. GCs encapsulate premature oocytes to form a follicle which enables the progression of oocytes through the first stages of meiosis in the human embryo. Our overall aim to model gametogenesis using hIPSCs therefore requires the in vitro differentiation of both oocytes and granulosa-like cells. The signals mediating differentiation towards granulosa-like cells are currently not known. To find and understand the signals that guide granulosa cell differentiation in vivo, we analyzed single cell RNA sequencing data from human embryos. Currently we are testing various identified signaling molecules, culture conditions (2D vs 3D) and assessing the methylation and transcriptomic landscapes of our obtained GC-like cells. Next, we aim to combine our GC-like cells with female hIPSC-derived primordial germ cell-like cells to model human oogenesis in a way previously unattainable.

Keywords: gametogenesis, embryo-model, somatic niche

3:45 PM – 4:45 PM

119

THE DYNAMIC CONTROL OF RETROVIRAL SUPPRESSION DURING THE TRANSITION INTO PLURIPOTENCY

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Pluripotency experiences dynamic transitions from the inner cell mass (ICM) of the blastocyst to the onset of somitogenesis, encompassing the naive and primed states in mouse embryonic stem cells (ESCs). These states manifest distinct developmental potentials, gene expressions, and chromatin structures. The transition from the naive to the primed state in ESCs triggers alterations in the distribution of H3K9me3 and turnover of H3.3. Despite H3.3's involvement in integrating into endogenous retrovirus (ERV) sequences, its precise role in retroviral silencing within ESCs remains unclear. The dynamics of chromatin, particularly H3.3, change during this transition, but the epigenetic differences that govern the naive and primed states, particularly in the silencing of retroviral elements, are not well understood.
Our study focused on evaluating H3.3's impact on retroviral sequence transcription in mouse ESCs. We compared the transcriptional statuses of retroviral sequences between the naive and primed pluripotent states using wild-type (wt) and H3.3 null ESCs. Our findings demonstrated that MLV infection disrupted the repression of retroviral sequences in naive 2i-ESCs after the depletion of H3.3, while wt ESCs effectively suppressed viral expression. In contrast, primed EpiSCs infected with MLV exhibited reduced disparities between wt and H3.3 null cells, with elevated transcription observed in both the MLV insert and several transposable elements (TEs). Furthermore, MLV silencing persisted when cells were infected in a naive state and subsequently transitioned to a primed state, possibly due to DNA methylation and the presence of H3K9me3 in primed ESCs. In conclusion, our study highlights the dynamic regulation of retroviral silencing during the transition between naive and primed pluripotent states. These findings provide insights into the role of H3.3 in retroviral regulation and underscore the complexity of epigenetic mechanisms governing pluripotency.

Keywords: retroviral silencing, chromatin dynamics, pluripotency

3:45 PM – 4:45 PM

121

MYOINOSITOL ENHANCES THE PORCINE EMBRYONIC DEVELOPMENT VIA ACTIVATING THE NRF2/HO-1 PATHWAY

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Myo-inositol (Myo-Ins), a type of inositol that possesses antioxidative properties, positively correlates with embryonic development in mammals. However, there is a lack of research on Myo-Ins supplementation in the in vitro culture medium of porcine embryos. Therefore, we investigated the impact of Myo-Ins inclusion in porcine zygotic medium (PZM3) with different concentrations (0, 5, 10, and 20 mM) for 7 days and porcine embryos were checked on day 2 for cleavage and on day 7 for blastocysts formation rates. Myo-Ins supplementation at 20 mM significantly enhanced the blastocyst formation rate compared to the control group. However, neither the cleavage rate nor the total cell number showed a significant increase compared to the control group. The Myo-Ins treatment leads to a reduction in apoptotic rate and apoptotic index in the blastocysts compared to those of the control group. Myo-Ins significantly decreased the reactive oxygen species (ROS) levels and drastically increased the glutathione levels (GSH) in 20 mM group on day 2 embryos at 4-5 cell staged as compared to the control group. Moreover, Myo-Ins significantly enhances antioxidant genes such as NRF2, HMOX1, and GCLC in the 20 mM treated blastocysts compared to the control group blastocysts. These results illustrate that the addition of Myo-Ins improves the developmental potential of porcine embryos through the activation of the Nrf2/HO-1 pathway.

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Keywords: myo-inositol, embryos, oxidative stress

3:45 PM – 4:45 PM

123

DEVELOPMENTAL REGULATION OF ERK SIGNALING BY MITOTIC KINASES

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At the blastocyst stage, the embryo invades the uterine and the pluripotent epiblast cells get reshaped from an amorphous ball into a polarized cup-shaped epithelium. At the same time, the transiently established naïve pluripotency is dismantled, transforming into a more developmentally advanced post-implantation pluripotent state. The major driver of the differentiation process is the autocrine Fgf/Mek/Erk singling which promotes the exit of naïve pluripotency. However, inhibiting Mek alone is not sufficient to maintain the cells in the naïve state suggesting the contribution of additional, as yet unidentified, pro-differentiation signals. Using a 3D embryonic stem cells (ESC) based model of epiblast development we established an automated pipeline for cell culture and analysis and performed a large-scale functional screen to identify factors regulating the development of the pluripotent lineage. We found that the mitotic kinases – Aurora kinase A (Aurka) and Polo-like kinase (PLK) positively regulate Erk signalling in a Mek-independent manner. We determined that Aurka and Erk form a complex in ESCs and suppression of Aurka activity delays the exit of naïve pluripotency. Moreover, a combination of Aurka and Mek inhibition enabled the de novo derivation of ESC lines from mouse embryos. Interestingly, during cell division, we found that phospho-Erk (pErk) localizes on the spindle poles at prophase and metaphase, gradually decreasing its signal at anaphase and telophase. Proximity biotinylation analysis of the pErk interactome identified the formation of PLK/pErk complex in metaphase cells. Moreover, inhibition of PLK, but not Mek or Aurka, resulted in a substantial decrease in Erk phosphorylation at the mitotic spindle. In summary, our findings demonstrate that the pluripotent cells continuously generate and perceive two types of Erk-mediated pro-differentiation cues - autocrine signals of the Fgf/Mek cascade and cell-intrinsic cues via the mitotic kinase Aurka and PLK. Altogether, these signals promote the exit of naïve pluripotency, driving the transient pace of embryonic development.

Keywords: naïve pluripotency, 3D in vitro model, Erk signaling

3:45 PM – 4:45 PM

125

SMALL MOLECULES MEDIATED DIRECT AND EFFICIENT INDUCTION OF MOUSE INTEGRATED EMBRYO MODEL THAT REACH MID-LATE STREAK STAGE

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The recent emergence of stem-cell-derived embryo models opens exciting opportunities to promote fundamental understanding of mammalian development and advance regenerative medicine. However, current embryo models require either transgene or sorting and mixing individual lineages. Here, we report a non-genetic, reporter-free, and non-sorting approach to generate mouse integrated embryo model by small compounds induction directly and solely from stem cells. Our chemical induced embryo model (ciEmbryoid) displays key events during gastrulation from pre-streak to early streak，mid-streak to late-streak morphologically and molecularly. Our results demonstrate that chemical small compounds trigger all three blastocyst lineages to reconstitute embryogenesis. Thus, chemical Embryoids are a powerful in vitro model for dissecting development.

Keywords: integrated embryo model, chemical/small molecules-induced embryo, mid-late streak stage

3:45 PM – 4:45 PM

127

DECIPHERING THE ROLE OF THE CORE PLURIPOTENCY TRANSCRIPTION FACTORS OCT4 AND SOX2 IN THE TISSUE-SCALE ORGANISATION OF THE EMBRYONIC LINEAGE

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Blastocyst formation is a critical step in mammalian embryogenesis. Defects in the lineage segregation and tissue scale organisation of the blastocyst are detrimental to embryonic development. The molecular control that coordinates the tissue morphogenesis and cell fate dynamics in the early embryo is still poorly understood. On a cellular level, a major driver of embryonic morphogenesis is the establishment of epithelial polarity, which takes place at specific developmental time points.

While the extraembryonic tissues establish epithelial polarity during the pre-implantation stages of development, the pluripotent cells of the epiblast undergo epithelialisation only after implantation. The developmental significance and the factors that control this sequence of events are still elusive. We aim to decipher this process, focusing on the transcriptional regulation and tissue morphogenesis of the pluripotent lineage. Analysis of genome occupancy dynamics and loss of function effects at sequential stages of the early embryogenesis revealed that the developmental timing of epiblast polarisation is controlled by the molecular cooperativity of the core pluripotency transcription factors Oct4 and Sox2. Our results indicate that the pluripotent cells are actively sustained in an apolar state, allowing proper tissue segregation of the epiblast and the primitive endoderm and hence, enabling the further development of the embryo. Thus, our study defined the physiological role of Oct4/Sox2 cooperativity in tissue morphogenesis and cell fate dynamics in the early mouse embryo.

Keywords: epithelial polarity, Oct4, Sox2

3:45 PM – 4:45 PM

129

BAP-TREATED HUMAN EMBRYONIC STEM CELLS CHIMERIZE WITH THE MOUSE BLASTOCYST AND CONTRIBUTE TO BOTH THE PLACENTA AND EMBRYO

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It has been reported that human embryonic stem cells (hESCs) treated with BMP4 and inhibitors of TGFb signaling (A83-01) and FGF signaling (PD173074), named BAP, can efficiently differentiate to trophoblasts (TBs) in vitro. However, markers for amniotic cells and mesodermal progenitors were also detected in the BAP-induced cells (BAP cells). Due to restricted access to human embryos, the developmental potential of the BAP cells in vivo remains unknown. Here, we found that, following intra-uterine transplantation of BAP cells into a surrogate mouse, the cells contributed to the placenta. Injection of BAP cells into the mouse blastocyst allowed them to penetrate the trophectoderm. After implantation of the injected blastocysts into surrogate mice, human cells were found at E14 in the placental labyrinth, junction zones, and even near the uterine decidua, expressed placental markers, and secreted human chorionic gonadotropin. Surprisingly, BAP cells also contributed to cartilages of the chimeric embryo with some expressing the chondrogenic marker SOX9. Deleting MSX2, a mesodermal determinant, restricted the contribution of BAP cells to the placenta. Thus, we conclude that BAP cells contain not only TBs as the majority but also amniotic and mesodermal cells, and can chimerize with the mouse blastocyst and contribute to both the placenta and cartilages of the chimera. Chimerization with the mouse blastocyst is a sensitive assay to report the fate of the non-pluripotent cells.

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Keywords: human embryonic stem cells, human trophoblasts, human-mouse chimera

3:45 PM – 4:45 PM

131

MITOCHONDRIAL THYMIDINE METABOLISM AND DYNAMIC CHANGES IN MTDNA COPY NUMBER DURING INDUCED PLURIPOTENCY

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Reprogramming somatic cells into human induced pluripotent stem cells (hiPSCs) entails profound intracellular changes, including modifications in mitochondrial metabolism and a decrease in mitochondrial DNA (mtDNA) copy number. However, the mechanisms underlying the decline in mtDNA copy number during reprogramming remain uncharted. In this investigation, we seek to elucidate this mechanism. Through a meta-analysis of numerous RNA sequencing datasets, we identify the genes accountable for the reduction in mtDNA. We delve into the function of these identified genes and scrutinize their regulatory mechanisms. Particularly, the thymidine kinase 2 (TK2) gene, required for mitochondrial DNA synthesis, found in the mitochondria, exhibits diminished expression in human pluripotent stem cells (hPSCs) compared to somatic cells. TK2 is substantially downregulated during reprogramming and significantly upregulated during differentiation. Collectively, the reduction in TK2 influences the decrease in mtDNA copy number and intricately participates in shaping metabolic characteristics in hPSCs. These findings enhance comprehension of the reprogramming process and hold potential for enhancing reprogramming efficiency.

Funding Source: This result was supported by National Research Foundation of Korea (Grant No. RS-2023-00220207) and the Korean Fund for Regenerative Medicine (KFRM) grant funded by the Korea government (KFRM-RS-2022-00070557).

Keywords: reprogramming, mitochondrial DNA, thymidine kinase 2 (TK2)

3:45 PM – 4:45 PM

133

DISENTANGLING THE ENTANGLEMENT: ENZYME-BASED AGGREGATE DISSOCIATION AND THEIR EFFECTS ON ECM

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Emerging induced pluripotent stem cell (iPSC) derived cell therapies for a wide range of clinical indications highlight the demand for the production of iPSCs in large quantities. The need for generating and expanding iPSCs can clearly be addressed by large scale expansion in bioreactors. However, this strategy is prone to induce cellular stress due to a process including changes in cell culture from 2D single-cells to 3D aggregates and back again. Therefore, harvest optimization is inevitable. While doing so, we would like to understand the underlying

biology regarding the enzyme-based dissociation and its aftermath on iPSC identity and performance. Here, we explored the aggregate biology before/after dissociation by investigating the Caspase-3/7 activity, Annexin V exposure, nuclear and mitochondrial ROS elements as well as couple of ECM members.

Keywords: iPSC expansion, cell stress, cell viability

3:45 PM – 4:45 PM

135

EXPLORING TRANSCRIPTIONAL VARIABILITY DURING EARLY EMBRYOGENESIS

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In the early stages of embryonic development, epiblast cells exhibit pluripotency and are capable of differentiating into all cell types. During gastrulation, these cells break symmetry and restrict their differentiation potential by self-organizing into the endoderm, mesoderm, or ectoderm germ layers. Even though embryogenesis is a robust process, the molecular regulation underlying this development can be quite variable. This heterogeneity manifests at the single-cell level through dynamic changes in gene and protein expressions, along with epigenetic modifications. Transcriptional bursting, a molecular phenomenon characterized by sporadic bursts of transcription, contributes to this diversity by introducing noise into gene expression profiles. This molecular noise might be able to prime choices of differentiation within a seemingly homogeneous cell population. To explore this, we utilize mouse gastruloids, grown from ES cells, as an in vitro model of gastrulation. Following the establishment of the most optimal culture conditions for gastruloid formation, we applied single-cell nascent RNA sequencing to identify stochastic genes associated with cell fate decisions during symmetry breaking. Additionally, initial findings from bulk RNA sequencing have underscored the importance of noise regulation during early embryogenesis.

Funding Source: NWO XL mouse gastruloid consortium

Keywords: nascent single-cell RNA-sequencing, gastruloid, embryogenesis

3:45 PM – 4:45 PM

137

MECHANO-OSMOTIC SIGNALS CONTROL CHROMATIN STATE AND EXIT FROM PLURIPOTENCY

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The cell nucleus exhibits dramatic shape changes during differentiation and lineage commitment. Embryonic stem cells exhibit nuclear flattening upon exit from pluripotency, a morphological change necessary for this transition, implicating nuclear shape as an important regulator in cell fate decisions. In the 3D human embryo microenvironment, both physical deformations as well as osmotic pressure differences extrinsically deform the cell nucleus, but how these morphological transitions are integrated through signaling networks during cell state transitions is unclear. We investigated the regulation of the cell response to mechanical and osmotic forces using human induced pluripotent stem cells (iPSCs) as a model system. We found that removing pluripotency factors triggered rapid nuclear volume loss and nuclear envelope fluctuations. Consistently, inducing nuclear flattening and volume loss by extrinsic compression was sufficient to induce a chromatin state primed for pluripotency exit. However, in contrast to biochemically-induced differentiation, this primed chromatin state was transient and accompanied by a pluripotency growth factor-driven transcriptional circuitry that restored pluripotency. In the absence of these growth factors, chromatin priming by mechano-osmotic forces was sufficient to accelerate pluripotency exit. Thus, osmotic signaling is associated with and enhances stem cell differentiation and can be mimicked by extrinsic mechanical stress or counteracted by pluripotency-promoting growth factors. These findings establish a critical regulatory mechanism that integrates nuclear mechanics and volume with biochemical signaling and chromatin state to control cell fate transitions.

Keywords: chromatin, nuclear mechanics, mechanotransduction

3:45 PM – 4:45 PM

139

THE SPECIFICATION AND LINEAGE RESTRICTION OF PRIMORDIAL GERM CELLS IS DEPENDENT ON TGF BETA/NODAL SIGNALING

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The establishment of the human germline, occurring around week 2-3 of embryonic development, involves the emergence of primordial germ cells (PGCs) induced by bone morphogenetic proteins (BMPs). However, the mechanisms governing the separation of the germline from other BMP-induced somatic lineages remain unclear. Furthermore, even after specification, PGCs may not be fully committed to the germ cell lineage, as evidenced by their ability to generate pluripotent embryonic germ cells (EGCs) in both mice and humans. This phenomenon of lineage plasticity extends to PGC-like cells (PGCLCs) derived from pluripotent stem cells (PSCs), which tend to lose germ cell identity in prolonged culture, hampering research on germ cell maturation. Recently we demonstrated that supplementing extracellular matrix (ECM) components with BMP4 treatment enables the generation of human PGC-like cells (PGCLCs) from induced pluripotent stem cells (iPSCs) with approximately 50% efficiency. Expanding on this work, we present evidence that TGFβ/Nodal signaling controls the specification of PGCLCs and subsequently maintains their germ cell fate. Modulating TGFβ/Nodal signaling either by blocking or strongly inducing it disrupts PGCLC formation, whereas establishing low levels of endogenous TGFβ/Nodal signaling enhances PGCLC induction to 75%-80%. Following specification, modulation of TGFβ/Nodal signaling combined with ECM is crucial for preserving the germ cell fate. Using this combination, we demonstrate feeder-free maintenance and proliferation of isolated PGCLCs. Throughout extended culture, PGCLCs sustain the expression of key PGC markers, including SOX17, TFAP2C, and POU5F1, while also upregulating CD38 and SUSD2, indicative of their transition to migratory-stage germ cells. Furthermore, we observe clear morphological changes during extended culture, with PGCLCs developing protruding filopodia that are characteristic of migrating cells. Our study identifies the optimal levels of TGFβ/Nodal during and after specification PGCLC, highlighting the importance of TGFβ/Nodal in maintaining the germ cell lineage. These findings pave the way for the efficient generation and culture of PGCLCs, offering a new human in vitro model for investigating the intricate processes of germ cell migration and maturation.

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Keywords: PGC, gametogenesis, primordial germ cell

3:45 PM – 4:45 PM

141

THE INTERPLAY OF GEOMETRY AND ENVIRONMENT IN REGULATING EPITHELIAL TO MESENCHYMAL TRANSITION (EMT) DYNAMICS IN HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPSC)

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The epithelial to mesenchymal transition (EMT) is a cellular state change that is difficult to compare across the diverse set of biological contexts and model systems in which it is observed, from normal embryo development to pathological cancer metastasis, studied both in-vivo and in-vitro. To understand how differences in starting state affect the EMT, we compared EMT induced across four models of hiPSC culture with different growth geometries using existing and forth-coming fluorescently labeled cell lines from the Allen Cell Collection (allencell.org). We found that initial geometry affected migration timing with hiPSCs from flat 2D colonies migrating earlier than those grown as basal-side out, 3D hollow spheres, or “lumenoids.” We also found an association between the onset of cell migration and the time that the mesoendoderm marker Eomesodermin reached peak expression. Migration onset occurred slightly after peak Eomesodermin expression in 3D models but occurred at a similar time as peak Eomesodermin expression in 2D models. In contrast, the drop in expression of the stemness marker SOX2 was similar across hiPSC models. Interestingly, vimentin and E-Cadherin expression and organization differed in the initial hiPSC states and took on distinct patterns of change during EMT depending on starting model. However, the expression and organization of these proteins became similar across the models at later timepoints when cells were migratory. Finally, we visualized the cell environment by performing all imaging experiments in the presence of a human-specific anti-collagen IV antibody to reveal the dynamics of the basement membrane (BM) in live cells. This revealed a 3D shell of collagen IV built with lumenoid formation and changes in the BM with EMT, including deformities, hole formation, and breakdown. These findings allow us to relate differences and dynamic changes in the cell environment to behavioral, molecular, and structural variations found in EMT and will provide insight into a more holistic understanding of cell state transitions.

Keywords: EMT, hiPSC, state

3:45 PM – 4:45 PM

143

STUDYING THE EVOLUTION OF GENE EXPRESSION IN EARLY PRIMATE DEVELOPMENT

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The foundation of many human phenotypes is established during early embryonic development. In order to obtain a better understanding of the underlying molecular processes, experimental systems are required that enable the measuring of gene expression dynamics during early development. Additionally, to gain more insights into evolutionary and developmental mechanisms, comparative analysis of human and non-human primates (NHPs) can provide valuable and unique functional information. However, a major challenge in such studies is obtaining homologous cells from multiple species, which can be difficult, especially during developmental stages. For this purpose, we reprogrammed either urine-derived stem cells or fibroblasts to induced pluripotent stem cells (iPSCs) using a modified transduction method of a commercially available Sendai virus vector system. With this method, we were able to generate iPSCs from Human, Gorilla, Orangutan, Baboon, Vervet, Cynomolgus and Rhesus Macaque, which can be grown feeder-free under the same culture conditions. We then differentiated iPSCs from four of those primate species to embryoid bodies (EBs), which can be considered as a model for early embryonic development as they develop cells of all three germ layers. At day 8 and 16 of EB differentiation, we performed 10X scRNA-sequencing, resulting in a dataset of in total ~85,000 cells with high cell type diversity in each species, ranging from cardiac mesoderm to neural cells. This poses the challenge to integrate data across batches and species to identify comparable cell types. To address this, we established a pipeline to process and render the data comparable. Therefore, we first pooled the cells from different species before generating the sequencing libraries and then computationally demultiplexed the species and individuals to minimize technical batch effects. Subsequently, we developed an approach to assign corresponding cell types across species based on data integration at different levels and reciprocal classification. This resulted in a total of 16 corresponding cell types, with 10 of them being present in at least three of the four species. With these, we were able to reconstruct developmental trajectories that will allow us to study gene expression dynamics during early development across primates.

Funding Source: This work was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project number: 458247426

Keywords: primate iPSCs, embryoid bodies, scRNA-sequencing

3:45 PM – 4:45 PM

145

DERIVATION OF EXTENDED POTENTIAL STEM CELLS FROM PORCINE CLONED EMBRYOS

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The pig has emerged as a valuable agricultural resource and a preclinical therapy model of humans. Despite their biomedical importance, porcine cloned embryos have yet to yield extended potential stem cells (EPSCs), which contribute to both embryonic and extra-embryonic cell lineages. Herein, we attempted to establish porcine EPSCs from nuclear transfer blastocysts (EPSCsNT) cultured in a modified EPSCs medium. A total of 5 ESC lines, exhibiting dome-shaped colonies with single-cell passaging capability, were obtained from 48 cloned blastocysts. After prolonged passaging, these cell lines still showed normal karyotype and expression of core pluripotency marker proteins under feeder conditions. Furthermore, through transcriptomic analysis, we verified that our EPSCsNT showed the properties of intermediate formative state, which is between naïve and primed, and highly expressed EPSCs signature genes, such as DNMT3A, DUSP6, and CHD7. To determine the differentiation potential, we spontaneously differentiated EPSCsNT cells. After four days of differentiation, we observe two distinct structures: embryoid bodies (EBs) and blastocyst-like structures. EBs exhibit marker proteins indicative of three germ layers and express various embryonic lineage genes. Additionally, these structures display limited expressions of CDX2 and KRT7, along with cytosolic expression of TEAD4 proteins. Conversely, blastocyst-like structures demonstrate detection of CDX2 and GATA6 proteins comparable to NT blastocysts. Essentially, the EPSCsNT we have established possess the capability to differentiate into both embryonic and extra-embryonic cell lineages. In conclusion, our study presents empirical evidence supporting the feasibility of obtaining porcine NT-derived EPSCs, which hold promise for generating blastoids, and these cell lines may find extensive applications in translational research within biotechnology and regenerative medicine.

Funding Source: This work was supported by grants from the National Research Foundation of Korea (NRF) funded by the Korean Government (No. 2021R1C1C2007132).

Keywords: porcine extended potential stem cells, blastocyst-like structures, cloned embryos

3:45 PM – 4:45 PM

147

TOWARDS GENERATING PLURIPOTENT STEM CELL-DERIVED HUMAN POST-IMPLANTATION EMBRYO MODELS

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The development of mammalian embryos accompanies sequential and dynamic morphogenetic events, starting from a totipotent zygote that is generated via sperm-egg fusion and culminating in a fully developed organism. Errors in the process of this early embryogenesis lead to pregnancy loss. Thus, unraveling the mechanism behind early human development is crucial not only for basic biology but also for clinical practice. However, although the advent of artificial reproductive technology sheds light on the study of pre-implantation human development, there remains a significant knowledge gap in our mechanistic understanding of human post-implantation development because of limited access to the embryo samples and ethical and technical challenges in recapitulating post-implantation development bia in vitro culture of donated blastocyst samples. In this study, we sought to mimic a 3D human post-implantation embryo-like structures by aggregating wildtype PSCs and two extra-embryonic cell types induced from naïve PSC via transcriptional factor overexpression. We generated inducible GATA6 (iGATA6) cells for the hypoblast lineage and iCDX2, iGATA3, and iYAP1-5SA cells for trophoblast lineage. Upon DOX induction, iGATA6 and iYAP1-5SA naïve (5iLA & PXGL) lost pluripotent markers including OCT4/SOX2 and upregulated hypoblast markers such as SOX17/GATA4/FOXA2 and trophoblast markers such as CDX2/GATA3/TFAP2A within three days, respectively. As iCDX2 and iGATA3 underwent massive cell death upon Dox administration, we used iYAP1-5SA cells for the trophoblast lineage. Upon aggregation with induced hypoblast- and trophoblast-like cells, however, wildtype naïve PSC did not form any cavity, although they undergo self-sorting to form epiblast and hypoblast compartments surrounded by trophoblast-like cells. Then, we tested several stem cell types for the epiblast compartment and found that capacitated 5iLA naïve PSCs generated a bilaminar-like structure with a cavity. Primed PSCs also underwent self-organization and bilaminar-like structure formation. By eight days after aggregation, BRA+ mesoderm-like cells were specified. However, the structures lacked several lineages and tissues, and thus, further optimization is needed to recapitulate human post-implantation embryo structures.

Funding Source: This work is supported by: the New York Stem Cell Foundation (NYSCF) and a Discovery and Innovation Grant from the American Society for Reproductive Medicine (ASRM) Research Institute.

Keywords: pluripotent stem cell, transgene-dependent induction of extra-embryonic cell types, post-implantation embryo model

3:45 PM – 4:45 PM

149

DECIPHERING THE MECHANISMS OF EMBRYO COLONIZATION BY MOUSE AND CHIMPANZEE PLURIPOTENT STEM CELLS AT SINGLE-CELL RESOLUTION

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Naïve pluripotent stem cells (PSC) possess the ability to re-enter normal development and generate chimeric fetuses in rodents. However, naïve PSCs from non-rodent species exhibit a significantly less efficient capacity to colonize embryos. Currently, our understanding of the mechanisms involved in chimera formation is limited. To investigate these mechanisms, we generated inter-species chimeras using mouse and chimpanzee PSCs. For this purpose, we injected 5-10 mouse PSCs or chimpanzee PSCs into rabbit embryos. The injection of mouse and chimpanzee PSCs led to almost 100% of chimerism in each condition. We then performed single-cell RNA sequencing on the inter-species chimeric embryos 48 hours post-injection. Concurrently, we sequenced mouse and chimpanzee PSCs before injection, as well as non-injected rabbit embryos, serving as controls. We initially focused on transcriptomic changes observed in the injected cells, revealing adaptive alterations in the transcriptome of chimpanzee and mouse PSCs. Gene ontology and KEGG pathway analysis identified the enrichment of PI3K signaling in chimpanzee PSCs upon injection into host embryos. Functional inhibition of PI3K in chimpanzee PSCs led to a drastic reduction in chimerism, confirming its role in blastocyst colonization. In parallel, we observed the enrichment of cell adhesion-related genes in both mouse and chimpanzee PSCs after injection, indicating shared processes among these species for embryo colonization. Subsequently, we examined transcriptomic changes in host embryos. Although there were minimal global transcriptomic differences between non-chimeric and chimeric host embryos, lineage identification revealed a reduced number of epiblast cells in embryos injected with donor cells, suggesting the involvement of cell competition mechanisms during the colonization process. Immunostaining on chimeric embryos confirmed the reduction in the number of host embryo epiblast cells, compensated by the injected PSCs. These findings suggest that both PSCs and host embryos undergo adaptations for successful colonization and reveal a significant role of the PI3K signaling pathway in chimeric competency. Ongoing analyses aim to identify potential regulators of these adaptive changes during chimera formation.

Keywords: naïve pluripotent stem cells, chimerism, non-human primates

3:45 PM – 4:45 PM

151

UNCOVERING EPIGENETIC AND METABOLIC REGULATORS OF LINEAGE FATE DURING HUMAN PRE-IMPLANTATION DEVELOPMENT

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Understanding the fundamental principles underlying lineage fate and patterning in the early mammalian embryo remains a key question in developmental biology. While many studies have focused on the role of transcription factors (TFs), emerging evidence underscores an interplay between metabolism and chromatin states that have the potential to alter transcriptional outcomes, adding a layer of complexity and regulation to these events. Although valuable insights have been extrapolated from rodent studies, it is pivotal to distinguish between the mechanisms that are shared between mammal species, and those unique to humans. Notwithstanding, due to a lack of methods to efficiently perturb gene expression at early stages of human development, our understanding of the mechanisms orchestrating human embryo patterning remains largely elusive. To this end, we developed CRISPRi Blastoids - a tractable in vitro model to knock-down genes of interest (GOI) in the context of a 3D model of early human development. Using a human embryonic stem cell (hESC) line with Dox-inducible expression of catalytically inactive Cas9 (dCas9), we established a tool that allows an inducible knock-down (KD) of target genes during blastoid formation. Furthermore, by adjusting the timing and duration of Dox treatment, the perturbation can be finely tuned, expanding the range of possibilities of this tool. Drawing upon transcriptomic data of human embryos and in vitro models, we have selected a set of candidate target genes to uncover epigenetic and metabolic barriers towards specification. Taken together, this model allows us to dissect the function of single genes during the first days of development, and will also enable us to interrogate how genetic, epigenetic and metabolic regulation is intertwined.

Keywords: early human development, epigenetics, lineage fate

3:45 PM – 4:45 PM

153

THE EMERGENCE SOX AND POU TRANSCRIPTION FACTORS PREDATE THE ORIGINS OF ANIMALS AND STEM CELLS

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Pluripotent stem cells are a hallmark of animal multicellularity, and, consistently, stem cell-associated Sox and POU transcription factors were considered animal innovations. However, we discovered Sox and POU members in the closest unicellular relatives of animals. We show that unicellular Sox binds DNA like mammalian Sox2 and can induce pluripotent stem cells. The capacity to form stem cells is shared by the ancestrally reconstructed sequences Ur-Sox factors. In contrast, unicellular POU bind DNA differently than animal POUs and cannot induce pluripotency. Our findings imply that the evolution of stem cells exploited a pre-existing set of transcription factors, entailing critical changes in DNA specificity of POU and the exaptation of the ancestral capacity to interact with Sox transcription factors.

Keywords: evolution of pluripotency, origin of animals, pioneer factors

3:45 PM – 4:45 PM

155

TRIM28 REGULATION OF LTR ELEMENTS SAFEGUARDS THE PGC EPIGENOME DURING SEX DETERMINATION

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Derivation of gametes from Pluripotent Stem Cells (PSCs) in vitro remains inefficient, with few in vitro derived Primordial Germ Cell-Like Cells (PGCLCs) maturing and undergoing gametogenesis. We hypothesized this may be due to inefficiencies in epigenetic reprogramming during sex determination in PGCs, thus requiring a better

understanding of the regulators of germline sex-determination to better facilitate the transition in vitro. Here, we asked how Transposable Elements (TEs) are controlled during sex determination in PGCs, as TEs of the LTR subclass have been shown to have cis-regulatory roles in developing tissues. We reasoned LTR elements may have regulatory roles in PGCs, and that these would have to be carefully balanced against their mutagenic potential. We hypothesized that LTR control in PGCs was achieved by Trim28 (Kap1), an epigenetic scaffolding protein which coordinates formation of repressive heterochromatin at targeted loci. We tested our hypothesis in vivo using a PGC-specific TRIM28 conditional knockout (TCKO) mouse model, focusing on the transition from early PGCs to sex-determined PGCs, corresponding to E10.5 through E14.5. We found that TE expression across this transition, from E11.5 to E13.5, is highly patterned and sex-specific, implying active dynamic regulation of TEs during this window. Supporting our hypothesis, we found that knockout of TRIM28 in PGCs disrupted this patterning. At the chromatin level, ATAC-seq identified sex-specific TRIM28 dependent LTRs normally silenced during sex determination. We next wanted to determine if TRIM28 safeguards the transition to sex-determined PGC. To do so, we examined Dazl expression. Dazl is thought to extinguish the early PGC program, capacitating PGCs to undergo sex determination. We found that Dazl expression was heterogenous in TCKO PGCs, but that only TCKO males failed to downregulate the early PGC program prior to sex determination. In line with this finding, male TCKO PGCs fail to differentiate into pro-spermatogonia, while female TCKO PGCs inefficiently differentiate into meiotic germ cells. Collectively, these results demonstrate that TRIM28 orchestrates sex-specific LTR regulation which safeguards the capacity to transition from early to sex-determined PGCs, thus licensing their epigenome for gametogenesis.

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Keywords: PGC, TRIM28/KAP1, germline

3:45 PM – 4:45 PM

157

ENHANCING THE POTENTIAL OF 3D GASTRULOID EMBRYO MODELS: STEERING BODY PLAN FORMATION AND MESODERM SPECIFICATION

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Gastrulation is an essential process in mammalian development in which the endodermal and mesodermal germ layers are generated in coordination with the emerging body plan. The various sub-types of mesoderm that appear during gastrulation are spatially organized along the co-developing embryonic body axes: the centrally located axial midline mesoderm is bilaterally flanked by the paraxial mesoderm (which forms the somites), the intermediate mesoderm (which generates the gonads and embryonic kidneys), the lateral plate mesoderm (which forms the heart, blood vessels, and definitive blood, including haematopoietic stem cells), and the extra-embryonic mesoderm.In recent years, we and others developed 3D gastruloids, non-integrated stem cell-based embryo models, which can be used to study gastrulation in vitro in a high-throughput manner. Previous characterizations revealed that gastruloids recapitulate key aspects of gastrula-stage embryos, including germ layer specification and axial organization. Here, through deeper analysis of published single-cell and spatial transcriptomics datasets, we show that 3D gastruloid models to date are however biased in their mesodermal output, with preferential formation of paraxial mesoderm, in detriment of intermediate, lateral plate, and extra-embryonic mesoderm. Moreover, our analysis confirms that current 3D gastruloid models are posteriorized and have limited capacity to model anterior mesodermal fates. To steer 3D gastruloids into alternative mesodermal and anterior-posterior fates, we performed a systematic screen of signaling molecules thought to participate in intermediate/lateral plate mesoderm and anterior fate specification in mouse embryos. By combining microscopy with spatial and global transcriptional profiling, we define combinatorial levels of WNT, TGF-beta, and FGF signaling that result in modified gastruloids displaying higher levels of intermediate/lateral mesoderm (e.g. Osr1 and Lhx1) and anterior-related (e.g. Gata6) gene expression than regular gastruloids. Our results 1) extend our understanding of modularity and cell fate establishment during embryonic development and 2) improve the versatility of the gastruloid model in generating the full complement of mesodermal outputs, enhancing its translational potential.

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Keywords: 3D gastruloids (non-integrated stem cell-based embryo models), mammalian body plan formation, mesoderm specification

3:45 PM – 4:45 PM

159

FOXO TRANSCRIPTION FACTORS ACTUATE THE EXIT FROM NAÏVE PLURIPOTENCY

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Naïve pluripotency is sustained by a self-reinforcing gene regulatory network (GRN) composed of core and naïve pluripotency specific transcription factors (TFs). Upon exit from the naïve state, ES cells enter differentiation by transitioning through formative, post-implantation-like pluripotency. This is a crucial cell fate decision, initiating the generation of an entire animal from a small population of pluripotent cells. The exit from naive pluripotency is instructed by a set of signalling pathways, but how exactly changes in pathway-activities are translated into decommissioning the naïve and the initiation of the formative GRN is still largely unknown. One of the key input signals into pluripotency-progression is funnelled through the kinase AKT. Accordingly, multiple components up- and downstream of AKT are top hits in genetic screens for factors required for the exit from naïve pluripotency. We show that a reduction of AKT-activity by the tumour suppressor PTEN is required for proper differentiation by controlling the activity of FoxO-family TFs.

FoxO TFs are well-known as regulators of longevity and metabolism; their involvement in cell fate transition is far less studied. We find that in naïve ESCs, and in the naïve epiblast in vivo, phosphorylated AKT acts as gatekeeper to stop FoxO TFs from entering the nucleus. Reduction of AKT-activity by PTEN at the onset of differentiation allows shuttling of FoxO TFs into the nucleus, where they enforce the transition from naïve to formative pluripotency by binding and activating enhancers for formative pluripotency specific cell-fate regulators, such as Otx2. At the same time, enhancers of key naïve specific TFs, such as Klf4, Nanog and Esrrb, are bound by FoxO TFs at the onset of differentiation. This is consistent with a dual role for FoxO TFs in decommissioning the old naïve, while establishing the new formative gene expression programme. Highlighting the pivotal role of FoxO TFs in the exit from pluripotency, they are both required and sufficient for the exit from naïve pluripotency. Our work identifies a central role for FoxO TFs in instructing early embryonic cell fate transitions and provides a mechanistic explanation for the role of AKT signalling during the exit from naïve pluripotency.

Keywords: pluripotency, formative pluripotency, FoxO transcription factors

3:45 PM – 4:45 PM

161

GENETIC RESCUE IN THE NORTHERN WHITE RHINOCEROS: COMPARISON OF INTER-INDIVIDUAL VARIABILITY IN PRIMORDIAL GERM CELL DIFFERENTIATION EFFICIENCY

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The world is currently facing the 6th mass extinction due to human activities such as poaching, habitat destruction and global warming. One species that is currently functionally extinct due to these activities is the northern white rhinoceros (NWR), with only two non-reproductive females remaining. The only way to save species this close to the brink of extinction is to incorporate advanced reproductive technologies to standard conservation practices. These methods may include such techniques as somatic cell nuclear transfer, ovum pickup with in vitro fertilization, and in vitro derived gametes with the resulting embryo transferred to a closely related surrogate. The San Diego Zoo Wildlife Alliance is the home of the Frozen Zoo® that contains over 11,000 fibroblast cell lines from 1,100 species/subspecies, including 12 genetically diverse NWR. We have generated iPSC lines from nine NWR and three southern white rhinoceros (SWR) to date for the generation of in vitro gametes. Using these lines we have explored the differentiation potential of seven NWR and three SWR lines for primordial germ cell (like-cells; PGCLC) production, the first step in gamete generation. We have compared both a human and a rhino specific differentiation protocol across all samples. We see inter-individual and intra-clonal variability in PGCLCs generation efficiency as marked by expression of the conserved key PGC markers PRDM1, TFAP2C, SOX17 and NANOS3 by quantitative RT-PCR. RNAseq analyses are presently exploring gene expression profiles and pathways generated by both protocols and potential regulatory processes with miRNAseq. Additional work is currently evaluating methods to increase the efficiency of PGCLC differentiation across all samples. In order to generate a sustainable, genetically diverse, population in the future, we will need to generate PGCLCs from all cryopreserved NWR in sufficient quantities to mature them to functional gametes. Here, we report the reproducible generation of PGCLCs across multiple individuals, clones and both sexes from NWR and SWR, providing increased material for the future genetic rescue of this species.

Keywords: primordial germ cells, genetic rescue, rhinoceros

3:45 PM – 4:45 PM

163

UNRAVELLING THE FUNCTION OF A NEW LONG NON-CODING RNA IN REGULATING PLURIPOTENT CELL STATES

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A class of RNAs, namely long non-coding RNAs (lncRNAs), are emerging as new players in gene regulation. They are 200nts or longer in length and they generally lack a coding potential. LncRNAs play various roles in regulating gene expression and are implicated in development as well as several diseases. However, detailed mechanisms of action of lncRNAs, especially in the context of pluripotency, are rarely investigated. Here, we identified a novel lncRNA, Tapir, that is highly expressed in mouse and human pluripotent stem cells and embryos. We demonstrate for the first time that Tapir lncRNA regulates the pluripotent cell state of embryonic stem cells (ESCs). ESCs are pluripotent stem cells essential for proper embryo development as they give rise to all the cell types of the body. Knock-down of Tapir in mouse ESCs leads to a decrease in pluripotency genes expression and affects splicing of several pluripotency-associated genes. Additionally, overexpression of Tapir lncRNA accelerates the reprogramming of differentiated cells towards induced pluripotent stem cells, further demonstrating its role in the regulation of the pluripotency gene network. Consistent with this result, using RNA sequencing we found that Tapir overexpression is accompanied by an early activation of immediate reprogramming responder genes, leading to a faster cell state change. We also identified a SINE retrotransposable element in Tapir sequence that may be responsible for its function, as overexpression of a SINE-depleted form of Tapir during reprogramming significantly altered the pluripotency-enhancing effect of Tapir. Finally, we found that Tapir interacts with several pluripotency-associated mRNAs, possibly through complementary SINE sequences. Although the expression levels of those interactors were not affected when knocking-down Tapir in ESCs, alternative splicing of these transcripts was modified, possibly dysregulating the pluripotency network. Finally, conservation of Tapir expression profile in embryo and ESCs, and to some extent of its sequence and exon-intron structure,

suggests that Tapir function could be conserved in human pluripotent stem cells. Overall, our study identifies a new player in the regulation of pluripotent cells states and highlights the underestimated role of lncRNAs in pluripotency.

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Keywords: pluripotency and iPSCs, embryonic development, long non-coding RNAs

3:45 PM – 4:45 PM

165

UNFOLDING THE MECHANISMS OF NLRP7 IN REGULATING HUMAN MATERNAL IMPRINTING

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Complete hydatidiform mole (CHM) is an abnormal human pregnancy in which trophoblast tissue overgrows without proper embryo in the uterus. Recurrent CHM has been found highly associated with biallelic mutations in NLRP7, a maternal effect protein gene, and maternal imprinting loss, indicating a possible role of NLRP7 in maternal imprinting. However, it remains largely unknown how NLRP7 regulates maternal imprinting. Given that maternal imprinting is thought to be established through two rounds of global DNA demethylation and methylation processes during oocyte and early embryonic development, we aim to investigate the function of NLRP7 on maternal imprinting during global demethylation and methylation processes using naïve conversion of human embryonic stem cells (hESCs) as an in vitro model which is known as a global demethylation process. Thus, primed to naïve conversion of hESCs provide us with a valuable model to study NLRP7 role on maternal imprinting during global methylation changes. Here we show that global DNA methylation is considerably reduced, and imprinting is lost upon naïve conversion of hESCs as previously reported. Furthermore, NLRP7 exhibits a very low expression in primed hESCs but dramatically upregulated after global demethylation and naïve conversion. Since NLRP7 protein is highly abundant in zygotes and preimplantation embryos, it was anticipated that low expression of NLRP7 may accounted for the loss of maternal imprinting during naïve conversion. To test this hypothesis, NLRP7 was ectopically expressed at high levels in primed hESCs (NLRP7-OE) and then convert these hESCs to naïve state. Interestingly, maternal imprinting was partially retained in the NLRP7-OE hESCs after naïve conversion while paternally imprinted gene was still lost. The experiments have been done repeatedly in more than one hESC lines with similar outcomes. Moreover, we have found that although NLRP7 is thought to be subcortical protein and the localisation of NLRP7 is predominantly in the cytoplasm, a small amount of NLRP7 can be detected near nuclear DNA, particularly at metaphase of the cell cycle when nuclear envelop disappears when NLRP7 is co-localized with spindle fibre. These results suggest that NLRP7 has a function to specifically protect maternal imprinting from global demethylation.

Keywords: naive conversion of hESCs, NLRP7, human maternal imprinting

3:45 PM – 4:45 PM

167

THE EFFECT OF THE MOUSE GASTRULOID CO-CULTURE WITH YOLK SAC-LIKE ORGANOIDS

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Mouse 3D gastruloids represent stem cell-derived, self-organized structures that feature aspects of gastrulation and early organogenesis. Despite the abilities of gastruloids to reconstruct aspects of early embryogenesis such as axis establishment and the initiation of gut, neural tube, and heart formation, they experience developmental limitations associated with the absence of extra-embryonic tissues. In particular, cardiovascular system formation in embryonic development is closely associated with the yolk sac. We therefore adopted an approach based on co-culturing gastruloids with murine yolk sac-like organoid (mYSLO). The mouse gastruloids were obtained according to standard protocol and cultivated in U-shaped 96-well plates until 168 hours. The mouse yolk sac-like organoids were added to the gastruloids at 46 hours post-aggregation, 2 hours before Chir 99021 pulse. At 168 h post-aggregation co-cultured and control gastruloids were examined for the heart beating activity, and distribution of Flk-1, CD31 and cTnT expression. The mYSLO were developed by 3D co-aggregation of embryoid bodies with mouse mesenchymal stem cells (OP9) and showed AFP, Sox17, and E-cadherine - positive cells along with CD31 and Flk1-positive cells. The mYSLO also expressed molecules associated with the induction of the embryonic heart and vascular formation (Ihh, Dkk1, Lefty1). The co-culture of mouse gastruloids with mYSLO led to a significant increase of beating heart-like structures in 168h gastruloids without the addition of cardiogenic factors. Using a Flk1-GFP reporter cell line we then tracked the formation of the vasculature and were able to distinguish the gastruloid-derived vessels from the ones originating from the mYSLO. The mYSLO-gastruloid co-culture is a promising strategy for studying embryonic-extraembryonic interactions during gastrulation/early organogenesis as well as a biologically relevant protocol modification for obtaining cardiogenic gastruloids.

Funding Source: SUMO (“Supervised Morphogenesis”), an international research consortium funded by the European Innovation Council Pathfinder Challenges program (grant agreement #101071203 duration 2023-2027); Scientia fellowship

Keywords: mouse gastruloids, mouse embryonic stem cells, development

3:45 PM – 4:45 PM

169

THE CHARACTERIZATION AND APPLICATION OF MONKEY NAÏVE EMBRYONIC STEM CELLS IN GENERATING MONKEY CHIMERA AND MONKEY EMBRYO MODEL

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A formal demonstration that mammalian pluripotent stem cells (PSCs) possess naive pluripotency is the generation of chimeric animals through early embryo complementation with homologous PSCs. Whereas such naive pluripotency has been well demonstrated in rodents, poor chimerism has been achieved in other species including non-human primates. Here, we have systematically tested various culture conditions for establishing monkey naive embryonic stem cells (ESCs) and optimized the procedures for chimeric embryo culture. This approach generated an aborted fetus and a live chimeric monkey with high donor cell contribution. A stringent characterization pipeline demonstrated that donor cells efficiently (up to 90%) incorporated into various tissues (including the gonads and placenta) of the chimeric monkeys. Besides, using monkey naïve ESCs, we also constructed cynomolgus monkey blastoids resembling blastocysts in morphology and transcriptomics. These blastoids can be in-vitro cultured to gastrulation stage with similar morphology and lineages composition to natural gastrulation-stage embryos. Transferring the monkey blastoids to surrogates achieves pregnancies, as indicated by progesterone levels and presence of early gestation sacs. Collectively, our chimeric monkey study has major implications for the study of primate naive pluripotency and genetic engineering of non-human primates, and the monkey embryo model study provides a useful system to dissect primate embryonic development without the same ethical concerns and access challenges in human embryo study.

Keywords: naïve pluripotency, chimeric monkey, monkey embryo model

3:45 PM – 4:45 PM

171

THE REVERSION OF MOUSE ESCS INTO NAÏVE GROUND STATE DOES NOT FOLLOW A LINEAR PATH

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Pluripotent stem cells were established from pre- and post-implantation embryos (ESC and EpiSC respectively). While all can differentiate in vitro into cells representative to the three germ layers, only ESCs can participate to chimeras. Moreover, mouse ESCs initially cultured in media containing serum and leukemia inhibitory factor (S/L) were found to be heterogeneous and exhibit fluctuating pluripotency states. However, when cultured in serum-free medium in the presence of two inhibitors (2iL) targeting MEK1/2 and GSK3α/β pathways, cells reach a homogeneous state called naïve ground state. Endogenous retroviruses (ERVs) are the hallmark of ancient retroviral integrations into genomes. Some ERVs are reactivated in early embryo development and their expression profile is specific to each ERV. The objective of this work is to decipher the kinetic of the reversion process from fluctuating pluripotency to homogeneous naïve ground states by exploiting ERV reactivation properties to label cells according to their pluripotency status and select cells with the highest chimeric potential. Fluorescent reporter genes whose expression is controlled by various ERV promoters were introduced into mESCs. The reversion of mESCs into the naïve ground state by transitioning from a S/L to a 2iL culture condition is followed by reporter gene expression analyses by flow cytometry. Each reporters have highly specific expression profiles depending on cell culture conditions and ERV promoters. We demonstrated by RNAseq and ERV reporter gene expressions that the reversion of mESCs into the naïve ground state by transitioning from S/L to 2iL condition takes several cell divisions and goes through several transient states including a 2C-like stage. Chimeric potential was assessed by microinjecting cells into embryo at the morulae stage and analyzing their colonization at blastocyst stage. Conclusion: These ERVs derived reporters hold considerable potential for identifying and subsequently isolating cells based on their different pluripotency status, enabling us to decipher the different phases of cell reversion into naïve ground state. Altogether, those reporters could efficiently help isolate PSCs from the embryos with the desired pluripotency state.

Keywords: pluripotent stem cells, endogenous retroviral element, ERV

3:45 PM – 4:45 PM

173

INVESTIGATING THE IMPACT OF MITOCHONDRIAL DNA VARIABILITY ON HUMAN PLURIPOTENT STEM CELL METABOLIC STATE AND FITNESS

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Mitochondria provide most of the energy to the cell and have their own genome, termed mitochondrial DNA (mtDNA). Each cell possesses multiple mtDNA copies and mutations affecting only some of these copies lead to heteroplasmy. Multiple selection mechanisms have been proposed to act on heteroplasmy levels, but their molecular mechanism of action remains poorly understood. To approach this problem in human embryonic stem cells (hESCs), we utilized a novel mtDNA editing tool that relies on a DddA-derived cytosine base editor. This allowed us to introduce mutations in mt-ND4, a Complex I component of the electron transport chain. Interestingly, we observed that although mutant cells proliferated at a similar rate to control cells, they displayed a deficiency in Complex I assembly and function but normal Complex IV activity. This was accompanied by mutant load-dependent decrease in oxygen consumption rate. Future work will investigate what compensatory mechanisms sustain proliferation in these cells and how this mutation affects the competitive ability of hESCs. In conclusion, by establishing a hESC-based mtDNA mutation cell model, we aim to provide a comprehensive understanding of the effects that mtDNA mutations have on metabolic performance and the mechanisms that select against these mutations during human development.

Funding Source: Medical Research Council

Keywords: cell competition, mitochondrial DNA editing, human embryonic stem cell

3:45 PM – 4:45 PM

175

IMPROVING DNA DAMAGE REPAIR WITH FARREROL ENHANCE THE DEVELOPMENT POTENTIAL OF SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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Reprogramming of somatic cell nuclear transfer (SCNT) requires a rapid transition of the donor nuclei genome from a somatic to an early embryonic state within 1-2 cell cycles in order to achieve the proper chromatin status to process further development. Consequently, the nuclear genome of the donor cell is subjected to immense pressure and becomes highly susceptible to DNA damage during SCNT reprogramming. Numerous studies have indicated that this transition is accompanied by significant replication-dependent DNA damage and genomic instability. In addition to combined epigenetic interventions, enhancing genome stability could be another potential approach for improving SCNT efficiency. Our previous study demonstrated that farrerol promotes DNA repair through homologous recombination (HR), thereby enhancing genome-editing efficiency. Here, using LiP-small molecule mapping (LiP-SMap) and surface plasmon resonance assay, we identified UCHL3 as the direct target protein of farrerol, with data revealing a strong affinity between farrerol and UCHL3 (Kd = 36.73 nM). Furthermore, we applied farrerol in SCNT-mediated cellular reprogramming and demonstrated that efficient HR repair plays a critical role in this process. We showed that farrerol directly binds to UCHL3 and activates its enzymatic activity, thereby promoting HR and improving SCNT efficiency during pre-implantation and post-implantation development.

Keywords: somatic cell nuclear transfer, farrerol, UCHL3

3:45 PM – 4:45 PM

177

IDENTIFYING NOVEL ESSENTIAL LONG NON-CODING RNA GENES IN HUMAN EMBRYONIC STEM CELLS THROUGH GENOME-WIDE SCREENING

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The human genome transcribes numerous long non-coding RNAs (lncRNAs), comprising sequences of over 200 nucleotides that lack the potential for translation into functional proteins. In recent years, lncRNAs have gained prominence as important regulators in diverse cellular processes under both normal and disease states. In light of their tissue and cell-type specific expression, they have been suggested to be exceptionally vital for human pluripotent stem cells, where lncRNAs involve in crucial aspects such as pluripotency maintenance, self-renewal and cell fate determination. However, the functionality and biological relevance of the majority of the lncRNAs remained elusive. In this work, we leveraged CRISPR interference technology in haploid human embryonic stem cells (hESCs) to generate robust genome-wide screening platform for lncRNAs. We have identified over 100 lncRNAs as essential in hESCs and about 150 to be growth-restricting. 57% of the essential lncRNAs are shown to reside in the nucleus. Our investigation also revealed two novel nuclear lncRNAs, exhibiting stem cell specificity and essentiality in hESCs. Their genomic composition is in the opposite strand of transcription factor genes central to the pluripotency network, implying on their potential mechanism of action as these

lncRNAs knock-down modified their coding counterpart's expression. Nonetheless, weighted gene co-expression analysis highlighted distinct functional roles for each of the lncRNAs, with one predominantly associated with proliferation whereas the other mainly with endoderm differentiation and WNT signaling pathway. Overall, these novel hESCs essential lncRNA genes should expand our understanding of the pluripotency network and provide new insights into the regulation of hESCs growth and cell state.

Keywords: human embryonic stem cells, pluripotency, long non-coding RNA

3:45 PM – 4:45 PM

179

HOW DO IMPRINTS COPE DURING REPROGRAMMING OF FEMALE AND MALE HUMAN IPSCS

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Reprogramming of somatic cells into induced Pluripotent Stem Cells (iPSCs) represents a significant advancement toward personalized approaches in disease modelling and cell-replacement therapies. However, a critical challenge lies in our inability to fully control the epigenetic fidelity within these cells. X-chromosome inactivation (XCI) and genomic imprinting are two key epigenetic phenomena, that are often disrupted in iPSCs by yet unclear reasons. Previously, we have shown that imprinting defects are remarkably common in mouse iPSCs, but their nature depends on the sex of donor cells and their response to culture conditions. These sex differences were explained by the phenomenon of inactive X chromosome reactivation (XCR) that occurs during female reprogramming. We are now focused on understanding whether dysregulation of imprinting in female and male human iPSCs follows the same trends or is explained by different parameters. Notably, mouse and human iPSCs do not share the same medium requirements for stem cell maintenance. Moreover, unlike mouse iPSCs, female human iPSCs do not undergo XCR. Nevertheless, they are renowned for the instability, specifically, the erosion of their inactive X. I will show our most recent data on the stability of imprinting and of XCI state of human iPSCs derived from peripheral blood mononuclear cells (PBMCs) using a non-integrative reprogramming approach under six different culture conditions. Our results show a signature of imprinting defects in human iPSCs and suggests that XCI erosion occurs earlier than anticipated. We will show whether different culture conditions can prevent imprinting defects and determine if the sex and XCI status of the donor cell affect imprinting fidelity. Unveiling the impact of culture conditions on XCI and imprinting stability will shed light on the molecular mechanism behind dysregulation of these two critical epigenetic events. Our findings are pivotal for refining reprogramming strategies and generate iPSCs with a stable epigenome, essential for preclinical and clinical applications.

Funding Source: This work is financed by national funds from Fundação para a Ciência e a Tecnologia, I.P (FCT) under a PhD fellowship - SFRH/BD/151251/2021 - and in the scope of the project 2022.01532.PTDC.

Keywords: stem cell epigenetics, reprogramming into iPSCs, culture conditions

3:45 PM – 4:45 PM

181

HUMAN POST-IMPLANTATION EMBRYO MODELS SOLELY GENERATED FROM MODULATING SIGNALING PATHWAYS IN PLURIPOTENT STEM CELLS

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Human post-implantation embryo development includes a series of lineage specifications and morphogenesis, but our knowledge of this period is impeded by the inaccessibility to the in vivo materials owing to technical barriers and ethical concerns. Recently, stem cell derived embryo like models are shedding light onto the field with emphasis on the reconstruction of both the lineages and the morphology. However, to achieve this most of the current models rely on mixing cells from different cultures, and thus lineage balance and synchronization can be an issue. Here, we report on the generation of integrated embryo like models from a single culture dish within 14 days. By modulating signaling pathways human pluripotent stem cells simultaneously give rise to all blastocyst lineages, and they can self-assemble into an embryo like structure containing both the embryonic and extraembryonic tissues in vitro. The obtained structure shows an organized morphogenesis including trophoblast like out layer, yolk sac cavity and extraembryonic mesoderm like compartment, apparent primitive streak like cell alignment, amniotic cavity, epiblast disk and bilaminar disk like compartment with high efficiency, which is reminiscent of a pre-gastrulating embryo. Transcriptomic analysis revealed the presence of primordial germ cells and confirms the embryo like structures resemble the natural Carnegie Stage 6-7 human embryos. We also identified clear anterior-posterior patterning, evidenced by the gradient expression of T, CER1, GATA4/6, COL6A1, MESP2 and CDH1/2. Our models provide an efficient flatform to generate integrated embryo like structures from a single cell source to recapitulate human post-implantation development.

Keywords: stem cell-based embryo model, STAT3 signaling, post implantation development

3:45 PM – 4:45 PM

183

METABOLIC CONTROL OF GERM LAYER PROPORTIONS THROUGH REGULATION OF NODAL AND WNT SIGNALLING

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Metabolic pathways can influence cell fate decisions, but their regulative role during embryonic development remains poorly understood. Here, we demonstrate an instructive role of glycolytic activity in regulating signalling pathways involved in mesoderm and endoderm specification. Using an mESC-based in vitro model for gastrulation, we found that glycolysis inhibition increases ectodermal cell fates at the expense of mesodermal and endodermal lineages. We demonstrate that this relationship is dose-dependent, enabling metabolic control of germ layer proportions through exogenous glucose levels. We further show that glycolysis acts as an upstream regulator of Nodal and Wnt signalling and that its influence on cell fate specification can be decoupled from its effects on growth. Finally, we confirm the universality of our findings using a human gastrulation model. Our work underscores the dependence of specific signalling pathways on metabolic conditions and provides mechanistic insight into the nutritional regulation of cell fate decision making.

Keywords: germ layer specification, glycolytic activity, nodal and Wnt signalling

3:45 PM – 4:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

185

INVESTIGATING THE DEVELOPMENTAL TRAJECTORY OF IPSC-DERIVED MACROPHAGES AND GAINING INSIGHTS INTO THEIR ONTOGENY

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Macrophages play a crucial role in the innate immune system, and their malfunction is associated with various human diseases, driving the exploration of macrophage-based cell therapies. We focus on advancing macrophage-based therapies by establishing a protocol for in vitro generation of macrophages from induced pluripotent stem cells (iPSCs). We aim to characterize the developmental trajectory leading to iPSC-derived macrophages (iMacs), utilizing single-cell RNA sequencing to unravel the myeloid lineage's ontogeny. Our findings reveal the emergence of progenitor cells with myeloid potential (MYB, CD34) from hemogenic endothelial cells (expressing CDH5, PECAM1, RUNX1) during differentiation and identify potential surface markers for distinguishing subpopulations for in-depth functional characterization. Flow cytometry confirms the emergence of CD34+ cells by day 4 of mesoderm priming, followed by distinct populations at day 7, including CD34+ LAIR1- (~7.7%) and CD34low LAIR1+ (~5.3%) cells. By day 10, mature restricted progenitors express markers such as CD36, FCGR1A, and SIGLEC1, among others. Follow-up functional studies are currently underway to further elucidate the roles and capabilities of these identified subpopulations. These insights not only contribute to understanding iPSC-derived macrophage development but also hold implications for enhancing therapeutic strategies. Furthermore, our ongoing studies involve assessing the plasticity and adaptability of iMacs in murine models, providing important insights for their application in disease treatment. This work addresses critical gaps in knowledge and pushes the field of macrophage-based cell therapies forward, offering promising avenues for translational research and therapeutic interventions in various diseases.

Keywords: iPSC-derived macrophages (iMacs), human induced pluripotent stem cells, development

3:45 PM – 4:45 PM

187

A DEVELOPMENTAL APPROACH TO MODEL HUMAN EMBRYONIC HEMATOPOIESIS IN VITRO BY INDUCING ELONGATION

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Human pluripotent stem cell (hPSC) differentiation is a promising model of human embryonic development. However, when it comes to embryonic hematopoiesis, it is difficult to characterize, as it occurs in waves at different anatomical locations and timepoints during development. Using hPSCs, we combine models for embryo elongation with hematopoietic cell induction to model the developmental context in which the different hematopoietic waves occur by modulating WNT signaling. Cells are aggregated in suspension to form embryoid bodies (EBs) and treated with a pulse of WNT inducer CHIR99021 at different concentrations. High-throughput imaging shows that variations in WNT signaling at aggregation influence the morphology and initial elongation of the EBs. Morphologically, the EBs show increased elongation, confirmed molecularly by elevated expression of caudal genes T, HOXA9, CDX1, and CDX4, over time and in response to CHIR. By day 3, the EBs differentiate into nascent and caudal mesoderm, hence an embryonic context for both primitive and definitive hematopoiesis is obtained. As the EBs differentiate the hemogenic endothelium population (CD43-, CD45-, VEcad+, CD34+, CD90+, CD73-) appears at day 8 and increases by day 13. There is also evidence of endothelial to hematopoietic transition between these timepoints as hematopoietic stem cell-like (HSC-like, CD43/CD45+, CD34+, CD90+) cells are identified by day 13. We observe an increase in the frequency of HSC-like cells and multilineage colony-forming potential at higher CHIR concentrations. Conversely, lower CHIR conditions gave rise to less HSC-like cells and more GYPA+ erythroid cells, indicating a primitive hematopoietic wave. Our results suggest a link between the initial elongation of the EBs and the hematopoietic cell populations obtained, making it a promising model to mimic the different waves of hematopoiesis taking place in both the yolk sac and the embryo proper.

Funding Source: This work was supported by the Swedish Childhood Cancer Foundation, AG Fonden, Hasselblad Foundation, the Swedish Research Council and Sahlgrenska Academy.

Keywords: developmental hematopoiesis, elongation, human pluripotent stem cells

3:45 PM – 4:45 PM

189

HUMAN ENGINEERED UNIVERSAL IPSC-DERIVED ENDOTHELIAL CELLS CAN HAVE IMMUNE COMPROMISED ABILITIES IN IN VITRO AND PAD ANIMAL MODEL

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Despite the increase in the number of peripheral artery disease (PAD) patients, there has been limited progress in medical treatments. Human induced pluripotent stem cells (hiPSCs) are considered a valuable resource for therapeutic strategies. However allogeneic transplantation of hiPSCs derivatives has the potential to elicit the patient's immune response and lead to graft rejection. To address these challenges, we developed universal hiPSCs that do not trigger immune rejection in any patient. We diminished the immunogenicity of hiPSCs through the inactivation of major histocompatibility complex (MHC) class I and II genes to evade T cells. As MHC class knockout renders cells susceptible to NK cell attack, we over-expressed the 'don't eat me signal' CD24. We first validated this strategy by isolating CD4+ and CD8+ T cells from human umbilical cord blood (UCB) and activating the T cells with the hiPSC-derived endothelial cells in vitro. Our results showed that T cell activation was markedly decreased in the universal hiPSC-derived endothelial cells (U-ECs) compared to the wild type hiPSC-derived endothelial cells (WT-ECs). Additionally, to verify the abilities to evade NK cell attack, we isolated NK cells from human UCB and observed their interaction with hiPSC-derived ECs. The degranulation and LDH release of NK cells in U-ECs were significantly decreased compared to WT-ECs, indicating that U-ECs effectively suppressed the NK cells. Based on these in vitro data, we transplanted hiPSC-derived ECs into humanized mouse models generated by injecting CD34+ hematopoietic stem cells from human UCB into NSG mice. Using IVIS imaging, we observed that U-ECs survived for longer periods after transplantation compared to the WT-ECs, while evading immune rejections. These findings suggest that human engineered U-ECs can be utilized as an "off-the-shelf" cell therapy in PAD patients.

Funding Source: This study was partially supported by the Research Institute for Veterinary Science, Seoul National University, and Korean Fund for Regenerative Medicine (KFRM) grant (22A0101L1-11).

Keywords: human induced pluripotent stem cell-derived endothelial cells, hypoimmunogenic, humanized mouse

3:45 PM – 4:45 PM

191

DISCOVERY AND CHARACTERIZATION OF MYELOID-DERIVED GROWTH FACTOR NRG1-VII USING IPSC-DERIVED MACROPHAGES

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Macrophages are specialized phagocytes found in most human tissues, where they regulate homeostasis, innate immune response and can direct tissue repair. Recently, myeloid-derived growth factor NRG1 has been reported to support epithelial stem cell proliferation and differentiation in gut organoids. As NRG1 is a highly processed locus with six known classes of isoform regulated in a tissue-specific manner, we sought to understand which class was expressed by myeloid cells. Here, we demonstrate that macrophages produce NRG1-VII, a previously uncharacterized class of NRG1 and further characterized the regulation of NRG1 isoforms in iPSC-derived monocyte and macrophages. NRG1–VII is expressed from a novel transcriptional start site (TSS) that seems to be exclusive to hematopoietic cells. Using nanopore long read sequencing, we have identified at least 5 potential protein coding transcripts arising from this TSS, caused by differential use of transcriptional ends or alternative splicing. Blood isolated monocytes express isoforms with a cryptic internal exon (poison exon), and so is a candidate for nonsense mediated decay. We hypothesize that NRG1 is just one example of the uncharacterized molecular diversity that explains the functional complexity of myeloid cells and their adaptability to their environment. Examples of myeloid-isoforms of VEGFA, WNT5A, OSM and HB-EGF, among others, are regulated by different pathogenic and environmental cues in iPSC- derived macrophages. Our future work would try to describe more of these exclusive myeloid isoforms, their regulation at different levels and the functional implications of the end products.

Funding Source: GCI Travel support scheme

Keywords: macrophages, growth factors, NRG1

3:45 PM – 4:45 PM

193

TRANSCRIPTION FACTOR BLUEPRINTS UNDERLYING DENDRITIC CELL DIVERSITY

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Dendritic cells (DCs) are a heterogeneous family that orchestrates immune responses. Conventional type 1 DCs (cDC1) are defined as cross-presenting cells, cDC type 2 (cDC2) cells govern type 1, 2 and 3 immunity, and plasmacytoid DCs (pDCs) are critical for antiviral responses. Understanding how DC diversity is generated is crucial to predict and promote immune responses, but the transcription factors (TFs) driving DC subset identity and functional divergence remain unclear. Previously, we identified PU.1, IRF8 and BATF3 as sufficient to induce cDC1s from fibroblasts, opening opportunities to understand DC diversity with reprogramming. Here, we uncovered cDC2 and pDC fate-inducing TF codes using combinatorial overexpression in Clec9a reporter mouse fibroblasts, which specifically marks the DC lineage. We anchored our screen in PU.1 for cDC2 and IRF8 for pDC induction, because of their roles and expression patterns. Regarding cDC2, overexpression of PU.1, IRF4 and PRDM1 (PIP) activated the Clec9a reporter and cDC2 lineage surface marker Sirpα. For pDC induction, SPIB, IRF8 and IKZF2 (SII) activated the Clec9a as well as the pDC-specific hCD2 reporter and expression of the pDC marker B220. Single-cell transcriptomics validated the acquisition of lineage-specific programs and revealed that combined action of the three TFs is crucial to defining subset identity. PIP-induced cells resembled pro-inflammatory cDC2Bs harboring an interferon gene signature and cross-presented antigens to naive CD8+ T-cells while SII-induced cells exhibited an immature pDC identity. When transferred into melanoma models in vivo, both PIP- and SII-induced cells increased animal survival and promoted anti-tumor immunity. While tumors treated with induced cDC2s showed infiltration of B-cells, neutrophils, and CD4+ T cells, induced pDCs promoted myeloid-cell infiltration. Mechanistically, cooperative engagement of the three TFs at the onset of reprogramming mediates distinct DC sub-programs. While ETS family TFs show pioneer activity across DC reprogramming, the engaged targets and the dominant TF are dependent on the subset. Collectively, our findings contribute to define the drivers of DC specification, heterogeneity, and function, leading to novel approaches to generate patient-tailored DC subsets for immunotherapy.

Funding Source: This project was co-funded by Cancerfonden, the Swedish Research Council, the European Research Council, and FCT. LH-O is supported by an FCT doctoral fellowship (2020.06447.BD).

Keywords: dendritic cells, cell reprogramming, transcription factors

3:45 PM – 4:45 PM

195

ORIGINS OF HEMATOPOIETIC STEM CELL MEMORIES

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The hematopoietic system generates billions of new blood and immune cells daily, a process which is regulated by hematopoietic stem and progenitor cells (HSPCs). At the single cell level, hematopoietic stem cells (HSCs) are functionally heterogeneous in terms of the rate at which they differentiate and the different type and numbers of blood cells they generate, creating lineage biases. These HSC biases are imprinted and propagated within each HSC clone and are maintained even after multiple transplantations. Due to the strong epigenetic component underlying these behaviors, we define them as clonal memories. Since HSC memories may have relevant biological implications, is crucial to understand the principles by which these memories are established and when they are generated during development. From their emergence until adulthood, HSCs experience a dramatic shift in their environmental context, which could be a key factor in the imprinting of their clonal memories. Here, using single-cell lineage tracing, we mapped the function and state of thousands of individual mouse HSCs spanning from the earliest mature fetal stages until adulthood. We determined that adult myeloid-biased HSCs are intrinsically programmed around birth, before cells migrate into the adult bone marrow. In turn, fetal HSCs, prior to migration, maintained their non-myeloid biased fetal-like program even 4 months after transplantation into adult bone marrow. Finally, our single-cell data revealed an unexpected transient perinatal HSC state, which interestingly coincides with the developmental window in which HSC migrate to the bone marrow. By tracing the lineage of this state, we found an epigenetic repression of lymphoid-differentiation programs in a subset of clones during development. In sum, we characterized the developmental origin of myeloid-biased HSCs and a new developmental HSC-state associated with lymphoid restriction, which may have important connotations in tissue homeostasis and disease.

Keywords: hematopoietic stem cells, fate biases, developmental hematopoiesis

3:45 PM – 4:45 PM

197

EARLY DEVELOPMENT OF GRANULOCYTES IN THE HUMAN FETUS

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Until recently, in healthy pregnancies the fetus was assumed to be protected from bacteria and other microorganisms. However, recent work has suggested the exposure of the fetus to bacteria in normal pregnancy, and infections that involve the extraembryonic tissues and fetus can occur with potential dire consequences for the developing fetus. Granulocytic blood cell lineages play an important role in combating infection, but when and in which tissues these cells arise has not been clearly elucidated. We investigated human prenatal development of granulocytes with a particular focus on the role of the liver and bone marrow during midgestation in the production of neutrophils, eosinophils, mast cells, and basophils. Tissues were obtained anonymously, with written consent, from women undergoing elective abortion. A spectrum of gestational ages were analyzed using multi-parameter flow cytometric analyses. Although the liver is primarily an erythropoietic organ during midgestation, myeloid and lymphoid progenitors are known to be present. We observed the presence of CD15+CD66b+ neutrophils with a high side-light scatter profile in the liver, bone marrow, and spleen as early as 16 weeks' gestation, demonstrating that the liver can produce neutrophils. Mast cells expressing high levels of CD117 (CD117++) and low levels of (CD203c+) were observed among leukocytes expressing intermediate levels of CD45, in both the liver and the spleen. Basophils expressing CD123 and CD203c, but lacking CD117 expression, were observed in the liver. The basophil and mast cell populations were found among cells with a low side-light scatter. Examination of other tissues found evidence of mast cells in the bone marrow, heart, lung, spleen, and skin. Basophils were also apparent in these tissues except for unclear data obtained from skin samples. Eosinophils development was also observed in midgestation bone morrow using IL-5Rα, CD193, and Siglec-8 as specific markers. Examination of extraembryonic tissues for the presence of granulocytes is ongoing. We show that granulocytic cells are produced in both the fetal liver and bone marrow. Although granulocyte numbers are low in the fetal circulation, these cells may help protect from infection and the hematopoietic tissues have the potential to increase production in the case of infection.

Funding Source: Supported by the National Institute Of Allergy And Infectious Diseases of the NIH under Award Number R01AI168322 and the California Institute for Regenerative Medicine Bridges to Stem Cell Training grant (TB1-01188).

Keywords: granulocytes, mast cells, fetal development

3:45 PM – 4:45 PM

199

DECIPHERING THE ROLE OF EOMES IN YOLK-SAC HAEMATOPOIESIS USING SINGLE-CELL MULTI-OMICS

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Advances in our understanding of the gene regulatory networks involved in hematopoietic commitment during embryogenesis have spurred on the invention of novel methods to induce hematopoietic progenitors from pluripotent stem cells. Recently, we identified Eomesodermin (Eomes), a T-box transcription factor, as a key regulator of murine developmental haematopoiesis. Our work suggested that Eomes regulates the hemogenic competence of yolk-sac mesoderm by opening a suite of hematopoietic enhancers at early stages of gastrulation. To shed light on the Eomes-dependent regulatory mechanisms involved in this process, we performed single-cell RNA-seq of chimeric Eomes mutant embryos (E8.5). Eomes-null embryonic stem cells (ES) contributed to the allantois endothelial lineage but failed to generate yolk-sac blood and endothelial cells. Additionally, we generated an Eomes-degron mouse ES line, which allowed us to deplete Eomes protein rapidly during hematoendothelial differentiation in vitro. Using this system, we generated a comprehensive single-cell multiomic dataset (RNA + ATAC) during the time-window when Eomes is required for hematopoietic progenitor development. Preliminary analyses of these data also suggest endothelial cells predominantly arise via allantois-like precursors in Eomes-depleted cultures. Altogether, this study provides a detailed time-resolved role for Eomes during developmental haematopoiesis revealing that Eomes is a key regulator of extra-embryonic yolk-sac mesoderm formation.

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Keywords: hemogenic endothelium, development, multiomics

3:45 PM – 4:45 PM

201

IMMUNE CONTROL OF ANIMAL GROWTH IN CONTEXT OF DIETARY STRESS AND HOMEOSTASIS

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The precise developmental control ensures functional and proportionate adult body and organ size. Recent study from our lab highlights involvement of immune cells to control animal size. However, mechanism remains elusive. Through an in-vivo RNAi screen of 700 genes in Drosophila using high sugar diet (HSD) as sensitised model, we identified 30 genes majorly linked to metabolism affects animal size. In homeostasis, changing immune cell metabolic state from low glycolytic to high glycolysis via hypoxia inducible factor 1alpha (Hif1a) and lactate dehydrogenase (Ldh) gain-of-function decreases wing imaginal disc size. Mechanistically, immune cell lactate production seems to control size of imaginal tissue. Functionally wingdisc show high STAT activity, low translation rate and defective ribosome biogenesis. Our finding show immune cells have specific control on animal size by regulating the imaginal tissue size during development.

Funding Source: DBT-Welcome India Alliance Senior Research Fellowship. Core Fund Institute for Stem Cell Science and Regenerative Medicine.

Keywords: metabolism, growth, animal size

3:45 PM – 4:45 PM

203

SIGLEC-7 EXPRESSION AFFECTED LINEAGE COMMITMENT DURING HEMATOPOIESIS

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Recently, it has been demonstrated that surface receptor, sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7), was regulated via histone deacetylase (HDAC1) transcriptional during megakaryocyte (MK) differentiation. And enriching surface expression of Siglec-7 positively enhanced MK maturation and platelet formation along hematopoiesis. In addition to platelet biosynthesis, accumulating evidence suggests that MKs are also involved in other pathophysiological processes, including HSC quiescence, inflammation response, and immunity regulation in both human and mouse. Knowing the Siglec-7 can be detected on both myeloid and lymphoid cells, for example, platelet and natural killer (NK) cell, respectively, we were interested in whether the Siglec-7 expression can affect the lineage commitment at the early stage of

hematopoiesis. We examined the proportion of common myeloid progenitor (CMP), common lymphoid progenitor (CLP), granulocyte-macrophage progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP) by inducing S7-OE CD34+ hematopoietic stem cells (HSCs) and mock control along MK differentiation. At the early stage of the differentiation, we found the Siglec-7 expression enhanced the lymphoid differentiation, as evidenced of that more CLPs were detected in the S7-OE group but no such effect was observed in the CMP population. On the other hand, in the myeloid lineage, Siglec-7 level significantly promoted GMP differentiation, but did not show same result on MEP, a progenitor for both erythrocytes and platelets. Interestingly, we have found the elevated HSC-niche related factor, platelet factor 4 (PF4), could be observed in S7-OE cells. Taken together, these results suggested that Siglec-7 on HSC-derived MKs may possess multiple functions, including the promotion of MK differentiation, regulation for lineage commitment, and HSC niche regulation.

Funding Source: NSTC 112-2320-B-A49-046

Keywords: MK differentiation, Siglec-7, HSC niche

3:45 PM – 4:45 PM

205

FROM FLOW TO FORM: UNDERSTANDING HUMAN INDUCED PLURIPOTENT STEM CELL DERIVED ENDOTHELIAL CELL RESPONSE TO SHEAR STRESS

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The Allen Institute for Cell Science aims to understand the principles by which human induced pluripotent stem cells (hiPSCs) establish and maintain robust dynamic localization of cellular structures, and how they transition between states during differentiation and disease. Endothelial cells (ECs) are essential for vascular development and function. They are subject to shear stress as blood flows through the vessels they line, which in turn influences their structure, function, and morphology. To explore how variation in an environmental cue such as shear stress influences cellular organization, we differentiate endogenously tagged hiPSC lines from the Allen Cell Collection (www.allencell.org) into ECs. We found that the alignment of hiPSC-ECs varies with the magnitude of shear stress applied using an ibidi Pump System. At low shear stresses (0.8-6 dyn/cm2), cells align parallel to fluid flow, whereas at high stress (15 dyn/cm2), they align orthogonally. In contrast, intermediate stresses (9 or 11 dyn/cm2) induce mixed alignment. We found the response of hiPSC-ECs to different magnitudes of shear stress also depended upon if they had previously been exposed to flow. The application of shear stress has been shown to affect the morphology, motility, polarity, and mechanical coupling of ECs. We are currently using this method in conjunction with immunofluorescence and live cell imaging of tagged hiPSC-ECs to investigate how the cytoskeleton, focal adhesions, polarized structures, and adherens junctions of these cells change in response to this cue. We also developed a method for segmenting the whole cell and nucleus of hiPSC-ECs, which allows for detailed analysis of morphology and subcellular organization from 3D images of cells expressing a mEGFP-tagged protein representing a structure of interest. As a next step, we plan to introduce genetic perturbations to hiPSC-ECs via iCRISPRi to target key proteins vital for intracellular structure and function. Ultimately, understanding how shear stress as an environmental factor affects the alignment, morphology, and subcellular organization of hiPSC-ECs will enhance our comprehension of endothelial cell biology.

Keywords: endothelial cells, shear stress, imaging

3:45 PM – 4:45 PM

207

EXPLORING NEW DEPTHS: ENHANCED IPSC-BASED ERYTHROPOIESIS IN 3D CULTURE

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Erythropoiesis is a tightly regulated and complex process that results in the production of 200 billion red blood cells per day. Uncovering the molecular mediators of hematopoietic regulation is key in understanding bone marrow physiology and erythroid development. Moreover, accurate in vitro modeling of blood disorders is necessary to identify disease mechanisms and new therapeutic targets. Erythroid differentiation from induced pluripotent stem cells (iPSCs) is well-known to provide an abundant and tractable in vitro cell system; however, iPSC-based erythroid cultures are hindered by incomplete differentiation, particularly with primitive cell signatures and limited terminal erythropoiesis. Here, we have engineered a three-dimensional (3D) microenvironment to present human stem cells with a more physiologically relevant milieu, aiming to support hematopoiesis and long-term culture and identify novel, biophysical mediators of red blood cell regulation. Porous and biocompatible polyurethane scaffolds were produced in-house and coated with extracellular matrix protein prior to seeding with hematopoietic stem and progenitor cells differentiated from healthy donor-derived iPSCs. Cells were harvested throughout erythroid differentiation comparing 3D culture against conventional suspension culture. Collection at multiple timepoints enabled characterization of major erythroid maturation stages via surface marker profile and May Grünewald-Giemsa staining. Fourteen days of erythroid culture in 3D scaffolds revealed an increase in late-stage erythroid populations as well as enhanced cell enucleation compared to control cells grown in well plates. These results suggest a role for biophysical regulation in terminal erythropoiesis that is uniquely recapitulated in the 3D microenvironment. FACS purification of a full panorama of erythroid populations for further high-throughput analyses is aiming to dissect the underlying players and pathways present exclusively in 3D and then target these in routine cell culture to enhance terminal differentiation. Ultimately,

our work will advance our understanding of erythroid biology as well as bring us closer to generating functional blood components from iPSCs for disease modeling and therapeutic applications.

Keywords: 3D culture, induced pluripotent stem cells, erythropoiesis

3:45 PM – 4:45 PM

209

GENERATION OF HYPOIMMUNE HEMATOPOIETIC PROGENITORS FROM HLA CLASS-I AND CLASS-II NULL IPSC LINE

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Over recent years, the field of regenerative medicine has witnessed a notable surge in the adoption of cell-based therapies. Among these innovative treatments are hematopoietic cell transplantation, CAR-T cell therapy, and CAR-NK cell therapy, which hold promise for addressing various diseases, particularly cancer. Nonetheless, a significant challenge in allogeneic transplantation is the potential development of graft-versus-host disease (GVHD), a serious condition where the transplanted immune cells attack the recipient's tissues. To mitigate the risk of GVHD there is a growing interest in utilizing hypoimmune cells. Induced pluripotent stem cells (iPSCs) have emerged as a versatile platform for generating ample quantities of the cells with the uniform genetic background. In this study, we present data on the generation of hypoimmune cells from iPSCs. Notably, we have previously utilized this line to generate HLA class-I and class-II null mesenchymal stem cells (MSCs). Specifically, we demonstrate the successful derivation of CD34+ hematopoietic progenitor cells and CD5+/CD7+ myeloid progenitor from HLA class-I and class -II null iPSC line. These progenitors exhibit the capability to further differentiate into T and NK cells. Additionally, we evaluated the differentiation efficiency of this genetically modified iPSC line in comparison to its parental counterpart. In conclusion, our findings underscore the promising prospects of utilizing HLA class-I and class-II null iPSC lines as a renewable and hypoimmune cell source for allogeneic transplantation, particularly in the realm of cancer treatment. By harnessing the regenerative power of iPSC technology, we endeavor to pave the way for safer and more effective therapies with the potential to revolutionize medical practice.

Funding Source: This project was funded by the Maryland Stem Cell Research Fund, through its Commercialization Program, award number: 2023-MSCRFCO-6157

Keywords: iPSC, hypoimmune, HLA class-I and class-II null

3:45 PM – 4:45 PM

TOPIC: KIDNEY

211

AMNIOTIC FLUID ORGANOIDS AS PERSONALIZED TOOLS FOR REAL-TIME MODELING OF THE DEVELOPING FETUS

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Despite recent biomedical advances, major knowledge gaps regarding human development remain. Accordingly, many developmental disorders lack effective treatments, representing a huge clinical burden. These gaps result from human fetuses being largely inaccessible for analysis. Here, by culturing fetal cells present in human amniotic fluid (AF) in defined three-dimensional conditions, we specifically select for kidney or lung progenitors, which give rise to single cell-derived personalized fetal kidney and lung organoids (AFKO and AFLO, respectively). Using a battery of immunostaining, functional assays and single cell-based transcriptomics, we show AF organoids to recapitulate the respective fetal organs in cell composition and lineages, transcriptome and function. Namely, AFKO harbor key fetal kidney cell

populations, including nephrogenic, urothelial and stromal lineages, and are capable of endocytosing albumin. Moreover, upon inhibition of PAX2, a key renal developmental gene and a common cause of renal anomalies, a clear phenotype mirroring the human defect is observed. Strikingly, upon injection into the nephrogenic cortex of human fetal kidney explants, AFKO-derived SIX1+ progenitors integrate into the host progenitor niche and contribute to developing nephrons. Concomitantly, AFLO comprise both alveolar cells and most airway cell types in a typical pseudostratified structure. In addition, AFLO show functional CFTR channels and upregulate surfactant expression upon corticosteroid treatment, thus mimicking the physiological effect to these agents. Overall, this platform represents a new personalized tool that can be applied to virtually any fetus in real-time (i.e., while it is in-utero), affording unprecedented options in studying development, uncovering mechanisms of fetal pathologies (e.g., congenital anomalies, infections, chemical teratogens, and prematurity-related complications), and tailoring treatments for these pathologies. Importantly, since AF contains cells from additional tissues (e.g., skin and gastrointestinal tract), and is derived in a procedure that is already routinely performed in many patients, this platform may well become a broadly applicable tool in both developmental biology and in fetal medicine.

Funding Source: Sheba Medical Center Physician-Researcher Program, Israel Science Fund, US-Israel Binational Science Foundation, Israel Ministry of Science and Technology, Israeli Estates Committee, Alrov Fund.

Keywords: amniotic fluid, kidney organoids, lung organoids

3:45 PM – 4:45 PM

213

MODULATING KIDNEY MICRO-ORGANOIDS TO ENHANCE NEPHRON FORMATION

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Stem cell-derived human models of the kidney have potential in disease modelling, drug screening, and ultimately the capacity to engineer renal replacement tissue, all of which may enable the development of new treatments for kidney disease. These micro-tissues contain patterning and segmenting nephrons surrounded by renal stroma and an endothelial component. Numerous protocols have been described for the generation of kidney organoids, containing subtle variations in the identity and ratio of component cell types. Previously, we have described an approach for generating suspension culture micro-organoids. These are a cost effective and feasible approach to scaling out the production of nephrons. However, in comparison to standard organoids, patterning of the nephrons in micro-organoids differ, as does the stroma to nephron ratio. This study aims to assess how varying the signalling environment in micro-organoids can modulate nephron number, patterning, and maturity. To characterise the impact that modification in growth factor conditions has on nephron morphology and downstream signalling events, we utilise a combination of reporter stem cell lines, 3D imaging techniques and transcriptional analysis. This research will enable the enhanced use of micro-organoids for application in drug screening or for functional transplantation, aiding the development of methods that allow us to generate organoids suitable for therapeutic application.

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Keywords: kidney micro-organoids, nephron, morphology

3:45 PM – 4:45 PM

TOPIC: LIVER

215

ROLE OF BIOMATERIALS IN LONG TERM STABLE CULTURE SYSTEM FOR HEPATIC PROGENITOR/ STEM CELL

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Hepatic Progenitor Stem Cells are a rich cell source for cell transplantation treatment of liver diseases because of their high proliferative ability, small size also easy integration into the liver plate after transplantation. However, HPSCs isolated in vitro are prone to lose their high proliferative ability and bidirectional differentiation potential, which has brought great obstacles to further research. Therefore, the purpose of this study is to isolate the HPSCs culture system in vitro. This study can accumulate experience for the further study of the pluripotency maintenance mechanism of HPSCs and lay a biological foundation for clinical application. The number of HPSCs in fetal liver was negatively correlated with the development maturity of liver tissue. HPSCs were isolated from mouse fetal liver by density gradient centrifugation. The cell population abundance of DLK1+ labeled cells was detected by flow cytometry. The results showed that the abundance of DLK1+ labeled cells isolated from E11.5 fetal liver was significantly higher than that of other liver development stages. Immunofluorescence staining results showed that the isolated cells with large nucleoplasm small cell volume and small oval shape, the cells were doubly labeled with DLK1+/AFP+. In addition, extracellular matrix and cytokines were studied, effects of these compounds on in vitro amplification and culture systems were tested. Gelatin and Fibronectin significantly promoted the adhesion and expansion of HPSCs. Gelatin and Fibronectin with HGF, FGF2, FGF10, Nicotinamide, and R_Spondin1 were used to establish a system for long-term stable culture of HSCs in vitro. The effects of different combination conditions on the proliferation of HPSCs were detected

by CCK8. it was found that cell colonies composed of tightly arranged small cells appeared during culture, continuous observation revealed an enlarged cell colony. HPSCs in this study were successfully isolated and had high proliferation capacity. In the study of in vitro amplification culture, the primary HPSCs could be maintained for amplification culture for up to 7 days. The proliferation of HPSCs was significantly increased in the culture system supplemented with Gelatin or Fibronectin HGF, FGF2, and FGF10.

Keywords: stem cell, hepatic progenitor cell, long-term

3:45 PM – 4:45 PM

217

UNRAVELING THE ROLE OF WNT SIGNALLING IN LIVER ORGANOGENESIS USING HUMAN HEPATOBLAST ORGANOIDS

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Hepatoblasts are the foetal stem cells of the liver and play a key role in organogenesis. These stem cells have the capacity to differentiate into hepatocytes and cholangiocytes which represent the main functional cell types of the liver. Lineage tracing and genetic studies in the mouse have shown the association of Wnt signalling with proliferation and differentiation of hepatoblasts. However, the exact function of this pathway in hepatic development and cell fate choice is not fully uncovered, especially in human where access to primary tissues is challenging. Here, we took advantage of human hepatoblast organoids (HBOs) to investigate the role of Wnt in self-renewal and cell fate decisions. Notably, HBOs display a transcriptomic profile characteristic of hepatoblasts and they maintain the capacity to differentiate into hepatocytes and biliary cells. Here, we first showed that Wnt plays a key role in hepatoblast self-renewal in vitro by maintaining their proliferative state through regulation of cell cycle-related genes. However, Wnt was not sufficient to block differentiation of HBOs into hepatocytes or cholangiocytes. Finally, using single-cell transcriptomic analyses, we found that Wnt signalling activity correlates with proliferation of hepatoblasts in the human foetal liver, thereby suggesting that the role for Wnt could be conserved in vivo. Taken together, our results support a model where Wnt signalling acts to preserve the proliferative, self-renewal capacity of hepatoblasts without being sufficient to maintain their bipotent state. These results could open new investigations to further understand the role of Wnt signalling in other adult stem cells and developmental progenitors, where Wnt is also known to drive tissue homeostasis and cell fate decisions.

Keywords: organoid, foetal liver stem cell, Wnt signalling

3:45 PM – 4:45 PM

219

CYCLIC MECHANICAL STIMULI WITH A STRAIN-CONTROLLABLE DEVICE INDUCES ANGIOCRINE SIGNALS DURING LIVER BUD STAGE

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Liver organoids derived from human pluripotent stem cells (hPSCs) serve as a resource for understanding liver development and creating transplantable livers, necessitating vascularization for successful integration with recipients. However, the conventional self-condensation approach for organoid generation fails to induce a perfusable vascularized structure. To address this issue, we have focused on the liver bud's developmental environment, a crucial stage for liver vascularization. Recent studies emphasize the role of cyclic mechanical stretch (cMS) in promoting vasculogenesis during liver development and regeneration, suggesting that cMS could similarly enhance this process in the liver bud stage. Here we show that an organ-on-a-chip (OoC) platform allows to mimic the mechanical environment of liver development, and investigate the role of cMS in liver vasculogenesis. The OoC platform made of polydimethylsiloxane (PDMS) is pneumatically actuated to exert cyclical stretch on the PDMS membrane, under which the cells are cultured, thereby mimicking the mechanical environment present during liver development. Then, we generate liver organoids, specifically targeting the liver bud's cellular constituents: septum transversum mesenchyme (STM), endothelial cells (ECs), and hepatic endoderm (HE). Our differentiation method efficiently yields 99.8% and 88.9% of STM and ECs from hPSCs, positive for their respective markers, compared to 4.7% and 11.3% obtained using a conventional method. We also induced 99.0% HE from hPSCs, positive for the marker with a conventional method. Before demonstrating that cMS induces vasculogenesis in liver organoid, we show that cMS enhances angiocrine signals in HE with our OoC. RNA sequencing revealed that the expression of angiocrine signals, including hepatocyte growth factor (HGF) and matrix metallopeptidase 9 (MMP9), were increased by 1 Hz and 10% strain cMS. The expression and secretion of HGF and MMP9 were also increased. These results allow us to investigate that cMS could induce vasculature in liver organoid with enhanced angiocrine signals, with liver organoid containing STM and ECs. Our findings provide new insights into

the mechanical factors in liver development and directions for future improvements in the engineered liver and transplantation.

Keywords: liver organoid, cyclic mechanical stretch, organ-on-a-chip

3:45 PM – 4:45 PM

221

GENERATION OF HEPATOCYTE-LIKE CELLS FROM HUMAN VENTRAL FOREGUT STEM CELLS

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The visceral organs are formed during embryonic development from the endoderm germ layer. In particular, the liver and pancreas are derived from the embryonic region known as the ventral foregut (VFG) endoderm or anterior intestinal portal lip in human. While the generation of functional liver cell types has been an important thrust of stem cell biology for both the treatment of degenerative disease and for drug screening, it has been difficult to produce robust, mature cell types. Here, we exploit human embryonic stem cell-derived ventral foregut (VFG) progenitors to generate improved hepatocyte-like cells. VFG cells exhibit expansion-dependent increases in differentiation efficiency to liver progenitors and we characterize their capacity to produce 3D hepatocytes-like cells (HLC). Based on the secretion of albumin, uptake of low-density lipoprotein and glycogen, and rifampicin-induced CYP3A4 P450 activity we demonstrate that VFG-derived HLCs are generated both more efficiently and with enhanced functionality. Using a newly generated CYP3A4 reporter cell line and clinically defined CYP inducers, we isolated mature hepatocytes and generated a tool that can be used for drug-drug interaction studies. Our results demonstrate that expansion of lineage-specific intermediates is an important step in achieving high-fidelity in vitro differentiation, producing improved, economical liver cells that can be used in disease treatment and toxicology tests.

Keywords: liver, development, organoids

3:45 PM – 4:45 PM

223

A NOVEL ORGANOID MODEL OF EARLY HUMAN LIVER BUD DEVELOPMENT AND HEPATOBLAST SPECIFICATION

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Understanding the liver in health and disease is vital for the development of new therapies against liver diseases, such as the production of hiPSC-derived hepatocytes for regenerative medicine. For that, understanding early liver organogenesis is pivotal since it will provide essential information about liver cells specification and their functional maturation. However, early human organogenesis remains difficult to study due to technical and ethical challenges. To address these major questions, we have developed a novel 3D culture system allowing for differentiation of human induced Pluripotent Stem Cells (hiPSCs) into self-organising human Hepatic Bud organoids or HepBuds. This method relies on a heterogenous differentiation containing anterior/posterior foregut cells, cardiomyocytes and endothelial cells as shown by immunostaining and singe cell transcriptomic analyses. After 3D seeding, these populations self-organise into complex budding structures which recapitulate the initial organogenesis of the liver bud. This includes a “bud” containing hepatoblast (ALB+AFP+HNF4A+) and a “trunk” containing hepato-pancreato-biliary progenitors (KRT19+CDX-2+PDX1+HNF1B+) and extra-hepatic cholangiocytes (ACE2+MUC13+). Thus, our HepBud organoids are composed of the multiple cell types produced during the early organogenesis of the hepato-biliary system. Finally, we decided to demonstrate the functional relevance of these organoids by deriving hepatoblast organoids (iHBOs). For that, HepBuds underwent serial passaging in culture conditions supporting hepatoblast self-renewal. We observed the formation of homogenous self-renewing organoids expressing hepatoblast markers. These iHBOs were able to self-renew for an additional 8 passages while maintaining their morphological characteristics and displayed a capacity to differentiate into intra-hepatic ductal plate cholangiocytes and hepatocytes. Critically, we also show that hiPSC-derived hepatoblast share many features with their primary counterparts. Taken together, these results show that our model of liver bud development provides a new platform not only to study hepatobiliary lineage specification in vitro but also to produce cell types with a clinical interest.

Funding Source: Wellcome Trust

Keywords: liver bud, liver, organoid

3:45 PM – 4:45 PM

225

OPTIMIZING HUMAN HEPATIC ORGANOID DEVELOPMENT: LEVERAGING IPSC DIFFERENTIATION AND ADVANCED ANALYTICAL PLATFORMS WITH RUO GROWTH FACTORS AND CYTOKINES

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Increasing requirement for the generation of species-specific, physiologically relevant systems for the study of disease and development has led to the expanded use of 3D models, such as organoids. These biologically accurate tissue models can be expensive and time-consuming to produce, necessitating efficient and reliable methods for their development, monitoring, and characterization. Here we present methods for differentiation of iPSCs to human hepatic cells and further development into human hepatic organoids using Sartorius RUO Growth Factors and Cytokines. During this workflow, cell growth and morphology were monitored using the Incucyte® Live-Cell Analysis System and marker expression was characterized on the iQue® Advanced Flow Cytometry Platform. Image analysis of iPSC development during differentiation revealed morphological changes associated with loss of pluripotency, while hepatic cell characteristics were visualized during the workflow. Confirmation using surface marker analysis showed loss of pluripotency marker expression as iPSCs progressed along the differentiation pathway, and increased expression of hepatocyte markers throughout differentiation (CD99 > 80%, CD184 > 30%). Mature hepatocytes were established after 25 days, and endpoint analysis confirmed a four-fold increase in CYP enzyme activity compared to source iPSCs. Quantification of iPSC-derived hepatic organoid morphology and growth indicated healthy organoids with ideal characteristics capable of high levels of propagation in culture. Surface marker expression provided similar profiles for hepatic organoids and mature hepatocytes, with high expression of CD99 (>95%) and >40% expression of CD184. LGR5 expression in hepatic organoids (25%) was also observed. CYP enzyme activity analysis of hepatic organoids showed a five-fold increase compared to source iPSCs. Comparison of Sartorius-developed RUO hepatic organoid media with a commercial alternative showed increased growth with double the maximum diameter of organoids in the RUO media after 7 days in culture (1.2 mm vs 0.6 mm, respectively), with conserved organoid counts across both media types. These data exemplify the use of Sartorius RUO Growth Factors and Cytokines for development of human iPSC-derived hepatocytes and hepatic organoids.

Keywords: iPSCs, organoid, differentiation

3:45 PM – 4:45 PM

227

POSITIVE AUTOREGULATION OF SOX17 IS CRITICAL FOR SOX17 DOSAGE-DEPENDENT FORMATION OF THE GALLBLADDER AND EXTRA-HEPATIC BILE DUCTS

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The mammalian biliary system channels, stores and releases bile produced by hepatocytes into the duodenum via the gallbladder, hepatic, cystic and common ducts. Expression of the SRY-box transcription factor 17 (Sox17) in an endodermal region caudal to the hepatic diverticulum during late gastrulation is critical for formation of the gallbladder, extrahepatic bile ducts (EHBDs), and ventral pancreas. We reported previously that a conserved 126 bp sequence upstream of transcription start site 2 (TSS2) is essential for hepato-pancreato-biliary (HPB) development. However, further analysis was not pursued due to the region functioning both as the TSS2 promoter and RNA-processing site for TSS1-generated transcripts. Instead, we used CRISPR/Cas9 mutagenesis in mice to disrupt three putative cis-regulatory elements (CREs) upstream of TSS2 without affecting RNA processing from TSS1: mut3 and mut5 (a possible SOX17 binding site) were nine bp transversions that targeted single CREs, whereas a fortuitous Δ50 bp deletion generated by microhomology-mediated repair eliminated three CREs. Mice with these mutations were viable but exhibited defects in HPB development of variable severity: Sox17mut3/mut3 animals showed no defects, Sox17mut5/mut5 animals have hypomorphic gallbladders, and Sox17Δ50/Δ50 animals completely lack their gallbladders and cystic ducts. Fusion gene and DNA binding analyses confirmed that the mut5 allele disrupts binding of SOX17, thereby establishing the existence of a positive Sox17 autoregulatory loop necessary for EHBD development. Immunostaining and transcriptomic analysis of HPB progenitor cells in Sox17Δ50/Δ50 embryos and gallbladders from adult Sox17mut5/mut5 animals revealed many Sox17-dependent genes, including Cited1, a transcriptional coactivator associated with self-renewing progenitor cells. Finally, compound heterozygous mice containing a Δ50 and Sox17 null allele died shortly after birth due to EHBD atresia. These findings further establish the critical role of Sox17 in EHBD development and suggest the existence of low-affinity SOX17 binding sites that may require higher amounts of SOX17 in specific HPB progenitor cells for proper spatiotemporal activation of target genes during gallbladder and EHBD formation.

Keywords: gallbladder, organogenesis, gene expression

3:45 PM – 4:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

229

FETAL BOVINE MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE SELF-ASSEMBLE WITH TESTICULAR CELLS AND EXPRESS GERM CELL MARKERS IN TESTICULAR ORGANOIDS

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Mesenchymal stem cells (MSC) exert both self-renewal and mesodermal differentiation, which make them potentially candidates for in vitro derivation of gametes. This technology may constitute experimental therapies for assisting human and animal reproduction, and in the improvement of livestock genetic traits, and conservation of endangered animals. Organoids simulate a cellular niche and provide a spatial support for in vitro differentiation studies. In this study, we evaluated the integration of fetal bovine MSCs derived from adipose tissue (AT-MSCs) in testicular organoids (TOs), and their potential for germ cell differentiation. Leydig, Sertoli, and peritubular myoid cells previously isolated from bovine testes (n=6) were used for TO assembly. PKH26-positive AT-MSCs (PKH26+AT-MSCs) were cultured with testicular cells at a concentration of 1x106 cells per well in Ultra Low Attachment U-shape bottom (ULA) plates. From day 3 to day 24 of culture TOs with PKH26+AT-MSCs (TOs+AT-MSCs) were cultured with different concentrations of testosterone (0.2; 2 and 20 µM) and 1 µM of retinoic acid. Germ cell differentiation of AT-MSCs was evaluated by colocalization of DAZL, STRA8 and UCHL1 in TOs+AT-MSCs and controls. Untreated controls and TOs+AT-MSCs treated with 0.2 µM of testosterone decreased ($P< 0.05$) mean diameters from day 7 to 24 of culture (523 ± 51.3 ± 9 µm to 335 ± 29.7 µm; 438 ± 9 µm to 335 ± 40 µm, respectively) compared to testosterone treated TOs+AT-MSCs. DAZL was colocalized with PKH26+AT-MSCs in TOs+AT-MSCs treated with 0.2 and 2 µM of testosterone at days 14 and 24 of culture, respectively. STRA8 was colocalized with PKH26+AT-MSCs in TOs+AT-MSCs exposed to 2 and 20 µM of testosterone at days 14 and 24 of culture. Moreover, colocalization of UCHL1 and PKH26+AT-MSCs was observed after treatment of TOs+AT-MSCs with 0.2 and 2 µM at days 14 and 24 of culture. Colocalization of PKH26+AT-MSCs with germ cell markers DAZL, STRA8 and UCHL1 at different stages of TOs development suggest the potential of bovine fetal AT-MSCs for germ cell differentiation under in vitro conditions.

Funding Source: This study was supported by grants 1191114 and 1231145 from the National Research and Development Agency of Chile (ANID) from the Ministry of Education, Government of Chile.

Keywords: mesenchymal stem cell, testicular organoid, germ cell differentiation

3:45 PM – 4:45 PM

231

COMBINATION TREATMENT OF MESENCHYMAL STEM CELL THERAPY AND REDUCED GLUTATHIONE ENHANCES THERAPEUTIC EFFICACY BY INCREASING MSC REACTIVITY

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Mesenchymal stem cells (MSCs) have been expected as an effective treatment for intractable diseases. However, since MSC didn't meet the expectations, the need for the development of next-generation therapeutic agents has emerged. Glutathione (GSH) is one of the most powerful antioxidants in the body and is composed of the Cys-Gly-Glu peptides. It is well known that in order to maintain the stemness of mesenchymal stem cells (MSCs), they must be protected from oxidative stress. Recently, it has been published that showing GSH plays a significant role in maintaining stem cell function. However, it is still unclear whether GSH treatment can improve the stemness of MSCs. We performed experiments to verify stemness by treating human bone-marrow derived-MSC with GSH in vitro. As a result, we obtained that the stemness of MSC can be enhanced by treating it with GSH. Thus, we suggest that a combination treatment of GSH and MSC would be more effective than MSC alone. Furthermore, this finding is expected to contribute to the development of intractable drugs.

Keywords: glutathione, MSC, priming

3:45 PM – 4:45 PM

233

NEW GMP-COMPATIBLE IPSC-BASED DIFFERENTIATION WORKFLOW YIELDS MESENCHYMAL STROMAL CELLS (MSCS) WITH OUSTANDING PROLIFERATION POTENTIAL

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Mesenchymal stem cells (MSCs) have distinct characteristics and hold significant potential in cell therapy, regenerative medicine as well as in curing immune-mediated disorders. MSCs tend to show self-renewal and multipotent characteristics as well as immunomodulatory properties with low immunogenicity and tumorigenicity following cell transplantation. In addition, MSCs may serve as factories for producing large quantities of exosomes that may be used to exert immunomodulatory functions in patients. MSCs can be derived from a variety of different tissues or organs such as bone marrow, umbilical cord, fat

tissue and others. However, these primary MSCs show variability in their characteristics including their differentiation potential, expression of MSC-specific marker genes, and immunomodulatory activity. Furthermore, primary MSCs tend to have limited proliferation potential, which may necessitate gene manipulation to enable any applications at larger scale. To overcome these challenges and exclude donor-to-donor variations, it is desirable to identify an appropriate and reliable source of MSCs in clinical applications. Induced pluripotent stem cells (iPSCs) could present a promising alternative source to derive homogeneous populations of MSCs (iMSCs), which may potentially overcome the issues relevant for clinical applications. Here, we introduce a novel, simple and GMP-friendly approach for the directed differentiation of iPSCs toward iMSCs. This platform provides MSCs at high purity and with potential to proliferate for over 20 passages while yielding vast cell numbers and preserving multipotency. We showed that the differentiation of iMSCs from iPSCs and further expansion and upscaling of established iMSC lines is highly dependent on culture conditions and MSC media formulation employed as we find strong differences comparing commercial MSC media from different suppliers. Given its overall GMP-compatibility, this platform will enable the generation and banking of consistent batches of off-the-shelf iMSCs to feed upscaling approaches in clinical applications.

Keywords: mesenchymal stromal cells, differentiation workflow, iPSC

3:45 PM – 4:45 PM

TOPIC: MUSCULOSKELETAL

235

SPECIES CONSERVATION REVEALED BY COMPARATIVE ANALYSES OF SINGLE CELL TRANSCRIPTOMES OF MOUSE AND HUMAN IN VITRO ESC-DERIVED NOTOCHORDAL-LIKE CELLS

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The nucleus pulposus (NP) in the intervertebral disc serves a critical role in buffering the spine from mechanical loading. NP cells develop from the early embryonic notochord (NC), and NC-like cells persisting in the mature NP are thought to be progenitors essential for maintaining a healthy disc. During ageing, the composition of the NP changes: NC-like cells diminish, most of the cells appear chondrocyte-like and fibroblast-like, and the extracellular matrix becomes stiffened. These changes are believed to contribute to intervertebral disc degeneration (IDD). Thus, understanding human NC and NP cell development and their biological properties, is crucial, as it may facilitate establishment of treatments to maintain a healthy NP population. It is also important to derive NC and NP cells in vitro for fundamental research and therapeutic use. We describe the derivation of mouse and human NC cells from embryonic stem cells (ESCs) by stepwise induction using small molecules to manipulate signaling pathways (including Wnt and Nodal). We derived mouse NC-like cells that express canonical NC markers like T, Noto, Shh, and Foxa2, resembling in vivo E8.5 NC. The mouse NC-like cells can be further differentiated into NP-like cells that express extracellular matrix components such as Col2a1, Col9a1, Col6a3, Acan, and Hapln1, resembling in vivo NP cells. Similarly, NC-like cells resembling node-stage NC, could be derived from human ESCs. An integrated single-cell transcriptomic analysis on the derived NC- and NP-like cells and in vivo counterparts revealed mouse-human conserved gene expression during NC and NP cell differentiation. For instance, matrix metalloproteinases such as Mmp2, Mmp14, and Mmp15, are enriched in early NC cells, Hopx is expressed during the NC-to-NP transition and later in the NP, and Sox9 is expressed throughout NC and NP development. In sum, our study provided novel approaches to derive cells of the NC lineage from mouse and human ESCs. The significant mouse-human conservation in gene expression in early notochordal development can be exploited for in vivo developmental genetic studies relevant to human NC cells. Our system provides a platform for functional studies on the biology of the NC/NP cells, their role as progenitors and for identifying factors critical for their maintenance.

Funding Source: Collaborative Research Fund (C7044-19G), Health and Medical Research Fund (09201886)

Keywords: notochord, stem cells, mouse-human conservation

3:45 PM – 4:45 PM

237

RECAPITULATION OF HUMAN LIMB DEVELOPMENT WITH HUMAN INDUCED PLURIPOTENT STEM CELLS

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Although the study of human development is limited due to the ethical limitations and availability of samples for research, the use of human induced pluripotent stem cells (hiPSCs) offers a unique opportunity to study human development and its relevant genetic disorders. Recently, we established a protocol that leverages PRRX1–tdTomato reporter hiPSCs for the selective induction of expandable and ontogenetically defined PRRX1+ limb bud mesenchymal cells (Expandable limb bud mesenchymal cells, ExpLBM cells). Furthermore, the limb bud mesenchymal cells can be expanded in culture before directing them to undergo chondrogenesis. To characterize human limb development in vitro, here we report the cell signature at each differentiation stage in terms of transcriptome by RNA-seq, chromatin-accessibility by ATAC-seq, and chromatin modification by CUT&TAG analysis. Principal-component analysis (PCA) of the RNA-seq and ATAC-seq data revealed distinct transcriptomes and accessible chromatin signatures among each differentiation stage. RNA-seq analysis revealed that mRNA expression patterns at each stage were almost similar to those known in previous mouse and chick studies. Interestingly, ExpLBM cells revealed a high gene expression in the hindlimb marker (PITX1 and TBX4), but not in the

forelimb marker (TBX5). Chromatin-accessibility around hindlimb markers was observed in ExpLBM cells by ATAC-seq. CUT&TAG analysis clearly showed that H3K27me3 peak associated with gene repression was not observed around the hindlimb marker promoter region, but both H3K9ac and H3K27ac peaks associated with gene activation were detected. These results suggest that our guided differentiation scheme of hiPSCs into limb bud mesenchyme could be a promising research tool for understanding human limb skeletal formation.

Keywords: limb bud mesenchymal cells, ExpLBM cells, epigenetics

3:45 PM – 4:45 PM

239

ALGAE SCAFFOLD INTEGRATION WITHIN EMBRYONIC STEM CELL ORGANOIDS: ADVANCING SUSTAINABLE CULTIVATED SEAFOOD

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Cultivated seafood, produced by growing marine animal muscle tissue from stem cells in vitro, has emerged as a potential solution to address the sustainability, ethical, and environmental challenges of traditional animal aquaculture. One of the key challenges in cultivated fish and meat production is the development of a three-dimensional tissue structure that resembles conventional fish or non-aquatic meat. Scaffolds, which serve as structural templates for tissue formation, have shown promise in overcoming this challenge. This research project aims to develop a novel scaffold for the production of muscle organoids from embryonic stem cells (ESCs). The scaffold is based on seaweed, an abundant and sustainable resource that has been shown to have potential applications in the biomedical field. Algae may be a suitable scaffold for cultivated fish due to its original morphology, chemical components, and the potential to provide additional nutrients and flavor-enhancing agents. Additionally, the incorporation of algae in the final product may be more appealing to consumers due to their perceived "natural" connection to fish. We utilize the unique properties of these natural materials, such as biocompatibility, biodegradability, and favorable mechanical properties, to create a scaffold that can support the growth and differentiation of ESCs into muscle within organoids. As a first step, we demonstrated that algae processed particles can be fully incorporated into mouse ESC organoids without compromising the aggregation and differentiation abilities. We also found that algae integration can enhance the final size of the organoids while allowing the production of abundant muscle fibers. We are extending these results to fish cells-based organoids. The adoption of algae-based scaffolds may not only reduce production costs but also enhance reproducibility, ensuring a controlled growth environment for the ESCs to differentiate into muscle organoids. This could possibly support the scalability, commercial viability, and standardization of cultivated seafood production.

Keywords: embryonic stem cells, cultivated meat, scaffolding

3:45 PM – 4:45 PM

241

DELINEATING IN VITRO MYOGENESIS IN HUMAN

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Skeletal muscle makes up nearly half of the human body mass and is prone to numerous genetic and metabolic disorders, as well as cancer- and ageing-related wasting conditions. To study these conditions and to develop therapeutic strategies for them, robust and reliable in vitro models of skeletal muscle cells are required. Current protocols of inducing skeletal muscle cell identity range from differentiation of human pluripotent stem cells (hPSCs) to transdifferentiation of non-myogenic somatic cells into myogenic fate. These methodologies have proved to lead to inefficient induction of myogenic identity and thus have been highly variable in mimicking bona fide skeletal muscle cells. Here, we sought to delineate the state of the art of the in vitro models of human skeletal muscle. To this end, we performed a large-scale transcriptomics analysis of sixty published datasets, including bulk and single cell RNA sequencing and covering more than 400 samples to gain a better insight into the transcriptomic identities of various in vitro models of human skeletal muscle. The samples analyzed ranged from primary muscle cells isolated from biopsies to in vitro differentiated or transdifferentiated myogenic cells and immortalized cell lines. Our comprehensive analysis reveals the missing links in the in vitro models as compared to their in vivo-derived counterparts. We show that the myogenic cells derived from the differentiation of hPSCs or the transdifferentiation of non-myogenic cells differ from their in vivo-derived reference cells for the expression of a group of transcription factors, epigenetic factors and metabolic genes. In addition, we also profiled cell surface and secretome expression signatures in these in vitro myogenesis models, suggesting potential differences in their cell signaling dynamics. This large-scale meta-analysis serves as a robust reference to guide the future research of in vitro skeletal muscle models and highlights the necessity of studying the gene networks regulating the induction of this cell fate.

Keywords: human skeletal muscle cell identity, large-scale transcriptome analysis, in vitro myogenesis

3:45 PM – 4:45 PM

243

SCLERAXIS IS A NEGATIVE REGULATOR OF NUROGENESIS IN TENDONS

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Tendons, dynamic connective tissues crucial for musculoskeletal function, heavily rely on well established master regulators, including Scleraxis (Scx) for homeostasis. In this study, leveraging high-throughput RNA sequencing, we profiled the transcriptomic landscape of

Scx-deficient tendons. Scx knockout prompts extensive gene expression alterations, impacting key processes related to matrix organization, tenogenesis, and collagen synthesis. Strikingly, our investigation unveils an unexpected facet—myelinated nerves in Scx knockout tendons, suggesting an intriguing interplay between Scx and neural elements within the tendon microenvironment. KEGG pathway analysis of the down-regulated transcripts reveals Wnt-related pathways. Macrostructural experiments and Cybersort deconvolution analysis confirm the presence of axons and nerve cells in Scx knockout tendons. ChIP-seq analysis of Scx binding sites accentuates its pivotal role in orchestrating fundamental tendon processes. This study places Scleraxis in the context of neural elements within tendons, unraveling novel dimensions in the intricate regulatory network. Scx regulation of neural-related processes adds a new layer in understanding the neural-tendon dynamic, offering insights into tendon dysfunction and neural underpinnings.

Funding Source: This work was supported by the National Key Research and Development Program of China (2017YFA0104902), and by the National Natural Science Foundation of China (NSFC) grants 81330041 and 31830029.

Keywords: tendon, scleraxis, transcription factor

3:45 PM – 4:45 PM

TOPIC: NEURAL

245

SUSPENSION CULTURE-DERIVED ORGANOID MODELS OF CEREBRAL CORTEX DEVELOPMENT

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Organoids are self-assembled, three-dimensional structures derived from stem cells that recapitulate various morphological and functional aspects of in vivo tissues. In recent years, neural organoids have seen multiple advances in modeling a wide range of specific brain regions and diseases. However, current protocols rely on many chemical cues and a laborious ECM-embedding method that greatly limits throughput. Here we used Wnt modulation and suspension culture to generate suspension-derived neural organoids (sNOs), which proved significantly less laborious and enabled upscaling of organoid production with a high degree of homogeneity in morphology and size. While developing a suspension culture-based cardiac differentiation protocol, we serendipitously discovered that slight changes in conditions changed differentiation outcomes and led to the formation of sNOs. Embryoid bodies (EBs) were formed 24h post inoculation in magnetically stirred spinner flasks, followed by Wnt pathway activation and then inhibition. EBs initially formed neural rosettes, a population of neural progenitor cells (SOX2+, NES+), which developed over time into neural epithelium (ZO1+) forming ventricles throughout sNOs. With prolonged culture (Day 60-100) sNOs grew into large (~5 mm in diameter), homogeneous and dense structures revealing a cortical organization, which comprised a defined layered and column-like arrangement of neural progenitor cells and mature neurons (MAP2+, GABA+, SYN1+, VGLUT1+) radiating outwards, originating from neural epithelium. Furthermore, sNOs presented with a consistent outer epithelial layer (ZO1+) resembling the arachnoid barrier cells found in human brains, which is likely the reason why our suspension protocol does not require ECM supplementation for initial creation and maintenance of sNO. Here we present a simplified yet enhanced methodology to produce scalable and reproducible sNOs.

Keywords: organoids, suspension culture, cortex development

3:45 PM – 4:45 PM

247

EFFICIENT EMBRYOID-BASED METHOD TO IMPROVE GENERATION OF OTIC VESICLES FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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The early stages of human inner ear development involve the assembly of cells emerging from various embryonic cell lineages. There is a growing need to develop in vitro models of the human inner ear to mimic both its structure and function outside the organism. However, due to a lack of human inner ear biopsies, one of the main strategies relies on recapitulating the embryonic development of the inner ear from human induced pluripotent stem cells (hiPSCs) for cell therapy, drug testing and other applications. Nevertheless, our understanding of the human early otic/placodal microenvironment where multipotent otic cell progenitors arise and develop remains limited, posing a significant obstacle to progress. To address this, we adapted a previously published protocol of otic organoids to improve embryoid body-based otic induction from hiPSCs using different culture plate formats and optimal hydrogel dilution throughout the culture, to preserve the integrity and cell viability of cells forming EBs and otic vesicle-like structures. Furthermore, using a Sox2-GFP reporter hiPSC line at different stages of differentiation allowed us tracking the progressive evolution from pluripotency to otic progenitors within emerging otic/placodal structures after exposure to specific signaling modulators. Morphological characterizations, upregulated transcript levels and protein expression of a comprehensive panel of known otic markers revealed a robust otic/placodal induction and improved otic vesicle-like structures generation between day 11 and 60 of culture. In summary, we report an adapted and efficient 3D in vitro strategy to understand the mechanisms of human otic development, and to evaluate the effects of novel therapeutic approaches for inner ear hair cell protection and regeneration.

Funding Source: Supported by the SATT, AxLR Ref N°23/0021

Keywords: induced pluripotent stem cells, otic progenitor cells, otic organoids

3:45 PM – 4:45 PM

249

MODELING SECONDARY NEURULATION BY ORGANOIDS

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Congenital syndromes affecting the lumbosacral region of the neural tube occur in approximately 1 per 1,000 births. While pluripotent stem cell (PSC)-organoid model primary neurulation, a gap exists in modeling the lumbosacral secondary neural tube (SNT). Utilizing PSC-derived self-renewing neuromesodermal stem cells (NMSCs) that are similar to SOX2 and T (Brachyury) double-positive chordoneural hinge progenitors, we developed organoids displaying features of the SNT. These organoids were characterized by a caudal-like pole exhibiting SOX2 and TBXT double-positive cells that differentiated into multiple lumens displaying PAX6, N-cadherin, and ZO-1. The opposite pole exhibited a single lumen comprising SOX2+PAX6+ neuroepithelial-like cells. The multiple lumens site displayed exclusively CDX2 expression, which is a posterior marker expressed by axial progenitors. Additionally, HOX genes have been posteriorized to HOX10-11 in these organoids. We propose a model of lumbar-sacral morphogenesis in NMSCs akin to the caudal human SNT.

Keywords: lumbosacral neural tube, secondary neurulation, organoid models

3:45 PM – 4:45 PM

251

MOUSE AND HUMAN MIDBRAIN FLOORPLATE RADIAL GLIA SUBTYPES CONTROL DOPAMINERGIC NEURON DEVELOPMENT BY DISTINCT MECHANISMS

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of midbrain dopaminergic (mDA) neurons in the substantia nigra. With neural grafting of mDA progenitors derived from human pluripotent stem cells (hPSCs) emerging as a promising treatment option, understanding mDA neuron development has become crucial for advancing PD cell transplantation therapies. Radial glia (Rgl) cells in the ventral midbrain (VM) floorplate have been proposed as potential mDA progenitors, but also as non-neurogenic signaling cells. Recently, single-cell RNA-sequencing (scRNA-seq) analysis has identified several subtypes of transient Rgl cell types in the developing mouse and human VM. In this study, we sought to answer what functions these different midbrain floorplate Rgl subtypes serve, whether their functions are redundant or highly specialized, and how they regulate mDA neuron development. To address these questions, we took advantage of unbiased single-cell and bulk RNA-seq data from the developing mouse and human VM and analyzed the transcriptome of endogenous midbrain floorplate Rgl. Our study identifies Rgl1 and Rgl3 as the two main Rgl cells contributing to mDA neuron development, with each cell type serving a specialized function. We find that the Rgl1 transcriptome is defined by a neurogenic network centered on the transcription factor Arntl/Bmal1, whereas Rgl3 expressed multiple cell extrinsic signaling factors, including growth factors, morphogens, and extracellular matrix. To experimentally validate our findings, we use human embryonic and neuroepithelial stem cells to demonstrate that cell intrinsic factors expressed by Rgl1 regulate mDA neurogenesis whereas cell extrinsic factors expressed by Rgl3 regulate diverse aspects of mDA neuron development. Our results thus suggest a role of Rgl1 as a mDA progenitor, and of Rgl3 as a signaling cell in the VM. Moreover, we identify key intrinsic and extrinsic factors that promote the generation of human mDA neurons from hPSCs, thus offering the potential for advancing PD cell transplantation therapy.

Funding Source: Swedish Research Council, Swedish Foundation for Strategic Research, European Commission, PreciseCell PD, Knut and Alice Wallenberg Foundation, Karolinska Institutet, Hjärnfonden, Cancerfonden and Chan Zuckerberg Initiative.

Keywords: dopaminergic neuron development, single-cell RNA-sequencing, human pluripotent stem cell engineering

3:45 PM – 4:45 PM

253

REPRESSION OF HERV-K NEGATIVELY IMPACTS ASTROCYTE DEVELOPMENT

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One of the major challenges towards deciphering the blueprint of life is to understand how functional elements of the genome influence key biological processes. However, the functions of large parts of the human genome including human endogenous retroviral (HERV) elements remain elusive. We could recently show that activation of one specific HERV family, namely HERV-K(HML-2), negatively impacts cortical neuronal development. However, thorough analyses of a larger number of HERV groups are missing to understand their functional contribution to the development of diverse brain cell types beside neurons. Transcriptomics analysis of post-mortem patient samples demonstrated that HERV groups are differentially expressed between astrocytes and neurons. In particular, we uncovered a substantial upregulation of several HERV-K elements in astrocytes compared to neurons. To unravel the functional contribution of HERV-K to astrocyte development, we applied CRISPR-interference targeting multiple LTRs of the HERV-K group in neuronal progenitor cells. Excitingly, inhibition of HERV-K resulted in a significant decrease in the astrocytic markers GFAP and AQP4 upon differentiation into astrocytes. In contrast, transcriptional repression of HERV-K LTRs had had no effect on neuronal differentiation. HERV-K transcriptional repression also influenced the expression of several cellular genes involved in astrocytic development. In particular, we revealed a C2H2-type ZNF as a promising downstream target, and transcriptional downregulation of the C2H2-type ZNF also resulted in impaired astrocyte development providing mechanistic insight into the identified HERV-K—astrocyte differentiation axis. In conclusion, our study unveils a previously unknown function of HERV-K in astrocyte development, with implications for brain-specific cellular differentiation. These findings contribute to a deeper understanding of the intricate regulatory mechanisms governing astrocyte differentiation and neurodevelopmental processes.

Keywords: astrocyte development, human endogenous retroviruses (HERV), transposable elements

3:45 PM – 4:45 PM

255

SMACKSEQ UNVEILS HUMAN BRAIN EVOLUTION VIA COMBINATORIAL PAIRED GENES ACTIVATION AND KNOCK-OUT IN INDUCED PLURIPOTENT STEM CELLS AND CORTICAL BRAIN ORGANOIDS

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The detailed genomic comparison of modern humans with extinct hominins reveals critical evolutionary variations, yet understanding their impact on brain development remains elusive. High-coverage genomes from our closest extinct relatives could illuminate potential phenotypic differences crucial for the human condition but the functional validation of these genetic variants has been constrained by current gene-editing technologies. We focused on high-frequency missense mutations and implemented SMACKseq, a novel CRISPR-Cas9-based technique, to target multiple genes and investigate their regulatory interactions in cortical brain organoids, revealing insights into the combinatorial genetic mechanisms driving human brain evolution. Our findings demonstrate the potential of simultaneous gene activation and knockout in elucidating complex genetic interactions, highlighting the importance of gene regulation and the effects of high-frequency segregating mutations. This study lays the groundwork for future research aimed at a comprehensive understanding of the genetic foundations of human brain development and evolution.

Keywords: human evolution, brain development, CRISPR-based methods

3:45 PM – 4:45 PM

257

HIGH THROUGHPUT SCREENING OF TRANSCRIPTION FACTORS FOR SUBTYPE-SPECIFIC NEURONAL PROGRAMMING

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The bHLH transcription factor (TF) Neurogenin-2 (NGN2) can be used to program human induced pluripotent stem cells (hiPSCs) into populations of induced glutamatergic neuronal fate (NGN2-iNeurons). Due to the simplicity of their generation and the availability of iPSCs from different genetic/disease backgrounds, NGN2-iNs provide an interesting and unique opportunity for disease modeling and drug screening in a field otherwise limited by the availability of human primary material. However, and despite recent advances in neuronal programming, NGN2-iNs are heterogenous and cannot be assigned to defined identities within the human brain at high confidence. To capitalize on their full potential as a model system, it is therefore necessary to further improve their programming. In this study, we establish a single-cell RNA-sequencing based screen to identify neuron subtypes that arise after overexpression of neuron subtype-specific TFs on top of the well-characterized NGN2-iN platform. We use lentiviral infection to integrate a doxycycline-inducible expression construct into NGN2-iN hiPSCs, which allowed us to express both NGN2 and a target TF simultaneously. Our expression construct contains a fluorescent reporter gene (GFP), a TF coding sequence (CDS) and a feature capture sequence directly downstream of TF. The feature capture sequence is compatible with 10X genomics feature barcoding technology and enabled us to simultaneously capture transcriptomes and exogenous TF identities from single cells. Using this approach, we investigated 160 TFs in pooled format and identified 25 TFs, whose overexpression results in distinct transcriptomic changes compared to control NGN2-iNs including the upregulation of noradrenergic, dopaminergic or glycinergic markers, or the gain of regionally specific glutaminergic markers such as SLC17A7. To analyze the TF induced neuronal fate, we systematically compared our data to a primary human neuron reference atlas. Our study provides a strategy for programming distinct human neuron subtypes from hiPSCs, which will greatly benefit both disease modeling as well as drug discovery. Our approach can be applied to any other cell type to identify TF combinations for subtype specific cell programming.

Keywords: neuronal programming, NGN2-iNeurons, single cell transcriptomics

3:45 PM – 4:45 PM

259

DECIPHERING GROWTH CONTROL IN THE DEVELOPING HUMAN BRAIN

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Brain development comprises neural stem cells that initially divide symmetrically to expand the neural progenitor pool before dividing asymmetrically to produce differentiated cell types. Variability in the timing of neural stem cells to shift between these division modes generates heterogeneity in neural stem cell lineages that give rise to the developing brain. Cerebral organoids have emerged as an in vitro 3D model system recapitulating important aspects of human brain development. Recent experiments in brain organoids using lineage tracing, computational modelling and scRNA-seq have demonstrated how human brain tissue development follows a dynamic growth pattern. Lineages sizes were shown to be variable in size with large lineages maintaining a pool of symmetrically dividing cells well into neurogenesis. Experiments in chimeric organoids containing both control and growth perturbed cells have additionally revealed that wildtype cells can compensate for growth defects in neighboring lineages. This indicates that neural stem cell fate is plastic and that mechanisms of tissue-mediated growth control exist in the developing human brain. We will perform a comprehensive investigation into tissue-mediated growth control in brain organoids. Chemical ablation assays in various chimeric organoids are performed to characterize the replenishment capacity of long-lasting neural stem cells at different stages of organoid development and neurogenesis. Replenishing cells will be analyzed by microscopic and transcriptomic approaches. Additionally, tissue-mediated growth control will be probed using CRISPR-based loss-of-function genetics. Taken together these experiments will shed light on mechanisms of tissue-mediated growth control in human brain organoids important for robust brain development.

Keywords: neural development, brain organoids

3:45 PM – 4:45 PM

261

DIRECT REPROGRAMMING OF NG2 GLIA TO NEURONS USING A SEQUENTIAL EXPRESSION SYSTEM

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Direct reprogramming of somatic cell fate provides the capacity to mechanistically investigate regulation of cell lineage determination during development and offers the potential to be harnessed for therapeutic uses. As neurons are post-mitotic cells that are not replaced, neurological disorders and injuries often result in irreversible damage to the central nervous system. The capacity to target an existing dividing cell for conversion to neuronal fate has been previously demonstrated. However, current approaches to direct reprogramming using forced single or multiple instructive genes do not allow for gene expression in a temporally sequential manner. This can result in low efficiency of cellular reprogramming with a loss of targeted neuronal cells. Here, we describe a novel strategy using NG2 glial progenitor cells (GPCs) for neuronal reprogramming with the aim to ultimately restore lost functionality in the damaged brain. Our study focuses on a regulatable gene expression system to control timing of key genes instructive for neuronal reprogramming, survival, and subtype specification. This novel switch expression system should more closely mimic the in-vivo sequence allowing for more robust and targeted reprogramming. Previously, we have demonstrated the successful reprogramming of GPCs into neurons with the acquisition of neuronal markers and electrophysiological properties. In this study we have implemented an improved reliable switch vector system that results in better understanding the effects of the expression duration of pioneering transcription factors on cell survival, reprogramming efficiency, and lineage specification. Our results suggest that a shorter duration of expression may lead to increase in neuronal conversion. Our approach represents a significant step forward by introducing a novel strategy for reprogramming of GPCs into neurons. The potential implications of this approach extend beyond theoretical advancements, offering a promising avenue for the development of innovative therapies for various neurological disorders and injury. The findings from our study may pave a way for future clinical applications to improve the quality of life for those affected by neurodegenerative conditions.

Funding Source: RO1 NS100514

Keywords: neuronal replacement, glial reprogramming, NG2 glia

3:45 PM – 4:45 PM

263

A SOX2 ENHANCER CLUSTER REQUIRED FOR THE ANTERIOR REGIONAL IDENTITY OF NEURAL STEM CELLS

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Embryonic development depends on spatially and temporally orchestrated gene regulatory networks. Expressed in neural stem and progenitor cells (NSPCs), the transcription factor sex-determining region Y box 2 (Sox2) is critical for embryogenesis and stem cell maintenance in neural development. Whereas the enhancers of Sox2 are well defined in early embryogenesis, little is known about Sox2 gene regulation in the neural lineage. Using functional genome data, we identify an enhancer cluster region that regulates Sox2 transcription in NSPCs derived from mouse embryonic stem cells (ESCs). By generating allelic mutants using CRISPR-Cas9 mediated deletions, we show that this proximal enhancer cluster, termed Sox2 regulatory regions 2-18 (SRR2-18), is a cis regulator of Sox2 transcription during neural differentiation. Transcriptome analyses demonstrate that loss of even one copy of SRR2-18 disrupts the anterior regional identity of NSPCs. Biallelic deletion of this Sox2 neural enhancer cluster causes reduced SOX2 protein function and perturbs chromatin accessibility genome-wide further affecting the expression of neurodevelopmental and anterior-posterior regionalization genes. Furthermore, homozygous NSPC deletants exhibit cell-type autonomous self-renewal defects and impaired differentiation into cell types found in the brain. Altogether, our data define a cis-regulatory enhancer cluster controlling Sox2 transcription in the neural lineage and highlight the sensitivity of NSPCs to Sox2 dosage which controls the anterior-posterior regionalization response.

Keywords: neural stem cells, enhancer, transcription factor

3:45 PM – 4:45 PM

265

IN VITRO DERIVATION OF VENTRAL MESENCEPHALON PROGENITORS FROM PIG PLURIPOTENT STEM CELLS

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Pigs are considered as a valuable non-primate model in neuroscience because their gyrencephalic brains more resemble human brains in anatomy, growth, and development compared to the lissencephalic brains of rodents. We adapted a floor-plate transition-based method to derive ventral mesencephalic (VM) progenitors from pig pluripotent stem cells (PSCs). This differentiation protocol includes ventralization with SHH and casualization using GSK3 inhibitor CHIR99021 (CH) and FGF8 addition. As in humans, the absence of GSK3 inhibition during pig VM patterning resulted in the upregulation of forebrain-related genes (FOXG1 and NKX2.1) and high concentration of CH (3.0 μκ) promoted expression of hindbrain markers (HOXA2 and GBX2). On the other hand, 1.5 μκ CH supplementation caused progenitors toward midbrain fate based on high transcript levels of caudal VM marker genes and the presence of EN1-positive cells at day 16. The modification of differentiation protocol using high concentration of SHH (500 ng/ml) improved pig VM identity by increasing FOXA2-positive cells and reducing PAX6 expression. Through in vitro maturation of the VM progenitors, we observed neural dendrites and confirmed a robust expression of midbrain dopaminergic (mDA) neuron-specific genes (EN1, SNCA, TH, KCNJ6, and CALB1) in differentiated cells on day 28. Furthermore, the presence of mDA markers such as TH, DAT, and Nurr1 and synaptic proteins were observed in immunostaining analysis. In this study, we established stem cell-derived pig VM progenitors for the first time and confirmed their maturation capacities. Such progenitor cells could be used to study human brain disorders such as Parkinson's diseases in combination with in vivo pig models.

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Keywords: pig pluripotent stem cells, ventral mesencephalic progenitors, midbrain dopaminergic neuron

3:45 PM – 4:45 PM

267

UNDERSTANDING MIDBRAIN DOPAMINERGIC CELL FATE ACQUISITION USING MIDBRAIN-LIKE ORGANOIDS FOR PARKINSON'S DISEASE CELL THERAPY

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Midbrain dopaminergic (mDA) neurons in the substantia nigra are preferentially degenerated in Parkinson's disease (PD), leading to motor deficits and a broad range of non-motor symptoms. As no curative treatment currently exists for PD, cell replacement therapy involving the transplantation of dopaminergic neuron precursors into patient brains represents a promising mode of treatment. Several studies have shown a range of transcriptional subtypes that exist among mDA neurons, with implications for their eventual axonal targets and synaptic function. Grafts that contain contaminating cell types such as serotonergic neurons may lead to off-target effects and reduce treatment efficacy. Using human midbrain-like organoids (MLOs) derived from human pluripotent stem cells, we define major cell types of the midbrain and delineate its developmental trajectories using single cell transcriptomics. We compare these developmental programs to the fetal midbrain and show that MLOs are able to recapitulate the cell type diversity of the midbrain seen in vivo, and are transcriptionally similar to their age-matched counterparts in the fetal brain. We also note the appearance of the A9-like dopaminergic neuronal population affected in PD, which becomes functionally mature within 6 months in culture. Through our analysis, we identify transcriptional programs that are upregulated in dopaminergic precursors. Furthermore, we transplanted dopaminergic neuron precursors derived from MLOs into substantia nigra-lesioned mice models, showing that MLOs have therapeutic potential to correct motor deficits in a mouse model of PD. These results can be used to guide stem-cell therapies of PD, and produce better quality grafts of dopaminergic neuronal progenitors.

Keywords: midbrain organoids, Parkinson's disease, cell therapy

3:45 PM – 4:45 PM

269

NOTCH SIGNALLING DYNAMICS REGULATE TRUNK NEURAL CREST FORMATION FROM NEUROMESODERMAL PROGENITORS

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The spinal cord, peripheral neurons, and skeletal muscles originate from the transiently proliferating neuromesodermal progenitors (NMPs) in embryo axis development. Generating human NMPs from pluripotent stem cells (PSCs) enable the study of signalling cascades in posterior body development. Based on self-renewing axial stem cells (AxSCs) that we derived from human PSCs, we differentiated dorsal neurons via intermediate neural tube and trunk neural crest cell populations. We activated notch signalling by inducing the notch intracellular domain fragment during AxSCs differentiation, resulting in the up-regulation of the neural crest markers SOX10, PAX3, and TFAP2A and the peripheral glial cell markers NGFR, GPR126, and TSPAN2. Furthermore, these cells resemble neural crest progenitors in morphology and migratory capacity. These findings indicate a role for notch signalling in neural crest and glial cell development and can be leveraged for embryogenesis research and modelling neurocristopathies.

Keywords: notch, neural crest, neuromesodermal progenitors

3:45 PM – 4:45 PM

271

GENERATION AND CHARACTERIZATION OF VENTRAL NEURAL TUBE PROGENITORS FROM HUMAN NEUROMESODERMAL PROGENITOR-LIKE CELLS

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A challenge in current differentiation protocols of human pluripotent stem cells towards spinal cord neurons is enriching and efficiently expanding specific domains along the dorsoventral axis. Using novel stem cell lines representing human neuromesodermal progenitors (NMPs) and their downstream neural tube progenitors (NTPs), we screened matrixes of morphogens and agonists for Wnt, Sonic Hedgehog, FGF2 and retinoic acid to identify self-renewing populations of ventral NTPs. We discovered that retinoic acid facilitates the stabilization of ventral NTPs and their self-renewal. Conversely, other conditions revealed factors that support NTP plasticity in their dorsoventral identity, enabling them to change their identity between dorsal and ventral while continuing self-renew. These findings highlight the plasticity of NMPs and their NTP progeny, providing insight into early development of the caudal neural tube which is generated from NMPs. NTPs which are stable, and yet plastic in their identity when desired could benefit disease modeling and developmental research.

Keywords: neuromesodermal progenitors, neural tube development, dorsoventral spinal cord identity

3:45 PM – 4:45 PM

273

DERIVATION OF A STABLE ISTHMIC-LIKE PROGENITOR WITH HIGH CAPACITY FOR DOPAMINERGIC NEURON PRODUCTION IN VITRO AND IN VIVO

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The isthmic organizer is a major signaling hub in the developing vertebrate brain that plays a critical role in early embryonic development. This signaling center, located at the midbrain-hindbrain boundary, orchestrates the formation of neighboring brain regions and cell types. Recently, we have succeeded in identifying and stabilizing a multipotent neural plate border progenitor, termed induced neural plate border stem cells (iNBSCs). iNBSCs can be obtained by direct reprogramming from somatic cells under specific culture conditions and without permanent transgene integration, and retain the potential to differentiate into both central and peripheral nervous system cell types. In this study, by carefully modulating the WNT-, SHH-, and FGF-signaling pathways in iNBSCs, we have successfully isolated and characterized a novel stable human neural progenitor, which expresses distinct markers of the isthmic organizer region. This cell population, termed isthmic region-like neural progenitor cells (istNPC), exhibits robust self-renewal, stable culture, and clonal growth, while expressing key isthmic region markers such as EN1, PAX2, PAX5, and FGF8. Consistent with their regional identity, istNPCs can be efficiently directed by specific cues to give rise to specific mid-hindbrain neuronal lineages, such as dopaminergic and serotonergic neurons, with high efficiency in vitro and in vivo. In addition, we demonstrate the release of dopamine and its processing products in culture, confirming the functional endpoint of differentiation. The direct conversion of human somatic cells into neural progenitors representing the isthmic region holds immense potential for studying early human neural development. In addition, this advance represents an encouraging step toward rapid and efficient production of dopaminergic neurons through direct reprogramming of neural progenitors, which may hold promise for future cell replacement therapy applications.

Keywords: neural progenitor, isthmic organizer, dopaminergic progenitor

3:45 PM – 4:45 PM

275

DEVELOPING AN IN VITRO MODEL TO UNDERSTAND THE MOLECULAR MECHANISMS OF PAEDIATRIC GLIOMA

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Diffuse midline glioma (DMG) is a lethal paediatric midline cancer lacking effective treatments. Recent studies revealed that approximately 90% of these tumours harbour the pK27M substitution mutation in histone 3 (H3). Moreover, 20% of DMG tumours are associated with mutations in both histone variant 3.1 and ACVR1 genes. While these genetic lesions primarily occur in the pons, their synergistic role in oncogenesis remains unclear. We hypothesise that the histone mutation arrests glial progenitor cells in an undifferentiated state, while mutant ACVR1 drives proliferation. To address this, I have developed a reproducible protocol to differentiate hESCs into rostral hindbrain neural progenitors and astrocytes. Ongoing research aims to fine-tune the protocol for oligodendrocyte precursor cell generation. In parallel, we have introduced the mutant histone and ACVR1 separately into hESCs and are exploring the consequence of their activation throughout differentiation. In the future, we will introduce both mutations in combination and explore the consequences of activating these mutations during differentiation. By pinpointing the cell of origin and understanding molecular mechanisms, we aim to develop targeted therapies for DMG.

Keywords: differentiation, hindbrain, glia

3:45 PM – 4:45 PM

277

EPIGENETIC REGULATION OF DEVELOPMENTAL TIMING IN HUMAN EMBRYONIC STEM CELLS

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Embryonic development follows a series of events at a particular order and speed. While the order of events is evolutionarily conserved across mammalian species, the duration of many developmental processes is species-specific, which is considerably longer in humans compared to rodents and other primates. This difference in developmental timing is recapitulated in embryonic stem cells (ESCs), suggesting a cell-autonomous timing mechanism. Cross-species comparisons have shed light on biological pathways, such as protein turnover and metabolic processes, that can account for species-specific timing of segmentation clock and neuronal development. However, it remains unclear whether these pathways constitute a general timing mechanism that is applicable throughout embryonic development, and what the genetic basis of such timing mechanism is. To understand the genetic basis, we first performed a whole-genome CRISPR knockout (KO) screen to uncover regulators of neuroectoderm differentiation speed, which identified two chromatin regulators: 1) Menin, a co-factor for H3K4 methyltransferase, and 2) core subunits of Polycomb Repressive Complex 2 (PRC2), including SUZ12 and EED. We found that Menin or SUZ12 loss-of-function accelerates expression of neuroectoderm genes during differentiation by altering the balance of H3K4me3 and H3K27me3 on gene promoters. Menin KO selectively increases H3K4me3 on developmental genes without significantly affecting H3K27me3 level, whereas SUZ12 KO erases H3K27me3 globally and induces a general increase in H3K4me3. In both cases, the shifted balance towards H3K4me3 on developmental genes leads to increased chromatin accessibility and allows for faster gene activation. To examine whether the function of speed regulation by Menin and SUZ12 is broadly applicable, we then tested differentiations in other cell types and developmental stages. We found both KOs can accelerate neurogenesis, and SUZ12 KO can also accelerate the differentiation of other germ layers. Our results highlight a deterministic role of chromatin landscape in setting human developmental timing. Future work will investigate the interplay between epigenetic regulation and mechanisms identified from cross-species studies to achieve an integrative understanding of species-specific timing.

Keywords: developmental timing, epigenetics, human embryonic stem cells

3:45 PM – 4:45 PM

279

GLYCOLYTIC METABOLISM DETERMINES GLIAL FATE IN HUMAN DORSAL ROOT GANGLION ORGANOIDS

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Dorsal root ganglion (DRG) consists of well-organized sensory neurons and satellite glia, playing essential roles in transmitting sensations of touch, pain, itch, temperature, and spatial position. Notably, multiple congenital sensory neuropathies with defective DRG formation are highly associated with metabolic abnormalities, suggesting an active involvement of carbon metabolism during DRG development. However, the specific features and the functions of metabolic regulation in developing DRG remain unclear. To establish more relevant models that closely resemble human DRGs, we generated DRG organoids(DRGOs) from human pluripotent stem cells (hPSCs). Our findings demonstrate that these DRGOs express specific lineage markers and undergo self-organization into neuron-glia ganglia, with satellite glia enveloping neuronal bodies, resembling the structure of native DRGs. During DRGO development, we identified distinct metabolic features across bipotent progenitor, sensory neuron, and glial cell stages, which is highly conserved between human DRGOs and chicken DRG. We

observed that glycolysis is highly active in DRG progenitors and glial-biased lineage and barely detectable in sensory neuronal populations. Inhibition of glycolysis in the DRG, either chemically or genetically, resulted in lineage-biased segregation, leading to an increase in sensory neurons and a decrease in satellite glia formation, both in vivo and in vitro. Intriguingly, glycolytic inhibition in glia also disrupted sensory axon outgrowth, suggesting a non-cell autonomous mechanism, indicating a non-cell autonomous mechanism, likely via metabolic support from satellite glia. All these results suggest that a delicate glycolytic activity is required in coordinating complex lineage segregation programs during DRG development.

Keywords: dorsal root ganglion organoids, peripheral nervous system, glycolysis

3:45 PM – 4:45 PM

281

DISSECTING THE ROLE OF VASCULAR LEPTOMENINGEAL CELLS (VLMCS) IN HPSC-DERIVED GRAFTS FOR PARKINSON DISEASE

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Parkinson's disease (PD) is the second most common degenerative disorder and affects more than 8.5 million people worldwide. PD is caused by the focal and selective degeneration of midbrain dopaminergic (DA) neurons. The restricted location of the pathology and the focused loss of dopaminergic innervation make PD an attractive target for cell replacement therapies. Ventral midbrain (VM) progenitors derived from human pluripotent stem cells (hPSCs) represent a scalable, renewable and readily available cell source for transplantation. Using these cells, ongoing clinical trials aim to restore physiological levels of dopamine in the striatum, which in turn would restore motor function in patients with PD. These transplants contain a heterogenous mix of cell types including DA neurons, astrocytes and vascular leptomeningal cells (VLMCs). While the therapeutic DA neurons have been extensively studied in xenografting studies using fetal and stem cell derived neurons, the occurrence and impact of other cell types in the graft has not been systematically assessed. While at least some of these non-DA cells in the graft likely contribute to graft viability and function, it is currently unknown to what degree graft cell composition affects maturation and precise phenotype of the therapeutic neurons, which are key parameters for graft functionality. We explore the specific role of VLMCs in VM-derived grafts in two ways: first, we map the heterogeneity of VLMCs and their interactions with other cell types in a preclinical xenograft model of PD and second, we engineered lineage-restricted hPSC lines devoid of the ability to differentiate into VLMCs. To this end, we selected VLMC-specific promoters to drive Cre recombinase expression. This system coupled with CRISPR-Cas9 allows us to target essential genes in cells fated to become VLMCs and thus eliminating this population for differentiated VM-patterned cultures. Using this system, we will elucidate the role of non-neuronal cell types in hPSC-derived DA grafts and determine the contribution of VLMCs to graft functionality. A better understanding of graft composition and function would potentially lead to enhanced cell replacement therapy in the future.

Keywords: vascular leptomeningeal cells, graft composition, cell replacement therapies

3:45 PM – 4:45 PM

283

NEURAL REPROGRAMMING OF HUMAN GLIA IN BRAIN ORGANOTYPIC CULTURE

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GABAergic interneurons play a key role in balancing neural circuits in the human brain. The loss or damage of interneurons is associated with an imbalance in the optimal functioning of the neural circuitry, triggering neurological disorders such as epilepsy, schizophrenia or autism. There are attempts to restore the GABAergic interneurons through cell replacement therapies with hope for restoring normal neuronal function but to generate specific interneurons has shown to be difficult in vitro. We have previously successfully generated human GABAergic interneurons with subtype specificity in vitro using direct reprogramming of glia progenitor cells (GPCs). Direct reprogramming allows convertion of a somatic cell into neurons with ectopic expression of neuronal genes and bypasses the stem cell stage and thereby provides potential for in vivo application. Here, we aim to apply our reprogramming protocol into an ex vivo system using organotypic cultures from mouse or human brain. This is an alternative to the living mouse that allow more efficient use of animals as well as a unique possibility of assessing neural integration in clinically relevant human brain tissue. To assess whether human GPCs can survive and reprogram into interneurons in an ex vivo system, they were transplanted into mouse organotypic cortical cultures and kept for up to 4 weeks. Preliminary data shows that adult mouse tissue was kept viable without detection of apoptosis for up to 4 weeks. Moreover, the human glia survived and migrated into the culture in both adult (15 weeks) and pup tissue (P10). Ongoing experiments are evaluating whether the human glia can reprogram into interneurons in situ using doxycycline inducible system. The long-term goal is to assess if human reprogrammed interneurons can synaptically integrate and mature in mouse and/or human organotypic cultures. Interneuron in vivo reprogramming represents a promising approach for cell replacement therapy, offering potential to restore neural circuitry and improve patient outcomes in interneuron-related diseases.

Funding Source: Knut and Alice Wallenberg Foundation and Swedish Research Council.

Keywords: glia progenitor cells, interneurons, organotypic culture

3:45 PM – 4:45 PM

285

MORPHOLOGICAL DYNAMICS OF BASAL RADIAL GLIA CELLS IN HUMAN BRAIN ORGANOIDS

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The cerebral cortex, and particularly its evolutionary most recent part, the neocortex shape the foundation of higher cognitive functions in the human brain. The overall expansion of the human neocortex is guided by the higher proliferation capacity of a specialized subtype of neural progenitors called basal or outer radial glia cells (bRGs). Morphologically these cells can be classified into six different morphotypes, based on the number and apico-basal orientation of their processes. A dominant morphological feature of bRGs are long radial processes that extend to the cortical plate of the developing neocortex. The radial processes are linked to the proliferative capacity of bRG as they underlie the interaction of bRG with their surrounding extracellular environment. Furthermore, radial processes act as a scaffold structure for neuronal migration. However, the dynamics of bRGs morphology have been poorly assessed since the abundance of this cell type is strikingly low in the key animal model, the mouse. Here we show that human brain organoids derived from human pluripotent stem cells recapitulate key aspects of cortical development, allowing for the observation of bRG dynamics in a more controlled and accessible experimental setting. We show that human brain organoids recapitulate the distribution and the morphological heterogeneity of bRGs observed in human fetal tissue. Additionally, by utilizing advanced imaging techniques, we investigate the spatiotemporal dynamics of bRG morphotypes, emphasizing their distinctive features, with the aim to understand their contribution to the overall neuronal output in human brain development. The implications of these findings can potentially extend to the study of neurodevelopmental disorders, as deviations in bRG morphology have been associated with conditions like microcephaly.

Keywords: brain organoids, neural progenitor cells, neurodevelopment

3:45 PM – 4:45 PM

287

DEFINING THE TRAJECTORY OF VASCULAR PROGENITOR CELLS IN HUMAN BRAIN DEVELOPMENT

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Brain blood vessel cells play key roles in both physiological and pathological states. Recent studies focused on the adult human and mouse brain have described subtypes of brain vascular cells. However, there are critical gaps in our understanding of the origin and stages of vascular cell maturation, particularly in the human brain. We used Fluorescence Activated Cell Sorting (FACS) and single-cell transcriptomics to define endothelial and mural cell subtypes in the human brain during the second trimester. To determine their putative developmental trajectories, we performed RNAvelocity analysis, which revealed smooth muscle cells as potential mural stem cells which give rise to classic pericytes. To test their potential in vitro, we performed FACS and cell culture comparing smooth muscle cell mural cells to other subtypes. We identified potential cell surface markers for smooth muscle cells to purify them with FACS; we then purified smooth muscle cells and classic pericytes and cultured them on collagen in smooth muscle cell media or pericyte media. After 1-2 weeks in culture, we performed immunocytochemistry to determine the mural cell subtypes. Cells purified as smooth muscle cells gave rise to smooth muscle cells and other cell types over time, but pericytes did not have the ability to produce many smooth muscle cells. These preliminary results are consistent with our hypothesis that the smooth muscle cells are the progenitors that give rise to classic pericytes. To define the spatiotemporal dynamics of human brain mural stem cells, we then performed HiPlex RNAscope with twelve RNA probes, including smooth muscle cell, pericyte, fibroblast, and mitotic markers. Preliminary results show expression of pericyte markers at the ventricular wall in 21GW human brain, and smooth muscle cell markers are less abundantly expressed, mainly in larger blood vessels in the deep brain structures. Surprisingly, the smooth muscle cell markers are not expressed at 15GW. The results indicate that smooth muscle cell markers may be lowly expressed at earlier gestational ages, or that the mural cell progenitor may be an as-yet undefined cell.

Funding Source: NIH K08 NS116161, California Institute of Regenerative Medicine (CIRM) Bridges Science Master's Fellowship (SFSU EDUC2-12693).

Keywords: nerurovasculature, smooth muscle cells, lineage tracing

3:45 PM – 4:45 PM

289

THE PATHOGENESIS OF COMMON GJB2 MUTATIONS ASSOCIATED WITH HUMAN HEREDITARY DEAFNESS IN MICE

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Mutations in the GJB2 gene, specifically the 35delG and 235delC mutations, are the most prevalent genetic causes of non-syndromic hereditary deafness in humans. Despite the homozygous lethality of Gjb2 mutations in mice, there are currently no perfect mouse models that accurately replicate Gjb2 mutations from patients, hindering the study of human hereditary deafness and the unraveling of its pathogenesis. In this study, we successfully created heterozygous Gjb2+/35delG and Gjb2+/235delC mutant mice using advanced androgenic haploid embryonic stem cell (AG-haESC)-mediated semi-cloning technology. These mice exhibited normal hearing by postnatal day (P)28. To further explore the role of Gjb2, we first established a Gjb235delG/35delG embryonic stem cell line from the inner cell mass of the blastocyst. Then, we generated Gjb2 35delG homozygous mice through enhanced tetraploid embryo complementation. This model revealed the indispensable role of GJB2 in mouse placenta development, with mice experiencing profound hearing loss similar to human patients by P14, shortly after the onset of hearing. Mechanistic analyses indicated that the Gjb2 35delG mutation disrupts the formation of intercellular gap junction channels, impacting the mechanoelectrical transduction of the cochlea without affecting the survival and function of hair cells and supporting cells. Our technical advancements allowed the successful construction of heterozygous 35delG and 235delC mice, providing valuable models for studying hearing function in heterozygous carriers. The significance of this study lies in the generation of Gjb2 35delG homozygous mice through tetraploid complementation, faithfully reproducing the phenotypes of DFNB1A-related human hereditary deafness and shedding light on the pathogenesis of the disease. Overall, our research offers ideal mouse models for comprehending the pathogenic mechanisms of DFNB1A-related hereditary deafness, opening new avenues for investigating potential treatments for this condition.

Keywords: tetraploid embryo complementation, hereditary deafness, hair cell development

3:45 PM – 4:45 PM

291

EXPLORING THE ROLE OF PROLACTIN IN MURINE PLURIPOTENCY AND NEURAL DIFFERENTIATION: INSIGHT INTO EARLY DEVELOPMENT AND CORTICOGENESIS

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Prolactin (PRL), a hormone with over 300 physiological functions, has been implicated in the regulation of maternal behavior and neurogenesis. Despite its roles in adults, its potential involvement in early development remains poorly understood. This study aimed to investigate the effect of PRL on murine pluripotency and its differentiation towards the cortical lineage in vitro. Initially, the presence of the PRL receptor (PRLr) in mESCs cultured under serum-free conditions promoting ground pluripotency was assessed, compared to serum conditions allowing the coexistence of ground and primed states. A decrease in PRLr expression in serum-cultured cells was observed, possibly related to population heterogeneity. Subsequently, the dynamics of PRLr expression for 144 hours after removing pluripotency maintenance conditions were monitored, revealing a shift in receptor distribution from membranal/cytoplasmic to cytoplasmic/nuclear. Furthermore, PRL treatment in pluripotent cells did not maintain the population of Nanog+ cells, unlike Oct4+ and Sox2+ cells, which remained similar to the control group at concentrations of 0.2 and 0.4nM of the hormone. On the other hand, PRLr expression was detected during all stages of the differentiation. Co-localization of Oct4+, Nestin+, Tuj1+ and NeuN+ positive cells with PRLr throughout immunofluorescence was observed. We did not observe any effect of PRL on neural stem cells proliferation. However, an increase in immature neurons (Tuj1+) and mature neurons (Map2+) was observed with concentrations of 6 and 2nM of PRL, respectively. Additionally, we observed an increase in Tbr1 and NeuN-positive cells with concentrations of 0.2nM and 6nM, respectively. This effect was particularly evident when PRL was administrated during early differentiation. Moreover, neurons treated with 6nM of PRL exhibited enhanced dendritic complexity and increases distance from the soma, suggesting potential effects on neuronal morphology. Overall, our findings provide insight into the roles of PRL and its receptors in murine pluripotency and neural differentiation, suggesting potential implications for early development and corticogenesis. These results contribute to advancing our understanding of the regulatory mechanism governing stem cells biology and neurodevelopment.

Funding Source: INPer 212250-3230-21214-03-16.

Keywords: prolactin, stem cells, cerebral cortex

3:45 PM – 4:45 PM

293

GENOME-WIDE SCREENING REVEALS ESSENTIAL ROLES FOR HOX GENES AND IMPRINTED GENES DURING NEURONAL DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

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Imprinted genes are expressed in uniparental fashion and their transcription is controlled by differentially methylated regions. Genomic imprinting regulates early human development and is involved in multiple disorders. Previous works from our lab discovered that human embryonic stem cells (ESCs) that contain either only paternal epigenome (androgenetic ESCs) or only maternal epigenome (parthenogenetic ESCs) have unbalanced contribution to the different organs, and the maternal epigenome is essential for the proper development of the brain. We have recently generated a loss-of-function library in haploid human embryonic stem cells (hESCs) by targeting 18,000 protein coding genes with over 180,000 sgRNAs. Using this library, we identified essential genes for the normal growth and survival of hESCs and their differentiation into the embryonic germ layers. To uncover the role of imprinting in early human development we utilized the genome-wide loss-of-function library which we differentiated into neurons and established the essentialome of imprinted genes during neurogenesis, that maternally expressed genes are non-essential in pluripotent cells and their differentiated germ layers, yet several imprinted genes are essential for neuronal development. These include Beckwith-Wiedemann syndrome and Angelman syndrome related genes, for which we suggest a novel regulatory pathway. In addition, we found that in addition to imprinting disorders, a large group of neurological conditions, including neurodegenerative disorders, manifest early neuronal differentiation phenotypes. Moreover, the essential transcription factors that we identified include several HOX genes demonstrating synergistic roles and surprising non-redundant functions for HOXA6 and HOXB6 paralogs during neuronal differentiation. Overall, our work identifies essential pathways for neuronal differentiation and stage-specific phenotypes of neurological disorders, clarifying the effect of maternal genes in neuronal development and neural imprinting disorders and aiding in the identification of novel therapeutic agents.

Keywords: parental imprinting, neuronal differentiation, genome-wide CRISPR screening

3:45 PM – 4:45 PM

295

IN-VITRO GENETIC PERTURBATION OF HUMAN NEURAL PROGENITOR CELLS USING CRISPRI

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Subtle genomic and epigenomic differences between humans and nonhuman-primates (NHP) are enough to translate into remarkable cognitive differences. Human brains have higher complexity, connectivity and proliferative capacities that facilitate neocortical expansion. Nonetheless, the study of the genetic modulation behind these differences has been challenged by ethical, legal, and methodological limitations. Here, we attempted to surpass these limitations by using state-of-the-art technologies like neural progenitor cells (NPCs) derived from human induced pluripotent stem cells (hiPSCs), and gene perturbation. Especifically, the aim was to derive functional and inducible dCas9-KRAB NPCs from iPSCs, using small molecules and knock down the expression of NPAS3, ARHGAP11B, and FOXO3, all previously shown to be upregulated in human neurodevelopment compared to NHP. Candidate genes were knocked down in hNPCs with Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi), a gene editing variation that represses gene expression with the binding of dCas9-KRAB to the Transcription Start Site of the gene targeted. We show that the knock-down of FOXO3 affects the expression of genes related to autophagosome biogenesis and mitophagy. Furthermore, it accelerates neurogenesis, possibly through ASCL1 desinhibition. The findings of this project will not only contribute to unraveling the mechanisms behind the specializations of the human brain, but can have implications in disease modeling, regenerative medicine, and aging processes.

Funding Source: Austrian Science Fund (FWF, I 4791-B, I 5184, TAI 801, and SFB F7810)

Keywords: neurodevelopment, neurobiology, CRISPR

3:45 PM – 4:45 PM

297

RETINAL ORGANOIDS: NEW TOOLS TO STUDY RETINAL DYSTROPHIES AND DEGENERATION

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Millions of people worldwide suffer from retinal degeneration and blindness. Exploring novel therapies for retinal diseases is pivotal, but mice are nocturnal animals with poor visual acuity and have a rod-dominated retina without macula. Therefore, new 3D in vitro models as retinal organoids are needed. iPSCs serve as fundamental starting material for retinal organoid generation. We compare two previous published protocols, AMASS and CYST, for their efficacy in producing retinal organoids. In the AMASS protocol, a single-cell suspension initiates embryoid body formation in microwells, while the CYST protocol involves the formation of cell aggregates floated in Matrigel to form cysts with a single lumen. Following initial culture, embryoid bodies are adhered to Matrigel-coated plates, while cysts are plated on a Matrigel-coated plate and after a week epithelial cell sheets were detached using dispase. Isolation of retinal neuroepithelium in the AMASS protocol by scraping and epithelial domains in the CYST protocol by manual isolation occurs at specific time points, guided by the development of retinal structure. Both protocols employ retinal differentiation medium to induce retinal differentiation until day 200. Five retinal organoid (RO) batches of each method were generated and compared. Bright-field images of ROs were acquired daily to monitor growth. Histological analyses including H&E staining and immunofluorescence were performed on specific time points. AMASS generated ROs expressed OTX2 and PAX6 during neuroepithelium stage on day 36. By day 82, PAX6 expression persisted, while CRX emerged. On day 111, CRX and OPN1SW were detected, while NRL and RHO were only weakly expressed. By day 130, CRX, NRL and OPN1SW showed strong positivity, while RHO barely detectable. In contrast, CYST-generated ROs e showed OTX2 and PAX6 expression at day 25. CRX was observed by day 80, whereas NRL remained undetectable. AMASS and CYST retinal organoids were successfully developed from iPSCs. Data indicate that the two protocols differ during development. With that, degeneration of the retina induced with cytokines and to investigate Müller glial cell response can be studied in 3D.

Keywords: retinal organoids, retinal dystrophies and retinal degeneration, cytokines

3:45 PM – 4:45 PM

299

DEVELOPMENT AND CHARACTERIZATION OF 3D MICROFLUIDIC FABRICATED LAMINATED CORTICAL NEURONAL TISSUE

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We are interested in developing pre-assembled 3D-layered human neuronal cortical constructs that can be used to study human cortical development and regeneration. We hypothesize that the laminated pre-assembled constructs with multiple cortical neuronal cell types may provide a better understanding of the neural circuit formation within the human cortical column. Firstly, we established a protocol to generate human iPSCs-derived neurons with upper- and lower-cortical layer characteristics. The efficacy of differentiation was assessed by gene expression profiling and immunohistochemistry. Moreover, spontaneous activity of the neuronal network was measured with Ca2+ imaging, and subsequent analysis of Ca2+ activity showed distinct neuronal activity patterns of iPSCs derived upper- and lower-layer cortical neurons. Additionally, many neurons with glutamatergic and a few with GABAergic phenotypes were identified in the culture. These responded to the application of neurotransmitter blockers indicating the presence of inhibitory post-synaptic currents (IPSCs) and excitatory post-synaptic currents (EPSCs), respectively. Subsequent implantation of the differentiated lower- or upper-cortical neurons into the cerebral cortex of p8 mice demonstrated layer-specific axonal projections, with the lower-cortical neurons projecting into sub-cerebral regions and the upper-cortical neurons projecting to the cortex and striatum. We used a microfluidic technique to fabricate two-layered 3D assembles comprising distinct upper- and lower-layer cortical neuronal compartments. These 3D assembles showed long-term survival, proliferation, and differentiation into layer-specific fates in vitro. Through analysis of Ca2+ activity of these layered tissues, two modules have been identified. The nodes belonging to both modules were distributed through the entire layered construct with some hubs integrated into most of the subnetworks suggesting the organization of complex and interconnected neural networks within the layered construct. These 3D structures formed of human iPSCs-derived neurons provide a promising avenue for anatomical and functional studies of developing human cortical circuits and may be used in the future as bioengineered tissue implants for traumatic brain injury recovery.

Funding Source: Oxford Martin School

Keywords: iPSCs-derived neurons, neuronal circuits, tissue engineering

3:45 PM – 4:45 PM

301

CHARACTERIZING A NOVEL HUMAN ARCUATE NUCLEUS DIFFERENTIATION PROTOCOL USING SINGLE-CELL RNA SEQUENCING

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The hypothalamus, located at the interface of peripheral and neural inputs, maintains fundamental life processes, including energy intake and expenditure, through a collection of anatomically distinct neuronal populations named hypothalamic nuclei. With the increasing prevalence of metabolic disorders, particular attention has turned to the tuberal region of the hypothalamus, which contains appetite-regulating POMC and AGRP neuronal subtypes within the arcuate nucleus (ARC) along with nutrient-sensing glial-like cells tanycytes. In this study, we apply single-cell and single-nuclei RNA sequencing at different developmental stages to characterize a novel differentiation protocol to generate ARC cultures from human embryonic stem cells. The time-course analysis revealed that our ARC progenitors upon maturation gave rise to tanycytes as well as AGRP, PNOC, and GHRH neurons, and multiple POMC neuron subtypes. We further identified favorable conditions for maturation of the progenitors towards either tanycytes or neuronal lineages, and we found that the tanycytes in the cultures functioned as the primary responders to FGF1 – a growth factor known to induce sustained remission from hyperglycemia in diabetic rodents through actions on the hypothalamus. Single-cell reference mapping to a human hypothalamic scRNAseq dataset confirmed that the cell populations in our ARC cultures closely resembled their in-vivo counterparts, making these cultures a valuable platform for developmental lineage studies, disease modeling, and drug screening.

Keywords: scRNA-seq, hypothalamus, transcriptomics

3:45 PM – 4:45 PM

303

SPATIALLY RESOLVED SINGLE-CELL ANALYSIS REVEALS EARLY PRENATAL SPECIFICATION OF HUMAN CORTICAL LAYERS AND AREAS

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The human cerebral cortex, essential for advanced cognitive functions, comprises six distinct layers and dozens of functionally specialized areas. These structures are defined both molecularly, by diverse neuronal and glial cell subtypes, and structurally, through intricate spatial organization. Although single-cell transcriptomics studies have advanced molecular characterization of human cortical development, a critical gap exists due to the loss of spatial context during cell dissociation. Here, we utilized multiplexed error-robust fluorescence in situ hybridization (MERFISH), augmented with deep-learning based single-cell segmentation, to examine the molecular, cellular, and cytoarchitectural development of human fetal cortex with spatially resolved single-cell resolution. Our extensive spatial atlas, encompassing over 16 million single cells, spans nine cortical areas across four time points in the second and third trimesters. We uncovered the early formation of the six-layer structure, identifiable by layer-restricted distribution of excitatory neuronal subtypes in mid-gestation, long before the emergence of cytoarchitectural layers. Notably, while most cortical areas displayed a gradient-like distribution of neuronal subtypes along the frontal-occipital axis, a striking exception was found at the sharp molecular border between the primary and secondary visual cortices at gestational week 20. At this distinct border, we discovered an abrupt binary shift in neuronal subtype specification, challenging the notion that continuous morphogen gradients dictate mid-gestation cortical arealization. Furthermore, incorporating single-nuclei RNA-sequencing and in situ whole-transcriptomics analysis, we elucidated the signaling pathways and gene regulatory networks underpinning the highly localized post-migratory specification in the primary visual cortex. Collectively, our findings underscore the pivotal roles of spatial organization in directing the molecular specification of cortical layers and areas. This work not only provides a valuable resource for the field, but also establishes a spatially resolved single-cell analysis paradigm that paves the way for building a comprehensive developmental atlas of the human brain.

Keywords: cerebral cortex development, spatial transcriptomics, single cell analysis

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

305

MECHANISMS BEHIND THE FAILURE TO DIFFERENTIATE TO RETINAL PIGMENT EPITHELIUM BY ANEUPLOID HPSC

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Age-related macular degeneration (AMD) is a significant global health challenge, persistently ranking as a primary cause of visual impairment and blindness among the aging population. One promising therapeutic strategy involves retinal pigment epithelium (RPE) transplantation using cells derived from human pluripotent stem cells (hPSCs). However, clinical concern arises due to hPSCs's susceptibility to genomic instability and the potential oncogenic hazards associated with transplanting aneuploid hPSC-derived cells. Despite this, the functional consequences of these genetic variants remain inadequately characterized, and a precise assessment of the associated risk in both research and clinical contexts is currently lacking. In this study, we discovered that two recurrent abnormalities—loss of chromosome 18q (hPSCdel18q) and gain of chromosome 20q (hPSCdup20q) exhibited decreased differentiation potential into RPE. Time-course gene-expression analysis during the onset of differentiation and following 5 weeks revealed that while hPSCdup20q do not activate the eye field transcription program, hPSCdel18q exhibited delayed progression towards the RPE fate. To gain deeper insight into the mis-specified cell types associated with these genetic abnormalities, we conducted single-cell RNA sequencing. Our analysis identified a potential misspecification of hPSCdel18q and hPSCdup20q into amnion cells and immature neuron-like cell types. Interestingly, we found cell populations retaining pluripotent characteristics. We tested the modulation of the WNT, BMP and Activin/Nodal/TGFβ pathways and found that administering SB, an Activin/Nodal/TGFβ signaling pathway inhibitor during the initial 10 days of treatment enhanced RPE yield in these aneuploid cell lines, but does not appear to fully correct the phenotype. This finding suggests that the Activin/Nodal/TGFβ pathway might contribute to impaired RPE differentiation in mutant cell lines. Finally, competition assays revealed that hPSCdup20q retain their growth advantage during differentiation, leading to the enrichment of hPSCdup20q during RPE differentiation when co-cultured with their isogenically balanced counterpart.

Keywords: pluripotent stem cells, aneuploid, retinal pigment epithelium

3:45 PM – 4:45 PM

307

TRANSCRIPTOMIC ANALYSIS OF HIPSCS CULTURED IN HOMEMADE, WEEKEND-FREE CULTURE MEDIA

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High quality and reproducible hiPSCs culture is key for several applications such as investigating the mechanisms of differentiation in various cell types. Experimental results are influenced by the media used, which has proved to have an impact on morphology, metabolism, and cell cycle. To better define these differences, we compared the transcriptomic profile of the characterized WTC11 male healthy donor hiPSCs line grown in commercial Essential 8 (cE8) and in two different in-house media: homemade Essential 8 (hE8) and the recently developed B8 formula (which substitutes TGFβ-3 instead of TGFβ-1 and adds NRG1). For both hE8 and B8, we adopted a commercially available source of animal-free thermostable FGF2: a 145 amino acid truncated variant of FGF2-G3 (patented for commercial use by Enantis/Masaryk University). We performed bulk RNA sequencing on three biological replicates of hiPSCs adapted to culture in each medium for five passages. Principal component analysis clustered the replicates for the three media, confirming culture and data reproducibility. We performed gene ontology and gene set enrichment analyses for differentially expressed genes. Cells grown in B8 appear to be primed towards mesoderm as indicated by upregulation in heart, muscle, blood vessels, and kidney developmental processes. On the opposite side, cE8 hiPSC seem to be primed for neuronal development, as their transcriptomes are instead enriched for genes involved in neurogenesis, nervous system processes, and neurotransmitters secretion; molecular function enrichments confirm a higher expression of ion channels. hE8 grown hiPSCs appear to be unbiased. By analysing the pluripotency signaling pathways we found a significant upregulation of Nodal and BMP4 in B8, and of Wnt signaling in cE8 and hE8, that overall seem to have the most comparable transcriptomic profiles. We also analysed the differences in cell cycle propensity by calculating the score of each phase using marker genes; cells grown in B8 seem to have a lower rate of proliferation. These results were also extended by proliferation assays carried out with the Click-iT EdU kit. Concluding, while cE8, hE8 and B8 all maintain pluripotency, they induce subtle changes in transcriptomes that are reminiscent of epiblast regionalization and could influence downstream applications.

Keywords: hiPSCs, medium optimisation, bulk RNA sequencing

3:45 PM – 4:45 PM

309

EXPLORING THE ROLE OF RNA MODIFICATIONS ON TRANSPOSABLE ELEMENT CONTROL

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Transposable Elements (TEs) are repetitive DNA sequences that comprise a significant part of eukaryotic genomes and have evolved as regulatory elements capable of shaping the host´s transcriptional landscape. Rigorous transcriptional regulation of TEs is a crucial process, as derepression and mobilization of these repetitive elements can lead to detrimental effects including cancer, and autoimmune diseases. Besides the role of epigenetic modifications in TE control, the novel regulatory layer encompassing RNA modifications (a.k.a. the epitranscriptome) emerges as a key player involved in TEs regulation. While the role of N6-methyladenosine (m6A) in the posttranscriptional control of repetitive elements has been previously investigated, the contribution of the 5-methylcytidine (m5C) remains largely unexplored. Here, by employing genome-editing techniques (CRISPR-Cas9), we performed loss-of-function assays on m5C RNA methyltransferases in embryonic stem cells (ESCs). By combining in silico approaches with Next Generation Sequencing (RNA-seq and BS-seq) and mass spectrometry techniques, we analyzed the transcriptional and epitranscriptomic dynamics of TEs upon m5C loss in pluripotent cells. Our study highlights a novel epitranscriptomic mechanism involved in TE control, expanding the functional repertoire of m5C RNA modification.

Keywords: transposable elements, epitranscriptomic, methylation

3:45 PM – 4:45 PM

311

ESTABLISHMENT AND MULTI-OMICS OF HUMAN AXIAL STEM CELLS

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Axial elongation is orchestrated by neuromesodermal progenitors (NMPs) and their descendants. By mimicking the signaling environment of the stem-zone region where NMPs and other axial progenitors reside, we have established two types of axial stem cells (AxSCs) showing no teratoma formation. Dual activation of WNT and FGF, and inhibition of TGF-β signaling (CFS condition), were utilized to generate NM-AxSCs resembling NMPs, while exclusion of FGF activation (CS condition) induces N-AxSCs corresponding to posterior dorsal neural tube progenitors. Both types of AxSCs sustain their self-renewing activity and identity confirmed by single-cell transcriptomics and proteomics. NM-AxSCs are capable of producing dorsoventral spinal cord neurons, neural crest, and myocytes. Mouse NM-AxSCs established by the CFS condition further confirmed the potential of these cells by integrating into neural tube and somites. N-AxSCs are able to differentiate into dorsal spinal cord neurons and neural crest. AxSCs unveil ontogeny and promises modeling and therapy without transgenesis in neuropathies.

Keywords: axial stem cells, neuromesodermal progenitors, posterior development

3:45 PM – 4:45 PM

313

A COMPUTATIONAL TOOLKIT FOR SINGLE CELL ANALYSIS OF MOUSE EMBRYOS AND IN VITRO EMBRYO MODELS

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Embryo models, ranging from embryoids to synthetic embryos, are yielding mechanistic insights many developmental phenomenon. In this context, single cell omics has primarily been used to characterize the broad transcriptional resemblance of models to in vivo embryos. However, if appropriate computational tools and resources were available and easy-to-use, more questions could be readily addressed including those concerning cell type composition, lineage fidelity, and the identity of genes and pathways that differ between models and their in vivo counterparts. However, no robust and easy-to-use tool exists that would allow researchers to readily answer these and related questions. To fill in this gap, we have: - Refined and optimized the annotation of mouse gastrulation scRNA-seq to facilitate cross-study comparison - Made our species-independent cell-typing method SingleCellNet compatible with Scanpy and Anndata - Added visualization functionality that displays a sample's cell type composition and quality in comparison to reference in vivo embryos - Added functionality to identify genes and pathways that track with or diverge from in vivo embryos - Curated genes sets relevant to embryonic development, including ChIP-Seq defined targets of signaling pathway effectors Our easy-to-use Python package allows for powerful analyses. For example, we assessed the extent to which two embryo models (and several flavors of each) vary in cell type composition and lineage fidelity. This led to a general observation that virtually all models generate a substantial proportion of cells that lack a well-defined transcriptome as compared to those of the in vivo embryo. Because these cells modestly resemble some embryonic lineages, they may inflate one's impression of the overall fidelity of embryo models using standard scRNAseq integration methods. Using our toolkit, we show that these cells are distinct in signatures of specific developmental processes and are therefore unlikely to reflect technical artifacts. Moreover, in some cases we have identified downstream targets of canonical signaling pathways that distinguish these cells and thus point the way for strategies to enhance their transcriptional fidelity.

Funding Source: R35GM124725

Keywords: embryo models, single cell omics, computation

3:45 PM – 4:45 PM

315

DNA HYPOMETHYLATION DIVERTS LINEAGE TRAJECTORIES IN 3D GASTRULOIDS

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Epigenetic chromatin modifications such as DNA methylation are important during mammalian embryonic development for reinforcing lineage-specific gene expression as cells differentiate. The pluripotent, self-renewing state of cultured mouse embryonic stem cells is surprisingly tolerant to changes in epigenetic regulation. However, when cells start to differentiate, loss of DNA methylation becomes incompatible with healthy embryonic development. The question remains both when and why do cells become dependent on DNA methylation during development. Stem cell-derived in vitro models have enabled a shift away from the requirement of real embryos to study these transitions —for example, three-dimensional gastruloid models can mimic aspects of mouse development, recapitulating many attributes of axial tissue patterning. We used this system to investigate the loss of DNA methylation using chemical inhibition and transcriptional depletion of maintenance methyltransferase enzyme DNMT1. Similar to their in vivo counterparts, hypomethylated gastruloids exhibit developmental delay reflective of the arrest observed around the gastrulation stage in DNMT1-mutant embryos, and fail to establish consistent symmetry-breaking and tissue patterning. Expression analysis reveals persistence of pluripotency gene networks and mis-regulated Hox gene expression in addition to activation of DNA methylation-dependent genes. Unexpectedly, we also find signatures indicative of both primitive and definitive erythropoiesis in later-stage hypomethylated gastruloids, which are absent in gastruloids with wild-type methylation. Our data provide insight into the processes that are perturbed during differentiation in the absence of DNA methylation, as well as an indication of which lineages are most tolerant of global hypomethylation. This provides an opportunity to better understand the development of these lineages and the shift towards dependence on DNA methylation during embryo progression.

Funding Source: BBSRC Eastbio DTP

Keywords: DNA methylation, differentiation, gastruloid

3:45 PM – 4:45 PM

317

EXTRACELLULAR MATRIX MICROENVIRONMENT COLLABORATES WITH CELL MECHANICS TO GUIDE EARLY EMBRYONIC CELL FATE SELECTION

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Embryogenesis is a self-governed process of sequential cell fate transitions controlled by the interplay of morphogenesis and cell fate transitions, leading to the emergence of tissue shapes and patterns. The gene regulatory networks governing early embryonic fate transitions are relatively well understood, but how they are coordinated with cell and tissue shape transformations remain unclear. This project aims to understand the mechanisms of this coordination by addressing two fundamental open questions: 1) How do individual embryonic cells sense their position and couple this to fate selection? 2) What mechanisms relay the information of location to the transcriptional machinery? By combining various in vitro models of human gastrulation with quantitative imaging and sequencing, we show that the biochemical composition of the extracellular matrix impacts early lineage decisions. The signals from the microenvironment are relayed by the contractile actomyosin cytoskeleton to impact the mechanical deformation of the nucleus. Further, we observe that transient nuclear deformation directly causes changes in the transcriptional potential of cells with long lasting effects on epigenetic landscape. Together, this study establishes a link between physical forces and biochemical signalling for coordination of mammalian embryogenesis.

Keywords: cell fate decision, nuclear mechanics, extracellular matrix

3:45 PM – 4:45 PM

319

THE RIGHT CONTACTS MARK THE MOST EFFICIENT ROUTE TO PLURIPOTENCY - REVEALED BY SINGLE CELL CHROMATIN DYNAMICS

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In multicellular organisms, somatic cell identity is determined during development and remains resilient to change unless challenged by disease or injury. Remarkably, the over expression of the Yamanaka transcription factors Pou5f1 (Oct4), Sox2, Klf4, and c-Myc (OSKM) can convert cells from a somatic to pluripotent identity albeit a low efficiency of ~5% in two weeks. Using a rationally designed combination of small molecules that modify the somatic epigenome, to resemble that of pluripotent cells, we increased mouse reprogramming efficiency to ~42% in 6 days. We performed scATAC-seq analysis on a timecourse of reprogramming and found that AP1 motif accessibility is consecutively repressed while that of OCT4 and ESRRB is activated to enable pluripotency. Upon withdrawal of exogenous OSKM, the trophoblast master regulator TCFAP2C and its motifs are exclusively enriched in cells that successfully complete reprogramming in contrast to TEAD motifs that are enriched in cells that revert to the somatic state. High efficiency reprogramming to bonafide iPSCs rapidly opened regions that are enriched in KLF4 motifs, and CTCF associated motifs MAZ and PATZ1 that are central hubs for long range 3D- chromatin interactions. Therefore, we developed a versatile computational algorithm called scCisInt, that uses non- negative matrix factorization to define subclusters of cells and identifies cis elements whose accessibility are predictive of cluster-aggregated promoter accessibility. Using scCisInt we identified a set of long-range enhancers, marked by H3K27ac, that switch the promoters that they control in temporally distinct clusters. Using CRISPRi to inactivate these enhancers revealed an essential role for TCFAP2C in activating a miRNA290 cluster in moving poised cells to the pluripotent state and preventing reversion to the somatic state. Taken together we find that direct route to pluripotency requires long range chromatin reorganization orchestrated by a trophoblast transcription factor.

Keywords: scATAC-seq, reprogramming to iPSCs, computational method 3D interactions

3:45 PM – 4:45 PM

321

GENERATION AND VALIDATION OF SOX17-MKO2 REPORTER HUMAN EMBRYONIC STEM CELL LINES

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SOX17 (SRY-Box Transcription Factor 17), a member of the family of SOX (SRY-related HMG-box) transcription factors, plays important roles in the regulation of embryonic development and in the determination of the cell fate, such as definitive endoderm development, cardiovascular and hematopoietic development, primordial germ cell development, etc. Pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs), somatic cell nuclear transfer derived ESC (SCNT-ESCs) and induced pluripotent stem cells (iPSCs), have the abilities to self-renew and to differentiate into all cell types of the body. PSCs expressed fluorescent reporter protein are highly useful for visualization, monitoring and isolation of cells during various biological processes. In this study, we made in the lentiviral vector containing SOX17 promoter and monomeric Kusabira-Orange 2 fluorescent protein (mKO2), and transduced into human embryonic stem cell lines, CHA-hES 6 and CHA-hES 15. Established CHA-hES 6-SOX17-mKO2 and CHA-hES 15-SOX17-mKO2 cells showed normal karyotype and pluripotency. When we differentiated SOX17-mKO2 hESCs into definitive endoderm and primordial germ cell lineage, we observed the reporter mKO2 expression. Also, differentiation into pancreatic precursor cells (PPCs) from SOX17-mKO2 hESCs (hES-PPCs) was well monitored and hES-PPCs could regulate blood glucose levels when transplanted into streptozotocin-induced diabetic mouse model. From these results, we expect that CHA-hES 6-SOX17-mKO2 and CHA-hES 15-SOX17-mKO2 can be a useful tool for screening and monitoring the cell differentiation.

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Keywords: human pluripotent stem cells, SOX17, reporter cell line

3:45 PM – 4:45 PM

325

IMPROVED GENETIC STABILITY OF HUMAN PLURIPOTENT STEM CELL CULTURES PASSAGED AS SINGLE CELLS USING ETESR

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Genetic instability has been widely documented in human pluripotent stem cells (hPSCs). Recurrent cytogenetic abnormalities arise throughout culture and confer a selective advantage to genetically variant cells through mechanisms including resistance to cell death, increased cell proliferation and reduced differentiation capacity. We have previously shown that routine single-cell passaging of hPSCs can result in a high incidence of de novo genetic abnormalities and that eTeSR™, a novel hPSC maintenance medium developed to better support single-cell passaging, can significantly reduce the appearance of recurrent abnormalities. To further demonstrate the increased genomic stability observed when routine single-cell passaging hPSCs in eTeSR™, 24 clonal lines were derived from the H1 and H9 hPSC lines (48 clones total) using single-cell deposition. Each clone was then independently maintained for 20 weeks in either two commercially available control media (A & B) or eTeSR™. Genomic DNA was extracted and analyzed using single nucleotide polymorphism microarray. We observed that the H9 clones were more susceptible to acquiring cytogenetic changes with 83% of clones maintained in control media abnormal after 20 weeks compared to 48% of H1 clones. Of the 91 clonal lines maintained in control media, 60 (66%) had acquired at least one de novo abnormality compared to only 19 (26%) of the 74 samples maintained in eTeSR™. This can be attributed to fewer small structural variants (< 10,000 kb) observed in clones maintained in eTeSR™, with only 6 detected across 74 samples (8%) compared to 64 detected in 91 samples (70%)

maintained in control media. Strikingly, 46 of 91 (65%) samples maintained in control media harbored a gain of chromosome 20, with 43 instances presenting as a copy number variants that are often undetectable using G-band karyotyping. Conversely, this abnormality was not detected in any of the eTeSR™-maintained samples, a significant finding due to the prevalence of 20q11 duplications reported in large-scale studies that describe recurrent karyotypic changes in hPSCs. This study provides a better understanding of culture-acquired genetic aberrations in hPSCs and demonstrates that innovative media formulations, like eTeSR™, can address major challenges in the hPSC field.

Keywords: genetic stability, pluripotent, quality

3:45 PM – 4:45 PM

327

NOVEL ROLES OF OCT4/SOX2 DIMERIZATION IN SUBDOMAIN DYNAMICS AND LINEAGE SPECIFICATION

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The complexity of the mammalian genome is owed in part to multiple layers of transcriptional regulation. Sequence-specific DNA-binding transcription factors (TFs) often work in tandem to govern where and when specific genes are expressed or silenced, determining cell identity. Multidomain TFs contain compound structural elements that have evolved to act synergistically, enabling diverse functions through their combined action. However, our current understanding of the form-function relationship in essential multidomain TFs is incomplete, marking a significant gap in our ability to reprogram cell fate with precision and efficiency. Recent structural studies of OCT4/SOX2/DNA complexes, known to be crucial for regulating pluripotency, demonstrated that SOX2 cobinding differentially affects OCT4 DNA binding subdomains. Here we used mutagenesis to dissect the functionality of different OCT4 subdomains. While OCT4 binding alone and reprogramming abilities were equally disrupted by most mutations regardless of the subdomain location, SOX2 could partially rescue additional functions of POUHD but not POUS mutants. In addition, WT OCT4 was revealed to be capable of bridging two DNA molecules using its DNA binding subdomains, which could be enhanced by the presence of SOX2. Finally, ESCs carrying a mutation in the OCT4 POUHD subdomain were expandable and expressed pluripotency markers similar to WT controls, yet simultaneously expressed high levels of primitive endoderm markers, suggesting a novel role for the OCT4 POUHD in lineage specification. Together these data reveal crucial details about OCT4 structural domains in the presence of SOX2. These results have broader implications for cell fate determination, ultimately informing TF design to refine cell reprogramming techniques and enhance regenerative medicine strategies.

Keywords: pluripotency transcription factors, lineage specification, embryonic stem cells

3:45 PM – 4:45 PM

329

MITOCHONDRIA-DEPLETED PLURIPOTENT STEM CELLS FOR STUDYING MITOCHONDRIA BIOLOGY

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The symbiotic relationship between mitochondria and eukaryotes dates back ~1.7 billion years. Once an independent aerobic prokaryote, over hundreds of millions years of co-evolution as an intracellular symbiont, the mitochondrion has obtained a set of deeply integrated roles in eukaryotic physiology, far surpassing its stereotypical portrayal as an ATP-producing powerhouse of the cell. The mitochondrion is now recognized to play major and essential roles in various cellular processes, ranging from the regulation of cell death and signal transduction, to mediating cell differentiation and developmental timing. However, the extent to which eukaryotic physiology and development are dependent on mitochondria remains an open and fundamental question; a question that can only truly be answered by generating eukaryotic counterparts lacking the organelle. To better understand mitochondrial roles in early mammalian development, we adapted an approach of inducible mitochondria-depletion, called enforced mitophagy, to generate a panel of pluripotent stem cells (PSCs) devoid of mitochondria. These cells provide us with a unique angle to study mitochondrial biology in in vitro models of one of the earliest stages of life. In addition, we have leveraged mitochondria-depleted PSCs for generating interspecies composite cells between human and non-human primate PSCs (i.e., chimpanzee, bonobo, gorilla, and orangutan) that contain either human mitochondria or non-human primate mitochondria. These composite cells maintain pluripotency and provide us with an exciting tool to study how species-specific differences in mitochondria may drive developmental differences between species. Finally, we have devised a method for transgenic depletion of mitochondria in vivo, opening the door for exciting studies involving the modulation of mitochondria copy number in tissues and animals.

Funding Source: NINDS (5F31NS125906-02) NYSCF NIGMS(GM138565-01A1) NICHD (HD103627-01A1) Welch Foundation (I-2088)

Keywords: mitochondria, pluripotency, development

3:45 PM – 4:45 PM

331

UNLOCKING HUMAN PRIMORDIAL GERM CELL-LIKE CELL REPROGRAMMING AND MOUSE IN UTERO MODELING OF GERM CELL TUMORS

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Sacrococcygeal teratomas (SCTs) are the most common tumor in newborns, characterized by significant perinatal morbidity and lack of reliable biomarkers for recurrence. SCTs are believed to originate from apoptosis-resistant gamete-precursors, called primordial germ cells (PGCs), defining them as germ cell tumors. Mouse PGCs can revert to pluripotent cells called embryonic germ cells (EGCs) when exposed to a cocktail of growth factors; however, establishing an in vitro model of human EGCs has been challenging. We seek to understand the unique tumor environment of SCTs and to model the formation of SCTs in vitro using iPSC models. We performed single cell sequencing of SCTs isolated from 4 infants (31,000 cells total). We performed spatial transcriptomic analysis of 2 aggressive SCTs requiring prenatal resection. To understand the relationship between PGCs and EGCs, we used a human PGC-like cell line (hPGCLC) and performed bulk RNA-sequencing during differentiation of PGCLCs to EGC-like cells (hEGCLCs). Single cell sequencing of patient samples revealed a diverse cellular composition: fibroblasts, smooth muscle, neuro/glia, epithelial, endothelial, mast cells, macrophages, and T-cells, with populations of proliferative cells across all cell types. Using RNA-velocity tools we found that these proliferative cells are the node of development for the rest of the clusters. We detected an overrepresentation of developmental signaling pathways (WNT, EGF, FGF, LIF), suggesting a dynamic tumor microenvironment is crucial for SCT maintenance. Bulk RNA-seq during hPGCLC to hEGCLC preprograming revealed upregulation of pluripotency genes. We found dynamic changes in the expression of the germ-cell related transcription factor DMRT1 after 3 days, suggesting the importance of epigenetics on the reprogramming of the cells.We created a scRNA-seq and spatial transcriptomic atlas of patients with SCTs, employing a variety of analytical approaches to classify heterogeneous cells. These studies are the first to identify the important tumor microenvironment of SCTs and the developmental trajectories occurring with the reprogramming of hPGCs towards pluripotency. These results can aid biomarker development to predict tumor recurrence and provide potential targetable pathways to treat aggressive SCTs.

Funding Source: This research was supported by supported by a pilot grant from the UCSF HDFCCC and UCSF CTSI. It was also supported by an NIH NRSA F31-Diversity Fellowship (# F31-CA284719)

Keywords: germ cell tumors, single cell and bulk RNA sequencing and spatial transcriptomics, primordial germ cell like cells

3:45 PM – 4:45 PM

333

NEUROECTODERM SPECIFICATION REQUIRES TET3 CATALYTIC ACTIVITY TO ACTIVATE NEURAL GENES AND NONCATALYTIC FUNCTIONS TO SUPRESS MESODERMAL PROGRAMS

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The ten-eleven translocation family of proteins (TET1/2/3) are epigenetic enzymes that regulate genes by promoting DNA demethylation (catalytic activity) and partnering with regulatory proteins (non-catalytic functions). Unlike Tet1 and Tet2, Tet3 is not expressed in mouse embryonic stem cells (mESCs) but is induced upon mESC differentiation. However, the significance of its dual roles in lineage specification is less defined. By generating TET3 catalytic mutant (Tet3m/m) and knockout (Tet3–/–) mESCs and differentiating them to neuroectoderm (NE), we have identified distinct catalytic dependent and independent roles of TET3 in NE specification. We found that TET3-mediated DNA demethylation is important for activation of neural genes such as Neurog2 and Sox1. In contrast, non-catalytic functions of TET3 are likely involved in suppressing mesodermal programs during differentiation toward NE. Surprisingly, several DMRs were hypomethylated and largely associated to aberrantly upregulated genes in Tet3–/– cells. This included several mesodermal genes such as Myl9 and Wnt10a. We found the maintenance methyltransferase, Dnmt1, as a direct target of TET3, which is downregulated in TET3 deficient NE cells. Our data suggests that one possible mechanism of TET3-mediated suppression of mesodermal genes is through direct regulation of Dnmt1 independent of TET3 catalytic activity. Our findings establish that the catalytic dependent and independent roles of TET3 have distinct contributions to NE specification with potential implications in development.

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Keywords: DNA methylation, embryonic stem cell, primitive neuroectoderm

3:45 PM – 4:45 PM

335

UNRAVELING THE IMPACT OF HOLLIDAY JUNCTION RESOLVASES ON PLURIPOTENCY

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Pluripotent stem cells (PSCs) hold a great potential in regenerative medicine due to their ability to self-renew and differentiate into all cell types of the adult organism. However, despite the promising advancements in technology, the clinical application of PSCs still faces significant challenges, including the risk of genome instability leading to potential tumorigenicity. Therefore, it becomes imperative to understand the mechanisms regulating DNA repair in PSCs to facilitate the complete development of PSCs-based therapies. Homologous recombination (HR) is one of the two major DNA damage repair (DDR) pathways with a crucial role in repairing DNA double-strand (DS) breaks within PSCs, ensuring the maintenance of genome integrity. Holliday junctions (HJs) serve as intermediate repair DNA structures that may emerge in the course of HR and represent a potential risk for chromosomal maintenance if not adequately resolved. By employing both loss-of-function and gain-of-function approaches, we have investigated the role of HJ resolvases in pluripotency maintenance. We demonstrate the key importance of HJ elimination from the PSC genome to maintain the pluripotent state. Furthermore, transcription-wide analysis of HJ resolvases-depleted PSCs revealed deregulation both in coding genes and transposable elements, which can represent a threat to the host genome. Hence, our results support the importance of a tight control of HJ intermediates during DNA repair for the conservation of the pluripotent state, key for unleashing the full potential of these cells for clinical purposes.

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Keywords: DNA damage repair, pluripotent stem cells, homologous recombination

3:45 PM – 4:45 PM

337

POTENTIAL MALIGNANCY OF STEM CELLS: IDENTIFICATION AND CHARACTERIZATION OF YOLK SAC ELEMENTS IN EXPERIMENTAL TERATOMAS

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Concern about the safety of pluripotent stem cell-based therapies, predominantly related to potential malignant transformation, is a big drawback in bringing these therapies to the clinic. The teratoma assay—a method in which stem cells are xenografted into immuno-compromised mice—is currently the accepted (gold) standard for risk assessment. A pluripotent stem cell line is considered malignant when embryonal carcinoma-like cells are present in the xenograft. Elements of yolk sac tumor(-like cells) might also be present, but the significance of these elements for the safety of stem cell products is unclear. Human germ cell tumors containing yolk sac elements are considered highly malignant. This study aims to develop tools for identifying and further characterizing yolk sac tumor-like elements in experimental teratoma (xenograft-derived or in vitro generated) and in primary germ cell tumors. To this end, single-cell RNA expression profiles will be obtained from previously generated paraffin-embedded samples. This eliminates the need to re-perform xenograft assays, reducing costs, time and animal lives. Preliminary data shows that RNA of sufficient quality can be obtained from 20-year-old samples. The final goal is to find new transcriptional or molecular markers for stem cell-derived yolk sac-like elements and identify malignancy-related molecular pathways of value for risk stratification of pluripotent stem cell products.

Keywords: yolk sac, single-cell RNA sequencing, germ cell tumour

3:45 PM – 4:45 PM

339

VGLL1 CONTROLS HUMAN TROPHECTODERM LINEAGE SPECIFICATION

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The placenta is the largest fetal supporting organ. It originates from the trophectoderm (TE) and constitutes, together with the pluripotent inner cell mass (ICM), the first lineage bifurcation event during development. In contrast to rodents, the mechanisms underlying human trophectoderm and early placenta specification are understudied due to ethical barriers and the scarcity of embryos. Recent reports have shown that human pluripotent stem cells (PSCs) can differentiate into trophectoderm (TE)-like cells (TELCs) and trophoblast stem cells (TSCs), offering a valuable in vitro model to study early placenta specification. In this study, we demonstrate that the transcriptional cofactor VGLL1 (vestigial like family member 1), which is highly expressed during human and non-human primate TE specification in vivo but is negligibly expressed in mouse, is a critical regulator of cell fate determination and self-renewal in human TELCs and TSCs derived from naïve PSCs. Mechanistically, VGLL1 partners with the transcription factor TEAD4 (TEA domain transcription factor 4) to regulate chromatin accessibility at target gene loci including cell cycle genes and TE/TSC-related genes through histone acetylation. In addition to YAP and WWTR1 (TAZ), VGLL1 acting as a core cofactor of TEAD4 enhances flexibility to safeguard the development of human TELCs. Besides, we found that VGLL1/TEAD4 complex acts synergistically with GATA3 and TFAP2C. Our work is relevant to understand primate early embryogenesis and how it differs from other mammalian species. We have uncovered a key regulatory mechanism of human early placenta formation whose further characterization may facilitate a deeper understanding of both normal and abnormal developmental processes.

Keywords: human pluripotent stem cells, trophectoderm, VGLL1

3:45 PM – 4:45 PM

341

INDUCED PLURIPOTENT STEM CELLS FROM HUMAN SKIN BIOPSIES WITHIN THREE WEEKS VIA NON-INTEGRATIVE REPROGRAMMING

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The rise of human induced pluripotent stem cells (hiPSCs) is unquestioned and iPSCs hold great potential for wide-reaching biomedical applications both in allogenic and autologous cell therapy. However, many traditional techniques used for iPSC generation suffer from low efficiencies and long procedures, and can entail the risk for genomic integration limiting their use in clinical settings. Here, we present a complete workflow covering the primary sample collection, reprogramming and iPSC line characterization. The workflow mitigates the problems mentioned above by using transient mRNA reprogramming that is one of the fastest and most efficient reprogramming technologies available today. Fibroblasts from five healthy donors (age 50 – 62) were isolated from abdominal skin via mechanical and enzymatic dissociation. The cells were expanded for only one passage before starting the reprogramming, eliminating the need for extensive upstream expansion of fibroblast and thus minimizing the risk for cultivation induced aberrations. For reprogramming cells were transfected for five consecutive days with a mix of transcription factors. During a short growth phase iPSC colonies formed with high efficiency and could be further expanded into stable monoclonal cell lines after an iPSC enrichment. We derived and phenotyped 36 iPSC lines with low intra- and inter-donor variability. Generated iPSCs showed characteristic stem cell morphology, marker expression and could successfully differentiate into all three germ layers. Thus, all tested cell lines met our phenotypic criteria. From a safety perspective, the cells were tested for genomic integrity by digital PCR detecting over 93% of recurrent abnormalities in iPSCs, and for their oncogenic potential by whole exome sequencing. Timewise, the entire procedure is very fast resulting in fully reprogrammed cells after three weeks, and in iPSC lines ready for banking, molecular characterization and further differentiation after six weeks, preventing cells from being in culture for unnecessary long time. The described protocol allows the efficient and reliable integration-free generation of high quality monoclonal iPSCs from human skin punch biopsies and will therefore facilitate the generation of iPSCs from individual donors.

Keywords: reprogramming, personalized medicine, pluripotency

3:45 PM – 4:45 PM

343

UNRAVELING THE IMPACT OF DIET-INDUCED OBESITY ON EFFECTIVE SOMATIC CELL REPROGRAMMING TO PLURIPOTENCY

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The global nutritional crisis, characterized by unhealthy dietary patterns and diet-induced obesity (DIO), presents a pressing challenge. While prior research has predominantly focused on somatic cell reprogramming for induced pluripotent stem cells (iPSCs) in mice fed standard diets, understanding the impact of DIO on iPSC generation is essential, given the pervasive prevalence of obesity. Here, we investigate the influence of DIO on cell reprogramming by applying the Yamanaka factors to fibroblasts sourced from mice subjected to either standard or high-fat diets. Our findings reveal the detrimental effects of DIO on iPSC generation, highlighting substantial alterations in the expression of key epigenetic and epitranscriptomic regulators required for successful iPSC induction. Employing a comprehensive multi-omics approach, we elucidate alterations in spliceosome dynamics, chromatin structure, and the metabolome that contribute to restricting cellular plasticity at the initial stages of reprogramming imposed by obesity. Ultimately, our findings underscore the intricate interplay between diet-induced obesity and somatic cell reprogramming, offering insights to enhance the efficiency of reprogramming strategies for therapeutic applications.

Funding Source: Research from the M.F. laboratory was supported by the Spanish Agencia Estatal de Investigacion (PID2019-105739GB-I00/AEI/10.13039/501100011033 and PID2022-143105NB- I00/AEI/10.13039/501100011033/FEDER, UE).

Keywords: obesity, reprogramming, iPSCs

3:45 PM – 4:45 PM

345

UNDERSTANDING THE ROLE OF TP53 IN THE MALIGNANT BEHAVIOR OF HUMAN PLURIPOTENT STEM CELLS

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The malignant potential of human pluripotent stem cells (PSC) is not fully understood and its assessment currently relies on the evaluation of the cells' behavior in vivo upon their engraftment into mice (teratoma assay). Resulting tumors are histologically identical to human (malignant) Germ Cell tumors (hGCT), and thus the malignant potential of the engrafted lines are assessed according to hGCT pathology. In vitro however, long-term culture of PSCs can lead to (epi)genetic drift, potentially activating processes that resemble malignant transformation. Here we investigated the relevance of TP53 mutations in the malignant transformation of hPSC, a key gene regulating cell cycle control and (epi)genetic stability and often found mutated in PSCs due to culture adaptation by comparing the implications of its depletion in hPSC and hGCT. With this goal, TP53 knockout (KO) cell lines were generated through CRISPR-Cas9 technology of representatives of an embryonic (H9) and an induced (Lu07) PSC line, as well as previously two hGCT lines (2102Ep, NCCIT). Phenotypically, TP53-KO cell lines displayed proliferation rates similar to those of their isogenic wild-type counterparts, despite showing a greater colony formation capacity and ability to grow in an anchorage-free environment. We demonstrate that despite the loss of TP53 expression and related depletion of P21 expression (both mRNA & protein), the KO lines did not show changes in expression of the pluripotency and malignancy-associated miRNA-371 and miRNA-373. Similarly, we did not observe changes in the expression levels of pluripotency markers (OCT4, SSEA3, TRA-1-60, NANOG) in hPSCs, and bulk RNAseq did not show difference in expression of malignancy-associated genes. We also explored the effect of TP53 depletion on cisplatin, gemcitabine and navitoclax sensitivity, DNA-damaging agents commonly used in clinics for the treatment of hGCTs. Loss of TP53 led to increase of cisplatin and gemcitabine resistance of PSC lines, similar to clinical observations in hGCTs. Overall, this study shows that TP53 mutations in PSCs in vitro can lead to a greater resilience of the cells and causing clinically relevant malignancy-associated traits, while not necessarily triggering their full oncogenic transformation.

Keywords: pluripotent stem cells, malignancy, TP53

3:45 PM – 4:45 PM

347

HUMAN BLOOD MONOCYTES AND IPSC-MICROGLIA EXHIBIT DIFFERING PROTEOMIC, TRANSCRIPTIONAL AND FUNCTIONAL PROPERTIES FOLLOWING ENGRAFTMENT WITHIN THE ADULT MAMMALIAN BRAIN

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Hematopoietic stem cell transplantation (HSCT) is increasingly being tested as a potential therapy for neurological disorders. The premise of this approach is that HSCT-derived monocytes may infiltrate the brain and differentiate into “microglia-like” cells. Recent advances in the differentiation of induced pluripotent stem cells (iPSCs) into microglia, provide a potential new source of therapeutic cells. However, many questions remain regarding the similarities and differences between microglia and monocytes (MNs) following engraftment within the brain. To compare the engraftment potential and transcriptional and functional profiles of iPSC-microglia (iMGs) and human monocytes following transplantation into the CNS, we developed a novel xenotransplantation-compatible mouse model that lacks endogenous murine microglia (MITRG-FIRE mice). iMGs and blood monocytes from four male patients were transplanted into the cortex of adult male MITRG-FIRE mice. Four months later, we utilized single cell RNA sequencing, TMT-MS proteomics, and histological and biochemical approaches to compare the phenotype and function of engrafted human monocytes and microglia. Immunohistochemical analysis revealed near-complete chimerism of human iMGs and human monocytes throughout the brain. Four months after transplantation, monocyte-derived cells express several microglial-related makers and yet continue to exhibit significant differences in marker expression and cell morphology. In addition, scRNA seq, multiplex cytokine ELISAs, and proteomics analysis reveal distinct and persistent differences in mRNA and protein levels. Taken together, these results offer novel insights with implications for the future development of myeloid cell-replacement therapies that aim to address microglial-associated neurological diseases.

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Keywords: human microglia, human monocytes, chimeric mouse model

3:45 PM – 4:45 PM

349

INTEGRATIVE PROTEOMIC PROFILING OF MOUSE PLURIPOTENT STATES REVEALS CRITICAL DEVELOPMENTAL DIFFERENCES BETWEEN MALE AND FEMALE DEVELOPMENT

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During embryonic development, the chromatin undergoes rapid reshaping to allow lineage specification. As such, a comprehensive analysis of the chromatome at different states of pluripotency improves the understanding of gene regulation during development. In this study, we generate a comprehensive atlas of the chromatin-associated proteome, post-transcriptional histone modification (hPTM), and DNA methylation across major pluripotency states of mouse male ESCs, including naïve, metastable, formative, and primed pluripotency. We used this reference map of key proteomic features to compare with female mESCs, which have been suggested to show critical differences in developmental timing. Additionally, we use it as a reference map to compare with naïve and primed human ESCs. Our comprehensive analysis unveils that the Polycomb-repressive complex 2 (PRC2) and its product H3K27me3 are highly abundant in naïve states and gradually downregulated in subsequent states. Conversely, G9a and H3K9me2/3 exhibit low abundance in naïve states but are upregulated in later stages. Naïve pluripotency is characterized by a hypomethylation DNA state and the absence of DNA methylation factors DNMT 3A/B, while DNA methylation is progressively rebuilt in metastable, formative, and primed pluripotency. In female mESCs, the metastable pluripotent state shows male naïve like- features, including DNA hypomethylation, less chromatin binding of DNA methylation factors, and a higher abundance of H3K27me3 but a lower abundance of H3K9me2/3. In human ESCs, naïve pluripotency shares a similar feature of a high abundance of H3K27me3 with mouse, but H3K9me2/3 has no significant difference between naïve and primed hESCs. Human and mouse naïve stem cells both show a high DNMT3L chromatin binding, but human naïve ESCs retain the DNMT3A/B binding. Altogether, our findings contribute to the generation of a comprehensive chromatome atlas for major pluripotency states and provide insights into developmental mechanisms and gender-based sex-specific chromatome features, and species-specific chromatome features.

Keywords: pluripotency stem cells, chromatin-associated proteome, hPTM, DNA methylation, naïve, metastable, formative, primed

3:45 PM – 4:45 PM

351

SETTING UP GENOME EDITING: FEEDBACK FROM THE IPSC CORE FACILITY OF NANTES

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Induced pluripotent stem cells (iPSCs) are reprogrammed from a patient or healthy control and then differentiated into a cell type of interest. The phenotype of iPSCs is compared to the phenotype of the healthy control cells. In those condition cells do not have the exact same genetic background. By using genome editing with for example CRISPR/Cas9 technology we can create isogenic lines that can be compared for relevant phenotype. This can be done by correcting a pathogenic mutation in patient-derived cells or by introducing a mutation into healthy control cells. Here we present the implementation of genome editing in our iPSC core facility, with optimized CAS9:gRNA:ssODN stoechimetry. The CorEuStem network was instrumental to support us establishing a fast and efficient editing pipeline, especially the CRG (Barcelona) and MDC (Berlin) core facilities.

Funding Source: Région Pays de la Loire

Keywords: iPSC, gene editing, CRISPR/Cas9

3:45 PM – 4:45 PM

353

MRNA REPROGRAMMING: BACK IN THE GAME

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Somatic cell reprogramming is now established as a major strategy to study human genetics. The main method for reprogramming somatic cells is to use Sendai vectors encoding the reprogramming factors. Despite its efficacy, mRNA reprogramming suffered from discontinuation of reagents, lack of “one-protocol-that-works-for-all ”, complicated culture steps in the Week-ends. Leveraging a new reprogramming kit, we improved our previously published protocol to reprogram faster . Here, we present the results of 6 patient fibroblast reprogramming using our new protocol, and discuss the influence of reprogramming parameters, QC of the clones and give an over-all feedback on the place of mRNA reprogramming in our core facility.

Funding Source: Region pays de la Loire

Keywords: hiPSC, mRNA reprogramming, fibroblast

3:45 PM – 4:45 PM

355

EXPLORING THE INTERPLAY BETWEEN WNT-SIGNALLING AND CHROMATIN BIOLOGY THROUGH CHD4-NURD PHOSPHORYLATION IN MOUSE PLURIPOTENT STEM CELLS

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Despite the known importance of WNT signalling in mammalian development and various cancers, its downstream mechanistic effect on chromatin regulation remains less understood. When WNT signalling is activated, Glycogen Synthase Kinase 3 (GSK3) is inhibited, leading to dephosphorylation and nuclear accumulation of β-catenin, where it regulates WNT-target genes. However, GSK3 is a major kinase with multiple client proteins, and how phosphorylation of additional chromatin proteins downstream of GSK3 regulates stem cell identity remains poorly understood. Using phospho-proteomic approaches we found that CHD4-NuRD, a de-acetylating complex important in lineage differentiation and a tumour-suppressor, is de-phosphorylated downstream of WNT-signalling in mouse embryonic stem cells (mESCs). To unravel the overall CHD4 function, we generated Chd4-knockout mESCs and observed profound upregulation of primitive endoderm markers, implying a repressive role of CHD4 for endoderm lineage specification. To address how differential phosphorylation of CHD4-NuRD complex downstream of WNT signalling is involved in chromatin and stem cell biology, we used gene editing and engineered mESCs expressing different Chd4 phospho-variants. Our findings connect WNT signalling

and CHD4-NuRD complex, two key players with opposite functions in stem cell biology, providing key insights into how signal transduction cascades control chromatin functions and cell fate decisions in stem cells.

Funding Source: Department for Economy (DfE)

Keywords: CHD4, WNT signalling, mouse embryonic stem cells

3:45 PM – 4:45 PM

357

REPROGRAMMING ACTIVATES 55 SPECIFIC POLYMORPHIC, FUNCTIONAL LINE-1 LOCI IN PLURIPOTENT STEM CELL GENOMES THAT ARE MOBILIZED IN HUMAN TUMOR CELLS AND TISSUES

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Reprogramming induced epigenome remodeling in human induced pluripotent stem cells (hiPSCs) was shown to result in the mobilization of endogenous retrotransposons LINE-1 [L1], Alu and SVA, and intronic L1 de novo insertions occuring during reprogramming or hiPSC cultivation, interfered with host gene expression. Here, we set out to identify those specific loci among the 1 Mio L1 insertions in the genome that encode retrotransposition-competent L1 (RC-L1) elements which are expressed and mobilized in human pluripotent stem cells (hPSCs), causing mutagenic insertions. To this end, we generated individualized, custom-tailored genomes of two human embryonic stem cell lines and six hiPSC lines and their parental cells by mapping all fixed and polymorphic RC-L1 loci of these cell lines, and identified defined subsets of 13 fixed and 104 polymorphic RC-L1 loci in the various cell lines. To quantify transcripts expressed from the L1-specific promoter of RC-L1 loci, we applied RNA-seq and the 5'RACE method combined with PacBio sequencing, which facilitated mapping of L1-specific RNA reads to unique genomic L1 loci. We mapped those RC-L1 loci in hiPSCs that are expressed and/or transcriptionally upregulated in the pluripotent state identifying potential source elements that are responsible for L1-mediated retrotransposition in hPSCs. Subsets of only 29-63 of the 78-97 cell line-specific polymorphic RC-L1 loci were found activated in hiPSCs relative to their parental cells. One to six specific RC-L1 loci were responsible for ~50 % of all RC-L1 transcripts. Unexpectedly, 75 out of 117 RC-L1 loci identified in PSCs were shown to be mobilized in cancer cells. Out of these 75 L1 source loci, 55 were also expressed in hPSCs. We confirmed their retrotransposition activity in hPSCs by isolating these individual L1 elements form their hPSC genomes and quantifying their retrotransposition activity applying L1 retrotransposition reporter assays. In sum, we uncovered small subsets of 22-54 specific RC-L1 loci that are transcribed and mobilized in each analyzed hPSC line and include L1 source loci reported to be responsible for deleterious endogenous mobilization events observed in lung, HNC, prostate and colon cancer. Our findings underscore the significant potential of expressed RC-L1 elements as endogenous mutagens in hPSC.

Keywords: pluripotent stem cells, LINE-1 retrotransposition, genomic destabilization

3:45 PM – 4:45 PM

359

UTR-MEDIATED TRANSLATIONAL REGULATION IN HUMAN ESC LINEAGE DIFFERENTIATION

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Translational regulation provides fine-tuned and precise control over gene expression, but the global translational dynamics and the role of translational regulation during lineage differentiation are still ambiguous. Here we investigate whether and how translational regulation contributes to human embryonic stem cell (ESC) differentiation into various lineages, including neuroectoderm (NE) and definitive endoderm (DE) as well as the derivates including early-stage pancreatic progenitors (PP1) and late-stage pancreatic progenitors (PP2). We adopt a pioneering experimental strategy by integrating ribosome profiling (Ribo-seq) and RNA sequencing (RNA-seq) technologies to map the translatome and transcriptome throughout ESC lineage differentiation. Subsequently, we develop a method to illuminate the discrepancies between the transcriptome and translatome datasets. During differentiation, genes related to morphogenesis predominantly undergo transcriptional regulation, while crucial pathways including WNT and MAPK signaling are subjected combined transcriptional and translational regulation. Notably, pathways related to mRNA translation itself and several metabolic pathways significantly engage in translational regulation. Moreover, we present evidence suggesting that sequence features of untranslated regions (UTRs) notably influence translation efficiency. Genes having shorter 5'UTRs and strong Kozak consensus sequences tend to exhibit higher translation efficiency in ESCs, but not in other cell types. In addition, we find that short open reading frames in 5'UTR region, codon usage bias and the repertoire of RNA-binding proteins also show certain correlations with translation efficiency. Currently, we are exploring the underlying mechanisms and seeking to pinpoint

feasible practical applications for these insights. Taken together, the present study not only highlights the pivotal role of 5'UTR sequences in dictating translation efficiency, but also emphasizes the necessity of translational regulation in cell fate determination, transcending the traditional dichotomy of gene expression as solely 'on' or 'off'.

Keywords: translational regulation, human embryonic stem cell, lineage differentiation

3:45 PM – 4:45 PM

361

NOVEL PATHOGENESIS OF DIAMOND-BLACKFAN ANEMIA EXPLORED THROUGH REGULATION OF PROTEOSTASIS

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Diamond-Blackfan anemia (DBA) is a congenital bone marrow failure syndrome characterized by a significant decrease in red blood cells and physical abnormalities, including craniofacial malformations. DBA results from heterozygous mutations in ribosomal protein (RP) genes, with over 20 causative genetic mutations identified to date. However, approximately 40% of the patients do not exhibit these mutations, suggesting the presence of other causative genes or mechanisms. Furthermore, many aspects of DBA, including treatment, genetic diagnosis, and mechanisms of onset, remain unclear. We conducted large-scale trans-omics analyses of various cell types. We confirmed that all reported DBA causative genes have higher protein levels in human induced pluripotent stem cells (hiPSCs) than in differentiated cells, independent of mRNA levels. We then generated and compared hiPSCs derived from patients with DBA with the most frequent mutations in DBA patients such as RPS19, RPL11, and RPL5. Among them, RPL5 and RPL11 -DBA-hiPSCs showed defective differentiation potential in the early mesoderm. Furthermore, although the protein levels of RPs in DBA-hiPSCs were comparable to those in healthy hiPSCs, they decreased during mesodermal differentiation. These findings suggest that DBA genes undergo different quantity control between hiPSCs and differentiated cells. Importantly, this regulation of protein levels occurs post-transcriptionally but not during transcription. This study proposes a novel mechanism for the onset of DBA through the regulation of proteostasis and leads to a better understanding of common principles underlying mutation-independent DBA pathogenesis.

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Keywords: proteome, Diamond Blackfan anemia, ribosome

3:45 PM – 4:45 PM

363

FULL-LENGTH LAMININS ARE CRUCIAL FOR RECREATING THE CELLULAR NICHE IN VITRO

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Laminins are an extracellular matrix (ECM) family of 16 different protein isoforms. The expressions of the laminins are temporally and spatially specific, being essential for tissue formation and homeostasis. The laminins are within all tissues of the body, particularly concentrated in the formation and maintenance of the basement membrane (BM). Intact laminins are essential, since mutations in genes encoding laminins can cause a wide spectrum of disorders called "laminopathies". Mutations in different domains of the laminin protein result in BM weakness which can result in tissue-specific dysfunctions, affecting for example muscle, kidney, nerve, skin, and eye. Examples of such congenital disorders caused by laminin chain mutations are Pierson syndrome and Epidermolysis bullosa. In vivo, most laminin receptors are integrins and non-integrins including dystroglycan, syndecan, and Lutheran blood group glycoprotein. Multiple essential growth factors (GF) can bind with high affinity to the complex laminin molecules. Interactions between GFs and ECM are essential for controlling GF release kinetics of Intact laminins (full-length). Laminins are large trimeric proteins of about 800 kDa, with all multiple binding sites, crucial for building the correct network and capable of self-assembly, which provide a key structure element of BMs. Thus, the intact trimeric laminin complex is necessary for the bioactivity levels of the protein. Truncated laminin proteins are not produced by healthy tissue and lack essential components for healthy tissue homeostasis. We have compared the survival, proliferation, and migration of human PSCs, on truncated laminins to intact full-length laminin protein isoform 521. We can demonstrate, a robust and enhanced survival, proliferation, and migration, on the full-length laminin. It even allows for single-cell seeding and omits the need for ROCKi. The cells are highly migratory on full-length laminin-521, reaching a 100% closure compared to commercial truncated laminin products, which reach a maximal closure of 50%. In conclusion, full-length laminin-521 mimics the natural cell microenvironment for PSCs in vitro, which is crucial for PSC culture and development of successful differentiation protocols, predictable disease models, and effective gene editing.

Keywords: full-length laminin, laminin 521-LN521, microenvironment

3:45 PM – 4:45 PM

TOPIC: PANCREAS

365

IDENTIFICATION OF BETA CELL DIFFERENTIATION-PRONE IPSC CLONE FOR A CELL THERAPY IN DIABETES

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Induced pluripotent stem cells (iPSCs)-derived β cells are promising candidates for the cell therapy of type 1 diabetes. However, cellular reprogramming generates different iPSC clones with unpredictable differentiation potential from the same donor. This project aims to identify an early-stage gene signature for the selection of the best β cell differentiation-prone iPSC clone. Eleven iPSC clones generated from the same donor were differentiated in vitro into cells of the definitive endoderm (DE). Six clones were also differentiated into mature β cells and differentiation efficiency assessed by flow cytometry at iPSC, DE, pancreatic precursor (PP) and β cell stages using stage specific markers. A gene expression analysis was performed at iPSC stage evaluating 770 genes involved in stemness and trilineage specification. Five differentially expressed genes (DEGs) were validated by qPCR and a z-score based on the over-all expression was calculated to establish a ranking between clones. Gene expression analysis showed that at iPSC stage the 11 clones clustered in two different groups (Gr1 and Gr2). Flow cytometry showed that clones belonging to Gr2 were more prone to endoderm differentiation. In fact, at DE stage clones belonging to Gr2 had more Cxcr4 positive cells compared to Gr1 clones (Gr1 79.36±3.9% vs Gr2 90.54± 2.39%; P = 0.016) and at PP stage Pdx1 and Nkx6.1 were higher in Gr2 clones (Pdx1, Gr1: 43.0±10.3% vs Gr2: 79.6±3.9%, P= 0.008; Nkx6.1, Gr1: 23.2±7.7% vs Gr2: 53.0±8.0%, P= 0.02). Gr1 and Gr2 differentially express 73 genes (-1.5< LogFC >1.5; p-Adj 0.05) mainly involved in naïve/primed state, endoderm lineage and glutamine, amino acid and fatty acid metabolism. From 73 DEGs, 5 genes, involved in metabolism and in pluripotency, were selected and tested in qPCR for correlation with differentiation propensity. A positive Pearson correlation was observed between z-score of these 5 genes, calculated at iPSC stage, and flow cytometry results at DE, PP and β cell stages, showing the possibility to discriminate the best iPSC clones already at pluripotency stage. This study proposes a suitable tool for patient-personalized autologous cell therapy.

Keywords: iPSC, β cell, gene expression

3:45 PM – 4:45 PM

367

INVESTIGATING HUMAN PANCREAS DEVELOPMENT USING A HIGH-CONTENT SCREEN ON ORGANOIDS

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During development, cell-fate decision is coupled with morphogenesis of the organ. Nonetheless, pancreas organogenesis during human development remains largely unexplored due to lack of three-dimensional in vitro model systems. We have recently established a three-dimensional culture system for long-term maintenance of pancreatic progenitors, derived from human pluripotent stem cells, as spheroids (Gonçalves C et al, Nat Comm 2021). This organoid system enables recapitulation of the developmental processes in 3D. My project aims to understand the mechanisms controlling cell fate and morphogenesis of the developing human pancreas using organoid models. To identify pathways regulating progenitor expansion, differentiation, and morphogenesis, we developed an image-based high-content screening assay on pancreatic progenitor-spheroids in 384-well plate format. We performed a primary screen against a library of 548 annotated molecules that target cell-signaling pathways. Readouts of the screen were two reporters for PDX1 as a proxy for progenitor and NEUROG3 as endocrine lineage marker, along with DAPI to monitor cell identity and numbers, and Phalloidin to track morphology. Image segmentation, feature extraction, and phenotypic profiling focused on nuclear as well as organoid parameters for intensity, shape, number, and morphology of the objects. Based on these profiles, we developed a pipeline to quantify the heterogeneity and changes in the spheroid morphology. We flagged 54 compounds that modified cell fate and/or morphological features of the spheroids. In particular, we identified molecules that affected PDX1 expression as well as lumen morphologies. Hit validation and mechanism deconvolution are in progress to explore novel pathways regulating pancreas development. Overall, we have established a robust image-based high-content screening assay with cell identity and morphological readouts that can be applied to many other organoid model systems. Further investigations on mechanisms of action will help in understanding the pathways governing the in vivo developmental trajectories. Understanding the expansion and differentiation of pancreatic progenitors would be extremely valuable for therapeutic studies pertaining to pancreatic disorders like diabetes and cancer.

Keywords: pancreas development, organogenesis, high-content screen

3:45 PM – 4:45 PM

369

NOTCH CONTROLS PANCREATIC CELL FATE DECISIONS VIA HES1-MEDIATED DEEP REPRESSION OF GENE REGULATORY NETWORKS

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Notch signaling controls developmental cell fate choices in many organs including pancreas. Using human embryonic stem cell-derived pancreatic progenitor cells (hESC-PPs) as a model system, we find that Notch-induced HES1 represses differentiation-inducing gene regulatory networks (GRNs), through a “deep” GRN architecture. Transcriptomic profiling of wild type, HES1-, NEUROG3-, and HES1; NEUROG3-deficient hESC-PPs together with identification of genomic HES1 binding locations suggests that pancreatic endocrine and acinar fates are directly suppressed by HES1, not only through repression of the bHLH master regulator genes NEUROG3 and PTF1A, but also via direct repression of downstream NGN3 and PTF1A target genes. Moreover, combined loss of HES1 and NEUROG3 leads to marked upregulation of markers associated with pancreatic alpha cells as well as ATOH1 and ASCL1, two HES1-bound, bHLH master regulator genes of non-pancreatic GRNs. scRNA-seq revealed that ATOH1 and ASCL1 are expressed in discrete cell populations with transcriptomic signatures indicating a mixed lineage with features of pancreatic and stomach/intestinal secretory lineages, suggesting that HES1 and NEUROG3 cooperatively suppress emergence of inappropriate gene expression programs in pancreatic progenitors. During pancreas development, alpha cell differentiation is believed to be Neurog3-dependent, however further investigation revealed that in a mouse model deficient for both Hes1 and Neurog3, glucagon positive cells can be detected at embryonic day (E)12.5 and E15.5, suggesting that this can be circumvented and lead to activation of the GRN governing alpha cell development. Furthermore, in hESC-PPS we have identified a transcription factor that can drive endocrinogenesis in the absence of NEUROG3. Our results provide new insights into Notch controlled cell fate decisions that will aid directed differentiation of stem cells to clinically useful cell types.

Funding Source: ReNEW is funded by a grant from the Novo Nordisk Foundation, grant: NNF21CC0073729. Heidi K. Mjoseng is the recipient of a fellowship from the Novo Nordisk Foundation, Copenhagen Bioscience PhD Programme, grant: NNF19SA0035442.

Keywords: Notch signalling, HES1, gene regulation

3:45 PM – 4:45 PM

371

WNT/PLANAR CELL POLARITY SIGNALING ORCHESTRATES CILIOGENESIS AND CELL-CYCLE EXIT DURING ENDOCRINE LINEAGE COMMITMENT

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Insulin-producing beta cell replacement holds great promise for diabetes therapy. However, pluripotent stem cell (PSC) differentiation protocols are still inefficient. Especially, endocrine progenitor induction is a bottle neck, as it is a highly complex process orchestrated by a multitude of intertwined factors, such as morphogenetic changes during pancreas development as well as extrinsic and intrinsic cues like cytoskeletal rearrangements. One candidate pathway that regulates actin and microtubule dynamics and potentially cell fate decisions is the Wnt/Planar cell polarity pathway (PCP). Using our knock-in FltpVenus/+ reporter mouse line (FVR) that monitors Wnt/PCP activity, we elucidated a crucial role for Wnt/PCP signaling in beta cell maturation and in the formation of endocrine cells from intestinal stem cells. To understand how morphogenesis and tissue architecture are linked to endocrinogenesis in the pancreas and to translate our in vivo findings from mice to human, we generated an Insulin (INS-)C-peptide-Cherry; FVR double reporter iPSC line. Combined single-nuclei RNA/single-nuclei ATAC sequencing and single-cell RNA sequencing of FVR+ enriched cells at endocrine induction stage during pancreatic iPSC differentiation, showed that acquisition of WNT/PCP signaling marks the emergence of endocrine progenitors (EPs) as well as the exit from cell cycle during endocrinogenesis. Pseudo- and lifetime analysis resolved endocrine differentiation at an unprecedented resolution and described novel EP subtypes. Strikingly, we identified an EP specific ciliary program driven by the transcription factor FOXJ1 potentially regulating

endocrinogenesis and cell cycle exit in WNT/PCP activated cells. Genetic manipulation of FOXJ1 will provide a deeper understanding how ciliogenesis, planar polarization cues and lineage commitment are linked in the emergence of endocrine cells. Taken together, our findings identify an important role of WNT/PCP-mediated cytoskeletal rearrangements to allow cilia formation during in vitro endocrinogenesis. As cilia are associated with cell crosstalk and signaling in development, unraveling their specific role upon endocrine induction will further enhance our understanding of the formation of hormone producing cells in vitro and in vivo.

Keywords: endocrine induction, iPSC derived pancreatic islet like clusters, ciliogenesis

3:45 PM – 4:45 PM

373

A GENOME-WIDE CRISPR SCREEN IDENTIFIES GENETIC REGULATORS OF MITOCHONDRIAL GLUCOSE RESPONSE IN HUMAN PLURIPOTENT STEM CELL-DERIVED BETA-CELLS

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Found in the pancreatic islets, β-cells are vital regulators of blood glucose homeostasis via tightly controlled insulin release. β-cell dysfunction or loss causes diabetes, affecting over 500 million adults worldwide. Human pluripotent stem cell-derived islets (SC-islets) are in vitro-differentiated cultures that include cell types akin to those found in the human pancreatic islet. This includes stem cell-derived β-cells (SC-β-cells), which are promising as a source of replacement cells for transplantation and an in vitro model to explore mechanisms of diabetes and test therapies. However, current methodology for generating SC-β-cells fails to produce cells functionally equivalent to primary human β-cells. Of particular significance is the lack of the characteristic robust respiratory response to glucose stimulation, a crucial driver of insulin secretion. Here, we conducted a CRISPR/Cas9-based genome-wide loss-of-function screen to unbiasedly identify genes relevant to SC-β-cell mitochondrial glucose response. In this screen, SC-islets were labeled with cell type-specific surface markers and sorted via fluorescence-activated cell sorting (FACS). Additionally, using a mitochondrial membrane-potential based dye, subpopulations of SC-β-cells were FACS separated based on their mitochondrial activation during glucose stimulation. These distinct populations were compared to identify gene knockouts that influenced the mitochondrial glucose response. In this manner, we identified 330 negative and 833 positive regulators of this responsiveness. Among these candidate regulators are genes that are involved in a variety of pathways and functions such as cell cycle phase transitions, chromatin binding regulation, B-lymphocyte cell activation and proliferation, and cellular respiration. Of interest, key folate cycle enzymes are identified in this screen as potentially relevant to β-cell respiratory function, a feature not previously characterized in either primary β-cells or in vitro cultures. Collectively, the findings of this screen unveil many new avenues to explore in the quest towards the improvement of methodologies for derivation of functional SC-β-cells.

Funding Source: This project is funded by: NIH UH3 DK122639, 1R03DK138495-01, and DRC Project #42

Keywords: human pluripotent stem cell-derived islets, genome-wide CRISPR screen, beta cell metabolism

3:45 PM – 4:45 PM

375

LOSS OF ONECUT1 ALTERS PANCREATIC EXOCRINE DEVELOPMENT

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Recently, we demonstrated that ONECUT1 controls a transcriptional and epigenetic machinery regulating endocrine development which is implicated in a spectrum of diabetes encompassing monogenic forms, both recessive and dominant, as well as multifactorial inheritance. Interestingly, ONECUT1 mutations causing monogenic recessive syndromic diabetes were not only characterized by endocrine dysfunction but also intrauterine growth retardation, pancreas hypoplasia (including exocrine insufficiency) and gallbladder agenesis/hypoplasia, consistent with findings from mouse knockout (KO) models. However, detailed knowledge of transcriptional regulation in human pancreas remains limited. To delve deeper into the role of ONECUT1 in human pancreatic exocrine development, we utilized a human embryonic stem cell (hESC) derived pancreatic duct-like organoid (PDLO) model. Both wildtype (WT) and ONECUT1 knockout (KO) cell lines were subjected to differentiation into pancreatic progenitor cells (PP), followed by maturation into PDLOs within a 3D matrigel culture. Throughout this process staged ATAC- and RNA-sequencing were conducted, alongside functional analysis. Our data indicate that ONECUT1 plays a crucial role not only in pancreatic endocrine development, but also in establishing all three lineages contributing to the human pancreas. Loss of this gene results in altered exocrine development characterized by formation of large, cyst-like ducts and changes in morphology and functionality. KO PDLOs exhibited impaired polarity, leading to a redistribution of CFTR channels on both the luminal and basal side of the cells. Transplantation of PPs onto the porcine urinary bladder (PUB) revealed a bias towards ductal

cells, resulting in a reduced number of acinar cells and endocrine cells. This observation is supported by the early expression of the maturation marker cytokeratin 7 in PDLO cultures. Moreover, analysis of RNA-sequencing data revealed a disturbed developmental program, consistent with results from immunofluorescence staining. In summary, our findings suggest that ONECUT1 plays a vital role in pancreatic organogenesis. This methodology provides a valuable tool for characterizing pancreatic diseases and offers insights into the role of ONECUT1 in pancreatic development and pathology.

Keywords: pancreatic duct-like organoids, pluripotent stem cells, in vitro differentiation

TRACK: SOMATIC STEM CELLS, CANCER AND REGENERATION (SSCCR)

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

377

ASTROCYTIC FATE REPROGRAMMING IN GLIOBLASTOMA FOR A THERAPEUTIC APPLICATION

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Glioblastoma is the most common malignant brain tumor and poses significant therapeutic challenges due to its invasive nature and limited treatment options. Conventional therapies exhibit modest success against this aggressive and immunosuppressive cancer, prompting exploration of alternative strategies. In this project, we explored direct cell fate reprogramming as a therapy for glioblastoma. Direct cell reprogramming is the conversion of somatic cell fate to a different cell identity without passing through a pluripotent intermediate state. By inducing overexpression of the transcription factors Sox9 and Nfib, our group has successfully converted fibroblasts to astrocytes and we demonstrated that human glioma cell lines can similarly be converted to astrocyte-like cells with low proliferative activity. Converted cells have a lower tumor-initiating ability when injected in immunodeficient mice, proving the potential of this treatment as a therapy against glioblastoma. To transition to a model that better recapitulates the human disease, we chose to test mouse glioma cell lines that can be injected orthotopically in syngeneic mouse models of glioblastoma. Syngeneic mouse models allow the study of glioma in a host with intact immune system. To validate the treatment on different mouse cell lines, we selected combinations of transcription factors along diverse glioma lines and expression cassettes. The results are evaluated through a comprehensive marker analysis, which includes proliferation and astrocytic markers. Preliminary results show an increase of the astrocytic marker S100B after treatment in mouse glioma cells, and a downregulation of proliferation. This validation is key to understanding how the combinations of transcription factors change glioma cells fate and their interactions with the immune system. It paves the way to translate these findings to human primary glioblastoma material, including 3D cultures from glioblastoma patients, and for future clinical applications.

Keywords: cell fate reprogramming, cancer, astrocyte

3:45 PM – 4:45 PM

TOPIC: CARDIAC

379

TREATMENT OF CARDIAC PROGENITOR CELLS WITH 17BETA-ESTRADIOL INCREASES THEIR THERAPEUTIC POTENTIAL IN THE TREATMENT OF HEART FAILURE

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The postnatal mammalian heart exhibits very limited regenerative capacity. Nevertheless, cellular turnover occurs in the heart, and the existence of cardiac progenitor cells (CPCs) has been reported. Although CPCs can differentiate into all cardiac lineages, clinical studies have reported poor cell survival and inefficient engraftment of transplanted CPCs into host tissue. In this study, we hypothesized that pretreatment with 17β-estradiol (E2) enhances the cardiac regeneration properties of CPCs in heart failure (HF). We observed a significant increase in CPC migration upon treatment with E2 in vitro, accompanied by upregulated expressions of Cxcr4/Sdf-1 and EMT markers. Meanwhile, we showed that E2 also targets the mitochondria and modifies their dynamics. Expression of mitochondrial fission proteins Fis1 and Drp1 decreased, while Mfn2 to Mfn1 ratio (mitochondrial fusion) and LC3-II to-I ratio (autophagy) increased in E2-pretreated CPCs (E2-CPCs) compared to untreated CPCs (C-CPCs). Furthermore, we found that E2 reduces the fluidity of the CPC mitochondrial membrane, which may be associated with increased mitochondrial fusion. Male E2-CPCs or C-CPCs were injected into the myocardium of female mice with isoproterenol-induced HF. E2 pretreatment resulted in greater retention of Y-chromosome+/Tnni3- CPCs on day ten after transplantation. This increased cellular retention in the infarct zone was correlated with heightened fluidity of the plasma membrane. E2-CPCs induced more

significant myocyte proliferation and differentiation in recipient hearts compared to the C-CPCs group by increasing expressions of Ki67, Cxcr4, Tnni3, and the angiogenesis marker vWF. The expression levels of Col1a1 and NFATc4 decreased more profoundly in the E2-CPCs group than in the C-CPCs group. E2-CPC transplantation significantly reduced intracellular lipid accumulation and collagen deposition in recipients' hearts. Finally, our electrocardiogram data showed significant cardiac function recovery in the E2-CPCs group compared to the C-CPCs group. Overall, our approach demonstrates that E2 significantly enhances CPC regenerative potential, ultimately improving cardiac function through mediating differentiation, EMT, membrane lipid composition, angiogenesis, fibrosis, and mitochondrial kinetics.

Funding Source: This work was supported by the TUBITAK ARDEB 3501 (Project No. 220S910).

Keywords: cardiac progenitor cells, 17β-estradiol, heart failure

3:45 PM – 4:45 PM

381

MACROPHAGES SUPPRESS CARDIAC REPROGRAMMING OF FIBROBLASTS IN VIVO VIA IFN-MEDIATED INTERCELLULAR SELF-STIMULATING CIRCUIT

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Direct conversion of cardiac fibroblasts (CFs) to cardiomyocytes (CMs) in vivo to regenerate heart tissue is an attractive approach. After myocardial infarction (MI), heart repair proceeds with an inflammation stage initiated by monocytes infiltration of the infarct zone establishing an immune microenvironment. However, whether and how the MI microenvironment influences the reprogramming of CFs remains unclear. Here, we found that in comparison with cardiac fibroblasts (CFs) cultured in vitro, CFs that transplanted into infarct region of MI mouse models resisted to cardiac reprogramming. RNA-seq analysis revealed upregulation of interferon (IFN) response genes in transplanted CFs, and subsequent inhibition of the IFN receptors increased reprogramming efficiency in vivo. Macrophage-secreted IFN-β was identified as the dominant upstream signaling factor after MI. CFs treated with macrophage-conditioned medium containing IFN-β displayed reduced reprogramming efficiency, while macrophage depletion or blocking the IFN signaling pathway after MI increased reprogramming efficiency in vivo. Co-IP, BiFC and Cut-tag assays showed that phosphorylated STAT1 downstream of IFN signaling in CFs could interact with the reprogramming factor GATA4 and inhibit the GATA4 chromatin occupancy in cardiac genes. Furthermore, upregulation of IFN-IFNAR-pSTAT1 signaling could stimulate CFs secretion of CCL2/7/12 chemokines, subsequently recruiting IFN-β-secreting macrophages. Together, these immune cells further activate STAT1 phosphorylation, enhancing CCL2/7/12 secretion and immune cell recruitment, ultimately forming a self-reinforcing positive feedback loop between CFs and macrophages via IFN-IFNAR-pSTAT1 that inhibits cardiac reprogramming in vivo. Cumulatively, our findings uncover an intercellular self-stimulating inflammatory circuit as a microenvironmental molecular barrier of in situ cardiac reprogramming that needs to be overcome for regenerative medicine applications.

Keywords: reprogramming, heart regeneration, macrophage

3:45 PM – 4:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

383

ROLE OF BMAL1 AND YAP INTERACTION IN EPIDERMAL CELLS DURING SKIN AGING

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Aging can be defined as a decline or loss of adaptation during the increase of age, caused by a progressive deterioration of functions and integrity. This occurs in tissues such as the skin, which presents deep structural and functional changes during aging. However, the precise alterations occurring in each skin compartment, as well as their origin and consequences are still not completely understood. Here, we describe a functional interaction between BMAL1, the main transcription factor of the circadian clock machinery and YAP, a downstream effector of the Hippo pathway crucial for mechanotransduction processes. We show that in aged epidermal cells BMAL1 binds to the regulatory areas of a different set of genes, which are enriched in those with inflammatory functions. Moreover, we report that YAP shares many BMAL1-bound genomic locations, and that this adjacent binding happens mainly in enhancer regions. Additionally, we show that there is an increase in YAP activity in aged epidermis, the outermost layer of the skin. So far, with these results we hypothesize that age-associated changes in skin reshape the chromatin landscape of aged epidermal cells, directly impacting on these cells' transcriptional profile and function. We are currently disentangling the influence that YAP exerts on BMAL1 genomic relocation and transcriptional activation, and what are the age-associated signals that promote this phenomenon.

Keywords: aging, epidermis, epigenetics

3:45 PM – 4:45 PM

385

CUTANEOUS SCHWANN CELL REGENERATIVE CONTRIBUTION

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Aging causes both structural and functional alterations in the skin, leading to its overall decline. Schwann cells are glial cells of the peripheral nervous system and crucial for supporting peripheral nerves, including those of the skin. Schwann cells are also known as key players in the skin wound healing process, and their depletion causes delayed wound closure in murine skin. Chronic wounds are a significant health problem within the elderly, causing higher rates of infection and increased medical intervention. Preliminary single cell RNA-sequencing analysis revealed the presense of novel schwann cell population during wound healing process. Actually, we hypothesize that malfunction of aged cutaneous Schwann cells contributes to the age-associated defective wound healing, through a perturbation in their regenerative function. Hence, we are addressing this gap of knowledge by functionally characterizing aged cutaneous Schwann cells. With single cell RNA-sequencing of purified Schwann cells from adult and aged mouse back skin, we will obtain insights into the changes suffered by Schwann cells during aging. Dissecting those changes will be the starting point towards deciphering the role of these underestimated skin-resident cells in the impaired regenerative potential of aged skin.

Keywords: Schwann, regeneration, skin

ABSTRACT WITHDRAWN

ABSTRACT WITHDRAWN

3:45 PM – 4:45 PM

389

METABOLIC UNDERPINNINGS OF TUMOR-INITIATING STEM CELLS AND CANCER-ASSOCIATED FIBROBLAST NICHE IN LEFT AND RIGHT COLORECTAL CANCER

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Clinically, left and right CRC are regarded as two separate disease entities, yet little is known about how the metabolic features of the left (distal) and right (proximal) colon stem cells (p-colSCs and d-colSCs) may contribute to the region-specific colorectal cancer (CRC) development. Using scRNA-seq analyses for CRC patients, we identify metabolic rate-limiting enzyme encoding genes (RLEs) distinguishing tissue-resident versus tumor-resident colSCs, where mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) is the most down-regulated RLE in tumor-resident colSCs. Deletion of HMGCS2 promotes Wnt independency and induces fibroblast formation in tumor organoids derived from d-colSCs but not from p-colSCs. Our proteome analyses suggest that loss of HMGCS2 promotes oxidative-stress-induced senescence in the proximal colon while stimulating respiratory metabolism favoring regenerative cell-fate transition in the distal colon. Notably, with one copy deletion of tumor suppressor APC, the metabolic perturbation results in locally invasive tumors displaying robust

infiltration of inflammatory cancer-associated fibroblast (CAF) in the distal colon and rectum, while having minimal impact on the proximal colon. Our findings highlight the fundamental differences between p-colSCs and d-colSCs in responses to the metabolic perturbation and its influence on the tumor-initiating stem cells and cancer-associated fibroblast niche, potentially contributing to the region-specific colorectal cancer (CRC) development.

Keywords: colorectal cancer, intestinal stem cell, cancer-associated fibroblast

3:45 PM – 4:45 PM

391

A BITTERSWEET SYMPHONY: ABERRANT OGLCNAC ORCHESTRATES THE PROGRESSION OF PULMONARY FIBROSIS

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Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease characterized by the accumulation of fibrotic tissue, leading to impaired lung function, reduced quality of life, and ultimately, death. While the etiology of IPF remains elusive, emerging evidence suggests that metabolic dysregulation plays a vital role in disease progression and compromised repair capacity. In this study, we developed 3D in vitro models of healthy- and IPF-derived distal lung airway organoids to decipher fundamental mechanisms underlying aberrant tissue repair. Our findings demonstrated that these IPF organoids fully recapitulate key IPF phenotypes, including the expression of pro-fibrotic genes, secretion of IPF clinical biomarkers, and the presence of aberrant basal cells (KRT17+/COL1A1+). Furthermore, exposure to fibrotic stimuli exacerbated these IPF-like features. Intriguingly, multi-omic analysis unveiled a pivotal role for dysregulated O-linked β-N-acetylglucosamine (OGlcNAc) as a metabolic rheostat, influencing mitochondrial dynamics and fibrotic fate decisions through the regulation of JUNB. Targeting OGlcNAc on JUNB, using either pharmacological or gene editing approaches, mitigated the pro-fibrotic response and restored homeostasis. These findings highlight the significance of metabolic reprogramming mediated by OGlcNAc in shaping regenerative capacity and aberrant repair processes in IPF, shedding light on potential therapeutic avenues for this challenging disease.

Funding Source: Funded by Boehringer Ingelheim

Keywords: idiopathic pulmonary fibrosis, metabolic dysregulation, OGlcNAc

3:45 PM – 4:45 PM

393

DESIGN PRINCIPLES OF SMALL INTESTINAL CRYPT MAINTENANCE

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The intestinal epithelium has a remarkably high turnover in homeostasis. It remains unresolved how turnover is orchestrated at the cellular level and how the behavior of stem and progenitor cells ensures tissue maintenance. To address this, we combined quantitative fate mapping in three complementary mouse models with mathematical modeling and single-cell RNA sequencing. Our integrated approach generated a spatially and temporally defined model of crypt maintenance that is based on two cycling populations: crypt-based columnar (CBC) and transit amplifying (TA) cells. Validation experiments substantiated the predictions from the model revealing TA cells as the major contributor to the absorptive lineage, while balanced CBC cell fate choices controlled the numbers of cells in the secretory lineage. By unraveling these mechanisms, we gain insights into the process of tissue turnover and provide direct evidence to support the notion of CBC cells as the major driver of the intestinal epithelium replenishment.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine is supported by a Novo Nordisk Foundation grant (NNF21CC0073729).

Keywords: small intestinal epithelium, tissue maintenance, mathematical modeling

3:45 PM – 4:45 PM

395

FUNCTIONAL FIBROBLAST HETEROGENEITY IN THE OESOPHAGEAL STEM CELL NICHE DURING CANCER PROGRESSION

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Esophageal Squamous Cell Carcinomas (ESCCs) develop through mutations in epithelial progenitor cells. In addition, microenvironmental signals either promote or inhibit tumor initiation. Fibroblasts are a key component of the stromal microenvironment, but how distinct subpopulations of fibroblasts impact ESCC development is currently not well understood. We identified and functionally characterized fibroblast heterogeneity in the esophagus during homeostasis and tumor development. We find that TROY-Positive (POS) fibroblasts are differentially distributed from proximal to distal and are in direct contact with the esophageal epithelium. To understand the dynamics of TROY-POS fibroblasts in esophageal cancer progression, we treated mice with the carcinogen 4NQO. We find a spatial reorganization of the stromal niche early in the development of ESCC, where TROY-POS fibroblasts loose contact with the epithelium and are interspersed with immune and endothelial cells. Organoid co-cultures and xenografting of Sox2-P53ΔKrasG12D transformed cells reveal that TROY-POS fibroblasts suppress both organoid growth of healthy progenitor cells and tumor growth of transformed cells. In addition, ablation of TROY-POS fibroblasts during 4NQO induced ESCC progression, resulted in expansion of the epithelium into the stroma. Transcriptional profiling showed that TROY-POS fibroblasts express extracellular matrix components enriched in Col1a1 expression, providing a stiff matrix which can supress epithelial invasion. Our results identify a TROY-POS fibroblast subpopulation which restricts esophageal epithelial growth normally, as well as during tumor development. Dissecting the role of fibroblast subpopulations during esophageal cancer progression is of importance to improve therapeutic strategies.

Funding Source: This study was supported by ERC Starting Grant TroyCAN (851241), Cancerfonden (19007Pj) and Cancerfonden Postdoctoral Position Grant (22 0538)

Keywords: fibroblasts, Esophageal Squamous Cell Carcinoma, stem cells

3:45 PM – 4:45 PM

397

THREE-DIMENSIONAL GENOME ARCHITECTURE COORDINATES KEY REGULATORS OF LINEAGE SPECIFICATION IN MAMMARY EPITHELIAL CELLS

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Although lineage-specific genes have been identified in the mammary gland, little is known about the contribution of 3D genome organization to gene regulation in the epithelium. Here we describe the chromatin landscape of the three major epithelial subsets through integration of long- and short-range chromatin interactions, accessibility, histone modifications and gene expression. We find that lineage specific marker genes display unique epigenetic modifications, achieving lineage specification within the mammary gland. Basal genes display exquisite lineage-specificity via coupling of faithful promoter and distal enhancer activity. Luminal-specific genes show widespread promoter priming in basal cells. Cell-specificity in luminal progenitors is largely mediated through extensive chromatin interactions with super-enhancers in gene-body regions, in addition to interactions with polycomb silencer elements. Genes enriched in mature luminal cells strangely display increased bivalent chromatin across their promoters which are linked to distal active enhancers through low-level chromatin interactivity. Finally, histone modifications were largely homogenous between active and quiescent basal cells, with chromatin accessibility proving the most dynamic epigenetic factor. Interestingly, genes enriched in quiescent cells were highly enriched for polycomb across both basal populations. This work provides a comprehensive resource for understanding the role of higher-order chromatin interactions in cell-fate specification and differentiation in the adult mouse mammary gland.

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Keywords: mammary gland development, epigenetics and chromatin looping, transcription regulation

3:45 PM – 4:45 PM

399

THE ROLE OF TRANSFERRIN 2 IN HOMEOSTASIS AND AGEING OF THE DROSOPHILA MIDGUT

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Loss of the balance between cell death and production, which often occurs with old age, can lead to a variety of diseases. As the intestine is a tissue with a high turnover rate, homeostatic control is especially important. Stem cells are responsible for maintaining tissue homeostasis, and previous work from our lab and others has shown that proteins secreted by the stem cells play important roles in the control of Drosophila intestinal homeostasis. A screen of conserved intestinal stem and progenitor cell-secreted proteins identified Transferrin 2 (Tsf2) as a regulator of homeostasis. We have found that changing the levels of Tsf2 expression in stem and progenitor cells in the Drosophila midgut disrupts homeostasis, and that Tsf2 expression changes with age. Furthermore, although Tsf2 acts as a septate junction protein in other tissues, changing expression levels in the Drosophila midgut does not impact its barrier function, suggesting an alternative mechanism of action. Our findings may have relevance to human health and disease, as Melanotransferrin, the mammalian homologue of Tsf2, has been associated with tumorigenesis.

Funding Source: BBSRC, UKRI

Keywords: Drosophila, intestinal stem cells (ISCs), homeostasis

3:45 PM – 4:45 PM

401

HUMAN CORNEAL ENDOTHELIAL CELL EXPANSION ON A RGD FUNCTIONALIZED CHITIN SCAFFOLD

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Donor corneal transplantation is the only available treatment for patients with corneal endothelium dysfunction to date. However, the worldwide donor corneal shortage, patient's discomfort to cadaveric transplantation and corneas' unusability due to the increasing refractive surgery are becoming non-negligible issues. This has led to investigate alternative approaches for treating corneal endothelial diseases. In particular, corneal endothelial tissue engineering is an emerging therapeutic approach that involves the use of human corneal endothelial cells (HCEnCs) combined with a biomaterial to create tissue engineered grafts for transplantation. This study investigated the use of an innovative chitin scaffold functionalized with a synthetic RGD (Arg-Gly-Asp) peptide as a potential substrate for HCEnCs adhesion and expansion. Importantly, scaffold characteristics were optimized to produce a substrate with biomechanical properties resembling the human corneal stroma for transparency, 3D structure, stiffness, and mechanical strength. The RGD functionalized chitin scaffold ensured a complete adhesion and growth of HCEnCs as a confluent monolayer. HCEnCs retained their unique polygonal morphology and expressed typical epithelial cell markers of adult human corneal endothelium such as cytokeratin (CK)-8, CK-18 and CK-19, markers involved in the maintenance of corneal transparency such as Na+K+/ATPase and Aquaporin, and the tight junction markers ZO-1 and N-Cadherin essential for maintaining the proper structure of the corneal endothelium. Therefore, our RGD functionalized chitin scaffold was able to preserve both the characteristic HCEnCs phenotype and the functionality of the cell monolayer. In addition, the vitality and apoptosis assays showed no signs of cytotoxicity indicating that chitin scaffold didn't affect HCEnCs viability and activity. This study is a proof of concept for considering RGD functionalized chitin scaffold as a potential carrier for cultured HCEnCs in order to produce a suitable tissue engineered endothelial graft and, from a future perspective, a three-dimensional human cornea with all its layers (epithelium + stroma + endothelium). If successful, this elegant approach has the potential to increase access to corneal therapy by treating multiple patients.

Keywords: corneal endothelium, tissue engineering, cell therapy

3:45 PM – 4:45 PM

403

EPIDERMAL CLOCK INTEGRATION AND GATING OF BRAIN SIGNALS GUARANTEES SKIN HOMEOSTASIS

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In mammals, an integrated network of molecular oscillators drives daily rhythms of tissue-specific homeostatic processes. This circadian clock network maintains tissue health and is compromised during ageing, disease and by lifestyle choices, such as diet and exercise. However, critical properties of this systemic network, such as which tissues communicate to coordinate their respective programs of daily physiology, and the exact homeostatic processes requiring each communication pathway, remain undefined. To dissect daily inter-tissue communication, we have constructed in mice a minimal clock network comprising only two nodes: the peripheral epidermal clock and the central brain clock. By circadian transcriptomic and functional characterization of this isolated connection, we have identified a previously unknown gatekeeping function of the peripheral tissue clock with respect to systemic inputs. That is, the epidermal clock concurrently integrates and corrects brain signals to ensure timely execution of epidermal daily physiology. Specifying the integrative arm of the clock, we identify that timely cell cycle termination in the epidermal stem cell compartment is dependent upon incorporation of clock-driven signals originating from the brain. Unexpectedly, and in contrast, the epidermal clock corrects potentially disruptive feeding-related signals to ensure that DNA replication of epidermal stem cells occurs at the optimum time of day. Together, we present a novel approach for cataloguing the systemic dependencies of a given tissue, and in turn identify an essential gate-keeping function of the circadian clock in epidermal cells that guarantees tissue homeostasis.

Keywords: circadian rhythms, epidermal stem cells, systemic communication

3:45 PM – 4:45 PM

405

SPATIAL RECONSTRUCTION OF SINGLE TELOCYTES ALONG THE CRYPT-VILLUS AXIS DEFINES THE MOLECULAR BASIS OF INTESTINAL HOMEOSTASIS

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Epithelial tissues, display a well-organized structure into distinct domains or regions, with each region being associated with specific functions. However, the molecular and cellular mechanisms orchestrating these intricate and coordinated functional zonation, essential for supporting tissue homeostasis, remain a subject of ongoing debate. Recent studies in the intestine have identified a continuous subepithelial network of specialized stromal cells called telocytes that constitute the intestinal stem cell niche. Telocytes expressing the transcription factor FOXL1, accompany both stem cells and all their derivatives along the crypt-villus axis throughout the entire differentiation process1-3. Here, we combined single cell RNA sequencing together with spatial approaches to create a genome wide topology map of single telocytes along the intestinal crypt-villus axis. This integrated approach reveal four clusters of telocytes; one along the crypt and three along the villus epithelium- namely, villus-base, villus-mid and villus-tip. Remarkably, crypt telocytes display high expression of niche-supporting signalling molecules, including canonical Wnt ligands, Rspondins, the Bmp-inhibitor Gremlin1and Wnt receptors. At the villus base, telocytes exhibit the expression of smooth muscle actin (Acta2), the cell adhesion molecule Alcam, and genes associated with the regulation of both cell proliferation and cell differentiation. Intriguingly, telocytes located at the mid-villus express canonical Wnts along with Rspondin3, as well as various chemokine and cytokine genes. On the other hand, telocytes at the villus tip are actively involved in Bmp and Egfr signalling. In summary, our data indicate that telocyte function correlate with epithelial functions, thereby defining the molecular basis for intestinal homeostasis.

Funding Source: ISF 1997/19, NSFC-ISF 3436/20

Keywords: stem cell-ciche, stroma, regeneration

3:45 PM – 4:45 PM

407

MELANOMA STEM CELLS POLARIZE MACROPHAGES TOWARDS A MIXED M1/M2 PHENOTYPE

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Cancer stem cells (CSCs) actively contribute to the aggressiveness of melanoma and possess the ability to influence immune cells, for instance by polarizing macrophages towards a pro-tumor phenotype. Macrophages are a highly heterogeneous myeloid cell population, well known to facilitate melanoma growth and dissemination. However, the impact of melanoma stem cells (SCs) on macrophages biology has not been completely elucidated yet. The main purpose of our study was to explore whether melanoma stem cells (SCs) can reprogram macrophage phenotype. To this aim, we utilized an in vitro model of human macrophages by using THP-1 monocyte cell line, differentiated into macrophages (M0) using phorbol 12-myristate 13-acetate (PMA). M0 macrophages were exposed to the conditioned medium (SCs-CM) of A375 and WM115 melanoma SCs, and subsequently, the macrophage

phenotype was analyzed using flow cytometry, real-time PCR, and ELISA assay. We observed that melanoma SCs can influence macrophage polarization by increasing the expression of Arginase 1 while decreasing the levels of CD163, two well-known M2 markers associated with cancer immunosuppression and progression. Interestingly, RT-PCR analysis revealed an upregulation of both M1 and M2 markers, including IL-6, IL-12B, STAT1, IL-10, VEGF, and MARCO, in macrophages after exposure to melanoma SCs-CM compared to untreated controls. At the same time, in treated macrophages we found a reduction in the levels of M1 markers, such as IL-1β and TNF-α, as well as the M2 marker TGF-β. Accordingly, ELISA data showed a higher secretion of IL-6, IL-10, and MMP-2 compared the control counterpart, reinforcing the idea of the acquisition of an M1/M2 mixed phenotype in macrophages treated with melanoma SCs-CM. To functionally characterized the phenotype of melanoma SCs-activated macrophages, we exploited the ability of macrophages to influence NK cells cytotoxicity in vitro. SCs-treated macrophages did not have any effect on NK cells cytotoxic activity compared to controls, suggesting that, although expressing pro-inflammatory mediators, they do not have the ability to mount a proper immune response. This study deepened our understanding of how melanoma SCs influence macrophages, revealing their capacity to induce a combination of M1 and M2 phenotypes.

Keywords: melanoma stem cells, macrophage polarization, immune environment

3:45 PM – 4:45 PM

409

DECIPHERING THE TRANCRIPTOMIC AND EPIGENETIC PROFILES OF CLINICAL-GRADE LIMBAL AND CONJUNCTIVAL STEM CELLS AT THE SINGLE CELL LEVEL

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Stem cells of the ocular surface notably differ in their role, topology, and differentiation potential. Limbal stem cells (LSC) regenerate the cornea and reside in the limbus while conjunctival stem cells (CjSC) renew the conjunctival epithelium that contributes to the maintenance of the tear film although the knowledge regarding their location and features is still very faint. However, a better characterization of these populations would greatly help to shed light on ocular surface regenerative processes and for the treatment of many conditions, such as LSC deficiency (LSCD), especially when both eyes are involved, thus, the stem cells are absent or functionally altered. To date, analyses of these tissues at the single-cell level have only been shown considering in vivo samples, where the cells are mostly in a differentiated status. Therefore, a comprehensive description of these stem cell cultures is needed to improve their proper in vitro cultivation for regenerative purposes and especially since specific CjSC markers have not been demonstrated yet. To address this knowledge gap, we examined cultured human limbal and conjunctival samples in a clinical-grade system to deeply describe the low differentiated cell populations of these tissues by using single-cell RNA-seq and ATAC-seq analyses. Clustering and pseudotime trajectory led to the identification of numerous clusters containing cells identified as stem, in a quiescent or activated status, proliferative and early differentiated. Differential Expressed Genes (DEGs) were identified for each cluster revealing a plethora of new candidate markers. Moreover, enrichment analysis allowed us to identify the major pathways involved in stem cell self-renewal and differentiation for both epithelia. Finally, these findings help us to delineate a stem cell gene signature for LSC and CjSC, which is of great importance for further in vitro and in vivo studies with clinical purposes. In conclusion, reaching a Gene Regulatory Network description for LSC by this multiomic approach may bring to an in vitro transdifferentiation protocol especially useful for LSCD bilateral treatments.

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Keywords: cornea, conjunctiva, ocular surface

3:45 PM – 4:45 PM

411

CHARACTERIZATION OF THE TRNAS OF LUNG CANCER STEM CELLS

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Lung cancer remains a major cause of cancer-related deaths worldwide with unfavourable prognosis mainly due to the late stage of disease at presentation. Malignant phenotype in lung cancer is sustained by transformed alveolar epithelial type II (ATII) lung cancer stem cells (CSCs) that have the potential of initiating lung cancer formation, mediate metastasis formation and support resistance to therapy. A growing amount of evidence suggest that mRNA translation regulation and tRNA modifications play a key role in supporting cancer establishment. Therefore, we postulate that the expression of a specific signature of tRNA enzymes permits the establishment lung cancer stem cells by sustaining proteome rewiring upon KRAS oncogenic transformation. To investigate this hypothesis, we optimize human healthy lung spheroids and KRASG12S lung cancer spheroids cultures to increase the expression of stemness markers, anchor-free survival and EMT markers. We then performed a set of drop-out CRISPR-Cas9 pool screens of all the known human tRNA enzymes to identify new mRNA translation modulators of lung CSC. Through this approach, we highlighted five tRNA enzymes responsible for three different types of tRNA modifications that specifically impact stem-like proprieties of KRASG12S lung cancer cells but not of healthy cells. To validate our targets, we depleted the tRNA enzymes by shRNA and quantified CSC fitness by following spheroid formation and the capability of cells to undergo EMT. Our preliminary results indicate that the loss of our targets impacts lung CSC fitness. Currently, we are generating tRNA-seq, RNA-seq and proteomic datasets of KRASG12S lung cancer spheroids depleted or not of our targets to better characterize the impact of our targets on lung CSC proteome establishment.

Keywords: stem cells, progenitors cells, alveolar type II cells, lung adenocarcinoma

3:45 PM – 4:45 PM

413

REGENERATION OF A MATURE AIRWAY EPITHELIUM ON A PERFUSABLE CLINICAL-GRADE SCAFFOLD

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Tracheal and bronchial reconstruction represents the only therapeutic option in cases of wide circumferential damage, where conventional surgical procedures prove ineffective. Yet, despite long-standing interest, none of the tested reconstructive approaches has evolved into a well-established clinical practice. Tissue engineering attempts aimed at regenerating respiratory tracts must prioritize the identification of a culture system allowing for safe and efficient expansion of airway epithelial cells and a scaffold suitable for cells' growth and physiological differentiation. Here, we showcased the ability of human primary airway epithelial cells to undergo safe and efficient expansion while preserving the stem cell compartment using a clinically validated culture system. We also demonstrated the in vitro capacity of cultured airway epithelial cells to rapidly repair a wound and act as a physical barrier. Moreover, when cultivated on a collagen-based clinically-approved scaffold, expanded airway epithelial cells retained their proliferative, clonogenic and differentiative potential without exhibiting acute or chronic cytotoxic effects. Of note, through an organotypic model mimicking in vivo conditions, we accomplished the complete reconstruction of a mature airway epithelium onto the scaffold. Essential to determining the competence of the selected biomaterial to integrate within the recipient and guarantee graft survival after transplantation in vivo, we proved the scaffold's ability to host a functional vascular network once implanted in a murine model. These findings demonstrate the feasibility of regenerating a self-renewing functional airway epithelium and developing an autologous clinical-grade tissue-engineered product. With adequate 3D scale-up and integration with other cell types (cartilage cells), this study holds promise to attain the regeneration of long-lived autologous respiratory tracts.

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Keywords: airway regeneration, tissue engineering, clinical-grade scaffold

3:45 PM – 4:45 PM

415

ERBB-FAMILY RECEPTORS REGULATE HUMAN AIRWAY SUBMUCOSAL GLAND MORPHOGENETIC BEHAVIOR AND LINEAGE OUTCOMES

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Cystic fibrosis, asthma, and chronic bronchitis are all hypersecretory disorders of the airways characterized by increased secretions from the submucosal glands (SMGs.) The SMGs seemingly constitute a protected niche for putative stem/progenitor populations capable of repairing airway and glandular epithelia. However, the full complement of SMG cellular identities remains dubious, in part due to the unique complexity of airway SMGs in humans. Further understanding of the cellular lineages and niche interactions that govern SMG stem/progenitor behavior is instrumental for development of therapeutic strategies to address disorders afflicting the airways. We used single-cell and spatial mRNA sequencing to profile gene expression of human airway submucosa derived from the intercartilaginous zone of donor tracheae and bronchi. Single-cell trajectories and receptor-ligand interaction were analyzed via the STREAM pipeline and iTALK package, respectively. The impact of identified ligand interactions was assessed using a novel human SMG epithelial organoid model. Our transcriptomic dataset captures a diverse collection of epithelial, mesenchymal, immune, and endothelial lineages comprising a multitude of cell types and states, including multiple distinct fibroblast and secretory populations and divergent transitioning epithelial precursors. Interrogation of the human airway submucosa reactome via receptor-ligand analysis revealed that mobilization and differentiation of SMG stem/progenitor cells is mediated by ERBB family members, namely EGFR and ERBB3. During expansion of human SMG epithelial organoids, ERBB ligands HBEGF and NRG1 promote branching morphogenesis of undifferentiated stem/progenitor cells. Conversely, treatment of SMG organoids with EGFR or ERBB3 inhibitors results in attenuated growth. Further, AREG and EREG increase secretory cell differentiation. Regulation of glandular stem/progenitor activation and fate outcomes has important translational implications for airway hypersecretory disorders, in which glandular hypertrophy and hyperplasia are pathophysiological hallmarks. Our novel SMG organoids provide a tractable system for disease modeling and drug discovery, which we leveraged to functionally validate ERBB-regulated SMG stem/progenitor behavior.

Funding Source: Cystic Fibrosis Foundation Rose Hills Foundation - Broad Stem Cell Research Center Training Program

Keywords: airway submucosal glands, cystic fibrosis, organoid modeling

3:45 PM – 4:45 PM

417

BIOMECHANICAL REGULATION OF THE HAIR FOLLICLE ADULT STEM CELL NICHE SELF-ORGANIZATION

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Adult stem cell niches in rapidly self-renewing tissues such as skin and intestine exhibit dynamic behavior. This involves balancing proliferation, differentiation and exit from the niche, while preserving niche integrity and stable stem cell numbers. Cell dynamics generates fluctuations in mechanical forces. However, how adult stem cells integrate these physical cues with biochemical signals to maintain niche homeostasis and to determine cell behaviors remains unclear. Using hair follicle stem cell (HFSC) organoids as model, we have utilized live imaging to quantify self-organizing principles of stem cell niche dynamics. We observe equilibrium-like behavior where HFSCs self-renew and generate differentiated progeny to generate equal proportions of these cell states. At the same time, HFSCs and their direct progenitors physically segregate into distinct compartments by engaging sorting-like behavior. Analysis of junctional composition and tension as well as nuclear-mechanical properties has revealed cell state-specific differences that potentially underlie this self-organizing behavior. This project highlights how cellular mechanics are linked to stem cell niche homeostasis.

Keywords: adult stem cell niche, self-organization, mechanobiology

3:45 PM – 4:45 PM

419

CHARACTERIZING THE BASAL CELL POPULATION AND CLONAL DYNAMICS IN THE HUMAN LUNG

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Basal cells are stem cells of the pseudostratified airway epithelium in the human lung. During homeostasis, they are responsible for self-renewal and differentiation into multiple airway epithelial cell types and are required for regenerative responses after injury. One key aim for regenerative medicine is to improve the repair response of basal cells in chronic lung diseases. However, there remains a critical knowledge gap in understanding the heterogeneity and long-term clonal dynamics of the basal cell population and its differences during development and homeostasis. This research seeks to address that knowledge gap by functionally assessing and creating a quantitative model of stem cell turnover and clonal dynamics in human fetal tissue, as well as adult human airway epithelium. We hypothesize that a novel tissue culture system preserving the 3D architecture of epithelial tissue while reaching a steady state will mimic the airway epithelial niche at homeostasis. To see if our in vitro model recapitulates the normal airway epithelium, we are comparing with in vivo data using immunofluorescence, scRNA-seq, and qPCR. We are using clonal analysis and lineage-tracing techniques to build a quantitative model of cellular turnover and heterogeneity within the basal cell population. This work addresses unanswered fundamental questions in developmental biology, as well as practical knowledge that will be critical to proper implementation of targeted stem cell therapeutics in the human lung.

Funding Source: Gurdon Institute, Wellcome Trust, Cancer Research UK, Medical Research Council, and Gates Cambridge

Keywords: lung development and homeostasis, stem cells, lineage-tracing and clonal dynamics

3:45 PM – 4:45 PM

421

INTEGRATED STRESS RESPONSE IN INTESTINAL STEM CELLS: MOLECULAR MECHANISMS AND ROLE IN INTESTINAL HOMEOSTASIS

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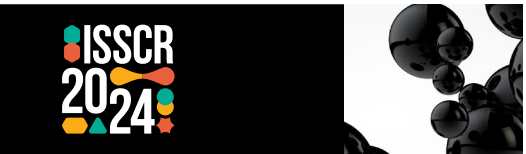
The intestinal epithelium provides a selective boundary between the animal and its environment, allowing absorption of nutrients while protecting other tissues from pathogens and toxins. It consists of differentiated cell types, such as absorptive enterocytes and hormone-secreting enteroendocrine cells, and their self-renewal is orchestrated by intestinal stem cells (ISCs). ISC functions are regulated by environmental factors, such as nutrition, as well as intrinsic signaling networks that aim to keep intestinal epithelium under homeostasis. How externally and intrinsically induced molecular responses influence ISCs, remain incompletely understood. Cellular protein homeostasis, proteostasis, can be lost under several conditions, such as upon amino acid imbalance or aging. Loss of cellular proteostasis activates stress response pathways, including the integrated stress response (ISR). Activation of ISR leads to phosphorylation of eukaryotic initiation factor 2-α (eIF2α), which inhibits translation of most proteins, thus relieving the risk of impaired proteostasis. ISR also activates transcription factor ATF4, which restores the cellular homeostasis activating stress response genes. Since the activity of translation is a key driver of cell growth, activation of ISR in somatic cells leads to inhibition of growth. Unexpectedly, we have discovered that phosphorylation of eIF2α levels is elevated during normal ISC differentiation into absorptive enterocyte fate in Drosophila. Furthermore, loss of proteostasis in ISCs, both genetically or pharmacologically, leads to dysplastic phenotype with hyperproliferation and increased enterocyte differentiation from ISCs. We hypothesize that this ISR mediated signaling network would be a double-edged sword, allowing maintenance of ISC homeostasis when the frequency of



impaired proteostasis is low, but leading to uncontrolled differentiation and dysplasia when too prevalent. Collectively, this work is expected to uncover a new mechanism involved in dynamic control of intestinal turnover and homeostasis.

Keywords: intestinal stem cell, metabolism, homeostasis

3:45 PM – 4:45 PM

423

ARE E-CIGARETTES SAFER THAN COMBUSTIBLE TOBACCO ON HUMAN TYPE II ALVEOLAR CELLS AND MESENCHYMAL STEM CELLS?

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Electronic cigarettes (e-cigarettes) are promoted as safer alternatives to combustible cigarettes. However, the health burden and effects of their long-term use remain unidentified. This study evaluates the extent and reversibility of cellular damage following exposure to cigarette and e-cigarette smoke aerosols, in vitro, of human type II alveolar cells (A549) and bone marrow-derived mesenchymal stem cells (BM-MSCs). The survival, levels of reactive oxygen species (ROS) generation and expression of epithelial-to-mesenchymal transition (EMT) markers are reported for A549 cells. BM-MSCs are evaluated for their survival and their differentiation potential into the osteogenic lineage. Cigarette smoke aerosols irreversibly reduce cell proliferation in A549 cells and enhance ROS production. E-cigarette smoke aerosols also permanently reduce proliferation of A549 cells, downregulated the gene expression of connexin 43 and E-cadherin and upregulated that of N-cadherin; promoting EMT. The integrity of BM-MSC- mediated cellular repair, described in terms of BM-MSC proliferation and osteogenic differentiation, was compromised by both cigarette and e-cigarette aerosol extracts. However, BM-MSCs were able to recover their proliferative ability and osteogenic differentiation upon e-cigarette, but not combustible cigarette, washout. The cellular damage caused by cigarette aerosols is irreversible in A549 cells and on BM-MSCs; while e cigarettes were associated with less alterations and greater, albeit incomplete, recovery of some cellular functions after smoke washout. These results shed light on the safety of e-cigarettes, attempt to explain long-term illnesses in chronic smokers and feed into future public policies on tobacco control.

Funding Source: This work was partly supported by a grant (ARG/FHS/22-23/002) from the University of Balamand.

Keywords: electronic cigarettes, combustible cigarettes, in vitro models of cell damage

3:45 PM – 4:45 PM

425

SOX2-HIGH/NTRK2-HIGH MARK A TPA-NON-RESPONSIVE SUBPOPULATION OF NASOPHARYNGEAL CARCINOMA CELLS

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The pervasive occurrence of nasopharyngeal carcinoma (NPC) is intricately linked with Epstein-Barr virus (EBV) infection, making EBV and its associated pathways promising therapeutic targets for NPC and other EBV-related cancers. Lytic induction therapy, an emerging virus-targeted therapeutic strategy, capitalizes on the presence of EBV in tumor cells to specifically induce cytotoxicity against EBV-associated malignancies. Despite the expanding repertoire of compounds designed to induce EBV lytic reactivation, achieving universal induction across all infected cells remains elusive. The inherent heterogeneity of tumor cells likely contributes to this variability. In this study, we used a recently generated EBV-positive NPC cell line, named NPC43, as the in-vitro model for NPC, and employed single-cell transcriptomics to delineate the diverse responses of NPC43 cells to lytic induction of EBV. Our longitudinal monitoring revealed a distinctive lytic induction non-responsive cellular state characterized by elevated expression of SOX2 and NTRK2. Cells in this state exhibited phenotypic similarities to cancer stem cells (CSCs), and we verified the roles of SOX2 and NTRK2 in manifesting these phenotypes. This discovery underscores a limitation in the lytic induction efficacy, suggesting that not all tumor cells are susceptible to this treatment, highlighting the importance of integrating lytic induction with other targeted approaches during therapy.

Funding Source: Hong Kong RGC CRF C4001-1 The Innovation and Technology Fund (MRP/036/21X) Research Grant Council (Areas of Excellence Scheme – AoE/M-401/20; Collaborative Research Fund - C4001-18GF) Health and Medical Research Fund (08191046)

Keywords: lytic induction therapy in nasopharyngeal carcinoma, therapy resistant cancer cells identification with scRNA-seq, cancer stem cells phenotypes

3:45 PM – 4:45 PM

427

LGR5 AS A NEW POSITIVE MARKER FOR THE ENRICHMENT OF HUMAN LIMBAL CORNEAL EPITHELIAL STEM/PROGENITOR CELLS

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Limbal stem cells (LSCs) attribute to the maintenance of the corneal epithelium and are endowed with a capacity for self-renewal and proliferative potential. However, specific markers for limbal stem/progenitor cells have not been identified. LGR5 (Leucine-rich repeat-containing G-protein coupled receptor 5), a target of Wnt signaling, has been found in other organs and used as an adult stem cell marker. In this study, we investigated whether LGR5 can be used as a limbal stem cell marker. Post-keratoplasty discards of human corneal-limbal tissues from unidentifiable cadavers obtained were divided into 10 equal segments, cut into 0.5-mm-wide limbal strips, and cultured in air–liquid interface conditions on Transwell plates. Limbal epithelial cells were separated and analyzed for LGR5-positive cells using FACS. FACS were used to fractionate positive/negative cells and to confirm the expression of markers related to colony formation and stemness. Over-expressed genes in LGR5-positive cells were confirmed by PCR analysis. The expression of LGR5 was confirmed by immunofluorescence staining from the corneal center to the limbus, as well as in the basal layer of the limbus. LGR5 was observed where known other markers p63α, CK15, ABCG2, and ABCB5 were positive. After 12-14 days of limbal explant culture and isolation, the ratio of LGR5 positive cells by FACS was 7.3 ± 2.7%. Colony-forming efficiency (CFE) of LGR5 positive/negative cells were significantly higher in LGR5 positive cells than negative cells. Additionally, PCR analysis demonstrated that fractionated LGR5 positive cells showed significant increases of other stemness-related markers p63α, ABCG2, and ABCB5 and proliferation marker Ki67. The expression of LGR5 in human limbal tissues was confirmed, as well as increases in CFE and stemness of LGR5 positive cells in limbal explant cultures. Thus, LGR5 demonstrates potential to be utilized as a new limbal stem/progenitor cell marker in the future.

Keywords: limbal stem cell, LGR5, corneal epithelial

3:45 PM – 4:45 PM

429

HETEROGENEITY OF INVASIVE BREAST CANCER CELLS AND THEIR ROLE IN VASCULAR MIMICRY

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Human breast cancer (BC) is the most commonly diagnosed cancer in women worldwide and one of the leading causes of cancer-related deaths. Activation of partial or more complete epithelial-to-mesenchymal transition (EMT) in cancer cells enhances the acquisition of invasive behavior and the appearance of circulating tumor cells (CTC) in the blood. EMT has also been associated with cancer stem cell (CSC) properties, self-renewal capabilities, resistance to conventional therapies, and a tendency for post-treatment recurrence. Recent studies have shown that cancer stem cells (CSCs) and epithelial-to-endothelial transition (EET), a subtype of epithelial-to-mesenchymal transition (EMT), can promote the process of vasculogenic mimicry (VM), a newly defined pattern of tumor microvascularization by which aggressive tumor cells can form vessel-like structures themselves. However, the biological features of tumor cells that form VM and their role in tumor metastasis remain unknown. Therefore, we recently developed a reporter system using a human breast cancer model to label and track tumor cells undergoing EMT in vivo. RNA-seq analysis of highly invasive cancer cells isolated using this system revealed enrichment in genes essential for cellular movement, cell invasion, and, interestingly, tumor-vasculature interactions. Our analysis of tumors with a reporter system let us identify a rare population of cancer cells with increased plasticity, expressing endothelial marker MCAM, and participating in vascular mimicry. MCAM-positive breast cancer cells may represent cells with stem cell potential, capable of self-renewal and differentiation into endothelial-like, critically involved in tumor vascular mimicry and metastasis. Moreover, specific targeting of this cell population and related molecular pathways could thus provide novel strategies to eradicate cancers currently resistant to conventional therapy.

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Keywords: chaoatulin, invasion, vascular mimicry

3:45 PM – 4:45 PM

433

IDENTIFICATION OF A NOVEL ALVEOLAR EPITHELIAL CELL TYPE 2 AND ALVEOLAR MACROPHAGE POPULATION GENERATED BY TRANS DIFFERENTIATION FOLLOWING INJURY

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During homeostasis, the adult lung epithelium is mostly quiescent but upon infection or injury to the epithelium, specific pathways and cell populations are activated to restore the lung architecture. Failure to do this, results in scarring and impaired lung function. Mechanism associated with normal tissue repair following injury remain poorly understood. We utilized a CRISPR-Cas9 mouse line (CARLIN) to combinatorial capture populations as well as molecular profiles associated with tissue repair following naphthalene induced injury at the single cell level. Following injury, tissue repair was identified to be initiated through the recruitment and assembly of the Nlrp3 inflammasome complex by the alveolar macrophage population. We further identified two novel populations: subset of the alveolar macrophage and alveolar epithelial cell type 2 population both specifically co expressing leukocyte as well as alveolar epithelial cell markers, suggesting a shared development path. Lineage tracing further revealed that the novel alveolar epithelial cell type 2 population is likely emerging from the alveolar macrophage. Altogether, our data suggest that the alveolar macrophage may play a role in regenerating the lung epithelia following injury.

Keywords: tissue repair, lung epithelium, lineage tracing

3:45 PM – 4:45 PM

435

EXPLOITING THE REGENERATION POTENTIAL OF THE NAIL MINI-ORGAN STEM CELLS IN DIGITS RESTORATION

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The digit tips of rodents and primates are recognized for their endogenous regeneration capability, including the digit's nail plate (NP), epidermis, nerves and bone. The crucial role in orchestrating the activation of such regenerative response is attributed to two autonomous populations of skin stem cells (SCs): the highly proliferative stem cells of the nail matrix (NSCs), that in normal conditions replenish the NP, and the slow-cycling population of the nail proximal fold stem cells (NPFSCs). NPFSCs display bifunctional properties, by contributing to the peri-nail epidermis under the normal homeostasis and the hard nail structure upon injury. The main purpose of our work was to define these cells and explore their molecular characteristics in order to understand the critical role of the bone morphogenetic protein (BMP) and Wnt signaling pathways in directing and regulating their fates. We have successfully isolated and established culture conditions for marked NPFSCs, which were further harvested and engrafted into amputated digit tips of immunocompromised mouse models, with the aim of stimulating the regenerative responses in regeneration-incompetent injuries. Such injuries involve removing whole visible nail plate, over 50% of the distal phalanx and the nail matrix's distal region with the Wntless-expressing zone - required for the initiation of the Wnt signaling. However our research revealed that simultaneous overexpression of the BMP pathway could prevent such regeneration failure through the enhanced involvement of the NPFSCs in the digit restoration. Indeed, our gain-of-function (GoF) mouse models, with overexpressed BMP signaling pathway in keratinocytes, have shown abnormal overgrowth of nail structures and facilitated regeneration after both - distal and proximal amputation. Conversely, loss of the BMP pathway was associated with irregular and thinner NP structure, hyperplastic nail bed (NB), loss of the keratinous zone (KZ) and reduction of the nail matrix cells' proliferating abilities. In conclusion, our research may help fill the gaps in the current understanding of nail biology and could not only offer novel forms of treatment for patients with nail and digit defects but, in a broader sense, might also provide new regenerative therapies for amputees in the future.

Funding Source: National Science Centre, Poland (NCN), Opus Grant #2019/33/B/NZ3/02966; Foundation for Polish Science (FNP) co-financed by the EU under the European Regional Development Fund, Team Grant #POIR.04.04.00-00-4222/17-00

Keywords: nail proximal fold stem cells (NPFSCs), bone morphogenetic protein (BMP) signaling, digit tip regeneration

3:45 PM – 4:45 PM

437

EPITHELIAL-STROMAL COMMUNICATION VIA BMP SIGNALLING IS A CRITICAL REGULATOR OF SITE SPECIFICITY IN THE MOUSE ORAL MUCOSA

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The mouth or oral cavity consists of the tongue, cheeks, gums, and palate. Without these specialized tissues, breathing, eating, tasting, and speaking would not be possible. As a multifunctional organ, the oral cavity has a high degree of structural heterogeneity. Each oral site is unique and contains distinct niches for oral epithelial stem cells. Despite their importance, our understanding of these oral sites and their stem cell niches remains limited. Here, we use genetic mouse

models, single-cell and spatial transcriptomics, and organoid technology to define the molecular mechanisms governing oral site-specific stemness. Using a Ki67 knock-in mouse model, we found that proliferation in the basal layer of the oral epithelium is site-specific, with the dorsal tongue being the most proliferative tissue and the buccal mucosa being the least proliferative. We then performed single-cell and spatial transcriptomics to generate a comprehensive cell atlas of all major oral sites in the mouse. Our oral cell atlas comprises more than 60,000 cells from major cell lineages, including epithelial, stromal, and immune cells. Sub-clustering of epithelial cells confirmed the heterogeneous molecular signatures of site-specific differentiation. However, the transcriptomes of epithelial basal cells appeared surprisingly homogeneous. Ligand-receptor analysis revealed robust communication between epithelial basal cells and stromal cells. Subsequent sub-clustering of stromal cells revealed that oral fibroblasts have site-specific gene expression profiles, including expression of bone morphogenetic protein (BMP) signaling molecules. Palatal fibroblasts expressed high levels of BMP7, whereas BMP5 transcripts were enriched in tongue fibroblasts. To functionally assess the role of BMP signaling, we established site-specific epithelial organoid cultures. Organoid-forming efficiency assays showed that tongue and palate organoids were sensitive to BMP inhibition, whereas buccal mucosa organoids grew independently of BMP modulation. Our comprehensive cell atlas of the mouse oral mucosa revealed a critical role of epithelial-stromal communication via BMP signaling for site-specific stemness. It represents a rich resource for dissecting other key modulators of site specificity in the mouse oral mucosa.

Funding Source: The Kretzschmar group is funded by the German Cancer Aid (via MSNZ Würzburg/NG3) and the European Union (ERC Starting Grant 101042738/OralNiche).

Keywords: single-cell sequencing, oral mucosa, organoid technology

3:45 PM – 4:45 PM

439

NOTCH SIGNALING MAINTAINS SALIVARY GLAND STEM/PROGENITOR CELL ACTIVITY AFTER IRRADIATION

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Radiotherapy is a standard treatment for approximately 75% of patients suffering from head and neck cancer. However, the co-irradiation of surrounding non-tumor tissue leads in 40% of patients severe side effects, such as xerostomia (or dry mouth syndrome), significantly impairing quality of life. Proton therapy, currently used to treat more than 500.000 patients/year, offers a higher precision in targeting the tumor than conventional photon-based radiotherapy. However, the biological response of normal stem/progenitor cells to proton and photon irradiation remains poorly understood. To investigate and compare the potential differences between these radiation types, we used a murine stem cell-derived salivary gland organoid (SGO) model. We performed single-cell RNA sequencing to identify transcriptomic changes at the single-cell level. Analysis of 7 and 11-day-old SGOs showed the presence of a population of salivary gland stem/progenitor cells defined by Sox9 and Foxc1; progenitor cells defined by Cd44, Itgb1 and Trp63; basal duct cells defined by Krt6a and Krt5; luminal duct cells defined by Krt8/18; proliferating cells defined by Mki67 and non-proliferating cells defined by Cdkn1a. Interestingly, irradiation led to a reduction of the stem/progenitor cell populations and an increase in the more differentiated ones. Nevertheless, pseudobulk and gene set enrichment analyses showed a significant upregulation of genes related to Notch signaling and enrichment of development-related processes at 2 days following irradiation, particularly within the stem/progenitor cell clusters. Activation of the Notch pathway is important for stem cell maintenance and proliferation following irradiation. Indeed, cell-cell interaction analysis showed a higher upregulation of Notch signaling in proton-irradiated SGOs, which also maintained a higher self-renewal capacity, measured as secondary organoid forming efficiency (OFE). Notably, inhibition of Notch signaling reduced the OFE of irradiated SGOs, while its activation increased the OFE upon photon irradiation. In conclusion, our study demonstrates the involvement of Notch in the maintenance of stem/progenitor cells following irradiation and provides a potential advantage of proton therapy over conventional photon-based radiotherapy.

Funding Source: This work was supported by the Dutch Cancer Society KWF Grant nr: 12092

Keywords: salivary gland organoids, Notch signaling, irradiation

3:45 PM – 4:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

441

NOTCH SWITCHES OFF HEDGEHOG SIGNALLING VIA CUL3-ARI-1 E3-E3 TEAM TAGGING FOR OVARIAN GERMLINE STEM CELL NICHE SPECIFICATION

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The balance between stem cell renewal and differentiation is critical for tissue homeostasis, and that is largely regulated by stem cell niches, a specialized microenvironment composed of specific groups of cells. The stem cell maintenance niche recruits and maintains stem cell pool, while the stem cell differentiation niche promotes stem cell differentiation. However, little is known about how these niches are formed. Here, we report that off and on states of Hedgehog (Hh) signalling determine the fate of maintenance (cap cells) and differentiation niche (escort cells), respectively, of the Drosophila ovarian germline stem cell (GSC). During development, Hh signaling is activated in intermingled

cells (ICs, niche precursors), while it is absent in cap cells (CpCs) but remains strongly active in escort cells (ECs). Activating Hh signalling in ICs results in cap cell reduction, and induces EC-like cells that eventually shift toward the escort cell fate. On the other hand, suppressing Hh signalling in ICs induces CpC-like cells where escort cells acquire the cap cell marker, leading to a germ cell differentiation defect characterized by spectrosome-containing cells (SCCs). In terms of the underlying mechanism, we also report that Notch signalling suppresses Hh signalling in cap cells via Cullin3 (Cul3)-HIB-mediated proteasomal degradation. From a small-scale RNAi screening for Cul3 regulators combined with in silico promoter analysis, we find that Notch signalling may regulate Ubc10 (an E2 ubiquitin-conjugating enzyme) expression, and Ubc10 knockdown in ICs phenocopies the above-described phenotypes. Interestingly, Ubc10 with its E3 partner, Ari-1 (Ari-1-Ubc10) is proposed to coordinate with Cul3-HIB complex for protein degradation as the E3-E3 team tagging model. Although more studies are needed to pinpoint the involved mechanisms, our studies have added knowledge on the establishment of stem cell niches, and that may be applied to other stem cell systems.

Funding Source: Grant: the Ministry of Science and Technology, Taiwan (107-2311-B-001-004-MY3)

Keywords: germline stem cell niche specification, Notch-Hedgehog signalling crosstalk, Cul3-Ari-1 E3 E3 team tagging

3:45 PM – 4:45 PM

443

COMPARISON OF CELLULAR STRESS ENDURANCE USING STEM CELLS FROM MULTIPLE SPECIES

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Cellular senescence, a key hallmark of aging, is triggered by cellular stress. Paraspeckles, mammalian nuclear membraneless organelles, are associated with development and cellular stress response. The long non-coding RNA (lncRNA) NEAT1 acts as a scaffold for RNA-binding proteins, forming paraspeckles. We utilized a phylogenetic computational and cellular modeling approach to analyze how paraspeckles influence cellular stress response. We uncovered the mammalian NEAT1 archetypes across over 500 mammalian species and identified consensus tRNA-like motifs and triple helix domains, transposable elements, and Neat1 isoforms. Given sequence feature dissimilarities of rodents and primates' NEAT1 gene, we are conducting cross-species genomic replacement in mouse embryonic stem cells through genomic editing to explore the relationship between cellular stress endurance and NEAT1 -paraspeckle axis.

Keywords: NEAT1, paraspeckle, cellular stress response

3:45 PM – 4:45 PM

445

THE ROLE OF CHROMODOMAIN PROTEIN CDYL2 IN SPERMATOGENESIS

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During spermatogenesis, chromatin structure is dramatically changed, and epigenetic regulation plays an important role. Chromodomain proteins recognize methylated histones and are involved in epigenetic regulation as leader proteins. CDYL2 is a chromatin regulator with a chromodomain whose role in spermatogenesis remains unclear. Here, we focus on CDYL2 and aim to clarify its role in spermatogenesis by generating and analyzing KO mice. First, we analyzed CDYL2 expression in wild-type mice to obtain basic information about CDYL2. CDYL2 is ubiquitously expressed throughout body tissues, especially in the cerebellum, testis, and ovary. In the testis, CDYL2 was found to be expressed in undifferentiated spermatogonia and Sertoli cells. Next, KO mice were generated by genome editing and phenotypic analysis was conducted. Since KO mice were fertile and showed normal spermatogenesis, we focused our analysis on sperm stem cells (SSCs) rather than Sertoli cells. However, when we observed SSC colonies by using seminiferous tubules whole-mount staining, wild-type cells were composed of a single cell, whereas the deficient-type formed characteristic colonies composed of five to six cells. These findings suggest that stem cell differentiation is inhibited in KO mice and that stem cells accumulate or are actively proliferating, indicating that CDYL2 may be involved in stem cell regulation. However, the mechanism by which CDYL2 regulates stem cells has yet to be elucidated. In the future, we would like to confirm the reproductive ability of CDYL2 KO mice and investigate the detailed molecular mechanism by RNA sequencing analysis to clarify the relationship between stem cell regulation by CDYL2 and stem cell senescence.

Keywords: epigenetics, spermatogenesis, sperm stem cells

3:45 PM – 4:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

447

DISSECTING ENDOTOME INDUCTION OF HSC EMERGENCE VIA RETINOIC ACID SIGNALLING

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Haematopoietic stem cells (HSCs) are self-renewing stem cells capable of replenishing all blood lineages. In all vertebrate embryos that have been studied, definitive HSCs are generated initially within the dorsal aorta (DA) of the embryonic vasculature by a series of poorly understood inductive events. Previous studies have identified that signalling relayed from adjacent somites coordinates HSC induction via the deployment of a specific endothelial precursor population, which arises within a sub-compartment of the zebrafish somite termed the endotome. Endothelial cells of the endotome are specified within the nascent somite by the activity of the homeobox gene meox1 and express unique molecular makers. Specified endotomal cells consequently migrate and colonize the DA, where they induce HSC formation. However, the molecular nature of the endotome remains poorly described as does the nature of the signals secreted from these cells that regulate HSC induction from the nascent hemogenic endothelium of the dorsal aorta. We describe the molecular nature of the endotome through bulk and single cell RNA seq approaches and determine its trajectory of differentiation within the nascent zebrafish vasculature. These studies reinforce the unique cellular and molecular profiles of these somite derived endothelial cells and implicate retinoic signalling as an endotome derived signal regulating HSC induction. Through this signalling cascade endotome-derived signalling facilitates HSC induction from the nascent haemogenic endothelium.

Keywords: hematopoeitic stem cells, endotome, Meox1, Aldh1a2, retinoic acid signalling pathway, hematopoeitic stem cells, endotome, meox1, endothelial cells, retinoic acid, Aldh1a2, single cell transcriptomics, hematopoeitic stem cells, endotome, Aldh1a2

3:45 PM – 4:45 PM

449

IDENTIFICATION OF CELLS OF LEUKEMIC STEM CELL ORIGIN WITH NON CANONICAL REGENERATIVE PROPERTIES

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Despite most acute myeloid leukemia (AML) patients entering remission following chemotherapy, long term remission rates remain poor due to rare surviving leukemic cells. These cells initiate disease re-occurrence, called relapse, which is often fatal. The enduring cells believed to be responsible for relapsed AML disease are termed leukemia stem cells (LSCs) and forms the basis of canonical regeneration of AML. However, the causal and direct evidence to support LSC's role in relapse disease is inconsistent. Here, to better characterize the cellular and molecular characteristics of AML regeneration post chemotherapy, we temporally applied transcriptomics at single cell resolution within a patient derived xenograft (PDX) modelling system throughout a chemotherapy time-course. Using this novel approach, we reveal a unique cell population tightly correlative to AML regeneration, termed AML Regeneration Enriched Cells (RECs), while demonstrating LSCs are not correlative this process. RECs are molecularly and functionally distinct from LSCs, and unlike LSCs are consistently defined by co-expression of cell surface markers. Based on their in-situ proximity to primitive CD34 expressing cells, RECs were functionally examined and demonstrated the ability to augment or reduce leukemic stem cell driven regeneration in vivo based on transfusion or depletion, respectively. Furthermore, RECs are prognostic for overall/event free patient survival as well predictive of treatment failure in AML patient cohorts, and therefore pose as a novel biomarker and therapeutic target to better the clinical management of AML patients. These findings reveal RECs as a previously unknown functional catalyst of LSC driven regeneration, founding the concept of "non-canonical" framework of AML regeneration.

Funding Source: Canadian Cancer Society, Canadian Institutes of Health Research

Keywords: acute myeloid leukeima, cancer regeneration, cancer stem cells

3:45 PM – 4:45 PM

451

INVESTIGATION ON THE EFFECTS OF THE CCL5-CCR5 AXIS ON MYELOID BIAS IN AGED HEMATOPOIETIC STEM CELLS

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Past research has identified that long-term hematopoietic stem cells (HSCs) experience a loss in potency in mammals with old age and become more myeloid biased (My-bi), implying that changes in the stem cell level can be the cause of immune decline and increased incidence of myeloid malignancies in older individuals. Although several cell-surface molecules have been identified to mark My-bi HSCs, the role of chemokine receptors on aging of the hematopoietic system remains largely unknown. Our work shows that the bone marrow HSC and progenitor populations that express CCR5 expand with age in mice and humans. We further demonstrate that the disruption of this axis through antibody blocking of CCL5 binding to CCR5, and antibody depletion of CCR5+ cells results in a decrease of CD150high My-bi HSCs in the bone marrow and myeloid cells in the peripheral blood. Finally, we demonstrate that serial transplantation of old CCR5+ KLS to lethally irradiated young mice causes an increase in the frequency of myeloid cells, suggesting that CCR5 marks myeloid biased HSCs. Through improving our understanding of the role of the CCL5-CCR5 axis in hematopoietic stem cells, we can create new therapeutic interventions to restore balanced lineage of blood cells in aged systems.

Keywords: myeloid-bias, CCR5, aging

3:45 PM – 4:45 PM

453

MACROPHAGE CALRETICULIN MARKS EFFECTOR MACROPHAGES AND CAN BE MODULATED TO PROMOTE PROGRAMMED CELL REMOVAL

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Macrophages are significant contributors to tissue homeostasis, and their functional plasticity is critical for their response to clear damaged, diseased, and unwanted cells, including pre-malignant and/or diseased stem and progenitor stem cell populations. Recent studies have begun to characterize the diverse phenotypic spectrum of macrophage subpopulations, revealing unique gene expression signatures, surface markers, and functions across subtypes. Our studies have shown macrophage Calreticulin to be a key component of programmed cell removal, where it can function as an 'eat me' signal and lead to phagocytosis of target cells. However, it has not been determined whether all macrophage subtypes can carry out Calreticulin-mediated programmed cell removal. Here, we interrogate the heterogeneity of macrophages with respect to their levels of Calreticulin mRNA, cell surface expression, and phagocytic capacity. Our findings reveal Calreticulin transcripts to be increased in phagocytosing macrophages compared to non-phagocytosing macrophages. We also find that polarization into 'pro-inflammatory' M1-like macrophages with a TLR agonist significantly increases cell surface Calreticulin and phagocytosis. On the other hand, polarization into "anti-inflammatory" M2-like macrophages show decreased levels of Calreticulin and reduced target cell clearance. These findings suggest Calreticulin expression to be induced by effector macrophages, and that its modulation may be an effective strategy for enhancing or inhibiting programmed cell removal for a diverse set of target cells including disease-causing stem cell clones.

Keywords: macrophage heterogeneity, calreticulin, programmed cell removal

3:45 PM – 4:45 PM

455

HALLMARKS OF APOBEC3 MUTAGENESIS IN NORMAL, PRE-LEUKEMIC, AND MYELOPROLIFERATIVE NEOPLASM STEM AND PROGENITOR CELL POPULATIONS

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Changes in gene expression and subsequent editing patterns of APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like) cytosine deaminases are present in solid tumor cancer evolution, however, the context specificity and mechanisms by which

APOBEC3 enzymes promote cancer initiation and progression require further elucidation, especially in the hematopoietic niche. Building on advanced sequencing data of healthy, pre-leukemic, and myeloproliferative neoplasm patient samples FACS sorted into stem and progenitor populations, we seek to uncover normal and malignant patterns of APOBEC3C overexpression in hematopoietic cells. Overexpression of lentiviral APOBEC3C and an editase-mutated APOBEC3C in sorted normal cord blood, healthy bone marrow, and MPN patient hematopoietic stem and progenitor cells (HSPCs) allows us to examine the distinct role of RNA and DNA editing in both normal hemopoietic stem cell fate determination as well as the malignant transformation of cancer stem cells. In addition to APOBEC3, we are exploring the upregulation of adenosine deaminase acting on RNA1 (ADAR1), as we have previously shown that these two innate immune deaminases are synchronously upregulated in the high-risk myelofibrosis (MF) stem cell population as compared to normal aged bone marrow. These overexpression studies show novel differential gene expression changes, RNA hyper-editing sites, and DNA mutation signatures induced by APOBEC3 mutagenesis, which can be cross-referenced to gene expression and mutation signatures seen in hematopoietic malignancies and solid tumor cancers. Gene set enrichment analysis performed on this dataset has exposed numerous deregulated pathways brought on by amplified levels of APOBEC3, including changes in splicing pathways. We will use this comprehensive study of APOBEC3 deregulation to uncover predictive biomarkers and hallmarks of malignant mutagenesis in cancer stem cells.

Keywords: cancer stem cells, hematopoiesis, RNA/DNA editing

3:45 PM – 4:45 PM

457

THE RNA BINDING PROTEIN TRIM71 IS A DEVELOPMENTAL REGULATOR OF HUMAN HEMATOPOIETIC STEM CELL FUNCTION

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Hematopoietic stem cells (HSCs) differentiate into all mature blood cells, while also renewing to maintain the HSC pool. During early development, HSCs rapidly divide and renew to expand a few de novo generated HSCs into the adult stem cell pool. While crucial for the formation of the blood system, the mechanisms that drive this proliferative state remains poorly studied in the human context. RNA binding proteins (RBPs) are essential post-transcriptional determinants of stem cell function, and thus may represent a novel set of developmental HSC regulators. Here, we show that RNA processes are enriched in human HSCs during early development, and further identify the RBP TRIM71 as elevated in fetal HSCs compared to neonatal or adult HSCs. In vitro lentiviral knockdown of TRIM71 in fetal CD34+ hematopoietic stem and progenitor cells (HSPCs) results in impaired proliferation and differentiation, while in vivo xenotransplantation showed reduced reconstitution and reproduced differentiation defects, demonstrating that TRIM71 is essential for FL HSCs. We next investigated whether TRIM71 could re-establish a fetal identity when expressed in a more mature HSC type. Interestingly, neonatal cord blood (CB) CD34+ HSPCs overexpressing TRIM71 showed increased competitiveness, cell cycling and protein synthesis in vitro, reminiscent of the highly active nature of FL HSCs. Moreover, RNA-sequencing revealed a significant enrichment of a fetal HSC signature, further supporting the acquisition of a fetal identity. Intriguingly, TRIM71-overexpressing CB xenografts were devoid of HSCs, possibly due to more mature HSCs being unable to cope with fetal-like proliferative over long time-periods. Given that p21, the canonical target of TRIM71 repression, is reduced in its expression in FL HSCs compared to mature HSCs, and is downregulated upon TRIM71 overexpression, we propose a model where enhanced TRIM71 expression in FL HSCs suppresses the G1 checkpoint, allowing for rapid cycling and renewal. Upon TRIM71 downregulation during HSC maturation, the G1 checkpoint becomes increasingly functional, resulting in more restrained adult HSCs. Altogether, our findings provide an example of the cross-developmental influence of post-transcriptional mechanisms regulating stem cell function and the establishment of tissues.

Funding Source: Stem Cell Network, Canadian Institutes for Health Research

Keywords: hematopoietic stem cell, development, RNA-binding proteins

3:45 PM – 4:45 PM

459

SENESCENCE-DRIVEN INFLAMMATION IS REGULATED BY THE ADAPTIVE IMMUNE SYSTEM AND IMMUNE CHECKPOINTS

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We previously showed that oncogene (BRAFV600E) expression in human hematopoietic stem and progenitor cells (HSPCs) triggers cellular senescence, resulting in permanent cell cycle arrest and the onset of the senescence-associated secretory phenotype (SASP). SASP boosts the secretion of pro-inflammatory cytokines to favor the clearance of senescent cells (SCs) through activation of immune cells.

However, inefficient clearance of SCs may result in chronic inflammation which has been associated with poor immune function, increased risk of malignancy, and accelerated aging. Therefore, understanding the factors involved in the persistence of SCs is paramount to improve therapies for their clearance. To investigate this aspect, we generated two mouse models of histiocytosis through the transplantation of mouse HSPCs expressing BrafV600E in immune-deficient (NSG) or immune-competent (C57) mice. In NSG mice (n=12), BrafV600E-expressing HSPCs caused dose-dependent lethality (Median = 36 days), while 40% of WT mice (n=7) survived up to 500 days and eliminated SCs. Both models showed histiocytosis, impaired hematopoiesis, and SASP, but NSG mice had immune suppressive IL-9 and IL-10 levels correlating positively with the amount of SCs, while in WT mice CCL-2 and IL-6 inversely correlated with the number of recipient B and T cells. RNA-seq data revealed upregulation of MHC Class II molecules in both models, implying higher antigen presentation process in SCs. Intriguingly, WT mice cells upregulated previously published SCs signatures while NSG did not, despite showing other SCs markers (Cdkn2a). NSG SCs upregulated immune checkpoint genes (Il10, Pdcd1), while WT SCs also upregulated immune response genes (Cd274, Icosl, Cd80, Cd86). This data hinted that recipient B and T cells, only present in WT mice, recognized and eliminated SCs, dampening the inflammation. We tested our hypothesis by performing the same experiment on Rag1KO mice, which lack B and T cells. Indeed, all Rag1KO mice transplanted with oncogene expressing HSPCs died just as NSG, with no elimination of SCs. Thus, we identified two components involved in the clearance of SCs. Firstly, the adaptive immunological background of the host can eliminate SCs. Secondly, SCs may increase the expression of immune checkpoints to escape immunological clearance.

Funding Source: Telethon Foundation

Keywords: senescence, histiocytosis, HSPCs

3:45 PM – 4:45 PM

461

REDUCING NUCLEAR STRETCHING REJUVENATES AGED HEMATOPOIETIC STEM CELLS

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Biomechanical changes might contribute to the decreased regenerative capacity of aged stem cells. Small RhoGTPases are key regulators of mechanosignalling, but their specific role for mechanotransduction in stem cell aging remains unknown. Here, we show that a specific small RhoGTPase is necessary to survive mechanical insults in hematopoietic stem cells, which cell intrinsically induce its activation. Interestingly, we measure a 2-fold higher activity level of this small RhoGTPase in aged hematopoietic stem cells, associated with altered nuclear architecture. Reducing the activity of this small RhoGTPase with a selective inhibitor influenced nuclear mechanostransduction and improved the regenerative capacity of aged stem cells in vivo. Moreover, we developed an imaged-based computational framework to reveal the chromatin changes that are reverted in aged stem cells upon treatment with the selective small RhoGTPase inhibitor. Reducing the smallRhoGTPase activity in aged stem cells decreased chromatin accessibility and transcription of several repetitive elements (REs), downregulating inflammation and interferon response and inducing partial reprogramming. Overall, our data support that an intrinsic small RhoGTPase mechanosignalling axis is necessary for adult stem cells to survive mechanical insults and can be pharmacologically targeted to rejuvenate aged stem cell function.

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Keywords: stem cell aging, stem cell rejuvenation, nuclear biomechanics

3:45 PM – 4:45 PM

463

THE ROLE OF VASCULAR ENDOTHELIAL STEM CELLS IN THE REGENERATION OF THE BONE MARROW VASCULAR NICHE

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Bone marrow (BM) vascular endothelial cells serve a critical role in regulating and supporting the function and regenerative capacity of hematopoietic stem and progenitor cells. Moreover, the BM vascular network has been shown to couple angiogenesis and osteogenesis. Despite its importance in several physiological and pathophysiological conditions, the understanding of the underlying mechanisms of vascular regeneration in the BM is extremely limited. Recently, a cell population with vascular stem cell potential was identified in the liver tissue, termed vascular endothelial stem cells (VESCs). VESCs were characterized by the expression of CD157/BST1. Here we characterize CD157+ BM endothelial cells (BMECs) and examine their number and localization in distinct blood vessel subtypes. To investigate their stem cell potential, we performed functional assays to measure their self-renewal and differentiation capacity. By label retaining assays and by intra-femoral vascular injury studies, we identified CD157+ BMECs localized in close proximity to the injured area and actively contributing to the formation of new vessels. Taken together, our findings support the existence of an endothelial cell population with stem cell properties within the BM vasculature, that significantly contributes to its regeneration after injury and over time.

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Keywords: endothelial stem cells, bone marrow, regeneration

3:45 PM – 4:45 PM

465

CIRCULATING HAEMATOPOIETIC STEM AND PROGENITOR CELL SHOW THYMUS SEEDING PROPERTIES AND ACTIVELY CONTRIBUTE TO HAEMATOPOIESIS

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Although most hematopoietic stem/progenitor cells(HSPC) reside in the bone marrow(BM), few circulating HSPC(cHSPC) are in the peripheral blood(PB) at steady state. We previously found that healthy donor(H-D)-derived cHSPC are phenotypically, transcriptionally and functionally enriched for multi-potent, erythroid and lymphoid progenitors. cHSPC subsets are low-cycling but primed for differentiation, suggesting their pre-activated state. However, cHSPC in vivo dynamics in and out the BM in humans are poorly elucidated. To investigate the migratory fate of cHSPC subsets, we generated a Human Organ-Resident (HuOR) HSPC dataset based on published scRNAseq datasets of HD-organs. Lymphoid cHSPC showed high transcriptional similarity with HuOR thymic HSPC. Moreover, PB multi-lymphoid progenitors(MLP) highly expressed a gene signature of thymus seeding progenitors type 1(TSP1) and showed a higher transcriptional and functional T cell commitment with respect to BM-MLP. Pseudotime analyses showed a differentiation trajectory of BM-MLP to PB TSP1-high MLP and then thymic lymphoid progenitors, implying that PB TSP1-high MLP connect the BM with the thymus. To study PB vs. BM HSPC in vivo dynamics, we exploited integration site(IS) clonal tracking of cHSPC, BM-HSPC subsets derived from 2 distinct BM sites, and mature PB lineages isolated from 8 HSPC-gene therapy(GT) patients at steady-state hematopoiesis(>2 years post-GT). In line with their enriched expression of TSP1 signature, cHSPC shared a higher number of IS with T cells than BM-HSPC. Moreover, among all HSPC subsets, lymphoid BM-HSPC had the highest IS sharing with cHSPC, further supporting their increased propensity to egress the BM. IS shared between 2 BM distant sites were re-captured at higher frequencies in PB than not shared IS, implying an active role of cHSPC in clonal re-distribution among BM niches. Finally, among all HSPC subsets, primitive HSPC showed the highest sharing with these re-circulating clones, suggesting their higher propensity to re-enter into the BM. Altogether, our findings suggested the key function of trafficking MLP in thymus seeding to contribute to T lymphopoiesis. Moreover, our IS analysis unveiled the dynamics of HSPC trafficking in humans by supporting their fundamental role in hematopoietic homeostasis.

Keywords: circulating hematopoietic stem cells, human hematopoiesis, extramedullary hematopoiesis

3:45 PM – 4:45 PM

467

INVESTIGATING THE FETAL-TO-ADULT TRANSITION OF HOXB5-EXPRESSING HEMATOPOIETIC STEM CELLS

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The emergence of hematopoietic stem cells (HSCs) in the fetal liver indicates a crucial transitional stage, where they shift from a more primitive state to a more mature and specialized state. During this wave of hematopoiesis, HSCs in the fetal liver rapidly expand and actively contribute to the generation of various blood cell lineages before the bone marrow takes over hematopoietic function. Our lab previously reported Hoxb5 to be a marker of adult long-term HSCs and we have now investigated the role of Hoxb5 in delineating definitive HSCs within the fetal liver and their fate into adulthood. Single-cell RNA sequencing revealed that Hoxb5 is more highly expressed in fetal liver HSCs than adult HSCs. Utilizing our Hoxb5-mCherry reporter mice, we find that the fetal liver contains Hoxb5-mCherry+ HSCs with peak frequency at E14.5. Through comprehensive lineage tracing using Hoxb5-CreERT; R26VT2/GK3 rainbow reporter mice, we observe that induction with 4-hydroxy-tamoxifen (4-OHT) at E14.5 results in the long-term persistence of labeled HSCs into the adult bone marrow. Transplantation of E14.5 fetal liver Hoxb5-mCherry+ HSCs additionally reveals capacity for long-term reconstitution with multilineage potential. These findings shed light on the ontogeny and fate of long-term HSCs, implicating Hoxb5 as a key marker for identifying and tracking HSCs from fetal stages to adulthood.

Keywords: hematopoietic stem cells, hematopoiesis, development

3:45 PM – 4:45 PM

469

HEMATOPOIETIC STEM CELL REJUVENATION BY AN ENGINEERED BONE MARROW NICHE REVERSES HEMATOPOIETIC AND SYSTEMIC AGING

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Hematopoietic stem cells (HSCs) sit at the apex of the hematopoietic system, the functionality of which declines dramatically with age. Our recent study found that bone marrow stiffening is a novel hallmark of hematopoietic aging, and that reconstitution of a soft bone marrow niche by 3D co-culture of bone marrow stromal cells (BMSCs) and HSCs in a soft hydrogel robustly promotes HSC rejuvenation (Cell Stem Cell, 30: 378–395). However, whether HSC mobilization, ex vivo rejuvenation and self-transplantation is a feasible strategy to reverse hematopoietic or systemic aging is still unknown. To test this hypothesis, we mobilized middle-aged (12-month-old) mouse bone marrow HSCs into the peripheral blood (mPB-HSC), and sorted them to perform 3D co-culture with BMSCs. Cell polarity and mitochondrial membrane potential analyses revealed that mPB-HSCs were significantly rejuvenated after 3D co-culture. Consistent with this, the long-term multilineage reconstitution capacity of 3D co-cultured mPB-HSCs was also significantly increased as compared to uncultured mPB-HSCs. Next, we compared three nontoxic bone marrow transplantation protocols reported by previous studies, and found that G-CSF/AMD3100/BIO5192 administration (G7AB) in middle-aged mice facilitate efficient engraftment of young (2-month-old) HSCs. Finally, we mobilized HSCs from middle-aged mice to perform 3D co-culture with BMSCs for 7 days, followed by self-transplantation using the G7AB protocol. Compared to control middle-aged mice, self-transplantation of ex vivo rejuvenated mPB-HSCs significantly increased lymphopoiesis and decreased myelopoiesis, suggesting rejuvenation of the hematopoietic system. Secondary bone marrow transplantation confirmed that the long-term reconstitution capacity of HSCs was significantly restored. More importantly, self-transplantation of ex vivo rejuvenated mPB-HSCs also promoted systemic rejuvenation as determined by multiple behavioral and metabolic analyses. Taken together, our study provided proof-of-concept evidence that HSC mobilization, ex vivo rejuvenation and self-transplantation is a promising strategy to reverse hematopoietic aging, which could promote systemic rejuvenation and healthy aging.

Keywords: hematopoietic stem cells, systemic rejuvenation, healthy aging

3:45 PM – 4:45 PM

471

SOMATIC ASXL1 MUTATIONS RESULT IN INEFFECTIVE CLONAL ERYTHROPOIESIS WITH A DYSREGULATED EPIGENETIC LANDSCAPE

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Clonal hematopoiesis (CH) due to acquired mutations in hematopoietic stem and progenitor cells (HSPCs) is a risk factor for blood cancers. Of the various factors considered in progression from CH to leukemia, two most predictive are features of red blood cells – an increase in their average size and their size distribution. As CH is not associated with quantitative changes of red cell production, we posed that qualitative changes in erythropoietic output is a reflection of clonal erythropoiesis from the mutant HSPCs. To test this hypothesis, we induced mosaic somatic mutations in asxl1 with CRISPR-Cas9 mutagenesis in zebrafish embryos with uniquely labeled HSPCs, and sorted mutant dominant HSPC clones from adults for transposase-accessible chromatin with sequencing (ATAC-seq) and gene expression (RNA-seq) assays. About 4.2% of peaks decreased by 2-fold in asxl1-mutant clones compared to controls, composed of peaks at or around genes enriched in erythropoietic program. This included peaks at major transcription factors such as tal1 and gata1a, genes such as alas2 and cahz, and the globin regulatory region with enhancers in nprl3 gene. RNA-seq on sorted asxl1-mutant dominant HSPC clones showed downregulated genes of erythroid lineage such as hbba1, hbaa1, alas2 and cahz, although less pronounced or no significant changes were noted in erythroid transcription factors. Developmental mapping of erythroid lineage in asxl1 crispants showed no differences in gata1a erythroid progenitors at 3 days post fertilization (dpf) and 14dpf, but decreased frequency at 28dpf ($p< 0.05$). We observed reduction of globin switch lcr expression starting at 14dpf ($p< 0.01$). In complementary studies we induced ASXL1 CRISPR-Cas9 mutagenesis in human CD34+ HSPCs and tested them in erythroid differentiation assays. We observed significantly reduced proerythroblast formation in ASXL1 mutant cells compared to controls. These data suggest that asxl1-mutant clones have an erythropoietic lineage defect with reduced epigenetic accessibility at lineage-specific loci with selective changes in gene expression. We surmise that the ineffective clonal erythropoiesis characterized by large cells lead to increased mean volume and increased size distribution, a pathobiologic link between red cell features and malignant potential of CH.

Funding Source: Howard Hughes Medical Institute (LIZ); The Burroughs Wellcome Fund (SA).

Keywords: clonal hematopoiesis, erythropoiesis, zebrafish

3:45 PM – 4:45 PM

TOPIC: KIDNEY

475

TONSIL-DERIVED MESENCHYMAL STEM CELLS PROTECT THE KIDNEY FROM GENTAMICIN-INDUCED ACUTE KIDNEY INJURY (AKI) BY AN AMELIORATION OF OXIDATIVE STRESS AND APOPTOSIS

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Gentamicin (GM)-induced AKI occurs in 10-20% of treated patients. GM is accumulated in renal epithelial cells, which results in the loss of the brush border, apoptosis and overt necrosis of renal tubules. Recent developments in stem cell research have shown a promise for the treat¬ment of AKI, however the mechanisms underlying the improvement in kidney function provided by stem cell therapy remain unclear. Tonsil-derived MSCs (T-MSCs) isolated from discarded tonsils after tonsillectomy are reported to protect the liver from acute liver injury. The aim of this study is to investigate the therapeutic potential of T-MSCs in gentamicin-induced AKI. Twenty male Sprague-Dawley rats were divided into four groups: Control, GM (140 mg/kg/day, intraperitoneal injection for 10 days), GM+T-MSCs (1x107 cells, intravenous T-MSC injection at 1 day after the 1st GM injection), and T-MSC group. To examine the intra-renal localization of T-MSCs, T-MSCs were labeled with PKH-26 red fluorescence before infusion. Measurement of BUN, Cr, proteinuria and histologic analysis including TUNEL staining were performed on 16 days of GM injection. Effect of T-MSC on renal tubular cells was also evaluated using a transwell co-culture system of NRK cells and T-MSC. Intracellular ROS was analyzed by measuring NOX activity, H2O2 generation, NOX mRNA expressions with DCF-DA staining. In GM+T-MSC group, BUN, Cr, proteinuria were lower with less tubulointerstitial fibrosis compared to GM group. T-MSCs injection decreased apoptotic cells and the expression of Bax, cytochrome C, and cleaved caspase and increased Bcl-2. T-MSCs suppressed oxidative stress as reflected by a decrease in the level of urinary 8-OHdG with an increase in antioxidant enzymes (glutathione peroxidase and catalase) in the kidneys. Anti-human nuclei and PHK-26 staining demonstrated the localization of T-MSCs in the tubules of renal cortex. In-vitro study revealed that T-MSC or T-MSC-conditioned media ameliorated GM-induced NOX-1 expression, H2O2 generation, and apoptosis of NRK cells. T-MSCs ameliorate GM-induced AKI, which is mediated by direct engraftment into the damaged renal tubules and paracrine effects of T-MSCs, exerting anti-apoptotic and anti-oxidative effects.

Funding Source: This research was supported by the Korean Fund for Regenerative Medicine (KFRM) grant funded by the Korea government (the Ministry of Science and ICT, the Ministry of Health & Welfare). (23A0201L1)

Keywords: tonsil-derived mesenchymal stem cells, gentamicin, acute kidney injury

3:45 PM – 4:45 PM

477

LOCALIZATION OF ADMINISTERED MSC IN PORCINE KIDNEYS REVEALED THROUGH CRYOVIZ IMAGING

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In this study, we employed CryoViz Imaging, a serial sectioning and block-face imaging technology that acquires 3-D microscopic color anatomical and molecular fluorescence volumes from large frozen tissue blocks, to evaluate the efficiency and tissue distribution of fluorescently labeled MSC after renal intra-arterial delivery to a porcine renal ischemia reperfusion model. The regenerative capabilities of mesenchymal stromal cells (MSC) make them suitable for renal regenerative therapy. The most common delivery route of MSC is via intravenous infusion, which is associated with off-target distribution. Renal intra-arterial delivery offers a targeted therapy but limited knowledge is available regarding the fate of MSC delivered via this route. MSC were isolated from adipose tissue of healthy male pigs, fluorescently labeled with Qtracker 655 (deep red), and infused into the renal artery of female pigs. A total of 10M MSC were infused. Flow cytometry showed ~2-4M MSC per kidney. Both healthy and ischemic kidneys (30 minutes and 8 hours post infusion) were excised, cut in half, embedded in a histological medium, flash frozen in liquid N2, and CryoViz imaged. Dual imaging mode (Brightfield and Fluorescence) was employed with an in-plane pixel size of 10 microns, and a section thickness of 40 microns. A long-pass fluorescence filter optimized to detect Qtracker 655 (Excitation: 460-490nm, Emission: >510nm) was employed. We observed that MSC were successfully delivered to both healthy and ischemic kidneys. Majority of MSC remained in the kidney for at least up to 8 hours. Qdot-labelled MSC were detected in renal cortex and dissociated renal medulla, however, MSC numbers were 10-fold lower in renal medulla than those found in the renal cortex. CryoViz Imaging has revealed that targeted MSC delivery via the renal artery is a feasible route to deliver MSC to the kidney. Upon infusion, MSC are distributed throughout the kidney, located mostly in renal cortex and particularly inside glomeruli. MSC are retained in the renal cortex independently of their metabolic state after renal intra-arterial infusion, presumably through a passive mechanism and after infusion they survive for at least 8 hours.

Keywords: 3-D tissue block-face imaging, porcine kidneys, mesenchymal stromal cells

3:45 PM – 4:45 PM

TOPIC: LIVER

479

CRYOPRESERVED HEPATOCYTE ORGANOIDS RETAIN FUNCTION BETTER THAN HEPATOCYTES

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Hepatocyte organoids (HOs) outperform bile duct epithelial cell organoids in functional versatility despite having a shorter lifespan. While optimization of culture methods has addressed this limitation, it remains a potential obstacle to broader applications in research and therapy. This study focuses on extending the lifespan of pig Hos and assessing their viability and functionality after one year of cryopreservation. Apoptosis and liver function gene analyses were conducted to evaluate the impact of adipose-derived mesenchymal stem cell (A-MSC) co-culture on cryoresistance before and after freezing. Following one year of cryopreservation, HOs were either cultured alone or co-cultured with ASCs in Matrigel. Co-cultured HOs exhibited a higher post-thaw development rate compared to those cultured alone. P21 expression increased regardless of pre-freezing culture conditions. ALB and CYP3A29 expression patterns resembled non-frozen HOs, with consistent effects from co-culture. Epithelial cell adhesion molecule (EpCAM) expression surged post-freezing. This study demonstrates that HOs maintain liver function post-preservation, exhibiting increased EpCAM expression and enhanced regenerative capacity against cryoinjury. These findings position HOs as a valuable cell source for animal drug development and research on human disease remediation.

Funding Source: This work was supported by the Cooperative Research Program for Agriculture Science and Technology Development, National Institute of Animal Science (grant number: PJ015872).

Keywords: cryopreservation, hepatocyte organoid, adipose tissue derived mesenchymal stem cells

3:45 PM – 4:45 PM

481

ELUCIDATION OF THE ROLE OF PLASTICITY FACTORS IN HUMAN LIVER REGENERATION

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Mechanisms driving Liver regeneration is well-documented in cases of acute liver injury while the regenerative processes occurring in chronic diseases remain controversial. Indeed, animal studies have proposed three potential models: i) hepatocytes and cholangiocytes may revert to a liver cell progenitor state, restoring the corresponding cell compartment; ii) cholangiocytes may transdifferentiate into hepatocytes, and vice versa; iii) hepatic stem cells can be activated, leading to the generation of fresh liver cells. Nevertheless, these mechanisms have not been verified in humans, primarily due to technical and ethical constraints. To overcome these challenges, we mapped at the single cell level the progression of metabolic dysfunction-associated steatotic liver disease (MASLD) and looked for potential regenerative occurrences. Our investigations revealed that hepatocytes and cholangiocytes can transdifferentiate into one another during chronic injury. This process involves a set of transcription factors, potentially crucial for acquiring the plasticity necessary for such cellular transdifferentiation. To test this hypothesis, we validated the upregulation of these plasticity factors in transdifferentiating cholangiocytes/hepatocytes in vivo by immunostaining of human tissue slides. Additionally, we performed in vitro gain-of-function experiments by overexpressing candidate factors in intrahepatic cholangiocyte organoids obtained from patients with end-stage liver disease and hepatocyte-like cells derived from human induced pluripotent stem cells. Phenotypic assessments confirmed the involvement of these factors in intrahepatic cholangiocyte organoids and induced pluripotent stem cell-generated hepatocyte plasticity. Collectively, our findings partially elucidate the molecular mechanisms governing regeneration during chronic liver disease, thereby paving the way for the development of novel therapies aimed at promoting tissue repair in cases of chronic injury.

Keywords: liver regeneration, cell plasticity, chronic liver disease

3:45 PM – 4:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

483

TO INVESTIGATE THE ROLE OF MELATONIN IN THE PRECONDITIONING OF ADIPOSE-DERIVED STEM CELLS REGARDING EXTRACELLULAR VESICLE SECRETION

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The properties of adipose-derived stem cells (ADSC) are well known, however their potential application in increasingly specific and targeted therapies is still under investigation. The applicability of ADSC is currently directed towards their paracrine activity, which is exerted through the secretion of extracellular vesicles (EVs), the role of which is being explored. EVs are a heterogeneous population of vesicles of different sizes that transport various compounds including proteins and nucleic acids that can be transferred into recipient cells. Having a tool that could enhance the secretion of EVs would be beneficial for studying their biological activity and investigating alternative therapies for challenging medical conditions. Melatonin has been extensively studied. This compound has effects that are useful in anticancer therapy. It ameliorates inflammation or oxidative stress. Melatonin receptors have been found in the brain, adipose tissue, amnion, bone marrow. Their expression was confirmed in ADSCs. The influence of melatonin on ADSC activity is not clearly defined. We hypothesize that melatonin may be useful in preconditioning ADSCs for their potential use in therapy. This study investigated the efficacy of EVs secretion by ADSCs under the influence of melatonin and its receptor antagonists. ADSC phenotype was assessed by flow cytometry and adipo-, chondro- and osteogenesis assay. EVs were isolated from conditioned medium (CM) by differential centrifugation. The study groups included cells treated with melatonin, melatonin and luzindole. Untreated cells were used as control. EVs were identified by immunogold staining and imaged by S-TEM. EV populations were characterized cytometrically by magnetic bead-based method and assessed by nanoparticle tracing analysis (NTA). Flow cytometry and differentiation assay confirmed a normal phenotype of ADSCs. S-TEM revealed EV-like structures and the presence of gold particles indicative of positively stained EVs against CD9 antigen. Magnetic bead-based cytometric examination and NTA showed differences between the study groups. In conclusion, ADSC secrete EVs that can be isolated from conditioned medium. Melatonin significantly affects ADSC activity and may be an important agent in regulating the formation and secretion of EVs.

Funding Source: Medical University of Silesia in Katowice - BNW-2-060/K/3/I Metropolitan Fund for Science Support - RW/18/2024

Keywords: extracellular vesicles, adipose derived stem cells, melatonin

3:45 PM – 4:45 PM

485

SPATIAL TRANSCRIPTOMICS REVEAL RESTING ZONE QUIESCENCE AND NOVEL GENE EXPRESSION PROFILES WITHIN THE HUMAN GROWTH PLATE

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In childhood and adolescence, humans grow taller due to the actions of specialized organs known as growth plates, located near the ends of our elongating tubular bones. These growth plates contain chondrocytes organized into distinct zones, orchestrating the deposition and mineralization of cartilage matrix to form a scaffold for bone formation. Recent studies in mice suggest the existence of a skeletal stem cell niche within postnatal growth plates. Yet, a comprehensive understanding of the regulatory factors governing these stem cells and their niche remains elusive. While deciphering the genetic profiles of growth plate chondrocytes and the secondary ossification centre holds promise for unravelling the molecular mechanisms of growth regulation, obtaining cells from these intricate tissues poses a significant challenge. We employed a combination of two Spatially Resolved Transcriptomic (SRT) technologies to overcome this obstacle. Our findings revealed the presence of a subset of chondrocytes in the resting zone exhibiting functional quiescence, characterized by low RNA levels, specific sub-cellular RNA localization, abundant heterochromatin, and the compromised capacity to exit the G0 phase under certain conditions. Additionally, we identified and validated novel markers for all growth plate zones and the secondary ossification centre, shedding light on both established and novel regulators of human growth plate function. In conclusion, our study illuminates key mechanisms driving human growth plate function, revealing quiescent chondrocytes in the resting zone, and unveiling novel markers for growth plate zones and the secondary ossification centre.

Funding Source: Swedish Research Council Karolinska Institutet KID strategic research areas (SFO)

Keywords: skeletal stem cell, human growth plate, quiescence

3:45 PM – 4:45 PM

487

AFFECTING ADSC BEHAVIOUR: EFFECT OF MCF7 EXHAUSTED MEDIUM ON DIFFERENTIATION

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Lipofilling is a technique used in reconstructive and aesthetic surgery, also used for the treatment of defects after demolitive or conservative surgery such as mastectomy. The regenerative potential of lipofilling can be ascribed to stem cells resident in the adipose tissue. Indeed, adipose tissue represents a valuable source of mesenchymal stem cells suitable for regenerative therapies, being easily harvested and propagated in vitro. They can be conditioned in vitro by chemical and physical factors, able to affect different molecular mechanisms as proliferation, senescence, differentiation and migration of Adipose-derived stem cells (ADSCs). In this study, we exposed stem cells from adipose tissue (ADSCs) obtained from plastic surgery procedures to an exhausted medium from the breast cancer cell line (MCF-7) to assess whether soluble factors released by these cells may be able to induce changes in stem cell behaviour. Therefore, we decided to analyse the expression of stemness-related genes, the cell cycle regulators, the main markers of adipogenic and osteogenic differentiation. We also analysed the 3 miRNAs (mir145, mir148 and mir185) related to cell proliferation and differentiation. From the results obtained, we can deduce that the depleted medium was more likely to maintain a stem phenotype and sometimes to switch to a highly undifferentiated proliferative type of behaviour.

Keywords: adipose-derived stem cells, stem cells differentiation, conditioned media

3:45 PM – 4:45 PM

489

DYNAMIC CULTIVATION OF HUMAN MESENCHYMAL STEM/STROMAL CELLS FOR THE PRODUCTION OF EXTRACELLULAR VESICLES IN A 3D BIOREACTOR SYSTEM

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3D cell culture and hypoxia have been demonstrated to increase the therapeutic effects of mesenchymal stem/stromal cells (MSCs)-derived extracellular vesicles (EVs). In this study, a process for the production of MSC-EVs in a novel 3D bioreactor system under normoxic and hypoxic conditions was established and the resulting EVs were characterized. Human adipose-derived MSCs were seeded and cultured on a 3D membrane in the VITVO® bioreactor system for 7 days. Afterwards, MSC-EVs were isolated and characterized via fluorescence nanoparticle tracking analysis, flow cytometry with staining against annexin V (Anx5) as a marker for EVs exposing phosphatidylserine, as well as CD73 and CD90 as MSC surface markers. Cultivation of MSC in the VITVO® bioreactor system demonstrated a higher concentration of MSC-EVs from the 3D bioreactor compared to static 2D culture under normoxic and hypoxic conditions, respectively. Also, the particle-to-protein ratio as a measure for the purity of EVs was significantly elevated in EVs from 3D bioreactor culture. Total MSC-EVs as well as CD73-CD90+ MSC-EVs were elevated in 2D normoxic conditions. Furthermore, cell viability of MSCs was increased under hypoxic conditions, whereas EV concentration and size did not differ significantly between normoxic and hypoxic conditions. The production of MSC-EVs in a 3D bioreactor system under hypoxic conditions resulted in increased EV concentration and purity. This system could be especially useful in screening culture conditions for the production of 3D-derived MSC-EVs.

Funding Source: This work was partially funded by the Lower Saxony Ministry for Science and Culture.

Keywords: mesenchymal stem cell extracellular vesicles, bioreactor, hypoxia

3:45 PM – 4:45 PM

491

ESTROGEN PROMOTES BONE FORMATION THROUGH UPREGULATION OF OSTEOLECTIN EXPRESSION IN LEPR+ CELLS

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Maintaining bone homeostasis requires a delicate balance between formation and resorption. Utilizing a Tet-on LepR-Cre allele, we traced LepR+ bone marrow stromal cells upon doxycycline induction, confirming their pivotal role as the primary source of osteoblasts throughout adulthood as previously reported (Cell Stem Cell 15:154). Investigating menopausal osteoporosis, characterized by estrogen deficiency, we found that estradiol significantly enhanced osteoblast formation from LepR+ cells, while ovariectomy and estrogen deprivation markedly diminished it in female mice. These results suggest a direct effect of estrogen on LepR+ cells to promote bone formation. Notably, we observed highly enriched expression of the estrogen receptor Esr1 in LepR+ cells. Subsequent conditional deletion of Esr1 in LepR+ cells using Tet-on LepR-Cre, initiated at 2 months of age, did not impact hematopoiesis but led to early-onset osteoporosis, particularly in females. Following RNA-seq analysis of LepR+ cells, we identified multiple bone growth factors whose expression is regulated by Esr1. One such factor is Osteolectin, which promotes osteogenic differentiation of LepR+ cells (eLife 5:e18782, eLife 8:e42274). These findings prompted us to explore the therapeutic potential of Osteolectin administration in mitigating the detrimental effects of estrogen deprivation-induced osteoporosis. Indeed, administration of recombinant Osteolectin promoted bone formation and rescued ovariectomy-induced osteoporosis in mice. Moreover, women experiencing menopause or premature ovarian failure exhibit significantly reduced serum Osteolectin levels. Thus, our results propose LepR+ cells as crucial mediators of reduced bone formation in menopausal osteoporosis, highlighting them as potential therapeutic targets. Furthermore, supplementation with recombinant Osteolectin could serve as a viable therapeutic intervention for restoring bone health in postmenopausal women.

Keywords: LepR+ cells, osteoporosis, estrogen

3:45 PM – 4:45 PM

493

CHANGES IN METABOLIC AND SECRETOME PROFILE OF MULTIPOTENT MESENCHYMAL STROMAL CELLS SPHEROIDS

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Multipotent mesenchymal stromal cells (MSCs) are currently in the focus of extensive research due to their therapeutic properties, such as multilineage differentiation capacity, immunomodulatory and paracrine activity. Compared to standard monolayer cultures, the 3D spheroid cultivation of MSCs may closely recapitulate the native microenvironment by facilitating cell-cell interactions, which improves the therapeutic properties of cells. The in-depth analysis of the metabolic/metabolomics changes that occurred during the 3D spheroid formation may help discover mechanisms of this enhanced therapeutic potential. In this study, we compared the metabolic activity, metabolomic and lipidomic profile, secretome composition and gene expression of human adipose tissue-derived MSCs (AT-MSCs) in 2D and 3D spheroid culture conditions. We detected a significant increase in the content of EGF, FGF-2, HGF, PIGF, LIF, SCF, and VEGF-A in the conditioned medium produced by spheroid cultures compared to monolayer. The metabolic activity was significantly decreased in 3D spheroids. A comparative examination of metabolomics data acquired from spheroids and monolayer cultures showed decreased content of membrane-associated lipids (cholesterol, cholesterol ethers), with a simultaneous increase in the content of triglycerides in 3D cultured cells. In conclusion, we show that the MSCs within 3D spheroids undergo metabolic adaptations, reorganization of intracellular lipid composition, and simultaneous increase in paracrine activity, confirming the potential of using MSC spheroids in regenerative medicine applications. All human tissue donors provided their written informed consent before any intervention. All studies involving human tissues or cells were approved by the Ethics Committee of the Institute of Experimental Medicine of the CAS, Prague, Czech Republic. All methods were performed in accordance with the relevant ethical guidelines and regulations.

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Keywords: spheroids, metabolomics, secretome profile of spheroids

3:45 PM – 4:45 PM

495

METFORMIN IMPROVES THE OSTEOGENIC DIFFERENTIATION ABILITY OF PLACENTA-DERIVED HUMAN MESENCHYMAL STEM CELLS

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Mesenchymal stem cells derived from the placenta (PL-MSCs) hold promise for regenerative medicine due to their immune privilege and regenerative properties. However, their osteogenic differentiation potential remains inconsistent. Metformin, a first-line drug for type 2 diabetes, has been shown to impact bone regeneration, yet its effects on PL-MSCs remain unclear. This study aimed to investigate the effects of metformin on the viability and osteogenic differentiation of PL-MSCs. All experimental procedures were conducted in accordance with the Declaration of Helsinki and the Belmont Report. This study was approved by the Human Research Ethics Committee of Thammasat University (Medicine). PL-MSCs were isolated from the human placenta and characterized. Cells were treated with metformin and viability was assessed using an MTT assay. Osteogenic differentiation was evaluated through alkaline phosphatase (ALP) activity assay, Alizarin red staining, and gene expression analysis. The results demonstrated that metformin stimulated ALP activity, mineralized nodule formation, and upregulated osteogenic markers. Additionally, metformin activated the AMPK pathway in a dose-dependent manner. These findings suggest that metformin enhances the osteogenic differentiation of PL-MSCs via the AMPK pathway, highlighting its potential for bone repair in orthopedic applications.

Funding Source: This work was supported by the Thailand Science Research and Innovation Fundamental Fund and the Center of Excellence in Stem Cell Research and Innovation, Thammasat University.

Keywords: metformin, mesenchymal stem cells, osteogenic differentiation

3:45 PM – 4:45 PM

497

AGE-RELATED DIFFERENCES ON CELLULAR CHARACTERISTICS OF MESENCHYMAL STEM CELLS, AND DNA METHYLATION PATTERNS AND RNA EXPRESSION LEVELS OF WHOLE BLOOD FROM 5- AND 10-YEAR-OLD BEAGLE DOGS

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Aging not only implies that the remaining lifespan is relatively short but also presents a remarkable risk of disease onset and a decline in the stem cell capacity within the body. Telomere length and telomerase activity are key in the cellular understanding of aging, serving as crucial indicators of cellular senescence and longevity. While human medicine has seen extensive research on telomeres and telomerase on mesenchymal stem cells (MSCs), veterinary studies remain limited. Also, though MSCs are promising for age-related disease treatment, the link between donor age and MSC characteristics remains unclear. This study aims to compare the cellular characteristics of MSCs from beagle dogs aged 5 and 10 years with molecular data from RNA sequencing (RNA-seq) and DNA methylation analysis of blood samples from these groups. MSCs were derived from adipose tissue of beagle dogs aged 5 and 10 years, characterized based on the International Society for Cell & Gene Therapy criteria, and assessed for senescence through proliferation, migration, and β-galactosidase staining. TERT gene expression levels, indicating telomerase activity, were measured via RT-qPCR. Additionally, RNA-seq and DNA methylation analyses were conducted, using RSEM software and MethylDackel for analysis of TERT gene expression and methylation level, respectively. The results of these investigations revealed that MSCs derived from the 10-year-old beagles demonstrated significantly lower proliferation and migration abilities ($p< 0.05$) and exhibited a higher level of senescence, as evidenced by β-galactosidase staining, when compared to those derived from the 5-year-old beagles. Interestingly, there was no significant difference in the levels of TERT gene expression between the two groups as determined by RT-qPCR analysis. These findings were confirmed by RNA seq and DNA methylation analyses, validating the age-related differences in MSCs characteristics and senescence. In summary, different biological characteristics and telomerase expression patterns are exhibited in different aged donors. The findings provide a foundation for further investigations into age-related changes in MSCs, and their implications in regenerative medicine and aging-related diseases.

Funding Source: Cooperative Research Program for Agriculture Science & Technology Development (Project no. RS-2022-RD010282), National Institute of Animal Science, Rural Development Administration, Republic of Korea

Keywords: age-related factors, canine, mesenchymal stem cells

3:45 PM – 4:45 PM

499

DISCOVERY OF BIOMARKERS FOR PREDICTING TREATMENT RESPONSIVENESS IN ACUTE MYOCARDIAL INFARCTION MSC THERAPY: TOWARDS NEXT-GENERATION STEM CELL THERAPEUTICS

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Acute myocardial infarction (AMI) is a common cause of sudden death, with an initial mortality rate of approximately 30%, and it remains a dangerous disease with a hospital mortality rate ranging from 5-10%, despite aggressive treatment. Muscle damage resulting from AMI is irreversible, leading to complications such as heart failure, arrhythmias, pericarditis, and mitral regurgitation. Even after successful reperfusion therapy, long-term complications such as heart failure and arrhythmias may persist, necessitating ongoing treatment, but so far, appropriate medications have not been developed. Stem cell therapies such as Hearticellgram®-AMI have been developed, and their safety and efficacy have been validated through numerous studies. However, direct mechanisms of action beyond indirect effects and left ventricular muscle function recovery have not been elucidated, thus the therapeutic effects cannot predict the varying causes among patients. This study aims to compare and analyze mesenchymal stem cells (MSCs) from individuals treated with stem cell therapy to identify biomarkers that can predict treatment efficacy by elucidating differences, and ultimately, to elucidate the mechanism of treatment responsiveness. We classified patients with acute myocardial infarction (AMI) who received MSC therapy into Responder (R) and Non-responder (NR) groups based on treatment outcomes. Analyzing the MSCs from the patients, we demonstrated that treatment responsiveness differs not in the treatment itself but in the fundamental source of the treatment. Additionally, we identified candidate biomarkers through Next Generation Sequencing of R and NR samples, and confirmed the functions of MSCs. Furthermore, we not only demonstrated in vitro models that MSCs regulating the expression of these biomarker genes can improve myocardial infarction but also elucidated their mechanism of action. Ultimately, we have discovered biomarkers predictive of AMI MSC therapy and elucidated their mechanism, and these findings are anticipated to serve as the foundation for the development of next-generation stem cell therapies to overcome the high unmet need in acute myocardial infarction.

Keywords: acute myocardiac infarction, mesenchymal ctem cell therapy, biomarker

3:45 PM – 4:45 PM

501

DECELLULARIZED HUMAN AMNIOTIC MEMBRANE SCAFFOLD ENHANCES ADIPOSE MESENCHYMAL STROMAL CELLS REGENERATIVE CAPACITIES VIA MODULATING MITOCHONDRIAL BIOENERGETICS

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The human amniotic membrane (hAM) has been applied as a scaffold in tissue engineering to sustain stem cells and enhance their regenerative capacities. We investigated the molecular and biochemical regulations of mesenchymal stromal cells (MSCs) cultured on hAM scaffold in a three-dimensional setting. Culture of adipose-MSCs (AMSCs) and bone marrow-MSCs (BMSCs) on decellularized hAM showed significant improvement in their viability and proliferative capacity and enhanced AMSCs' adipogenic and osteogenic differentiation. MSCs cultured on hAM scaffold showed less apoptosis, while the pro-angiogenic potential and inflammatory responses were altered, with upregulated mesenchymal markers in AMSCs. The hAM scaffold modulated cellular respiration by upregulating glycolysis in MSCs as evidenced by increased glucose consumption, cellular pyruvate and lactate production, and upregulation of glycolysis markers. These metabolic changes modulated mitochondrial oxidative phosphorylation (OXPHOS) and altered the production of reactive oxygen species (ROS), expression of OXPHOS markers, and total antioxidant capacity. They also significantly boosted the urea cycle and altered mitochondrial ultrastructure. These findings provide evidence for the favorable properties of hAM as a biomimetic scaffold for enhancing the in-vitro functionality of MSCs and supporting their potential usefulness in clinical applications.

Funding Source: This work is funded by the Science and Technology Development Fund (STDF) (FLUG grant No. 46721).

Keywords: amniotic membrane, bone marrow stem cells, mitochondrial bioenergetics

3:45 PM – 4:45 PM

503

EFFECTS OF RAPAMYCIN IN MESENCHYMAL STEM CELLS (MSC) EXPANSION AND DIFFERENTIATION

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Rapamycin is a metabolite produced by several species of actinomycetes, and it is primarily used as an immunosuppressant due to its ability to inhibit T-cell proliferation. Rapamycin also modifies PI3K/mTOR activity and intracellular oxidate stress (ROS), while it has been linked with increased life expectancy and reduced aging. In mesenchymal stem cells (MSC) context, rapamycin affects metabolism, growth and differentiation, but there is a lack of consensus regarding whether rapamycin promotes or inhibits these processes. Therefore, the aim of this work is to elucidate the effect of rapamycin in human MSCs (hMSCs). To do this, cells were cultured in presence or absence of rapamycin to define its effect in regulating cellular oxidative stress and ROS. Then, cells were cultured in extended periods of time in presence or absence of rapamycin to define its effect on hMSC growth. Finally, hMSCs were cultured in differentiation media in presence or absence of rapamycin to define its effect in cell differentiation capacity, to adipose and bone, and evaluated by histology and gene expression studies. The results indicate that Rapamycin treatment reduces ROS in hMSCs. We also observed a significant increase in cell growth and lifespan in presence of rapamycin. In terms of differentiation, rapamycin at differentiation media consistently impedes osteogenic and adipogenic differentiation. Cells expanded with rapamycin can differentiate properly in initial subcultures, while cells long-term expanded with rapamycin show total abrogation of cell differentiation potential in contrast to their control counterparts. Altogether, data suggest that at hMSCs Rapamycin reduces ROS and increases cell cycle, while its inhibitory effect in cell differentiation ability is related to cell expansion status. This result may explain the contradictory reports observed in bibliography regarding rapamycin effect in MSC differentiation. This study may help to clarify the effects, capabilities and limitations of using rapamycin in mesenchymal stem cells context.

Keywords: mesenchymal stem cells, rapamycin, differentiation

3:45 PM – 4:45 PM

505

DENTAL FOLLICLE: A SOURCE OF MESENCHYMAL STEM CELLS

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Dental follicle is a remnant from the early stages of development of the tooth and represents loose connective tissue lined with double epithelium found in retained teeth. Dental follicle mesenchymal stem cells (DFMSCs) can be isolated and cultivated in appropriate conditions. The aim of this study is to isolate DFMSCs and to reveal their ability to express mesenchymal and epithelial markers and to differentiate into various cell types. DFMSCs were isolated and seeded at standard cell culture conditions. We aimed to reveal the presence of epithelial cells in the dental follicle after incubation in keratinocytes cell culture media. Cell morphology and expression of stem cell specific and tissue specific markers were determined via fluorescent microscopy. Osteogenic, adipogenic, chondrogenic and epithelial differentiation capacity of the isolated cells was stimulated. For this purpose specific cell culture media were applied. Stem cell differentiation ability was detected via immunofluorescence, Alizarin red, Oil red and Alcian blue staining. We managed to isolate both mesenchymal and epithelial cells from dental follicle. Microscopically epithelial cells presented with round shape and larger size and were tightly clustered compared to mesenchymal stem cells in the same cell culture flask. Our cells reveal positive expression for some epithelial markers. DFMSCs were expressing stem cell markers and were capable in appropriate conditions to differentiate into various cell types, including osteoblasts, chondroblasts, adipocytes and epithelial cells. There is a pool of mesenchymal stem cells in the dental follicle capable of multilineage differentiation – mesodermal and epithelial.

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Keywords: MSC, characterization, dental follicle

3:45 PM – 4:45 PM

507

NOVEL BREAST RECONSTRUCTION USING EX VIVO AMPLIFIED MONONUCLEAR CELLS AND ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Breast cancer ranks first among cancer incidence in women, and the number of patients is increasing. Breast reconstruction is becoming increasingly important, since concerns have been raised about the decrease in QOL resulting from mastectomy. However, breast reconstruction using fat grafts presents challenges, such as fat necrosis due to inadequate blood flow, resulting in reduced engraftment rates. Many methods have been investigated to supplement adipose tissue with adipose-derived mesenchymal stem cells (ASCs) to promote rapid vascularization of the transplanted tissue. However, there are still limitation to vascularization of fat grafted tissue only with ASC transplantation. Ex vivo cultured amplified mononuclear cells (RE01) were developed as a cell population with highly vascular and tissue regenerative properties produced from small amount of peripheral blood. We have recently conducted the phase 1 physician based clinical trial using RE01 for patients with non-healing lower extremity ulcers resulted from Buerger's disease or collagen disease. We hypothesize that combining RE01 with ASCs may promote stronger angiogenesis and improve the fat grafting rate and also reduce the number of ASCs required. In this study, we examined the angiogenic and adipogenic potential of RE01 co-cultured ASCs in vitro and in vivo. ASCs isolated from human adipose tissue were co-cultured with RE01 produced from peripheral blood of healthy volunteers. In vitro experiments showed that ASCs co-cultured with RE01 promoted angiogenesis and adipogenesis compared to normal ASCs. Fat grafting experiments using nude mice demonstrated that the fat engraftment rate was significantly improved in the mixed group of ASCs co-cultured with RE01. The number of blood vessels and fat quality of transplanted adipose tissue were also promoted in this group, suggesting that RE01 co-cultured ASCs is highly effective in fat transplantation. The results of in vitro and in vivo experiments suggest that ASCs co-cultured with RE01 may be useful in improving the engraftment rate of fat grafts via their high angiogenic potential.

Funding Source: JSPS KAKENHI Grant Number 21K16919

Keywords: peripheral blood mononuclear cells, mesenchymal stem cell, fat graft

3:45 PM – 4:45 PM

509

EFFECT OF CYTOKINE PRIMING ON MULTIPOTENT MESENCHYMAL STROMAL CELLS: A MULTI-OMICS EVALUATION

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Priming with inflammatory mediators has been proven to increase the immunomodulatory activity of multipotent mesenchymal stromal cells (MSCs) and improve their survival in the harsh microenvironment. This approach is widely used in preclinical and clinical studies to enhance cell therapeutic potential. Primed MSCs show altered metabolic state and paracrine activity. However, the non-inflammatory aspects of this modulation remain insufficiently explored. In the current study, we assessed the effect of priming with cytokines (TNFα and IFNγ) on transcriptome, secretome, metabolomics and differentiation potency of Wharton jelly-derived MSCs. The gene expression analysis confirmed prominent upregulation of pathways connected to immune response. The most pronounced changes were linked to tryptophan breakdown via the kynurenine pathway. It was associated with the energetic shift of cells through activation of both glycolysis and oxidative phosphorylation. Priming caused upregulation of several lipid classes, including phosphatidylserines, phosphatidylethanolamines, phosphatidylinositols, cholesteryl-esters, and free fatty acids, which suggests anabolic reactions and membrane build-up in treated cells. The level of free amino acids, in contrast, was decreased in primed cultures compared to the control group. The composition of cytokine-stimulated MSC secretome confirmed their immunomodulatory tuning, but no significant difference was detected in the production of angio/neurotrophic growth factors (FGF, BDNF, HGF, bNGF, EGF, LIF, SCF or VEGF-A). Priming suppressed induced osteogenic and adipogenic differentiation of MSCs, revealing a negative impact on differentiation capacity. The most pronounced downregulation after cytokine treatment and following induction was observed in ALPL and CEBPA expression. In conclusion, our study suggests that cytokine priming may have limited potential in certain cell therapeutic areas due to its negative impact on MSC differentiation capacity and negligible effect on non-immune growth factor production.

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Keywords: cytokine priming, multipotent mesenchymal stem cells, omics

3:45 PM – 4:45 PM

511

ADHESION AND EXTRACELLULAR MATRIX MOLECULES IN ORAL MESENCHYMAL STEM CELL CULTURES

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Detailed characterization and comparison of the ECM and adhesion profile of different mesenchymal stem cell cultures is not yet performed. The aim of this research is to isolate primary mesenchymal cell cultures from adult (DPSC) and deciduous tooth pulp (SHED), periodontal ligament (PDL), dental apical papilla (SCAP), bone marrow (BMC) and gingiva (GMSC) and to characterize them for expression of specific extracellular matrix and adhesion molecules using RT Real time PCR. DPSC, SHED, PDL, SCAP, BMC, GMSC were isolated from unadulterated dental, alveolar bone and gingival ex-plants from patients undergoing routine surgical operations after acquiring informed consent. Ex-plants were digested in 3 mg/ml Collagenase I/ Dispase and seeded in culture flasks with DMEM / 20% FBS and antibiotics. Total RNA was extracted with RNeasy Plus Mini kit, cDNA was synthesized with RT2 First Strand Kit and analyzed with RT2 Profiler PRC Array, Human Extracellular Matrix & Adhesion Molecules Array. Analysis of adhesion and ECM markers showed significant differences between different oral mesenchymal stem cell cultures, particularly Collagen I was with highest expression in BMC and lowest in SCAP. Analysis of integrins showed similarity for the most abandoned β subunit is ITGB1 followed by ITGB5. ITGB3 on other hand were mostly expressed by dental mesenchymal stem cells and of α subunits, ITGA8 was the most abandoned except in PDL and BMC where ITGAV and ITGA5 prevailed respectively. Stem cell related ITGA1 and ITGA6 were expressed in DPSC, RDL and

SCAP and ITGA6 was much lower than ITGA1 in other three sell types. Comparison of MMPs showed highest amounts of MMP2 and MMP14 in all cells with significant difference in other minor expressed types. Cluster analysis of total examined gene expression revealed similar expression profiles of SCAP and PDL cells, differing significantly from BMC and GMSC, whereas DPSC and SHED showed bigger similarities with BMC and GMSC respectfully. Oral tissues contain a set of different mesenchymal stem cells, having different adhesion and ECM profile, thus possessing different stem cell properties. This understanding would refine the knowledge about adult stem cells and there role in healthy and pathological conditions and may lead to development of regenerative and cell therapy methods.

Funding Source: Project BG-RRP-2.004-0004-C01 financed by Bulgarian National Science Fund. The research is financed by the Bulgarian national plan for recovery and resilience.

Keywords: characterization, oral stem cells, ECM

3:45 PM – 4:45 PM

515

TROPOELASTIN PRESERVES MSC PHENOTYPE AND DELAYS REPLICATIVE SENESCENCE

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The persistence of senescent mesenchymal stromal cells (MSCs) gives rise to a range of chronic conditions in vivo, and contributes to poor clinical outcomes in MSC therapies. Although a decline in matrix integrity is evident in age-related pathologies such as fibrosis, osteoarthritis, and aortic stiffness, few studies have dissected the effects of extracellular matrix signals on cellular aging. Elastin loss and dysregulation is present in deteriorating mesodermal tissue of the skin and vasculature during aging. Conversely, supplementation of the elastin monomer, tropoelastin, promotes MSC regenerative functions, such as proliferation, migration, and differentiation. Given its association with MSC youth and functionality, we posit that tropoelastin modulates MSC senescence. Indeed, RNA sequencing revealed concurrent upregulation of proliferative genes and downregulation of genes implicated with cellular senescence by tropoelastin. Furthermore, we show that these effects persist in continuous culture of MSCs. Cells expanded with tropoelastin retain greater youth-associated cellular functions of differentiation and paracrine secretion, maintain phenotypes of low senescence-associated beta-galactosidase activity, small cell size, and low aging marker expression throughout various stages of replicative aging. Tropoelastin likely reduces MSC senescence by downregulating oncogenes p16 and p21, and upregulates cell cycle progression mediated by phosphorylated retinoblastoma protein. Our results indicate that despite exerting strong mitogenic effects, tropoelastin protects against MSC senescence during replicative aging, demonstrating the capability of youth-associated extracellular matrix components to modulate cellular aging. This work sheds new light on the role traditionally structural matrix proteins play in driving cellular fitness and age-associated functions.

Funding Source: This work is supported by Australian Research Council Discovery Project Grant and National Health and Medical Research Investigator Grant.

Keywords: senescence, mesenchymal stromal cells, tropoelastin

3:45 PM – 4:45 PM

517

COMPARISON OF MESENCHYMAL STEM CELLS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS (IPSC-MSC) AND UMBILICAL CORD (UC-MSC) IN IMMUNOMODULATORY FUNCTIONS

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Mesenchymal stem cell (MSC) therapy is currently being actively investigated in clinical trials. Their anti-inflammatory activities and abilities to home to damaged tissue allow wide applications in different diseases. With their confirmed abilities to suppress the immune system, MSC was first applied in clinical trials in treating graft-versus-host disease (GvHD). MSCs from different sources have their uniqueness and can behave very differently. Among those, MSCs derived from traceable cell sources such as induced pluripotent stem cells (iPSCs) are preferably considered in clinical cases, due to the ease of mass production, unlimited cell sources supply, and better quality-control when translating into good manufacturing practice (GMP)-compliant cell therapeutic products compared to tissue-derived MSCs. We previously reported that iPSC-MSCs have advantages over bone marrow MSCs such as their higher proliferation capacity, and they can better promote angiogenesis in the ischemia hindlimb mouse model. To further assess the potential use of iPSC-MSCs over tissue-derived MSCs, we aim to compare iPSC-MSCs with umbilical cord (UC)-MSCs. We first studied their similarities and differences by both bulk RNA sequencing and single-cell RNA sequencing analysis and pinpointed their immunoregulatory characteristics. We also examined their immunomodulatory effects by assessing their abilities to suppress T-cell proliferation in vitro and compared the RNA expression levels of common immunoregulatory proteins after co-culture studies. From both aspects, we found that both iPSC-MSCs and UC-MSCs have comparable immunosuppression potential. With the advantage of being differentiated from pluripotent stem cells, we believe that iPSC-MSCs is a better alternative in terms of immunomodulation for different autoimmune diseases in clinical applications.

Keywords: mesenchymal stem cells (MSC), immunomodulation, iPSC-MSC

3:45 PM – 4:45 PM

519

ESTABLISHMENT OF HUMAN EXFOLIATED DECIDUOUS TEETH STEM CELL LINE BY ENHANCING TELOMERASE ACTIVITY

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The current study aims to transform and establish the cell line of stem cells isolated from human exfoliated deciduous teeth (SHED), a subtype of mesenchymal stem cells, through ectopic expression of human telomerase reverse transcriptase (hTERT) using a retroviral method. To establish and achieve our goal, stem cells from the remnant pulp of deciduous teeth have been immortalized by retroviral-mediated ectopic expression of the telomerase catalytic subunit. The transformed cells were characterized for their growth characteristics, and their telomerase activity was analyzed using quantitative gene expression. The cells were analyzed for CDKN2A gene to determine the tumorigenic potential of transduction as well as analysis of short tandem repeats of 16 loci were performed for the authentication of virus transduction. The transformed SHED cells with hTERT depicted enhanced hTERT gene expression without changing the fibroblast-like morphology. The evaluated growth characteristics have shown that the SHED cell-line exhibited higher population doubling time, level, and cell migration activity with respect to controls. The STR profiling of fifteen loci was similar to primary cells while, at CSF1PO loci that there was a deletion of allele 13 in both SHED-hTERT and mock control. Whilst both SHED-hTERT and mock control depicted deletion therefore, suggesting no adverse effect due to hTERT integration in genome. Also, there is no change in CDKN2A gene expression. The hTERT immortalized stem cells from human exfoliated deciduous teeth cell-line exhibit stemness, multi-potency, and growth characteristics of primary cells and maintain continuous cell line growth characteristics.

Keywords: mesenchymal stem cells, SHED Cell-line, ectopic hTERT expression

3:45 PM – 4:45 PM

521

DECOUPLING CANCER CELLS FROM MESENCHYMAL STEM CELLS TO REGAIN NK SENSITIVITY

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Circulating tumor cells (CTCs) shed from primary tumors must overcome the cytotoxicity of immune cells, particularly natural killer (NK) cells, to form metastasis. The tumor microenvironment (TME) protects tumor cells from the cytotoxicity of immune cells partially executed by cancer-associated mesenchymal stromal cells (MSCs). However, the mechanisms by which MSCs influence the NK resistance of CTCs remain poorly understood. This study demonstrates that MSCs enhance NK resistance of cancer cells in a gap junction-dependent manner, thereby promoting survival and metastatic seeding of CTCs in immune-compromised mice. Tumor cells crosstalk with MSCs through an intercellular cGAS-cGAMP-STING signaling loop, leading to increased production of interferon-BETA(IFNBETA) by MSCs. IFNBETA reversely enhances the type I IFN (IFN-I) signaling in tumor cells and hence their expression of human leukocyte antigens class I (HLA-I) on the cell surface, protecting the tumor cells from NK cytotoxicity. Disrupting the loop results in the reversal of NK sensitivity in tumor cells and a decrease in tumor metastasis. Moreover, positive correlations between IFN-I signaling, HLA-I expression, and NK tolerance were found in human tumor samples. Thus, the NK-resistant signaling loop between tumor cells and MSCs may serve as a novel therapeutic target.

Funding Source: Ministry of Science and Technology National Key R&D grant National Science Foundation China general grant Macau Science and Technology Development Fund grants University of Macau grants

Keywords: mesenchymal stromal cells, circulating tumor cells, natural killer cells

3:45 PM – 4:45 PM

523

DEVELOPMENT OF SELF-MULTILAYERED CELL SHEETS FROM DENTAL PULP STEM CELLS WITH SUPERIOR MECHANICAL AND OSTEOGENIC CAPABILITIES

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We have successfully isolated and expanded human dental pulp stem cells (DPSCs) under xenogeneic serum-free culture conditions (XFM). Furthermore, we developed a culture method that enables DPSCs to form self-multilayered cell sheets (self-CS, patent pending), aimed at enhancing regenerative therapy applications. This study sought to characterize self-CS and identify its potential in regenerative therapy. Initially, we optimized the seeding density and culture duration under XFM conditions to produce self-CS. The optimally seeded DPSCs formed cell sheets with a multilayered structure after a specified period. Compared to conventional cell sheets generated on temperature-responsive culture dishes, self-CS exhibited significantly greater mechanical strength. The self-CS contained mesenchymal stem cell marker-positive cells and comprised multiple types of collagen, including types 1, 4, and 7. In vitro osteogenic differentiation induction led to the formation of large calcified nodules by self-CS. Furthermore, when mixed with hydroxyapatite/β-tricalcium phosphate and implanted subcutaneously in immunodeficient mice, significant bone-like hard tissue formation was observed without any cellular tumorigenesis. Our findings demonstrate a novel culture method for developing unique cell sheets with spontaneous multilayered structures. The self-multilayered cell sheet structure offers high mechanical strength and malleability, alongside potent osteogenic capacity both in vitro and in vivo. Self-CS holds promise for application in bone regenerative medicine, leveraging advanced cell sheet engineering techniques.

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Keywords: mesenchymal stem cells, cell sheet engineering, dental pulp stem cells

3:45 PM – 4:45 PM

525

THERAPEUTIC POTENTIAL OF ADIPOSE-DERIVED STEM CELL-CONDITIONED MEDIUM AND EXTRACELLULAR VESICLES IN SKIN INJURY MODEL

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Radiotherapy (RT) is one of three major treatments for malignant tumors. More than 50% of patients with malignant tumors undergo RT. Over 95% of patients undergoing RT may develop some form of radiation dermatitis or radiation-induced skin injury. This condition can affect the patient's quality of life during and after treatment. If severe, there is a risk of limiting the radiation dose or interrupting the treatment plan, which may negatively affect the treatment outcome. We established an in vitro model of radiation-induced skin injury using human dermal fibroblasts (HDFs), examined and analyzed extracellular vesicles of adipose-derived stem cells (ADSC-EVs), characterized ADSC-EVs. We observed the effects of conditioned medium of ADSCs (ADSC-CM) and ADSC-EVs on irradiated HDFs and evaluated the mechanisms of the beneficial effects of ADSC-CM and ADSC-EVs on radiation-induced skin injury. Collagen gene expression was compared by qPCR to evaluate an in vitro model of radiation-induced skin injury established with HDFs. Wound healing in the radiation-induced skin injury model by ADSC-CM and ADSC-EVs treatment were analyzed by gene expression, collagen synthesis and cell proliferation assay. ADSC-EVs promoted proliferation of HDFs in a dose-dependent manner. ADSC-CM and ADSC-EVs showed significant benefit in the treatment of radiation-induced skin injury. Our established in vitro model of radiation-induced skin injury showed that ADSC-CM and ADSC-EVs play a beneficial role in the healing process; ADSC-CM and ADSC-EVs may be promising therapeutic strategies in the treatment of radiation-induced skin injury.

Funding Source: This research was partially supported by JST SPRING, grant number JPMJSP2124.

Keywords: adipose-derived stem cells, extracellular vesicles, radiotherapy

3:45 PM – 4:45 PM

527

NOTCH ACTIVATION IS ASSOCIATED WITH QUIESCENCE IN A LEPTIN RECEPTOR EXPRESSING ENDOMETRIAL MESENCHYMAL STEM/STROMAL CELLS SUBPOPULATION

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Endometrial stem cells are responsible for the continual cycles of shedding, regeneration and repair throughout a woman's reproductive years. Quiescence is regulated by both intrinsic cellular factors and extrinsic environmental factors. Extrinsic factors such as Notch signal are vital in maintaining somatic stem cells. Our group demonstrated that activation of Notch signaling better maintained quiescence of human endometrial stem cells. The co-expression of two perivascular marker CD140b+CD146+ was used to isolate endometrial mesenchymal stromal/stem cells (eMSC). Using single cell sequencing, we identified abundant leptin receptor (LEPR) expressing cells in an eMSC subpopulation (SP3) located at the root of trajectory analysis. In this study, we hypothesized that the LEPR+ eMSC were in a quiescence state that could be differentiated into mature stromal cells, and tested the quiescence features of LEPR+ eMSC in two models. The LEPR+ eMSC underwent differentiation rapidly losing their CD140b+CD146+ phenotypic markers in culture. In the first model, when LEPR+ eMSC were subjected to serum starvation, the phenotypic expression and colony forming ability were effectively enhanced when compared to the control group. Upon re-stimulation with serum, the cells transited from a resting state to an active state. However, serum starvation decreased cell viability with to increased apoptosis. In the second model, activation of Notch signaling by Notch ligand JAG1 increased phenotypic expression without compromising viability. Using a published endometrial cell single-cell dataset, we found enrichment of several NOTCH genes in the LEPR+ eMSC. Experiments using qPCR indicated significantly more abundant expression of HES1, NOTCH1 and NOTCH4 in the LEPR+ eMSC when compared with the stromal cells, LEPR- eMSC and eMSC. The findings show the importance of Notch signaling in controlling the dynamics of endometrial stem cells and its implications on stem cell behavior.

Funding Source: This project was supported by the National Natural Science Foundation of China/ Research Grants Council Joint Research Scheme (N_HKU 734/20).

Keywords: endometrium, mesenchymal str0omal cells, quiescence

3:45 PM – 4:45 PM

TOPIC: MUSCULOSKELETAL

529

DMD PATIENT-DERIVED SKELETAL MUSCLE ORGANOIDS EXHIBIT A DEVELOPMENTAL DELAY OF MYOGENIC PROGENITOR MATURATION

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Duchenne muscular dystrophy (DMD) is one of the most common muscular diseases affecting worldwide 1 in 3500-5000 new-born boys. Constant skeletal muscle weakness and loss as well as reduced muscle regeneration capacity leads to increased fibrosis over time. Satellite cells (SC) are the skeletal muscle stem cells for adult muscle maintenance and regeneration. As SC enter a cycle of asymmetric division, dystrophin is essential for the establishment of cell polarity. In DMD dystrophin is mutated or missing which leads to asymmetric cell division failure and ultimately to cell cycle arrest in SC. To overcome the restricted availability of muscle biopsies from DMD patients along their disease progression we have established a 3D organoid system which delivers a stable population of skeletal muscle progenitors to investigate human myogenesis during early embryonic and fetal development. We have performed organoid cultures from three DMD patient-derived iPSC lines, subsequent scRNAseq analysis and in vivo transplantation of CD82-positive myogenic progenitors in immune deficient mice. DMD myotubes from these progenitors showed to have a smaller size compared to healthy controls in an in vivo environment. scRNAseq analysis of DMD versus healthy skeletal muscle organoids indicates a reduced myogenic progenitor population that suffers from constant activation and differentiation as well as a trend towards embryonic and immature myotubes. Mapping our data on the human myogenic reference atlas together with primary SC scRNAseq data indicates a more immature developmental stage of DMD organoids derived myogenic progenitors. Our organoid system provides a promising model to study muscular dystrophies in vitro esp. in their early developmental onset and a method to overcome the bottleneck of limited patient material for skeletal muscle disease modelling.

Funding Source: Deutsche Duchenne Stiftung, Duchenne Deutschland e.V.

Keywords: skeletal muscle organoids, myogenic progenitors, Duchenne muscular dystrophy

3:45 PM – 4:45 PM

531

FOXP2+ PERICHONDRIAL STEM CELLS ORCHESTRATE LONG BONE DEVELOPMENT AND REPAIR

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It is widely accepted that the adult skeleton is maintained by multiple region-specific skeletal stem cells (SSCs), including bone marrow, periosteal and growth plate SSCs. However, whether these postnatal SSCs share a common embryonic origin, and whether SSCs exist in the perichondrium remain elusive. By performing genetic lineage tracing, cell ablation and single-cell RNA-seq (scRNA-seq) analyses, we identified a novel population of Foxp2+ perichondrial stem cells (PcSCs) in the embryonic and postnatal long bones. Foxp2+ cells first appeared in E11.5 limb bud mesenchyme surrounding the primordial cartilage template, and subsequently localized in E13.5 perichondrium. Foxp2+ cells gave rise to chondrocytes and osteoblasts, as well as bone marrow, periosteal, and growth plate SSCs in postnatal long bones. In P3 mice, Foxp2+ cells specifically localized in the Groove of Ranvier surrounding growth plate, which exhibited active chondrogenic (while migrating towards the articular cartilage and resting zone of growth plate) and osteogenic (while invading the primary and secondary ossification center) potential. Adult Foxp2+ cells were relatively quiescent under homeostasis but could be activated to generate chondrocytes and osteoblasts upon bone fracture or articular injury. Genetic ablation of Foxp2+ cells in P3 mice caused significantly decreased growth plate thickness and bone mass. Ablation of Foxp2+ cells in adult mice led to delayed repair of articular cartilage. scRNA-seq revealed that perichondrial Foxp2+ SSCs (CD200+CD105-), or PcSCs, exhibited the highest self-renewal capacity in adult long bones, which could undergo trilineage differentiation in vitro, and generate bone, cartilage and bone marrow microenvironment upon renal transplantation. Intriguingly, ex vivo cell culture and in situ atomic force microscopy revealed that Foxp2 expression was negatively regulated by matrix stiffness, which explained why Foxp2+ cells were specifically found in the perichondrium but not periosteum. More importantly, human PcSCs were also found in the perichondrium of young adults, suggesting that they are evolutionarily conserved. Overall, this study demonstrated that PcSCs are the embryonic origin of postnatal SSCs and a novel stem cell population that orchestrate skeletal development and repair.

Funding Source: National Natural Science Foundation of China (82302706) National Key R&D Program of China (2022YFA1103200)

Keywords: skeletal stem cells, perichondrial stem cells, skeletal development and repair

3:45 PM – 4:45 PM

533

WNT/BETA-CATENIN SIGNALING REGULATES OSTEOBLAST DEDIFFERENTIATION DURING ZEBRAFISH FIN REGENERATION

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Cellular dedifferentiation is an important phenomenon that is invoked to form source cells for regeneration in various vertebrate species and organs. We define dedifferentiation as the process by which a differentiated cell reverts back to a less differentiated state and becomes proliferation-competent. In response to fin amputation, osteoblasts (OB) proximal to the amputation plane start to dedifferentiate, become proliferative and migrate to form part of the regeneration blastema. In zebrafish, various signaling pathways have been shown to be required for fin regeneration, but only retinoic acid and its upstream regulator NF-κB signaling, have been described as regulators of OB dedifferentiation. Our aim here is to better characterize the molecular changes occurring in dedifferentiating osteoblasts. To do that we have established a protocol to isolate and sort differentiated and dedifferentiated osteoblasts from caudal fins using the bglap:eGFP transgenic fish line, and then performed RNA sequencing at 12 hours and 1 day post amputation (dpa). qRT-PCR and HCR in situ hybridization confirmed that known differentiation markers are downregulated during dedifferentiation. Importantly, we find that transcriptional changes in dedifferentiating OBs are massive, with hundreds of genes being up- and downregulated. Interestingly, GSEA indicated that the Wnt/beta-catenin pathway is positively enriched within dedifferentiating OBs by 1 dpa. While our and other labs have described important roles for Wnt signaling at later stages of fin regeneration, including in OB re-differentiation, a potential function in the regulation of OB dedifferentiation has not been uncovered. To address this, we have used a hs:Axin1-YFP transgenic line, where axin1, a Wnt signaling inhibitor, is overexpressed after heat-shock. Intriguingly, using qRT-PCR and HCR in situ hybridization we found that Wnt signaling inhibition reduced OB dedifferentiation. Then we used Cre-lox approach to get more cell-type specific insights into Wnt signaling effects on OB dedifferentiation. Therefore, it would be interesting to investigate Wnt signaling pathway regulation of OB dedifferentiation in more detail by finding targets and regulatory networks, and also the interaction between osteoblasts and epidermal cells.

Keywords: fin regeneration, dedifferentiation, osteoblast

3:45 PM – 4:45 PM

535

FACILITATION OF THE HEALING OF MEDICATION-RELATED OSTEONECROSIS OF THE JAW USING OSTEOGENICALLY STIMULATED GINGIVA-DERIVED STEM CELLS-LADEN GELATIN METHACRYLATE-POLYDOPAMINE HYDROGEL

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Medication-related osteonecrosis of the jaws (MRONJ) is a serious complication in patients taking antiresorptive agents such as bisphosphonate and poses negative impacts on patients life quality. To reduce the occurrence of MRONJ and recover the regeneration capability of the MRONJ-affected sites, gingiva-derived stem cells (GSCs)-laden gelatin methacrylate-polydopamine (GelMA-PD) hydrogel have been proposed according to the multipotency and of GSCs and outstanding biocompatibility and adhesion property of GelMA-PD. GSCs were harvested from Sprague-Dawley rats and showed capabilities for osteogenesis, chrondrogenesis, and adipogenesis. >99% GSCs were double positive for CD73 and CD90, < 1% GSCs were negative for CD11b on the passages 3 and 5. Osseous defects were surgically created on the maxilla of Sprague-Dawley rats with bisphosphonate pretreatment to simulate MRONJ-affected sites and were unfilled or filled with GelMA-PD alone, unstimulated GSC-laden GelMA-PD (UGG), or osteogenically stimulated GSC-laden GelMA-PD (OGG) for 3 weeks. The results revealed that soft tissue coverage were accelerated, with reduced sequestrum formation in the UGG and OGG groups. The immunohistochemical evidence showed that RANK expression was downregulated in the unfilled group but appeared to be recovered, with upregulated bone sialoprotein expression in the OGG group. In conclusion, osteogenically stimulated GSCs-laden GelMA-PD was capable of facilitating the mucosal healing and recovering osteoblastogenesis-osteoclastogenesis coupling and could be a feasible strategy for treating MRONJ-affected sites.

Funding Source: National Science and Technology Council (Taipei, Taiwan): 110-2314-B-002-109-MY3, 113-2314-B-002-039

Keywords: medication-related osteonecrosis of the jaw, mesenchymal stem cell, bone tissue engineering

3:45 PM – 4:45 PM

537

DISSECTING THE ROLE OF MYF6 IN FACILITATING FIBROBLAST CONVERSION INTO FUNCTIONAL MYOGENIC PROGENITOR CELLS

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Conversion of fibroblasts into muscle cells in vitro by overexpression of the transcription factor MyoD has been widely employed in past decades. However, the potential of other myogenic regulatory factors (MRFs), including Myf5, Myf6 and Myog, to elicit a similar myogenic conversion has been rarely studied. To address this disparity, we set out to investigate the role of MRFs during fibroblast conversion into skeletal muscle stem and differentiated cells. Surprisingly, we found that Myf6, a transcription factor associated with late-stage myogenesis, efficiently converted fibroblasts into myotubes alone, or alternatively induced myogenic progenitor cells (iMPCs) in the presence of small molecules. Myf6-derived iMPCs expanded for more than 10 passages while expressing a suite of differentiation genes, in addition to muscle stem cell markers, most notably Pax7. To investigate reprogramming dynamics in a defined transgenic system, we generated a novel dox-inducible Myf6 overexpression mouse model. This system enabled us to dissect reprogramming kinetics and transcriptomic changes that occurred during Myf6-induced cell-fate change. Mechanistically, we found that subjecting MyoD-KO fibroblasts to Myf6 and small molecules gave rise to expandable iMPCs, indicating that MyoD is dispensable for iMPC formation and self-renewal. Similarly, we found that iMPCs can be derived using MyoD and small molecules in Myf6-KO fibroblasts. Collectively, these results uncovered redundancy between MRFs' function, highlighting an unexpected potency for Myf6 in the induction of muscle stem cell fate in vitro.

Funding Source: SNSF Eccellenza Grant

Keywords: direct lineage reprogramming, myogenic regeneration, muscle stem cells

3:45 PM – 4:45 PM

TOPIC: NEURAL

539

OPTIMIZING CRISPRA-GRNA EXPRESSION IN MOUSE PRIMARY ASTROCYTES THROUGH ECOTROPIC LENTIVIRAL VECTORS

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CRISPR-Cas9-based gene activation (CRISPRa) of transcription factor genes has emerged as a powerful tool in cellular reprogramming studies. Its remarkable multiplexing capacity and ability to directly target endogenous loci make it particularly promising for developing cell therapeutic strategies for neurological pathologies. However, existing gene delivery systems for CRISPRa-gRNA expression (such as liposome-mediated transfection, lentivirus (VSV-G pseudotyped), retrovirus and Adeno-associated virus) could not allow us to carry out barrier-free and fast screening of candidate gRNA (guide RNA) in mammalian primary cell culture systems. This limitation is especially pertinent in primary astrocytes, which are extensively utilized as an in vitro model system for studying astrocyte-to-neuron direct reprogramming. To carry out easy and rapid gRNA screening, we investigated the potential of delivering lentiviral gRNA vectors to in vitro primary astrocyte cultures using BSL-1 (Biological safety level) ecotropic lentivirus (ELV). Through packaging lentiviral-gRNA vectors with murine ecotropic envelope in Hek293T cells via a three-plasmid transfection system, we successfully transduced the ELV to mouse primary astrocytes. Subsequently, we validated the activation of ELV-gRNA targeted gene. Moreover, we demonstrated the feasibility of directly reprogramming astrocytes into neuron-like cells mediated by ELV-Ascl1-gRNAs. Unlike the transient

expression of gRNA achieved through liposome-mediated transfection, our approach enables long-term and stable activation of target gene in primary astrocyte culture. Similarly, in contrast to the strict BSL-2 requirement of lentivirus and retrovirus, and the long-term time requirement of AAV, ELV approach is much easier to implement in most labs. Therefore, we propose a pipeline for delivering CRISPRa-gRNA to mouse primary astrocyte via BSL-1 ELV. This application of ELV pipeline might significantly facilitate the validation of optimal gRNAs for further in vivo reprogramming studies in a more convenient manner.

Keywords: CRISPRa-gRNA, primary astrocytes, ecotropic lenti-virus

3:45 PM – 4:45 PM

541

EFFICIENT GENERATION OF OLIGODENDROCYTE FROM IPSC-DERIVED NEURAL PRECURSOR CELLS

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Oligodendrocyte loss in the central nervous system causes demyelinating diseases such as multiple sclerosis and several leukodystrophies. Yet effective pharmaceutical drugs which fundamentally cure those diseases are unavailable and thus cell replacement therapy is considered to be an alternative strategy. Oligodendrocyte progenitor cells (OPCs) which are a cellular source of oligodendrocytes have been generated from induced pluripotent stem cells (iPSCs). However, this process is shown to be time-consuming and exhibits a limited efficiency. Here, we have developed an efficient method by which over 80% OPCs can be generated from iPSC-derived neural precursor cells (NPCs) within 45 days. We first generate neural progenitor cells (NPCs) from human iPSCs through dual SMAD/GSK3 inhibition. These NPCs express a number of NPC makers including SOX1, SOX2, PAX6, and NESTIN and can differentiate into OLIG2+ and NKX2.2+ cells within 4 days. These cells are then mechanically dissociated into small clumps and cultured them in the ultra-low-attachment plate to allow forming spheroids till day 22. The spheroids are then plated into Poly-L-ornithine/Laminin coated-dishes and further cultured in OPC medium till day 45. During this period, OPCs which are positive for PDGFRα, SOX10, NG2, and O4 are migrated out from the edges of spheroids and highly proliferative. Isolated PDGFRα+/SOX10+/O4+ OPCs by cell sorting can be terminally differentiated to myelin basic protein–positive (MBP+) oligodendrocytes. The MBP+ oligodendrocytes are capable of tightly ensheathing axons of neurons and nanofibers. Overall, the method presented herein enables OPC generation in a very rapid and efficient manner. The iPSC-derived OPCs can be utilized for preclinical and clinical trials to investigate therapeutic potential of demyelination diseases.

Keywords: induced pluripotent stem cells, neural precursor cells, oligodendrocytes

3:45 PM – 4:45 PM

543

LINGO-1 DEFICENCY NSC-DERIVED NEURAL TISSUEOID ENHANCED ITS RETENTION AND NEURONAL RELAY IN SPINAL CORD TRANSECTED COMPLETELY

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Self-organizing neural stem cell (NSC)-derived tissueoids are a promising option to model spinal cord development and repair injury. However, retention and integration ability in vivo still needs to be improved. We aimed to determine if genetically engineered neural tissueoids could exhibit increased retention and relay properties and improved cell therapeutic efficacy. Gene expression screening revealed that LINGO-1 increased significantly in injured and donor neurons. Moreover, LINGO-1 accelerated programmed cell death by increasing the cle-caspase-3 and interacted with TrkC to decrease the phosphorylation of the PI3K/AKT/CREB axis for synaptic degradation. Based on CRISPR-Cas9 system, LINGO-1 deficiency NSC-derived tissueoids were generated with an apoptosis resistance. In situ implantation of LINGO-1 deficiency tissueoids dramatically promoted neuronal connectivity. Numerous green fluorescent protein positive donors were retained in the injury and graft site, promoted 5-hydroxytryptamine and calcitonin gene-related peptide regeneration, received corticocortical and supraspinal inputs, and drove the coordinated movement of the hindlimbs. This strategy demonstrates that inhibiting LINGO-1 in tissueoid is a promising strategy for responding to injury stimuli and highly intensifying host circuit effects in large spinal injury cavities.

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Keywords: neural stem cell-derived tissueoid, spinal cord injury

3:45 PM – 4:45 PM

545

NON-CANONICAL HES1 ACTIVATION AND ITS IMPLICATIONS IN THE MAINTENANCE OF STEMNESS AND DEVELOPMENT

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Hes1 is a crucial transcription factor that plays a fundamental role in stem cell maintenance and differentiation. Conventionally, Hes1 activation has been associated with the canonical Notch signaling pathway. Along with other reports, we found the existence of Notch-Independent Hes1 (NIHes1) activation in mouse neocortex. To investigate the possible activators of NIHes1 we performed a reverse ChIP assay of the promoter, followed by mass spectrometric analysis. From this, we found that transcription factors such as βcatenin, Smad and Tead1 can bind to the NIHes1 promoter. GSEA analysis of identified transcription factors showed the enrichment of Wnt, Hippo, TGFβ and BMP signaling pathways. To validate this, we carried out a luciferase assay with the NIHes1 promoter and specific blockers of selected pathways, which showed that β-catenin mediated Wnt signaling can activate NIHes1 and thereby maintain stemness. In addition, upon analyzing Single-cell sequencing data of two cell and four cell embryos, we found that Hes1 and βcatenin are expressed at the 4-cell stage, while none of the canonical notch downstream effectors such as Hes5 and Hey1 are expressed. This pointed towards a non-canonical mode of Hes1 activation during early embryonic development. To study the significance of this observation, we generated NIHes1 fl/fl; Rosa Cre ERT2 mice. Ablation of NIHes1 from the Morula stage in-vitro showed significant cleavage abnormalities, which indicate the important role of NIHes1 in early embryonic development and the possible role of Wnt signaling in its activation.

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Keywords: stem cell maintenance, Hes1, embryonic development

3:45 PM – 4:45 PM

547

RETINAL REMODELING AND EXTRACELLULAR MATIRX PERFORMANCE IN ZEBRAFISH: REGENERATIVE CAPACITY UNDER SCRUTINY

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Zebrafish exhibit remarkable regenerative abilities in various tissues, including the retina. In this study, we investigated the dynamics of retinal remodeling in zebrafish using a laser injury model. Thereby, understanding the intricate mechanisms involved in the formation of the extracellular matrix (ECM), as well as the genes responsible for adhesion molecules and ECM proteins, is crucial for determining whether successful regeneration or permanent scar formation occurs. Hereby, we observed that the zebrafish retina exhibited initial transient scar deposition following laser injury, but did not develop chronic fibrotic scarring. Despite the regenerative capacity of the zebrafish retina, incomplete regeneration of the retinal pigment epithelium (RPE) was observed even at late time points. Proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, showed biphasic expression pattern in Müller cells during the regenerative phase. A peak of PCNA-positive cells was evident in the outer nuclear layer (ONL) and inner nuclear layer (INL) at 7 and 79 days post injury (dpi). Interestingly, gliotic scar formation, characterized by the concentration of glial fibrillary acidic protein (GFAP) in the scar region, did not occur. Instead, GFAP up-regulation was observed in all Müller cells, suggesting a different pattern of glial response to injury. Paired box protein 6 (Pax-6), pivotal transcription factor for retina formation, exhibited a triphasic expression pattern, with notable peaks at 5, 10 and 23 dpi, predominantly in the ONL. Screening revealed the up-regulation of 61 genes associated with ECM at 7 dpi. This included the up-regulation of collagen types 1, 4, and 12, indicating their involvement in the repair process. In summary, our findings supported the hypothesis that the zebrafish retina undergoes continuous regeneration following damage. We did not observe evidence of chronic retinal scarring in the zebrafish model, nor did we observe complete regeneration. Additionally, the up-regulation of collagen type 12, which has not been previously described, raises new research questions regarding retinal remodeling and might contribute to the development of new approaches to replicate the regenerative processes observed in zebrafish.

Funding Source: Hanela-Stiftung Aarau, Switzerland

Keywords: retina, regeneration, zebrafish

3:45 PM – 4:45 PM

549

TRANSPLANTATION OF HIPSC-DERIVED NEURAL NETWORK TISSUEOID COMBINED WITH ELECTROACUPUNCTURE PROMOTES REPAIRATION OF BEAGLE SPINAL CORD INJURY

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The application of human induced pluripotent stem cell (hiPSC) transplantation in the treatment of spinal cord injury (SCI) has shown significant translation promise, but the therapeutic efficacy also encounters some challenges, including uncontrolled cell fate, low efficiency of neuronal differentiation and poor survival of donor cells. In this study, to increase the neuronal differentiation and grafted cell colonization with avoiding a risk of tumor formation, we first constructed a hiPSC-derived neural network tissueoid (hiPSC-NNT) with synaptic transmission potential by using tissue engineering techniques and 4D culture in vitro. And then the hiPSC-NNT was transplanted into the injury area after the completely transected SCI of beagle to investigate whether electroacupuncture (EA) treatment could enhance the survival of hiPSC-NNT and integration of one with host tissue, which further promotes the movement functional recovery of paralyzed hindlimbs. Our results in vivo showed that EA improved the inflammatory microenvironment in injured spinal cord, and facilitated the survival and neuronal differentiation of hiPSC-NNT in the injury/graft area and integration of hiPSC-NNT with host neural network. The hiPSC-NNT transplantation combined with EA stimulation synergistically promoted axonal regeneration, especially, the descending propriospinal tract. Furthermore, the results of the neural tracing, MRI, electrophysiology and behavioral test demonstrated that the hiPSC-NNT transplantation combined with EA treatment better reconstruct the spinal cord neural pathway and enhanced

hiPSC-NNT to play a neuron relay role, so as to further improved the movement ability of paralyzed hindlimbs. The present study provides a novel combination treatment strategy for reconstructing spinal cord neural pathway after SCI.

Funding Source: The National Natural Science Foundation of China (Grant No. 81891003, 82374579) electroacupuncture

3:45 PM – 4:45 PM

551

IMPACT OF THE REACTIVE CELLULAR ENVIRONMENT ON IN VIVO LINEAGE REPROGRAMMING IN THE INJURED ADULT MOUSE CORTEX

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Direct conversion of non-neuronal cells, such as glial cells, into induced neurons (iNs) is a promising strategy to reinstate neurogenesis in the mammalian brain after injury. Our recent work shows that Ascl1 fails to give rise to iNs in vivo, while its phosphodeficient mutant, Ascl1SA6, combined with Bcl2, efficiently induces neuronal conversion in the postnatal cortex. This suggests that the local milieu may impact the reprogramming outcome. Here, we investigated the reprogramming potential of Ascl1SA6 in the injured adult brain and the interactions between iNs and their local cellular milieu. Stab-wound injury was performed in the adult mouse cortex. Three days later, proliferative reactive glia was transduced with retroviruses encoding for Ascl1 or Ascl1SA6 and Bcl2. Twelve days post-injection (dpi), Ascl1 increased the proportion of oligodendrocyte precursor cells, while Ascl1SA6 enabled the generation of iNs in the injured adult cortex. However, the number of iNs was reduced at 70 dpi compared to 28 dpi, suggesting limited long-term survival.To assess the interactions between iNs and their local milieu, we measured the extent of astroglia, microglia, and infiltrating monocytes at the reprogramming site by immunohistochemistry (IHC). We found that reactive astrocytes, infiltrating monocytes, and microglia accumulated at the reprogramming site. Moreover, injury-induced reactive astrocytes can acquire a neurotoxic profile. To detect whether neurotoxic astroglia was present at the reprogramming site, complement 3 (C3) expression in reactive glia was assessed by RNAscope and IHC. Notably, some C3-expressing reactive astrocytes were found at the reprogramming site. Such astrocytes may limit neuronal conversion, maturation and survival of iNs. We next questioned whether neurotoxic astroglia was targeted for neuronal conversion. Thus, EdU cell proliferation assay was performed at the time of retroviral delivery (3 days post-injury). We found that most proliferating reactive astrocytes expressed C3, suggesting that neurotoxic astrocytes may be targeted for lineage reprogramming. Future work will reveal whether depleting the local milieu of neurotoxic reactive astrocytes affects the reprogramming outcome. Altogether, our work may provide further insight towards ameliorating reprogramming.

Funding Source: Welcome Trust, CRC 1080, ReALity, ERA-NET NEURON, ERC

Keywords: in vivo lineage reprogramming, neuroinflammation, neuroregeneration

3:45 PM – 4:45 PM

553

A PLATFORM FOR STUDYING ASTROCYTE TO NEURON TRANSDIFFERENTIATION IN HUMAN CELLS

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Neuronal cell death is a hallmark of neurodegenerative diseases. The loss of neurons leads to motor dysfunction, memory deficits and cognitive decline in patients. To restore a declining neuronal population, astrocytes can potentially be converted into functional neurons in situ. Various strategies for this astrocyte-to-neuron (AtN) transdifferentiation have been described, including overexpression of pro-neuronal transcription factors (TFs) such as ASCL1 as well as gene knockdown of the RNA-binding protein PTBP1. To date, most studies investigating AtN conversion use primary mouse astrocytes as a model system.
To increase the translatability of the obtained results to humans, we generated a toolbox to investigate AtN transdifferentiation in vitro using human induced pluripotent stem cell (hiPSC)-derived and human primary astrocytes. These cell types were characterized based on their expression of canonical astrocyte markers and used to conduct a proof-of-concept (PoC) study recapitulating AtN transdifferentiation. We used lentiviral overexpression of the pro-neuronal TFs ASCL1, NEUROG2, NEUROD1 and DLX2 as well as gene knockdown of PTBP1 and evaluated morphological and transcriptional changes to assess the potential of AtN transdifferentiation technology. We obtained positive PoC for TF-mediated but not for PTBP1-based AtN transdifferentiation in vitro. Of the tested TFs, overexpression of ASCL1 promoted AtN conversion the most, leading to a strong induction of the early neuron marker DCX and the more advanced neuron marker MAPT. These changes in expression were corroborated by corresponding immunocytochemistry stainings which also highlighted the distinct neuronal morphology of the newly converted cells. Our results highlight the feasibility of studying AtN transdifferentiation in human cell culture systems which offer access to large numbers of cells for extensive testing and improved translatability.

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Keywords: astrocyte to neuron transdifferentiation, direct conversion, cell reprogramming, gene therapy, neurodegeneration

3:45 PM – 4:45 PM

555

REGIONAL HETEROGENEITY OF ACTIVATED NEURAL STEM CELLS IN THE SUBEPENDYMA OF THE ADULT FOREBRAIN IS INDEPENDENT OF THE NICHE

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The largest pool of adult mammalian neural stem cells (NSCs) is found in the subependyma lining the walls (medial; M, lateral; L, and dorsal; D walls) of the forebrain lateral ventricles. Each wall has a distinct cellular composition and contains NSCs. Heterogeneity within the NSC pool is also observed with NSCs in quiescent (qNSCs) and activated (aNSCs) states. To date, the interplay between intrinsic and extrinsic factors that regulate these states has not been fully elucidated. Single-cell RNA sequencing (scRNAseq) has characterized NSCs and their niches, but no study thus far has provided a comparison between all three walls. Furthermore, functional assays that demonstrate regional heterogeneity of NSC behaviours and the relationship to their respective niches, are lacking. Here, we combine scRNAseq with in vitro neurosphere assays (that generate clonally derived colonies from aNSCs), to characterize intrinsic/extrinsic regulators of NSC heterogeneity. We found that the L wall generated the greatest number of neurospheres relative to the M and D walls. Co-culturing cells from each wall with niches from other walls did not influence neurosphere number, suggesting extrinsic factors were not able to override intrinsic neurosphere forming capacity. scRNAseq was conducted across the M, L, and D walls to assess regional heterogeneity of NSCs and their niches. The L wall was enriched for aNSCs – identified as high Egfr, Fabp7, Dll1 and Egr1 expressing cells. Conversely, the M and D walls were significantly enriched in Gfap, the most highly expressed gene in qNSCs (cells also expressing Clu, Id2, and Aldoc), suggesting regional differences in neurosphere formation may be due to differing qNSC/aNSC pool sizes. We used fluorescence-activated cell (FAC) sorting to demonstrate enrichment of EGFR-hi cells in the L wall, consistent with the presence of aNSCs. As predicted, EGFR-hi cells were the primary source of neurosphere forming cells when plated in the neurosphere assay, whereas FAC sorting for GFAP-hi expressing cells resulted in no neurosphere formation. Hence, aNSCs are enriched in the L wall, and the neurosphere forming capacity of aNSCs is not overcome by niche derived factors. Identifying regional heterogeneity of NSCs and their niches provides insight into the factors that regulate cell state.

Funding Source: Ontario Graduate Scholarship, Carlton and Marguerite Smith Medical Research Fellowship, James F. Crothers Family Fellowship in Peripheral Nerve Damage, Margaret & Howard Gamble Research Grant

Keywords: neural stem cell, scRNAseq, heterogeneity

3:45 PM – 4:45 PM

557

ARE RAT EMBRYONIC NEURAL STEM CELLS A GOOD CHOICE TO GENERATE A BRAIN ORGANOID IN VIVO?

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Neural stem cell (NSC) injection has been investigated as a therapy to repair brain tissue in neurological diseases. In temporal lobe epilepsy (TLE), intracerebral injection of GABAergic progenitors reduced seizure frequency with limited effects on cognitive impairments. To overcome these limitations, we investigated a stem cell therapy using cells able to develop in excitatory and inhibitory neurons and in glial cells, a condition necessary to reestablish excitatory/inhibitory balance and to improve cognition. We previously showed that embryonic NSCs extracted from the rodent hippocampal subgranular zone generate functional organoids in vitro. Here, we want to investigate the possibility to use these cells to generate a hippocampal organoid in vivo. We injected fluid pre-tissue to obtain a less invasive approach to generate in situ a brain organoid in rats. Rat NSCs were transplanted into the hippocampus, one of the highly damaged area in TLE. NSC viability and maturation were assess over time by immunofluorescence. To improve survival and maturation conditions in the pathological brain (the rat pilocarpine model of TLE), the bioactive polymer alginate was added to the pre-tissue injected in the epileptic rat brain. Our results showed poor cell survival rates and immature phenotype in both acute and chronic phase with no substantial differences between naïve and epileptic group injected with fluid pre-tissue in the ventral CA3 (vCA3). The bioactive polymer added to the pathological condition did not improve neither NSC viability nor development, exhibiting outcomes similar to those observed in the control group not receiving alginate. To assess the in vivo neurogenic potential of the NSC, naïve adult rats were transplanted with NSCs into the vCA3 and into the dorsal dentate gyrus (dDG), the area well known as a NSC niche and for neurogenesis. In this scenario, cell viability was slightly improved but still not sufficient to obtain a functional neuronal network within organoid cells. These data suggested that the NSC survival and differentiation in vivo was limited both in physiological and pathological conditions. Taken together our results highlight the limitation of using embryonic NSCs for the in

vivo generation of organoids, considering the difficulties to obtain an adequate number of alive mature neurons.

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Keywords: temporal lobe epilepsy, subgranular zone-derived rat neural stem cells, in vivo organoids

3:45 PM – 4:45 PM

559

NEUROGENIC MATURATION OF NEURAL STEM CELLS DERIVED HUMAN STEM CELLS FROM APICAL PAPILLA UNDER 3D-NEUROSPHERE INDUCTION USING CHICK EMBRYONIC DORSAL ROOT GANGLIA-CONDITIONED MEDIUM

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Stem cell-based therapy could emerge as a promising approach for neurodegenerative disease to regenerate functional neurons. Recently, the neural stem cells derived from human stem cells from apical pallia (NSCs-hSCAPs) were successfully induced under 3D neurosphere formation and exhibited the potential to differentiate into neuronal cells. Interestingly, the conditioned medium collected from embryonic chick dorsal root ganglia (DRG-CM) contained endogenous neurotrophic factors to promote neuronal differentiation. Taken together, this study aims to demonstrate the neurogenic maturation of NSCs-hSCAPs and highlight the potential of using DRG-CM to enhance neurogenic maturation. These findings demonstrated that NSCs-hSCAPs were induced under 3D-neurosphere formation, positively stained the Nissl body (the typical neuronal marker), and expressed the SOX2 and Nestin, representing the early neural stage. The dorsal root ganglia (DRG) were isolated from chick embryonic E9, consisting of the Cresyl violet positive cells, which revealed the Nissl substance, weakly expressed SOX2/Nestin, and intensely exhibited neuronal maturation-associated markers (B-III Tubulin, MAP2, and SV2A), respectively. Moreover, these cells presented the dynamic change of Ca2+ to indicate functional activity. Consequently, the DRG-CM, which might contain the secreted-endogenous neurotrophic factors, was collected to supplement the neurogenic maturation of the NSCs-hSCAPs. Significantly, the NSCs-hSCAPs were differentiated into neuronal cells, which exhibited neuronal processes with a round cell body. These neuronal cells were specifically expressed as neuronal maturation-associated markers. Moreover, the intracellular calcium oscillation demonstrated the functional activity of the maturated neuronal cells, which presented a higher intensity of Ca2+ than the hSCAPs. Interestingly, the DRG-CM can enhance neurogenic maturation, resulting in an increase of 45.59% in neuronal cells. Furthermore, each neurite and total neurite outgrowth had higher elongations than the control group. Finally, these findings suggested the potential of using NSCs-hSCAPs and DRG-CM to generate functional neuronal cells as the stem cell-based therapy for further transplantation in treating neurodegenerative disease.

Keywords: functional neurons, dorsal root ganglia, dental-derived stem cells

3:45 PM – 4:45 PM

561

HUMAN BRAIN ORGANOID MODEL FOR STUDYING RADIATION-INDUCED EFFECTS ON NEURAL STEM CELLS AND NEUROGENESIS

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The adult brain contains neural stem cells that generate new neurons throughout life in a process known as neurogenesis. Adult neurogenesis is critical for new learning and memory as well as mood control. Neural stem cells are sensitive to ionizing radiation that induces their apoptosis, increases oxidative stress levels, decreases proliferation, and ultimately, impairs neurogenesis and associated learning and memory. Preserving neural stem cells could help prevent these neurogenesis-associated pathologies following irradiation. We studied the effects of proton radiation on human brain organoids and evaluated potential therapeutics to rescue the decline in neural stem cells and neurogenesis. We exposed 25-30day-old organoids to a single dose of 0.5 Gy using a clinical proton beam. We then examined alterations in apoptosis, cell proliferation, organoid size, the transcriptome and the metabolome at different time points over 4 weeks post-irradiation. We identified an increase in apoptosis and decrease in cell proliferation that resulted in a reduced organoid size by day 10 after irradiation. We observed a significant increase in the expression of genes linked to the P53 signaling and apoptosis pathways as well as a decrease in the expression of genes linked to cell cycle, lipid synthesis, and oxidative phosphorylation. To rescue radiation-induced decline in neurogenesis, we targeted nuclear receptor NR2E1, which plays a critical role in regulating neural stem cell self-renewal and neurogenesis. We developed synthetic small molecules that mimic the action of the NR2E1 endogenous ligand, leading to increased neurogenesis. Pretreatment of organoids with the synthetic NR2E1 agonist did not appear to affect neural stem cell proliferation but led to an increased expression of DCX, a marker of immature neurons, 3 days post-irradiation, suggesting that the drug is effective at stimulating neurogenesis. This work demonstrates molecular and cellular impacts of proton irradiation on human brain organoids and opens a new avenue for potential rescue of neurogenesis following irradiation.

Funding Source: NNX16AO69A (RAD01013) COUNTERACTING SPACE RADIATION BY TARGETING NEUROGENESIS IN A HUMAN BRAIN ORGANOID MODEL

Keywords: brain organoid, radiation, neurogenesis

3:45 PM – 4:45 PM

563

WEIGHTED CO-EXPRESSION NETWORKS REVEAL GENE CLUSTERS WITH TIGHT INTERACTIONS DURING AXOLOTL REGENERATION PROCESS

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Recent advances in single-cell transcriptomics allow the discovery of cell-to-cell communication mechanisms during vertebrate brain regeneration. In this work, we found that co-expressed gene communities coordinate with those in other cell types, providing the basis for detailed studies of brain regeneration regulatory mechanisms. Ambystoma mexicanum, also known as axolotl, is a type of salamander with the uncommon ability to regenerate large portions of its body, including the adult brain. Through this animal model, we can understand brain regeneration with the potential to extract their regulation mechanisms for health applications. In previous studies, researchers removed the axolotl's telencephalon (the foremost part of the brain) and observed their regeneration with a single-cell spatial transcriptomic technique. This cutting-edge technique gives us information of the genes expressed for each cell of a specific sample with geographical localization data. By studying the genes expressed we can learn each cell identity (or type) and how they behave and interact with each other within the tissue. This level of molecular detail in space-time had never been done before. Their results included a big load of data and a few suggestions of gene interactions across cell types. In this work we present a novel approach to analyze all this data with hdWGCNA. This is a bioinformatic tool starts with co-expressed genes as modules in every cell type. For each module, we identify its summarized expressed genes or eigengenes. We then traced the eigengene network, finding those with highest functional relevance. Within this framework of cellular functions, we looked for cell-to-cell communication between similar modules from different cell types that are located nearby. Our findings indicate module interdependence, with changes in gene composition altering other modules. Furthermore, only a few modules are directly related to regeneration, suggesting that there are key regulators of the process with cell-to-cell communication and the relative presence of neighboring cell types are crucial for regeneration.

Keywords: brain regeneration, co-expression networks, cell-to-cell comunication

3:45 PM – 4:45 PM

565

INTEGRATION-DEPENDENT EFFECTS OF A PIGGYBAC DOXYCYCLINE-INDUCIBLE EXPRESSION VECTOR ON NEURAL STEM CELL PROLIFERATION AND DIFFERENTIATION

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To perform inducible gain-of-function approaches in neural stem cells (NSCs), we generated transgenic cell lines using NSC cultures derived from the adult mouse brain and the epB-puro-TT transposable vector. This vector contains two piggyBac terminal repeats flanking a cassette that drives constitutive expression of a puromycin-resistance gene and doxycycline-inducible expression of a gene of interest. Co-transfection with the piggyBac transposase leads to efficient genomic integration of the epB-puro-TT cassette; transgenic cells are then selectively expanded by culture in the presence of puromycin. Through this approach, we generated two transgenic NSC lines with inducible expression of the homeobox gene Dbx2, and two control lines expressing either a Dbx2 sequence bearing a nonsense mutation or the original epB-puro-TT cassette without Dbx2. These cell lines were cultured in media supporting NSC proliferation or differentiation, with or without doxycycline. Surprisingly, all the lines showed a strong inhibition of cell proliferation and neuronal differentiation upon doxycycline treatment; however, these effects were not observed in a transgenic NSC line obtained by low-efficiency integration of the epB-puro-TT vector without the transposase. Of note, most of the transposase-dependent integration sites of epB-puro-TT in NSCs were mapped within transcriptionally active genes, which showed significant transcriptional changes upon doxycycline treatment. Taken together, these results show that transposase-mediated integration of piggyBac expression vectors in NSCs can affect their proliferation and differentiation, and suggest that transcriptional alterations in the genes targeted by the integration may underlie these effects. The role of these genes in NSC regulation deserves further investigation.

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Keywords: neural stem cells, cell proliferation and differentiation, piggyBac transposable vectors

3:45 PM – 4:45 PM

TOPIC: NO TISSUE SPECIFICITY

567

P53 MEDIATES MECHANICAL ELIMINATION OF LOSER CELLS IN HUMAN EMBRYONIC STEM CELL COMPETITION

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Cell replacement therapies rely on the possibility to expand human stem cells in vitro, harnessing their ability to self-renew and to differentiate into a variety of cell types. However, it has been shown that human embryonic stem cells (hESCs) in culture acquire mutations in the tumour suppressor gene TP53 and display clonal dominance and expansion, posing a risk for their use in therapy. The mechanisms leading to this positive selection are unknown. We have previously shown that differential activity of p53 across cells can induce mechanical cell competition, whereby low-p53 cells eliminate high-p53 cells due to differential sensitivity to mechanical compaction and cellular crowding. We therefore set out to investigate if p53 activity can also lead to cell competition in hESCs and if this is modulated by mechanical inputs. Extended live cell imaging shows that TP53 mutant hESCs (hES-P53KO) eliminate hES-WT cells. Competition is effected via apoptosis induction, as inhibiting apoptosis significantly rescues hES-WT cells elimination. It further requires direct cell contact, as in co-cultures of hES-P53KO and hES-WT cells where contact is prevented, we do not observe cell competition. This indicates that exchange of soluble factors and metabolites is not sufficient to induce cell competition. To ask if hES-P53KO competition is mediated by mechanical interactions, we cultured cells on deformable membranes to measure their resistance to cell compaction. Interestingly, while hES-WT cells displayed apoptosis induction upon cell compaction, hES-P53KO cells were resistant to compaction-induced apoptosis. This suggests that hES-P53KO outcompete hES-WT mechanically, due to differential sensitivity to cell compaction. To further investigate the mechanism by which hES-P53KO cells induce elimination of hES-WT cells, we analysed the transcriptome of hES-WT cells undergoing competition with hES-P53KO cells and identified several genes and pathways differentially expressed in competing hES-WT cells vs naïve hES-WT cells. Their characterisation will help identify actionable targets to control p53 mediated cell competition, making cell replacement therapies safer and potentially preventing the expansion of p53 mutant stem cells in cancerous and pre-cancerous lesions.

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Keywords: ES cell-competition, mechanical compaction, apoptosis

3:45 PM – 4:45 PM

569

GLYCOENGINEERED BIO-CLICKABLE SPHEROIDAL UNITS FOR GENERATING HIERARCHICAL LIVING MATERIALS

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Spheroids have emerged as valuable platforms in disease modeling and bottom-up tissue engineering applications. Despite major advancements, the assembly and tunability of spheroids remains largely restricted by their random cell aggregation. Uncoupling cell density from the spheroids' assembly process can unlock a higher control in size and downstream processability. For this purpose, cell surface modification toolboxes are valuable to control cellular coupling and spheroids' formation in chemistry-controlled time scales, and compatible with biological processes. As such, we employed metabolic glycoengineering to install click-chemistry moieties in cells and combined them with a DBCO-modified extracellular matrix-based polymer, to rapidly create spherical living beads with tunable cell densities, and independent of cell number dimensions. Bio-clickable living units, formed through bioorthogonal connections, can be rapidly assembled and offered improved handleability, as well as heightened cellular viability. This innovative technology was successfully applied across different cellular units, including stem cells, endothelial and cancer cells, showcasing its applicability across diverse biological contexts. Conventional spheroids and beads across the different cell types were compared, revealing that while spheroids exhibit size dependency on cell number, our platform demonstrates rapid assembly and maintains a consistent size independent of cell number. The developed beads also displayed self-healing capabilities, allowing for the assembly of intricate multicellular constructs, opening new avenues for the development of complex tissue models. The fabricated constructs showed structural integrity and evolvability over extended culture periods, providing also a highly resilient and handleable platform. Beyond providing a faster, more efficient and controllable alternative to spheroid fabrication, this technology provides means to decouple the living units' dimensions from cell density, which is currently unattainable with conventional scaffold-free spheroid production methodologies. These units aim to unlock on-demand processing for hierarchic living materials, vital for tissue engineering, with reproducibility and controllable cell density.

Keywords: living materials, metabolic glycoengineering, click-chemistry

3:45 PM – 4:45 PM

571

A NOVEL BONE MARROW SINGLE CELL ATLAS OF MECHANOTRANSDUCTION IN MICROGRAVITY, NORMAL GRAVITY, EXERCISE, AND HINDLIMB UNLOADING FOR WT AND CDKN1A-NULL REGENERATIVE MICE

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Mechanical loading of adult stem cell progenitors is a key factor in modulating their proliferation, differentiation, and tissue regenerative potential, with loading generally promoting tissue formation and unloading mediating tissue loss. To address the role of mechanotransduction in maintaining stem cell-based tissue regenerative health, we generated a single cell transcriptomic atlas mapping responses of femur bone marrow mesenchymal and hematopoietic lineages to a range of altered mechanical loading conditions, both in WT B1629SF2/J mice as well as the p21/CDKN1A-null regenerative mice. We selected the femur marrow compartment as a model because of the diversity and high numbers of stem cell progenitor stages present, and because it undergoes static and cyclic hydrostatic pressure loading associated with weight-bearing and ambulation. Our study included normally loaded mice at 1g, unloading in microgravity during spaceflight as well as tail suspension hindlimb unloading, and voluntary running wheel exercise. Basal, one-year natural aging, and habitat controls were also conducted. Overall, the atlas encompasses 18 different experimental conditions with N=3 mice per condition and includes more than 500,000 single cell expressomes. Key specific findings include: increased mature reticulocyte populations in aging and unloading mice compared to active mice; greater hematopoietic and mesenchymal differentiating progenitors cluster identification in CDKN1A-null samples, especially the exercise model; distinct pseudotime cell trajectory shifts in the hematopoietic lineage for spaceflight and unloaded mice; and shifts in monocytic cell populations toward osteoclastic bone degenerative lineages in unloading and spaceflight samples. Overall mechanical loading shows increased marrow progenitor population differentiation while unloading is associated with increased CDKN1A expression and maintenance of marrow population stemness. Deletion of CDKN1A appears to remove a negative check on progenitor lineages commitment to differentiation. Finally, the Bone Marrow Mechanotransduction Single Cell Atlas will serve as a reference tool for studying marrow regenerative responses across a range of mechanical environments as they relate to CDKN1A status.

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Keywords: mechanotransduction, marrow, single-cell

3:45 PM – 4:45 PM

573

EXPOSURE OF STEM CELL POPULATIONS FROM TUMORS WITH MASS CYTOMETRY

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Therapy resistance, metastasis, and relapse represent major challenges in achieving a complete cure for cancer. These properties are attributed to cancer-associated stem cells (CSCs), which can employ developmental pathways for self-renewal and plasticity. However, detecting and targeting CSCs is immensely difficult, posing a significant obstacle in cancer treatment. Currently, there are no clinical tools to detect heterogeneous stem cell populations from patient tumor samples. To address this problem, we have developed an extensive Cytometry by Time of Flight (CyTOF) PLURIpanel. This technology utilizes lanthanide metal-tagged antibodies with mass cytometry, enabling simultaneous usage of a large marker panel—comprising 40-50 markers—ideal for detecting rare cells within heterogeneous mixtures. Our design, the PLURIpanel includes all the best-known pluripotency and CSC markers for which we have carefully selected and optimized antibodies. Preliminary data from the PLURIpanel is highly encouraging. For instance, we can accurately identify SSEA4+/SSEA5+/CD44+/CD133+ subpopulations within cancer cell lines like Caco-2, known for harboring tumor-initiating cancer stem cells. Notably, this subpopulation would evade detection in conventional CSC analyses relying solely on CD133+/CD44+ markers. Misdirected therapy causes unnecessary suffering for numerous patients, highlighting the critical importance of accurately assessing CSC presence and quantity within tumors. Our novel approach aims to develop a diagnostic tool capable of quantitatively detecting all CSC subtypes within heterogeneous tumors, independent of cancer type. With PLURIpanel, we strive to improve the information available for clinicians for successful treatment selection and prediction of treatment efficacy, metastasis capacity, and incidence of relapse.

Keywords: mass cytometry, pluripotency and CSC markers, diagnostic tool

3:45 PM – 4:45 PM

575

AMPHIREGULIN MEDIATES IMMUNE-SUPPRESSIVE FUNCTIONS IN AMNIOTIC CELLS/TISSUE VIA THE COX-2/PGE2/EP4 AXIS

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Inflammation plays a crucial role in initiating tendon regeneration by coordinating immune cells recruitment. Nevertheless, an immune imbalance often results in tissue fibrosis or chronic inflammation, compromising the recovery of tendon homeostasis after injury. Amniotic epithelial cells (AEC), collected from amniotic membranes (AM) at middle gestation, exhibit a remarkable tendon pro-regenerative influence, particularly in mitigating the early inflammatory response. However, their clinical application is limited due to a lack of knowledge of the underlying molecular mechanisms. This study aims to verify whether the interplay between the COX-2/PGE2/EP4 and the Amphiregulin (AREG)/EGFR axes plays a role in mediating the crosstalk among immune cells and AEC/AM, as observed for other stem and cancer cell models. Results reveal, for the first time, that AREG is constitutively secreted by AEC or AM, exerting a powerful immunosuppressive effect by inhibiting PBMCs-PHA activation (up to 90%) and the NFAT pathway in CD3/CD28-stimulated Jurkat cells (up to 60%). Furthermore, this protein proved to be a key soluble mediator for blunting acute inflammatory responses in vivo, experimentally induced in Tg(lyz:DsRED2) zebrafish larvae. Notably, AEC and AM release of AREG can be enhanced by external inputs such as pro-inflammatory (LPS) and stretching. Of note, these stimuli do not directly affect AREG synthesis. Instead, they firstly interfere with COX-2 expression and, in turn, via PGE2/EP4 signaling stimulate AREG secretion. These findings offer valuable insights for targeted cell-free therapeutic interventions, exploiting the paracrine influence of AEC/AM to enhance host-protective responses through inflammation control, thus promoting tissue regeneration.

Funding Source: This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 955685 (www.p4fit.eu).

Keywords: amniotic epithelial cells, amphiregulin, immunomodulation

3:45 PM – 4:45 PM

577

INSIGHTS FROM SPONGE SINGLE-CELL TRANSCRIPTOMICS INTO STEMNESS, TRANSDIFFERENTIATION AND INJURY RESPONSE

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Rapid and accurate activation of wound healing and regeneration in response to mechanical injury has obvious benefits for the survival and health of animals. Yet, while the majority of animals have the capacity to heal wounds, morphologically complex animals such as vertebrates and insects often lack the capacity to regenerate tissues and organs. In contrast, sponges have simple body plans and strong regenerative powers that are highlighted by quick tissue repair after injury and the ability to rebuild the body from dissociated cells. In many sponge species, regeneration involves archaeocytes, widely recognized as stem cells. Archaeocytes are not found in calcareous sponges yet, where choanocytes (sponge-specific cells characterized by the presence of flagellum and collar apparatus involved in filter-feeding) transdifferentiate in response to injury. Despite their apparently highly differentiated nature, choanocytes are thought to play the role of somatic stem cells and appear to be totipotent in calcareous sponges. The molecular and cellular mechanisms allowing choanocytes to switch between their filter-feeding and stem cell roles, linked to the high regenerative capacity of sponges, remain to be elucidated. To gain insight into these mechanisms, we have generated single-cell transcriptome data for Sycon capricorn, an Australian calcareous sponge species. Computational clustering of cells followed by in situ hybridization experiments with identified marker genes allowed the identification of cell types and cell states, such as choanocytes undergoing proliferation. As our dataset includes samples from immediately after dissociation up to 90 minutes after injury, we have been able to trace injury response at single-cell resolution. Strikingly, we observed loss of proliferation markers within an hour after injury, and concomitant emergence of novel cell states appearing to undergo transdifferentiation from choanocytes. The results corroborate the stemness of choanocytes and hint at their role in closing the wound during healing. With further analyses in progress, we expect that findings from this project will provide an in-depth understanding of tissue repair mechanisms in sponges and a foundation for regeneration studies in other more complex systems, including those with medical importance.

Funding Source: ANU Research School of Biology International Scholarship (406a/2016) ANU HDR Fee Remission Merit Scholarship ARC Centre of Excellence for Coral Reef Studies

Keywords: wound healing, simple body plan of sponge, cell transdifferentiation

3:45 PM – 4:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

579

INVESTIGATING THE INFLUENCE OF CALRETICULIN MUTATIONS ON MACROPHAGE EFFECTOR FUNCTION

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Myeloproliferative neoplasms are a group of disorders characterized by the overproduction of blood cells in the bone marrow. CALR mutations are one of the three main driver mutations found in MPNs, along with JAK2 and MPL mutations. These mutations in CALR are frequently observed in patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF), which are subtypes of MPNs. The presence of CALR mutations in HSCs contributes to the clonal expansion of abnormal hematopoietic cells, leading to the characteristic features of MPNs, such as increased production of platelets (thrombocythemia) or fibrosis of the bone marrow (myelofibrosis). While the primary impact of calreticulin (CALR) mutations in Myeloproliferative Neoplasms (MPNs) is on hematopoietic stem cells (HSCs) and the myeloid lineage, emerging evidence suggests that CALR mutations may also have indirect effects on macrophages. Our recent studies have challenged a conventional understanding by revealing macrophages as the primary source of CALR, which can label unwanted cells and facilitate their clearance. Despite its typical retention within cells, we find that CALR can be translocated and secreted by healthy donor-derived macrophages upon activation. We also find that secreted CALR levels correlate with efficient target cell clearance. Given that CALR mutations in MPN result in a frameshift and the creation of a new C-terminal peptide sequence, we hypothesized that programmed cell removal by macrophages may be affected. By introducing CALR mutations into macrophages, we observe reduced target cell clearance suggesting a potential alteration in macrophage function due to CALR mutations in MPNs. Our findings may provide additional insights towards understanding how CALR mutations at the HSC level affect downstream myeloid cells.

Keywords: myeloproliferative neoplasms, calreticulin, macrophages

TRACK: PLURIPOTENCY AND DEVELOPMENT (PD)

Session III: Even

4:45 PM – 5:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

102

EPIGENETIC LANDSCAPE INSTRUCTS PRECISE TRANSCRIPTION FACTOR REGULOME DURING EMBRYONIC LINEAGE SPECIFICATION

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The totipotent zygote undergoes continuous division followed by cell-fate decision and lineage-specific differentiation to generate multiple cell types in different organisms as well as in extraembryonic tissues. Early lineage specification events are generally believed to be regulated by specific transcription factors (TFs) and epigenetic information. Due to the technical restrictions of traditional ChIP-based methods, how transcription factors (TFs) regulate embryonic cell differentiation in vivo remains elusive, with their regulomes primarily inferred from cultured cells. These static 2D-cultured cell lines fail to faithfully mimic the highly dynamic epigenetic environment during early embryogenesis, therefore leading to inaccurate conclusions. Here, we investigated the TF regulomes during mouse embryonic lineage specification from blastocyst formation to gastrulation by an ultra-low-input CUT&RUN strategy. We deciphered the remarkable dynamics of TFAP2C binding in collaboration with highly adaptable lineage regulators such as NANOG and CDX2 and underscored the central role of such specific TF regulomes in assuring irreversible cell fate changes in both intraembryonic and extraembryonic lineages. In addition, the de novo formation of H3K9me3-dependent heterochromatin in the extraembryonic ectoderm during implantation serves as an effective repression mechanism to antagonize TFAP2C binding to crucial developmental genes, which generally display a unique super bivalent chromatin state in vivo and engage in pivotal developmental functions of the embryonic lineage. Consequently, the restricted TF binding to these primed genomic loci persistently forestalls their transcription in later gastrulation stages and thereby sustains precise extraembryonic lineage identity. In summary, our study not only revealed the highly adaptable TF regulomes during mammalian peri-implantation but also provided an unprecedented perspective of TF binding and epigenetic environment during early lineage specification.

Funding Source: This work was supported by the National Key R&D Program of China (2020YFA0509800 (R.G.), 2021YFA1100300 (J.C. and C.J.)), the National Natural Science Foundation of China (32270855 (R.G.), 32270856 (J.C.), 32100652 (G.Y.)).

Keywords: lineage specification, transcription factor, epigenetic landscape

4:45 PM – 5:45 PM

104

HIPSC-DERIVED IN VITRO MODEL OF HUMAN OOGENESIS TO STUDY (EPI)GENOME REGULATION AND CELL-CELL SIGNALING

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As part of the Dutch HipGametes consortium, we aim to recapitulate step-by-step in vitro gametogenesis through differentiation of hiPSCs into functional gametes, to unravel basic and human-specific aspects of this process. At present, the field is able to generate human primordial germ cell-like cells (hPGCLCs), which we aim to mature, using (combinations of) 2D/3D-culture systems as well as co-cultures with mouse fetal somatic gonadal cells. In silico analyses of published and own generated sequencing data, combined with whole genome gene and enhancer activity tracing technology, will help us to design more optimal in vitro differentiation conditions including growth factors and matrix components. Using this model, we can study for example X chromosome dynamics during oogenesis. Furthermore, we plan to use this model to study outstanding questions regarding human oocyte development and meiosis. To be able to compare the in vitro generated oocytes to in vivo oocytes, we are currently studying proteins involved in DNA recombination and chromosome pairing in human fetal oocytes, using the super-resolution microscopy technique dSTORM. Eventually, our in vitro tools, once validated, should facilitate functional analyses of key players in human oocyte meiotic prophase progression, as well as studying (epi)genome regulation and cell-cell signaling during this process.

Keywords: oogenesis, co-cultures, super-resolution microscopy

4:45 PM – 5:45 PM

106

TET ACTIVITY SAFEGUARDS PLURIPOTENCY THROUGH EMBRYONIC DORMANCY

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Dormancy is an essential biological process for the propagation of many life forms through generations and stressful conditions. Early embryos of many mammals are preservable for weeks to months within the uterus in a dormant state called diapause, which can be induced in vitro through mTOR inhibition. Cellular strategies that safeguard original cell identity within the silent genomic landscape of dormancy are not known. We show here that the protection of cis-regulatory elements from silencing is key to maintaining pluripotency in the dormant state. We reveal a TET - transcription factor (TF) axis, in which TET-mediated DNA demethylation and recruitment of methylation-sensitive TFs drive transcriptionally-inert chromatin adaptations during dormancy transition. Perturbation of TET activity compromises pluripotency and survival of embryos under dormancy, whereas its enhancement improves survival rates. Our results reveal an essential mechanism for propagating the cellular identity of dormant cells, with implications for regeneration and disease.

Keywords: dormancy, pluripotency, enhancer

4:45 PM – 5:45 PM

108

UNRAVELLING THE ROLE OF SWI/SNF-ATPASES IN PREIMPLANTATION HUMAN EMBRYOGENESIS

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Chromatin regulation is one of the key processes in cell biology and is paramount for cellular identity. However, despite major advances in our understanding of chromatin processes in mammalian development, our knowledge of the role of chromatin regulators in early human embryogenesis remains limited. One of the primary regulators of early embryogenesis in mammals is the chromatin-remodeling complex SWI/SNF. However, key differences exist between murine and human embryonic development, and the role of the SWI/SNF complex in early human embryonic cell fate decisions is currently still unknown. Here, we use human naïve pluripotent stem cells to investigate the role of the SWI/SNF complex in human pluripotency, trophectoderm specification, blastoid formation, and, embryonic development. First, we analysed the expression patterns of SWI/SNF-ATPases, SMARCA2/4, using immunofluorescence and existing transcriptome datasets. We found that SMARCA2/4 show unique developmental expression patterns. Next, we perturbed SMARCA2/4 in human blastoids and found that trophectoderm development is enhanced upon SMARCA2/4 depletion. Moreover, we will present our efforts to determine the effects of SMARCA2/4 depletion in trophectoderm differentiation and preimplantation embryos, and the mechanisms underlying cell fate specification. Collectively, these results suggest an important role of the SWI/SNF complex in the regulation of early cell fate decisions in early human embryogenesis.

Funding Source: Fonds Wetenschappelijk Onderzoek (FWO) PhD fellowship for fundamental research

Keywords: human blastoids, cell fate specification, chromatin remodelling

4:45 PM – 5:45 PM

112

HAPLOID-GENETIC SCREENING OF TROPHECTODERM SPECIFICATION IDENTIFIES DYRK1A AS A REPRESSOR OF TOTIPOTENT-LIKE STATUS

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Trophectoderm (TE) and the inner cell mass (ICM) are the first two lineages in murine embryogenesis and cannot naturally transit to each other. The barriers between them are unclear and fascinating. Embryonic stem cells (ESCs) and trophoblast stem cells (TSCs) retain the identities of ICM and TE, respectively, and thus are ideal platforms to investigate these lineages in vitro. Here, we develop a loss-of-function genetic screening in haploid ESCs and reveal many mutations involved in the conversion of TSCs. The disruption of either Catip or Dyrk1a (candidates) in ESCs facilitates the conversion of TSCs. According to transcriptome analysis, we find that the repression of Dyrk1a activates totipotency, which is a possible reason for TE specification. Dyrk1a-null ESCs can contribute to embryonic and extraembryonic tissues in chimeras and can efficiently form blastocyst-like structures, indicating their totipotent developmental abilities. These findings provide insights into the mechanisms underlying cell fate alternation in embryogenesis.

Keywords: haploid, genetic screening, totipotent

4:45 PM – 5:45 PM

114

NODAL SIGNALLING IS NOT REQUIRED TO MAINTAIN THE PLURIPOTENT EPIBLAST IN PRE-IMPLANTATION HUMAN DEVELOPMENT

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The human blastocyst contains the pluripotent epiblast, from which naïve human embryonic stem cells (hESCs) can be derived. In naïve hESCs, ACTIVIN/NODAL signalling maintains expression of the transcription factor NANOG and is required for their in vitro propagation. It is unknown whether this reflects a functional requirement for epiblast development in embryos. Here, we characterised NODAL signalling activity during pre-implantation human development. We showed that NANOG is an early molecular marker restricted to the nascent human pluripotent epiblast and was initiated prior to the onset of NODAL signalling. We further demonstrated that expression of pluripotency-associated transcription factors NANOG, SOX2, and KLF17 are maintained in the epiblast in the absence of NODAL signalling activity. Genome-wide transcriptional analysis showed that NODAL signalling inhibition impacted neither on NANOG transcription nor the wider pluripotency-associated gene regulatory network. These data suggest differences in the signalling requirements regulating pluripotency in the pre-implantation human epiblast compared to existing naïve hESCs culture.

Keywords: human blastocyst, NODAL signalling, pluripotency maintenance in vivo

4:45 PM – 5:45 PM

116

LONG NON-CODING RNAS IN ENDODERM DIFFERENTIATION OF PLURIPOTENT STEM CELLS

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Long non-coding RNAs (lncRNAs) have emerged as important regulators in a variety of biological processes, including embryonic development and cellular differentiation. Nevertheless, many lncRNAs have not yet been functionally characterized, especially at the initial steps of endoderm differentiation. Recent studies from our group have revealed that nearly 25% of transcripts expressed during endoderm differentiation of human embryonic stem cells (hESCs) are lncRNAs, although less than 0.5% have a function established in this germ layer. Thus, characterization of endoderm-specific lncRNAs is essential for understanding how endoderm differentiation is regulated, as well as for determination of lncRNAs crucial for this cell process. Therefore, this study aims to evaluate the role of lncRNAs during endoderm differentiation of pluripotent stem cells. For this purpose, knockdown by RNA interference of selected lncRNAs will be performed in human pluripotent stem cells (hiPSCs) undergoing endoderm differentiation. Four lncRNAs of interest (LINC01467, ENSG286468, MAGEB17-AS1 and LINC00295) were selected from RNA-sequencing data of polysome-bound and total RNA of hESCs differentiated to endoderm, following four criteria of analysis: upregulation (fold change: log2≥2; FRD≤0.01), high number of transcripts per million (>500 counts), expression restricted to endoderm and absence of function described in stem cells. hiPSCs submitted to endoderm differentiation exhibited an efficiency of differentiation >80% (D4), and validation of selected lncRNAs demonstrated an increase of approximately 100 times in relative expression of LINC01467 and ENSG286468 compared to undifferentiated cells (D0). Moreover, the expression pattern of both lncRNAs follows the progression of endoderm differentiation, indicating that their expression is tightly associated to endoderm induction. In addition, LINC01467 was shown to be expressed in the cytoplasm of endoderm cells and associated with light polysomes. hiPSCs cells exhibited high transfection efficiency with control small interference RNA (siRNA). Knockdown of LINC01467 is currently being performed, which will improve our understanding of gene expression regulation of lncRNAs during endoderm differentiation.

Funding Source: This work was supported by CNPq - National Center of Science and Technology for Regenerative Medicine/REGENERA grant number 465656/2014-5, CNPq PROEP/ICC grant number 442353/2019-7.

Keywords: endoderm differentiation, lncRNAs, human induced pluripotent stem cells

4:45 PM – 5:45 PM

118

POST-TRANSCRIPTIONAL GENE REGULATION BY CCR4-NOT STABILIZES THE PLURIPOTENT STATE AND SAFEGUARDS STEM CELL FATE

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The Ccr4-Not complex is the main deadenylase in eukaryotic cells, and it can regulate mRNA poly(A)-tail length to dictate mRNA stability. Ccr4-Not plays important roles in both physiological and pathological processes, as many subunits in the complex have been implicated in development and diseases. However, the underlying mechanism is not fully understood. To systematically dissect its function, we generated a series of mouse embryonic stem cell (ESC) lines with constitutive or conditional deletion of the core subunits in the complex. We examined the impact of individual or combinatorial deletion of Ccr4-Not components on ESC maintenance, integrity of the complex, mRNA poly(A)-tail length, synthesis, half-life, and steady-state level using cell biology, biochemical, genetic and genomic approaches. We found that disruption of the complex or the deanylase subunits results in ESC differentiation. Mechanistically, we show that Ccr4-Not disruption causes global increases in mRNA-tail length and half-life. In turn, the increased mRNA stability preferentially elevates the steady-state level of transcripts that are normally short-lived and lowly expressed, most of which are involved in differentiation and development. In contrast, the steady-state levels of transcripts that are long-lived or highly expressed, such as housekeeping or pluripotency genes, are largely dependent on

transcriptional output and are not significantly affected by changes in mRNA half-life. Thus, we conclude that Ccr4-Not-dependent mRNA poly(A)-regulation represses differentiation and developmental gene expression in ESCs at the post-transcriptional level, stabilizing and safeguarding the pluripotent state.Our work provides new insights to the role of individual components in the assembly and function of the Ccr4-Not complex. More importantly, it uncovers a novel and essential post-transcriptional mechanism that maintains the pluripotent state in ESCs. As Ccr4-Not plays critical roles in a large number of developmental processes, we propose that Ccr4-Not-mediated poly(A)-tail length regulation may serve as an integral component in cell fate determination during development and diseases.

Keywords: post-transcriptional regulation, stem cell fate, pluripotency

4:45 PM – 5:45 PM

120

INTERLEUKIN-7 PROMOTES THE STABLE ESTABLISHMENT OF EMBRYONIC STEM CELLS BY INCREASING THE PORCINE INNER CELL MASS

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Interleukin-7 (IL-7) is a crucial factor for cell development, proliferation, and survival. Although IL-7 plays a significant role in porcine oocyte maturation, its role in embryonic development is not yet fully understood. In this study, we investigated whether IL-7 supplementation affects the formation of inner cell mass (ICM) in blastocysts and, consequently, the efficiency of establishing porcine embryonic stem cells (pESCs). First, we treated IL-7 and fetal bovine serum (FBS) during in vitro culture (IVC) after parthenogenetic activation (PA) to investigate whether they were associated with porcine blastocyst formation and ICM enhancement. The FBS-treated groups showed a significantly higher ($p < 0.05$) proportion of hatched blastocysts compared to the control group. Specifically, the IL-7 + FBS group had significantly higher ($p < 0.05$) hatched blastocysts than the FBS group. Then, we demonstrated that treatment with IL-7 and FBS during IVC affected the ICM marker SOX2+ cells, ICM ratio, and pAKT expression. Finally, we evaluated the efficiency of pESC establishment using only hatched blastocysts from each group. On day 2 after seeding, there was no significant difference in attachment efficiency between the two groups. However, the proportion of blastocysts that formed colonies was significantly higher ($p < 0.05$) in the IL-7 + FBS-treated group compared to the FBS group. The levels of gene expression related to core pluripotency markers (OCT4, SOX2, and NANOG) were significantly upregulated ($p < 0.05$) in pESCs derived from blastocysts treated with IL-7. In conclusion, the results suggest that IL-7 supplementation enhances the ICM ratio during porcine embryonic development in vitro. This finding may help establish a stable core pluripotency of pESC.

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Keywords: Interleukin-7, inner cell mass, porcine embryonic stem cells

4:45 PM – 5:45 PM

122

UNRAVELING THE ROLE OF DNMT3L IN HUMAN NAÏVE PLURIPOTENT STEM CELLS

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During early human embryogenesis, the complex coordination of cellular differentiation and lineage specification is closely linked with various epigenetic processes, such as DNA methylation. DNMT3L, a non-catalytic member of the DNA methyltransferase family, facilitates the establishment of DNA methylation patterns during early embryogenesis and germ cell development by interacting with other members of the DNMT family, particularly DNMT3A and DNMT3B. While extensively studied in mice, the role of DNMT3L in early human development and in human naive pluripotency remains unknown. We show that DNMT3L is highly expressed in the human naïve epiblast and trophectoderm of human embryos with nuclear accumulation. This finding stands in contrast to previously published immunofluorescence data on human embryos. To determine the role of DNMT3L in naive human pluripotency, we generate DNMT3L knockout naive human pluripotent stem cell (hPSC) lines using CRISPR/Cas9. The global transcriptome of the DNMT3L knockout cells is similar to wild type naive human pluripotent stem cells. However, the expression of several key pluripotency genes is slightly decreased in DNMT3L knockout hPSCs. Our results suggest that DNMT3L is dispensable to maintain naive human pluripotency. Interestingly, our transposable element analyses revealed an increased expression of hominid-specific transposons upon DNMT3L knockout. Our data revealed the reduction in global DNA methylation in the DNMT3L knockout naive hPSCs compared to their wild-type counterparts. This finding highlights the differences in the role of DNMT3L between humans and mice. Furthermore, we also show that DNMT3L knockout facilitates naive hPSCs to trophectoderm differentiation, suggesting that DNMT3L potentially prevents differentiation of naive hPSCs into trophectoderm. In addition, blastoids generated from DNMT3L knockout cells exhibited smaller sizes and improper segregation of epiblast and trophectoderm. Taken together, our study shows that DNMT3L is required to maintain the DNA methylation in naive hPSCs, but is not required for self-renewal. In addition, DNMT3L knockout impairs transposon expression and the differentiation of naive hPSCs towards the trophectoderm lineage.

Funding Source: FWO, KU Leuven

Keywords: DNMT3L, DNA methylation, human naive pluripotent stem cells

4:45 PM – 5:45 PM

124

MODULATION OF HDAC ACTIVITY DIRECTLY REPROGRAMMES EMBRYONIC STEM CELL TO TROPHOBLAST STEM CELL

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Embryonic stem cells (ESCs) can differentiate into all cell types of each of the embryonic germ layers. ESCs can also generate totipotent 2C-like cells and trophectodermal cells. However, these latter transitions occur at low frequency due to epigenetic barriers, the nature of which is not fully understood. Here, we show that mouse ESCs treated with sodium butyrate (NaB), an HDAC inhibitor, have a greatly increased 2C-like cell population and can transdifferentiate into trophoblast stem cells (TSCs). Importantly, this ESC to TSC transition is a direct reprogramming event that does not require transition through a 2C-like state. Mechanistically, NaB inhibits Class I histone deacetylases activities in LSD1-HDAC1/2 corepressor complex, increases acetylation levels in the regulatory regions of both 2C and TSC specific genes and promotes their expression. Interestingly, NaB-treated cells acquire the capacity to generate blastocyst-like structures that can develop beyond the implantation stage invitro and form deciduae in vivo. These results uncover how epigenetics restrict the totipotent and trophectoderm fate in mouse ESCs.

Keywords: HDAC inhibitor, 2C-like cells, mouse trophoblast stem cells

4:45 PM – 5:45 PM

128

IDENTIFICATION OF NOVEL CANDIDATE REGULATORS OF HUMAN NAIVE PLURIPOTENCY BY MEANS OF A GENETIC SWITCH UTILIZING THE CHIMERIC RECEPTOR G-CSFR:GP130

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The conversion of human pluripotent stem cells (hPSCs) from the primed to the naive state is notably challenging as only a few cells in a population can successfully execute this transformation. Furthermore, the molecular mechanisms initiating naive reprogramming remain largely unclear. We devised a system utilizing hPSCs that express a genetic switch to induce the transition from primed to naïve state. This switch relies on the co-expression of a hormone-dependent STAT3-ERT2 and a chimeric GCSF:GP130 receptor, which combines the extracellular domain of GCSF-R with the cytoplasmic domain of the GP130 signal transducer. Initially, we created hPSCs that express both STAT3-ERT2 and either the wild type (WT) GCSF:GP130 receptor or its mutants, each unable to recruit and activate one of the downstream effectors of GP130: JAK, STAT3, SHP2, HCK, and YES, respectively. The cell line with the WT receptor could be reprogrammed to the naïve state in the presence of 4'OHT + GCSF. hPSCs expressing STAT3, SHP2, HCK and YES binding-deficient chimeric receptors retained their self-renewal capability in media supplemented with 4'OHT + GCSF, whereas hPSCs with a JAK binding-deficient receptor lost this ability and differentiated. A loss-of-function approach using shRNAs targeting each JAK family kinase confirmed JAK1's critical role in naive conversion. To identify early response genes activated at the beginning of reprogramming, we performed RNA-sequencing analysis on cells treated with 4'OHT + GCSF for 24 hours and identified differentially expressed genes (DEGs). Among these DEGs, 27 genes were also expressed in the ICM and epiblast of human blastocysts. RNA interference targeting each of these 27 genes pinpointed IFI16, IFITM1, IFITM3, SSP1, CD44, BHLHE40, PROM1, FABP5, VWA1 and SERPING1 as potential regulators of the primed-to-naïve transition. A loss-of-function approach using

CRISPR-OFF is being employed to explore the role of these genes in the primed-to-naïve state conversion.

Funding Source: This work was supported by the Fondation pour la Recherche Médicale (DEQ20170336757 to P.S.), and the LabEx DEVweCAN (ANR-10-LABX-0061 to P.S.). It has benefited from the facilities and expertise of the PrimaStem platform.

Keywords: primed to naive state conversion, chimeric receptor G-CSFR:GP130, novel regulators of human naive pluripotency

4:45 PM – 5:45 PM

130

GENERATION OF SYSTEMIC CHIMERAS WITH RABBIT INDUCED PLURIPOTENT STEM CELLS REPROGRAMMED WITH KLF2, ERAS AND PRMT6

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Embryo-derived and induced pluripotent stem cells (ESCs and iPSCs, respectively) exist in two different states, designated naive and primed. Naïve and primed states differ by the signalling pathways, transcription factors and epigenetic regulators that hold the cells in one of either state. In rabbits, ESCs and iPSCs only exist in the primed state of pluripotency.We developed a strategy that led to identify factors capable of reprogramming rabbit iPSCs to a naïve-like, embryo-colonization competent state. We conducted an unbiased screening of a cDNA library encoding a panel of 36 factors, including transcription factors, epigenetic regulators and signalling molecules associated with naïve-state pluripotency in rodents and primates. All the factors were randomly introduced into rabbit primed iPSCs by means of lentivector infection, followed by clonogenic growth in a FGF2/KOSR-deprived culture media containing LIF, activin A, PKC and WNT inhibitors. The three transgenes most frequently detected were PRMT6 that encodes an arginine methyl transferase, ERAS that encodes a GTPase, and KLF2 that encodes Krüppel-like factor 2. To investigate the transgene action, KLF2, ERAS and PRMT6 were overexpressed, either separately, in pairwise combinations, or all three together, in rabbit iPSC cells, either constitutively or with induction. KLF2 was necessary and sufficient to sustain self-renewal in a FGF2/KOSR-deprived culture regimen. The addition of both ERAS and PRMT6 led to the appearance of a subset of cells expressing the naïve state-specific marker CD75 at a high level. These CD75high cell population underwent epigenetic reconfiguration typical of naïve-state pluripotency including reactivation of the 2nd X-chromosome. Remarkably, the CD75high cells gained the ability to produce chimeric embryos and fetuses, showing a high contribution in all major organs. Our results describe for the first systemic chimeras in the rabbit species, similar to the chimeras produced in mice with ES cells.

Funding Source: Oryctocell ANR-18-CE13-023, CHROMNESS ANR-21-CE20-0018-01, DEQ20170336757, INGESTEM ANR-11-INBS0009, IHU-B CESAME ANR-10-IBHU-003, LabEx (REVIVE ANR-10-LABX-73, "DEVweCAN" ANR-10-LABX-0061, "CORTEX" ANR-11-LABX-0042)

Keywords: pluripotency, naïve-state, systemic chimera

4:45 PM – 5:45 PM

132

OPTIMIZED TESTICULAR ORGANOID WITH ADVANCED IN VITRO SPERMATOGENESIS IN MICE

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Spermatogenesis is a strictly ordered and highly complicated process. Primordial germ cell-like cells (PGCLCs) induced from pluripotent stem cells (PSCs) can generate functional gametes when transplanted into mouse. However, the transplantation strategy largely impedes the further clinical application of PGCLCs. Thus, achieving complete in vitro gametogenesis with functional sperms stands as a milestone in reproductive regeneration. Currently, the male mPGCLCs in the reconstituted testis (rTestis) can develop to the germline stem cells-like cells (GSCLCs) for further development. Yet little attention has been paid on the reconstitution and in vitro culture system. Here, by testing multiple cytokines, antioxidents and extracellular matrixes, we developed an optimized in vitro reconstitution and culture system. The IF and FACS results showed that our new system can support the in vitro cultured neonatal testis for longer period with a significantly higher haploid efficiency. Besides, the reconstituted neonatal testis can also produce sperms with the optimized system. Finally, we applied the system into rTestis composed of PGCLCs and the in vitro embryonic male gonadal somatic cells. The rTestis, although harbored some teratomas, can be cultured for more than 2 months with a relatively good situation, and the germ cells in there can develop up to spermatocytes showed by IF staining. Our work will provide an in vitro new model for studying mammalian meiosis and may pave the way for the complete in vitro spermatogenesis in the future with translational potential.

Keywords: testicular organoid, in vitro gametogenesis, reproductive regeneration

4:45 PM – 5:45 PM

134

ELUCIDATING THE ROLE OF DELETED IN LIVER CANCER 1 (DLC1) IN HUMAN NEURAL CREST DEVELOPMENT

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Neural crest cells (NCCs) are multipotent embryonic stem-like cells that originate from the neural plate border (NPB) along the neural axis and give rise to diverse derivatives. It is well-established that NC development involves a complex transcriptional network but our previous studies in chick embryos revealed an unprecedented role of RhoGAP Deleted in liver cancer 1 (DLC1) in cranial NC formation. To extend these findings to humans, we used human-induced pluripotent stem cells to investigate the role of DLC1 in NC development. Our results demonstrate that DLC1 isoform 2 (DLC1-i2) expression is initiated in NPB-like cells expressing NPB markers and is maintained in early NCCs while DLC1 isoform 1 (DLC1-i1) expression is initiated and continues alongside the expression of NC markers. Knockdown of both isoforms led to reduced expression levels of NPB markers and NC markers. In contrast, DLC1-i1 overexpression (OE) promoted the formation of NC-like cells, while DLC1-i2 OE maintained cells in an NPB state. These findings highlight the distinct functions of DLC1-i2 and DLC1-i1 in the process of NPB establishment and NC specification respectively. Our study indicates that human DLC1 functions similarly to its chick counterpart during early NC development, and the switching of DLC1 isoforms is crucial for the transition from NPB-like cells to committed NCCs. To investigate the underlying molecular mechanism of DLC1 in NC development, a mass spectrometry analysis identified IGF2BP2, which is known for its role in mRNA stabilization, as a potential co-factor of DLC1. Notably, while DLC1 knockdown did not affect the mRNA expression of IGF2BP2, it did lead to a reduction in FOXD3 mRNA expression and a decrease in crestosphere size. This aligns with the reported role of FOXD3 in regulating the self-renewal capacity of NC stem-like cells, as NCCs overexpressing FOXD3 produced larger crestospheres compared to the vehicle control group. Moreover, RNA immunoprecipitation (RIP)-qPCR demonstrated that DLC1 and IGF2BP2 protein bind to FOXD3 mRNA. Collectively, these findings suggest that DLC1 interacts with IGF2BP2 to stabilize FOXD3 mRNA, thereby playing a crucial role in regulating the self-renewal capacity of NC stem-cell cells.

Keywords: neural crest, deleted in liver cancer 1 (DLC1), neural plate border

4:45 PM – 5:45 PM

136

SINGLE-CELL ROADMAP REVEALING THE FORMATION OF THE SPERMATOGONIAL STEM CELL COMPARTMENT IN THE MARMOSET MONKEY

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The differentiation of human gonocytes into spermatogonial stem cells, which form the basis for spermatogenesis, occurs in synchrony with testicular somatic cell maturation. These processes remain poorly understood due to limited access to immature human testes. We address this knowledge gap by using the common marmoset monkey (Callithrix jacchus) as a model for human testicular development. We have analyzed the transcriptional profiles of ~26,000 neonatal, prepubertal, and adult germ and testicular somatic cells obtained by single cell RNA-sequencing. Additionally, findings were associated with functional histomorphometric analyses of testicular tissues obtained for monkey and human. Expression of PIWIL4 and NANOS3 represent relevant benchmarks of differentiation and were therefore used as markers to delineate the developmental track of spermatogonial stem cells. We found that in prepubertal tissues, a PIWIL4+ spermatogonial cluster is predated by two transcriptional substates, including NANOS3+ spermatogonia, connecting neonatal germ cells to the most undifferentiated adult spermatogonial stem cells. Comparative analysis between adult marmoset and human demonstrates that the spermatogonial compartment makeup is similar between the two species. With regard to the somatic cells, we found that neonatal Leydig cells already produce androgens, as demonstrated by expression of steroidogenic genes. However, the somatic environment first becomes responsive to androgens in prepubertal tissues, as exemplified by expression of the androgen receptor. In summary, we provide basic data on the synergistic somatic and germ

cell differentiation from neonatal to the prepubertal marmoset testis and provide the hitherto missing link in the differentiation trajectory of spermatogonial stem cells.

Funding Source: This work was supported by the Innovative Medizinische Forschung (IMF) granted by the Medical Faculty of the University Münster to N.N. and S.L. (LA112010).

Keywords: male germ cells, development, single cell RNA sequencing

4:45 PM – 5:45 PM

138

TROPHOBLAST STEM CELLS: DECODE THE BLUEPRINT OF EARLY PLACENTATION

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A specialized population of stem cells called trophoblasts organize and establish the placenta architecture to sustain a fetus during human pregnancy. Current human trophoblast stem cell (TSC) models produce impure trophoblast populations that cannot make all lineage subtypes. Overlap in the expression of TSC markers (i.e., GATA3, TP63, TFAP2C) across the trophoblast lineage makes identifying TSC insufficient. Therefore, we defined the novel molecular features of human TSC to inform our high-throughput bioengineering tool for generating TSC in vitro. First, we obtained publicly available single-cell transcriptomic sequencing data of three-dimensional human embryos from post-fertilization days six to fourteen to determine the hallmarks of early TSC. Second, we assessed biologically relevant stem cell pathways required for placental development to detect novel markers of TSC. Next, we tested our fluorescently activated cell sorting (FACS) strategy to isolate TSC from chemically induced naïve pluripotent stem cells and first-trimester human primary chorionic villous tissue samples. Our results show that FOXM1+AXIN2+ TSC arises on day seven of post-fertilization human embryos. We found a population of trophoblast progenitors not previously reported. FACS isolation experiments with induced TSC and primary tissue samples validate our list of novel TSC surface markers (i.e., GREM2, NRP1). In addition, we optimize a TSC maintenance media for this rare population of TSC. We provide the first biologically relevant developmental map of early trophoblasts utilizing stem cell biology approaches in the human placenta. Altogether, a reproducible method for making TSC in vitro is vital for uncovering mechanisms of placental dysfunction. This work lays the foundation for future therapies and diagnostic tools to improve Maternal-Fetal Medicine.

Keywords: trophoblasts, human placenta, organogenesis

4:45 PM – 5:45 PM

140

FUNCTIONAL INVESTIGATION OF HYPOBLAST LINEAGE SPECIFICATION AND DIFFERENTIATION

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Hypoblast development in the human embryo has remained enigmatic. The hypoblast is established at the late blastocyst stage, when the inner cell mass segregates into epiblast and hypoblast. Upon implantation, hypoblast rapidly diversifies into visceral endoderm, lining the cavity side of the embryonic epiblast, primary- and subsequently secondary yolk sac endoderm and, potentially, extraembryonic reticulum. All of these lineages are essential for embryo development. However, the signaling cues during human hypoblast lineage specification events remain elusive. Here we use human naïve pluripotent stem cells to model hypoblast lineage specification events in vitro. Signaling pathways are modulated and identities of differentiated cells are profiled using systematically identified lineage marker genes from embryo datasets. Candidate lineage specifier genes are ablated to examine their roles in lineage acquisition. This study investigates the signaling and genetic requirements in the specification of hypoblast lineages and aims to shed light on the key mechanisms in hypoblast development in the early embryo.

Keywords: hypoblast, extraembryonic endoderm, naïve pluripotent stem cells

4:45 PM – 5:45 PM

142

DIFFERENTIAL MORPHOGENIC INDUCTION FOLLOWED BY CONTROLLER AGGREGATION FOR ENHANCED HUMAN GASTRULOID FORMATION

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Stem cell-derived embryo models, including gastruloids, offer a versatile platform to investigate aspects of embryonic developmental stages and processes that are commonly in the the “black box” of embryo development. Until now, robust and reproducible protocols to produce human gastruloids have been lacking. Therefore, we developed robust protocols for anteriorized human gastruloids with the goal of reaching the stages where organ induction occurs. Using RUES2-GLR human embryonic stem cells containing markers for the three germ layers, we first focused on optimising the input conditions by carefully controlling growth and environmental cues. Using a semi-automated system combined with ML, we monitored how input conditions impact germ layer formation and gastruloid development. We then developed a ML

supervised differential aggregation platform (DAP) where batches of morphogen treated RUES2-GLR are combined to control the presence and ratio of germ layers in the developing gastruloids. Obtained gastruloids were monitored by confocal microscopy, RT-PCR and scRNAseq. This strategy led to significantly improved gastruloid development and forms the basis for a controlled extended gastruloid culture which is a prerequisite for reaching organogenesis.

Funding Source: Funded by European Union (Horizon-EIC-2021-PathfinderChallenges-01 101071203, SUMO)

Keywords: gastruloids, embryo development, embryonic pluripotent stem cells

4:45 PM – 5:45 PM

144

UNVEILING THE ROLE OF THY1 IN EPIDERMAL PATTERN FORMATION: A NOVEL INSIGHT INTO YAP-MEDIATED HAIR FOLLICLE DEVELOPMENT

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Understanding the intricate mechanisms governing epidermal pattern formation, particularly within ectodermal appendages like hair follicles, is a key pursuit in developmental biology. Reciprocal interactions between mesenchymal and epithelial cells regulate hair follicle growth and development through various signalling pathways, yet the underlying molecular pathways remain elusive. Our investigation focuses on Thy1, a cell surface protein tethered via a glycosylphosphatidylinositol (GPI) anchor, which plays a multifaceted role in cell-cell and cell-matrix adhesion. We found that Thy1 significantly influences hair follicle development through the YAP (Yes-associated protein) mediated signalling pathway. In-vivo deletion experiments revealed a marked increase in hair follicle numbers upon Thy1 deletion, attributable to the activation of the YAP/Integrin-β1/Src module. Additionally, employing ultrasound-guided lentiviral delivery and pharmacological inhibition, we elucidated the pronounced regulatory role of YAP in hair follicle morphogenesis, further delineating the Thy1-dependent phenotypes. Our findings underscore the pivotal role of YAP activity in regulating hair follicle development, shedding light on potential therapeutic implications for hair-related disorders and presenting exciting prospects in the realm of regenerative medicine applications.

Keywords: hair follicle morphogenesis, hair follicles developmental biology Thy1 yes-associated protein (YAP) signaling pathways, developmental biology hair follicle morphogenesis hair follicle YAP developmental biology hair follicle YAP

4:45 PM – 5:45 PM

148

ROLE OF NETRIN-1 SIGNALING IN HUMAN NAIVE PLURIPOTENCY

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A role for Netrin signaling in the control of human pluripotency has never been documented. Our analysis of published RNA sequencing data from human preimplantation embryos showed early expression of Netrin-1 (NTN1) and two of its receptors, NEOGENIN (NEO) and UNC5B. The inner cell mass and epiblast exhibited a salt-and-pepper pattern of expression, indicating that Netrin-1 signaling is implicated in regulating naïve pluripotency. To investigate the role of Netrin-1 signaling in naïve pluripotency further, we generated human pluripotent stem cell (hPSC) lines: one expressing green fluorescent protein (GFP) as a control, another overexpressing wild-type NTN1 (wtNTN1), and three additional lines expressing mutant NTN1 variants incapable of binding to either NEO, UNC5H, or both receptor families. We then conducted single-cell RNA sequencing on these cell lines under two distinct experimental conditions: i) in the naive state of pluripotency, using the PXGL protocol, and ii) during the exit from naive pluripotency by deprivation of growth factors. Our findings indicate that i) cells overexpressing wtNTN1 show higher levels of naive markers and lower expression of primed markers compared to control cells, and ii) after 48 hours without growth factors, cells expressing wtNTN1 maintain elevated levels of naive markers, along with reduced expression of primed and lineage markers. These results were confirmed using dox-inducible wild-type and mutant NTN1. These results imply that overexpression of NTN1 enhances naive pluripotency in human PSCs and delays their exit from this state. Intriguingly, human PSC lines expressing mutant forms of NTN1, which cannot bind to either the NEO, UNC5H, or both receptor families, displayed a transcriptomic profile similar to cells overexpressing wtNTN1. This indicates that neither receptor is crucial, suggesting the involvement of an unidentified third receptor in this process.

Keywords: Netrin-1, pluripotency, human

4:45 PM – 5:45 PM

150

DEFINING THE CONTRIBUTION OF BIOCHEMICAL AND GEOMETRICAL CUES DURING CELL FATE PATTERNING IN A MINIMAL MODEL OF HUMAN ANTERIOR PRIMITIVE STREAK FORMATION

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How biochemical signals and mechanical cues interplay to coordinate cell fate patterning during early development remains to be uncovered. Our lab has recently introduced a minimal in vitro system using hESC confined onto micropatterns to elicit the emergence of spatially organised anterior primitive streak cell fates. Using this system, we have identified the temporal signalling sequence involving WNT, NODAL and BMP which is crucial to define differentiation decisions. Importantly, we also found that colony size modulates cell fate proportions as well as their ability to undergo morphogenetic movements resembling embryonic axial elongation. Manipulating the curvature of colony boundaries shows that concave versus convex borders dictate the physical properties of epithelial layers. These properties, in turn dictate the positioning of the endodermal domain. Overall, our result provide novel insights into the role of tissue geometry and scale in defining the balanced proportion of tissue progenitors and their spatial destination during gastrulation.

Keywords: gastrulation, morphogenesis, geometry

4:45 PM – 5:45 PM

152

GENERATING A REPORTER FOR 2C-LIKE CELLS IN HUMAN PLURIPOTENT STEM CELLS TO INVESTIGATE THE INITIAL STAGES OF HUMAN DEVELOPMENT

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Transcriptomic analyses of naive human pluripotent stem cell cultures have unveiled the existence of rare cells resembling 8-cell stage embryos post-zygotic genome activation. Despite successful experimental enrichment and transcriptional identification of these 8-cell-like cells (8CLCs), challenges persist in their isolation and long-term culture. Notably, 8CLCs do not depict developmental stages preceding zygotic genome activation (ZGA). This study aimed to identify genetic elements marking cells resembling early human embryonic cells before major ZGA. Utilizing expression data from preimplantation human embryos, we identified transposable elements with higher expression in human 2-cell embryos compared to other developmental stages. Subsequently, fluorescent marker-containing reporter systems were generated based on these transposable elements and introduced into the CLYBL safe harbor region. To demonstrate their functionality, we experimentally activated these reporters by using a CRISPR/Cas9-based system. These novel reporter cell lines will provide a platform to investigate the presence of 2C-like cells within human pluripotent stem cell populations and may facilitate identification of factors that may induce human 2C-like cells.

Funding Source: This study was supported by TUBITAK project 121Z292

Keywords: 2-cell like cell, iPSC genetıc reporter

4:45 PM – 5:45 PM

154

MARMOSET AND HUMAN TROPHOBLAST STEM CELLS DIFFER IN SIGNALLING REQUIREMENTS AND RECAPITULATE TROPHOBLAST INVASION

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Defects in placental invasion have been linked to numerous pregnancy complications including preeclampsia. The diversity in implantation strategies amongst primates provides a promising avenue to examine early trophoblast invasion by contrasting trophoblast differentiation regulation across different species. For instance, embryos of the marmoset, a small New World monkey, implant superficially and exhibit delayed extravillous trophoblast formation compared to human. Here, we illuminated the pre- to postimplantation transition of the trophoblast lineage in the marmoset embryo in vivo. We differentiated marmoset naïve pluripotent stem cells (PSC) into trophoblast stem cells (TSC), which exhibited trophoblast-specific transcriptome and methylome, differentiation potential and long-term self-renewal. Notably, human TSC culture conditions failed to support marmoset TSC derivation and promoted an extraembryonic mesoderm fate. We show that combined MEK, TGFβ/NODAL and histone deacetylase inhibition stabilizes a periimplantation trophectoderm-like state in marmoset TSCs, which readily form polarized trophoblast spheroids in vitro. Both, marmoset and human TSCs readily acquire extravillous trophoblast-like identity in response to NRG1 and TGFβ/NODAL inhibition. However, in contrast

to marmoset, human naive PSCs gradually differentiate to extravillous trophoblast in marmoset TSCs culture medium, requiring WNT agonism to stabilize the cytotrophoblast state. Our work presents a paradigm to harness the evolutionary divergence in implantation strategies to elucidate human trophoblast development and invasion.

Keywords: trophoblast stem cells, invasive trophoblast non-human primate

4:45 PM – 5:45 PM

160

DMSO DERIVES TROPHECTODERM AND CLONAL BLASTOID AND REVEALS NOVEL PATHWAYS UNDERLYING HUMAN BLASTOCOEL FORMATION

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Human naïve pluripotent stem cells (nPSCs) can differentiate into extra-embryonic trophectoderm (TE), a critical step in the generation of the integrated embryo model termed blastoid. The current paradigm of blastoid generation necessitates the aggregation of many nPSCs treated with cocktails of inhibitors and growth factors, or genetic modifications to initiate TE differentiation. The presence of complex crosstalk among pathways and cellular heterogeneity in these models complicates mechanistic study and genetic screens. Here, we show that a single small molecule, dimethyl sulfoxide (DMSO), potently induces TE differentiation in PXGL, HENSM, and 8CLC nPSCs in basal medium without pharmacological and genetic perturbations. DMSO induces efficient blastoid generation, even in the absence of any cocktail in multiple nPSC lines. DMSO blastoids mimic pre- and post-implantation development, including the formation of proamniotic cavity. Mechanistic studies showed that PKC signaling and cell cycle regulation underlie effects of DMSO and revealed novel pathways required for human blastocoel formation. Lastly, DMSO enables single nPSC-derived clonal blastoids, which could facilitate genetic screens for mechanistic understanding of human embryogenesis.

Funding Source: This work was supported by the King Abdullah University of Science and Technology (KAUST) Office of Sponsored Research (OSR) under Award No. BAS/1/1080-01 (ML).

Keywords: blastoid, blastocoel, trophectoderm

4:45 PM – 5:45 PM

162

RETINOIC ACID REGULATES INTRACELLULAR K+ VIA RCAN2 AND KCNK5B TO CONTROL THE VERTEBRATE EMBRYONIC FIN/LIMB BUD PROGRAM USING CA2+-MEDIATED SIGNALING

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K+ channels regulate morphogens to scale adult vertebrate appendages, but little is known about what regulates the channels and how they control morphogen expression. Using the zebrafish pectoral fin bud as a model for early vertebrate fin/limb development, we found that K+ channels also scale this anatomical structure, and we determined how one K+-leak channel, Kcnk5b, integrates into its developmental program. From FLIM measurements of a FRET-based K+ sensor, we observed coordinated decreases in intracellular K+ levels during bud growth, and overexpression of K+-leak channels in vivo coordinately increased bud proportions. Retinoic acid, which can enhance fin/limb bud growth, decreased K+ in bud tissues and up-regulated regulator of calcineurin (rcan2). rcan2 overexpression increased bud growth and decreased K+, while CRISPR-Cas9 targeting of rcan2 decreased growth and increased K+. We observed similar results in the adult caudal fins. Moreover, CRISPR targeting of Kcnk5b revealed that Rcan2-mediated growth was dependent on the Kcnk5b. We also found that Kcnk5b enhanced depolarization in fin bud cells via Na+ channels and that this enhanced depolarization was required for Kcnk5b-enhanced growth. Lastly, Kcnk5b-induced shha transcription and bud growth required IP3R-mediated Ca2+ release and CaMKK activity. Thus, we provide a mechanism for how retinoic acid via rcan2 can regulate K+-channel activity to scale a vertebrate appendage via intercellular Ca2+ signaling.

Funding Source: This work was supported by National Natural Science Foundation of China grants (32070820 and 32270881), the Shanghai Science and Technology Commission (22ZR1441200)

Keywords: vertebrate bud development, intracellular K+, retinoic acid

4:45 PM – 5:45 PM

164

NETRIN1 AND ITS RECEPTORS AS NOVEL REGULATORS OF EPIBLAST MORPHOGENESIS

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Deciphering early embryogenesis is crucial for reproductive biology as it will enable to understand and harness the numerous factors underlying pregnancy. The implantation of the embryo is a determining moment in early embryonic development. The identity of the embryonic stem cells (mESCs) also evolves with the deconstruction of the pluripotency gene regulation network supporting the naïve pluripotent state and the acquisition of the lineage-primed formative state. This dynamic progression of pluripotency has been extensively explored in the past 30 years but the molecular mechanisms that dictate the morphogenesis of the blastocyst during implantation remain unclear. We aim at understanding how the epiblast, an unorganized cluster of pluripotent stem cells, converts to a polarized epithelium, a prerequisite for lumenogenesis of the pre-amniotic cavity. We previously unveiled Netrin1, and its receptors Neo1 and Unc5b, as crucial regulators of naïve pluripotency and somatic cell reprogramming. Despite this finding, the role of Netrin1 and its receptors in the embryo upon implantation is still unexplored. By combining the study of mESCs and 3D in vitro models of epiblast development, we found that while the loss of Netrin1 and its receptors did not impact the pluripotency transition of mESCs, it greatly impairs the formation of the pre-apical patch, the first step of lumen formation in 3D epiblast-like models. Interestingly, in the pre-apical patch and in the lumen of 3D epiblast-like structures, both Netrin1 receptors colocalize with E-cadherin, a major regulator of polarization and lumenogenesis in the epiblast upon implantation. Moreover, in Netrin1-KO, Neo1-KO and Unc5b-KO mESCs generated using CRISPR/Cas9, we observed default in E-cadherin expression in RNAseq, but also in its localization and stability with the Retention Using Selective Hooks (RUSH) Assay for Protein Vesicle Tracking. We are currently exploring the role of Netrin1/Neo1 /Unc5b in vivo using conditional knockout mouse embryos. Investigating the molecular mechanisms behind the potential regulation of E-cadherin by Netrin1 and its receptors in epiblast morphogenesis could lead to a greater understanding of this important step of early embryogenesis, considered the “black box” of early development.

Keywords: early embryo development naïve pluripotency primed pluripotency mouse embryonic stem cells, epiblast morphogenesis polarisation lumenogenesis epiblast-like 3D model, E-cadherin cell-cell junctions trafficking

4:45 PM – 5:45 PM

166

ZCCHC8 GOVERNS RETROTRANSPOSON L1 SILENCING DURING SPERMATOGENESIS

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Over-activation of transposon elements (TEs) was a potential risk for male reproduction. During spermatogenesis, transposon elements were strictly regulated by several mechanisms including piRNA-mediated DNA methylation, post-transcriptional cleavage and histone modifications. Here we report that zinc finger protein ZCCHC8, a core factor of the nuclear exosome targeting (NEXT) complex, who is responsible for the nuclear RNA surveillance, is required for TE silencing during spermatogenesis. Loss of ZCCHC8 results in delayed meiotic progress and reduced production of round sperms. We found transcripts of several young long-interspersed nuclear elements (L1), which were directly targeted by ZCCHC8, were upregulated in both spermatogonia stem cells (SSCs) and pachytene spermatocytes (PS) of Zcchc8 null testes. Interestingly, DNA methylation at these young L1 subfamilies, was gradually lost from SSC to PS cells in ZCCHC8 KO testes, together with up regulation of active histone modification H3K4me3, independent of piRNA pathway. Afterall, we demonstrate ZCCHC8, governs TE silencing through multiple regulating ways not only degrading L1 transcripts, but also affect the chromatin landscape and epigenetic modifications at specific young L1 genomic sites.

Keywords: NEXT complex, spermatogenesis, retrotransposon silencing

4:45 PM – 5:45 PM

168

IN VITRO ORGANOGENESIS OF CHEMICALLY INDUCED MOUSE STEM CELL-DERIVED EMBRYO MODEL WITHIN MICROWELLS

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The series of morphogenetic events governing mouse embryogenesis, spanning implantation to organogenesis, has been a captivating area of study to understand normal development and related disorders. Over the past decade, significant progress has been made in developing various integrated and non-integrated in vitro embryo models, faithfully replicating different stages and aspects of embryo development. These

models serve as tractable, comparable, and scalable tools to study self-organization, model developmental disorders, and test pharmaceutical drugs. Recently, we demonstrated the formation of a chemically-induced embryo model within microwells, mimicking epiblast (Epi) and extraembryonic endoderm (XEn) co-development, progressing to form a pro-amniotic cavity (PAC), termed XEn/EpiCs, resembling a partial E5.5 embryo. Here, we illustrate the developmental progression of this non-integrated XEn/EpiC model in serum-free medium to spontaneously undergo gastrulation. Continued culture resulted in pronounced complexity reminiscent of organogenesis, including a cluster of beating cardiomyocytes that express early heart field markers. Notably, the XEn compartment further differentiated into a fluid-filled sac enveloping the embryonic compartment, mimicking a yolk sac tissue. Later, the beating heart/yolk sac-like structures progressed to undergo in vitro vasculogenesis forming blood islands, comprising blood, endothelial cells and macrophages, identified using RNA sequencing, TEM and flow cytometry. Interestingly, the yolk sac-like compartment developed a CD31+ and VEGFRa+ vasculature network that passed distally through the yolk sac. Overall, in the absence of trophoblast signals and serum, these non-integrated structures recapitulated the developmental complexity of early organogenesis encased within a yolk sac-like vesicle. This study represents the first demonstration of a non-integrated embryo model that fully self-organizes through sequential morphogenetic steps from pre-implantation lineage specification leading to organogenesis. We further translate the entire development of this model within custom thermoformed microwells enabling the tracking of structures, lineage tracing, and high-throughput screening for developmental toxicity and disorders.

Keywords: stem cell-based embryo model, microwells, beating heart/yolk sac-like structures

4:45 PM – 5:45 PM

170

SINGLE-CELL TRACKING OF REPROGRAMMING CELLS INDUCED BY CELL FUSION

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Lineage-committed cells can be induced to reprogram to pluripotency albeit generally at a low frequency and taking days before showing signs of reprogramming. Several reports indicate that reprogramming processes are tightly linked to cell cycle progression, although the exact contribution of each cell cycle phase remains largely unknown. This is partly because monitoring cells for many rounds of the cell cycle after exposing them to reprogramming factors is technically challenging, especially without prior knowledge of which cells will undergo reprogramming. Therefore, we employed a cell fusion system, known to induce reprogramming of the somatic nuclei towards pluripotency in approximately 10% of the fused population within the first round of the cell cycle. By establishing a system to isolate fused cells, track, and evaluate reprogramming levels through the analysis of pluripotency-associated gene expression using single-molecule RNA-FISH, we cataloged the first few cycles of fused cells and linked this information to the gene-expression profiles. We found that the speed of progression through the first round of the cell cycle post-fusion already impacts the induction of pluripotency-associated genes in the somatic nuclei. Furthermore, we show that pluripotency-associated genes are expressed at different times relative to cell cycle stages. Our finding suggests that certain cell cycle phases may facilitate or hinder the activation of a subset of pluripotency-associated genes, implicating the importance of temporal coordination between these processes. These insights could have implications for optimizing reprogramming strategies.

Keywords: cell fusion, reprogramming, cell cycle

4:45 PM – 5:45 PM

172

PROFILING OF THE PROXIMAL INTERACTOME OF THE EPIGENETIC POLYCOMB REPRESSIVE COMPLEXES REVEALS WIDE FUNCTIONAL CROSSTALK IN MOUSE PLURIPOTENCY

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Tight control of gene expression, achieved through the concerted action of transcription factors and chromatin modifiers, is critical for embryonic development. Polycomb repressive complexes PRC1 and PRC2 are multiprotein epigenetic complexes that play essential roles in establishing and maintaining cell identity during embryogenesis by regulating gene expression through modification of histones. While PRC2 and PRC1 complex members have been extensively characterized, recent findings suggest that activity of these complexes can be modulated by proteins that bind PRC1 and PRC2 weakly, transiently or that only interact with these complexes through ancillary proteins. To identify such proteins, we profiled the proximal interactome (proxeome) of the PRC1 and PRC2 catalytic subunits RNF2 and EZH2, respectively, in mouse embryonic stem cells (mESCs) using CRISPaint for endogenously (mini)TurboID tagging. This revealed >100 proteins proximal to

PRC1 and PRC2 in mESCs, which mainly comprise transcription factors, transcriptional regulators and RNA binding proteins. Notably, the EZH2 proxeome included both PRC complexes, while the RNF2 proxeome only identified PRC1 subunits. Using small molecule inhibitors, we show that the proximity of some proteins, but not the PRC1 subunits, is dependent on the catalytic activity of PRC2. More than half of the PRC2 proximal proteins are shared with PRC1, revealing the molecular constitution of Polycomb chromatin domains. We identified several pluripotency-associated transcription factors as proximal to the PRC complexes, including NANOG, for which follow-up studies showed an intriguing interplay with PRC2. Finally, we compared the dynamic PRC2 and PRC1 proximal interactomes between ground-state mESCs, serum-cultured mESCs and embryoid bodies. We find a range of stage-specific interactors, which might be associated the redistribution of PRC1 and PRC2 as observed during early mouse embryogenesis. Altogether, our comprehensive analysis uncovers the dynamic PRC1 and PRC2 proxeome during development, and the interplay between both complexes. It showcases how epigenetic factors interact with key transcription factors at different developmental stages to establish and maintain cellular identity.

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Keywords: embryonic development, polycomb repressive complexes, proximity labeling proteomics

4:45 PM – 5:45 PM

174

NRF1 PROMOTES PRIMORDIAL GERM CELL DEVELOPMENT, PROLIFERATION, AND SURVIVAL

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Primordial germ cells (PGCs) are the germline precursors that give rise to oocytes and sperm, ensuring the continuation of life. While the PGC specification is extensively studied, it remains elusive how the PGC population is sustained and expanded after they migrate to embryonic gonads before birth. In this study, we demonstrate that NRF1, a known regulator for mitochondrial metabolism, plays critical roles in post-migrating PGC development. As a methylation-sensitive transcription factor, NRF1 protein level gradually increases in post-migrating, hypomethylated PGCs during embryonic development. Enforced expression of NRF1 actively drives PGC derivation from pluripotent stem cells. Using whole genome transcriptome profiling and ChIP-seq analyses, we further reveal that NRF1 directly regulates key signaling molecules in PGC formation, transcription factors in proliferation and cell cycle, and enzymes in mitochondrial metabolism. Importantly, conditional Nrf1 knockout from embryonic germ cells leads to impaired PGC proliferation and survival. Overall, our findings highlight an essential requirement of NRF1 in regulating a broad transcriptional network to support post-migrating PGC development both in vitro and in vivo.

Keywords: primordial germ cells, pluripotent stem cells, NRF1

4:45 PM – 5:45 PM

176

MODELING HUMAN X-CHROMOSOME INACTIVATION USING BLASTOIDS

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In Eutherian mammals, XX females inactivate one of their X chromosomes to achieve dosage compensation with XY males in a process called X-chromosome inactivation (XCI). Much of the work investigating XCI has been done in mice, however prior to implantation, the X chromosome status differs between species. Further study will help determine what mechanisms differ between species and the consequences of these differences on human fertility and health. As human XCI begins around implantation, it is difficult to study in vivo. A recently developed in vitro model of human blastocysts, known as blastoids, may allow us to study this challenging period of human development. Our aim was to characterize blastoid formation as a model of human XCI initiation, which could then be used to test the mechanisms of XCI in humans. Analyzing pre-existing blastoid scRNA-seq datasets, we found a lineage-specific decrease in X chromosome expression during blastoid development, matching what we see in human embryo datasets across pre- and post-implantation development. We are currently using immunofluorescence and RNA-FISH to investigate the allelic expression of XIST and other X-linked genes in human blastoids, in a lineage specific manner. XIST RNA is expressed biallelically in blastoids at a rate that is higher than that found in naive pluripotent stem cells, but still lower than that found in embryos. Additionally we see lineage specific differences in the rate of biallelic XIST expression, with epiblast having more biallelic cells than trophectoderm. We also used immunofluorescence to examine whether condensed foci for the heterochromatic marks H3K27me3 and H2AK119ub were found in blastoids. We found these heterochromatin marks to have more foci in epiblast than trophectoderm. Altogether, our data shows the potential of human blastoids to model human XCI, enabling the further investigation of this crucial time point during development and how it differs between the sexes and species.

Funding Source: Fonds Wetenschappelijk Onderzoek (FWO)

Keywords: X-chromosome inactivation, blastoids, epigenetics

4:45 PM – 5:45 PM

178

DOUBLE TROUBLE: MODELLING MONOCHORIONIC TWINNING IN HUMAN BLASTOIDS

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The birth of identical twins is an intriguing phenomenon that occurs across many mammalian species. While it is known that at least 1 in 250 livebirths in humans are monozygotic (i.e., identical) twins (the incidence varies depending on ethnicity and geographical location) and that 70-75% of these twins are monochorionic (sharing a placenta), next to nothing is known about the underlying causes and mechanisms of twinning. It is clear however that monochorionic twin pregnancies, which result from blastocysts with a dual inner cell mass, suffer from a higher risk of morbidity. Since twinning is a rare event and human blastocysts are scarce, investigating the causes of this morbidity is practically limited. In this study, we show the generation of monochorionic twins in human blastoids, integrated models of the blastocyst. Using thermoformed microwell platforms, we screened for conditions that increase the yield of twin blastoids to 16%. We confirmed twin blastoids contain two opposed epiblast-like clusters, each adjacent to a distinct polar TE-like region within the shared TE-like cyst, compared their morphology to that of their singleton counterparts and tracked the splitting of the initially single ICM-like cluster into two during cavitation through time-lapse imaging. Additionally, using an endometrium-on-chip we precisely measured their ability to mimic adhesion to the uterine wall as a functional feature and observed the persistence of two epiblast-like clusters in post-implantation culture. All in all, we demonstrate the utility of blastoids to investigate rare events during early embryogenesis and provide a model for monochorionic twinning that can be used to study how twins arise and what causes their increased risk for morbidity.

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Keywords: twin embryo models, blastoids, thermoformed microwell platforms

4:45 PM – 5:45 PM

180

HIGH EFFICIENT CYNOMOLGUS MONKEY EMBRYO MODEL OF GASTRULATION FROM NAIVE ES CELLS

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Our understanding of primate gastrulation is hindered by ethical constraints and limited access to in vivo embryos. Stem cell-based embryo models offer a promising avenue for investigating embryonic development in vitro. Non-human primates possess reproductive and embryonic developmental characteristics more similar to humans, making them ideal animal models for studying human reproductive and embryonic development. In our previous studies, we successfully constructed non-human primate blastocyst model also known as blastoid using cynomolgus macaque monkey embryonic stem cells, which possesses the developmental potential of triggering early pregnancy reactions in vivo and developing into gastrula-like structures in vitro with a very low probability. Here, we developed a 3D suspension culture system for prolonged in vitro cultivation of blastoids, markedly enhancing the efficiency of obtaining D17 gastrula-like structures to approximately 50%. This innovative system enables real-time monitoring of morphological changes during monkey blastoid development from D9 to D17, coupled with experiments such as morphological analysis, immunofluorescence staining, and single-cell sequencing, further confirming their morphological and transcriptomic similarity to corresponding developmental stages in vivo in primates. Our research provides an excellent research system for studying the process of embryonic development from blastocyst to gastrulation in primates in vitro, which allows for gene editing at the stem cell level, facilitating the study of relevant mechanisms, and providing a suitable in vitro research model for screening drug targets and treating developmental defects-related diseases.

Keywords: non human primate, embryonic stem cell, embryo model

4:45 PM – 5:45 PM

182

INTEGRIN SIGNALING IN PLURIPOTENT CELLS ACTS AS A GATEKEEPER OF MOUSE GERMLINE ENTRY

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Primordial germ cells (PGCs) are the precursors of gametes and the sole mechanism by which animals transmit genetic information across generations. In the mouse embryo, the transcriptional and epigenetic regulation of PGC specification has been extensively characterized. However, the initial event that triggers the soma-germline segregation remains poorly understood. Here, we uncover a critical role for the basement membrane in regulating germline entry. We show that PGCs arise in a region of the mouse embryo that lacks contact with the basement membrane, and the addition of an exogenous extracellular matrix inhibits both PGC and PGC-like cell (PGCLC) specification in mouse embryos and stem cell models, respectively. Mechanistically, we demonstrate that the engagement of β1 integrin with laminin blocks PGCLC specification by preventing the Wnt signaling-dependent downregulation of the PGC transcriptional repressor Otx2. In this way, the physical segregation of cells away from the basement membrane acts as a morphogenetic fate switch that controls the soma-germline bifurcation.

Keywords: primordial germ cells, mouse embryo, extracellular matrix

4:45 PM – 5:45 PM

184

POLYUNSATURATED FATTY ACIDS RELATED ENZYME INHIBITION IMPACT ON CELLS VIABILITY AND EARLY DIFFERENTIATION

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Metabolic switches are key for cellular development, aiding growth and repair through environmental responses. In early embryos, these switches influence cell fate, impacting signaling, gene regulation, and differentiation. Understanding the role of metabolism in determining cell fate is crucial for deciphering embryonic development and guiding it in vitro. Despite recognizing the critical role of metabolism in cellular differentiation, distinguishing its specific contributions to energy production versus differentiation remains challenging, particularly in complex embryonic systems. To address this, we utilize a 3D embryo-like model, the gastruloid, as a simplified yet effective platform for dissecting these metabolic influences on cell fate. Previous studies have shown that blocking enzymes associated with polyunsaturated fatty acids (PUFAs) impacts cells differently across various stages and developmental pathways. Our research indicates that targeting PUFA-related enzymes can steer cell differentiation within our model towards the mesoderm layer. Early inhibition, before differentiation begins, resulted in slowed growth but enhanced mesodermal differentiation, especially when Delta-6-desaturase (D6D) was inhibited, while endodermal differentiation was diminished. Inhibiting D6D after differentiation had commenced led to a boost in mesoderm differentiation, preceded by a temporary reduction in the mesodermal regulator Brachyury. Our study provides evidence that manipulating metabolism through PUFA-related enzyme inhibition can affect cell differentiation, offering a new angle for tissue engineering and potentially contributing to the development of treatments for conditions associated with differentiation disorders. This approach may also refine our ability to guide differentiation in stem cell research, improving the precision of in vitro developmental models.

Keywords: gastruloids, metabolism, early development

4:45 PM – 5:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

186

DECIPHERING THE HUMAN EMBRYONIC STEM CELL DERIVED HAEMATOPOIESIS

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Haematopoietic stem cells are specialized stem cells with long-term repopulating potential capable of generating all blood and immune cell types. However, attempts at generating HSCs from pluripotent stem cells using cytokines and small molecules has met with little success, creating a need to identify the populations produced in culture and decipher how they differ from their in vivo counterparts. Using single cell transcriptomics, we characterised the repertoire of cell identities produced over time during the differentiation of human embryonic stem cells towards the haematopoietic lineage, to provide an understanding of how they correlate or differ from in vivo populations. We captured transient haematogenic endothelium and multipotent progenitor populations in culture, whose emergence are associated with increased transcription of genes involved in mitochondrial activity. Comparative stage analysis of embryonic data revealed a key requirement of glycolysis for the formation of the endothelium, followed by a switch to oxidative phosphorylation, prior to the emergence of the embryonic haematopoietic populations. In contrast, energy utilization by glycolysis remained low at all time points during in vitro derived haematopoiesis. Our data highlight a key need to reproduce metabolic requirements in culture, to model events happening in vivo.

Keywords: In vitro derived haematopoiesis, single cell analysis, metabolism

4:45 PM – 5:45 PM

188

GENERATING MELANOMA ANTIGEN-SPECIFIC ADOPTIVE IMMUNE RESPONSES FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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T-cell based cancer immunotherapies have demonstrated remarkable success against various cancers including melanoma. Thus, generating experimentally effective antigen-specific cytotoxic T-cells offers considerable promise for cancer immunotherapy. However, selection of suitable target antigen and low differentiation efficiency of naïve T cells poses a major obstacle in obtaining sufficient numbers of functional cytotoxic T-cells. Here, we utilized human induced pluripotent stem cells (iPSCs) as a tool to model human hematopoietic development in vitro. We developed a two-step approach to generate antigen-specific T-lymphocytes through cytokine mediated differentiation of iPSCs followed by in vitro priming with iPSCs derived antigen presenting monocytes and, finally, maturation in a 3D co-culture system with the thymic organoids. The resulting T-cells showed potent cytotoxic effects against HLA-A-matched primary melanoma cells in vitro. Moreover, this T-cell mediated cytotoxicity was accompanied by elevated interferon gamma production, a characteristic feature of effector responses of activated T-cells against tumors. In future experiments, T cell receptor (TCR) sequence analysis of these selectively activated T-cells will provide the foundation for generating novel neoantigen specific T-cells for clinical applications. Overall, the system provides a platform that may prove useful in answering experimental questions regarding T-cell development and provides a translational approach in generating tumor associated antigen specific T cell therapy.

Funding Source: The work is supported by the NIH grants P01 CA114046-13, R01 CA258113 and R01 CA255295 to Dr. Meenhard Herlyn.

Keywords: neoantigen specific T-cells, iPSCs, melanoma

4:45 PM – 5:45 PM

190

SENSORY CONTROL OF BLOOD PROGENITOR DEVELOPMENT VIA REDOX REGULATION IN DROSOPHILA

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Common myeloid progenitors (CMPs) in mammals maintain elevated reactive oxygen species (ROS). While this is developmentally established and is necessary for their differentiation and function, the regulation of ROS is critical for their normal development. Drosophila blood cells much akin to the vertebrate myeloid cells, in the larvae, arise from a pool of undifferentiated blood progenitor cells. These cells maintain elevated ROS as this sensitizes these progenitor cells to differentiation cues. Any reduction in ROS levels leads to loss of progenitor differentiation while any ectopic production of ROS leads to loss of progenitor homeostasis. Thus, much like CMPs, redox homeostasis in Drosophila blood progenitor cells is central to normal blood development and function. However, mechanisms underlying redox homeostasis during blood progenitor development are not clear. We find that blood development in Drosophila is strikingly influenced by sensory cues of olfactory in origin. While our previous findings have alluded to the role of animal odor sensing in immune priming, here, we present the developmental role of olfaction in blood progenitor redox balance. Upon olfactory stimulation, neuronally derived systemic GABA is utilized by blood progenitor cells and metabolized. We find that GABA metabolism is linked to limiting pyruvate's entry into the TCA cycle and this restricts TCA-derived precocious ROS production. Our findings also highlight a second important function of the olfactory-derived GABA in promoting pyruvate cycling to generate serine and cysteine. This enables the blood progenitor cells with a capacity to generate glutathione, a potent antioxidant. Together, this dual-modality keeps ROS levels in the blood progenitor cells in check. Consequently, in animals with olfactory dysfunction, the low GABA leads to loss of ROS homeostasis. These findings link animal odor-sensing to systemic moderation of blood progenitor metabolism and redox balance, which is central to their homeostatic development.

Keywords: hematopoiesis, metabolism, olfaction

4:45 PM – 5:45 PM

192

MODULATION OF FGF PATHWAY SIGNALING AND VASCULAR DIFFERENTIATION USING DESIGNED OLIGOMERIC ASSEMBLIES

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Cells progress through a specific series of lineage decisions during development that are spatially and temporally controlled. How cells precisely modulate outside signals to control their fate is an unanswered question in developmental biology. Growth factors and cytokines signal by binding to their receptors and driving association of the intracellular tyrosine kinase domains, initiating downstream signaling cascades. In particular, the FGF pathway has been shown to play a major role in regulating cell fate and survival. However, the differential function of different FGFR splice variants in developmental processes is unknown. To enable systematic exploration of how FGFR valency and geometry affect signaling outcomes, we designed cyclic homo-oligomers using repeat protein building blocks that can be modularly extended, and incorporated a de novo designed fibroblast growth factor receptor c-splice variant (FGFR1/2c) binding module into these scaffolds. These synthetic ligands exhibited potent valency- and geometry-dependent Ca2+ release and MAPK pathway activation. We also showed that these synthetic agonists have a capacity to drive endothelial cell differentiation through an FGF-mimetic trajectory. Vasculopathies are a critical defect occurring in multiple diseases. Using our designed agonists, we revealed distinct roles for two FGFR splice variants during early vascular development. The designed agonists, which activate signaling through the c-isoform of FGFRs 1/2, induce an endothelial cell fate, while using designed antagonists to restrict FGF signaling to the b-isoform induces a perivascular fate. Further in differentiation, the c-isoform-specific agonists promote the formation of arterial, rather than venous endothelium. Endothelial cells generated from human iPSCs through treatment with the designed FGFR1/2c agonists are functional and mature, and engraft to form vascular networks in mice. Thus, we show that designed FGFR1/2c agonists can be used to precisely reveal the function of FGFR variants in developmental processes. In general, designed RTK agonists can enable unraveling of the complexities of signaling in key developmental transitions, and their isoform-level specificity may permit enhanced tissue targeting in therapeutic applications of growth factors.

Keywords: de novo protein design, FGF signaling, vascular development

4:45 PM – 5:45 PM

194

STEPWISE TRANSCRIPTION FACTOR COOPERATION MEDIATED BY PARTNER SWITCHING IS REQUIRED FOR LINEAGE REPROGRAMMING

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Models of transcription factor (TF)-mediated reprogramming include pioneering, cooperative binding and sponge effects. However, the dynamic interplay and dependencies between TFs in reprogramming, particularly in direct conversions, remain largely unexplored. Dendritic cell (DC) reprogramming mediated by the cooperation of PU.1, IRF8 and BATF3 (PIB) provides a unique rapid, cell-cycle independent conversion system to address this question. We performed ChIP-seq in human dermal fibroblasts (HDFs) after individual, paired, or combined TF expression at the onset of reprogramming to understand binding co-dependencies. This revealed that IRF8 is dependent on PU.1 whereas BATF3 requires both factors for genomic engagement. Accordingly, PU.1:IRF8 determine initial genomic positioning, facilitate binding of BATF3 to sites marked by H3K27me3 and enable the establishment of cDC1-related promoter-associated chromatin loops. To track the dynamic changes in TF interdependency, we performed ChIP-seq on reprogrammed cells at days 6 and 9. Remarkably, this revealed a gradual shift in partner preference of IRF8 from PU.1 to BATF3. This switch was accompanied by loss of promiscuous binding by the pioneer factor PU.1 and an increase in on-target binding by IRF8:BATF3, exemplified by the engagement of XCR1 and the +49kb IRF8 super-enhancer. Pol-II ChIP-seq data confirmed this stepwise pattern, showing early disruption of fibroblast transcriptional network, rapid gene bursting mediated by PU.1, and subsequent elongation of DC-specific genes. The introduction of TF point mutations to disrupt pairwise interactions reduced the efficiency of reprogramming, highlighting the requirement for direct and stepwise cooperation. These findings support a cooperative model where TF partner switching plays a critical role for cell fate conversion. Ultimately, this molecular understanding may enhance the generation of DCs with improved efficiency and fidelity and offer new insights into the ontogeny of human DCs.

Keywords: transcription factor cooperation, lineage reprogramming, dendritic cell

4:45 PM – 5:45 PM

196

FEEDER-FREE 3D CULTURING FOR THE DIFFERENTIATION OF MINI YOLK SACS AND RED BLOOD CELLS FROM INDUCED PLURIPOTENT STEM CELLS

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Blood transfusions play a crucial role in modern medicine, but donated blood comes with challenges such as donor shortage and risk of disease transmission. Therefore, the technology of producing red blood cells in vitro with the same quality and function as natural red blood cells (RBC) is an attractive concept and seems indispensable. Human induced pluripotent stem cells(hiPSCs) are emerging as an alternative to overcome donation-dependent transfusions because they have less limitations in cell supply and can differentiate into mature erythrocytes in vitro in a laboratory setting. However, the production and expansion of pluripotent hematopoietic stem/progenitor cells (HPSCs) for clinical application remains a challenge. In particular, the use of feeder cells, such as OP9 cells, to achieve high-efficiency RBC differentiation carries a risk of contamination by heterologous pathogens during culture or in medium, which consequently limits its clinical use. In this study, efficiency was directly compared and analyzed using two experimental methods in the process of producing RBC from hiPSC without stromal cells; dissociation and three-dimensional(3D) culture. Our results confirmed that 3D cultures had a relatively higher efficiency in differentiating RBCs compared to dissociation, and therefore, we established a relatively simple and highly efficient protocol for differentiating RBCs from hiPSCs without using stromal cells. Our findings suggest the possibility of artificial blood production for future clinical transfusions.

Keywords: red blood cell, erythropoiesis, induced pluripotent stem cell

4:45 PM – 5:45 PM

198

SELF-SUSTAINING AND MULTIPOTENT HEMATOPOIESIS IN FETAL LIVER ORGANOIDS GENERATED FROM HUMAN PLURIPOTENT STEM CELLS

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Long-term hematopoiesis requires a niche proficient in cell-cell cross-talk, cytokine and chemokine gradients, blood vessels, macrophages, stroma and epithelium. In human development, the fetal liver serves as a major hematopoietic niche, yet is inaccessible for functional interrogation. Here, we integrated multiple syngeneic trajectories, including hematopoietic and niche-constituting lineages, to establish Fetal Liver Organoids (FLOs) from induced and embryonic pluripotent stem cells. We evaluated FLOs for functional hallmarks of hematopoietic niches, including self-sustaining hematopoieis. After pre-differentiation for ten days, FLO lineages self-organized and gave rise to CFU-GEMM colony-producing hematopoietic stem and progenitor cells (HSPCs) that are CD34+ CD43+ CD45+ CD90low CD10- CD135low CD144+ CD38low CD7+. A RUNX1-reporter line confirmed functional hematopoiesis. In long-term-experiments, CD34+ CD45RA- CD133/2+ CD38low FLO-HSPCs were maintained without cell loss over 9 weeks. Multiplex immunofluorescence and nanobody-labeling detected an endothelial-reticular system with partial CD34+ signature, Nestin+

pericytes, anti-inflammatory macrophages, and CXCL12+ hepatobiliary progenitors and stroma. We observed FLO-derived CXCL12-mediated HSPC-chemoattraction–a niche hallmark. Highly phagocytic macrophages self-sufficiently differentiated via CSFR-signaling and patrolled the FLO-periphery. Erythroblastic islands formed spontaneously. Lineage depletion and longitudinal single-cell RNA-sequencing identified cooperation with EPO-producing hepatobiliary epithelium. As described in the human fetal liver atlas, FLOs harbored neutrophil-myeloid progenitors that–upon G-CSF exposure–could be differentiated into bona fide neutrophils. Co-culture of FLOs in a B-cell assay gave rise to B1-like cells indicating lymphoid potential. Upon transplantation into vascularized and non-vascularized organ locations in NSG mice, FLOs maintained their niche architecture for over six weeks independent of host vasculature. In sum, we establish functional hallmarks of a hematopoietic niche in FLOs, including self-sustaining hematopoiesis and multipotent potential.

Funding Source: Research funding through the Emmy Noether Program (Milad Rezvani) of the DFG/German Research Foundation supported this project.

Keywords: hematopoiesis, hematopoietic niche, liver organoids

4:45 PM – 5:45 PM

200

THYMIC EPITHELIAL CELL FATE AND POTENCY IN EARLY ORGANOGENESIS ASSESSED BY SINGLE CELL TRANSCRIPTIONAL AND FUNCTIONAL ANALYSIS

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The thymus is the site of T cell development and repertoire selection, linked processes mediated largely by the array of highly specialised thymic epithelial cells (TEC) within the thymic stroma. Two main thymic compartments, the cortex and medulla, each have different functions: the cortex mediates T cell lineage commitment and differentiation, while the medulla imposes central tolerance on the developing repertoire. During development, cortical (c) and medullary (m) TEC arise from the third pharyngeal pouch endoderm. Current models suggest that within the thymic primordium most TEC exist in a bipotent thymic epithelial progenitor cell (TEPC) state able to generate both cTEC and mTEC, at least until embryonic day 12.5 (E12.5) in the mouse. This view however, has recently been challenged. We thus set out to investigate the fate and potency of TEC in the early thymus. Using single cell (sc) RNAseq we identified a candidate mTEC progenitor population at E12.5, consistent with recent reports, and predicted gene regulatory networks within this population. Via lineage-tracing we then demonstrated this population is mTEC fate-restricted. Additionally, using a single cell assay of potency, we established that most E11.5 and E12.5 progenitor TEC are cTEC-fated and showed that overnight culture causes most if not all E12.5 cTEC-fated TEPC to acquire functional bipotency. Finally, via further scRNAseq, we provide a likely molecular mechanism for this changed differentiation potential. Collectively, our data overturn the widely held view that a bipotent TEPC predominates in the E12.5 thymus, showing instead that sublineage-primed progenitors are present from the earliest stages of thymus organogenesis but that these early fetal TEPC exhibit cell-fate plasticity in response to extrinsic factors. These findings have implications for thymus-related clinical research, in particular for research focussed on generating TEC from pluripotent stem cells.

Funding Source: The School of Biological Sciences, University of Edinburgh, the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement number 602587, Leukaemia Research Fund, and the Wellcome Trust.

Keywords: thymus, epithelial cells**,** cell fate, potency, single cell transcriptome

4:45 PM – 5:45 PM

202

DYNAMIC TRACKING OF HEMATOPOIETIC STEM CELLS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

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Hematopoietic stem cells (HSCs) are a category of cells capable of differentiating into all types of mature blood cells. HSC transplantation is utilized for the treatment of a variety of hematological disorders, offering extensive clinical applications and therapeutic value. However, the ex vivo cultivation and acquisition of HSCs remain challenging issues in hematology research. In this study, we aim to enrich hematopoietic stem cells derived from human pluripotent stem cells (PSCs) by visualizing and tracking the dynamic journey of specific genes unique to HSCs. Through comprehensive bioinformatics analysis of extensive databases, we have identified the following genes as significantly important in the expression profile of HSCs: HOXA9, HLF, TCF16, CD34, and CD82. These genes have been found to play crucial roles in the regulation of maintenance, self-renewal, and differentiation of HSCs. We incorporated a fluorescent reporter system into the target genes of PSCs using CRISPR-Cas9 technology, which allows us to track the development of HSCs induced from PSCs. Then, we utilized PSCs to form embryoid bodies (EBs) and subsequently induced these EBs to differentiate into HSCs. It was observed that, beginning on the fourth day of differentiation, the cells started expressing surface markers specific to hematopoietic stem cells, accompanied by the expression of DsRed fluorescent protein, which persisted for five days before gradually diminishing. Moreover, during this period, qPCR revealed that there was a significant upregulation in the expression of genes associated with the mesoderm and hematopoietic and blood systems. Starting from the seventh day of differentiation, vesicle formation was observed within the EBs, which may indicate the initial stages of tri-germ layer development. These cells exhibit the capacity to form clonal colonies of various blood cell types and maintain self-renewal ex vivo. Our research for the first time captured the emergence of HSCs visually and provided an opportunity for the enrichment of HSCs, thus offering tantalizing prospects for treating blood disorders more effectively than ever before. This research will have direct implications for exploring more deeply mechanisms of HSCs' fate, dynamic development as well as the niche.

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Keywords: hematopoietic stem cells**,** CRISPR-Cas9 and fluorescent reporter system, embryoid body differentiation

4:45 PM – 5:45 PM

204

BLOOD-GENERATING HEART-FORMING ORGANOIDS (BG-HFOS) RECAPITULATE CO-DEVELOPMENT OF THE HUMAN HEMATOPOIETIC SYSTEM AND THE EMBRYONIC HEART

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Deriving in vitro hematopoietic stem and progenitor cells (HSCs/HPCs) is fundamental for therapeutic purposes yet challenging since their niche-like induction and stabilization in a proper developmental context is poorly understood. This is due to the limited knowledge of mechanisms controlling these processes due to barriers for investigating human embryogenesis in vivo. A human in vitro approach properly resembling the cellular and molecular aspects of hematopoietic development with neighboring tissues is therefore of interest. Our recently established human pluripotent stem cell (hPSC)-derived heart-forming organoids (HFOs) represent a complex, well-patterned multi-tissue model recapitulating key aspects of the interconnected heart, vasculature and foregut development. By modulating HFO differentiation by the stage-specific supplementation of hemato-endothelial factors, we here report the generation of blood-generating-HFOs (BG-HFOs). While maintaining their heart anlagen, these organoids comprise specific, morphologically distinct endothelial subtypes including a mesenchyme-embedded endothelial layer giving rise to hematopoietic

cells putatively via endothelial-to-hematopoietic transition (EHT). Our novel in vitro model thus reflects central aspects of the intra-embryonic region that gives rise to hematopoiesis in vivo. Single-cell RNA sequencing of BG-HFOs revealed gene expression signatures resembling HPCs as well as hematopoietic derivatives including erythroid, megakaryocytic and myeloid cells. Functional assays revealed that BG-HFO-derived hemato-endothelial cells display erythroid, myeloid and lymphoid potential. Together, the data suggest that our model simultaneously presents aspects of both primitive and definitive hematopoiesis. The study reveals the first human model of self-organized, morphologically structured co-development of cardiac, endothelial and multipotent-hematopoietic tissues, providing a valid tool for pharmacological assessments, promoting research on hematopoiesis development in vitro, and overcoming limitations of such studies in the human embryo.

Keywords: hPSC-derived organoid, hematopoiesis, cardiogenesis

4:45 PM – 5:45 PM

206

METABOLIC REGULATION OF BLOOD PROGENITOR HOMEOSTASIS AND HETEROGENEITY BY TCA CYCLE IN THE DEVELOPMENT OF LYMPH GLAND IN DROSOPHILA LARVAE

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Immunity as well as metabolism are quite old and extensively focused and sort after fields, but their inter-dependency, as Immune- metabolism, is being advocated very recently. This crosstalk infers how under immune compromised state, metabolic shift occurs and metabolites (a-KG, Succinate, Fumarate, etc.) takes on the tasks of proliferation, differentiation and activation of the concerned immune progenitors/cells. Recent work from our lab also highlighted GABA (released from brain) can elicit distinct immune cell population, under wasp infestation, which is nowhere to be seen in homeostasis (Madhwal et. al., 2020. Through our work we would like to switch the conventional paradigm of looking at TCA cycle as just an “intermediary step in the glucose metabolism for energy production” to the “reservoir of cardinal immune metabolites”. Metabolites are well known to get exchanged between the cellular compartments, which brands them competent for being signalling molecules. And what could be the better place for studying metabolites than TCA cycle, which is source as well as sink for all the three macromolecules of life. Conventionally TCA cycle, as the name suggest, always considered to be a cycle, but is it actually a cycle or an important junction of various cycles, so as to facilitate the exchange as well as maintenance of constant concentration of each metabolite. If the later is true, then each of the metabolites generated here can be an independent signalling molecules and directs the heterogeneity of different system. Here with our thorough analysis of TCA cycle in Drosophila larval blood cells, we would like to shed some light onto the crosstalk between the intermediary metabolites, development of blood progenitors, and the heterogeneity produced within blood cells. We are exploring the role of energy macromolecules (amino acids, lipids and carbohydrates) at different point of time in development influencing the lymph gland and later contribute to the conventional TCA cycle, by merging into each other. We have shown temporally, how initially only CS to alpha-Kdh arm of TCA cycle comes together to maintain the size of lymph gland by promoting the progenitors’ proliferation, which is later joined by the remaining steps to complete the cycle to regulate the differentiation of the lymph gland.

Funding Source: 1) UGC-JRF 2) DBT/Wellcome Trust India Alliance

Keywords: metabolism, drosophila, blood progenitors

4:45 PM – 5:45 PM

208

UNVEILING THE ROLE OF N6-METHYLADENOSINE IN VASCULAR ORGANOIDS

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Conventionally classified as tissues, blood vessels exhibit organ-like characteristics and functions crucial to understanding various disease pathologies. This research compares two established vessel organoid differentiation protocols. Furthermore, it investigates the influence of N6-methyladenosine (m6A) modification on VO development, providing significant information on vascular biology and pathophysiology. Differentiated vascular organoids (VO) were comprehensively characterized through various assays that included evaluation of cell morphology, gene expression profiling, and functional analyses. We compared the pharmacological agents used in the two differentiation protocols to elucidate the influence of modification of N6-methyladenosine (m6A) modification on vascular development. We conducted an in-depth examination of the expression patterns of m6A-associated genes. In addition, we devised a convolutional neural network (CNN) model to predict the developmental trajectory of VOs, improving our understanding of their intricate maturation processes. Both experimental protocols successfully generated vascular organoids (VO) that exhibit the appropriate cellular distribution, gene expression profiles, and functional attributes. In particular, Protocol 2 demonstrated a significantly higher yield rate of well-developed VOs (70%) compared to Protocol 1 (20%). Our analysis revealed a down-regulation of the expression of the METTL3, N6-methyladenosine (m6A) writer, in Protocol 2, which consequently resulted in reduced m6A levels. The introduction of the METTL3 inhibitor STM2457 in Protocol 1 effectively improved the expression of vascular genes. Furthermore, our convolutional neural network (CNN) model exhibited a commendable precision of 77.81% in distinguishing between different stages of VO development. This investigation juxtaposed two methodologies for the differentiation of vascular organoids (VO), elucidating the suppressive regulatory function of N6-methyladenosine (m6A) in vascular development. Furthermore, we pioneered the development of a convolutional neural network (CNN) model for prognosticating VO differentiation trajectories, presenting a novel computational tool with potential applications in VO research and clinical contexts.

Funding Source: This research was supported by the Ministry of Science and Technology (MOST) Project (NSTC 112-2320-B-A49 -049 -MY2).

Keywords: vascular organoids, N6-methyladenosine (m6A) modification, convolutional neural network (CNN) model

4:45 PM – 5:45 PM

210

MOLECULAR CONTROLS OF T-LINEAGE DIFFERENTIATION AND THYMUS-ENGRAFTMENT FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Currently, the primary sources of T cells for cell-based immunotherapies are mature T cells from peripheral blood mononuclear cells (PBMCs). Mature T cells are expanded in vitro and lentivirally-armed with chimeric antigen receptor (CAR) or other effector genes. This strategy requires autologous T cells or MHC-paired donors to avoid transplantation rejection, which limits its application. Induced pluripotent stem cells (iPSCs), which can be functionally differentiated into mature T lineage cells in vitro, can potentially serve as an alternative T cell source for immunotherapy. However, engraftment of either iPSC-derived hematopoietic stem cells (HSCs) or, progenitor T cells (Pro-T) has not been achieved. Alternatively, Pro-T can be envisioned as offering a way to generate new T cells in the host after adoptive cell transplant. The strategy of transplanting iPSCs-derived Pro-T is grounded on their potential ability to engraft the host thymus and develop into mature T cells via TCRβ-, positive-, and negative-selection events, leading to self-tolerant and MHC-restricted T cells. To address this, we analyzed single-cell RNA sequencing (scRNAseq) data from iPSC-derived Pro-T cells, which showed these cells to be similar to embryonic stage thymocytes rather than adult thymus Pro-T cells, with a signature of high Lin28b gene expression. Lin28b is known to inhibit the let7 microRNA family and down-regulate Erg transcription. Erg can promote the transcription of Gata2 and Runx1 to induce definitive hematopoiesis. We used fetal thymic organ cultures (FTOC) and RNA sequence data to show that Lin28b knockout (KO) iPSC-derived Pro-T is more similar to cord blood-derived Pro-T and displayed thymic engraftment ability. By further exploring the Lin28b's function in human T cell development, the Lin28b KO cells could be used to enhance iPSC-derived Pro-T engraftment ability and support its clinical application.

Funding Source: Funding was provided by Grant 1158417 from the Cancer Research Society and the Leukemia & Lymphoma Society of Canada.

Keywords: iPSCs, progenitor T cells, iPSC-derived Pro-T engraftment

4:45 PM – 5:45 PM

TOPIC: KIDNEY

212

TRACING THE ORIGIN OF MESENCHYMAL AMNIOTIC FLUID STEM CELLS TO THE DEVELOPING HUMAN KIDNEY TUBULAR EPITHELIUM

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Amniotic fluid stem cells (AFSCs) are fetal mesenchymal stem cells (MSCs) isolated throughout the second and third trimesters of pregnancy. They have a widely acknowledged regenerative medicine potential, however a lack of consensus on AFSCs origin and identity hinders their clinical translation. In this work, we employed a combination of comparative transcriptomic techniques to determine the anatomical origin and underlying processes governing AFSC generation. Firstly, we isolated MSCs from an array of fetal human tissues exposed or contributing to the amniotic fluid (AF) during gestation (n=3, 14-24 PCW). Subsequently, we compared our MSC library with AFSC lines isolated across a wide gestational age range (13-35 PCW). Greatest

similarity was observed between the AFSCs and fetal kidney-derived MSCs, validated by AFSC expression of a number of renal markers. Spatial transcriptomics of human fetal kidneys (n=3, 20 PCW) indicated that AFSCs are transcriptionally close to the nephron epithelia, within the early renal tubule structures. To validate our model, we generated scRNAseq cell maps of uncultured mouse (7,004 cells, n=13 amnios) and human (91,402 cells post-filtering, n=20 samples, 13-32 PCW) AF. Interestingly, both species showed absence of AF mesenchymal cell clusters in vivo. PAX8+ renal epithelial cells with nephron progenitor characteristics were consistently present in the human AF. This indicates that fetal kidney epithelial cells delaminate into the AF through the fetal urine. When cultured, some undergo Epithelial-to-Mesenchymal Transition (EMT), ultimately giving rise to what is commonly referred to as AFSCs. This EMT model is supported by the co-expression of epithelial and mesenchymal markers in cultured AFSCs, alongside EMT-related genes. This work improved our understanding of how AFSCs originate from the fetal renal tubular epithelium, providing new insights on the developmental processes behind their generation.

Funding Source: MFMG: H2020 Marie Skłodowska-Curie Fellowship (843265, AmnioticID) and Kidney Research UK (Paed_ST_005_220221129). PDC: National Institute for Health Research (NIHR-RP-2014-04-046), H2020 (668294, INTENS) and NIHR GOSH BRC.

Keywords: amniotic fluid stem cells, pithelial to mesenchimal transition, mesenchymal stromal cells

4:45 PM – 5:45 PM

214

DECODING AND DRIVING THE DISTAL NEPHRON PROGRAM IN KIDNEY ORGANOIDS

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In the United States, 33% of diabetic patients develop diabetic nephropathy (DN); the leading cause of end-stage renal disease (ESRD). Patients with DN have dysregulated functions in the distal nephron, a segment of the nephron where specialized macula densa cells control homeostatic regulation of salt, blood pressure, and water. How and when these cells form during development and the mechanisms underpinning their maturation and function are unknown. Kidney organoids fail to form normal distal cell types, which impedes efforts to develop human models of distal tubulopathies and regenerative therapeutics for ESRD patients. Here, we perform cross-species comparative analyses to delineate how distal cells form in vivo and replicate this process in a new human organoid protocol. Human and mouse nephrogenesis follows a deeply conserved developmental program: single cell RNA-seq and single nucleus ATAC-seq data show NPCs differentiate into a HNF1B+/POU3F3+/TFAP2A+ domain that prefigures putative distal precursors along a conserved developmental program. These data elucidate the later emergence of a transcriptionally distinct population within this HNF1B+ domain that expresses the metalloprotease PAPPA2 and solute carrier SLC12A1 – these proteins are detected in putative precursors of the macula densa and cortical thick ascending limb. Our new kidney organoid model generates PAPPA2+/SLC12A1+ enriched tubules stably cultured to day 28 of differentiation. To generate and sustain PAPPA2+/SLC12A1+ enriched organoids we systematically decode the requirement of β-catenin signaling dosages and durations, in combination with modifying a series of developmental signaling pathways. Synergism and additive pathway relationships show distalization is dependent on defined transcriptional states driven by finely tuned signaling events that generate maturing distal cell types. This model serves as a platform for delineating the genetic origin of congenital distal nephron abnormalities and provides a means to study pathophysiological mechanisms driving diabetic nephropathy.

Keywords: kidney organoids, nephron development, distal nephron

4:45 PM – 5:45 PM

TOPIC: LIVER

216

DELETION OF KINESIN FAMILY MEMBER 3B (KIF3B) CAUSES ABNORMAL DEVELOPMENT OF CHOLANGIOCYTES

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Biliary Atresia (BA) is a poorly understood devastating biliary disease of newborns. We have identified deleterious loss of function mutations (LOF) in cilia-related genes including KIF3B in 31.5% non-syndromic BA patients. KIF3B encodes Kinesin-like protein KIF3B that is a subunit of the anterograde intraflagellar transport motor protein kinesin-II in cholangiocyte primary cilia. Cholangiocyte has a single primary cilia, which functions physiologically as cellular antennae to detect and transmit signals that influence cholangiocyte function/development. In this study, we generated KIF3B+/- & KIF3B-/- human iPSC cells and differentiated them into biliary organoids to investigate the impacts of the KIF3B LOF mutation in biliary development in BA. Single-cell-RNA-seq analysis and immuno-staining showed that KIF3B+/- and KIF3B-/- iPSCs are less capable in the differentiation of hepatoblast and cholangiocyte progenitors (CPs). Individual cell AUC revealed down-regulation of Wnt, Notch and TGF-beta pathway activity, and a defective cell-cell interaction mediated by TGAV and ITGB8 (integrin αvβ8) in the KIF3B+/- and KIF3B-/- CPs. Furthermore, KIF3B+/- & KIF3B-/- biliary organoids were few, tiny and with abnormal or no primary cilia. Bulk/single cell-RNA-seq and immunostaining analysis of biliary organoids revealed a shift from cholangiocyte to hepatocyte differentiation in KIF3B+/- & KIF3B-/- biliary organoids, which is a resemblance of cholagiocytic development defects in BA liver tisse-derived biliary organoids. Taken together, our data indicate that KIF3B plays a key role in ciliogenesis and cholangiocyte development. In conclusion, primary cilia function/development influence cholangiocyte development, suggesting that defective ciliogenesis may contribute to BA pathogenesis.

Funding Source: Theme-Based Research Scheme(T12-712/21-R) and HMRF 09201836

Keywords: KIF3B, primary cilia, Biliary Atresia

4:45 PM – 5:45 PM

218

MIMICKING LIVER ZONATION THROUGH OXYGEN MODULATION IN IPSC-HLC CULTURES

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Establishing liver zonation in hepatic cell cultures is crucial for developing accurate in vitro liver models with zone-specific functionalities. This advancement enhances the utility of these models in various applications such as drug screening and disease modeling, providing a more accurate representation of human liver physiology. In the human liver, hepatocytes are arranged in hexagonal units called hepatic lobules, each further divided into three metabolically separate zones. This zonation can be attributed to the presence of various gradients, including oxygen, during development. The periportal zone, rich in oxygen, focuses on gluconeogenesis, cholesterol synthesis, and secretion of certain plasma proteins such as albumin. The mid-lobular zone, experiencing intermediate oxygen levels, maintains homeostasis, while the pericentral zone, low in oxygen, favors detoxification, lipogenesis, and glycolysis. Our study focuses on differentiating induced pluripotent stem cells (iPSCs) to hepatocyte-like cells (HLCs) under varied oxygen concentrations (1%, 5%, 10%) to emulate these distinct hepatic zones. For this purpose, we use OxyGenieTM mini-incubators and 1-well culture chambers from BioGenium Microsystems, which are designed for physiological oxygen studies. Additionally, specialized cover lids are employed to establish oxygen gradients within individual chambers. We also incorporate ratiometric oxygen imaging during the cell culture period to quantitatively assess the oxygen microenvironment inside the culture chambers. Metabolic activity of the iPSC-HLCs is evaluated through various methods including measurement of cytochrome P450 (CYP) enzyme activity, albumin production and urea synthesis. Furthermore, we analyze the expression of key metabolic zonated genes and use immunocytochemistry to identify hepatic and hypoxic markers. Our initial results from immunofluorescent staining of iPSC-HLCs show notable variations in the expression of zonated hepatic markers, including albumin and alpha-1 antitrypsin, in response to different oxygen environments.

Keywords: iPSC-HLCs, liver zonation, oxygen modulation

4:45 PM – 5:45 PM

220

THE MECHANICAL MICROENVIRONMENT IN INTRAHEPATIC BILE DUCT DIFFERENTIATION AND MORPHOGENESIS

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Chronic diseases affecting cholangiocytes, the epithelial cells of the bile ducts, account for a third of adult liver transplantations, a need exacerbated by the shortage of healthy donor organs. Addressing this, our research explores the therapeutic potential of human induced pluripotent stem cell (hiPSC)-derived cholangiocyte-like cells. Although they hold significant potential, these cells often retain a fetal phenotype, highlighting a gap in understanding the developmental mechanisms necessary for producing fully functional adult cholangiocytes. This includes understanding how the mechanical microenvironment, particularly the composition and stiffness of the extracellular matrix, affects cholangiocyte development. To address this issue, our study introduces a novel approach for generating functional hiPSC-derived cholangiocyte organoids through employing a synthetic hydrogel system. This system permits the precise modulation of extracellular matrix characteristics, addressing the issue of cell detachment encountered with previous methodologies. Through employment of this methodology, we discovered that collagen I functionalization significantly enhances intrahepatic cholangiocyte differentiation independently of substrate stiffness. Specifically, a high concentration of collagen I was associated with increased expression of biliary markers (KRT7/SOX9) and improved duct morphogenesis, whilst simultaneously reducing hepatic markers (ALB/AFP). These findings suggest a new pathway by which collagen I influences intrahepatic bile duct differentiation and morphogenesis. By uncovering a novel mechanism through which the extracellular matrix influences cholangiocyte development, our work presents a scalable platform for advancing our understanding of bile duct development at a biophysical level. This will enable the future generation of mature hiPSC-derived cholangiocyte-like cells, thereby contributing to the field of regenerative medicine.

Funding Source: Engineering and Physical Sciences Research Council (EPSRC)

Keywords: hydrogels, biophysics, extracellular matrix

4:45 PM – 5:45 PM

222

P53-MEDIATED CELL COMPETITION IS A WIDESPREAD MECHANISM OF CELL SELECTION IN SEVERAL CELL TYPES DERIVED FROM HUMAN STEM CELLS

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TP53 mutations confer clonal dominance in several tissues contributing to tumorigenesis. However, the mechanisms of clonal dominance are poorly understood and it has traditionally been proposed that heightened resistance to apoptosis/cell cycle arrest can account for clonal advantage. Our lab has previously shown that in some mammalian cultured epithelial cells p53 mutant cells can induce apoptosis of wild-type cells causing their outcompetition. This provides an alternative/additional mechanism for the establishment of clonal selection. P53 mediated clonal dominance has also been observed in human embryonic stem cells (hESCs) and we have recently shown that this is also due to cell competition and that TP53 mutant hESCs induce death of wild-type hESCs. Here we exploited the use hESCs to investigate if p53-mediated cell competition is maintained during differentiation along several lineages. We find that p53-mediated cell competition is maintained in neural stem cells and in cells differentiated into definitive endoderm (DE). Furthermore, we observe significant cell competition when we differentiate DE cells into hepatic progenitor cells (HPCs). Competition persists as HPCs undergo further differentiation into hepatocytes, though the strength of cell competition in hepatocytes is reduced. In conclusion, we find that p53-mediated cell competition is a widespread mechanism of cell selection in tissues. The implications of these findings extend to cancer biology, providing insights into why p53 mutations confer a growth advantage in cancers across many different tissues. Understanding the mechanisms of p53 cell competition could identify targets to prevent tissue repopulation in cancers or enhance it in cell replacement therapies.

Funding Source: This work is supported by WellcomeTrust Senior Research Fellowship 224675/Z/21/Z and Cancer Research UK Programme Foundation Award (Grant C38607/A26831).

Keywords: cell competition, TP53 mutations, clonal dominance

4:45 PM – 5:45 PM

224

DEVELOPMENTAL ANALYSIS AIMING FOR THE FORMATION OF TISSUE-LIKE STRUCTURE IN HUMAN IPSC-DERIVED LIVER ORGANOID

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The ultimate goal of organoid technologies is the ex vivo generation of human organs for use as alternatives in organ transplant and for disease modeling. Human induced pluripotent stem cells (hiPSC)-derived organoid has tissue-like structures and exhibit various organ-specific functions through the multiple cell communications. However, their structure is immature and similar to organ bud. For example, hiPSC-liver organoid has no hepatic cord along the sinusoidal vascular structure. Here, we analyzed the formation of liver primitive sinusoidal vasculature utilizing 4D (3D and temporal) imaging aiming for the application to hiPSC-organoid. We have identified the two important points in the formation of blood vessels by wholemount imaging as well as by liver-specific visualization using Imaris. First, the liver is avascular at E9.25, and second, blood vessels in the liver arise from a portion of the heart (sinus venous) after E9.25. Interestingly, recent report also revealed that endocardial cells derived from the sinus venosus is the main origin of blood vessels in the liver by lineage tracing analysis. Our results and previous report indicated that sinus venous-derived endocardial cells are crucial for the formation of hepatic blood vessels. Based on the above developmental findings, we hypothesized that the replacement of conventional vascular endothelial cells (EC) with venous sinus endodermal cells (Ecard), which constitute hiPSC liver organoids, could induce the formation of sinusoidal structures. Then, we attempted to induce differentiation of hiPSC-derived cardiac mesoderm into EC lineage and found that EC lineage markers were expressed (called as hiPSC-Ecard). hiPSC-Ecard also expressed human endocardial cell markers. In summary, we have identified that the sinus venosus is important for the formation of vasculature in the liver from a developmental analysis, and have also succeeded in inducing the hiPSC-Ecard. It is believed that if we can induce the formation of hepatic cords and sinusoidal structures using hiPSC-Ecard, we can recapitulate the interaction between hepatocytes and sinusoidal endothelial cells in vivo and create high-functional organoids.

Funding Source: the Ministry of Education, Culture, Sports, Science, and Technology (MEXT; 19K18034, 22K16440)

Keywords: iPS cells, organoid, liver

4:45 PM – 5:45 PM

226

UFMYLATION SAFEGUARDS LIVER DEVELOPMENT VIA REGULATING RIBOSOME HOMEOSTASIS

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The liver is a vital organ involved in numerous physiological processes, and a complex network regulates liver development. UFMylation, a novel ubiquitin-like modification, has been associated with various biological processes. Recent studies in mouse models have shown that UFMylation is necessary for liver development. However, the specific pathophysiological functions and molecular mechanisms of UFMylation in the human liver remain unclear. Here, we generated human embryonic stem cell (hESC)-derived hepatocyte-like cells (HLCs) and liver-bud organoids (LBs) and observed a gradual increase in the major components of the UFM1 cascade during hepatocyte differentiation. Individual deletion of UBA5, UFC1, UFL1, or CDK5RAP3 resulted in the unsuccessful generation of HLCs. Furthermore, we found that the E3 ligase adaptor CDK5RAP3 played a critical role in the maturation of human LBs. Single-cell RNA-Seq analysis revealed that the deletion of CDK5RAP3 disrupted ribosome homeostasis during hepatocyte maturation. Moreover, we developed a new strategy to identify UFMylated substrates in the mouse liver using a specific antibody that recognizes the lysine-glycine-valine (KGV) peptide. The ribosomal protein RPL26 was confirmed as a target of UFMylation in the liver, and that CDK5RAP3 is specifically responsible for UFMylation at the K132 site of RPL26. The deficient UFMylation on K132 impaired the generation of hESC-derived HLCs and disrupted lipid metabolism in hepatocytes. Further investigations revealed that CDK5RAP3 is required for 60S maturation to promote ribosome biogenesis (RiBi), and RPL26 UFMylation regulated ribosome assembly in the liver. Collectively, our findings suggest that UFMylation-associated ribosome homeostasis safeguards liver development. CDK5RAP3, an E3 adaptor for ribosomal UFMylation, is a novel RiBi factor. Moreover, targeting RPL26 UFMylation may hold therapeutic potential for NAFLD.

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Keywords: human liver development, UFMylation, ribosome homeostasis

4:45 PM – 5:45 PM

228

HEPATIC PROGENITOR CELL CYCLE REGULATION REQUIRES UHRF1 DURING ZEBRAFISH DEVELOPMENT

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Epigenomic inheritance and cell cycle progression is coordinated during progenitor cell differentiation. We found that loss of the DNA methylation factor, Uhrf1, in zebrafish embryos results in decreased expression of hepatocyte identity genes and blocks cell cycle progression. We found that uhrf1 mutant zebrafish embryos paradoxically have activation of pro-proliferative genes and increased number of hepatocytes in S-phase, but the liver fails to grow. These mutants also have a decreased in the expression of genes specific to hepatocyte identity during liver development. We discovered that Cdkn2a/b downregulation and persistent Cdk4/6 activation as the mechanism underlying the cellular and developmental phenotypes of uhrf1 mutants. uhrf1 mutant hepatocytes have replication stress, DNA damage and Atr activation. Palbociclib treatment of uhrf1 mutants prevented aberrant S-phase entry, reduced DNA damage, increased liver size and embryo survival. Inhibiting Atr reduced DNA replication and increased liver size in uhrf1 mutants, suggesting that Atr activation leads to dormant origin firing and prevents hepatocyte proliferation. Mutation of the DNA methyltransferase 1 (dnmt1) phenocopies the developmental and gene expression phenotypes, and also upregulates Cdk4/6 suggesting DNA hypomethylation as a mechanism of persistent Cdk4/6 activation during organogenesis. We conclude that the developmental defects caused by DNA hypomethylation are attributed to persistent Cdk4/6 activation, DNA replication stress, dormant origin firing and cell cycle inhibition.

Funding Source: NYUAD Faculty Research Fund

Keywords: zebrafish, liver development, DNA methylation

4:45 PM – 5:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

230

ADVANCING CULTIVATED PORK: THE ROLE OF PORCINE INDUCED PLURIPOTENT STEM CELLS IN SUSTAINABLE MEAT PRODUCTION

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Cultivated meat production, an emerging field, aims to meet the escalating global meat demand while addressing environmental and ethical issues linked to traditional animal farming. Porcine induced pluripotent stem cells (PiPSCs) have surfaced as a promising cell source for in vitro pork production, offering a sustainable and potentially more ethical alternative to conventional methods. The quality of biomass for cultivated meat production is a critical determinant of the final product's sensory attributes, nutritional value, and environmental footprint. Key metrics for assessing biomass quality include cell type selection, media composition, scale up/expansion strategies, robust differentiation protocols (to generate muscle and fat), and environmental impact of the manufacturing process. At Roslin Technologies, we have successfully reprogrammed and banked well-characterized PiPSC lines to ensure they are a viable source for generating pork tissue in vitro. These PiPSCs exhibit pluripotency markers SSEA-1, Oct3/4, Nanog, and Sox2, and retain trilineage potential. They commit to the mesodermal lineage to generate CD73, CD44, and CD105 positive mesenchymal stem cells (MSCs). Furthermore, PiPSCs can differentiate into PPAR gamma+, FABP+, and CEBP alpha+ adipocytes. They also demonstrate the potential to generate myoblasts. With a doubling time of less than 24 hours, PiPSCs are highly proliferative in culture, providing an inexhaustible cell source for meat production. We have successfully adapted PiPSCs to 3D suspension culture in the presence of defined media, achieving densities >10E6/ml using a fed-batch stirred tank reactor (STR) system. In conclusion, these PiPSCs, fulfill many stringent criteria for generating biomass for cultivated meat production by sustaining pluripotency, differentiation potential, rapid proliferation, scalability, high bioconversion rate, under optimized conditions.

Keywords: porcine iPSCs, cultivated meat, cellular agriculture

4:45 PM – 5:45 PM

232

DEVELOPMENT OF IN VITRO CULTURE PLATFORM TO INVESTIGATE THE EXTRACELLULAR MATRIX REMODELING POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS

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The ability of human mesenchymal stem cells (MSCs) to remodel and degrade extracellular matrix (ECM) are crucial in regulating excessive ECM accumulation, holding significant implications for MSC-based therapies. However, in the industrial manufacturing of MSCs, various culture conditions lead to fluctuations in the expansion process, ultimately influencing the potential of MSCs due to cellular heterogeneity. Thus, an evaluation method to assess the ECM remodeling potential of cultured MSCs is urgently needed. In this study, we developed a simple in vitro culture platform to evaluate the ECM remodeling potential of long-term cultured MSCs, using high-density collagen coated surface. Amniotic tissue derived MSCs with various population doubling (PD) were cultured on conventional collagen coated surface (conventional CL) and high-density (1.05 mg/cm^2) collagen coated surface (high-density CL). To substantiate the cells' ability to degrade ECM, PD=0 cells cultured on these surfaces were stained for CHP, a probe for detecting degraded collagen, along with MMP-1, cell-secreted enzyme involved in collagen degradation. Cells cultured on high-density CL surface showed remarkable ability to degrade collagen fibrils by secreting MMP, whereas on conventional CL surface, it was nearly nonexistent, suggesting that high-density CL surface was able to evaluate cell's ECM remodeling potential, which could not be evaluated using conventional CL surface. Lastly, PD=0 and PD=13.07 cells were stained with γH2AX, a DNA damage marker and CHP to quantitatively assess the ECM remodeling potential. This evaluation was based on distinguishing healthy cells (γH2AX-negative) and damaged cells (γH2AX-positive) in the context of ECM remodeling. Further evaluation of ECM remodeling potential of MSCs using different PDs revealed that, frequency of γH2AX-/CHP+ cells at PD=0 was 4.9-fold increase compared to PD=13.07, in which frequency of γH2AX+/CHP+ demonstrated a comparable pattern. This result suggests a decrease in ECM remodeling potential with an increasing number of PDs. Therefore, the utilization of high-density CL surface provides a practical means for investigating the ECM remodeling capabilities of ex vivo-expanded MSCs in vitro, potentially serving as a predictive platform for in vivo ECM remodeling effect.

Funding Source: Project Focused on Establishment of QbD-based control strategy and advanced core ecosystem in cell manufacturing from the Japan Agency for Medical Research and Development (AMED) under Grant Number JP20be0704001.

Keywords: human mesenchymal stem cells, collagen-coated surface, extracellular matrix remodeling

4:45 PM – 5:45 PM

234

INFLUENCE OF HGF SIGNALING AND FIBROSIS IN MESCENHYMAL STEM CELLS

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The aim of this research is to investigate the relationship between HGF stimulation and fibrosis. HGF is a protein that binds to the hepatocyte growth factor receptor and regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. Hepatocyte growth factor is secreted by mesenchymal cells and acts as a multi-functional cytokine on cells of mainly epithelial origin. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in tissue regeneration. Stem cells from apical papilla (SCAP) and stem cells from Periodontal ligament stem cells (PDLSCs) were collected after routine extractions in the Dental faculty (Medical university – Sofia) with informed consent obtained from patients. The cells were then isolated and cultured. The SCAPs and PDLSCs were placed in a 6-well plate. Four wells were treated with standard medium for 14 days, while two wells were treated with a medium containing 10 ng/ml HGF. The cells were then tested with Sircol™ biocolor. The study found that after 10 days of exposure to HGF, the PDLSCs began to detach, which was considered as a sign of the cells becoming apoptotic. However, the SCAPs were not affected in the same way and continued to thrive. No significant change in collagen production was observed. The results of this research suggest that there may be a relationship between HGF stimulation and fibrosis, as PDLSCs exposed to HGF began to detach, indicating the possibility of apoptosis. However, further research is needed to fully understand the relationship between HGF stimulation and fibrosis, and the potential implications for tissue regeneration. The study also showed that SCAPs were not affected in the same way as PDLSCs and continue to thrive under HGF stimulation and no significant change in collagen production was observed

Funding Source: Medical University Sofia

Keywords: HGF, SCAP, fibrosis

4:45 PM – 5:45 PM

TOPIC: MUSCULOSKELETAL

236

DECODING THE EPIGENETIC DETERMINANTS OF HUMAN SKELETAL DEVELOPMENT USING AN IN VIVO ORGANOID SYSTEM

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Endochondral ossification (EO) is the developmental pathway ruling the formation and repair of most of the skeletal system. This process is led by mesenchymal stromal/stem cells (MSCs), predominantly derived from the mesoderm layer. Firstly, MSCs condensate and form a collagen template in an early chondrogenic step. In a later step this cartilage mold will remodel and calcified leading to a mature bone organ hosting a hematopoietic compartment. Within, MSCs persist as regulators of the hematopoietic activity and maintaining bone homeostasis. Animal models have allowed identifying some of the key regulators of the EO process, including IHH/WNT signaling initiating chondrogenesis. Nevertheless, in human the precise epigenetic landscape and transcriptional drivers of EO remain cryptic. To this end, we here propose to exploit a tissue engineering approach, whereby human bone marrow derived MSCs are primed in vitro to form cartilage templates. Following in vivo implantation, tissues further developed into mature humanized mini bones through recapitulation of the latest EO steps. Throughout this process, we performed confocal microscopy, single cell RNA + ATAC seq and micro computerized tomography to study the human MSC differentiation and associated tissue formation. By collecting implants (chondrogenically primed and unprimed) 3 days post implantations, for confocal microscopy and single cell RNA + ATAC seq, we observe successful chondrogenic priming as shown by the activation and translocation of Sox9 and gene expression pattern in the chondrogenic primed tissues. In contrast Sc ATAC seq showed much greater cluster overlap across conditions. This suggests that hBM-MSCs fate decision may not require a considerable remodeling of the chromatin landscape for activation of the EO process. Our data also confirmed the formation of mature human ossicles within 4-6 weeks in vivo. Overall, these results show that we can use tissue engineering to study the early chondrogenic steps of the EO process, as well as investigate the epigenetic determinants of said process. Future research will allow us to investigate MSC population specific responses to chondrogenic priming.

Funding Source: The project was supported by the Knut and Alice Wallenberg Foundation, the Medical Faculty at Lund University, Region Skåne (to PB), the European Research Council (ERC) (Starting grant hOssicle #948588 to PB)

Keywords: endochondral ossification, epigenetic regulation, single cell RNA + ATAC seq

4:45 PM – 5:45 PM

238

IMPAIRED OSTEOGENESIS IN HUMAN INDUCED PLURIPOTENT STEM CELLS WITH ACETALDEHYDE DEHYDROGENASE 2 MUTATIONS

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Acetaldehyde dehydrogenase 2 (ALDH2) is the second enzyme involved in the breakdown of acetaldehyde into acetic acid during the process of alcohol metabolism. Roughly 40% of East Asians carry one or two ALDH2*2 alleles, and the presence of ALDH2 genetic mutations in individuals may affect the bone remodeling cycle owing to accumulation of acetaldehyde in the body. In this study, we investigated the effects of ALDH2 mutations on bone remodeling. In this study, we examined the effects of ALDH2 polymorphisms on in vitro osteogensis using human induced pluripotent stem cells (hiPSCs). We differentiated wild-type (ALDH2*1/*1-) and ALDH2*1/*2-genotyped hiPSCs into osteoblasts (OBs) and confirmed their osteoblastic characteristics. Acetaldehyde was administered to confirm the impact caused by the mutation during OB differentiation. Calcium deposits formed during osteogenesis were significantly decreased in ALDH2*1/*2 OBs. The expression of osteogenic markers were also decreased in acetaldehyde-treated OBs differentiated from the ALDH2*1/*2 hiPSCs. Furthermore, the impact of ALDH2 polymorphism and acetaldehyde-induced stress on inflammatory factors such as 4HNE and TNFα was confirmed. Our findings suggest that individuals with ALDH2 deficiency may face challenges in acetaldehyde breakdown, rendering them susceptible to disturbances in normal bone remodeling therefore, caution should be exercised regarding alcohol consumption. In this proof-of-concept study, we were able to suggest these findings as a result of a disease-in-a-dish concept using hiPSCs derived from individuals bearing a certain mutation. This study also shows the potential of patient-derived hiPSCs for disease modeling with a specific condition.

Funding Source: This work was supported by the National Research Foundation of Korea grant funded by the Korea government (NRF-2020R1A2C3004123, NRF-2019R1A5A2027588, and NRF-2021R1C1C2004688) and the Catholic Institute of Cell Therapy in 2024.

Keywords: induced pluripotent stem cell, acetaldehyde dehydrogenase 2, osteoblast

4:45 PM – 5:45 PM

242

MAKING MUSCLE: GENERATION OF INDUCED MYOGENIC PROGENITORS FROM AVIAN GERM CELLS

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Intense selection for rapid muscle growth in chickens has led to the development of several myopathies, adversely impacting animal welfare and causing large economic losses. Despite their impact, the molecular mechanisms underlying these myopathies remain elusive. This is largely due to the lack of relevant disease models, reporter lines, and cell lines. While pluripotent stem cells hold promise for studying muscle development in chickens, challenges in manipulating embryonic stem cells and a lack of readily available induced pluripotent stem cell lines have impeded their application. Primordial germ cells (PGCs) represent an attractive alternative source of pluripotent cells in chickens as they readily form pluripotent embryonic germ cells in culture, are easily isolated from any breed, can self-renew, and have established protocols for genetic manipulation. Our study demonstrates that overexpression of the master regulator factor MyoD with concomitant exposure to small molecules effectively reprograms PGCs into induced muscle progenitor-like cells (iMPCs). These iMPCs express key muscle progenitor markers Myf5, Pax3, and Pax7 and readily engraft into existing myofibers upon transplantation in vivo. To refine our culture conditions and further investigate the Pax7+ progenitors, we generated a Pax7-mCherry reporter line and utilized scRNA-seq from a developing chicken limb to identify pathways critical for maintaining muscle progenitors in vivo. Using this combined approach, we optimized culture conditions for iMPC expansion and revealed that purified Pax7+ progenitors possess multipotency, capable of differentiating into both myotubes and adipocytes. This study thus establishes a robust approach for generating muscle progenitor-like cells from germ cells to investigate muscle development and myopathies. Intriguingly, our work suggests the existence of a common Pax7+ progenitor population capable of differentiating into both myoblasts and adipocytes in chickens. This shared lineage offers a potential explanation for the fat infiltration observed in certain muscle myopathies, warranting further exploration.

Funding Source: University of Edinburgh and Cobb-Vantress

Keywords: primordial germ cells, skeletal muscle, chicken

4:45 PM – 5:45 PM

TOPIC: NEURAL

244

PROGRAMMING AREAL IDENTITY OF HUMAN CORTICAL NEURONS

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The human cerebral cortex is composed of hundreds of functional areas treating various modalities of information. These areas are composed of various types of neurons whose cell bodies, projections and connections are arranged in six layers. Their composition and organization can vary greatly from area to area. Cerebral cortex lesions originating from strokes or epilepsy focus ablations are usually spatially restrained and affect one or a few neighboring areas. With such a diversity in the cortex architecture, one can easily see the hurdles of producing the right type(s) of neurons to repair each area adequately. During development, the different types and subtypes of excitatory neurons are generated in a time- and space-specific fashion across the cerebral cortex. To study and identify key intrinsic factors of identity specification, I am using cortical organoids derived from human embryonic stem cells. Those organoids partially recapitulate dorsal telencephalon development. We produced our first single cell RNA-seq dataset on cortical organoids, revealing their cells' transcriptomic profile is very similar to their in vivo counterpart. Moreover, it shows they are more akin to the occipital cerebral cortex, consistently with histological assays. I will carry out a CRISPR activation screen focused on candidate transcription factors selected from differential expression analysis of single cell RNA-seq public datasets comparing areas of the developing cortex. Moving toward the screen, I have designed and generated lentiviruses for the delivery of guide RNAs that can successfully activate the expression of SP8 in my organoids. This transcription factor is expressed during the early development of the frontal cerebral cortex. The initial focus on this gene will be useful as a first proof of concept before attempting to activate several candidate genes. In the end, the results of the larger screen could help to produce neurons with a tailored identity to repair specific cortical areas.

Funding Source: The research leading to these results has received funding from the EU (H2020 NSC-Reconstruct 874758).

Keywords: cortical arealization, human cortical organoids, CRISPR activation

4:45 PM – 5:45 PM

246

DISSECTING THE ENZYMATIC ACTIVITIES OF TET ENZYMES IN NEURAL LINEAGE SPECIFICATION

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The Ten-Eleven Translocation (Tet1/2/3) enzymes promote DNA demethylation and are highly expressed in neural stem cells (NSCs). They catalyze the stepwise oxidization of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). These bases promote passive DNA demethylation or are removed by thymine DNA glycosylases to promote active demethylation. However, it is unknown how these individual enzymatic activites of Tets contribute to gene expression in the neural lineage. We have introduced a recently identified mutation in the Tet genes that specifically abrogates Tet's ability to formylate and carboxylate. Using this system, we have found that formylation and carboxylation vs hydroxylation have distinct roles in the regulation of neural genes and neural differentiation programs. Our findings help define the roles of 5hmC, 5fC, and 5caC in neural lineage specification with implications in neurodevelopmental disorders where Tets are mutated or the levels of these bases are perturbed.

Keywords: neural stem cells, Tet enzymes, DNA hydroxylation

4:45 PM – 5:45 PM

248

SUBSET-SPECIFIC GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELL-DERIVED MIDBRAIN DOPAMINERGIC NEURONS

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The generation of midbrain dopaminergic (mDA) neurons from human induced pluripotent stem cells (hiPSCs) holds great promise for the cure of human diseases. Dysfunction or degeneration of these neurons impairs various physiological processes, including motor control, reward mechanisms, and cognitive function, and is, thus, implicated in the pathogenesis of several debilitating neurological and psychiatric disorders, such as Parkinson's disease (PD), schizophrenia, and addiction. PD, the second most common age-related neurodegenerative disease, is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) of the brain, leading to a dopamine deficiency in the forebrain and characteristic motor symptoms, such as tremor, rigidity and bradykinesia. The successful generation of this specific subpopulation of dopaminergic neurons is of great interest for PD modeling and the development of preventive or therapeutic approaches to this disease. To date, most existing differentiation protocols have focused on the differentiation of hiPSCs into mDA neurons, but have not addressed the targeted generation of a specific subtype of these neurons. In vivo studies, including our own previous work, have shown that modulation of the WNT/β-catenin signaling pathway is critical for the subset-specific differentiation of mDA progenitors into mDA neurons. Two of the

most widely accepted protocols for differentiating mDA from hiPSCs focus on the early activation of this pathway by the continuous addition of either a high or low concentration of the GSK3 inhibitor CHIR99021. A subsequent differentiation protocol has demonstrated that altering the CHIR99021 concentration over time, thereby robustly activating the WNT/β-catenin pathway, has the potential to generate clinical grade mDA progenitors. Another group demonstrated that inhibition of the same signaling pathway during mDA differentiation increases the proportion of SNc mDA neurons in the cultures. This work presents the results of a combination of WNT/β-catenin pathway activation and inhibition during hiPSC differentiation into mDA progenitors. This study may provide a new avenue to the subset-specific generation of mDA neurons, in particular SNc mDA neurons, in the context of PD modeling and treatment.

Keywords: midbrain dopaminergic neurons, Parkinson´s disease, neuronal differentiation

4:45 PM – 5:45 PM

250

FUNCTIONAL DISSECTION OF M6A WRITER METTL3 IN NEURAL ORGANOIDS DERIVED FROM HUMAN EMBYONIC STEM CELLS

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Human embryonic stem cells (hESCs) possess the capacity to differentiate into all the cells in human bodies theoretically. In vitro differentiation of hESCs to functional cells represents the promising strategy for replacing old, injoured, or dead cells for regenerative medicine. Understanding the molecular mechanisms governing stem cell differentiation is key to generating hESC-derived functional differentiated cells. Transcriptional and epigenetic regualtion has been largely investigated in hESC maintenance and differentiation, however, regulation at post-transcriptional level remains largely unexplored. In this study, we aimed to characterize how m6A-mediated post-transcriptional regulation is involved in hESC differentiation. We first determined the m6A profilings in hESCs and hESC-derived ectoderm, mesoderm, and endoderm cells, elucidating the m6A dynamics and potential regulatory roles mediated by m6A modification. Next, we knocked out METTL3, the key player of m6A methyltransferase complex (m6A writers), in hESCs and discovered that METTL3 is required for the development of neural organoids. We will perform single cell RNA-seq and generate knockout mutants of key downstream genes to mechanistically dissect how METTL3 and m6A-mediated post-transcriptional regulate neural differentiation using organoid system.

Keywords: m6A modification, neural organoid, embryonic stem cells

4:45 PM – 5:45 PM

252

GENERATION OF HUMAN CEREBRAL ORGANOIDS WITH A STRUCTURED OUTER SUBVETRICULAR ZONE

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Mammalian outer radial glia (oRG) emerge as cortical progenitor cells that directly support the development of an enlarged outer subventricular zone (oSVZ) and, in turn, the expansion of the neocortex. The in vitro generation of oRG is essential to model and investigate the underlying mechanisms of human neocortical development and expansion. By activating the STAT3 pathway using LIF, which is usually not released in guided differentiated cortical organoids, we developed a cerebral organoid differentiation method from human pluripotent stem cells (hPSCs) that leads to the recapitulation of the expansion of a progenitor pool into the oSVZ. The structured oSVZ is composed of progenitor cells expressing specific oRG markers such as GFAP, LIFR and HOPX, which closely matches human oRG in vivo. The identity of the LIF-induced oRG population was confirmed by immunofluorescence staining, scRNA-sequencing and RNAScope analyses. The incorporation of hPSC-derived LIF-producing pericytes in cerebral organoids recapitulates the effects of LIF treatment, which suggests that the cellular

complexity of the cortical microenvironment, including non-neural cell types, endorses the appearance of oRG and provides a platform to routinely study oRG in hPSC-derived brain organoids.

Keywords: brain organoids, outer radial glia (oRG), neurodevelopment

4:45 PM – 5:45 PM

254

EARLIEST NEUROEPITHELIAL PROGENITOR CELL LINES DERIVED FROM HUMAN EMBRYONIC BRAIN TISSUE

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Cell lines derived from early stages of embryogenesis have played a pivotal role in advancing our understanding of mammalian development. Human neural progenitor cell cultures reported thus far reflect advanced stages of neural development such as rosette-like or radial glial cells during or post-closure of the neural tube and fall short to maintain clonal self-renewal over extended periods of time. Here, we report the successful isolation, long-term culture and detailed characterization of the earliest tissue-derived human neural cell population to date, mirroring human neuroepithelium at five weeks of in vivo development. Embryonic neural stem/progenitor cells (eNSPCs) are characterized by a distinct naive, non-polarized, pre-rosette stage phenotype, virtually unlimited self-renewal, and a broad capacity to differentiate into many lineages of both the central and peripheral nervous system. Single-cell RNA sequencing analyses reveal that eNSPCs are a homogeneous population of multipotent stem cells, rather than a mixture of committed neural progenitors exhibiting distinctive expression of SOX1, PAX2, PAX5 and EN2. Our findings establish eNSPCs as the earliest stabilized population of human brain stem/progenitor cells, advancing our understanding of human neurodevelopment and providing a novel source for potential applications in regenerative medicine.

Keywords: neural stem and progenitor cells, human embryogenesis, neuroepithelium

4:45 PM – 5:45 PM

256

SINGLE-CELL EPIGENOMICS AND SPATIOTEMPORAL TRANSCRIPTOMICS REVEAL HUMAN CEREBELLAR DEVELOPMENT

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Human cerebellar development is orchestrated by molecular regulatory net- works to achieve cytoarchitecture and coordinate motor and cognitive func- tions. Here, we combined single-cell transcriptomics, spatial transcriptomics and single cell chromatin accessibility states to systematically depict an inte- grative spatiotemporal landscape of human fetal cerebellar development. We revealed that combinations of transcription factors and cis-regulatory ele- ments (CREs) play roles in governing progenitor differentiation and cell fate determination along trajectories in a hierarchical manner, providing a gene expression regulatory map of cell fate and spatial information for these cells. We also illustrated that granule cells located in different regions of the cere- bellar cortex showed distinct molecular signatures regulated by different signals during development. Finally, we mapped single-nucleotide poly- morphisms (SNPs) of disorders related to cerebellar dysfunction and dis- covered that several disorder-associated genes showed spatiotemporal and cell type-specific expression patterns only in humans, indicating the cellular basis and possible mechanisms of the pathogenesis of neuropsychiatric disorders.

Keywords: cerebellar cortex development, single cell sequencing, spatial transcriptomics

4:45 PM – 5:45 PM

258

THERAPEUTIC POTENTIAL OF HUMAN IPSC-DERIVED PERIPHERAL NEURON/NERVE PROGENITORS ON A PERIPHERAL NERVE INJURY MODEL

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Research on peripheral nerve regeneration is lagging behind that of the central nervous system, but there are many patients and much progress is desired. Recently, human iPSC/ASC-derived induced peripheral neuron/nerve progenitor cells were developed by the National Institute of Advanced Industrial Science and Technology (AIST), Japan (Y Takayama, et al. Sci Rep. 2020 / Y Takayama, Y Shibuya, et al. PLoS ONE 2020). On the other hand, we have developed a model of facial nerve palsy which is advantageous, for example, for analysis using genetically engineered mice, modifying previously reported rat facial palsy. Using these cells and model, we launched an experimental system and project in collaboration with AIST to transplant iPSC-derived induced peripheral neuron/nerve progenitor cells into the mouse model of facial nerve palsy and evaluate their effect on peripheral nerve regeneration. In this pilot study, we evaluated the recovery of nerve function after transplantation of the iPSC-derived peripheral neuron/nerve progenitor cells into the mouse model of facial nerve palsy by evaluating nerve function which was facial scale evaluation modified by a previous paper and histological findings.

Funding Source: This research was partially supported by FY2021 TIA Collaborative Program Exploration and Promotion Project “Kakehashi

Keywords: iPSC-derived peripheral neuron/progenitor, peripheral nerve regeneration, murine model

4:45 PM – 5:45 PM

260

GERMLINE ASXL1 MUTATIONS IMPACT EARLY EMBRYOGENESIS THROUGH IMPAIRED NEURAL CREST MIGRATION IN A ZEBRAFISH MODEL OF BOHRING-OPITZ SYNDROME

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Bohring-Opitz Syndrome (BOS) is characterized by severe developmental anomalies attributed to germline mutations in the ASXL1 gene, and similar mutations are associated with clonal hematopoiesis and leukemia. Previously, we utilized genetically edited and patient iPSCs to show that ASXL1 truncating mutations impair neural crest migration. This suggested a mechanism for the craniofacial abnormalities, intellectual disabilities, and defects of the heart and gastrointestinal tract in BOS patients. Here, we generated zebrafish larvae with patient-like ASXL1 mutations, employing bioinformatic tools for sequence alignment and protein structure prediction. Our results demonstrated developmental delays and craniofacial deformities in the mutant zebrafish akin to BOS. Specifically, we examined mutations in three ASXL1 regions, resulting in differential phenotypic severity as is seen in BOS patients. Interestingly, these mutations led to the loss of a long intrinsically disordered region necessary for paraspeckle formation, membraneless organelles that are associated with hematopoiesis. These results substantiate the association of abnormal neural crest, hematopoiesis, and BOS.

Funding Source: ASXL Rare Research Endowment Foundation P.O. Box 4662, Portland, ME 04112, United States of America

Keywords: ASXL1, neural crest development, zebrafish rare disease modeling

4:45 PM – 5:45 PM

262

IRON DEFICIENCY ALTERS HUMAN VENTRAL FOREBRAIN ORGANOID DEVELOPMENT

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Iron deficiency is the most common micro-nutrient deficiency worldwide and is especially common amongst pregnant women. Gestational iron deficiency has been correlated with a wide range of timing, dose, and duration-dependent behavioral consequences for the developing fetus, many of which are unable to be substantially modified by postnatal iron supplementation. Despite these consequences, to date relatively little is known about the cellular and neurophysiological mechanisms through which iron deficiency acts to result in aberrant phenotypes. Previous work in our lab has demonstrated in a mouse model that iron deficiency during neurodevelopment alters inhibitory neural precursor cell fate within the medial ganglionic eminence (MGE), which further results in sustained alterations in inhibitory neuron subtype ratio in cortex that persist into adulthood. To examine whether these affects can be observed and experimentally modulated in a model of the developing human brain, we developed a model of iron deficiency using iPSC-derived human ventral forebrain organoids by supplementing apo- vs. holo-transferrin to the media. Using this model, we were able to demonstrate human-relevant iron levels for the duration of the organoid culture as measured in bulk via Inductively-Coupled Plasma Mass Spectrometry (ICPMS) as well as the spatial distribution of these metals via Laser Ablation ICPMS. These metallomics assays revealed that iron deficiency results in dyshomeostasis of not just iron, but other divalent metals as well, including Zinc and Copper. We next demonstrated using immunofluorescence that iron deficiency results in increased expression of the transcription factor Nkx2.1, a downstream target of Sonic Hedgehog that is associated with MGE-derived precursors. Furthermore, we observed an increased number of Nkx2.1+ precursors that were also positive for CoupTFII, a transcription factor associated with the neighboring caudal ganglionic eminence. Lastly, we examined gene expression via qRT-PCR and RNAscope. These experiments demonstrate that iron deficiency results in a dysregulation of lipid biosynthesis pathways, which are known to be required for appropriate signal transduction of the Sonic hedgehog pathway.

Keywords: organoid, neurodevelopment, iron deficiency

4:45 PM – 5:45 PM

264

CHARACTERIZATION OF RFX4 ROLE IN ACTIVE ENHANCERS THROUGHOUT THE DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO DOPAMINERGIC NEURONS

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Human pluripotent stem cells (hPSCs) have been successfully induced into midbrain dopaminergic (mDA) neurons using a floor-plate-based strategy that mimics mDA neuron development. The identity and functionality of the resulting mDA neurons have been largely characterized. Nevertheless, a comprehensive transcriptomic and epigenomic characterization is needed to understand the regulatory mechanisms driving mDA neuron specification. Gene expression is driven by an interplay of transcription factors, promoters, and epigenetic regulatory elements, like enhancers. In previous work, we determined a subset of mDA neuron-specific regulatory regions by mapping chromatin accessibility using ATAC-Seq. To further characterize open chromatin regions, we performed enhancer-specific histone modification (H3K27ac and H3K4me1) ChIP-Seq on in vitro generated neural precursors and mDA neurons. Transcription factor (TF) binding motif analysis suggested an important role of the RFX4 TF on mDA-specific enhancer regions. To gain insight into the regulatory mechanisms involved in mDA neuron specification, we mapped RFX4 chromatin binding sites using CUT&RUN on embryonic stem cells, and in vitro generated neural precursors and mDA neurons.

Funding Source: PAPIIT-UNAM IN213719, IN219122

Keywords: dopaminergic, enhancer, development

4:45 PM – 5:45 PM

266

BUILDING NEUROMUSCULAR ASSEMBLOIDS OF DISTINCT AXIAL LEVELS FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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During embryonic development, the post-cranial body axis is constructed from a highly proliferative population of axial progenitors (AxPs), including neuromesodermal progenitors (NMPs) located at the caudal tip of the embryo. NMPs simultaneously self-renew and generate essential cell lineages, including neural tube, neural crest, and presomitic mesoderm progenitor cells. There are currently no established protocols that describe the simultaneous in vitro derivation of presomitic mesoderm, and neural crest from human pluripotent stem cells (PSCs), which poses a significant challenge. We have addressed this gap by developing a monolayer protocol that can recapitulate the NMP lineage tree from human PSCs. Through modulation of growth factor conditions, we can generate and maintain NMPs as these go through colinear Hox activation and subsequently derive neural tube, neural crest, and presomitic mesoderm lineages. These lineages are further harnessed to build organoids, ultimately assembled into sophisticated 3D Posterior Axial Assembloids (PAXAs). The PAXAs can be maintained in vitro for months at a time, express mature neural, neural crest and mesodermal markers and display spontaneous contractions. Notably, we can direct the differentiation of neural tube progenitors towards a motor neuron fate. These motor neurons establish connections with muscle fibers derived from the presomitic mesoderm, forming functional synapses and neuromuscular junctions (NMJ). We believe that our in vitro system holds great potential for modeling human development and studying neuromuscular diseases such as amyotrophic lateral sclerosis (ALS), thereby paving the way for improved mechanistic understanding and potential therapeutic approaches.

Keywords: neuromuscular junction, neuromesodermal progenitors, assembloids

4:45 PM – 5:45 PM

268

STABLE AND EFFICIENT GENERATION OF FUNCTIONAL IPSC-DERIVED NEURAL PROGENITOR CELL ROSETTES THROUGH BOTULINUM HEMAGGLUTININ-MEDIATED REGULATION OF CELL BEHAVIOR

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To industrialize induced pluripotent stem cell (iPSC)-derived cell therapies, the need for robust and well-characterized bioprocesses for iPSC differentiation is becoming increasingly clear. However, developing culture strategies for bioprocesses remains challenging because there is no efficient way to control cell heterogeneity during the differentiation. In this study, we developed an effective and robust differentiation strategy for generation of neural progenitor cell rosettes from iPSCs incorporating botulinum hemagglutinin (HA). Treatment with HA suppressed the spatial heterogeneity of cells of iPSCs cultured under undirected differentiation conditions, resulting in the preservation of their pluripotency. Moreover, treatment with HA during neural progenitor differentiation combined with dual SMAD inhibition generated rosette-forming neural precursors surrounding the central lumen in monolayer cultures without detectable contamination by undifferentiated iPSC or derivatives of other germ layers. As the differentiation proceeded, the large networks of βIII tubulin+ neurons were found throughout the culture vessels. Cells cultured with HA generated a highly homogeneous population of PAX6- and SOX1-expressing neural progenitor cells with 8.4-fold higher yields of neural progenitor cells than untreated control cultures. Our results from the suppression of the spatial heterogeneity of cells using HA confirmed that neural differentiation induction utilizing dual SMAD inhibitors containing HA improve the yield and quality of neurons for the generation of functional neural rosettes, providing a valid tool for neuronal disease modelling and drug discovery applications.

Keywords: human induced pluripotent stem cells, neural progenitor cell rosettes, botulinum hemagglutinin

4:45 PM – 5:45 PM

270

EYES ON THE FUTURE: ILLUMINATING HUMAN RETINAL DEVELOPMENT WITH STEM CELL-DERIVED ORGANOIDS

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The retina serves as an invaluable model for investigating neural development due to its well-defined cellular composition and evolutionarily conserved developmental processes. Proper cell fate determination requires the precise spatiotemporal expression of thousands of genes, and errors in this intricate process lead to a myriad of devastating diseases. However, studying retinogenesis in humans has been challenging due to limited access to tissue. To overcome this challenge, various differentiation protocols have been devised to generate retinal organoids from human pluripotent stem cells. In this study, we present the results of a year-long characterization of one such differentiation protocol, focusing on production scaling and the developmental dynamics of retinal organoids generated from embryonic and induced pluripotent stem cells. Minor adjustments to the protocol yielded a remarkable improvement in efficiency, enabling the generation of over 200 retinal organoids per round of differentiation. Retinal organoids

were produced every other month over a 12-month period and were harvested in two batches separated by a one-month interval. A comprehensive array of analytical techniques including single-cell RNA sequencing, bulk RNA sequencing, transmission electron microscopy, and immunofluorescence, provided insights into the cellular diversity and specification of retinal organoids over time. Gene expression profiles and immunofluorescent labeling confirmed the presence of the major cell types of the neural retina. Our findings shed light on the temporal progression of retinal development within organoid models, serving as a valuable resource for deciphering the timing of key events during human retinogenesis. This comprehensive dataset enhances our understanding of human retinal development and underscores the utility of retinal organoids as a model system for studying neural development and disease.

Keywords: retinal development, organoid differentiation, single-cell RNA sequencing

4:45 PM – 5:45 PM

272

EXPLORING THE HUMAN EYE-FIELD AND EYE-SPOT GENE NETWORKS USING HUMAN INDUCED PLURIPOTENT STEM CELLS AND CHICKEN EMBRYOS

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A quarter of all childhood blindness is caused by congenital malformations of the eyes. We use chicken embryos to better understand retinal patterning in human development. In vertebrate embryos, the retinas of the eye are thought to develop from a single “eye field”; a domain of neural progenitor cells within the anterior neural plate (future forebrain). We identified a novel self-organising Turing network involving one of these eye-field factors, Pax6, which spontaneously generates eye-spots in vitro. We aim to classify the interactions between “eye-spot” and “eye-field” gene networks to answer the questions: How does the “eye-field” gene network relate to the “eye-spot” gene network? Does the “eye-field” gene network regulate the expression pattern of the Turing network or vice versa? Using a novel hybridisation chain reaction (HCR-FISH) approach to achieve spatial expression profiling as well as qRT-PCR and ATAC-Sequencing we aim to identify a timeline of activation and genomic regulatory elements modulating this gene network. With this approach, we have shown the expression of the Turing network in the developing optic vesicles of chicken embryos. In human iPSCs we have characterised the spontaneous formation of “eye-spots” following neural induction. We are now looking to expand this research into the human “eye-field” gene network and aiming to identify the interplay between the “eye-field” and the “eye-spots” generated by the Turing network. This work will deepen our fundamental understanding of how retinal patterning occurs during human development.

Keywords: developmental biology, human induced pluripotent stem cells, Turing network

4:45 PM – 5:45 PM

274

CELL FATE DECISIONS IN THE DEVELOPING HUMAN NEOCORTEX

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The human neocortex has undergone strong evolutionary expansion, largely due to an increased progenitor population, the basal radial glial (bRG) cells. These cells are responsible for the production of a diversity of cell types, but the successive cell fate decisions taken by individual progenitors remain unknown. Here, we developed a semi-automated live/fixed correlative imaging method to map bRG cell division modes in early fetal tissue and cerebral organoids. Through the live analysis of hundreds of dividing progenitors, we show that bRG cells undergo abundant symmetric amplifying divisions, and frequent self-consuming direct neurogenic divisions, bypassing intermediate progenitors. These direct neurogenic divisions are more abundant in the upper part of the subventricular zone. We furthermore demonstrate asymmetric Notch activation in the self-renewing daughter cells, independently of basal fiber inheritance. Our results reveal a remarkable conservation of fate decisions in cerebral organoids, supporting their value as models of early human neurogenesis.

Keywords: radial glial cells, cell fate decisions, cortical development

4:45 PM – 5:45 PM

276

SEQUENTIAL EMERGENCE AND CONTRACTION OF EPITHELIAL SUBTYPES IN THE PRENATAL HUMAN CHOROID PLEXUS REVEALED BY A STEM CELL MODEL

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As the blood-cerebrospinal fluid interface, the choroid plexus (ChP) mediates body-brain communication throughout life and has broad potential for CNS regenerative medicine. Despite this, relatively little is known about the human ChP or its epithelial cells (CPECs), including their lineages. Based on an earlier proof-of-concept method, we devised a simple, efficient, and scalable protocol for CPEC derivation from human pluripotent stem cells. In the absence of mesenchymal elements, derived CPECs (dCPECs) acquired canonical properties and displayed dynamic multiciliated phenotypes that impacted Aβ uptake and paralleled those seen in human tissues. Single dCPEC transcriptomes correlated well with human organoid and fetal CPECs, while pseudotemporal and cell cycle analyses revealed dCPEC, neuron, and neural progenitor lineages arising from neuroepithelial cells. In addition, transcriptome analyses defined metabolic (type 1) and ciliogenic dCPECs (type 2) at early stages, followed by type 1 diversification into anabolic-secretory (type 1a) and catabolic-absorptive subtypes (type 1b) as type 2 cells contracted. The sequential emergence and contraction of these subtypes was then confirmed in independent derivations and mapped to distinct prenatal stages using human tissues. These findings establish an improved protocol for deriving human CPECs, define their prenatal lineage dynamics, and lead to new models of ChP/CSF functions during human brain development.

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Keywords: choroid plexus, cerebrospinal fluid, lineage

4:45 PM – 5:45 PM

278

A CONTROLLABLE HUMAN SPINAL CORD MODEL WITH FULL DORSOVENTRAL PATTERNING

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Understanding embryonic development has vast scientific and translational implications. Advancements in stem cell technologies have enabled the development of human pluripotent stem cell (hPSC)-based embryo models promising for elucidating the mechanisms underlying human development and associated disorders. In vivo, embryonic tissues are patterned through interpreting concentration gradients of signaling molecules. However, the development of current embryo models relies heavily on spontaneous self-organization of stem cells. Such stochasticity during embryo model development significantly undermines their controllability and robustness, impeding their uses in fundamental or translational applications. Herein, we report the development of a microfluidic, hPSC-based system to recapitulate the patterning of a classic developmental model, the embryonic spinal cord (SC). In vivo, the SC is patterned into 13 transcriptionally distinct domains along the dorsoventral (DV) axis through interpreting antiparallel patterning signal gradients. Using our microfluidic system, we generated SC-like structures from hPSCs and further formed controllable antiparallel signal gradients over them. Excitingly, we were able to generate, for the first time, a human SC model exhibiting all 13 domains along the DV axis, with an unprecedented robustness and controllability of close to 100%. The identities of the 13 domains were validated against human embryonic SC data. Furthermore, using our model we studied the role of retinoic acid (RA) in SC DV patterning, which remains elusive, since in vivo studies have shown contradicting roles of RA in regulating both dorsal and ventral transcription factors. Data obtained from our model show that RA has an overall dorsalizing effect, suppressing the expansion of ventral markers during DV patterning of SC. Together, we have demonstrated that recapitulation of signaling gradients can greatly improve robustness and controllability of a hPSC-based SC model, which further allowed us to investigate the role of RA in SC DV patterning. Signal gradient-mediated patterning is a prevalent mechanism in embryo development. Thus, our embryo modeling technology will be useful for the study of other developmental systems.

Funding Source: National Institute of Health

Keywords: synthetic embryology, neurodevelopment, microfluidics

4:45 PM – 5:45 PM

280

LNCRNA NEAT1 PRODUCES PARASPECKLES THAT DIMINISH PROTEIN TRANSLATION AND ALTERS NEUROGENESIS

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The function of ribonucleoprotein (RNP) complexes in brain development is not fully understood. Here, we show that ectopic induction of the long non-coding RNA (lncRNA) NEAT1 in pluripotent stem cells (PSCs) assembles paraspeckles, one type of RNP complexes, and promotes nuclear retention of translation initiation factors, leading to protein translation inhibition. Cerebral organoids with ectopic induced paraspeckles form choroid plexus-like cysts. Further characterization of such choroid plexus-like cysts by single cell transcriptomics indicates a paraspeckle-driven cell fate change towards choroid plexus during brain development. This indicates an axis of neuronal differentiation regulation via paraspeckles diminishing protein translation.

Keywords: paraspeckle, protein translation, neurogenesis

4:45 PM – 5:45 PM

282

UNRAVELING THE FUNCTIONS OF THE TRANSCRIPTION FACTOR TBR2 IN HUMAN NEURODEVELOPMENT

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Among the different organs, the human brain has undoubtedly been the one most affected by evolution, with both morphological and functional changes. The human neocortex, in particular, is greatly expanded and exhibits increased complexity. The genetic mechanisms underlying the evolutionary changes in our neurodevelopment are, however, poorly understood. With the advent of human pluripotent stem cells (hPSC) in combination with the discovery of efficient gene editing technologies and the ability to generate organotypic hPSC-derived brain organoids we are now technologically equipped to decipher the molecular basis of the changes between our brain and that of our ancestors. In this project we apply gene editing in hPSC and thereof derived organoids to study the function of TBR2, a transcription factor selectively expressed in IPs and functionally required for SVZ neurogenesis, during early cortical development. Using CRISPR/Cas9 mediated gene editing we generated hPSC-TBR2-knockout (KO) lines. Following validation we applied a forebrain- and a cortical hem-type organoid protocols. When analyzing the transgenic organoids and isogenic controls we found that TBR2 is impacting in the forebrain on the generation and proliferation of the different types of intermediate progenitor cells including those expressing PPP1R17 or NHLH2. We also found that TBR2 is acting on the generation of the pre and subplate including the differentiation into pioneering neurons positive for the markers TBR1 and BHLHB5. In the cortical-hem-type organoids we found alterations in the developmental timing of cells expressing markers for the choroid plexus. By that our data suggests a role of TBR2 in forebrain and cortical hem development and proves that transgenic organoids represent a powerful tool to map gene function in brain development, to correlate genetics to functional phenotypes and to complement the long tradition of KO-models in developmental biology and neuroscience.

Keywords: neurodevelopment, TBR2, organoids

4:45 PM – 5:45 PM

284

CHARACTERIZATION OF EXOGENIC SPINAL NEURONS IN INTERSPECIES RAT-MOUSE CHIMERAS GENERATED VIA BLASTOCYST COMPLEMENTATION IN CRISPR/CAS9 GENE EDITED MOUSE EMBRYOS

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Spinal cord injury (SCI) causes severe motor and sensory loss, with no recognized treatment for functional recovery. Cell transplantation offers promise, but current approaches lack cell specificity, hindering integration with host tissue. Addressing this, recent technology enables precise gene editing in stem cell complemented embryos to generate region-specific spinal neurons. By targeting specific genes, we aim to create niches for exogenic motor and sensory spinal neurons that mimic authentic cells. The overall objective of this research is to identify specific genes in the generation of spinal cord neurons. We postulate that targeting select genes for deletion by gene editing can create a niche for the generation of exogenic motor and sensory neurons in the spinal cord, and these exogenic cells, in turn, recapitulate the phenotype of authentic cells. In this study we report findings of co-localized GFP positive donor rat cells in E12.5 Hhex knock-out mouse chimeric embryos throughout the rostral-caudal and dorso-ventral axes of the spinal cord. In our presentation we will exhibit the phenotypes of these exogenic rat neurons within the mouse spinal cord. These results contribute to the bench-to-bedside translation of stem cell therapeutics in spinal cord injury, and pave the way for targeted therapies to enhance locomotor circuits, improve sensation, and alleviate pain in SCI patients.

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Keywords: blastocyst complementation, spinal cord injury, interspecies chimera

4:45 PM – 5:45 PM

286

GENE REGULATORY NETWORK FOR PARVALBUMIN FATE DURING GLIA REPROGRAMMING

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GABAergic interneurons of parvalbumin (PV) expressing subtype have been shown to be lost or dysfunctional in many neurological disorders, such as schizophrenia and epilepsy. Therefore, strategies are evolving for generating PV interneurons in vitro for cell replacement therapy but to generate human PV neurons from e.g. stem cells have proven difficult. An appealing approach to generate neurons of different subtypes is via direct reprogramming, a process where somatic cells such as glia cell can be converted into neurons by overexpressing specific neuronal genes. The process bypasses the stem cell state thus allow for an in vivo approach with low risk of tumor formation. Using this technique, our lab has demonstrated that human glial precursor cells can be converted to GABAergic interneurons of PV phenotype in 2D cultures. In here, we want to unravel the gene regulatory network for PV reprogramming protocol. Thus, we convert human glial progenitor cells to GABAergic interneurons in a 3D culture system, that provides long-term maturation in vitro and more extensive synaptic connectivity. The 3D culture were transduced with GABAergic factors and performed single nuclei RNA sequencing at different time points during reprogramming. Data demonstrate that cell divergence begins to occur already at day 3, with neuronal gene expression at day 7 of reprogramming. Importantly we detect PV gene expression from day 7 with expression of crucial channels for their fast-spiking properties (SCNA1, CACNA1A). Using diffusion and latent time analysis, we further revealed the gene regulatory network for direct reprogramming towards PV cell fate, where important genes regulating PV expression were seen, e.g. RORA and GPHN. These have never been implicated in the reprogramming process before. Concluding, this interdisciplinary project provided a cohesive understanding of pathways and gene networks during direct reprogramming towards PV interneurons, that can be used for further development for PV interneurons towards cell replacement strategies.

Keywords: reprogramming, cell replacement, interneurons

4:45 PM – 5:45 PM

288

TOWARDS THE IDENTIFICATION OF MODULATORS OF THE METABOLIC SHIFT IN DIFFERENTIATING NEURAL STEM CELLS

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Stem cell metabolism plays a critical role in regulating the balance between self-renewal and differentiation by balancing intrinsic needs and extrinsic metabolic cues, thereby influencing stem cell fate. Metabolic pathways such as glycolysis and oxidative phosphorylation are intricately linked to the regulation of pluripotency, quiescence and proliferation in stem cells. Reprogramming stem cell metabolism, and therefore stem cell function, provides a strategy to improve stem cell therapy for various applications, including regenerative medicine and disease modelling. While metabolic profiling of stem cells has taken a leap forward with the advent of metabolomics, novel techniques need to be established to identify the key modulators of stem cell metabolism, particularly in the context of neural stem cell differentiation. Towards this goal, we have successfully established the use of genetically encoded, inducible metabolite biosensors for pyruvate and for the ratio of NADH/NAD+ in SH-SY5Y neuroblastoma cells to assess their metabolite landscape using fluorescence-activated cell sorting (FACS). Metabolite sensor intensity in SH-SY5Y cells scales according to cellular metabolite levels when altered by drugs (e.g. rotenone or 2-deoxy-glucose) or glucose starvation, as orthogonally validated by seahorse assays. We are currently establishing metabolite sensor-expressing human induced pluripotent stem cells (hiPSCs) that will be differentiated into neural stem cells and subjected to genome-wide CRISPRi screening to identify novel and essential regulators of energy metabolism, with a particular focus on RNA-binding proteins. The potential applications of a single-cell metabolic readout of key energy metabolites in hiPSCs are many, including the ability to monitor the response to reprogramming interventions, as well as studying the effects of ageing or damage to cells.

Keywords: hiPSCs, metabolic shift, metabolite sensors

4:45 PM – 5:45 PM

290

HOMEOTIC REGIONAL IDENTITY IN MOUSE AND HUMAN NEURAL STEM CELLS PROMOTES REGENERATIVE SUCCESS WHEN TRANSPLANTED IN SPINAL CORD INJURY

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Neural stem cells (NSC) are a viable transplantable therapeutic for sparing and replenishing the cellular niche lost after traumatic spinal cord injury (SCI). Recent experimental evidence suggests that a transplanted NSC with matching “regional” spinal cord identity to the injured cord can yield optimal cell integration and functional outcomes. A potential explanation for this regenerative success is the expression of homeotic genes (HOX). During Central Nervous System (CNS) development, HOX genes regionalize NSCs to ensure appropriate neuronal connectivity and promote motor and sensory functions. If HOX expression is maintained throughout the cellular transplantation process, this would suggest their developmental role is recapitulated and thus provide mechanistic explanation of regionally matching transplant successes. To begin, NSCs were dissected from the brain (b) and spinal cord (sc) of E12.5 mice, expanded in vitro, and transplanted in vivo. Notably, both bNSC and scNSC retained region-specific HOX markers (OTX2 & HOXB6, respectively) while in culture, and post-transplantation into naïve & injured spinal cords. Next, potential regenerative effects were evaluated in a clinically translational model using human induced pluripotent stem cell-NSC (hiPSC-NSCs). Prior to transplant, hiPSC-NSCs were exposed to a WNT activator and subsequently Retinoic Acid (RA) in vitro to promote expression of HOX genes (caudalization). Following transplant into an immunodeficient rat model (RNU) of cervical SCI, caudalized NSCs promoted tissue preservation and functional recovery in injured animals. Compared to non-regionalized NSCs, caudalized NSCs uniquely promoted a greater electrically evoked compound action potential (spNSC: 0.6 + 0.5mV; default NSC 0.1 + 0.06mV), suggesting an enhanced electrical conduction with endogenous cells across the injury site. In these animals, caudalized cell grafts also maintained HOX expression post-transplantation. Altogether, this work supports that region-specific NSC transplants provide an efficacious cell therapeutic for SCI, which can be mechanistically described through the maintenance of spinal cord specific HOX genes. This work implicates the importance of regional specificity of NSCs grafted in all contexts of CNS injury and degeneration.

Keywords: spinal cord neural stem cells, regional identity, spinal cord injury

4:45 PM – 5:45 PM

292

SINGLE-CELL DELINEATION OF LINEAGE TREE IN HUMAN CEREBELLAR ORGANOIDS

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Human pluripotent stem cell-derived cerebellar organoids have emerged as a promising in vitro system to interrogate the development and disease of this human brain region. Single-cell transcriptomics has allowed for highly resolved identifications of cell states within this system; however, uncovering the fate choices these cells take as they mature within this 3D culture system remains elusive. Here, we report the use of our reproducible cerebellar organoid model system in combination with emerging lineage tracing technologies to reveal the divergence of a common progenitor pool that leads to the generation of an inhibitory and excitatory lineages of the developing cerebellum. Our data elucidates how lineage and cell fate choices are determined in cerebellar organoids and poses a potential model to study lineage alterations in normal or perturbed cerebellar development.

Keywords: organoid, lineage tree, cerebellum

4:45 PM – 5:45 PM

294

FUNCTIONAL ANALYSIS OF FOXB1 DURING HUMAN PLURIPOTENT STEM CELL DIFFERENTIATION

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Forkhead box 1 (Foxb1) is expressed during the development of neural crest (NC). Rodent lineage tracing and knockout studies suggest that Foxb1 may functionally contribute to NC derivatives. FOXB1 is also expressed during early human pluripotent stem cell (hPSC) differentiation. However, the functional roles of FOXB1 during hPSC differentiation and in human pathologies are unknown. To answer these questions, we used hPSC as a model to study FOXB1 function in humans. We created multiple FOXB1 heterozygous and homozygous hPSC lines by targeting the exon 2 of FOXB1, which contained both the translational start site and the DNA binding domain sequence. We established that FOXB1 homozygous cell lines were functionally null. We coerced the differentiation of wild type and mutant cell lines towards various germ layer lineages, and found that knockout of FOXB1 did not affect mesoderm, endoderm, and neural specification, but resulted in accelerated NC induction. Interestingly, when knockout cells were first subjected to NC induction regime then switched over to neural induction, these cells displayed a reduced propensity for neural induction. To further define FOXB1's role in NC differentiation, we subjected FOXB1 knockout NC cells to peripheral neuronal and mesenchymal differentiation. We found that FOXB1 knockout cells displayed reduced expression of proneural genes POU4F1 and ASCL1, as well as autonomic neuronal genes TH and PHOX2A. We also observed an increased propensity of FOXB1 knockout cells to express mesenchymal genes. These analyses are consistent with a role of FOXB1 in maintaining and establishing early and late neuronal differentiation potential in human NC lineage, adding to the proposed function of Foxb1 as a neuronal survival factor in specific rodent CNS neurons. These data can also be interpreted as suggesting FOXB1 as a repressive factor for NC and mesenchymal cell types. There are currently no known human pathologies identified with FOXB1 mutations. Based on our findings, it is possible that FOXB1 mutations may lead to termination of human embryo development caused by early NC defects.

Keywords: neural crest, Forkhead box transcription factor, differentiation

4:45 PM – 5:45 PM

296

OPTIMIZING ASTROCYTIC LINEAGE MATURATION IN ADVANCED HPSC-DERIVED CORTICAL ASSEMBLOID

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Organs like the brain exemplify the species-specific differences that exist between humans and even our closest relatives, the great apes. Many aspects of human brain development and homeostasis are still obscure due to the great technical challenges of isolating and culturing human brain cells. The human pluripotent stem cells (hPSCs)-based technologies offer a solution to this challenge and provide an alternative experimental platform to study human brain development and homeostasis. The hPSC-derived brain organoids recapitulate some aspects of the cellular composition, architecture, and function of the human brain. However, hPSC-derived organoids often lack maturation features and, in the case of a guided differentiation protocol, cellular diversity. In this study, we generated a complex hPSC-derived brain assembloid that recapitulates the human brain microenvironment including outer radial glia, cortical neurons, interneurons, glial populations (astrocytes and microglia) and pericytes. In the previous organoid models established so far, the astrocytic lineage appears spontaneously only after 270 days. The goal of the project is to achieve a faster development of this compartment, using two different approaches: the overexpression of NFIA, an important nuclear factor that induces glial competency, in combination with the addition of LIF; the second method is the inhibition of EZH2, which triggers the glial compartment maturation.

Keywords: cortical assembloid, modeling, organoid

4:45 PM – 5:45 PM

298

AN ORGANOID MODEL TO STUDY FETAL HUMAN MICROGLIA DEVELOPMENT IN VITRO

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Microglia are specialized brain-resident macrophages that play crucial roles in brain development, homeostasis, and disease. However, until now, the ability to model interactions between the human brain environment and microglia has been severely limited. To overcome these limitations, we developed an approach to efficiently colonize human brain organoids by optimizing a guided human induced pluripotent stem cell-derived (iPSC) model of primitive hematopoiesis. We show that efficient colonization of various brain organoid models through erythromyeloid progenitors (EMPs) occurs in a time and stage-dependent manner. Colonizing EMPs are able to differentiate into early fetal but not fully mature microglia. We determined specific physical constraints pertaining to current organoid models that prevent organoid resident microglia from acquiring a bona fide in vivo identity and show that transplantation may allow to overcome these hurdles. Taken together, we have developed a scalable and reproducible approach to generate neuro-immune models that provide the basis for the study of fetal human microglia in health and disease.

Keywords: microglia, organoids, development

4:45 PM – 5:45 PM

300

DEVELOPING A 3D MODEL TO STUDY AXONAL DEGENERATION/REGENERATION OF MOTOR NEURONS DERIVED FROM MOUSE EMBRYONIC STEM CELLS

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The muscle contraction that leads our ability to move is mediated by the neurotransmitter release of motor neuros to the skeletal muscle. Physical injuries that affect these neurons or their axons prevent muscle stimulation, resulting in reduced mobility. The difficulties of using experimental animals to study the motor neuron changes after inducing axonal damage imply several challenges, including accessibility, long-range axons visualization through microscopy, etc. Thus, having an in vitro method which includes motor neurons and muscle cells could expand our basic knowledge to understand the events caused by physical injuries, that affect these neuron-muscle connections. We used a 3D model to study neuromuscular interactions, such as neuromuscular organoids or assembloids. We have started to use mouse embryonic stem cells that constitutively express Glial cell line-derived neurotrophic factor (GDNF), or control cells, to induce them to differentiate into motor neurons. In parallel, aggregates of C2C12 myoblasts were prepared to form neuromuscular assembloids. After joining neuronal and muscle aggregates, we could observe that axons from the the neural part contains GFP expression contact the muscular part. We are testing the optimal methods for labelling motor neurons reaching the muscle, to later cut such axons and study the effects of GDNF on regeneration. Our results will provide information about the molecular changes related to axonal regeneration of motor neurons and will give an alternative way to study neuromuscular interactions.

Funding Source: PAPIIT UNAM IN219122

Keywords: motor neuron axons, mouse neuronal and muscle aggregates, axon injury

4:45 PM – 5:45 PM

302

UNRAVELLING THE ROLE OF ARHGAP11A IN APICAL PROGENITOR CELL DELAMINATION DURING HUMAN CORTICOGENESIS

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During corticogenesis, apical progenitor cells (APs) can be found in the ventricular zone (VZ). With progression of brain development, APs delaminate from the ventricular surface to produce basal progenitors, which reside in the basally located subventricular zone where they generate cortical neurons. This process of delamination can involve symmetric or asymmetric cell division, and be affected by the orientation of the AP cleavage plane. However, the exact mechanisms regulating delamination remain poorly understood. Here, we show

that ARHGAP11A, a RhoGTPase-activating protein highly expressed in APs, plays a role in delamination. We used CRISPR/Cas9-based gene editing to generate human induced pluripotent stem cell (hiPSC) ARHGAP11A-knockout (KO) lines. Following validation, we applied a standardised forebrain-type organoid protocol. When analysing the KO organoids and isogenic controls, we observed enlarged ventricular surface areas of the neuroepithelium. Further, ARHGAP11A KO cells delaminated pre-maturely from the VZ and settled down basally to these structures, as observed in hybrid organoids composed of EGFP-labelled KO and isogenic control cells. In addition, we observed a misoriented cleavage plane of dividing APs in the KO. When investigating more mature organoids, we identified a trend towards a reduced abundance of S100β-positive cells, suggesting a reduction of astroglia differentiation. Hence, our data suggest that ARHGAP11A plays a key role in maintaining the AP pool by regulating the cleavage plane orientation and preventing pre-mature delamination, and support the validity of using human forebrain-type organoids as a model to study early brain development and corticogenesis.

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Keywords: forebrain organoids, corticogenesis, neural progenitor cells

4:45 PM – 5:45 PM

TOPIC: NO TISSUE SPECIFICITY

304

FUNCTIONAL ANALYSIS OF SALL3 IN THE REGULATION OF DIFFERENTIATION PROPENSITY OF HUMAN IPS CELLS

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Human induced pluripotent stem cells (hiPSCs) have the ability to differentiate into a variety of cells and self-renew in vitro. These two characteristics make hiPSCs promising for new applications in regenerative medicine and cell therapy. However, the ability of hiPSCs to differentiate into specific lineages varies greatly from cell line to cell line, and the cause of this variation is not clarified. In our previous studies, we explored genes correlated with differentiation propensity, and identified SALL3 as a gene that is highly expressed in lines that tend to differentiate into the ectoderm and lowly expressed in lines that tend to differentiate into the meso- and endoderm. The overexpression of SALL3 promoted differentiation to the ectoderm, while the suppression of SALL3 promoted differentiation to the meso- and endoderm, indicating that SALL3 is functionally involved in differentiation-directed differentiation. Furthermore, SALL3 binds to DNMT3B, a DNA methyltransferase, and inhibits gene body-specific DNA methylation of Wnt signaling-related genes. In this study, to examine further roles of SALL3 to regulate hiPSC differentiation propensity, we comprehensively searched for proteins that actually interact with SALL3 in hiPSCs by performing pull-down assays using FLAG-SALL3 and LC-MS/MS analysis. The results revealed that SALL3 interacts with several proteins in the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex, which regulates chromatin remodeling. Furthermore, by performing ATAC-seq analysis and histone modification analysis using ChIP-Seq, we analyzed epigenetic regulation involving SALL3, suggesting that SALL3 regulates genome-wide histone modifications, especially H3K27me3. This research will not only elucidate the differentiation mechanism of hiPSCs, but will also lead to the development of a quality evaluation method for hiPSCs as raw materials, which is highly useful for selection of hiPSCs and will greatly contribute to the realization of cell therapy.

Keywords: human pluripotent stem cells, differentiation propensity, SALL3

4:45 PM – 5:45 PM

306

DIGITAL PCR EXPRESSION ANALYSIS OF PLURIPOTENCY AND DIFFERENTIATION MARKERS IN HPSC CELLS

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Assessing the pluripotency and differentiation potential of human pluripotent stem cells (hPSCs) is critical for their applications in regenerative medicine and disease modeling to mitigate potential risks such as tumor formation or differentiation failure. To address this need, we developed a digital PCR expression assay to evaluate the expression of a carefully curated list of 16 genes. Four genes were selected for each of four developmental stages: pluripotency and the three embryonic germ layers (ectoderm, mesoderm, endoderm). The selection was carefully made based on recommendations from scientific societies such as the International Society for Stem Cell Research (ISSCR) and ISCBI, as well as relevant scientific publications. This assay provides scientists with an accurate understanding of the expression of pluripotency markers and the trilineage differentiation potential of their pluripotent stem cells. Our digital PCR platform enables precise quantification and analysis of gene expression levels, providing absolute measurement capabilities. Using digital PCR expression analysis, we accurately measured the expression levels of selected pluripotency and differentiation markers in hPSCs. Compared to other molecular techniques such as qPCR, digital PCR demonstrates increased sensitivity, quantitative accuracy, and the ability to detect low abundance gene expression levels. Our results highlight the utility of absolute quantification of gene expression by digital PCR in the assessment of pluripotency and differentiation markers in iPSCs. In addition, our results demonstrate a high degree of reproducibility, further strengthening the reliability of our assay. By using a carefully curated gene panel and advanced digital PCR technology, we obtain precise and consistent data, which is essential for the characterization of hPSCs.

Keywords: pluripotency, differentiation, three embryonic germ layers, digital PCR, absolute quantification

4:45 PM – 5:45 PM

308

PLURIPOTENT FACTORS DETERMINE THE LOCAL EFFICIENCY OF LATE ORIGINS OF REPLICATION IN MOUSE EMBRYONIC STEM CELLS

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During the cell cycle, genome duplication defines the course of S-phase and it follows a specific spatiotemporal program. DNA replication preferentially starts in specific loci known as origins of replication (ORIs) from which two bilateral forks arise and progress while copying DNA. At the genomic level, the temporal activation of ORIs, combined with the speed at which forks advance, segregate the genome in replication time domains, which are related to spatial topology and epigenetic inheritance. In mammals, contrary to yeast and bacteria, the selection and temporal firing of ORIs are poorly characterized and there is an ongoing debate regarding the stochasticity of the whole process. Embryonic stem cells (ESC) proliferate rapidly, and their cell cycle is marked by a short G1 phase, which may constrain their readiness to start and accomplish DNA duplication, potentially leading to replication stress, DNA damage and chromosomal instability. To deal with this, it is believed that ESCs schedule a surplus of ORIs, whose location and activation might be controlled by chromatin and signaling cues. We applied different synchronization methods combined with EdU-seq to monitor the activation of ORIs and fork progression in a real temporal manner. We located early ORIs at high resolution and, beyond to what is known until now, we also identified highly efficient ORIs in mid and late replication domains. We explored their mechanism of activation and found that, at the global level, the late replication program relies on a balance between the restraining action of ATR as part of the DNA damage checkpoint, and the redundant positive actions of DDK (CDC7 and DBF4) and CDK1/2. We further characterized these mid and late ORIs at the local genomic level by correlating their datasets with nascent transcriptomics and chromatin profiling. We found that they are distributed in large heterochromatic or poised regions, but preferentially located on islands of open chromatin. These small regions are kept accessible by the pioneer action of OCT4, whose specific depletion leads to the loss of firing activity emanating from these otherwise efficient origins of replication. Our results dissect the hierarchical arrangement between transcription factors at the local level and upstream global regulators on the control of DNA replication.

Keywords: DNA replication, origins of replication, OCT4

4:45 PM – 5:45 PM

310

P53 GOVERNS HUMAN TROPHOBLAST DIFFERENTIATION AND CELL FATE TRANSITION

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The TP53 tumor suppressor is the most commonly mutated gene in human cancers. p53 suppresses tumorigenesis by transcriptionally regulating a network for target genes that play roles in various cellular processes, including cell cycle progression, apoptosis, senescence and metabolism. Recent studies have underscored the importance of p53 in promoting cancer cell differentiation to suppress tumor growth, harkening back to studies that found p53 promotes lineage commitment in development. The placenta is the transient organ connecting the embryo to the mother during gestation, and mediating nutrient and oxygen supply to support fetal growth. Defects in placentation would lead to fetal growth restriction and even miscarriage. Trophoblasts are the cells composing of the placenta. Interestingly, these cells display hallmarks of cancer, such as avoiding immune destruction, activating invasion, and inducing angiogenesis. Here, using human embryonic stem cells and trophoblast stem cells, we uncovered the role of p53 in promoting trophoblast differentiation. Transcriptomic analysis suggested a delayed trophoblast differentiation from human embryonic stem cell in the absence of p53. RNA sequencing and ATAC sequencing in human trophoblast stem cells identified a p53-mediated trophoblast regulatory network through direct DNA binding, chromatin remodeling and epigenetic dynamics. Notably, single-cell transcriptomics analyses revealed the role of p53 in trophoblast cell fate transition. In vitro established human trophoblast stem cells demonstrated in vivo cytotrophoblast diversity, and p53 depletion resulted in a lineage transition from fusing cytotrophoblasts, the progenitors for syncytiotrophoblasts, to cell column cytotrophoblasts, the progenitors for extra-villous trophoblasts. Collectively, these findings illuminate a novel role of p53 in human trophoblast differentiation and cell fate transition, reflecting a fundamental role of p53 in orchestrating human placental development.

Funding Source: RGC GRF 2023-2024 (17104523), Hong Kong

Keywords: p53, trophoblast stem cells, differentiation

4:45 PM – 5:45 PM

312

THE HUMAN PLURIPOTENT STEM CELL REGISTRY: UPDATE AND MANDATING DATA REQUIREMENTS FOR TRUSTWORTHY CELL LINE DIGITAL IDENTITY ASSESSMENT

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The human pluripotent stem cell registry (https://hpscreg.eu) is a public worldwide database of human embryonic and induced pluripotent stem cells. The community-driven registry allows users to register their lines, issues a unique persistent identifier (ID), thus allowing for linkage with globally dispersed metadata associated with that line. Furthermore, the hPSCreg® also mandates the provision of a minimal set of mandatory data for each registered cell line to establish a digital identity. These include data on biological and genetic properties, as well as data to assess ethical provenance of the line. The submitted data undergo a largely manual validation process to check for data completeness and plausibility. If mandatory criteria are fulfilled, cell lines may be eligible for certificates attesting to ethical provenance and biological quality. These certificates can be requested directly from hPSCreg. Thus, the registry enables an immediate assessment of the digital identity through a strictly standardized process, establishing a trustworthy and transparent resource for human PSC – lines. It is discussed whether mandating data requirements reduces or improves utility of the data resource, as specifically ethics provenance information is often unavailable to the community. Solutions to reduce stringency while maintaining trustworthiness and utility are provided. Furthermore, conceptual expansion content of the Registry is presented.

Funding Source: European Commission Horizon Europe Programme under grant agreement no. 101074135

Keywords: stem cell registry, research reproducibility, FAIR stem cell data standards

4:45 PM – 5:45 PM

314

GAIN OF 1Q CONFERS AN MDM4-DRIVEN GROWTH ADVANTAGE TO UNDIFFERENTIATED AND DIFFERENTIATING HESC WHILE ALTERING THEIR DIFFERENTIATION CAPACITY

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Human pluripotent stem cells have become a promising research tool for transplantation and disease modeling due to their unique ability to differentiate to any cell type of the human body. In vitro propagation can induce certain chromosomal abnormalities in stem cells, which confers them with growth advantage and take-over of the culture. Gains of 1q chromosome are highly recurrent in human pluripotent stem cells. Currently, the effect of 1q abnormality on lineage specification is not yet understood. In this work, we show that gains of 1q impact the differentiation capacity to derivates of the three germ layers, leading to miss-specification to cranial placode and non-neural ectoderm during neuroectoderm differentiation and by lower expression of lineage specific markers in 64% of hepatoblast markers and 63% in cardiac progenitors markers. Competition assays show that the cells retain their selective advantage during differentiation. The population of cells with a 1q duplication increased from 11% to 45% during neuroectoderm differentiation, from 11,6% to 60,9% during hepatoblast differentiation and 10,8% to 33,5% during cardiac progenitor differentiation. Selective advantage is mediated by a higher expression of MDM4, a gene located in the common region of gain. MDM4 drives the winner phenotype of 1q cells in both the undifferentiated and differentiating state by reducing the cells' sensitivity to DNA-damage through decreased p53-mediated apoptosis. Finally, we find that cell density in culture plays a key role in promoting the competitive advantage of the cells by increasing DNA damage. When cells reach density above 265.224 cells/cm2, 1q cells with high DNA damage load keep proliferating while wild-type cells undergo apoptosis. Our work reveals that the selective advantage of 1q gain is retained during differentiation, showing that mosaic culture at the beginning of the differentiation can result in heterogeneous end population of poorly specified cells.

Funding Source: The Research Foundation Flanders (FWO)

Keywords: p53-mediated apoptosis, 1q aneuploidy, selective advantage during differentiaion

4:45 PM – 5:45 PM

316

MECHANOSENSITIVE RIBONUCLEOPROTEINS CONTRIBUTE TO THE HETEROGENEITY OF PLURIPOTENT STEM CELL COLONIES

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Pluripotent stem cell (PSC) colonies are inherently heterogeneous and the molecular determinants of such phenomenon, which affects their potency and self-renewal, are largely unknown. Heterogenous nuclear ribonucleoproteins (hnRNPs) belong to the RNA-binding protein family and their main function is to regulate alternative splicing (AS). We recently identified novel hnRNP-rich structures (speckles) which are located exclusively at the outer border of the nucleus of edge cells in human induced pluripotent stem cell (hiPSC) colonies. We hypothesized that the characteristic hnRNP distribution within the hiPSC colony might be involved in potency control at the post-transcriptional level. We used surface micropatterning to control hiPSC colonies shape and size and validate the accumulation of different hnRNPs at edge of the colony. Our co-immunoprecipitation data suggested that the members of hnRNP family form a higher protein structure complex with components of the splicing machinery. We also confirmed that the colony-edge hnRNP-rich speckles localization is sensitive to mechanical stimuli by treating the hiPSC colonies with intracellular tension inhibitors, which led to reversible disruption of the hnRNP colony-edge pattern. Finally, we proved the function of hnRNP-rich speckles accumulated in colony-edge hiPSC is to control AS by measuring the expression of 9 splicing variants of hnRNP prominent splicing target YAP1 in cells located at the edge or at the center of the colony. Moreover, using YAP1 wild type knock-down and overexpression of the 9 YAP1 splicing variants we identified 17 YAP1 downstream genes dependent on the YAP1 splicing under the control of hnRNP. The expression of each of them significantly varied between colony-edge and colony-center hiPSCs. In conclusion, given that hiPSC colonies are inherently heterogeneous, with cells located at the edge being exposed to different mechanical stimuli than those at the center of the colony, our data suggest that the formation of hnRNP-rich speckles might predict specific AS events involved in their potency.

Funding Source: MUQUABIS "Horizon Europe" Research and Innovation program under Grant Agreement 101070546 King's BHF Centre for Excellence Award (RE/18/2/34213)

Keywords: mechanosensing, heterogeneous nuclear ribonucleoproteins, RNA-binding proteins

4:45 PM – 5:45 PM

318

PERIODICITY OF NETRIN1-MEDIATED MECHANOREGULATION CONTROLS STEM CELL FATE

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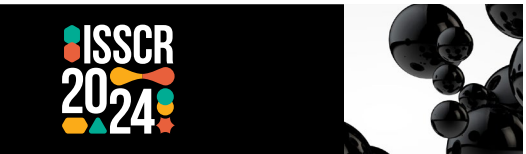
During mammalian development, pluripotent stem cells (PSCs) emerge from the inner cell mass (ICM) of preimplantation blastocysts to form the epiblast from which all the embryonic lineages arise. PSCs have an intrinsic heterogeneity to respond to external stimuli which constitute an important handicap for the application of stem cell–based therapies. A key regulator of this heterogeneity is the cell cycle, where PSCs in G1 phase are particularly prone to respond to external differentiation signals, while the S and G2M phases possess an intrinsic propensity toward the pluripotent state mediated by the p53 and Cyclin B1 regulators. Stem cell differentiation is also accompanied by changes in cell shape and mechanics. However, the precise link between mechanoregulation, cell cycle periodicity and stem cell fate remain largely unknown. Netrin-1 (Ntn1) is a laminin-like protein which controls the activity of embryonic, adult and cancer stem cells via its receptors Unc5b and Neo1. However, the molecular mechanisms governing Ntn1-mediated function in normal and pathological stem cells remain largely unknown. In this context, using a triple fluorescent Knock-in (TriC) to follow the expression of Ntn1, Neo1 and Unc5b, we found that these factors are dynamically expressed in PSCs with the co-existence of eight subpopulations of cells expressing different levels of the three proteins. By combining scRNA-seq on Embryoid Bodies (EBs) and artificial intelligence on teratoma we discovered that PSCs harboring different Ntn1 signaling activity are biased in their differentiation potential toward



specific germ layers. Intriguingly, the activity of the main signaling cascades controlling PSC self-renewal and differentiation remains constant during the cell cycle. Here we show that Ntn1 signaling is periodic and tightly regulated during the cell cycle where Clathrin-mediated endocytosis specifically regulate the lysosomal degradation of Ntn1, Neo1 and Unc5b in G1 phase. We found that this specific downregulation is responsible for molecular heterogeneity associated with cytoskeleton dynamics both in mouse and human pluripotent cells. Our data highlighted a broader role of Ntn1 as mechanoregulator helping to understand how cellular mechanics and cell cycle are molecularly linked to stem cell fate choice.

Keywords: mechanoregulation, molecular heterogeneity cell cycle, pluripotency

4:45 PM – 5:45 PM

320

MONITORING HUMAN EARLY OTIC DIFFERENTIATION FROM PLURIPOTENT STEM CELLS USING RAMAN SPECTROSCOPY

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Human induced pluripotent stem cells (hiPSCs) have the potential to provide some model systems for early stages of human ontogenesis. In the inner ear, these cells have been successively used to generate otic organoids containing hair cells, supporting cells, and neurons. However, much heterogeneity has been reported between different hiPSC lines used to model otic differentiation in vitro. This variation is partly due to each cell line exhibiting different endogenous levels of signalling pathway molecules involved in otic development. One key signalling molecule is bone morphogenetic protein 4 (BMP4), required for the specification of non-neural ectoderm (presumptive otic epithelium), which must be supplied to cells at the appropriate concentration to avoid unwanted differentiation towards neuronal (low BMP4) or surface ectodermal (high BMP4) fates. It is known that the amount of BMP4 required by cells for efficient otic induction must therefore be optimised for each hiPSC line. We differentiated hiPSCs towards otic cell fate using different concentrations of BMP4 to identify its optimal concentration for efficient otic sensory induction. Using qPCR and immunocytochemistry analyses, we observed the effects of different concentrations of BMP4 on otic cell fate specification in different hiPSC lines. Additionally, by using Raman spectroscopy, we detected the effects of different concentrations of BMP4 on fate specification based on the biochemical properties, composition, and amount of constituent molecules of cells undergoing early otic differentiation. This study confirms that Raman spectroscopy could discriminate between hiPSC otic derivatives with different proliferative and differentiation abilities, suggesting its further assessment for non-invasive label-free screening, identification, and isolation of otic sensory progenitors for inner ear cell-based strategies for hearing loss.

Funding Source: Supported by the Fondation Pour L'Audition (FPA), N° FPA RD-2022-8

Keywords: otic induction, otic sensory progenitors, Raman spectroscopy

4:45 PM – 5:45 PM

322

DERIVATION OF ACCESSIBLE, FULLY CONSENTED AND SEQUENCED HUMAN ES CELL LINE

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Human ESCs remain the gold standard of human pluripotency. However, despite their invaluable contribution to science and their broad distribution, there is a growing need for new hESC lines with up-to-date consents, culture conditions used for derivation guaranteeing genome stability, authorization to sequence the genome and banking at low passage. Here, we present our strategy to generate new hESC lines, fully sequenced, with consents allowing the generation of integrated stem cell-based embryo models. We also present methods to reprogram our primed hESC into naive hESCs and trophoblast stem cells, giving the opportunity to study pre- and post-implantation. Those hESCs will be deposited in cell banks and will be available to the scientific community. The "Infrastructure Organoid de Nantes" will use those lines as control lines and will publish their differentiation capacities in intestinal, heart, liver, retinal, muscular and kidney organoids.

Funding Source: Région Pays de la Loire

Keywords: hESC, hTSC, hNESC

4:45 PM – 5:45 PM

324

DEVELOPMENT OF A CLASSIFICATION PIPELINE FOR HUMAN PLURIPOTENT STEM CELLS

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Existing computational approaches for assessing induced pluripotent and embryonic stem cell (iPS and ES) lines rely on microarray technology. We developed a computational platform that is based on next-generation sequencing transcriptomics to evaluate the pluripotency and differentiation efficiency of iPS and ES cell lines. Specifically, we have generated a unified dataset of 40,000 published transcriptome libraries comprising iPS and ES cells, primary tissues, tumors, and cancer cell lines from multiple sources. Leveraging machine learning we developed a classifier that distinguishes iPS cell transcriptomes from other cell types, and, crucially, identifies cancer type specific gene expression patterns. We found that passage number, age, and sex minimally impact gene expression variance in pluripotency, while tissue of origin and technical factors contribute more significantly. Our pipeline provides an efficient and cost-effective quality assessment tool for ES cells, iPS

cells, and subsequent genetic editing and other manipulations utilized in research and manufacturing for clinical applications.

Keywords: transcriptomics, machine learning, pluripotency

4:45 PM – 5:45 PM

326

SELECTIVE TRANSLATION ORCHESTRATES KEY SIGNALING PATHWAYS IN PRIMED PLURIPOTENCY

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Pluripotent stem cell identities, such as differentiation and infinite proliferation, have long been understood within the frameworks of transcription factor networks, epigenomes, and signal transduction, yet remain unclear and fragmented. Directing attention toward translational regulation, as a bridge between these events, promises to yield new insights into previously unexplained mechanisms. Our functional CRISPR interference screening-based study revealed that EIF3D maintains primed pluripotency through selective translational regulation. The loss of EIF3D disrupts the balance of pluripotency-associated signaling pathways, impairing primed pluripotency. Moreover, we discovered that EIF3D ensures robust proliferation by controlling the translation of various p53 regulators, which maintain low p53 activity in the undifferentiated state. Therefore, this study establishes a paradigm for selective translational regulation as a defining feature of primed pluripotent stem cell identity.

Keywords: primed pluripotency, EIF3D, translational regulation

4:45 PM – 5:45 PM

328

CCN3, POSTN, AND PTHLH AS POTENTIAL KEY REGULATORS OF GENOMIC INTEGRITY AND CELLULAR SURVIVAL IN IPSCS

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Reprogramming human somatic cells into a pluripotent state, achieved through the activation of well defined transcriptional factors known as OSKM factors, offers significant potential for regenerative medicine. While OSKM factors are a robust reprogramming method, efficiency remains a challenge, with only a fraction of cells undergoing successful reprogramming. To address this, we explored genes related to genomic integrity and cellular survival, focusing on iPSCs (A53T-PD1) that displayed enhanced colony stability. Our investigation had revealed three candidate genes CCN3, POSTN, and PTHLH that exhibited differential expression levels and potential roles in iPSC stability. Subsequent analyses identified various protein interactions for these candidate genes. POSTN, significantly upregulated in A53T-PD1 iPSC line, showed interactions with extracellular matrix components and potential involvement in Wnt signaling. CCN3, also highly upregulated, demonstrated interactions with TP53, CDKN1A, and factors related to apoptosis and proliferation. PTHLH, while upregulated, exhibited interactions with CDK2 and genes involved in cell cycle regulation. RT-qPCR validation confirmed elevated CCN3 and PTHLH expression in A53T-PD1 iPSCs, aligning with RNA-seq findings. These genes' roles in preserving pluripotency and cellular stability require further exploration. In conclusion, we identified CCN3, POSTN, and PTHLH as potential contributors to genomic integrity and pluripotency maintenance in iPSCs. Their roles in DNA repair, apoptosis evasion, and signaling pathways could offer valuable insights for enhancing reprogramming efficiency and sustaining pluripotency. Further investigations are essential to unravel the mechanisms underlying their actions.

Keywords: iPSCs, ESCs, transcription factors, genomic integrity, cellular survival

4:45 PM – 5:45 PM

330

SYSTEMIC LIPIDS IMPACT EX VIVO CELL REPROGRAMMING INTO PLURIPOTENCY BY METABOLIC AND EPIGENETIC LANDSCAPE TRANSITIONS

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Cell reprogramming into induced pluripotent stem cells (iPSCs) is an attractive cell-based regenerative strategy, however, it still lacks efficiency as several barriers need to be overcome. Apart from bioenergetics, metabolites are emerging as key regulators of epigenetic events and gene expression programs driving stem cell fate decisions. In particular, glucose and lipids metabolism greatly impact histone acetylation and methylation. Despite its importance, our understanding of how systemic nutrition impacts metabolic and epigenetic determinants in cell reprogramming into pluripotency remains elusive. Here, we generated iPSCs from mouse adult skin fibroblasts that have been subjected to differential lipid dietary regimens and are characterizing the metabolic and chromatin transitions accompanying systemic lipids impact in ex vivo cell reprogramming. We observed a notable increase in body weight, serum glucose and triglycerides in animals subjected to high-fat (HFD), compared to low-fat (LFD) and control (CD) diet. Ex vivo primary fibroblast cultures, established from the adult mouse ear pinnae, presented lower expression of "activated fibrotic" markers in HFD and LFD-derived fibroblasts. Four-factor reprogramming (OKSM, Oct3/4, Klf4, Sox2, c-Myc) revealed decreased efficiency of iPSCs generated from HFD and LFD compared to CD adult skin fibroblasts, as quantified by alkaline phosphatase and expression of the early reprogramming marker SSEA1 at day 14. iPSCs expanded colonies could be maintained in the absence of feeders, with HFD-derived iPSCs presenting higher NANOG expression, increased levels of acetylated Histone H3 and more lipid droplets. Importantly, both LFD- and HFD- derived iPSCs produce teratomas with representation of the three developing layers and a clear enrichment in mesoderm lineage (immature bone tissue), compared to control teratomas. Our results suggest that systemic lipid exposure modulates ex vivo cell reprogramming into pluripotency and cell lineage commitment by long-term impact in cellular metabolism and epigenetics, which can be exploited for regenerative biology.

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Keywords: systemic lipids, iPSCs reprogramming, epigenetics

4:45 PM – 5:45 PM

332

HOW TO FIX YOUR STEM CELLS: RESETTING ABERRANT EPIGENETIC SIGNATURES IN PLURIPOTENT STEM CELLS RESTORES UNIVERSAL DIFFERENTIATION COMPETENCY

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Pluripotent stem cells (PSC) present unprecedented opportunity to study fate decision milestones that define development. Here we focus on how epiblast decides whether to become brain or body, and how fluctuations around this decision point in in vitro culture conditions affect developmental potential of human pluripotent stem cell lines. We selected a panel of human PSC lines experimentally confirmed to be either competent or non-competent to differentiate into cerebral organoids (CO) under unguided conditions. These lines were then characterised by proteomics, RNAseq, ATACseq and CUT&Tag. We identified a number of changes in chromatin accessibility, activity of pathways involved in cell adhesion and spreading, and metabolism in non-competent lines, which together suggested a posterior epiblast phenotype incompatible with cerebral differentiation. In some cells CO competency could be restored by culture on optimised coating matrix, richer culture medium or with canonical and non-canonical Wnt signalling inhibition. Cell lines resistant to modification of culture conditions could acquire cerebral competency by epigenetic re-setting with small molecules targeting chromatin modifiers. The re-set cells remained pluripotent, could reproducibly generate COs and differentiate into mesendoderm derivatives with the same or higher efficiency. We postulate that due to intrinsic variability or suboptimal culture conditions, PSCs acquire a range of developmental states characterised by specific epigenetic changes, some of which are incompatible with brain fate. Wnt inhibition maintains the most pluripotent state of primed stem cells and points towards how Wnt signalling determines the fate of the epiblast to become brain or body.

Keywords: epigenetics, epiblast, pluripotent stem cells

4:45 PM – 5:45 PM

334

A NOVEL ROUTE TO NAÏVE PLURIPOTENCY: THE AMPK-p38 AXIS IN HUMAN PLURIPOTENT STEM CELLS

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Human pluripotent stem cells (hPSCs) primarily exist in two stages of pluripotency: the 'naïve' and 'primed' stages. Representative pluripotent stem cells such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are typically found in the primed state. While various methods for inducing the naïve state have been reported, the comprehensive mechanisms behind this conversion have yet to be fully elucidated. This study revealed that activating a single pathway, specifically the AMPK pathway and its downstream target

p38, can induce conversion to the naïve state. Introduction of AMPK activators, such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) or metformin, or the use of a constitutively active p38 (CA-p38) in primed hPSCs, facilitated this conversion. These cells displayed characteristics of the naïve state, including specific gene and protein expression profiles, with changes in epigenetic status and mitochondrial activity being indicative of this state. Through the use of single-cell RNA sequencing and RNA velocity assays, the study documented the process of conversion, noting that intermediate cells during the transition exhibited features such as pluripotency markers, genes associated with naïve state induction, and differentiation inhibitory genes. These observations provide insights into the mechanism of reversion to the naïve state, offering valuable perspectives for further exploration of the naïve conversion process. Given the naïve state's association with lower levels of DNA methylation and the potential of DNA demethylation as an approach for rejuvenation, the roles of AMPK and metformin, already noted in rejuvenation research for their implications in promoting DNA demethylation, underscore the significance of this study. This suggests that AMPK activation and metformin could serve as a novel strategy in the pursuit of cellular rejuvenation and regeneration.

Keywords: human pluripotent stem cells, naïve pluripotency, AMPK-p38

4:45 PM – 5:45 PM

336

DECODING THE INFLUENCE OF AGING AND OBESITY ON A-TO-I RNA CODES IN IPSC FORMATION

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The conversion of differentiated cells into induced pluripotent stem cells (iPSCs) represents a promising avenue in regenerative medicine and serves as a valuable tool for investigating the molecular mechanisms underlying cell fate transitions. However, various factors, including cellular age and environmental variables such as dietary habits, notably diet-induced obesity, can significantly influence the efficiency of this reprogramming process and have long-term implications for the characteristics of resulting iPSCs. While our previous research has underscored the pivotal role of adenosine-to-inosine (A-to-I) RNA modification in determining cell identity during reprogramming, the specific mechanisms by which aging and obesity affect cell fate changes via A-to-I RNA alterations remain incompletely understood. In this study, we addressed this gap by employing an ex vivo mouse system combined with transcriptomic analysis to identify molecular signatures associated with A-to-I RNA modifications influenced by dietary changes during cellular aging. Notably, our approach revealed several RNA molecules encoding known key facilitators and barriers of reprogramming, whose editing patterns mediated by ADAR enzymes are altered in contexts of aging and obesity. Our findings contribute to a deeper understanding of the molecular mechanisms underlying cell fate transitions during induced pluripotency, particularly in the context of aging and obesity. By elucidating the impact of A-to-I RNA modifications on reprogramming efficiency, our study offers insights into potential strategies for optimizing iPSC generation and highlights the importance of considering environmental factors in regenerative medicine approaches.

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Keywords: induced pluripotency, RNA modifications, environmental factors

4:45 PM – 5:45 PM

338

GENERATION AND CHARACTERIZATION OF CLINICAL GRADE HUMAN MASTER CELL BANKS OF INDUCED PLURIPOTENT STEM CELLS (IPSC) WITH HIGH EXPANSION POTENTIAL

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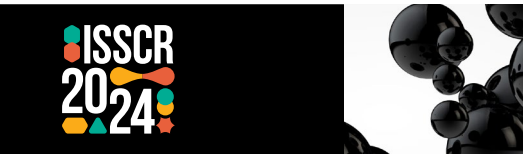
CiTHERA (Center for iPSC Therapies) is a state-of-the-art cGMP facility dedicated to generate the French clinically-compliant iPSC master cell banks from healthy haplotyped donors. We aim also to genetically modify iPSC clones as a platform to produce off-the-shelf cells of therapeutic relevance such as natural killer (iNK) cells, Macrophages(iMac) and T lymphocytes (iT) for the design of CAR therapies in oncology. To this end, we have developed cGMP-compatible protocols for the reprogramming of starting materials from healthy donors: (1) CD34+ cells from cord blood units, (2) total adult leukapheresis from platelet donors, (3) CD3+lymphocytes from peripheral blood. We used the CytoTune-iPS 2.1 Sendai Kit for reprogramming and the transduced cells were seeded on Biolaminin 521 Clinical Therapy Grade (CTG) coated dishes supplemented with Essential 8 Flex Medium. We generated and characterized 6 iPSC line (Cith-01-06). Five clones from each line were manually



selected based on the morphology, and expanded at low passages. PCR was performed to prove the absence of residual Sendai virus vectors. Clones free of Sendai virus genome was analyzed by karyotype and 1 clone per line was selected for full characterization and producing a Master Cell Bank. This was performed using the CliniMACS Prodigy®, as an automated cell feeding and harvesting closed system platform integrating GMP-compliant cell processing. After 14 manually in vitro cell passages, 1.106 iPS clone were plated into CliniMACS Prodigy® and expanded over 2 weeks using the StemMACs iPS-Brew XP Medium in 5 Cell-Stack dishes on coated by human recombinant laminin (L521-MX, Bio Lamina). After two passages, a final batch of 1.4 109 iPS cells was collected at the end of the process. This scalable protocol allowed the maintenance of genetic stability as showed by cytogenetic analyses as well as exome sequencing and pluripotency markers. The pluripotency was also shown by an efficient differentiation toward endodermal, mesodermal and ectodermal tri-lineage layers. Overall, this closed 2D-culture process is a safe and standardized scalable manufacturing platform for cGMP-grade iPSC expansion and can also be suitable for the generation of CAR-iPSC and iPSC-derived immune cells.

Funding Source: INGESTEM, ANR, INSERM, UNIVERSITY PARIS SACLAY, VAINCRE LE CANCER

Keywords: cGMP IPSC, manufacturing, reprogramming

4:45 PM – 5:45 PM

340

DISSECTING NODAL SIGNALLING IN ENDODERM SPECIFICATION USING MOUSE EMBRYONIC STEM CELLS

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The Nodal signalling pathway, mediated by complexes of SMAD2, SMAD3, and SMAD4 transcription factors (TFs), plays a crucial role in determining the balance between mesoderm and endoderm specification during early vertebrate embryogenesis. To elucidate their specific roles in eliciting Nodal responses and their crosstalk with other signalling pathways, I utilized CRISPR to knock out (KO) each SMAD gene in mouse embryonic stem cells (mESCs), followed by in vitro differentiation into anterior definitive endoderm (ADE). Results from qPCR, flow cytometry, and immunostaining revealed the critical importance of SMAD2 and SMAD4 in in vitro ADE differentiation, whilst SMAD3 appeared to be redundant. Next, to explore the transcriptional networks governing differentiation in wild type versus SMAD2-, SMAD3-, and SMAD4-null mESC lines, time course differentiation samples were subjected to single-cell RNA sequencing. Preliminary analysis indicated that besides their role in ADE differentiation, SMAD2 and SMAD4 play crucial roles in priming pluripotent mESCs for in vitro differentiation, with their absence leading to the emergence of new cell types dependent on retinoic acid and bone morphogenic protein. Additionally, analysis revealed a developmental delay in SMAD3 KO differentiation, suggesting, for the first time, its involvement in early development. Subsequent pseudotime trajectory analysis identified the key TFs and signalling pathways involved. Future experiments will entail chromatin profiling and assessment of SMAD enhancer binding to delineate the individual roles of each SMAD.

Keywords: nodal signalling, in vitro differentiation, endoderm specification

4:45 PM – 5:45 PM

342

DECIPHERING THE MECHANISMS OF DNMT3A RECRUITMENT TO SPECIFIC TARGET SITES: IMPLICATIONS FOR DE NOVO DNA METHYLATION IN DIRECTING CELL DIFFERENTIATION

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DNA methylation regulates gene expression and cellular identity during development. DNMT3A is a key player in establishing methylation patterns, particularly during early embryonic development and cellular differentiation. However, the precise targeting and recruitment of DNMT3A to specific genomic loci in different cell fates are poorly understood. This project aims to uncover the mechanisms behind DNMT3A recruitment to distinct target sites and its role in directing stem cell differentiation through DNA methylation. We hypothesize that DNMT3A's recruitment is facilitated by interactions with specific protein factors, such as transcription factors and histone marks. These interactions orchestrate diverse gene expression patterns, guiding cell differentiation. By employing tandem affinity purification with mass spectrometry, we identified cell fate-specific factors for further analysis. Genome-wide approaches, including CUT&RUN, CUT&TAG, and DNA methylation profiling, will investigate DNMT3A's binding patterns and its partners across various cellular contexts. Integrative analysis of the data may reveal co-binding events and enriched motifs at target sites. Cryo-EM techniques will be used to elucidate the structure of the DNMT3A protein complex and gain insights into its recruitment mechanism. Disrupting DNMT3A interactions based on structural information in different cellular contexts will allow us to examine the functional consequences. We will analyze gene expression profiles and DNA methylation patterns to determine if disruption leads to abnormal methylation landscapes and subsequently affects lineage-specific gene expression and cell differentiation. In summary, this study sheds light on the mechanisms of DNMT3A recruitment to specific target sites in different cell fates during stem cell differentiation. Understanding these mechanisms holds promise for manipulating cellular identity and advancing regenerative medicine and disease modeling.

Keywords: DNMT3A, DNA methylation, cell fate decision

4:45 PM – 5:45 PM

344

THE KEY COMPONENTS OF THE PLURIPOTENT MICROENVIRONMENT

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Human pluripotent stem cells (hPSCs) can be obtained from the inner cell mass of early-stage embryos or reprogrammed from somatic cells. The ability of these unique cells to differentiate into every cell type in the human body and self-renew indefinitely has led to their irreplaceable use in regenerative medicine, drug screenings, and disease modeling. Although the main factors that induce and maintain pluripotency have been known for many years, there are still large gaps in our knowledge about pluripotency regulation. HPSCs have a unique microenvironment that guides cell fate, adhesion, proliferation, survival, and differentiation. To maintain pluripotency, hPSCs must communicate with other cells, which could be mediated via active secretion. However, the secretome of hPSCs has not been studied using modern techniques that could provide new information on the regulatory factors of stemness. This project aims to fill the gaps with improved techniques and offer an extensive proteomic resource of hPSC-specific proteins. We have successfully generated exclusive data on the endogenously produced total and secreted proteins of hPSCs using the Stable Isotope Labelling by Amino acids in Cell culture (SILAC) method. SILAC was chosen for proteomic analysis because it is quantitative, unbiased, accurate, reproducible, and particularly suitable for studies involving subcellular fractionation and extracellular proteins. SILAC labeling also allows the discrimination of endogenous proteins from those of the culture media and matrix. Moreover, we have combined the proteomics data with deep DNA/RNA sequencing from the same samples, which allows the proteogenomics approach and the discovery of novel transcripts. Our preliminary data shows that hPSCs have multiple uncharacterized pluripotency-associated factors and unique and active secretion of proteins.

Keywords: human pluripotent stem cells, secretome, SILAC

4:45 PM – 5:45 PM

346

GENERATION AND CHARACTERIZATION OF INTERSPECIES WILD TYPE CHIMERIC EMBRYOS DURING DEVELOPMENT

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Organ demand currently outpaces the supply of organs for transplantation. Regenerative medicine has sought alternative methods to generate an inexhaustible source of organs and cells. One approach to generate exogenic organs and cells in chimeric models is blastocyst complementation. However, developing highly efficacious, competent, and gene specific intraspecies and interspecies chimeras has been challenging and the processes by which donor cells contribute or fail to contribute to various tissues is under active investigation. One area currently lacking robust data is analysis of cell types formed in wild type chimeras. Our study aims to characterize various cell types that are generated by the donor cells in rat-mouse and mouse-mouse wild type chimeras. We have found that GFP expressing donor cells have a widespread distribution throughout the developing chimeric embryo, with the latest collection date of E12.5. Notably, GFP-positive cells colocalized with cellular markers for microglia and macrophages in both intraspecies and interspecies chimeras. Preliminary findings showed that microglia were 20% and peripheral macrophages were 5% of the GFP-positive cells in intraspecies chimeric brains, while microglia were 1.3% and peripheral macrophages were about 0.3% of the GFP-positive cells in the interspecies chimeric brains. We also observed co-localization of GFP with hepatocytes and cardiac muscle cells. These findings suggest that blastocyst complementation is a novel tool that can generate a variety of cells without specification through genetic modification. It may also provide insight into cell types that are more susceptible to generation by surviving and proliferating donor cells during the blastocyst complementation process.

Funding Source: This work is supported by NIH Grant: R01-AI173804 (CFS and WLC).

Keywords: blastocyst complementation, interspecies chimeras, embryonic development

4:45 PM – 5:45 PM

348

CHARACTERIZING THE VARIABILITY IN ORGANIZATION AND SIZE AND SHAPE DYNAMICS OF THE HIPSC NUCLEUS

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The Allen Institute for Cell Science aims to understand the principles by which human induced pluripotent stem cells (hiPSCs) establish and maintain robust dynamic localization of cellular structures, and how they transition between states during differentiation and disease. We are taking an integrative approach to characterize the hiPSC nucleus across interphase in growing epithelial colonies by using live cell imaging together with genomic measurements. We previously generated gene-edited hiPSC lines that each express an endogenous, monoallelic EGFP-tagged protein, targeting 17 different nuclear proteins that range from major landmark structures (e.g. speckles and nucleoli) to specific chromatin loci (e.g. telomeres and DNA replication foci; see the Allen Cell Collection at www.allencell.org). Using these lines, we collected thousands of high resolution 3D images of single cells and adapted our previously developed computational analysis approach to map the average locations of 10 nuclear structures within the nucleus. We find clear patterns in the radial organization of these structures: nucleoli are most central with nuclear speckles localized outside of them; histones show enrichment at the periphery and near nucleoli but exclusion from the speckle-occupied region. Interestingly telomeres appear to be uniformly present except where peripheral histone enrichment occurs. To investigate nuclear dynamics, we developed an approach to extract high resolution nuclear segmentations from low resolution (low phototoxicity) 3D images of lamin B1 enabling 48 hr time-lapse imaging and tracking of nuclei across growing colonies of hiPSCs. We find that size, shape and growth dynamics of a nucleus depend on its initial size, its location within the colony, and the size of the colony. By analyzing lineage relationships (e.g. mother-daughter pairs or sister-sister pairs), we find that different aspects of nuclear growth (e.g. interphase duration, growth rate) show different strengths of heritability. We are using these findings, approaches and data as a basis for integrating with genomics datasets in collaborations across the 4D Nucleome Network and explorations of differentiation with a larger goal of understanding the functional consequences of the variability.

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Keywords: nucleus, lineage, imaging

4:45 PM – 5:45 PM

350

A SWITCH OF EXPRESSION OF TWO TRANSCRIPTION FACTORS REGULATES CELLULAR PLASTICITY DURING REPROGRAMMING AND TRANSFORMATION

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The generation of induced pluripotent stem cells (iPSC) by reprogramming and of cancer cells by oncogenic transformation are both multistep processes during which coordinated changes of cellular plasticity and identity occur. However, the molecular circuitries involved in both processes, their degree of analogy and of functional interplay remain poorly characterized. We are therefore interested in identifying factors that contribute to cellular identity maintenance and cellular plasticity acquisition during both processes. Studying the reprogramming and transforming roadmaps that lead to cellular identity loss in mouse embryonic fibroblasts (MEF) we have identified two candidate genes, Bcl11a and Bcl11b, previously described as cell fate regulators in immune cells. This study therefore aims to understand how these two transcription factors, subunits of the SWI/SNF complex, could be involved in regulating cellular identity and plasticity (i) and to identify the molecular mechanisms implied (ii). During the process of OSKM mediated reprogramming and oncogenic KRas triggered transformation, we showed that cellular identity loss in early intermediates is correlated with a switch in the expression of Bcl11b and Bcl11a. Moreover, using loss-of-function approaches, we revealed that the modulation of this balance controls the emergence of iPS and transformed cells. Furthermore, our scRNAseq analysis show that Bcl11a depleted cells do not undergo the reprogramming trajectory by not transiting from mesenchymal to epithelial state. We have therefore explored the redistribution on chromatin of Bcl11a and Bcl11b during reprogramming and transformation using ChIP seq analysis and have further investigated their interactome using immunoprecipitation mass spectroscopy analysis. Our data unveil that Bcl11a expression and redistribution in cellular intermediates constrains senescence contributing to reprogramming regulation. Altogether, this study provides insights into cellular identity and plasticity regulation for both regenerative and cancer biology.

Keywords: cellular identity, cellular plasticity, epigenetics, transcription factors, pluripotent reprogramming, oncogenic transformation iPSC, senescence

4:45 PM – 5:45 PM

352

10 YEARS OF REPROGRAMMING IN NANTES

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The hiPSC core facility of Nantes Université was created in 2012, with the task to provide hiPSC across France. Here, we present a compilation of the 200 patient lines that we reprogrammed across 10 years, discussing the reprogramming efficiency, the reprogramming pipelines and the QC results. We will specifically emphasize on genomic stability of the reprogrammed lines and rate of Sendai virus clearance. We will also present the perspective of improvements of our reprogramming pipeline.

Funding Source: Region Pays de la Loire

Keywords: reprogramming, hiPSC, genomic stability

4:45 PM – 5:45 PM

354

MLL1-MENIN COMPLEX IS A BARRIER TO SOMATIC CELL REPROGRAMING AND RESETTING TO NAÏVE STATE

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Expression of OCT4, SOX2, KLF4 and MYC (OSKM) can reprogram somatic cells to pluripotency that involves complete reset of the somatic cell identity. However, chromatin-based mechanisms that safeguard cellular identities act as barriers to reprogramming, resulting in low efficiency of cell fate conversions. To identify such barriers, a loss of function genetic screen was performed and MLL1 (KMT2A) was identified as a roadblock for reprogramming. In this work, we employed chemical and genetic approaches to characterize the effects of MLL1 in complex with MENIN on reprogramming of human somatic cells. First, we validated the barrier function of this complex via utilizing an inhibitor, VTP50469, that selectively targets MLL1-MENIN complex, which led to increased reprogramming efficiency. Next, we generated, through CRIPSR/Cas9, MLL1 and MEN1 knockout fibroblasts which displayed increased reprogramming efficiency. Combining DOT1L inhibition with VTP50469 treatment or MEN1 depletion did not further enhance reprogramming efficiency suggesting that these two chromatin factors may act through similar pathways. RNA-sequencing results showed that, several pluripotency genes which are usually expressed in late reprogramming were up-regulated and a large set of fibroblast-specific genes were down-regulated upon MENIN inhibition or depletion. The supplementation of the naive culture conditions with VTP50469 enhanced the induction of naive pluripotent stem cell markers suggesting that this complex acts as a barrier also during the resetting primed cells into naive state. Taken together, the results represented herein shows that MLL1-MENIN complex imposes barriers in the acquisition of cell identities of higher developmental potential.

Funding Source: This work was supported by TUBITAK grant 321S059.

Keywords: Mll1/Menin complex, naive pluripotency, reprogramming

4:45 PM – 5:45 PM

356

NSD1 AND SETD2 DEFICIENCY IN IPSCS FOR STUDYING H3K36-GUIDED DNA METHYLATION

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Interactions between histone modifications and DNA methylation might play a central role in regulation of the aging process. For example, dimethylation of H3K36 occurs predominantly in intergenic regions and seems to recruit DNMT3A, whereas trimethylation of H3K36 colocalizes with DNMT3B in active gene bodies. The main H3K36 histone methyl transferases are the nuclear receptor binding SET domain protein 1 (NSD1) and SET domain containing 2 (SETD2). Haploinsufficiency of NSD1 results in the overgrowth disorder SOTOS syndrome, which revealed aberrant DNA methylation signatures and an enhanced epigenetic age in the Horvath clock. Since the impact of H3K36 methylation in epigenetic mechanisms of aging is poorly understood, we aim for a better understanding of the interplay between methylation of H3K36 and age-associated DNA methylation. To this end, we generated knockout cell lines of NSD1 and SETD2 using CRISPR-Cas9 technology. The knockout was validated by Sanger Sequencing. Preliminary experiments indicated that cells modified in the PWWP domain downstream of the nuclear receptor interaction domains of NSD1 have a slower growth. Notably, modifications upstream of this PWWP domain did not affect growth and morphology. Immunofluorescence analysis indicated that NSD1 seems to relocate predominantly to the nucleus in NSD1 knockout cell lines. Deletions of 12 bp within the Set2 Rpb1 interacting domain of SETD2 seem to accelerate differentiation, as indicated by decreased Oct4 staining and morphological changes. We are currently further analyzing a potential differentiation bias with Pluripotency Screen, an epigenetic signature to track early germ layer specification. Taken together, we have successfully established iPSC lines with knockout of NSD1 and SETD2 and we will evaluate how this affects cellular differentiation, the epigenetic sequel and DNA methylation during aging.

Keywords: H3K36 methylation, induced pluripotent stem cells, CRISPR-Cas9

4:45 PM – 5:45 PM

358

DELETION OF GLUCOSYLCERAMIDE SYNTHASE IN HUMAN INDUCED PLURIPOTENT STEM CELLS ALTERS PLASMA MEMBRANE LIPID COMPOSITION

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Glycosphingolipids (GSLs) form a heterogeneous group of glycosylated membrane lipids, which are involved in basic cellular processes and can be associated with several diseases. GSLs are not essential at the level of embryonic stem cells but are required during development of multicellular organisms. Knowledge about the function of individual GSLs during the process of differentiation is scarce. To gain further insights, we generated CRISPR/Cas9 knockouts (KOs) of the first acting glucosyltransferase of GSL biosynthesis, glucosylceramide synthase (UGCG), in human induced pluripotent stem cells (hiPSCs). Analysis of GSL glycosylation by multiplexed capillary gel electrophoresis coupled to laser induced fluorescence detection (xCGE-LIF), clearly demonstrated the functional impairment of UGCG. Initial characterization of UGCG-KOs by morphology, growth behavior and stem cell marker expression did not reveal any anomalies when compared to WT hiPSCs. Differentiation into early progenitors of meso-, endo-, and ectoderm and analysis of differentiation efficacy based on characteristic cell surface markers resulted in comparable degrees of positive cell populations in UGCG-KO lines when compared to WT. For in vivo assessment of pluripotency, we performed teratoma analyses, which clearly confirmed pluripotency of the tested UGCG-KO hiPSC line. Comparative quantitative proteomic profiling of the explanted teratoma revealed 295 significantly regulated proteins between WT and UGCG-KO derived teratoma. As UGCG impairment likely interferes with lipid homeostasis, we performed global lipidomic analyses by MS to assess the impact of UGCG deletion on the plasma membrane lipidome of hiPSCs. This analysis revealed that sphingomyelin levels are significantly increased in mutant hiPSCs. Taken together, UGCG-KO hiPSCs hold great potential as a model system for the identification of specific GSL functions.

Funding Source: This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for FOR2953 (projectnumber: 409784463; project P9; BU 2920/4-2).

Keywords: glycosphingolipids, xCGE-LIF, membrane lipidome

4:45 PM – 5:45 PM

360

GENERATION OF A BIOACTIVE RECOMBINANT ZSCAN4 PROTEIN FOR UNDERSTANDING ITS ROLE IN REPROGRAMMING

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The reprogramming of somatic cells to generate induced pluripotent stem cells (iPSCs) has revolutionized the field of regenerative medicine. However, even after two decades, iPSCs are yet to be employed widely in therapy. Some of the key hurdles include the formation of transformed colonies, the generation of genetically unstable iPSCs, and so forth. A potential transcription factor, ZSCAN4 has been shown to be one of the promising candidates for overcoming these barriers. It has been reported that ZSCAN4 when included in the reprogramming cocktail of transcription factors, enhanced reprogramming efficiency, reduced single nucleotide polymorphisms in the iPSCs produced, and generated genetically stable mouse iPSCs. Additionally, inclusion of ZSCAN4 resulted in longer telomeres in the mouse iPSCs generated, thereby, maintaining a stable karyotype even in later passages. Our study aims to reveal the mechanistic role of ZSCAN4 in reprogramming of human fibroblasts. Firstly, we generated a recombinant ZSCAN4 protein, which has been expressed and purified under native conditions from a bacterial system with maximum purity. Secondly, the characterization of purified ZSCAN4 protein using Western blotting and Circular Dichroism spectroscopy was performed. Additionally, the influence of various factors such as storage buffer, storage temperature and presence of animal-derived products like fetal bovine serum, on the stability and solubility of the purified ZSCAN4 protein was carried out. Thirdly, we have validated the transduction ability and the functionality of the purified protein in mammalian cells using various assays. This functionally active, purified ZSCAN4 protein can be used in a time- and dose-dependent manner in the reprogramming process, to assess its stage-specific role. In addition, we have generated ZSCAN4 knockout human fibroblasts using a CRISPR-Cas9 toolbox. Further, we plan to employ these ZSCAN4-/- fibroblasts in reprogramming to further gain an in-depth knowledge on the vitality of ZSCAN4 during the reprogramming process. Therefore, this study will give insights into the importance of ZSCAN4 in reprogramming and maintenance of iPSCs. This will contribute to the generation of safer, clinical-grade iPSCs, which can eventually be utilized for therapeutic applications.

Keywords: reprogramming, CRISPR/Cas9, ZSCAN4

4:45 PM – 5:45 PM

362

METABOLIC SHIFTING IN THE TRANSITION FROM ADHERENT TO SUSPENSION CULTURES OF HUMAN PLURIPOTENT STEM CELLS

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Human pluripotent stem cells are traditionally grown in an adherent monolayer culture on matrix-coated tissue plastic (2D culture). Stem cells cultured in 2D culture systems are useful for small-scale differentiation and compound-screening. Suspension culture (3D culture) systems, on the other hand, offer greater scalability for generating large numbers of cells and are more compatible for industrial production of cell-replacement therapies. In 3D cultures, pluripotent stem cells spontaneously from aggregates; however, we have a limited understanding of the changes caused by the transition from 2D to 3D cultures. Therefore, we aim to understand the molecular changes that occur during this process and to determine parameters that influence the formation for 3D aggregates. To this end samples for proteomics and bulk RNA sequencing was collected at the earliest timepoints (6, 24, and 48hr) after inoculating cells into either 2D or a small-scale 3D culture system. Canonical pluripotency markers were highly expressed at all times; however, 3D cultures showed longer recovery compared to 2D. Using Gene Set Enrichment analysis on both proteomics and RNA sequencing, data suggested alteration in focal adhesion and gap junctions between 2D and 3D cultures. Interestingly, proteomic data revealed a shift from oxidative phosphorylation in 2D to a more glycolytic driven metabolism in 3D cultured stem cells. We extended this data by demonstrating increased Lactate production in 3D cultures as well as differentially expressed PDH and LDH protein levels assessed by SDS-PAGE/Western blot. Interestingly we observed increased OCR and ECAR in 3D cultures using the Seahorse XF analyzer. Future research will evaluate the metabolic shifting across human pluripotent stem cell lines, media, and 3D culture systems. Our data highlights significant changes in adhesive molecules and metabolism when transitioning from a 2D to 3D culture systems. Understanding the role of this changes could be used to further optimize 3D culture conditions for human pluripotent stem cells.

Funding Source: This project has been funded by the Innovation Fund Denmark and the Novo Nordisk fellowship program; novoSTAR.

Keywords: human pluripotent stem cells, metabolic shift in the transition from 2D to 3D, scalable culture systems

TOPIC: GERMLINE AND EARLY EMBRYO

364

DNA REPLICATION IN EARLY MAMMALIAN EMBRYOS IS PATTERNED, PREDISPOSING LAMINA-ASSOCIATED REGIONS TO FRAGILITY

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DNA replication in differentiated cells follows a defined program, but when and how it is established during mammalian development is not known. Here we show using single-cell sequencing, that both bovine and mouse cleavage stage embryos progress through S-phase in a defined pattern. Late replicating regions are associated with the nuclear lamina from the first cell cycle after fertilization, and contain few active origins, and few but long genes. Chromosome breaks, which form spontaneously in bovine embryos at sites concordant with human embryos, preferentially locate to late replicating regions. In mice, late replicating regions show enhanced fragility due to a sparsity of dormant origins that can be activated under conditions of replication stress. This pattern predisposes regions with long neuronal genes to fragility and genetic change prior to segregation of soma and germ line. Our studies show that the formation of early and late replicating regions is among the first layers of epigenetic regulation established on the mammalian genome after fertilization.

Keywords: preimplantation development, DNA replication timing, replication stress, replication origin, chromosome breakage

4:45 PM – 5:45 PM

TOPIC: PANCREAS

366

GLUCOSE REGULATED PROTEIN 94 (GRP94) IS INDISPENSABLE FOR ENDODERM FORMATION AND BETA CELL DIFFERENTIATION OF HUMAN INDUCIBLE PLURIPOTENT STEM CELLS

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Human inducible pluripotent stem cell (hiPSC)-derived insulin-producing cell therapy has been used in treating patients with type 1 diabetes and potentially for those with type 2 diabetes. Deciphering the roles of genes impacting hiPSC differentiation could enhance the

efficacy of this process. This study investigated the novel function and mechanisms of glucose-regulated protein 94 (GRP94) in human β cell development. Using a β cell differentiation protocol, bulk RNAseq analysis, and other tools, we sought to decipher the role of GRP94 in regulating the transition from pluripotency to definitive endoderm (DE) using two GRP94 deletion hiPSC cell lines. Our data show that GRP94 depletion specifically prevented the generation of β cells via inhibition of DE differentiation by reduced expressions of key transcriptional factors for DE formation and expression of ER stress-response genes with increased cell apoptosis in DE cells. GRP94 is essential in the regulation of gene expression related to protein processing in the ER, response to ER stress, and other functions of iPSC. Forced expression of GRP94 in knockout iPSCs reversed DE differentiation deficiency and rescued them from cell death. Further mechanistic analysis shows that activation of WNT/ β-catenin signaling pathway was decreased in the GRP94 KO cells with no significant change in TGF-β signaling. Our data support that GRP94 is essential for forming human definitive endoderm from hiPSCs via controlling expressions of critical factors required for DE formation and ER-stress-induced apoptosis; this process was also partly through the WNT/ β-catenin signaling pathway. Because human gastrulation and early lineage commitment cannot be studied in vivo, our study using wild type and GRP94 depleted hiPSCs provides valuable clues for understanding human endocrine and β cell development and may serve as novel tools to prevent pancreatic β cell death in the treatment of diabetes.

Keywords: iPSC, glucose regulated protein 94, endoderm differentiation

4:45 PM – 5:45 PM

368

A MULTIMODAL CROSS-SPECIES COMPARISON OF EMBRYONIC PANCREAS DEVELOPMENT

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Human embryonic development remains incompletely understood due to ethical concerns and practical limitations. Here we investigated whether pigs resemble human pancreas development better than rodents, and as such, might offer a valuable alternative large animal model system. We first annotated major developmental hallmarks and lineage markers of pig pancreas differentiation and morphogenesis throughout the 114-day gestation covering all three trimesters, which is practically not possible in human. Based on these details of pancreas organogenesis, we further generated a pig single-cell multiome atlas with temporal resolution across all three trimesters. Evolutionary comparisons with human and mouse time-resolved and integrated pancreas atlases revealed dynamic changes in progenitor populations and resolved differentiation trajectories and gene regulatory networks (GRNs) governing ductal, acinar and endocrine lineage acquisition. This analysis accentuated a close resemblance of pigs to humans in terms of developmental tempo, epigenetic and transcriptional regulation, gene expression patterns and GRNs. Most notably, we discovered the emergence of β-cell heterogeneity, owing to endocrine induction in pancreas progenitors with altered epigenetic and transcriptional identity evolving over time during pig pancreas development. Taken together, our study provides unprecedented evolutionary insights into pancreas development across species and developmental stages, highlighting conserved and species-specific differentiation principles and mechanisms.

Keywords: embryonic pancreas development, single cell multiomics, large animal model pig

4:45 PM – 5:45 PM

370

DIFFERENTIATION AND MORPHOGENETIC DYNAMICS OF HUMAN PANCREATIC PROGENITORS AND ENDOCRINE PROGENITORS DERIVED FROM PLURIPOTENT STEM CELLS

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The pancreas plays an essential role to control the metabolism of nutrients: endocrine pancreas regulates blood glucose by secreting hormones, such as insulin and glucagon, and exocrine pancreas produces and secretes digestive enzymes to duodenum. During organogenesis, in a coordinated manner, different cell types arise from pancreatic progenitor cells, and differentiated cells organize themselves and evolve into pancreatic morphology, so that the pancreas fulfils its function properly. Animal models, especially the mouse model, provide valuable information and insights on pancreas development, but a number of differences have been found in human compared to mouse. To better understand human endocrine pancreas development, we aimed to elucidate differentiation dynamics of human pancreatic progenitors and early steps of endocrine differentiation using embryonic stem cell-derived pancreatic cells and organoids. By tracing cells at the single cell level through live imaging using reporters in 2- and 3-dimensions, we observed that human cells exhibited slower molecular and cellular dynamics of pancreatic progenitors (PDX1) and endocrine progenitor (NEUROG3), compared to mice, in terms of progenitor renewal time and duration for transient endocrine progenitor expression to reach a peak over time. We also found heterogeneity in the peak levels of NEUROG3 expression among cells further differentiating into hormonal cells. For deeper analysis, we integrated data from live imaging tracking and single-cell transcriptomics by statistically correlating cellular dynamics to transcriptomic signatures based on the intensity of time-matched NEUROG3-reporter in individual cells. This integration revealed that newly emerging NEUROG3 cells in early phase of expression exhibited increased motility, compared to more differentiated cells. Furthermore, our data suggested that this accelerated motility might be regulated by KLK12, a serine protease transiently expressed in early phase of endocrine differentiation. Our study highlights the importance of using human systems to understand pancreatic endocrine differentiation for a better control over endocrine cell production as a therapeutic measures to treat diabetes mellitus.

Keywords: endocrine differentiation dynamics, live imaging, NEUROG3

4:45 PM – 5:45 PM

372

DECIPHERING TRANSCRIPTIONAL REGULATION DURING PANCREATIC DEVELOPMENT THROUGH PERTURB-SEQ AND ENHANCER MAPPING

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Insulin-secreting β cells in pancreatic islets are key regulators of blood glucose homeostasis. Alterations in β cell mass and function are hallmarks of diabetes. To better understand the disease progression and to uncover novel diabetes risk factors, it is important to first understand the developmental control of β cells. Although previous studies have made tremendous progress in identifying pancreatic genes and diabetes associated variants in putative enhancer regions, further studies are hampered by two major challenges: 1) establishing causal relationships between functional enhancers and their target genes, and 2) interrogating the underlying regulatory mechanisms at a network level. To overcome these hurdles, we plan to apply a two-pronged approach in our human pluripotent stem cell (hPSC)-β cell differentiation system. Specifically, we will map enhancers at key differentiation stages followed by pooled CRISPR screens with single-cell RNA sequencing readout (Perturb-seq) to identify functional pancreatic enhancers and their target genes. Meanwhile, we will perform Perturb-seq targeting all annotated TFs (n=1803) to generate gene regulatory networks at each differentiation stage. By integrating information collected from the above two parts, we aim to build a transcriptional regulatory atlas for β cell development. So far, we have generated three dimensional (3D) genomic maps at all key stages during hPSC-β cell differentiation using HiCAR, a newly developed assay that selectively captures high-resolution contacts anchored to open chromatin regions. These maps revealed extensive rewiring of genome-wide promoter-enhancer loops during β cell differentiation, allowing use to identify putative enhancers for β-cell developmental genes (e.g., PDX1 and ONECUT1). Additionally, we have conducted TF Perturb-seq in hPSCs and definitive endoderm (DE) cells. Results from hPSCs unveiled similar transcriptome changes upon perturbations of NANOG, EP300 and TADA2B, suggesting their potential synergetic activities. These initial efforts have proven the feasibility of using Perturb-seq to interrogate transcriptional networks in hPSC-derived β cells. Overall, results from this study will provide mechanistic insights into the transcriptional regulation of β-cell development.

Funding Source: DoD FY21 Peer Reviewed Medical Research Program Discovery Award, W81XWH21-PRMRP-DA

Keywords: β cell development, transcriptional regulatory network, Perturb-seq

4:45 PM – 5:45 PM

374

TARGETING THE CYTOSKELETON AT GUT TUBE STAGE TO ENHANCE BUDDING ORGAN DIFFERENTIATION

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During embryonic development, the blastocyst forms three germ layers: mesoderm, ectoderm, and endoderm. The latter undergoes intricate transformations, forming a gut tube, further regionalizing into foregut, midgut, and hindgut. The anterior foregut differentiates into organs like the liver, gallbladder, pancreas, and lungs through organ budding. Despite numerous efforts to differentiate human pluripotent stem cells (hPSCs) into endoderm-derived organs, challenges persist, with many protocols yielding impure cell populations. While the precise mechanisms governing structural processes during organ development are often overlooked, those occurring during human organ budding remain scarce. Here, we show that a variety of bioactive lipids, including lysophosphatidic acid (LPA) or sphingosylphosphorylcholine (SPC), significantly enhance pancreatic progenitor (PP) formation efficiency to virtual purity. Phase-dependent titration experiments identify a close time window - the gut tube stage during PP formation - being permissive to mediate this PP boost. Further testing found actin cytoskeleton dynamics, specifically its stabilization, as the underlying mechanism. Signaling-wise, we identified the small GTPase RhoA as the PP effect can be reversed upon RhoA inhibition but also by inhibiting the Rho-associated protein kinase (ROCK). We hypothesize that the observed effects on targeting the cytoskeleton are related to organ budding processes. Indeed, RhoA activation only improved PP differentiation when added between gut tube and pancreatic endoderm stages. Additionally, we incorporated RhoA activation in the differentiation of hPSCs into lung progenitors. Similar to pancreatic differentiation, RhoA activation substantially increased differentiation efficiency. Vice versa, during intestinal differentiation, RhoA activation prevented the formation of human intestinal organoids, representing non-budding organs. Future research will involve measuring cell viscoelastic properties and employing bioprinting techniques to create a precise model of PSC-derived gut tube. These approaches will offer innovative insights into the mechanisms underlying organogenesis, allowing for a deeper understanding of the dynamic processes involved in tissue formation and differentiation.

Keywords: pancreas differentiation, cytoskeleton remodelling, bioactive lipids

4:45 PM – 5:45 PM

376

EXPANSION OF VENTRAL FOREGUT PRIMES ENHANCER LANDSCAPE FOR ORGAN-SPECIFIC DIFFERENTIATION

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Cell proliferation is fundamental for almost all stages of development and differentiation that require an increase in cell number as a function of time. Although cell cycle phase has been associated with differentiation, the actual process of proliferation has not been considered as having a specific role. Here we exploit human embryonic stem cell-derived endodermal progenitors that we find are an in vitro model for the ventral foregut (VFG) stem cells, which can be readily transformed to either pancreatic or liver progenitors in 3D culture. VFG stem cells exhibit proliferation-dependent increases in differentiation efficiency to pancreatic progenitors linked to organ-specific enhancer priming at the level of chromatin accessibility and the decommissioning of lineage-inappropriate enhancers. This process depends on the transcription factor FOXA1 which may exploit its mitotic bookmarking activity. Our findings suggest that cell proliferation in embryonic development is about more than tissue expansion; it is required to ensure the equilibration of gene regulatory networks allowing cells to become primed for future differentiation. Expansion of lineage-specific intermediates may be an important step in achieving high-fidelity in vitro differentiation.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) is supported by a Novo Nordisk Foundation grant number NNF21CC0073729. This project is also supported by a Novo Nordisk Foundation grant number NNF21OC0070898

Keywords: cell proliferation, ventral foregut stem cell, enhancer priming

TRACK: SOMATIC STEM CELLS, CANCER AND REGENERATION (SSCCR)

4:45 PM – 5:45 PM

TOPIC: CARDIAC

378

MITOCHONDRIAL DYSFUNCTION AND ELECTROPHYSIOLOGICAL ABNORMALITIES IN SMA PATIENT-DERIVED IPSCS

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Spinal Muscular Atrophy (SMA) is a neurodegenerative disorder characterized by the progressive loss of motor neurons in the spinal cord, leading to muscle weakness and atrophy. While the genetic basis of SMA is well-established, recent research has unveiled the involvement of mitochondrial dysfunction in the pathophysiology of the disease. Some experimental data imply that the determination of the mitochondria dysfunctions at the developmental and progression stages in SMA pathogenesis may contribute to the cardiomyopathy. Therefore, here, we aimed to determine the impacts of SMN gene mutation on mitochondrial functions and electrophysiological properties of human induced pluripotent stem cells (hiPSCs) and hiPSC-derived ventricular cardiomyocytes (iPSC-vCM) from SMA patients. The hiPSCs were isolated from blood samples of SMA patients (0-2 years-of-age) and healthy-control donors. The function of mitochondria was examined by monitoring the reactive oxygen species (ROS) production ([ROS]i), mitochondrial membrane potential (MMP) and ATP in both control and SMA-iPSCs groups by using DCFDA, JC-1 dyes, and Luminescent Cell Viability Assay Kit respectively. Our data showed that cellular [ROS] i production was increased and MMP was significantly depolarized in SMA-iPSCs compared to control-iPSCs. Additionally, an increase in ATP levels has been observed in SMA groups compared to the control groups. iPSCs were differentiated into ventricular cardiomyocytes (iPSC-vCM) and characterized by qRT-PCR and immunofluorescence assays. Following characterization steps, patch-clamp analysis was performed to measure action potential parameters and voltage-dependent Na+, K+ and Ca2+ channel-currents. Our electrophysiological data demonstrated the SMN gene mutation induces changes in ionic mechanisms and electrical activities of iPSC-vCM through marked changes in channel currents. These results suggest that SMA patient-derived iPSCs shown the mitochondrial dysfunction leading to the electrophysiological abnormalities in iPSC-derived ventricular cardiomyocytes. Overall data may demonstate that membrane ion channels and mitochondria may be a potential therapeutic target for management of SMA-related heart failure.

Funding Source: This work was supported by the Ankara University Scientific Research Projects (BAP).

Keywords: Spinal Muscular Atrophy, induced pluripotent stem cells, mitochondria

4:45 PM – 5:45 PM

380

FUNCTIONAL VALIDATION OF DOXORUBICIN-INDUCED CARDIOTOXICITY-RELATED GENES

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Genome-wide association studies (GWAS) and candidate gene association studies (CGAS) have identified over 180 genetic variants statistically associated with anthracycline-induced cardiotoxicity (AIC). However, the lack of functional validation has hindered the clinical translation of these findings. We aimed to functionally validate all genes associated with AIC using human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs). Through a systemic literature search, we identified 80 genes containing variants significantly associated with AIC. Additionally, three more genes with potential roles in AIC (GSTM1, CBR1, and ERBB2) were included. Of these, 38 genes exhibited expression in human fetal heart, adult heart, and hiPSC-CMs. Using CRISPR/Cas9-based genome editing, each of these 38 genes were systematically knocked out in control hiPSC-CMs, and the resulting doxorubicin-induced cardiotoxicity (DIC) phenotype was assessed using hiPSC-CMs. Subsequently, functional assays were conducted for each gene knockout based on hypothesized mechanistic implications in DIC. Knockout of 26 genes increased the susceptibility of hiPSC-CMs to DIC. Notable genes included efflux transporters (ABCC10, ABCC2, ABCB4, ABCC5, and ABCC9), well-established DIC-associated genes (CBR1, CBR3, and RAC2), and GWAS-discovered genes (RARG and CELF4). Conversely, knockout of ATP2B1, HNMT, POR, CYBA, WDR4, and COL1A2 had no significant effect on the in vitro DIC phenotype of hiPSC-CMs. Furthermore, knockouts of the uptake transporters (SLC28A3, SLC22A17, and SLC28A1) demonstrated a protective effect against DIC. Our findings establish a comprehensive platform for the functional validation of DIC-associated genes, providing insights for future studies in DIC variant associations and potential mechanistic targets for the development of cardioprotective drugs.

Keywords: doxorubicin, human induced pluripotent stem cells, GWAS

4:45 PM – 5:45 PM

TOPIC: EPITHELIAL (INCLUDES SKIN, GUT, AND LUNG)

382

PLASTICITY OF ESOPHAGEAL PROGENITOR CELLS IS FINE-TUNED BY THEIR MICROENVIRONMENT IN VIVO

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In the esophagus, chronic acid reflux can trigger the replacement of squamous cells with columnar cells, leading to metaplasia which is a risk factor for esophageal adenocarcinoma. Esophageal keratinocytes can induce columnar metaplasia, demonstrating their plasticity. However, further studies are needed to characterize the mechanisms regulating the fate of esophageal progenitors. Our group previously elucidated the pivotal role of the transcription factor Sox9 in modulating esophageal cell plasticity downstream of the Hedgehog (HH) pathway in vivo. We showed that activation of the HH pathway due to chronic gastroesophageal reflux induces keratinocyte dedifferentiation into an embryonic-like state, followed by a squamous to columnar conversion in a Sox9-dependent manner. However, the mechanisms regulating Sox9 expression in esophageal progenitors remain unclear. Our current research shows that Sox9 is not directly regulated by HH. Instead, we show that HH activation in vivo modifies the cross-talk between esophageal keratinocytes and their microenvironment by inducing local modifications in the extracellular matrix allowing the release and activation of latent TGF-β. In addition, expression of the BMP inhibitor Follistatin is reduced while some BMP ligands are upregulated in esophageal keratinocytes following HH activation. These changes are associated with the activation of the TGF-β/BMP signaling in epithelial cells to induce Sox9 upregulation. Moreover, we were able to inhibit pharmacologically HH-induced Sox9 expression in esophageal keratinocytes and thus their plasticity in vivo. Interestingly, the drug had no impact on cell communication with their microenvironment, nor on TGF-β/BMP activation. Instead, stabilization of the Sox9 protein was inhibited by the action of the drug directly on the epithelial cells. In conclusion, the fine-tuning of Sox9 expression and keratinocyte plasticity in vivo is tightly regulated by the complex communication between esophageal epithelial cells and their microenvironment, and can be modulated using pharmacological tools.

Funding Source: FNRS TELEVIE

Keywords: esophagus, plasticity, organoids

4:45 PM – 5:45 PM

384

NANOTECHNOLOGY, NATURAL PRODUCTS AND SKIN STEM CELLS: A NOVEL COMBINATION TO COUNTERACT SKIN AGING

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Skin aging is related to long-term and over exposure to ultraviolet (UV) radiation, resulting in inflammation and tissue changes. Indeed, solar radiation causes oxidative stress, compromising the function of the epithelial barrier. The crucial role of UV-induced oxidative stress enhances the importance of employing intrinsic and extrinsic antioxidants to counteract their negative impact. Natural extracts, as H. italicum oil (HO), contain notable bioactive compounds recognized for their different pharmacological properties, as antioxidant and anti-inflammatory effects. The advent of nanotechnology, allowed the combination of nanoformulations with traditional treatments, improving the passive penetration of substances in the skin layers. Electrospinning is a useful technique for encapsulating bioactive compounds into multifunctional nanofibers that can be used for several applications in cosmetic and biomedical field. Within this context, we studied the biological properties of electrospun nanofibers, including Polyvinyl acetate (PVA) and Polyvinylpyrrolidone (PVP) encapsulated with HO (PVA-HO and PVP-HO respectively), also analyzed by scanning electron microscope (SEM). These polymers have been approved by the US Food and Drug Administration as a safe polymer for pharmaceutical and biomedical experiments. The activity of PVA-HO and PVP-HO was tested in vitro on Skin Stem Cells (SSCs) and BJ fibroblasts. In both cell lines, our results show that these functionalized nanofibers were able to increase proliferation and viability, counteracting aging triggered by UV stress. The molecular senescence program activated by UV exposure was counteracted by pretreatment with PVA-HO and PVP-HO. Moreover, the β-galactosidase assay, performed on stem cells, highlights the protective effect of both HO-functionalized nanofibers on aging induced by UV irradiation. Our results suggest that HO combined with PVA and PVP nanofibers, exhibit unique properties as controlled release, biocompatibility, and biodegradability. In conclusion, these nanofibers, safe for human health, could potentially be used as a medical device, making them excellent candidates for topical application.

Keywords: stem cells, nanofibers, skin aging

4:45 PM – 5:45 PM

386

THE REGULATORY ROLES OF ENTEROENDOCRINE CELL-DERIVED MELATONIN ON THE INTESTINAL HOMEOSTASIS AND REGENERATION

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Enteroendocrine cells (EECs), constituting merely 1% of the intestinal epithelium, represent a profound endocrine system in terms of hormone and bioactive molecule production. This research examines the influence of EEC-derived melatonin (M) on intestinal homeostasis and regenerative capabilities using intestinal organoids (IOs) and transgenic models. It was observed that melatonin hindered the budding process and proliferation capacity in murine IOs without inducing cell death. Notably, based on the bulk sequencing results and gene set enrichment analysis, M administration may activate pathways associated with injury response and a fetal-like regenerative process. Further, single-cell RNA sequencing revealed an increase in a specific subset of EECs with regenerative potential (characterized by high PAX6 and low Neurog3 expression) following M treatment. Recently, it has been shown that Prostaglandin E2 (PGE2) is one of the major paracrine signals to orchestrate the repair process during intestinal injury by inducing Clu/Ly6a+ regenerative stem cell (RSC) population in the intestinal epithelium. Intriguingly, M treatment was found to enhance the PGE2-driven augmentation of Ly6a+ RSC pools within IOs. To determine whether M administration can aid intestinal regeneration in vivo, we treated M to dextran sulfate sodium (DSS)-exposed mice and found that continuous treatment of M could improve the clinical symptoms of colitis. Finally, we further explored the impact of endogenous M by examining knockout mice deficient in AANAT, the rate-limiting enzyme crucial for M synthesis. It was revealed that the recovery pace from DSS-induced damage was slower in AANAT knockout mice compared to their wild-type counterparts. Therefore, these findings illuminate the potential of EEC-derived hormones in modulating the RSC population and augmenting the endogenous regenerative capacity.

Funding Source: This work was supported by Korean Fund for Regenerative Medicine (KFRM, 23A0205L1) and Starting growth Technological R&D Program (TIPS Program, (No. RS-2023-00302467)) funded by the Ministry of SMEs and Startups(MSS, Korea) in 2023.

Keywords: regeneration, organoid, revival stem cell

4:45 PM – 5:45 PM

388

SALIVARY GLAND CHIMERIC ORGANOIDS REPRESNT A NOVEL PLATFORM TO INVESTIGATE THE INTERACTION BETWEEN EPITHELIAL AND MESENCHYMAL CELLS

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Salivary gland organoids are considered useful tools for salivary gland regeneration research or modeling salivary gland diseases. However, current epithelial cell-prone culture of salivary gland organoids relies heavily on adult epithelial stem cells and excessively supplemented exogenous growth factors instead of mesenchyme, making them unsuitable for developmental stage salivary gland research and imposing limitations on their utility for drug discovery. To address these constraints, we have developed chimeric organoids (cSGO) by establishing salivary organoids from epithelial cells of postnatal day 1 murine with salivary mesenchyme obtained from embryonic day 14 murine. The confirmation of cSGO formation was achieved through immunofluorescent staining of characteristic markers (Vimentin, GFRA2, and CD31) for fibroblasts, neural cells, and endothelial cells, which constitute the majority of the mesenchyme. Notably, physical interactions between neural cells and organoids were observed. Through various inferences using single-cell transcriptome analysis, it was observed that in cSGOs, mesenchyme partially substitutes some growth factors. Additionally, compared to groups lacking both growth factors and mesenchyme, there is a higher proportion of cycling cells in the G2/M phase and basal cell population in cSGOs. Furthermore, differentiation into pro-acinar or acinar states appears to occur relatively less frequently. This is inferred to be due to the upregulation of inflammatory signals, represented by TNFA, in the immune cells comprising the cSGO organoids. To confirm whether inflammation inhibits differentiation into acinar cells, experiments were conducted under conditions lacking mesenchyme and growth factors using TNFA and dexamethasone. Treatment with TNFA downregulated the expression of proacinar genes such as AQP5 and BPIFA2. However, when treated with dexamethasone, recovery of expression was observed. Collectively, our study will provide an experimental platform for exploring postnatal salivary gland maturation and epithelial-to-mesenchymal interactions under a growth factor-deprived organoid culture system.

Keywords salivary gland, stem cell, organoid

4:45 PM – 5:45 PM

390

TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF GASTRIC FOVEOLAR CELL DIFFERENTIATION

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Mucus-producing foveolar/pit cells, which are located at the surface of gastric glands, are protecting the epithelium from microbes and the harsh luminal environment. Additionally, they play an important role during the gastric wound healing process and have been linked to several diseases. While it is already known which components these cells produce and secrete, little is known about the molecular regulation of their differentiation. Using human adult stem cell-derived gastric organoid cultures, we studied the molecular regulation of foveolar cell differentiation under homeostatic conditions. We compared various differentiation conditions by RNA-seq to select the most robust one, which caused the strongest upregulation of foveolar cell marker genes. Next, we wanted to identify transcription factors (TFs), which are important for foveolar cell differentiation or maintenance of their terminally differentiated state. Thus, I performed gene expression analysis on a differentiation time course experiment to detect up- and downregulated TFs, mapped open chromatin regions using ATAC-seq in differentiated cells to identify enriched TF motifs within these regions and performed a FACS-based CRISPR/Cas9 knockout (KO) screen targeting all known TFs. I could reproducibly identify candidate genes such as KLF3, KLF4 and PPARG by all three approaches in addition to hits found in individual experiments. Currently, I am further investigating these hits by generating single gene KO lines and analyzing their DNA binding pattern using CUT&RUN to gain mechanistic insights into their mode of action during foveolar cell differentiation. These findings contribute to a better molecular understanding of human foveolar cell differentiation and in the future could provide the basis for a refined treatment regimen to restore normal gastric homeostasis in patients suffering from abnormal foveolar cell behavior such as during gastric injury or Ménétrier's disease.

Keywords: human gastric organoids, foveolar cell differentiation, transcriptional and epigenetic regulation

4:45 PM – 5:45 PM

392

INHIBITION OF RAGE BY CA MITIGATES EMPHYSEMA AND PROMOTES ALVEOLAR REGENERATION IN COPD

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The association between emphysema and DAMP-RAGE signaling in COPD patients and therapeutic strategies are unknown. We first identified the expression of RAGE ligands and RAGE-binding signaling in COPD patients through GEO data. To demonstrate the relationship between RAGE and emphysema, we used a PPE-induced emphysema mouse model, AGER-/- mice, and Compound A (CA), a RAGE Inhibitor. We confirmed the association between RAGE and emphysema development by H&E and Western blot in mouse lung tissue and BALF. We also analyzed CSE-induced oxidative stress and inflammatory damage by CA in human alveolar epithelial cell lines and 3D alveolosphere culture. Our results showed that AGER-/- mice had reduced development of PPE-induced emphysema compared to WT. Therefore, we hypothesized that inhibition of RAGE could alleviate emphysema. In our data, CA attenuates PPE-induced alveolar macrophage infiltration and inflammation by inhibiting RAGE, reduces the activation of RAGE downstream mechanisms MAPK and NF-κB, and inhibits the expression of MMP2, thereby alleviating emphysema. Furthermore, we confirmed in alveolar epithelial cells via 2D culture and 3D alveolosphere that CA inhibits CSE-induced oxidative stress and inflammatory cytokines, suppressed p53, and activate ki67, which is involved in lung tissue repair and regeneration. In conclusion, blockade of DAMP-RAGE interaction by CA promotes alveolar epithelial cell proliferation through suppression of oxidative stress and inflammation and preserves alveolar wall by inhibition of MMP2 through regulation of MAPK, NF-κB in tissue, providing a promising therapeutic strategy to alleviate emphysema.

Funding Source: This research was supported by KEITI funded by the MOE and NRF-2020R1A5A8019180

Keywords: RAGE, COPD, alveolar epithelial cell

4:45 PM – 5:45 PM

394

THE CAUDALLY POLARIZED SUBPOPULATION OF THE BULGE AS A RESERVOIR FOR THE MOST QUIESCENT HAIR FOLLICLE STEM CELLS

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Hair follicles with stem cells located in the bulge undergo cyclic periods of regeneration, degeneration, and rest throughout adult life in mammals. The stem cells are maintained in a specialized microenvironment or niche and are regulated by internal and external signals, determining their dynamic behaviors in homeostasis and hair follicle regeneration. Depending on the niche surrounding, stem cell heterogeneity and compartmentalization within the bulge emerge as essential factors for hair follicle maintenance. Stem cells are thought to be aligned along the hair follicle's longitudinal axis, resulting in a quiescence gradient that decreases symmetrically from the top to the bottom region of the bulge along the HF. The bulge cells also have additional heterogeneity due to their relationship to the basement membrane. The critical question is still open: how do different components of the microenvironment influence stem cell compartmentalization, influencing their behavior and fate? Here, we show compartmentalized epidermal stem cells' transcriptional and anatomical contribution to the mouse hair follicle. Our study reveals a caudally polarized subpopulation of bulge hair follicle stem cells (HFSCs) and their interaction with the innervation of the skin. This subset is distinguished by a significantly higher quiescent score compared to the non-polarized bulge cells, as indicated by the expression patterns of crucial quiescence factors during HFSC activation at the telogen-to-anagen transition. This polarized subpopulation may serve as a reservoir for the most quiescent hair follicle stem cells, playing a crucial role in the self-renewal of the stem cell pool essential for tissue renewal and regeneration after injuries. The results obtained contribute to our understanding of the intricate bulge architecture, which is necessary for comprehending tissue homeostatic regulation and niche-mediated regeneration.

Funding Source: This work was funded by the Polish National Science Center (NCN) grants 2020/37/B/NZ5/03950 to A. Kobielak

Keywords: hair follicle stem cells, niche, polarization

4:45 PM – 5:45 PM

396

R-SPONDIN-YAP AXIS COORDINATES GLANDULAR REGENERATION IN THE STOMACH CORPUS BUT FUELS INFLAMMATION-DRIVEN METAPLASIA

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Stromal cells throughout the gastrointestinal tract secrete the signaling molecule R-spondin 3 (Rspo3) that amplifies Wnt signaling. Wnt signaling is essential for the proliferation and self-renewal of stem cells. Deregulation is linked to malignant tissue transformation. In the stomach corpus, regenerative kinetics and the function of the Wnt/Rspo3 signaling pathway are not well understood. We asked whether and how Rspo3 affects epithelial kinetics during homeostasis, epithelial regeneration, and in the context of Helicobacter pylori-associated metaplasia. Using conditional mouse models that enable manipulation of Rspo3 gene expression in gastric myofibroblasts together with single-molecule RNA ISH and microarray analysis, we investigated the effect of Rspo3 in corpus glands. To study its role in chief cell recovery, we applied a mouse model to simultaneously deplete Lgr5-expressing chief cells and stromal Rspo3. Using models of acute tamoxifen-induced and chronic Helicobacter pylori-driven corpus injury we explored the relevance of Rspo3 for tissue integrity and its role in injury recovery and the development of metaplasia. We found that stromal Rspo3 drives epithelial differentiation towards the chief cell lineage. Loss of Rspo3 expression reduced whereas overexpression of Rspo3 expanded the number of chief cells. Tamoxifen-induced loss of chief cells triggered overexpression of Rspo3 which was mandatory to initiate epithelial hyperproliferation and regeneration. In Rspo3 knockout mice, epithelial regeneration was insufficient and delayed. Chronic infection with Helicobacter pylori also induces chief cell loss. In mice that overexpress Rspo3, chronic Helicobacter pylori-driven inflammation locked the epithelium in a regenerative state that was accompanied by metaplastic changes. In addition, Rspo3 itself promoted epithelial immune cell infiltration, thereby perpetuating the pathology. Together, we identified Rspo3 as a critical endogenous

driver of epithelial differentiation and proliferation in the corpus. Rspo3 enables epithelial regeneration upon short-term tissue injury. However, the regenerative status persists during chronic inflammation predisposing to metaplasia. This work demonstrates the double-edged function of physiological signaling.

Keywords: gastric carcinogenesis, Wnt/Rspo3 signaling, bacterial infection

4:45 PM – 5:45 PM

398

IDENTIFICATION OF LONG-LIVED, MULTIPOTENT STEM CELLS AND THEIR NICHES IN THE HUMAN THYMUS

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Due to the intrinsic systemic nature of the immune system interconnecting all organs in our body, progressive immune senescence plays a major role in age-associated disorders. The thymus is a primary lymphoid organ underpinning adaptive immunity that provides defence against pathogens, cancer and establishes long-term immune memory. However, the thymus starts involuting in early life and continues into adulthood with atrophy in the elderly and yet it maintains a regenerative capacity. Current models for thymic regeneration are based on inducing injury though the underpinning mechanisms are unknown. In this study, we identify and characterize bona fide epithelial stem cells (SC) with multilineage differentiation potency in the post-natal thymus that are distinct from progenitors. Thymic SC are long-lived in humans and are defined by several unique cellular and molecular traits that are maintained in vitro where they self-renew and can be extensively expanded, akin to stem cells of constantly renewing tissues. These results were obtained thanks to a combination of single cell transcriptomic and proteomic analyses of thymic stroma, that also allowed the recognition of novel specialized populations. Finally, the identification of a distinctive molecular signature combined with spatial multi-omics of thymic SC allowed us to define for the first time the location and molecular features of their niches and design novel differentiation assays ex vivo. This offers a new perspective and conceptual advance to study the mechanisms of thymic involution and design new strategies for rejuvenating the adaptive immune system.

Funding Source: ERC-Stg No. 639429

Keywords: human thymus regeneration in vivo and in vitro, stem cell niche, single cell clonal analysis

4:45 PM – 5:45 PM

400

ASSESSING THE REGENERATIVE CAPACITY OF MOUSE SALIVARY GLANDS USING ORGANOID TECHNOLOGY

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Salivary gland regeneration after cancer therapy remains a major challenge due to treatment-induced glandular dysfunction. Understanding the mechanisms of salivary gland self-renewal is crucial for effective regenerative strategies. Here, we established epithelial organoid cultures of all major salivary glands to investigate their stemness and regenerative capacity. We purified epithelial cells from submandibular, sublingual, and parotid glands and cultured them under conditions conducive to organoid formation. We then characterized the morphology and molecular features of the resulting organoids using histology, immunohistochemistry, and real-time PCR. Our data show that organoids recapitulate gland-of-origin-specific features. For example, submandibular gland organoids are compartmentalized with ductal cell disposition, whereas sublingual gland organoids appear smaller and more compact with a large central mucus-containing lumen. We are currently performing transcriptomic analyses to determine whether the global gene expression profiles of the major salivary glands are maintained in organoid cultures in a gland-specific manner. We aim to use the organoid cultures to assess the stemness and regenerative potential of salivary glands following various challenges, including radio- and chemotherapy. Our goal is to elucidate the molecular mechanisms underlying salivary gland self-renewal and regeneration to pave the way for new therapies against glandular dysfunction and to improve patient outcomes following cancer treatment.

Funding Source: The Kretzschmar group is funded by the German Cancer Aid (via MSNZ Würzburg/NG3) and the European Union (ERC Starting Grant 101042738/OralNiche).

Keywords: salivary glands, organoid technology, tissue regeneration

4:45 PM – 5:45 PM

402

HARNESSING INTESTINAL STEM CELLS FROM HUMAN PLURIPOTENT STEM CELL-DERIVED INTESTINAL ORGANOIDS FOR APPLICATIONS IN REGENERATIVE MEDICINE

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Human Intestinal Organoids (hIOs) represent a promising approach in regenerative medicine, considering the crucial role played by intestinal stem cells (ISCs) in tissue regeneration. In this study, we isolated ISCs

from human pluripotent stem cell (hPSC)-derived hIOs and developed a novel method for long-term culture of these intestinal organoids. These ISCs were utilized as regenerative therapies using two distinct culture approaches: an extracellular matrix (ECM)-based method and a xenogeneic-free culture method, both demonstrating significant expansion capabilities. Our research successfully confirmed the engraftment of Matrigel ISC and xenogeneic-free ISC in a mouse model with EDTA-induced intestinal epithelial damage. Crucial validation of the efficacy of ISC transplantation in restoring damaged intestinal epithelial barriers was evidenced through endoscopic analysis, histological examination, mucin functionality assessment, and immunostaining with human-specific antibodies. Additionally, our study revealed the potential of xenogeneic-free ISCs integrated into the damaged colonic epithelium of mice with dextran sodium sulfate (DSS)-induced colitis, inducing intestinal regeneration. These findings highlight the feasibility of xenogeneic-free ISCs derived from hPSC-based hIOs as a viable strategy in regenerative medicine, particularly for the treatment of severe intestinal disorders.

Funding Source: This work was supported by the Korean Fund for Regenerative Medicine (KFRM) grant funded by the Korea government (21A0404L1), and a grant from the Technology Innovation Program (No. 20008777).

Keywords: human intestinal stem cell, xenogeneic-free culture, intestinal regeneration

4:45 PM – 5:45 PM

404

THE ROLE OF LGR5 POSITIVE STEM CELLS IN RECURRENCE OF COLORECTAL CANCER

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5-Fluorouracil (5-FU) remains the first-line treatment for colorectal cancer (CRC). Although 5-FU initially de-bulks the tumor mass, recurrence after chemotherapy is the barrier to effective clinical outcomes for CRC patients. Here, we demonstrate that p53 promotes WNT3 transcription, leading to activation of the WNT/β-catenin pathway in ApcMin/+/Lgr5EGFP mice, CRC patient-derived tumor organoids (PDTOs) and patient-derived tumor cells (PDCs). Through this regulation, 5-FU induces activation and enrichment of cancer stem cells (CSCs) in the residual tumors, contributing to recurrence after treatment. Combinatorial treatment of a WNT inhibitor and 5-FU effectively suppresses the CSCs and reduces tumor regrowth after discontinuation of treatment. These findings indicate p53 as a critical mediator of 5-FUinduced CSC activation via the WNT/β-catenin signaling pathway and highlight the significance of combinatorial treatment of WNT inhibitor and 5-FU as a compelling therapeutic strategy to improve the poor outcomes of current 5-FU-based therapies for CRC patients

Funding Source: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP) (grants 2019R1A2C3002751, 2018R1D1A1B07050189).

Keywords: LGR5+ cancer stem cells, cancer recurrence, intrinsic resistance

4:45 PM – 5:45 PM

406

INTERMITTENT FASTING-INDUCED LIPOLYSIS IN NICHE ADIPOCYTES DRIVES HAIR FOLLICLE STEM CELL APOPTOSIS

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Intermittent fasting has gained global popularity for its health benefits, but how it impacts somatic stem cells and tissue biology remains elusive. Here we report that commonly used intermittent fasting paradigms inhibit hair follicle regeneration by selectively inducing apoptosis of activated hair follicle stem cells (HFSCs) in mice. This effect is not caused by reduced calorie intake, circadian rhythm changes, or activation of the classical cellular nutrient-sensing mechanism. Instead, extended fasting activates crosstalk between adrenal glands and adipocytes in the skin, triggering the rapid release of free fatty acids into the niche. This disrupts normal stem cell metabolism and elevates cellular reactive oxygen species, causing oxidative stress and apoptosis. Surgical removal of adrenal glands, genetic inhibition of fatty acid oxidation in HFSCs, or enhancement of HFSCs' antioxidant capability prevents fasting-induced HFSC death. Our study uncovers a previously overlooked detrimental effect of intermittent fasting on somatic stem cells and tissue regeneration, and identifies interorgan communication between adrenal glands and stem cell niche that functions to eliminate activated stem cells and halt tissue regeneration during unstable nutrient supply.

Keywords: hair follicle stem cell, Intermittent fasting, stem cell metabolism

4:45 PM – 5:45 PM

408

RECOMBINANT LAMININ PROTEINS PROVIDE A BIOLOGICALLY RELEVANT NICHE FOR PRIMARY CELLS IN VITRO, MIMICKING THE NATURAL NICHE FOR BOTH ADULT STEM CELLS AND CANCER CELLS

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Major efforts have been made on cell culture protocols for cellular therapies, however, studies on cell substrates for pathologically relevant cell cultures are less frequent. Cancer cell models in particular are often based on cell-cell contacts leaving out the essential cell-extracellular matrix (ECM) responses, despite that 85% of all cancers are epithelial cancer in direct contact with the ECM of the basement membrane. Laminins are a large ECM protein family enriched within the basement membranes, underling epithelial tissues, and an essential part of the stem cell niches. The laminin proteins have multiple, often

cell type–specific functions, such as adhesion, differentiation, migration, phenotype maintenance, and resistance to apoptosis. We have analysed the expression patterns of the laminin proteins, in healthy and cancer tissues (19 cancers, from 7640 patient samples). We can demonstrate that there is a shift in laminin gene expressions within multiple cancer forms compared to healthy tissues. Both on protein and on relative expression level, within multiple major cancer types such as, lung adenocarcinoma, renal cancers, pancreatic cancer, glioma, and melanoma. The different laminin proteins have different activities and their presence in a tumour can have positive or negative impact on 5-year survival probability, for example, in lung adenocarcinoma where high LAMA3 expression is associated with a negative 5-year survival vs high LAMA2 expression is associated with a positive 5-year survival (P=0,00051 resp. P=0,0027, n=400). We further demonstrate that 53 commonly used lung adenocarcinoma cell lines primary express LAMA3 and LAMA5, and that providing laminin-332 and laminin-511 as substrate enables a lung carcinoma cell line to thrive in xenofree and defined conditions, removing the need for serum.

Keywords: cancer, extracellular matrix, microenvironment

4:45 PM – 5:45 PM

410

APPLICATION OF IN VITRO CULTURED MELANOCYTE PREPARATION PROCEDURE FOR MELANOCYTE TRANSPLANTATION

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Vitiligo, a prevalent pigment loss disorder, often undergoes various non-invasive treatments, such as local immunosuppression and ultraviolet light irradiation. Recently, the transplantation of cultured autologous melanocytes as a cell suspension has emerged as a promising therapeutic option. In this study, we obtained a residual skin specimen of approximately 1 to 2 cm^2 post-surgery. Subsequently, we dissociated it into single cells and cultured them in different Melanocyte Culture Mediums (MelM, ScienCell Research Laboratories; melanocyte growth medium M2, PromoCell) to selectively expand melanocytes at 37°C in a 5% CO2 incubator. We observed significant differences in melanocyte growth across various media, including variations in morphology, growth rate, melanocyte markers, and melanin production. Additionally, we investigated the expression differences of TYR, TRP1, c-kit, and other markers in different passages of melanocytes under each culture medium. Melanocytes cultured in MelM exhibited notably lower melanin content compared to those in melanocyte growth medium M2. Moreover, our study sheds light on the potential for enhancing melanocyte engraftment effects through careful consideration of culture conditions. The observed variations in melanocyte behavior and repigmentation outcomes emphasize the importance of selecting an optimal culture medium for the expansion of melanocytes prior to transplantation. These findings provide valuable insights into refining protocols for clinical melanocyte transplantation, offering a promising avenue for improving the efficacy of pigmentation treatments. In conclusion, our study establishes a foundation for future research aimed at advancing the field of melanocyte transplantation and optimizing treatment outcomes for patients with vitiligo.

Keywords: vitiligo, melanocyte preparation, melanocyte transplantation

4:45 PM – 5:45 PM

412

MIR-184/ATF3 AXIS IS REQUIRED FOR THE MAINTENANCE OF ACTICE LIMBAL STEM CELL STATE

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The cornea is an excellent stem cell (SC) model thanks to its transparency and segregated domains for SC populations in the peripheral limbal niche and differentiated cells in the central cornea. Recently, we identified two limbal SC (LSC) populations that reside in discrete zones. The outer quiescent LSCs (qLSCs) that serve as reservoir SC pool and the inner active LSCs (aLSCs) that maintain homeostasis. aLSCs are highly proliferative and renew the corneal epithelium by producing short-lived progenitors. As the latter cells move radially across the cornea, they gradually lose proliferative potential and differentiate. Very little is known about the mechanisms that allows the transition between SC and progenitor cell states in vivo. Interestingly, we discovered that microRNA-184 (miR-184), which its point mutation leads to congenital corneal disease and blindness, is differentially expressed by SC/progenitors and is absent in differentiated cells. In addition, Mir184-knockout (KO) animals showed a reduction in the expression of ATF3 and the transgenic reporter K15-GFP (GFP downstream to the K15 promoter), both of which mark aLSCs. Promoter analysis and in-vitro study suggests that miR-184 is required for ATF3 that positively regulates K15 promotor activity. In line, ATF3-KO mice displayed abnormal aLSC marker expression. Taken together, we propose that miR-184/ATF3 axis is required for the maintenance of aLSC state. A better understanding of the signaling upstream to miR-184 is required for the development of new treatments for corneal pathologies and to the broader understanding of SC fate decisions.

Keywords: corneal SC fate decisions, microRNA, corneal pathologies

4:45 PM – 5:45 PM

414

ORGANOID SCREEN IDENTIFIES TEMPORAL P53 ENHANCEMENT FOR THE ATTENUATION OF RADIATION-INDUCED SENESCENCE

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Radiotherapy treatment significantly improves the survival of head and neck cancer patients. However, it involves unavoidable irradiation of normal tissues surrounding the tumor, including the salivary glands. We have previously shown that radiation preferentially induces cellular senescence in the salivary gland stem/progenitor cell niche, contributing to hyposalivation. Clearance of senescent cells using senolytic drugs could be a therapeutic strategy to ameliorate radiotherapy-induced xerostomia.To develop a drug screening method to identify potent senolytic drugs in salivary gland organoids and test for regeneration capacity following senolytic treatment. (Sham-)irradiated salivary gland organoids were treated with different doses of candidate senolytic drugs. As a measure of senolytic activity, a Caspase-3/7 fluorogenic substrate was added together with the selected drugs. Increases in salivary gland organoid apoptosis following treatment, inferred from Caspase-3/7 activity, were detected in real-time using an IncuCyte S3. Drug concentrations that efficiently induced apoptosis in irradiated cells but were not or less toxic to unirradiated cells were selected to verify the elimination of senescent cells and corresponding changes in the organoid forming efficiency (OFE). The top candidate was tested in an in vivo model for radiation-induced hyposalivation. Of the senolytics tested, Nutlin3a, an MDM2 inhibitor, and P53 activator, improved organoid self-renewal potential after irradiation and had the most favourable toxicity profile. Accordingly, treatment with Nutlin3a reduced senescent markers in irradiated organoids and a transcriptional increase in p53 target genes. Retrograde ductal injection of Nutlin3a in irradiated mouse salivary glands elicited a regenerative response and resulted in a robust increase in saliva production as early as two weeks after administration. We conclude screening for drugs that show senolytic activity without hampering stem cell expansion may be a promising methodology for identifying compounds with potential applications in post-radiotherapy regenerative medicine.

Funding Source: This project was funded by the Dutch Cancer Society KWF Grant nr 12458.

Keywords: cellular senescence, senolytic, salivary gland

4:45 PM – 5:45 PM

416

TISSUE MORPHOLOGICAL CHANGES AND MECHANO-CHEMICAL COUPLING DURING INTESTINAL CRYPT MORPHOGENESIS

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Tissue patterning relies on the emergence of biological and anatomical structures of varying composition through a series of spatio-temporally coordinated events. To achieve this, cells need to sense both biochemical signals and mechanical cues and respond appropriately to generate higher order structures. Using recent advances in organoid and single-cell technologies, we can now understand how individual cells initiate patterning and collectively drive the emergence of complex structures. The small intestinal epithelium and the development of its stereotypical crypt villus architecture is a prototypical example of a coordinated and complex sequence of morphogenetic events suitable for studying mechanical modulation. Employing mouse intestinal organoids grown from single cells we have previously shown that crypt morphogenesis follows highly stereotyped morphological stages from stem cell niche formation to crypt budding. This process involves changes in tissue and cell shape, including an increase in curvature driven by actomyosin apical constriction and cell compaction. However, the detailed mechano-chemical process behind crypt morphogenesis remains to be elucidated. Using scRNAseq, we identified the calcium-dependent cytosolic phospholipase A2 (cPLA2) as a candidate responsible for the coupling between tissue curvature and actomyosin contractility during crypt morphogenesis and for maintaining crypt robustness. We found cell-specific expression of cPLA2 in Paneth cells and stem cells in organoids and in mice and showed that cPLA2 senses tissue shape changes in the intestinal crypt. We have also shown that chemical perturbation of cPLA2 results in perturbed crypt morphogenesis by affecting actomyosin contractility.

Keywords: intestinal organoids, morphogenesis, stem cell niche

4:45 PM – 5:45 PM

418

ORGANOID-BASED SCREENING IDENTIFIES THERAPEUTIC POTENTIAL OF SUMOYLATION INHIBITORS FOR INTESTINAL EPITHELIUM REGENERATION

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Intestinal organoids are complex 3D structures that mimic the cellular composition and architectural organization of the intestinal epithelium, thereby representing a tractable in vitro system to investigate tissue physiology. Interestingly, the self-organizing process of organoid formation from single cells recapitulates cellular and transcriptional aspects of epithelial regeneration in vivo. Here we demonstrate that mechanical disruption of organoid structures during conventional culture maintenance is sufficient to trigger a regenerative-like response independently of single cell dissociation. Using this simplified approach, we performed a small-molecule compound screening and identified ML792 - a selective inhibitor of the SUMOylation pathway - as potent enhancer of the regenerative response. Functional and transcriptomic profiling assays in vitro confirmed the effect of ML792 and a related compound, TAK981, on the regenerative capacity of intestinal epithelial cells. SUMOylome profiling was used to shed light into potential mechanism-of-action, and identified a diverse range of nuclear proteins that can be directly affected by the compounds. Finally, using an experimental murine injury, we showed that TAK981 enhances functional recovery vivo. Collectively, we demonstrate that the SUMOylation pathway modulates the epithelial-cell intrinsic regenerative response, and its transient inhibition may enhance tissue repair in ulcerative conditions.

Funding Source: The Novo Nordisk Foundation Center for Stem Cell Medicine is supported by Novo Nordisk Foundation grants (NNF21CC0073729).

Keywords: regeneration, colitis, SUMOylation

4:45 PM – 5:45 PM

420

TET3 REGULATES TERMINAL DIFFERENTIATION AT THE METABOLIC LEVEL

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TET-family members are crucial for cell lineage specification and their malfunction leads to skewed or abnormal cell fate commitment. Tet3 is ubiquitously expressed in differentiated cells and is essential for postnatal survival due to yet unknown reasons. Here, we used the intestinal epithelium as a model to study the role of TET3 in a hierarchical stem cell tissue. In order to do so, we performed a single cell RNA-seq analysis on the intestinal epithelium isolated from wild-type and Tet3 knockout mice. Our results showed that oxidative phosphorilation was one of the most negatively enriched pathways in the absence of TET3. Indeed, a large number of F1F0ATP synthase subunits were significantly downregulated in Tet3 knockout enterocytes leading to an ATP synthase assembly deficiency and a reduced ATP synthase activity. Finally, a metabolomics analysis demonstrated that the loss of TET3 compromises mitochondrial metabolic maturation and leads to a metabolic profile closely related to undifferentiated cells. Collectively, our study has revealed that the loss of TET3 impedes mitochondrial maturation and compromises terminal differentiation at the metabolic level.

Keywords: TET3 enzyme, oxidative phosphorilation, metabolic maturation

4:45 PM – 5:45 PM

422

ROLE OF TET3 AND 5HMC IN CELL FATE COMMITMENT

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The Tet gene family members, responsible of oxidizing 5mC into 5hmC, exhibit functional differences that remain to be defined. In this study, we have focused on deciphering the role of TET3 in cellular commitment since TET3 deficiency provokes developmental defects in multiple cell types. To this end, we have used the mouse intestinal epithelium as a model to investigate the function of TET3 during adult stem cell differentiation. A single cell RNA-seq profiling revealed that the majority of the Tet3 knockout enterocytes followed an aberrant differentiation trajectory and do not reach a full mature enterocyte identity. Next, a gene ontology analysis showed that oxidative phosphorylation was significantly impaired in TET3-depleted cells. Indeed, the expression of genes coding for mitochondrial F1F0-ATP synthase subunits was significantly reduced in the absence of TET3 and correlate with an increase in their methylation levels. Finally, an electron microscopy analysis demonstrated that mitochondria from Tet3 knockout enterocytes exhibited an immature phenotype in comparison with their wild-type counterparts. In conclusion, our study demonstrates that critical mitochondrial genes remain methylated upon TET3 loss leading to a mitochondrial dysfunction that prevents fully differentiation.

Keywords: Tet3 gene, DNA hydroxymethylation, mitochondrial dysfunction

4:45 PM – 5:45 PM

426

UNRAVELING THE BIOCHEMICAL MECHANISM OF DNA REPAIR IN EPITHELIAL STEM CELLS

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Permanent epidermal regeneration by means of cultured human keratinocytes requires the presence of an adequate number of long-lived self-renewing epidermal stem cells, generating transient amplifying progenitors and differentiated cells. Our recent efforts have delved deeper into understanding the biochemical mechanisms orchestrating self-renewal and differentiation of epithelial stem cells to foster their clinical application. We identified FOXM1 as a pivotal transcription factor essential for the self-renewal of holoclone-forming stem cells and acting downstream of YAP. Other than this, single-cell transcriptomic profiling of human epidermal stem cells highlighted the importance of DNA repair pathways, particularly relevant for the advancements in genome editing aimed at permanent genetic modifications. Indeed, we recently demonstrated that epidermal stem cells can be edited by an allele-specific Cas9-mediated knockout approach to treat the dominant form of Epidermolysis bullosa simplex, but gene addiction strategies in human primary stem cells are still challenging. We investigated DNA repair mechanisms by inducing double-strand breaks via x-ray irradiation in human primary keratinocytes enriched or depleted of epidermal stem cells. In instances of non-lethal high doses of DNA damage, epidermal stem cells exhibited apoptotic markers when DNA lesions remained unrepaired, suggesting the activation of a specific pathway of programmed cell death. FOXM1 appeared to play a crucial role in orchestrating this process, as its enforced expression supported the ability to undergo regulated cell death upon irradiation. When cells were able of rectifying DNA damage, we noted heightened activity of homologous recombination (and not non-homologous end joining) in epithelial stem cells, as compared to transient amplifying progenitors, indicating that epithelial stem cells might be also primed to homology-directed repair during gene editing approaches. These findings hold significant promise in advancing error-free gene editing techniques for a better control of gene editing therapeutic strategies to treat genetic skin diseases and would also provide insights into cancer progression.

Funding Source: European Research Council (ERC) Advanced Grant HOLO-GT (No. 101019289)

Keywords: epithelial stem cell, gene therapy, DNA repair

4:45 PM – 5:45 PM

428

REDUCED ENAMEL EPITHELIUM: A SOURCE OF STEM CELLS

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Our goal is to isolate and characterize epithelial cellular culture from reduced enamel epithelium in dental follicle of retained third molars Dental follicle of retained third molars were collected after routine extractions in Dental faculty (Medical university – Sofia) after informed consent was obtained from the patients. Follicle tissue was dissected with scalpel and minced, after which it was digested with 3 mg/ml Collagenase I / 4 mg/ml Dispase solution in PBS for 30 min. Heterogeneous cell culture was then trypsinised and mesenchymal cells were separated from epithelial cells. Further epithelial cells were grown in KBM-Gold medium (Lonza,Basel, Switzerland) supplemented according to the manufacturer's instructions. Epithelial cell cultures were characterized for expression of variety of dental specific and epithelial markers with immunofluorescence. After enzymatic digestion the follicle tissues were allowed to attach to the bottom of 6 cm. dishes and after 3-7 days we observed cells migrating and proliferating. We recognized two types

of cells: spindle like mesenchymal cells and oval, tightly lined next to each other epithelial cells. Trypsin dis-attached quickly the mesenchymal cells and the epithelial colonies remained attached longer.Morphological and immunofluorescent observations confirmed we managed to isolate epithelial cell culture, expressing CK10, CK14, P63, Amelogenin, Ameloblastin, Tuftelin. Dental follicles of retained third molars have viable epithelial cells, relative to the enamel epithelium and rests of Malassaez, which can be isolated and studied further.

Funding Source: Project BG-RRP-2.004-0004-C01 financed by Bulgarian National Science Fund. The research is financed by the Bulgarian national plan for recovery and resilience.

Keywords: epithelial stem cells, characterization, reduced enamel epithelium

4:45 PM – 5:45 PM

430

THE POLARITY PROTEIN ATYPICAL PKCλ SERVES AS A GATEKEEPER FOR FIELD CANCERIZATION AND SQUAMOUS CELL CARCINOMA FORMATION

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The mammalian epidermis forms an essential self-renewing barrier that protects against external hazards such as UV radiation, infections, and mechanical stress. To ensure lifelong protection, this barrier maintains tissue architecture and functional barrier homeostasis even in the presence of tumor-promoting mutations, e.g. in p53 . However, how epidermal stem cells prevent the progression of mutant p53 epidermal cells to actinic keratosis, defined by field cancerization and subsequent invasive squamous cell carcinoma (SCC), remains unclear. The polarity protein atypical protein kinase C (aPKCλ) is a key regulator of epidermal stem cell fate and homeostasis, acting as a rheostat for cell and tissue mechanics essential for skin barrier function. Our research demonstrates that epidermal aPKCλ function is essential to preserve physiological skin regeneration upon loss of p53. The combined loss of aPKCλ and p53 in the mouse epidermis upregulates epidermal Stat3 signaling, driving field cancerization and SCC formation. Initial scRNA-seq analysis of tumors suggests that epidermal basal cells may drive tumorigenesis accompanied by shifts in an immune cell population. To investigate how the loss of aPKCλ and p53 initiates field cancerization, we performed scRNA-seq analysis on young p21 mice without evident field cancerization. This analysis identified potential changes in mitochondrial activity and cell death regulation, which we are currently exploring further. Furthermore, since aPKCλ controls epidermal cell fate, we examined whether the loss of aPKCλ and/or p53 may regulate the epigenetic state of basal and suprabasal epidermal cells. Immunofluorescent staining for H3K9me3 and H3K4me3 markers, indicating a reduction in H3K4me3 levels and an increase in H3K9me3 levels, potentially impacting stem cell fate. To gain a comprehensive view of how aPKCλ and/or p53 change the epigenetic state and potentially alter cell fate, we will combine ChIP-seq with ATAC-seq. In summary, our results indicate that aPKCλ serves as a gatekeeper for field cancerization and skin cancer, preserving physiological skin regeneration in the face of oncogenic stress, potentially through guarding proper epidermal stem cell fate

Keywords: cell polarity, cell fate, squamous cell carcinoma

4:45 PM – 5:45 PM

432

DIETARY ARACHIDONIC ACID INDUCES STEM CELL PLASTICITY TO PROMOTE TISSUE RESILIENCE

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Intestinal epithelial cells acquire stem cell activity by dedifferentiation to promote regeneration in response to tissue damage, but it remains unclear whether such plasticity occurs under normal physiological conditions1. Here, we uncovered that a single dietary nutrient, arachidonic acid (AA), can induce stem cell plasticity under homeostatic conditions and protects intestinal epithelium from subsequent damaging insults. A systematic screen of fatty acids (FA) revealed a conserved pro-regenerative role for dietary arachidonic acid (AA) in eliciting stem cell plasticity in mice and human patient-derived organoids. Enriching AA in an isocaloric mouse diet elevates the regenerative fitness of intestinal epithelium and protects mice from abdominal irradiation or chemotherapy-induced tissue damage. AA emulates the regenerative response to tissue injury by activating an adaptive epithelial prostaglandin E2 (PGE2) – Ptger4 – cAMP – PKA signaling. Mechanistically, AA elicits epigenetic reprogramming by transcriptional regulators to promote stem cell plasticity and regenerative capacity. These results unveil a nutrient-triggered epigenetic mechanism for stem cell plasticity to promote resilience of epithelial tissues upon damage.

Keywords: stem cell plasticity, fatty acids, epigenetics

4:45 PM – 5:45 PM

434

ALTERNATING GENE OSCILLATION IN COMMON CORE GENE NETWORK REGULATED BY BMP-ID2 AXIS IN HAIR FOLLICLE STEM CELLS DETERMINES THEIR CYCLIC REGENERATION AND STEMNESS MAINTENANCE

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Previously, we revealed how regenerative hair cycle behavior in vivo is orchestrated by hair follicle stem cells (hfSCs) homeostasis as the result of the competitive balance between BMP and WNT pathways. Inhibition of BMP pathway (Bmpr1a-KO) leads to Id2 downregulation in hfSCs, identifying this transcription regulator (TR) as an effector of this signaling. Here, we generated Id2 gain of function (ID2-GoF) transgenic mouse line that allowed investigation of Id2 role in hfSCs. We showed that Id2 overexpression in hfSCs is sufficient to promote telogen with their prolonged quiescence. Performing RNA-seq on FACS isolated ID2-GoF hfSCs at 1st postnatal hair cycle allowed an intersection with common bulge signature genes in the quiescent hair follicles. With Id2 dependent gene set, we were able to recapitulate more than half of common bulge signature genes (51%), extending this pool with Bmpr1a-KO targets to 63%. Genes affected by Id2 overexpression are also BMP dependent (36%), emphasizing this TR's role in supporting the BMP-centered hfSCs transcriptional program. Surprisingly, 80% of common genes (370 of 460) are discordantly regulated in Id2GoF and Bmpr1aKO, indicating the intrinsic oscillation of a mutually opposite common core gene network which is regulated by synergistic BMP-Id2 axis interplay. Moreover, to mark all possible pSmad binding sites in genome-wide quiescent hfSCs we exploited of our previously generated transgenic mouse model expressing the constitutively active form of Bmpr1a (Bmpr1A-CA) and performed ChIP-seq analysis. Indeed, we observed that up to almost 60% of Id2-dependent genes have pSmad peaks assigned which were similar to Bmpr1aKO-affected genes. These results clearly show that Id2 is specifically targeted by BMP signaling pathway to expand the pool of affected genes to impose hfSCs quiescence transcriptional program with stemness maintenance. Thus, here, we discovered newly expanded network reciprocally regulated hfSCs genes dependent on BMP-ID2 axis hierarchy which beyond genes for Wnt/BMP pathways, indicated new genes in prostaglandin, cation channel and genes normally involved in T-cells and macrophages regulation. Finally, we show our effort to deconvolute BMP-centered transcriptional regulation program in hfSCs, investigating Id2-dependent promoter DNA-sequence.

Funding Source: Team Grant #POIR.04.04.00-00-4222/17-00 (to KK) and National Science Centre, Poland (NCN) Opus Grant 2015/19/B/NZ3/02948 (to KK), Opus Grant 2022/45/B/NZ3/03811 (to KK) PRELUDIUM Grant #2017/25/N/NZ3/02622 (to PD)

Keywords: hair follicle stem cells (hfSCs), hair regeneration, hfSCs stemness

4:45 PM – 5:45 PM

436

THE ROLE OF THE WNT SIGNALING MODULATOR, R-SPONDIN-3, IN HAIR MORPHOGENESIS, DEVELOPMENT AND REGENERATION CYCLE

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Our prior research revealed that the inhibition of Bone Morphogenetic Protein (BMP) signaling in hair follicle stem cells (hfSCs) prompts the precocious activation of quiescent hfSCs, resulting in profound changes in the Wingless–type MMTV Integration site (WNT) pathway activators expression, including R-spondin-3 (Rspo3). Therefore, here, we investigated Rspo3's role in the hair morphogenesis, development, and regeneration cycle using two transgenic mouse models. The first, with constitutive overexpression of Rspo3 (K14CrexRspo3-GoF) in the skin and its appendages, exhibited a phenotype with reduced hair follicle number and length along with changes in the hair types. Detailed analysis showed changes in the first morphogenetic cycle, including a shortened growth phase (anagen), followed by a prolonged destruction phase (catagen). Moreover, those mice had a reduced number of proliferating cells along with an increased number of apoptotic cells in the hair bulb, and changes in the hfSCs population. In vitro studies on keratinocytes isolated from those mice indicated their decreased ability to proliferate, migrate, and form colonies. Conversely, mice with Rspo3 overexpression only in hfSCs (K15CrePRxRspo3-GoF), activated during the postnatal hair cycle, exhibited premature anagen activation. RNA-sequencing of hfSCs from K15CrePRxRspo3-GoF mice allowed us to characterize the molecular mechanisms with target genes important for Rspo3 activation. Interestingly, a comparison of the results with upregulated signature genes representing quiescent hfSCs indicated an early activation of hfSCs in this model with more than half (52%) inversely regulated genes during stem cell activation. Moreover, a comparison of the result to our previously obtained data from the model with premature activation of hfSCs after BMP inhibition, showed approximately 80% gene consistency, with the same directional changes. Detailed analysis indicated that the overexpression of Rspo3 in hfSCs increased the canonical WNT pathway activity, and decreased the activity of the non-canonical WNT pathway along with the BMP pathway, resulting in precocious hfSCs activation and faster hair regeneration. This research confirms that Rspo3 plays a crucial role in the regulation of hair morphogenesis and hair regenerative cycle.

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Keywords: hair follicle stem cells, hair development, hair regeneration

4:45 PM – 5:45 PM

438

THERAPEUTIC POTENTIAL OF HUMAN DERMAL STEM CELL-DERIVED EXOSOMES IN IDIOPATHIC PULMONARY FIBROSIS

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Idiopathic pulmonary fibrosis (IPF) is a disease with incurable form that is characterized by fibrosis of the lung parenchyma caused by ongoing damage. As the fibrosis thickens due to repeated damage to the alveolar epithelium, the lung tissue loses the ability to exchange gases to supply oxygen to the cells. This leads to a poor patient prognosis and is fatal and incurable. This study was designed to investigate the therapeutic potential molecular mechanisms of lung injury by exosome (Exo) derived from human dermal stem cell-derived mesenchymal stem cells (HD-MSCs) in IPF. Exo have been found to transport bioactive molecules and are involved in a variety of physiological and pathological processes. We investigated the effects of HD-MSC-derived Exo (HD-MSC Exo) on mouse pulmonary fibrosis (PF) models in vitro and in vivo. The results showed that HD-MSC Exo inhibited bleomycin (BLM)-induced inflammation and pulmonary fibrosis by restoring normal alveolar structure and reducing both collagen accumulation and myofibroblast proliferation by in vitro and in vivo experiments. HD-MSC Exo was identified by nanoparticle tracking analysis (NTA) to quantify their number, imaged by transmission electron microscopy to confirm their morphology, and established by protein quantification by western blot. Treatment of HD-MSC Exo in a TGF-β and BLM-induced injury model in the A549 cell line inhibited inflammation and pulmonary fibrosis, which was confirmed by cell viability and cytotoxicity experiments. In addition, C57BL6 mice model of pulmonary fibrosis induced by BLM treatment with HD-MSC Exo intravenously (I.V.) and intratracheally (I.T.) and confirmed that HD-MSC Exo inhibited inflammation and pulmonary fibrosis by hematoxylin & eosin staining (H&E staining) and Masson's trichrome staining (MTS). These findings suggest that HD-MSC Exo treatment in a bleomycin-induced pulmonary fibrosis mouse model exhibited a promising anti-inflammatory and anti-fibrotic treatment modality of pulmonary fibrosis.

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Keywords: pulmonary fibrosis, mesenchymal stem cells, human dermal stem cell-derived MSC exosome

4:45 PM – 5:45 PM

440

KINOME-WIDE SCREEN IDENTIFIES MAPK PATHWAY AS A SPATIOTEMPORALLY TIMED REGULATOR OF HAIR FOLLICLE STEM CELL PLASTICITY

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Hair follicle stem cells (HFSC) undergo cyclic bouts of differentiation to fuel regeneration of the hair follicle throughout life. Notably, return of a subset of hair follicle progenitors to the stem cell state is requisite for long-term tissue maintenance but the mechanisms regulating this fate conversion are unclear. We recently established ex vivo niche conditions that support the growth and expansion of HFSC and recapitulates the regeneration of stem cells from early progenitors that occurs in vivo. To identify signaling pathways controlling progenitor cell plasticity we employed this system for a kinome-wide screen using a small molecule library of kinase inhibitors. Two activators of the Raf signaling pathway emerged as most robust hits, attenuating stem cell differentiation while increasing progenitor dedifferentiation through increased p42/44 MAPK (ERK) activity. Intravital imaging of ERK activity using reporter mice revealed that p42/44 activity propagates along the hair follicle in the stem cell niche upon progenitor dedifferentiation, which resolves upon HFSC niche repopulation. Dissection of the mechanisms of stem cell expansion versus dedifferentiation using live imaging of ERK activity in HFSC organoids revealed specific patterns in response to distinct upstream regulators with EGF provoking a rapid, ERK high state along with stem cell expansion, whereas FGF2 generated an oscillatory pattern of ERK activity and increased proportions of stem cells to progenitors. Together, our data establishes ERK signaling as a spatiotemporally timed cue for stem cell fate conversion and distinct roles of the upstream ligands EGF and FGF2 in regulating stem cell state and expansion by triggering specific patterns of ERK oscillations. Ultimately, the ability to modulate cellular plasticity to regenerate stem cells from progenitor cells has major clinical implications.

Keywords: somatic stem cells, hair follicle, cellular plasticity

4:45 PM – 5:45 PM

TOPIC: GERMLINE AND EARLY EMBRYO

442

LIPID ACCUMULATION PROMOTES LOSS OF HOMEOSTASIS IN THE MALE GERMLINE STEM CELL NICHE THROUGH DIFFERENTIATION

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The capacity of stem cells to self-renew or differentiate has been attributed to distinct metabolic states. In the Drosophila testis, we have shown that both germline stem cells (GSCs) and somatic cyst stem cells (CySCs) employ strategies to control lipid levels to promote stem cell maintenance. When lipid catabolism is impaired, neutral lipids accumulate in lipid droplets (LDs) and GSCs and CySCs exit the niche to differentiate, suggesting a role for lipid metabolism in the control of stem cell maintenance versus differentiation. However, the role of lipid metabolism in niche stromal cells is still unknown. Given the importance of niche cells in the maintenance and homeostasis of the resident stem cells they support, we are currently investigating the role of lipid metabolism in these cells. New data from our lab suggest that lipid accumulation is detrimental for the maintenance of “hub” cells, a main component of the stem cell niche in the testis. Strikingly, with age lipids accumulated as LDs in niche (hub) cells, which correlated with the loss of hub cell maintenance and function. Downregulation of the lipogenesis factor sterol regulatory element binding protein (SREBP) prevented LD accumulation in hub cells from older individuals, while ectopic activation of SREBP caused hub cell loss through their conversion into CySCs. While some mechanisms have been described to contribute to the conversion of hub cells into stem cells, this is the first time that a lipid metabolism gene has been implicated in such phenomenon. Our findings highlight a critical role for lipid homeostasis in stem cell maintenance, providing a framework for investigating the impact of aging and metabolic diseases on stem cell function and tissue homeostasis.

Keywords: lipid, metabolism, niche

4:45 PM – 5:45 PM

444

IN VIVO SCREENS REVEAL GENES ESSENTIAL FOR MALE MEIOSIS

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Meiosis is crucial to the successful progression of spermatogenesis, which is intricate and involves many momentous biological events. Abnormal meiosis directly leads to spermatogenesis abortion and male infertility. As spermatocytes can’t be long-term cultured in vitro, and some meiosis related genes are lethal, hindering meiosis research. To address these questions, we performed pooled in vivo CRISPR knockout screens in mouse spermatogonial stem cells (SSCs). After transplanting these SSCs into recipient mouse testes, we obtained spermatogonias (SPG) and spermatocytes containing various guide RNA. By comparing the guide RNA abundance distributions between SPG and spermatocytes, we found some genes loss generated meiosis arrest, resulting in sterility. Our study successfully conducted functional screening in the male reproductive system, providing a high-content phenotypic screening method for the male meiotic genes, and promoting the study and treatment of male infertility.

Keywords: meiosis, in vivo screen, SSCs

4:45 PM – 5:45 PM

446

NON-AUTONOMOUS CELL REDOX-PAIRS DICTATE NICHE HOMEOSTASIS IN MULTI-LINEAGE STEM POPULATIONS

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Cellular communication is a pivotal biological process, involves diverse signalling molecules, Reactive Oxygen Species (ROS) being one of them. ROS, predominantly derived from mitochondrial respiratory complexes, have evolved as key molecules influencing cell fate decisions like maintenance and differentiation. However, how these stem cells harmonize their redox potential by possessing a restrained oxidant system is not very clear. These redox-dependent events are mainly considered to be cell-intrinsic in nature, on contrary our observations indicate the involvement of these oxygen-derived entities as intercellular communicating agents. We addressed this fundamental question in multi-stem cell lineage-based niche architecture in Drosophila testis. In Drosophila germline, neighbouring Germline Stem Cells (GSCs) and Cystic Stem Cells (CySCs) maintain differential redox thresholds where CySCs having higher redox-state regulate physiological ROS levels of GSCs. Disruption of the intercellular redox equilibrium (by downregulating Sod1) between the two adjoining stem cell populations results in deregulated niche architecture and loss of GSCs. This mainly was attributed to loss of contact-based receptions and uncontrolled CySC proliferation due to ROS-mediated activation of self-renewing signals like EGFR signalling. Our observations hint towards the crucial role of intercellular redox gradients originating from somatic progenitors (CySCs) in niche stability where they function not only as a source of

their own maintenance cues but also serve as non-autonomous redox moderators of germline immortality. In contrast to GSCs, ROS imbalance in CySCs induced accelerated differentiation due to deregulation of PI3K/Tor and Hedgehog (Hh) pathways. Although, redox modulation of PI3K pathway components has earlier been shown, here we found Hh to be probably susceptible to redox regulation. Our study emphasizes the role of intercellular redox communication in biochemical adaptability and plasticity of different niches influencing stem cell fate, niche size and architecture to prevent stem cell loss and aging. This understanding is vital for stem cell maintenance, neurodegeneration, aging prevention, and advancements in therapy and regeneration.

Funding Source: Innovative Young Biotechnologist Award by DBT (BT/12/IYBA/2019/01), ECR Award by Science and Engineering Research Board (ECR/2018/000009), BHU-IoE grant (R/Dev/IoE/Incentive/2021-22/32452), DST-INSPIRE,UGC-CAS

Keywords: Reactive Oxygen Species (ROS), stemness maintenance and differentiation, niche homeostasis

4:45 PM – 5:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

448

CUSTOMIZING ECM-BASED BIOINKS FOR 3D PRINTING OF MULTI-SCALE VASCULAR NETWORKS COMPRISED OF IPSCS-DERIVED CELLS

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Tissue engineering for regenerative medicine hopes to provide a solution to the global donor-organ shortage. One method by which complex, three-dimensional tissues with multiple, spatially localized cell-types are commonly fabricated is via bioprinting, a method highly-regarded for its facility in integrating blood vessels into engineered tissues to solve mass transport and diffusion issues. Here, we describe the development of a novel blood vessel-specific bioink with adhesion motifs specific to endothelial cells (ECs). The bioink was formulated as a combination of decellularized ECM (dECM) hydrogel and modified alginate that was conjugated with a laminin adhesion molecule motif (YIGSR). This hybrid hydrogel was characterized for its mechanical properties, and biochemical composition. iPSCs were efficiently differentiated into endothelial cells and pericytes and were mixed with the hydrogel to form bioinks for printing blood vessels. Following investigation of the cell-hydrogel interactions, the hybrid hydrogel with the endothelial cell adhesion motifs, in conjunction with the pristine dECM hydrogel, was used to print large blood vessels with capillary beds in between. Matured vascular patches contained a multi-scale vascular network comprised of large blood vessels, with an average diameter of 700 µm, surrounded by a network of thin capillary-like structures with diameters of ~30 µm. iPSC-ECs formed a confluent monolayer with tightly connected cells covering the lumen of the large blood vessels. Additionally, the biological activity of this monolayer was demonstrated by the sprouting of ECs from the blood vessel walls. Throughout their length, bioprinted blood vessels displayed high shape-fidelity, with round and stable lumens. This work demonstrates an efficient differentiation to endothelial cells and pericytes and represents a proof-of-concept for a biologically relevant hydrogel with basement-membrane moieties that can be used to print fully confluent, barrier-forming blood vessels to vascularize any tissue. Furthermore, the system can be customized by modifying the peptide sequence added to the alginate molecules, varying the type of alginate and crosslinker concentration and type.

Keywords: tissue engineering, vascularization, iPSCs-derived endothelial cells & pericytes

4:45 PM – 5:45 PM

450

UNRAVELLING DETERMINISM IN HAEMATOPOIETIC RECONSTITUTION POST-TRANSPLANT

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Recent advancements have enabled the ex-vivo expansion of haematopoietic stem and progenitor cells (HSPCs) providing the potential for future therapeutic applications. As ex-vivo expansion of HSPCs results in functional and transcriptional heterogeneity, the relationship between the transcriptional program and subsequent cell fate outcome remains poorly understood. Here, we coupled an optimised method to expand murine HSPCs ex-vivo with a modified version of our expressed barcoding technology, SPLINTR-X (single-cell profiling and lineage tracing-X), which enabled persistent barcode expression ex-vivo and in-vivo. Barcoded HSPCs isolated from murine bone marrow (BM) were expanded for defined time periods of up to five weeks prior to transplantation into several mice. In these experiments, we preserved a portion of the culture just prior to transplantation and transplanted an identical population of HSPCs clones into several mice. This strategy enabled us to assess how the cell-intrinsic transcriptional properties of ex-vivo expanded HSPCs influences output and cell fate decisions in-vivo. Our findings suggest that despite extensive ex-vivo propagation, clonal engraftment and contribution to different haematopoietic lineages is remarkably preserved in sister-clones across different mice. We observed that the cell-intrinsic properties of HSPCs not only regulated the overall clonal output, but also dictate the proportional contribution of individual clones to distinct lineages in recipient mice. Of note, clones exhibiting multilineage reconstitution potential display the highest output. Moreover, despite changes in the BM microenvironment

induced by varying irradiation doses or transplantation into immuno-compromised mice, clonal behaviour was largely preserved. Single-cell RNAseq analysis of cells prior to transplantation revealed that the overall clonal behaviour in culture, as defined by the observed transcriptional drift between sister clones, was a better predictor of in-vivo fate outcome: rather than the transcriptional state of individual cells. Overall, our study provides new insights into how ex-vivo expansion of HSPCs influences output and cell fate in-vivo.

Keywords: hematopoietic reconstitution, cellular barcoding and lineage tracing, hematopoietic stem cells and microenvironment

4:45 PM – 5:45 PM

452

INVESTIGATING THE FETAL ORIGINS OF MYELOID-BIASED NEOGENIN-1+ HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSCs) play a crucial role in maintaining blood cell production throughout life and are essential for therapeutic interventions such as HSC transplantation. However, with aging, there is a dramatic shift in HSC lineage output towards myeloid cells, leading to compromised immune function and increased susceptibility to hematologic disorders. While this age-associated change has been largely attributed to alterations in the adult HSC compartment, emerging evidence suggests that lineage biases may originate during fetal development. Recent findings from our lab have identified a subset of HSCs marked by the surface marker Neogenin-1 (Neo1) and the transcription factor Hoxb5, which exhibit a myeloid-biased lineage output. Currently, it is not completely clear whether lineage bias occurs early in development or later in adulthood. RNA sequencing of fetal liver HSCs reveals that a subset of cells co-express Hoxb5 and Neo1. Utilizing our Hoxb5-mcherry reporter mice, we validate that Hoxb5+Neo1+ HSCs are present in the fetal liver at E14.5. Isolation and transplantation of these cells into lethally irradiated recipients shows increased myeloid output compared to Hoxb5+Neo1- HSCs, suggesting that myeloid priming in a subset of HSCs is established prior to the onset of adult hematopoiesis. Further, we utilized Hoxb5-CreERT; R26VT2/GK3 rainbow reporter mice to trace the fate of Hoxb5-expressing HSCs from E14.5 to adulthood. We find that one day post induction of 4-OHT, a subset of Hoxb5+Neo1+ HSCs are labeled. We then find that a fraction of Hoxb5+Neo1+ HSCs in the adult bone marrow retain labeling, suggesting their fetal origin. This study aims to uncover the developmental origins of my-HSCs and their implications for hematopoietic aging

Keywords: aging, hematopoiesis, hematopoietic stem cells

4:45 PM – 5:45 PM

454

THE SMALL RHO GTPASE CDC42 IN THE REGENERATION OF THE BONE MARROW VASCULATURE UPON AGING AND CHEMOTHERAPY

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Aging represents the main risk factor for the development of malignancies and chronic diseases such as cancer. The standard treatment of most cancers consists in chemotherapy regimens that severely alter the bone marrow (BM) niche, including the vascular network (Sacma et al, 2019). As the BM vasculature is critical to support an effective hematopoietic function, promoting the regeneration of the BM vascular niche upon aging and chemotherapy stands as a key strategy to improve the survival of the elderly. However, it is still not clearly known how different chemotherapy treatments might alter the aged BM vascular niche and if it is possible to improve its regeneration after chemotherapy. The small Rho GTPase Cdc42 has been reported to be overactivated upon aging and it is known to be critical for endothelial regeneration both in vivo and in vitro (Mammoto et al, 2019). Moreover, the inhibition of endothelial Cdc42 activity restores in vitro angiogenesis and decreases senescence in aged endothelial cells (Wang et al, 2007). Recently, we showed that a short systemic inhibition of Cdc42 activity with the specific inhibitor CASIN is sufficient to extend the health and life spans of aged mice by rejuvenating endogenous hematopoietic stem cells and restoring their BM vascular niche localization (Montserrat-Vasquez et al, 2022). Here, we hypothesize that the systemic CASIN treatment might be affecting also the aged BM vascular niche and that it might improve the regenerative capacities of the BM vessels, the hematopoietic stem cell function, and so increase the survival of the aged mice after chemotherapy. Altogether, our results will shed light on the molecular and cellular mechanisms of BM vessel regeneration and demonstrate whether Cdc42 is a relevant pharmacological target to improve the BM vascular niche regeneration upon aging and chemotherapy.

Keywords: bone marrow vasculature, aging, chemotherapy

4:45 PM – 5:45 PM

456

DYNAMIC DETECTION OF CLONAL MYELOPROLIFERATIVE NEOPLASM STEM CELL FITNESS UTILIZING RNA EDITING ADAR1 DEAMINASE ACTIVATION RESPONSIVE (READAR) PLATFORMS

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Myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell (HSC) derived disorders that can progress to acute myeloid leukemia (AML) at variable rates. We recently discovered inflammatory-cytokine-responsive ADAR1 overexpression in MPN derived HSCs during MPN progression. However, dynamic prediction of clonal MPN stem cell fitness and progression to AML remains a challenge. In this study, we performed longitudinal 150-gene next-generation sequencing (NGS) analyses for 129 MPN patients with a median follow-up time of 958 days (range 0-4214). On average, 6 NGS analyses (range 1-19) were performed for each patient. In addition, we developed single-cell RNA Editing ADAR1 Deaminase Activation Responsive (READAR) platforms to quantify ADAR1 activity using a novel ADAR1 NanoLuc-GFP reporter assay and whole transcriptome RNA editome analyses of FACS purified HSCs and hematopoietic progenitor cells (HPCs), along with survival and self-renewal assays. Within our cohort, 58.9% of patients were JAK2 V617F+, while mutations in CALR and ASXL1 were observed in 11.6% and 14.0% of cases, respectively. In total, 44.2% (N=57) of patients in our cohort received Inrebic, with 56% reaching the maximum dose of 400mg. In those with JAK2 V617F on Inrebic, 50% (n=19) experienced a reduction in the JAK2 V617F VAF from their peak value with a median drop of 16% (range 4-57) during follow-up. Our initial analyses using READAR platforms revealed increased RNA editing levels in JAK2 V617F+ patients from whole transcriptome sequencing analyses of FACS-purified HSCs compared with JAK2 wild-type patients. Lentiviral transduction of our ADAR1 NanoLuc-GFP reporter into CD34+ cells derived cells from MPN patients (N=7), enabled us to quantify ADAR1 activity in an ex vivo setting. Moreover, CD34+ cells derived from MPN patient samples (n=7) treated in stromal co-cultures with an ADAR1 inhibitor, Rebecsinib, demonstrated inhibition of self-renewal in replating assays, and a significant reduction in ADAR1-GFP reporter activity. Also, humanized ADAR1-GFP-luciferase reporter AML mouse models showed a significant reduction in ADAR1 activity and leukemia stem cell self-renewal following treatment. These READAR platform results suggest that ADAR1 activity can be utilized to predict clonal MPN stem cell fitness and progression.

Keywords: myeloproliferative neoplasm, hematopoietic stem cells, JAK2, ADAR1

4:45 PM – 5:45 PM

458

R SPONDIN 2 DICHOTOMOUSLY REGULATES HEMATOPOIETIC STEM CELL MAINTENANCE AND REGENERATION

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The mechanisms that govern hematopoietic stem cell (HSC) regeneration are poorly understood. Regenerating bone marrow endothelial cells (BM ECs) upregulate expression of R spondin 2 (Rspo2), a Wnt amplifying protein, following total body irradiation (TBI)(Nat Commun 2021;12(1):6990). We hypothesized that EC-derived Rspo2 regulates HSC regeneration. Cdh5-Cre-ERT2;Rspo2 fl/fl mice (EC-Rspo2 deficient mice) were compared with Cre(-);Rspo2 fl/fl mice that retain Rspo2 expression (EC-Rspo2+ mice). At day +10 post 500 cGy TBI, EC-Rspo2 deficient mice displayed decreased phenotypic BM HSCs (P=0.03) compared to EC-Rspo2+ mice. Recipient B6.SJL (CD45.1+) mice transplanted competitively with 5 x 105 BM cells from irradiated, EC-Rspo2 deficient mice (CD45.2+) displayed 5-fold decreased donor CD45.2+ engraftment at 16 weeks post-transplant compared to mice transplanted with BM from irradiated, EC-Rspo2+ mice (P=0.03, n=11-12/group). In homeostasis, EC-Rspo2 deficient mice and EC-Rspo2+ mice displayed no differences in blood counts, HSC percentages or HSC self-renewal capacity based on competitive BM transplantation assays. These data suggest that Rspo2, produced by BM ECs, regulates HSC regeneration, but is not essential for HSC maintenance. In complementary studies, we administered Rspo2, 10 µg, or saline SQ qOD, from D+1 to D+10, to C57BL/6 mice irradiated with 500 cGy TBI. At day +10, Rspo2-treated mice displayed 3-fold increased BM progenitors (P=0.001) and 10-fold increased HSCs (P=0.001) compared to controls. Recipient mice transplanted competitively with 1 x 106 BM cells

from irradiated, Rspo2-treated mice displayed 4-fold increased donor hematopoietic cell engraftment at 16 weeks compared to recipients of BM from irradiated, saline-treated mice (P=0.02, n=19/group). When we administered Rspo2 x 10 days to non-irradiated mice, we observed no change in blood counts, but decreased percentages of phenotypic HSCs compared to controls (P=0.0001) and mice transplanted competitively with 2 x 105 BM cells from Rspo2-treated mice demonstrated 3-fold decreased donor CD45.2+ cell engraftment at 16 weeks compared to mice transplanted with BM from saline-treated mice (P=0.005, n=5-7/group). Rspo2 promotes HSC regeneration following myelosuppression, but restrains HSC maintenance in homeostasis.

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Keywords: vascular, hematopoietic stem cell, regeneration

4:45 PM – 5:45 PM

460

DEVELOPMENTAL DYNAMICS OF ADULT B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (BCP-ALL) AT CLONAL LEVEL

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Adult B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is a neoplastic disease characterized by phenotypical and functional heterogeneity. However, the cellular organization underlying this heterogeneity is poorly understood. Heterogeneity can arise from both clonal evolution and/or from cell differentiation (leukemia stem cell paradigm). Beyond BCP-ALL, the quantification of the temporal dynamics of leukemia development remains generally challenging. BCP-ALL patient-derived long-term cultures are our model of choice to tackle this problem. We have developed lentiviral expressible barcode vectors that can be detected together with the transcriptomes and cell surface markers. We perform single-cell CITE-seq at different stages of leukemic development in NSG mice to track the progeny of individually barcoded cells (see abstract by A. Gessner et al.). In this work, we combine barcode and multiome information to uncover an unknown differentiation architecture and also to quantify the temporal dynamics of leukemic cell differentiation at clonal level. We identified several large subpopulations of cells which developed after transplantation. Analysis of their transcriptomes revealed that these clusters reflected different stages of B-cell development. Our analysis of cell barcodes further showed that cells could differentiate between these clusters. The barcode information also revealed the likely cluster of origin, which showed the largest clonal diversity and comprised cells from each of the clones. This suggests the existence of a differentiation hierarchy in BCP-ALL. Using machine learning, we then identified expression patterns predicting with high accuracy the differentiation potential of each barcoded cell within the most immature cell compartment. To determine the dynamical properties of the clones, we have designed a mathematical model that simulates the development of these subpopulations from a clonally diverse stem cell compartment. The model, fitted using Bayesian inference, could notably validate the most likely cluster of origin and estimate the variability in proliferation between clones and between clusters. Our approach reveals the existence of a differentiation hierarchy and yields a comprehensive mechanistic view of the disease development at a clonal level.

Keywords: differentiation, leukemic stem cell, clonal development

4:45 PM – 5:45 PM

462

UNRAVELING THE EFFECTS OF PROLIFERATIVE STRESS AND GENOTOXICITY IN HEMATOPOIETIC STEM CELLS IN VIVO

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Hematopoietic Stem and Progenitor Cells (HSPCs) from patients affected by inherited disorders can be corrected with the use of Gene Therapy (GT), providing long term therapeutic benefit upon reconstitution of the entire hematopoietic system. However, how the replication stress, aging, vector driven oncogene activation and cancer predisposing mutations may impact the processes of hematopoietic reconstitution remains uncertain. Here we characterized the clonal dynamics of hematopoietic reconstitution and the acquisition of somatic mutations of lymphoid and myeloid cells in mice transplanted with wild (WT) type HSPCs transduced either with a lentiviral vector with active long terminal repeats which is highly genotoxic (group WT Genotox N=25) or with the safer self-inactivating long terminal repeats (group WT Non Genotox N=24). Additionally, the same HSPC-GT strategy was applied using mouse HSCPs lacking the tumor suppressor Cdkn2a gene (group Cdkn2a Genotox N=24, group Cdkn2a Non Genotox N=23 and Cdkn2a mock transduced N=20). Blood composition and vector integration sites (IS) of B, T, and myeloid cells were monitored overtime (up to 2.5 years). Somatic mutations were identified analyzing the genomic portion of the mouse genome flanking each IS, and a new Mutation Index (MI) was developed to assess mutation accumulation rates. As expected, the group Cdkn2a Genotox showed an accelerated tumor onset when compared to control groups ($p < 0.0001$), caused by activation of Braf oncogene. Moreover, mice from all groups showed a marked myeloid skewing at the expense of lymphoid lineages at the latest time points, specifically in the group WT Genotox. More than 250,000 IS were

identified, corresponding to 9 Gb of sequence genomic information. We found that the MI in both Genotox groups was significantly higher when compared to Non Genotox groups ($p < 0.001$). Notably, myeloid clones exhibited a higher mutation frequency compared to B and T cell lineages. Moreover, the MI of the WT Genotox group in the myeloid compartment was significantly higher than Cdkn2a Genotox ($p < 0.01$). Overall, our data unveils a previously unappreciated effect of genotoxicity by vector insertions which have a profound negative impact on hematopoiesis and accumulation of somatic mutations even in absence of oncogenesis.

Keywords: hematopoietic reconstitution, somatic mutations, gene therapy

4:45 PM – 5:45 PM

464

IDENTIFICATION OF IMPORTANT FACTORS INVOLVED IN TP53-ASSOCIATED PATHWAYS IN EARLY STAGES OF HUMAN HEMATOPOIETIC DIFFERENTIATION

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The TP53-related pathway has been reported to be involved in cell proliferation, maturation and function in B cells and other tissues as well as the inhibition of cell death. Published data indicated that this pathway is also upregulated during early hematopoietic differentiation, but its detailed role in cells during this period remains unclear. Therefore, we used human cord blood-derived lineage(Lin)-CD34+ very immature hematopoietic progenitor cells to investigate their role in differentiation, particularly how they contribute to cell fate decision during early hematopoiesis. First, we amplified the most immature Lin-CD34+ cells from human cord blood-derived cells in vitro to knock down (KD) a gene known to be involved in the TP53 pathway at the early hematopoietic cell stage. After confirming that Lin-CD34+ cells amplified in this system maintained their ability as multipotent progenitor cells, including colony-forming capacity, the effect of KD was evaluated. The results showed that KD reduced the total number of colonies, particularly Mix (24.7±21.5 colonies to 5.78±3.27 colonies / 200 cells) and BFU-E (18.9±10.3 colonies to 11.1±10.9 colonies / 200 cells). These results indicate that the TP53 pathway is required for early progenitor cells to maintain their ability to differentiate into non-myelomonocytic lineages. We next evaluated whether this pathway is involved in commitment to each lineage cell and cell proliferation; KD reduced erythrocyte counts (from 27428±20889 to 3803±5242 / 100 cells) but not monocyte counts (from 192±91 to 286±270 / 100 cells). On the other hand, KD reduced the number of NK cells, as well as the percentage and number of NK cells expressing endogenous CD16 (from 25.2±8.4 to 6.64±10.7 % gated on CD56+ cells) (from 69313±20535 to 18958±34271 / 100 cells) in vitro. These results indicate that TP53-related factors affect lineage-specific cell differentiation and maturation, in addition to suppressing cell death, during the early stages of hematopoietic differentiation.

Keywords: hematopoietic progenitor cell, TP53, differentiation

4:45 PM – 5:45 PM

466

EVALUATION OF FEEDER-FREE METHODS FOR T CELL MANUFACTURING FROM HUMAN INDUCED PLURIPOTENT STEM CELLS (HIPSC)

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The emergence of human-induced pluripotent stem cell (hiPSC)-derived T (iT) cells marks a pioneering advancement in adoptive cell therapies, providing solutions to crucial limitations inherent in conventional autologous manufacturing methods. hiPSCs serve as a readily available source of therapeutic T cells, offering advantages of limitless expansion and genetic manipulation. However, current protocols for iT cell development are burdened by lengthy and labor-intensive processes. Additionally, a decrease in efficiency results from the efforts to develop clinically compatible protocols in xeno- and feeder-free conditions compared to the OP9-DL1/4 standard research protocol. To address this challenge, we have developed scalable and clinically compatible methods for generating hematopoietic stem/progenitor cells (HSPCs) using both monolayer (2D) and embryoid body (EB)-based (3D) protocols. We evaluated the efficiency of these protocols to generate CD34+CD43+ HSPCs capable of further feeder-free T cell differentiation, progressing towards the double-positive CD4+CD8+ population and eventually yielding functionally single-positive cytotoxic CD8αβ+ and helper CD4+ iT cells. We found that the monolayer method demonstrates the capability to yield CD34+CD43+ HSPCs within 8 days with higher efficiency than the EB-based protocol (1 iPSC-to-4 HSPC versus 1 iPSC-to-0.2 HSPC) and higher purity (60-80% CD34+ versus 10-40%). HSPCs generated by both methods displayed similar potential in producing CD4+CD8+ DP, CD8αβ+, and CD4+ iT cells in a feeder-free system (1 HSPC produced up to 150 DP, 750 CD8αβ+, and 150 CD4+ iT cells). Future studies aim to compare the functional potential of iT cells derived by the two different methodologies. Our results support further efforts to scale up these approaches to enable the high cell yield necessary for clinical-grade production.

Keywords: T cells, hematopoietic stem/progenitor cells, iPSC

4:45 PM – 5:45 PM

TOPIC: KIDNEY

468

KIDNEY REPAIR THROUGH CHEMICALLY-INDUCED RE-ESTABLISHMENT OF RENAL TUBULAR EPITHELIAL CELL IDENTITY

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In vivo partial reprogramming by transient overexpression of transcription factors has been shown to restore cell function in aged cells. Here, we investigated whether such partial reprogramming process can be induced to restore cell identity and functionality in maladaptive repairing renal tubular epithelial cells (RTECs) emerged after kidney injury, even with small molecule(s). A high-throughput imageomic and transcriptomic chemical screen in a RTEC injury model revealed that a kinase inhibitor Cer, can reverse injury-associated features, including cell senescence, partial epithelial-to-mesenchymal transition, and pro-fibrosis. In particular, Cer treatment results in restoration of master transcription factor expression, fatty acid metabolism, and transport functions, ultimately re-establishing cell identity in injured RTECs, especially in Vcam1+ failed-repair RTECs isolated from injured mouse kidney. Mechanistically, a less studied protein, BMP2K mediates such a Cer-induced RTEC revitalization process. BMP2K mediates phosphorylation of HNF1B, resulting in its proteasomal degradation, which can be blocked by Cer treatment or BMP2K knockdown. In mice with ischemia-induced acute kidney injury (AKI), Cer administration increases survival rates, improves renal function, and alleviates kidney damage in vivo. Conditional BMP2K knockout in RTECs results in reduced failed-repairing RTEC numbers and improved kidney function. Overall, this study illustrates a cellular revitalization strategy for regenerative medicine, via chemically-inducing the partial reprogramming of injured cells and restoring functional cell identity and function.

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Keywords: reprogramming, partial reprogramming, chemical reprogramming

4:45 PM – 5:45 PM

TOPIC: HEMATOPOIETIC, IMMUNE AND ENDOTHELIAL

470

LOSS OR MUTATION OF NPM1 LEADS TO MITOCHONDRIAL ALTERATIONS IN HEMATOPOIETIC STEM CELLS DRIVING MDS PATHOGENESIS

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Myelodysplastic syndromes (MDS) are a group of disorders characterized by dysfunctional hematopoietic stem cells (HSCs) and progenitor cells, resulting in ineffective hematopoiesis and a high risk of progression to acute myeloid leukemia (AML). The incidence of MDS increases with age. Abnormalities in mitochondrial physiology, which regulate critical cell-intrinsic signals for HSC maintenance, contribute to its aging and are frequently observed in MDS. Nucleophosmin 1 (NPM1), located on 5q35, is lost in approximately 10% of MDS cases associated with large 5q deletions and is the most commonly mutated gene in cytogenetically normal AML. While NPM1 is involved in multiple cellular processes, its role in mitochondrial physiology remains unexplored. In this study, we established a mouse model with conditional knockout (cKO) of Npm1 in the hematopoietic system, recapitulating human MDS phenotypes. Loss of Npm1 led to premature aging of HSCs and activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome. Our findings demonstrated aberrant mitochondrial activation in Npm1-deleted HSCs, as well as in an inducible humanized Npm1c (cytoplasmic Npm1) knock-in mouse model. Intracellular localization analysis also revealed that, in addition to the cytosol, NPM1 localizes to both mitochondria and mitochondria-associated membranes (MAM) fraction. MAMs are specialized domains enriched in crucial Ca2+ signaling elements, facilitating Ca2+ transfer between the endoplasmic reticulum (ER) and mitochondria. Consequently, MAMs can potentially lead to mitochondrial Ca2+ overload and destabilization, resulting in the release of mitochondria-derived ligands and subsequent activation of the NLRP3 inflammasome. Our study demonstrated the enrichment of both inflammatory and Ca2+ pathways in Npm1-deleted cells. Thus, pro-inflammatory signaling contributes to myeloid-biased hematopoiesis, which cooperates with the development of MDS-like disorders. In summary, our research highlights the role of NPM1 in maintaining mitochondrial integrity within HSCs and provides insights into the mechanisms underlying MDS pathogenesis. Understanding these processes may lead to the development of targeted therapies for MDS and related disorders.

Keywords: NPM1, mitochondria, inflammation

4:45 PM – 5:45 PM

472

WHOLE-GENOME SCREENING REVEALS CRITICAL ROLE FOR THE CHROMATIN READER ZMYND8 IN MEDIATING ANTI-CANCER IMMUNOTHERAPY THROUGH PD-L1

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Treatment of leukemia often involves the use of high doses of anthracyclines, such as doxorubicin (DXR), which results in severe side-effects including cardiotoxicity and development of secondary cancers. In our previous studies, DXR was shown to target therapy-resistant leukemia stem cells (LSCs) at low, generally non-toxic doses. Thus, we repurposed DXR as a targeted inhibitor rather than a broadly cytotoxic chemotherapy. In contrast to typical clinical usage, low-dose DXR treatment reduced expression of multiple immune checkpoint (IC) genes including PD-L1, which exposed LSCs to elimination by cytotoxic T cells. In particular, anti-PD-L1 treatment was shown to mimic and synergize with low DXR in targeting chemoresistant LSCs. Mechanistically, activated Wnt/ß-catenin was shown to bind multiple IC gene promoters, which was inhibited by low DXR. To investigate the distinct immunotherapeutic effects observed in low, sustained DXR treatment regimens vs high clinical doses, we performed whole-genome CRISPRi screens in leukemia cells. Differential gene analysis of top hits revealed dynamic immune cell changes, with lower DXR doses initiating innate immune responses but mid-range doses activating adaptive immune responses. Interestingly, genes modulating epigenetic effects were among the most prominently distinct in lower vs cytotoxic doses of DXR. Particularly, we identified ZMYND8, a chromatin reader with known roles in responding to DNA damage and tumor suppression. In a cytotoxic setting, knock-down of ZMYND8 conferred DXR resistance. Further analysis revealed that while low-dose DXR decreases PD-L1 expression, inhibition of ZMYND8 prevents PD-L1 repression by DXR. We also found that several Wnt signaling genes were resistant to cytotoxic doses but sensitized to low and mid-range doses. This coincides with our previous work demonstrating that low-dose DXR reduces Wnt signaling levels in LSCs, which are responsible for chemoresistance and immune escape. These results suggest that ZMYND8 is potentially responsible for DXR's efficacy by mediating its effect on PD-L1 in collaboration with the Wnt signaling pathway. Ultimately, we aim to reveal novel therapeutic strategies for harnessing the immune system to potentiate the response of low-dose DXR treatment providing less toxic cures for cancer.

Keywords: screen, immunotherapy, PD-L1

4:45 PM – 5:45 PM

TOPIC: KIDNEY

474

EFFECT OF TONSIL-DERIVED MESENCHYMAL STEM CELLS ON PHENOTYPE TRANSITION AND PERITONEAL FIBROSIS IN PERITONEAL MESOTHELIAL CELLS

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Mesenchymal stem cells (MSCs) are multipotent adult stem cells that have regenerative capability and exert paracrine actions on damaged tissues. The epithelial-to-mesenchymal transition (EMT) of mesothelial cells (MCs) is an early mechanism of peritoneal dysfunction in peritoneal dialysis (PD). MSCs have recently received a new attention due to its preventive effect on organ fibrosis by inhibiting EMT. Tonsil-derived MSCs (T-MSCs) can be isolated from the tonsillectomy of patients, and are reported to be effective in hepatic fibrosis. The aim of this study is to investigate the role of T-MSCs in TGFβ-induced EMT of human peritoneal mesothelial cells (HPMCs) and its mechanism. Transwell co-culture system was used in which MCs were cultured with T-MSCs or T-MSC-conditioned medium (T-MSC-CM). EMT was evaluated by the changes in morphology and markers of epithelial and mesenchymal cells. ROS generation was assessed by DCF-DA and MitoSoxR staining. Animal model of PD was established by daily infusion of 4.25% glucose-based dialysate with methylglyoxal for 3 weeks via intraperitoneal catheter in Sprague-Dawley rats. T-MSC (1.0 x 107cells, i.p.) was injected at 14 days of PD, and peritoneal tissue was isolated at 7 days of T-MSC injection. Markers of oxidative stress, ER-stress, apoptosis, and NLRP3 inflammasome were evaluated with peritoneal equilibrium test (PET) and histologic analysis. Co-culture of HPMCs and T-MSC or T-MSC-CM inhibited TGFβ-induced EMT (increased ZO-1 and E-cadherin and decreased α-SMA) and oxidative stress (decreased ROS generation). In PD+T-MSCs group, EMT and peritoneal fibrosis were ameliorated with a decreased expression of Bax, cleaved caspase, and 8-OHdG in peritoneal membrane with an increase in glutathione peroxidase and superoxide dismutase 2. T-MSC injection also led to a decrease in the markers of ER stress and NLRP3 inflammasome. T-MSCs treated rats had a higher D2/D0 glucose and a lower D2/P2 creatinine compared to PD only group. Anti-human nuclei staining revealed scattered positive staining along peritoneal mesothelial layer. T-MSCs represent a promising approach to prevent peritoneal fibrosis by providing anti-fibrosis and anti-oxidant effects in the peritoneal cavity and ameliorating the phenotype transition of peritoneal mesothelial cells.

Funding Source: This research was supported by the Korean Fund for Regenerative Medicine(KFRM) grant funded by the Korea government(the Ministry of Science and ICT, the Ministry of Health & Welfare). (23A0201L1)

Keywords: tonsil-derived mesenchymal stem cells, peritoneal fibrosis, peritoneal mesothelial cells

4:45 PM – 5:45 PM

476

GT AMELIORATES RENAL FIBROSIS AND SENESCENCE

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Accumulation of senescent cells and senescence-associated inflammatory responses have been characterized as main driving forces of renal fibrosis in disease-associated or age-related kidneys. Here, we uncovered the protective effects of GT against senescence and fibrosis induced by kidney injury and aging. Utilizing kidney injury models induced by cisplatin and adenine, along with naturally aged mice (23–24 months old), we demonstrated the capability of GT to attenuate kidney injury, senescence, and fibrosis. Mechanistically, GT decreased the levels of reactive oxygen species and suppressed the DNA damage response pathways, including p16, p21, and p53. Additionally, GT reduced the levels of senescence-associated secretory phenotype factors, resulting in decreased senescence of stem/progenitor cells. Taken together, our findings indicate that GT represents a novel pharmacological therapeutic candidate for kidney senescence and fibrosis.

Keywords: GT, renal fibrosis, renal fibrosis, sensescence

4:45 PM – 5:45 PM

TOPIC: LIVER

478

THE ROLE OF CELLULAR PLASTICITY IN HUMAN LIVER CANCER

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Liver cancer is frequently associated with metabolic dysfunction-associated steatotic liver disease (MASLD). The incidence of this chronic disorder is rapidly rising and thus, liver cancer poses a global health burden that remains unanswered due to the lack of treatment. Furthermore, the development of new therapeutics is currently impaired by a lack of knowledge concerning the mechanisms driving tumorigenesis in the chronically injured liver. Of particular interest, the cellular origin of intrahepatic cholangiocarcinoma (iCCA) and hepatocellular carcinoma (HCC) remains elusive. Here, we investigate the role of transdifferentiation occurring between hepatocytes and cholangiocytes during chronic disease in tumorigenesis. Using single-nuclei RNA-Sequencing (snRNA-Seq) on liver biopsies from patients with progressive MASLD, we have identified a population of bi-phenotypic cells originating from the transdifferentiation of cholangiocytes into hepatocytes. These cells express markers of both cell types and display a set of specific plasticity factors associated with liver cancer, thereby suggesting a role in malignant transformation. To validate this hypothesis, tumoral and non-tumoral biopsies were obtained from the same patients diagnosed with iCCA. Comprehensive gene and protein expression analyses confirmed the presence of bi-phenotypic cells in tumors. Additional analyses also confirmed the expression of several plasticity factors within cancer cells. These findings were validated in both primary tumor tissue and patient-derived tumoroids, supporting the utility of our in-vitro platform to study cellular plasticity in human liver cancer. Ongoing functional studies in tumoroids will reveal the molecular function of plasticity factors in the context of liver cancer development. Finally, sn-RNA-Seq mapping will provide insights into the transcriptomic landscape of cancer cells in-vivo and in-vitro, thereby addressing the importance of bi-phenotypic cells in cancer heterogeneity. In conclusion, our study describes novel mechanisms implicated in tumorigenesis within the context of chronic liver disease. These insights will improve our understanding of cancer progression and pave the way for the identification of new therapeutic agents.

Keywords: plasticity, liver cancer, patient-derived tumoroids

4:45 PM – 5:45 PM

480

ALTERATION OF METABOLIC ACTIVITY REGULATES MITOCHONDRIAL TEMPERATURE IN HEPATOCELLULAR CARCINOMA

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Oxidative phosphorylation (OXPHOS) is a key player in mitochondrial bioenergetic functions. In hepatocellular carcinoma (HCC), OXPHOS slows down or switches to glycolysis, via what is known as the Warburg effect. The cancer-induced altered respiratory mechanism was reported to affect mitochondrial temperature. We investigated the impact of this metabolic switch on the mitochondrial temperature in the HepG2 HCC cell line. Cancer cells were treated with metformin (N,N-dimethylbiguanide) to suppress glycolysis to emulate lower metabolically active cancer cells (Met-HepG2). The mitochondrial temperature was assessed using mito-thermo yellow (MTY) fluorescent dye, a thermo-probe used to detect the mitochondrial temperature by absorbing its radiant heat. Mito-tracker green (MTG) fluorescent dye was used to confirm mitochondrial localization. Our data showed lower MTY dye intensity in the Met-HepG2 treated group, indicating a significant increase in mitochondrial temperature compared to untreated HepG2 cells (HepG2). Genotypic analysis of the metabolic respiration gene expression showed significant down-regulation in glycolytic genes (ERR-$_{\gamma}$, HK2, PGK, ALDOC, TPI1, IDH1, and PKM2) in the Met-HepG2 cells compared to the HepG2 cells. ATP, reactive oxygen species, and NADPH production were significantly up-regulated in the Met-HepG2 group compared to the HepG2 group. Transmission electron microscopy showed fewer mitochondria with swollen elongated appearance and apoptotic bodies in the Met-HepG2 group. These data show a correlation between HepG2 altered metabolism and mitochondrial temperature and suggest that less metabolically active HepG2 cells are correlated with higher mitochondrial temperature. These data provide evidence for a possible role for mitochondrial temperature as a biomarker in HCC.

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Keywords: mitochondrial temperature, hepatocellular carcinoma (HCC), mitochondrial bioenergetics

4:45 PM – 5:45 PM

TOPIC: MESENCHYMAL STROMAL CELLS, ADIPOSE, AND CONNECTIVE TISSUE

482

DONOR AGE EFFECTS ON DENTAL PULP MESENCHYMAL STROMAL CELL CHARACTERISTICS

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The mesenchymal stromal cells (MSC) from dental pulp (DPSCs) have the potential for self-renewal and differentiation capacity in several cell lineages besides having a higher proliferation rate when compared with other MSCs sources. Due to their shared embryonic origin with the nervous system, DPSCs can potentially treat neurodegenerative diseases. To determine the optimal age for utilizing these cells in future studies, this research aimed to characterize DPSCs from different age groups and investigate age-related variations in their genetic stability and functional properties. The Local Research Ethics Committee approved this project. Healthy teeth from four age groups (n = 5, each group) were compared: Group A (20 to 25 years), Group B (30 to 35 years), Group C (40 to 45 years), and Group D (50 to 55 years). DPSCs were isolated, expanded in vitro, and characterized by differentiation and flow cytometry. The percentage of DPSCs differentiated for adipogenic lineage was analyzed. To evaluate the efficiency of these groups in forming colonies in vitro, the colony-forming units-fibroblast (CFU-F) assay was performed. The classical cytogenetics technique was carried out for genetic stability. The senescence and proliferation rate were assessed by flow cytometry. Statistical analysis was performed by Shapiro Wilk and Kruskal Wallis test, results were presented as mean and standard deviation. DPSCs showed MSC characteristics according to the ISCT criteria. Osteogenic differentiation was observed in all samples, but the adipogenic potential was limited. When evaluating the CFU-F capacity of these cells, significant differences were observed between Groups A, B and C compared to group D. The groups did not show clonal chromosomal abnormalities. When evaluating the senescence, group D exhibited a higher rate compared to Group A. Regarding proliferation, group D demonstrated a lower rate of cell proliferation compared to the other groups. All groups exhibited MSC characteristics and normal karyotypes. DPSCs from groups A, B, and C showed higher colony-forming efficiency, lower senescence rate, and higher proliferation rate than group D. These findings highlight the influence of age on DPSCs proliferation and senescence, emphasizing its significance as a criterion for future studies involving these cells.

Funding Source: Coordination for the Improvement of Higher Education Personnel CNPq Process 465656/2014-5 (INCT-REGENERA)

Keywords: aging, genetic stability, functional properties

4:45 PM – 5:45 PM

484

AGE OF THE DONOR AFFECTS THE NATURE OF IN VITRO CULTURED HUMAN DENTAL PULP CELLS

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The dental pulp stem cells (DPSCs) of six donors (three young donors aged < 19 years and three adult donors aged > 25 and < 30 years) were characterized for their stem cell marker expression and differentiation potential to study the effect of donor age on DPSCs in vitro. DPSCs were cultured in αMEM supplemented with 20% fetal calf serum (conventional conditions) or on fibronectin-coated flasks with neurobasal medium supplemented with B27, bFGF and EGF (alternative conditions). DPSCs were characterized by immunofluorescence staining to detect the neural crest/mesenchymal stem cells markers P75 and CD146, respectively. The differentiation potential was tested by the induction of DPSCs into osteogenic, adipogenic and glial lineages and then by detecting the corresponding markers osteocalcin, lipidtox and S100ß, respectively. The DPSCs of the young donors expressed CD146 only under the conventional conditions and expressed P75 regardless of the culture conditions. However, the DPSCs of adult donors expressed CD146 only under the alternative conditions and expressed P75 only under conventional conditions. Only the DPSCs of the young donors differentiated into the glial linage. The DPSCs of the adult donors differentiated more efficiently into the adipogenic linage. Osteogenic differentiation was comparable. Donor age affects the expression of stem cell markers and differentiation potential of DPSCs. Moreover, the effect of culture conditions on DPSCs is age dependent.

Funding Source: This study was kindly funded by the Deanship of Academic Research, the University of Jordan, Amman, Jordan.

Keywords: dental pulp stem cells aging, mesenchymal stem cells, neural crest stem cells, culture conditions

4:45 PM – 5:45 PM

486

SECRETOME COMPOSITION OF MESENCHYMAL STROMAL CELLS DERIVED FROM EXTRAEMBRYONIC TISSUES

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Understanding the unique features of the type specific MSCs' biology is vital for development biomedical products and optimizing treatment effectiveness. In our study, we analyzed the MSCs derived from extra-embryonic tissues (umbilical cord-derived MSCs (UC-MSCs), amniotic MSCs (A-MSCs) and placental MSCs (Pl-MSCs)) precisely focusing on secretome composition and efficiency of extracellular vesicles (EVs) production. All MSCs cultures exhibited a fibroblastic morphology, similarly high proliferation rate, displayed the CD73+, CD90+, CD105+, CD34-, CD45-, HLA-DR- phenotype and differentiated in adipogenic and osteogenic lineage. Growth factors and cytokines was determined in serum-free conditioned media (CM) after 48-hour MSCs cultivation using ELISA method. The NGF, NT3, NT4, IGFI, INFγ and TGFβ were undetectable in CM from all tested MSC types. The IL-10 was specific to Pl-MSCs secretome and detected at 0.7 ± 0.2 ng/ml level. Enrichment of IL-6 (343.03±23.01 pg/ml) was detected in CM of all MSC types. The highest level of bFGF was specific to Pl-MSCs (139.1±20.5 pg/ml) and A-MSCs (93.73 ± 15.20 pg/ml) CM, while in UC-MSCs was at the low level (46.4 ± 5.2 pg/ml). EGF was revealed at the extremely low level in Pl-MSCs (1.9 ± 0.5 pg/ml) and A-MSCs (1.3 ± 0.2 pg/ml), but not in UC-MSCs CM. The SCF was found at the similar level in UC-MSCs (20.3±1.5 pg/ml) and Pl-MSCs (18.6±2.1 pg/ml) CM, while in A-MSCs was significantly lower (6.5±0.5 pg/ml). All analyzed cell types secreted more than 1000 pg/ml VEGF with highest level in Pl-MSCs CM (8317±53 pg/ml). The GDNF was detected at the low level that not exceed 15 pg/ml in all MSCs-derived CM. The EVs were isolated from CM of studied MSCs using differential ultracentrifugation method with next NTA and protein analysis. The EVs in concentration of 191±2, 251±4 and 198.6±8 particles billions/ml were isolated from CM of UC-MSCs, Pl-MSCs and A-MSCs with average particles size 166.1±5.2 nm. Isolated EVs was positive on CD63 and CD81 expression. Thus, despite the similar anatomic origin and morphology extraembryonic MSCs vary in secretome composition. Pl-MSCs compare to UC-MSCs and A-MSCs secrete a richer profile of growth factors and cytokines and produce higher amount of EVs making them an attractive candidate for the development of cell-free biotechnological products.

Keywords: secretome, mesenchymal stromal cells derived from extraembryonic tissues, extracellular vesicles

4:45 PM – 5:45 PM

488

CANINE-IN-A-MOUSE PRE-CLINICAL ANIMAL MODEL TO ASSESS THE SAFETY OF CANINE MESENCHYMAL STEM CELL (MSC) ADMINISTRATION TO DOGS

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Cell therapy using MSCs is a promising modality and FDA has seen an increasing interest in companies pursuing approval for such therapies. However, current knowledge about MSC's mechanism of action (MoA) is primarily derived from pre-clinical work in murine models. There are advantages associated with murine models, however, the effect of xenogeneic MSCs on mice yields uninterpretable data due to incompatibility of exogenous MSCs and murine immune cells. Hence, murine models present a barrier to development of therapeutic veterinary products. Therefore, we hypothesize that a caninized mouse model would be a more stringent model to test MSC-based advanced therapies for canine patients. "Caninized mice", i.e., immunodeficient mice that support the development of canine immune systems by engraftment with canine leukocytes, better represent the canine immune system. To develop this system, we injected canine peripheral blood mononuclear cells (PBMCs) into NOG-EXL mice (NOD.Cg-Prkdcscid Il2rgtm1Sug Tg (SV40/HTLV-IL3,CSF2)10-7Jic/JicTac). We optimized the number of cells and route of administration required to obtain efficient engraftment the of canine PBMCs. Administration of 2 x 107 canine PBMCs intraperitoneally had the best engraftment, as measured by flow cytometry analysis of splenocytes. Post-engraftment, canine CD45+ cells were present in PBMC as early as seven days. Between 80-90 % of engrafted canine CD45+ cells were CD5+ T cells with a CD4:CD8 ratio ranging from 5:1 to 2:1. Additionally, we were able to detect canine T cells in spleen and bone marrow of transplanted mice. Four weeks post-engraftment, mice developed graft vs host disease (GvHD). Histologically, the spleens had extracapsular nodules, multifocal to coalescing nodules composed of blastic leukocytes with mitotic figures and scant cytoplasm. Myeloid and thrombocytic cells were also mildly increased in the splenic red pulp as well as in bone marrow. In summary, our studies suggest that the NOG-EXL mice allows engraftment of canine PBMCs and may provide a short-term model for investigating allogeneic MSC-based veterinary therapeutics. Disclaimer: This abstract reflects the views of the authors and should not be construed to represent FDA's views or policies

Funding Source: US FDA

Keywords: mesenchymal stem cells, pre-clinical animal model, caninized mice

4:45 PM – 5:45 PM

490

SENESCENCE-ASSOCIATED COMPETENT CHANGES IN MESENCHYMAL STEM/STROMAL CELLS

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Mesenchymal stem/stromal cells (MSCs) have immunomodulatory functions and are a promising source for cell-based regenerative medicine. Although the competence of tissue stem cells is known to be altered with aging, characteristic changes of MSCs in the clinical-scale expansion remain elusive. Cluster of differentiation (CD) 73 is widely expressed in MSCs and used to obtain a uniform MSC population. Human adipose-derived CD73 positive cells exhibited downregulation of inflammatory gene expressions compared with a heterogenous MSC population attached to the culture dish. Herein, we performed the longitudinal analyses of cell characterization, including cellular senescence, secretory phenotypes, and differentiation ability, in both CD73 positive cells and heterogenous MSCs. We found an upregulation of senescence-related gene expression and a reduction of the portion of Ki67 positive proliferation cells in CD73 positive cells and heterogenous MSCs after long-term culture for 120 days. The adipogenic and osteogenic differentiation abilities of these cells declined with long-term culture. MSCs secrete inflammatory cytokines, such as interleukin (IL)-6 and C-C motif chemokine ligand 2 (CCL2), after stimulation of lipopolysaccharide (LPS) and Poly (I:C). Notably, the cytokine production after LPS stimulation continued in post-senescence MSCs but not after Poly (I:C) stimulation. These results suggested that the alteration of secretory competence in the long-term culture of MSCs through a specific signal transduction change. We further demonstrated differences in gene expression after stimulations between uniform CD73 positive cells and heterogenous MSCs at 14 and 120 days of culture. Our findings indicate that senescence-associated competent changes (termed SACC) in the clinical-scale expansion of MSCs. The SACC, such as CCL2 secretion after Poly (I:C) stimulation, is a potential new quality control marker.

Keywords: cellular senescence, competent change, mesenchymal stem/stromal cells

4:45 PM – 5:45 PM

492

SEQUENTIAL SINGLE CELL TRANSCRIPTOME ANALYSES IDENTIFIED THE UNIQUE FIBROBLAST SUBSET WITH POTENTIAL OF MESENCHYMAL-TO-EPITHELIAL TRANSITION IN THE ENGRAFTED SKIN

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In the process of skin wound healing, fibroblasts recover the dermal environment by remodeling the extracellular matrix and contracting the wound surface. In addition, previous studies have shown that fibroblasts or mesenchymal stem cells are mobilized in the injury with epidermal necrosis, and have a plasticity to convert keratinocytes. We found that 1.2% of keratinocytes in the engrafted skin were the lineage of platelet-derived growth factor receptor alpha (PDGFRα) expressing mesenchymal cells. However, it remains unknown that the characters of the fibroblasts with the potential to mesenchymal-to-epithelial transition (MET). To identify these fibroblasts population during skin regeneration process, we used the skin engrafting mice model. Severe epidermal necrosis occurred broadly in the skin graft at post-operative day (POD) 3, then re-epithelialization and hair growth are observed until POD 15. Time-course analyses of single cell transcriptome of the graft for 15 days after surgery identified the unique fibroblasts population expressing keratinocyte markers including keratin 5, and MET related transcription factors, such as interferon regulatory factor 6 (IRF6) and transcription factor AP-2 alpha (TFAP2α). These fibroblasts contained two clusters, the one expressed dermal papilla markers and the other expressed stem cell antigen 1(Sca1) and CD34. Histologically, some dermal papilla cells showed keratin 5, protein zero and Wnt1 expressing cell lineage in physiological state, which indicated dermal papilla cells were the ectoderm derived mesenchymal cells. They proliferated significantly at about POD 6, and spread in papillary dermis mainly along the hair follicles. These data suggest that, in the condition with necrotic injury, the dermal papilla cells have the potential to convert keratinocytes and contribute epidermis regeneration.

Keywords: skin regeneration, mesenchymal-to-epithelial transition, wound healing

4:45 PM – 5:45 PM

494

UNRAVELING CELLULAR SENESCENCE OF DPSCS AND BMSCS IN RESPONSE TO PROLONGED IN VITRO CULTIVATION

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Cell senescence represents an ongoing and irreversible biological progression culminating in cell cycle arrest. Cells utilized in laboratory settings tend to accumulate debris over time, leading to diminished proliferation and impaired functionality. Mesenchymal stem cells (MSCs), widely distributed in various tissues and organs, hold significant promise for tissue regeneration. However, the isolation and further laboratory applications of these stem cells is typically constrained by limitations caused by the small number of obtained cells. This study aims to investigate cell proliferation and longevity, seeking a deeper understanding of the processes of cellular aging in adult oral mesenchymal stem cells (OMSCs). Dental pulp stem cells (DPSC) and bone marrow stem cells (BMSC), were isolated from non-fully developed third molars and bone fragments, obtained in routine dental extractions, using standard laboratory procedures. Tissues underwent enzymatic digestion, and cells were cultured in DMEM Dulbecco modified eagle media supplemented with 1% antibiotics and 10% FBS fetal bovine serum. Standard cell culture conditions were maintained, and media changes occurred every 2nd or 3rd day. Cells were passaged and counted at 80-90% confluence. MTT test for proliferation was administered. The cells underwent cultivation and passaging over several months, eventually reaching passage 27. Through a comparison of various passages of mesenchymal stem cells, we observed that they retained their vitality and exhibited stem cell-like characteristics beyond passage 27. There was no significant decline in their proliferation ability. The application of stem cells in regenerative medicine requires prior in vitro cultivation and multiplication. The cells isolated from non-fully developed third molars pulp demonstrate prolonged viability and show potential for further research.

Keywords: aging, DPSC, BMSC

4:45 PM – 5:45 PM

496

CANNABIDIOL PROMOTES SPONTANEOUS ADIPOGENESIS IN HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Over the years, human mesenchymal stem cells (hMSCs) have gained interest in the field of regenerative medicine, due to their immunomodulatory and differentiative abilities. Among the hMSCs, the adipose-derived MSCs (hASCs) seem to be very attractive, as they are obtained with minimally invasive procedures, and they are able to differentiate into specific lineages. The goal of many researchers is to modulate hMSCs biological properties to make them intriguing candidates in regenerative medicine field. The aim of our work was to evaluate the effects of cannabidiol (CBD, 0.1-10 μM) on hASCs, a molecule extensively studied in the neurological and pain fields, but little studied in the field of stem cells, despite its promising properties.Our studies revealed that CBD didn't significantly influence viability, proliferation and cell senescence of these cells, but it significantly promoted the spontaneous adipogenesis of hASCs, without using specific differentiative media, as revealed by the presence of lipid droplets in the cell cytoplasm. Adipogenic process was also confirmed by gene expression analysis of two early adipogenic markers, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (CEBPα), and immunofluorescence analyses of early and late adipogenic markers. These data lead to further research in this field to evaluate the promising role of CBD in hASC-mediated therapies.

Keywords: human adipose-derived mesenchymal stem cells, cannabidiol, spontaneous adipogenesis

4:45 PM – 5:45 PM

498

TRANSCRIPTION FACTOR HOX REGULATES MSC STEMNESS AS AN UPSTREAM FACTOR OF TWIST1

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Human mesenchymal stem cells (MSCs) represent one of the most used stem cells for clinical application due to multilineage differentiation potential, secretome and immunosuppression. Although MSCs have been used in over 1500 clinical trials to treat over 30 diseases, the molecular basis underlying MSC stemness still remains poorly understood, this greatly affects clinical applications of MSCs. To address this important question related to MSC biology, we established a step-wise, chemically defined and highly efficient iPSC-MSC platform, which enables us to study MSC development in vitro. By comparing gene expression profile during differentiation of iPSCs towards MSCs, we screened and identified one transcription factor HOX regulating MSC stemness. Knockdown of HOX abolished MSC proliferation and greatly decreased colony formation (CFU-F). Moreover, accelerated MSC senescence and a decrease in the expression of cell surface antigens linked to the MSC phenotype was observed, multi-linage differentiation was greatly impaired. HOX inhibitor consistently abolished MSC proliferation, CFU-F and compromised MSC differentiation potential. Notably, overexpression resulted in improved multi-lineage differentiation. HOX expression decreases with MSC important genes during in vitro expansion, such as Twist 1, RUNX2 and SOX9. ChIP-seq data showed that HOX binding sites at early passage of MSCs correlated with H4K4me3 binding sites for active genes, late passage of MSCs lose the most binding sites of early passage of MSCs. Most importantly, ChIP-seq data showed that HOX directly regulated Twist1, Twist1 overexpression partially rescued decreased MSC proliferation by HOX knockdown, showing Twist1 is a downstream factor of HOX. In addition, HOX reporter can be used to enrich good quality of MSCs expressing higher MSC important genes and displaying better differentiation potential. These data showed transcription factor HOX regulates MSC stemness as an upstream factor of Twist1. The identification of transcription factor HOX regulating MSC stemness not only deepens the understanding toward molecular basis of MSC stemness, but also provides a novel strategy for gene therapy using MSCs.

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Keywords: mesenchymal stem cells, stemness, transcription factor

4:45 PM – 5:45 PM

500

TECHNICAL DIFFICULTIES CONSIDERING THE ISOLATION OF EXOSOMES FROM MSCS' CULTURE MEDIUM - PRESENCE OF EXOGENOUS NANOPARTICLES IN WIDELY USED REAGENTS

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Mesenchymal stem/stromal cells (MSCs) exhibit unique therapeutic properties, which are mostly based on their secretory profile – they secrete various immunomodulatory, neuroprotective and proangiogenic factors. These properties seem to be connected to the release of exosomes, which in the in vitro culture are secreted directly into the medium. The released exosomes can later be isolated and further analysed. Basing on our experience with Wharton's-jelly derived mesenchymal stem/stromal cells (WJ-MCSs) and with the analysis of WJ-MSCs-derived exosomes, we observed that many of the reagents used in our lab for in vitro cell culture and exosome isolation contain exogenous nanoparticles. Therefore, we decided to perform an additional NTA analysis on the aforementioned reagents. We investigated the number of nanoparticles in - 1) Phosphate buffered saline - PBS (Sigma Aldrich) - 2) Dulbecco's Modified Eagle Medium – DMEM (Sigma Aldrich) - 3) Dulbecco's Modified Eagle Medium – DMEM (Gibco, ThermoFisher Scientific) - 4) Human Platelet Lysate – PLT Gold (Mill Creek Life Sciences) - 5) Full, standard culture medium - DMEM (Sigma Aldrich) + 1% AAS (antibiotic-antimycotic solution) + 5% PLT Gold. All reagents/media were sterile and freshly opened after having been sealed by manufacturer. We excluded any possibility of cross-contamination. After the first set of analyses, we have decided to eliminate the unwanted nanoparticles present in the aforementioned reagents and media by ultracentrifugation. After being centrifuged, the number of nanoparticles in the analysed reagents/media was once again investigated by NTA method. Moreover, we investigated whether double filtration through 0,1 µm-pore filters eliminates exosomes and other nanoparticles from PBS. It turns out that many widely used reagents contain exogenous nanoparticles, which interfere with the final outcome of the analysis – the exact number of exosomes secreted by cells themselves is difficult to establish. Manufacturers should therefore consider coming up with a solution either to reduce or eliminate exogenous nanoparticles in reagents they produce. Researchers should also keep in mind that the reagents they are using might contain exogenous nanoparticles and that their results considering the number of exosomes in a given sample might be unreliable.

Funding Source: This research was funded by National Science Centre grant number 2022/47/O/NZ3/01739.

Keywords: mesenchymal stem/stromal cells, exosomes, nanoparticles

4:45 PM – 5:45 PM

502

HUMAN POST-IMPLANTATION BLASTOCYST-LIKE CHARACTERISTICS OF NON-TUMORIGENIC MUSE CELLS ISOLATED FROM HUMAN UMBILICAL CORD

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Multilineage-differentiating stress enduring (Muse) cells, identified as cells positive for the pluripotent surface marker SSEA-3, are pluripotent-like endogenous stem cells located in the bone marrow (BM), peripheral blood, and organ connective tissues. The detailed characteristics of SSEA-3(+) cells in extraembryonic tissue, however, are unknown. Here, we demonstrated that similar to human-adult tissue-Muse cells collected from the BM, adipose tissue, and dermis as SSEA-3(+), human-umbilical cord (UC)-SSEA-3(+) cells express pluripotency markers, differentiate into triploblastic-lineage cells at a single cell level, migrate to damaged tissue, and exhibit low telomerase activity and non-tumorigenicity. Notably, ~20% of human-UC-SSEA-3(+) cells were negative for X-inactive specific transcript (XIST), a naïve pluripotent stem cell characteristic, whereas all human adult tissue-Muse cells are XIST-positive. Single-cell RNA sequencing revealed that the gene expression profile of human-UC-SSEA-3(+) cells was more similar to that of human post-implantation blastocysts than human-adult tissue-Muse cells. The DNA methylation level showed the same trend, and notably, the methylation levels in genes particularly related to differentiation were lower in human-UC-SSEA-3(+) cells than in human-adult tissue-Muse cells. Furthermore, human-UC-SSEA-3(+) cells newly express markers specific to extraembryonic-, germline-, and hematopoietic-lineages after differentiation induction in vitro whereas human-adult tissue-Muse cells respond only partially to the induction. Among various stem/progenitor cells in living bodies, those that exhibit properties similar to post-implantation blastocysts in a naïve state have not yet been found in humans. Easily accessible human-UC-SSEA-3(+) cells may be a valuable tool for studying early-stage human development and human reproductive medicine.

Keywords: umbilical cord, muse cells, human post-implantation blastocyst

4:45 PM – 5:45 PM

504

THE ENHANCER LANDSCAPE PREDETERMINES THE SKELETAL REGENERATION CAPACITY OF STROMAL CELLS

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Stromal cells are an attractive source for cell therapy and tissue engineering. Their widespread application faces challenges due to the functional diversity of available cell sources. Here we demonstrate that human stromal cells from bone marrow (BMSCs), but not white adipose tissue or umbilical cord form 3D cartilage discs in vitro, which could regenerate critical-size femoral defects in mice. Analyzing 246 transcriptomic datasets, we identified 2884 genes uniquely expressed in BMSCs, highlighting their distinct osteogenic and chondrogenic potential. The differential expression of RUNX3, DLX5, and ALPL underscores the distinct osteogenic and chondrogenic potential of BMSCs compared to other stromal cells. Beyond identifying transcriptional differences, we revealed that the regenerative capabilities of stromal cells are governed by the accessibility of specific enhancer and promoter regions, facilitating distinct osteochondral differentiation pathways. We discovered that binding sites for commonly expressed transcription factors, particularly of the Runt and bZIP families, were uniquely accessible in BMSCs, indicating an epigenetically predetermined differentiation potential that is intrinsic to the cell's origin. This predetermination enables common transcription factors to initiate distinct organ-specific transcriptional programs, thereby streamlining the identification of cell sources with high regeneration competence. Furthermore, our research demonstrates the pivotal role of viable human BMSCs in initiating defect healing through osteopontin secretion, contributing to the formation of a transient mineralized bone hard callus after transplantation into immunodeficient mice. This callus was eventually replaced by the recipient's bone during the final stages of tissue remodeling, highlighting the dynamic and interactive process of skeletal regeneration. These findings underscore the importance of epigenetic landscapes in stromal cell differentiation and regeneration, offering novel insights into optimizing cell source selection for therapeutic applications.

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Keywords: enhancer landscape, regenerative medicine, skeletal regeneration

4:45 PM – 5:45 PM

506

NAMPT-DEPENDENT ACSL5 ENHANCES FATTY ACID OXIDATION AND PRESERVES EXOSOMAL THERAPEUTIC EFFICACY IN TNF-α-EXPOSED MESENCHYMAL STEM CELLS

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The therapeutic effectiveness of mesenchymal stem cells (MSCs) is greatly influenced by senescence, a process that is worsened by inflammatory environment such exposure to tumor necrosis factor alpha (TNF-α). This study aims to fill the significant knowledge gap regarding the metabolic mechanisms that contribute to the senescence of umbilical cord blood-derived MSCs (UCB-MSCs). Specifically, it investigates the impact of nicotinamide phosphoribosyl transferase (NAMPT) on the regulation of fatty acid oxidation (FAO) and exosome secretion, plays a crucial role in preserving the anti-inflammatory effects and preventing UCB-MSC senescence. Our results show that senescent UCB-MSCs produce less efficient exosomes for immunomodulation and lowered NAMPT expression in senescent UCB-MSCs leads to an imbalance in NAD+ consumption and biosynthesis, promoting cellular senescence. In the cells exposed to TNF-α have increased NAMPT expression, which subsequently promotes the expression of acyl-CoA synthetase long chain family member 5 (ACSL5), an important enzyme in the FAO pathway. This metabolic alteration not only inhibits the intracellular fatty acid accumulation within UCB-MSCs, but also impacts the release and immunomodulatory function of exosomes secreted from UCB-MSCs exposed to TNF-α. However, the exosome derived from the NAMPT-silenced UCB-MSCs have reduced anti-inflammatory effects in collagen-induced arthritic mice model. We conclude that NAMPT-mediated metabolic adaptation is essential for preserving the therapeutic efficacy of exosomes secreted from UCB-MSCs, highlighting a novel avenue to prevent MSC senescence through metabolic regulation. Our research elucidates the mechanisms by which NAMPT and ACSL5 promote a metabolic alteration in the UCB-MSCs exposed to TNF-α, broadening our comprehension of MSC biology, and unlocking novel prospects for therapeutic interventions in regenerative medicine.

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Keywords: mesenchymal stem cells, fatty acid oxidation, nicotinamide phosphoribosyl transferase, ACSL5, exosome

4:45 PM – 5:45 PM

508

UNRAVELING HARMONY: DISRUPTED BONE MARROW STEM CELL NICHE IN CLONAL HEMATOPOIESIS AND MYELODYSPLASIA

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Aging hematopoietic stem cells (HSCs) exhibit lineage bias and can accumulate somatic mutations, fostering competitive advantage and causing clonal expansion, known as clonal hematopoiesis (CH). Moreover, aging alters the function and composition of the bone marrow (BM) niche, including a lineage skew of the multipotent stromal cells (MSCs) towards adipocytes. These intrinsic and extrinsic defects can contribute to blood malignancies including myelodysplastic syndromes (MDS). The human BM stem cell niche architecture and its remodeling during CH onset and MDS progression is poorly understood. To investigate this, we conducted a comprehensive study utilizing single-cell multiomics, proteomics, and multiplexed imaging to explore the pathological interactions among HSCs, stroma, and T cells in matched BM liquid and bone biopsies from a longitudinal CH/MDS study. Bone imaging revealed significant niche remodeling in CH compared to healthy donors, marked by emergent inflammatory MSCs and expanded sinusoidal endothelium. These changes intensified in MDS with a buildup of exhausted regulatory T cells. scRNA-seq supported these observations and identified specific inflammatory subsets in CH/MDS including cytokine-producing HSCs, stress-induced MSC subpopulations, and increased IFN-responsive cytotoxic T cells. To reveal the clonal diversity of the T cell subsets, we employed T cell receptor repertoire analysis (scTCR). Moreover, we used single-cell long-read sequencing (RaCH-seq) to identify mutated HSCs, allowing us to computationally unveil MDS-specific cross-talk between mutant HSCs, inflammatory stromal cells, and T cells. Lastly, proteomics analysis from a BM-MDS co-culture model replicated these inflammatory and pro-angiogenic processes, underscoring their significance in stroma remodeling in MDS. Summarized, our multiomics and imaging analyses discovered novel insights into BM niche adaptations under inflammatory stress during CH and MDS progression. Our findings not

only elucidate pathophysiological changes in chronic blood diseases but also deepen our general understanding of the human BM stem cell niche composition.

Keywords: bone marrow niche, clonal hematopoiesis and MDS, single cell multiomics

4:45 PM – 5:45 PM

510

HUMAN PLACENTA-DERIVED MESENCHYMAL STEM CELLS STIMULATE NEURONAL REGENERATION BY PROMOTING AXON GROWTH AND RESTORING NEURONAL ACTIVITY

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In the last decades, mesenchymal stem cells (MSCs) have become the cornerstone of cellular therapy due to their unique characteristics. Specifically human placenta-derived mesenchymal stem cells (hPMSCs) are highlighted for their unique features, including ease to isolate, non-invasive techniques for large scale cell production, significant immunomodulatory capacity, and a high ability to migrate to injuries. Researchers are exploring innovative techniques to overcome the low regenerative capacity of Central Nervous System (CNS) neurons, with one promising avenue being the development of tailored mesenchymal stem cell therapies capable of promoting neural repair and recovery. In this context, we have evaluated hPMSCs as candidates for CNS lesion regeneration using a skillful co-culture model system. Indeed, we have demonstrated the hPMSCs ability to stimulate damaged rat-retina neurons regeneration by promoting axon growth and restoring neuronal activity both under normoxia and hypoxia conditions. With our model we have obtained neuronal regeneration values of 10%–14% and axonal length per neuron rates of 19-26, µm/neuron. To assess whether the regenerative capabilities of hPMSCs are contact-dependent effects or it is mediated through paracrine mechanisms, we carried out transwell co-culture and conditioned medium experiments confirming the role of secreted factors in axonal regeneration. It was found that hPMSCs produce brain derived, neurotrophic factor (BDNF), nerve-growth factor (NGF) and Neurotrophin-3 (NT-3), involved in the process of neuronal regeneration and restoration of the physiological activity of neurons. In effect, we confirmed the success of our treatment using the patch clamp technique to study ionic currents in individual isolated living cells demonstrating that in our model the regenerated neurons are electrophysiologically active, firing action potentials. The outcomes of our neuronal regeneration studies, combined with the axon-regenerating capabilities exhibited by mesenchymal stem cells derived from the placenta, present a hopeful outlook for the potential therapeutic application of hPMSCs in the treatment of neurological disorders

Keywords: mesenchymal stem cells, neuroregeneration, hypoxia

4:45 PM – 5:45 PM

514

GLIA-LIKE CELLS INDUCED FROM HUMAN MESENCHYMAL STEM CELLS EXHIBIT ENHANCED THERAPEUTIC EFFECTS FOR CHRONIC ISCHEMIC STROKE RECOVERY

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The escalating incidence of stroke, predominantly cerebral infarction, poses a substantial burden, with a considerable proportion of patients experiencing severe neurological aftereffects. However, effective treatments for chronic cerebral infarction are lacking. While stem cell therapy holds theoretical promise for brain regeneration and plasticity, clinical trials, particularly involving mesenchymal stem cells (MSCs), have fallen short of demonstrating significant therapeutic efficacy. Therefore, exploring additional strategies to augment MSC potential or therapeutic efficacy may yield significant advancements in MSC applications, presenting enhanced clinical opportunities in the future. This study aims to explore the therapeutic potential of glia-like cells induced without genetic modifications from human mesenchymal stem cells (ghMSCs) in alleviating chronic sequelae resulting from cerebral infarction. Transcriptomic analysis revealed the acquisition of neuroprotective astrocytic characteristics by ghMSCs. Both ex vivo simulations using organotypic brain slice cultures and in vivo experiments demonstrated superior neuroregenerative and neuroprotective effects of

ghMSCs compared to hMSCs. The dose-dependent efficacy of ghMSCs in restoring neurobehavioral functions and reducing chronic brain infarction were also observed. The observed beneficial effects were attenuated by a CXCR2 antagonist, implicating the involvement of the CXCR2 signaling pathway in mediating these effects. In conclusion, this study suggests the potential of ghMSCs in treating refractory sequelae resulting from chronic cerebral infarction, with CXCR2 playing a crucial role in the underlying therapeutic mechanisms.

Funding Source: Korea Health Technology R&D Project through the KHIDI funded by Ministry of Health & Welfare (HI20C0253, HU21C0113, HU21C0007), NRF of Korea grant by the Ministry of Science and ICT (2022R1A2C1091804, RS-2023-00278819)

Keywords: chronic cerebral infarction, glia, neuroregeneration

4:45 PM – 5:45 PM

516

AGING IMPAIRS THE ABILITY OF EXTRACELLULAR VESICLES DERIVED FROM MESENCHYMAL STEM CELLS PROTECTING SKIN CELLS FROM ULTRA-VIOLET (UV)-INDUCE DAMAGE

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Destructive effects of UV from the sun on the skin cells are known as the main cause of skin aging and cancers, which incidence is noticeably increasing in many countries. Extracellular vesicles (EV) derived from stem cell, especially mesenchymal stem cell (MSC), possesses therapeutic abilities in protecting skin cells from UV-induced damage. In addition, although the effects of aging on the regenerative abilities of MSC have been reported, whether aging affects the functions of MSC-EV on skin protection is still obscured. In the present study, we compared the effects of EV derived from MSC isolated from adipose tissue of elderly donors (eAT-EV) and those from young donors, including infant-adipose-tissue MSC (iAT-EV) and Wharton's jelly MSC of umbilical cord (WJ-EV), on the protection of dermal fibroblasts and keratinocytes (HaCaT) from UV-induced damage. Under UV exposure, while fibroblasts and keratinocytes showed elevated ROS level, DNA damage and apoptosis rate, treatment with EV reduced these damages. Interestingly, the iAT-EV and WJ-EV showed greater rescuing effects than eAT-EV, suggesting the impairment of eAT-EV's ability due to aging. To clarify the protecting mechanisms of young donor MSC-EV, we further examined the anti-inflammation and anti-oxidation functions of WJ-EV. The results showed that cells incorporating WJ-EV exhibited increased Sirt1/FoxO1/CAT,SOD1,SOD2 level under UV exposure. Of note, in the coculture of keratinocytes and fibroblast using a transwell system, keratinocytes-incorporated-WJ-EV showed protective effects on the lower chamber's fibroblasts, which indicated the indirect effects of WJ-EV throughout recipient cells. Furthermore, we performed the small RNA sequencing of WJ-EV and examined the relationship between highly expressed miRNAs and anti-oxidation factors. By using Target Scan and miRDB, we suggested the role of miR-122 and miR-381 in targeting the Sirt1 inhibitor TP53. Collectively, younger sources of MSC-EV could reduce the damage of UV exposure in comparison to the older one, and induce anti-oxidative responses. The profile of miRNAs in WJ-EV and their potential targets might broaden the functions of miRNAs. As the limit in the sources of infant adipose tissue, Wharton's Jelly MSC-EV could be a promising source for further applications in the future.

Keywords: UV damage, extracellular vesicle, skin photoaging

4:45 PM – 5:45 PM

518

SKIN REGENERATION EFFECTS OF 3D CULTURED CANINE ADIPOSE TISSUE-DERIVED STEM CELLS

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Mesenchymal stem cells (MSCs) are being applied in the treatment of inflammatory diseases and tissue regeneration due to their ability to secrete cytokines and growth factors, which regulate inflammation and promote tissue regeneration. The secretion pattern of cytokines and growth factors may vary depending on the culture conditions of MSCs, potentially leading to improved therapeutic effects. Therefore, research on the culture conditions of MSCs continues. Recently, attempts have been made to create spheroids or organoids through 3D culture methods to mimic biological environments for therapeutic applications. However, therapeutic applications of 3D-cultured MSCs have not yet been explored in veterinary medicine. After obtaining canine adipose tissue-derived mesenchymal stem cells (cAT-MSCs), we cultured cAT-MSC spheroids using ultra-low adhesion plates. We observed an increase in the expression of VEGF-A and bFGF mRNA levels in MSCs during 3D culture (VEGF-A, $p < 0.01$; bFGF, $p < 0.05$). As these growth factors influence tissue regeneration and angiogenesis, we applied 3D-cultured cAT-MSC spheroids therapeutically to dogs with skin tissue loss due to trauma. We seeded 2x10^6 cells on an ultra-low adhesion plate and cultured them for 48 hours to obtain cAT-MSC spheroids. These spheroids were then diluted in 0.9% normal saline to a concentration of approximately 300 spheroids/ml. We performed subcutaneous injections of 100 µl around the lesion periphery, totaling 2 ml (approximately 600 spheroids). After six sessions conducted at two-week intervals, no adverse effects were observed, and improvement in the lesion with exposed muscles and blood vessels was noted. This study represents the first attempt to therapeutically apply 3D-cultured cAT-MSC spheroids to dogs, confirming their effectiveness in skin regeneration. Therefore, we believe that therapeutic application of cAT-MSC spheroids is feasible, and based on the increased expression of growth factors, they can be applied as a superior treatment compared to 2D culture methods.

Keywords: mesechymal stem cell, 3D culture, skin regeneration

4:45 PM – 5:45 PM

520

IN VITRO AGEING CHANGES THE GENE EXPRESSION AND SECRETOME PROTEOMIC PROFILE OF CANINE ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

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Canine adipose-derived mesenchymal stem cells (cAD-MSCs) are a prospective therapeutic tool in veterinary regenerative medicine due to their quantity and regeneration potential. In comparison to cell therapy, recent research highlights the stem cells' paracrine molecules, known as the secretome, and their therapeutic benefits. However, issues with the long-term in vitro culture and regenerative properties of cAD-MSCs have been raised. This study aims to investigate how in vitro ageing affects the gene expression of cAD-MSCs and the secretome proteomic profile. Consequently, samples of abdominal adipose tissue from nine healthy female canines were obtained. After extraction and propagation, trilineage differentiation and flow cytometry were utilized to confirm the multipotency and stem cell immunophenotype of undifferentiated cAD-MSCs, respectively. To identify changes in gene expression and secretome proteome throughout in vitro ageing, RT-qPCR array profiling and LC-MS/MS analysis were carried out in passages 3 and 6. The results confirmed the preserved multipotency and stem cell immunophenotype of cAD-MSCs during in vitro propagation. However, prolonged propagation led to morphological cellular changes, including enlargement and increased granularity of the cAD-MSCs. Furthermore, gene expression profiling resulted in a significant downregulation of two genes related to MSCs. In addition, proteomic investigation of the cAD-MSCs secretome revealed significant downregulation in 26 proteins and upregulation in 11 proteins that influence relevant regenerative protein pathways. To the best of our knowledge, this work offers the first insight into the alterations in secretome proteome that arise with the in vitro ageing of cAD-MSCs. Our findings reveal significant changes that, in conclusion, imply the regenerative ability of cAD-MSCs and their secretome may be reduced due to in vitro ageing. Therefore, early passages should be considered when using these cells for therapeutic purposes.

Funding Source: The Croatian Science Foundation (UIP-2019-04-2178) provided funding for this study. The European Regional Development Fund (CluK KK.01.1.1.02.0016) provided partial support for proteomic analysis.

Keywords: canine adipose-derived mesenchymal stem cell, gene expression, secretome

4:45 PM – 5:45 PM

522

HEAT SHOCK ACCELERATES BOVINE MSC ADIPOGENESIS FOR CULTURED MEAT

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Mesenchymal stem cells (MSCs) offer great potential as a source for cultured meat (CM) production. The well-documented ease of extracting these cells from various tissues makes them highly attractive. The capacity of MSCs for multilineage differentiation, alongside their relatively simple and economical cultivation methods, positions them as valuable assets in the CM industry. However, the limited capacity of MSCs to renew themselves and their slow rate of differentiation present significant challenges for scalable production. This necessitates the advancement of culture and differentiation techniques. Expanding on our previous investigation that suggested a brief exposure to heat shock (HS) could expedite differentiation, we further explore this hypothesis. Our current findings demonstrate that a short thermal shock significantly accelerates the adipogenic differentiation of bovine MSCs. Additionally, cells that undergo HS preconditioning not only differentiate faster but also exhibit an improved ability to generate fat cells. We propose that optimizing MSC adipogenesis protocols through a straightforward and cost-effective HS pretreatment could revolutionize their application in the CM industry. This advancement holds promise for streamlining differentiation processes, reducing production timelines, increasing yield, and enhancing both meat quality and differentiation efficiency in CM production.

Keywords: mesenchymal stem cells, adipogenic differentiation, heat shock

4:45 PM – 5:45 PM

524

CELL LINEAGE ANALYSIS OF CD73-POSITIVE CELLS IN OSTEOCHONDRAL TISSUE

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The bone marrow microenvironment, the niche, is known to be tightly regulated by a variety of cells and plays an important role in maintaining hematopoietic stem cell (HSC) homeostasis. However, the developmental mechanisms and maturation processes of the cell populations that comprise the niches remain largely unknown. In a previous study, we generated CD73 reporter mice to characterize mesenchymal stem cells (MSCs) in vivo and found that MSCs expressing high levels of CD73 localize in the bone marrow and support the HSC niche. Analysis using a mouse fracture model showed that endogenous CD73-positive cells migrated to fracture sites and differentiated into bone and chondrocytes. Furthermore, some CD73-positive cells contributed to neovascularization, suggesting that they are involved in niche remodeling. Cell lineage analysis of CD73-positive cells using inducible Cre system showed that labeled cells appeared from embryonic day 14.5, and their progenies were observed in the bone, articular cartilage, and synovium. The labeled cells in articular cartilage tissue expressed Sox9, suggesting that they are chondrogenic progenitors. The labeled cells also localized perivascular regions in the metaphysis and showed osteoprogenitor marker osterix. These cells migrated into the cortical bone and differentiated into osteocytes as the mice matured.These results indicate that CD73-positive MSCs are a population with high proliferative and osteochondrogenic differentiation potential. These cells play an important role in osteochondrogenesis and niche remodeling during bone injury. Further cell lineage analysis will be performed to analyze the CD73-positive cells constituting the osteochondral niche comprehensively.

Keywords: mesenchymal stem cells, niche, osteochondral tissue

4:45 PM – 5:45 PM

526

PRIMING BONE MARROW MESENCHYMAL STEM CELLS WITH HEPATOCYTE GROWTH FACTOR (HGF-MSC) AMELIORATES BLEOMYCİN-INDUCED PULMONARY FIBROSIS IN MICE

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MSCs hold immense potential in regenerative medicine. However, their systemic administration faces challenges, including poor retention and potential damage during delivery. Various priming strategies have been explored to enhance MSC effects, of those, priming with HGF emerges as a promising candidate. In this study, efficacy of HGF-MSCs was compared with MSCs grown in standard medium in pulmonary fibrosis C57Bl/6 mouse model induced by intratracheally administered bleomycin (BLM). The cells were characterized by immunophenotyping, secretion profile, differentiation capacity and scratch assay. Cell transplantation was performed iv with two different doses (5 x 105 or 1x 106, cells) on the 7th day of BLM administration. The treatment groups were as follows: HGF-MSC, MSC and PBS. Samples were collected at the 7th- and 14th-day following cell transplantation to evaluate their effects. On the 7th day with 5x105 cells, both HGF-MSCs and MSCs exhibited trends toward improving airway compliance and reducing bronchoalveolar lavage fluid (BAL) lymphocyte cell ratio. The 14th day of HGF-MSC treatment showed superior efficacy on airway compliance [0.025±0.002 ml/cmH2O, HGF-MSC; 0.023±0.002 ml/cmH2O, MSC vs. 0.017±0.002 ml/cmH2O, PBS (n=6 for each group)], on total protein content [1002.73±255.12 mg/ml, HGF-MSC (n=6); 1251.67±196.19 mg/ml, MSC (n=6); vs. 1639.32±105.87 mg/ml, PBS (n=5)], but the histopathologic scores of the cell treated groups were similar to the PBS ones. TNF-α levels were decreased dramatically with both treatments [55.88±14.65 pg/ml, HGF-MSC; 49.58±6.31 pg/ml, MSC vs. 128.39±38.01 pg/ml, PBS (n=5 for each group)]. Moreover, Col-1 protein levels ameliorated with both cell treatments. When the dose was increased to 1x106 cells, fibrosis score reduction was prominent on the 7th day [3.00±3.00, HGF-MSC (n=3); 4.00±1.16, MSC (n=3) vs 6.00±0.84, PBS (n=5)] but not on the 14th day, indicating an early therapeutic window. Overall, our findings underscore the potential of HGF preconditioning to enhance the therapeutic efficacy of MSCs in pulmonary fibrosis, mainly by anti-inflammatory effect. It is noteworthy to mention that there is a clue

for antifibrotic influence with higher dose. Further research is warranted to elucidate dose and time optimization for clinical translation.

Funding Source: This work was supported by Health Institutes of Türkiye, TUSEB-4569

Keywords: pulmonary fibrosis, mesenchymal stem cell priming, Hepatocyte Growth Factor

4:45 PM – 5:45 PM

TOPIC: MUSCULOSKELETAL

528

ACTIVATION OF SYMPATHETIC SIGNALING-MEDIATED FIBRO/ADIPOGENIC PROGENITORS EGRESSION FROM SKELETAL MUSCLE DRIVES STROKE-RELATED SARCOPENIA

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Patients surviving stroke usually experience rapid muscle wasting and an increased risk of physical disability. Although multifactorial interactions including malnutrition, disuse, systemic catabolic imbalance and neurohormonal dysregulation are considered to contribute to the progression of stroke-related sarcopenia, the underlying mechanisms of this brain-muscle crosstalk remain elusive. Muscle-resident fibro/adipogenic progenitors (FAPs) are indispensable for maintaining muscle homeostasis and function as initial sensors of external perturbations. In the present study, we illustrate that FAPs rapidly respond to the overactive sympathetic nervous system (SNS), and egress from the muscle niche into circulation during the acute phase of stroke. FAP-specific ablation of the adrenergic receptor Adrb2 appreciably ameliorates stroke-related sarcopenia, highlighting the central role of SNS-mediated FAPs loss in its pathogenesis. Mechanistically, augmented norepinephrine (NE) release initiates FAPs mobilization through activating pro-migratory signals as well as degrading ECM components. Using transcriptomic profiling, we further characterize insulin growth factor-1 (IGF-1) as a key anti-trophic executive factor predominantly derived from FAPs, whose expression is repressed during stroke progression. Collectively, our work establishes that SNS-mediated loss of FAPs and subsequent compromised IGF-1 secretion mainly contribute to sarcopenia in stroke mice. Targeting this mechanism by early anti-sympathetic treatment with propranolol and exogenous IGF-1 administration could effectively rejuvenate muscle homeostasis and restore muscle mass after stroke.

Funding Source: National Natural Science Foundation of China (82101367), Natural Science Foundation of Guangdong Province (2022A1515012370) and Young Talent Support Project of Guangzhou Association for Science and Technology (QT-2023-054).

Keywords: stroke-related sarcopenia, sympathetic nervous system, fibro/adipogenic progenitors

4:45 PM – 5:45 PM

530

MOUSE LEMUR AS A NEW PRIMATE MODEL FOR ADULT STEM CELL BIOLOGY

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Animal models have transformed our understanding of biology. Because established models do not recapitulate all aspects of human and disease biology, there is a need for new animal models. Mouse lemurs are the smallest, fastest reproducing, and among the most abundant primates, and an emerging model organism for primate biology, behavior, health, and conservation. Here, we identify and functionally validate populations of muscle stem cells (MuSCs) and fibro-adipogenic progenitors (FAPs) in a new primate model, the grey mouse lemur Microcebus murinus. We find that mouse lemur MuSCs and FAPs are more similar to their human counterpart than to the mouse one, in terms of molecular profiles and activation kinetics. By combining cell phenotyping with cross-species molecular profiling and functional assays, we identify new roles for spermidine metabolism in MuSC function and for Complement Factor D (CFD) in primate FAP differentiation. Finally, we find that the expression pattern of muscle disease genes in mouse lemur cells more closely resembles that in human cells than in mouse cells. We propose mouse lemur as a new model organism to study human stem cell and disease biology.

Keywords: non-human primate, quiescence, muscle stem cells

4:45 PM – 5:45 PM

532

THE EAR PINNA REGENERATION IN MAMMALS

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Regeneration is not evenly distributed in nature, with some animals regenerating extensively while others cannot. Mammals such as mice and humans usually retain limited regenerative capacities. The molecular mechanisms underlying such limited regeneration remain elusive. Interestingly, mammalian ear pinna displays great diversity in the ability to regenerate full-thickness holes punched through the organ. For example, rabbits can regenerate the damaged ear pinna within 30 days, while mice fail to close the ear hole even at 90 days post injury. Using multi-omics and genetic lineage tracing, we identified the cell source for ear pinna regeneration and the key molecular changes

between rabbits and mice. We will show the evolutionary mechanisms underlying such molecular changes.

Keywords: cartilage regeneration, ear pinna regeneration, evolution

4:45 PM – 5:45 PM

534

SKELETAL MUSCLE STEM CELL EXHAUSTION WITH AGEING DUE TO PREMATURE PRIMING

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Skeletal muscle has a remarkable capacity to regenerate thanks to muscle stem cells (MuSCs; satellite cells), which are quiescent unless damage occurs. Ageing negatively affects the relative cell numbers and regenerative capacity of MuSCs; however, the mechanisms behind these age-related changes are mostly unclear in humans. Here, we have generated a single-cell/single-nucleus transcriptomic and chromatin accessibility map of human limb skeletal muscles encompassing over 387,000 cells/nuclei from individuals ranging from 15 to 99 years of age with distinct fitness and frailty levels. Our findings show that aged MuSCs lose their stemness and functionality due to persistent activation of pathways related to inflammation, mitochondrial dysregulation, and cell damage, resulting in reduced promptness for proliferative responses. All this greatly blunts the repair processes in old muscles and facilitates the onset of sarcopenia. Based on cross-comparison with genetic data, we also identified key elements of chromatin architecture that mark susceptibility to sarcopenia. Our study provides a basis for the discovery of novel targets in the skeletal muscle that are amenable to medical, pharmacological and lifestyle interventions in late life.

Keywords: ageing, human skeletal muscle, multimodal omics

Clinical Trial ID number: CElm 28/2019, 2020-ICE-90

4:45 PM – 5:45 PM

536

GROWTH AND DIFFERENTIATION CAPACITY OF SALMON MUSCLE PROGENITORS FOR CULTIVATED SEAFOOD PRODUCTION

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Controlling cell growth and differentiation is a crucial skill in the field of cellular agriculture and establishing long-term fish myogenic/adipogenic cell lines poses several challenges. Fish cell cultures exhibit slower growth and metabolic rates compared to mammalian cell cultures. While the methodologies and tools for the maintenance and characterization of mammalian stem cells are well understood, so far little is available for piscine counterparts. Understanding essential signaling network requirements and making appropriate adjustments in culture conditions are crucial if fish cells are to achieve their full potential. In this study, we were able to isolate, expand, and differentiate salmon muscle progenitor cells in a special medium that contained %5 FCS. Salmon muscle progenitors were expanded over passages with a PDT of less than 60 hours without the use of additional fish serum or embryo extract. Cells were differentiated towards myotubes and differentiation is characterized by morphology, muscle-specific protein, and gene expression. Salmon stem cells form the basis for the successful development of structured cultivated seafood products like salmon filets. In this study, we were able to isolate, expand, differentiate, and increase the protein content of salmon muscle tissue progenitors efficiently. A progenitor cell line establishment from salmon muscle tissue with a high proliferative/differentiation capacity holds great potential for the development of cost-efficient cultivated fish products.

Keywords: muscle stem cells, salmon, myogenic differentiation

4:45 PM – 5:45 PM

TOPIC: NEURAL

538

ASTROCYTES CONTROL QUIESCENT NSC REACTIVATION VIA GPCR SIGNALING-MEDIATED F-ACTIN REMODELING

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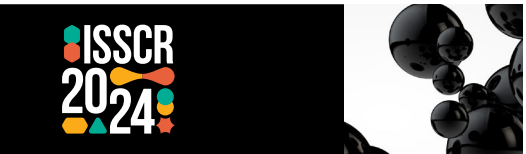
The transitioning of neural stem cells (NSCs) between quiescent and proliferative states is fundamental for brain development and homeostasis. Defects in NSC reactivation are associated with neurodevelopmental disorders. Drosophila quiescent NSCs extend an actin-rich primary protrusion toward the neuropil. However, the function of the actin cytoskeleton during NSC reactivation is unknown. Here, we reveal the fine F-actin structures in the protrusions of quiescent NSCs by expansion and super-resolution microscopy. We show that F-actin polymerization promotes the nuclear translocation of Mrtf, a microcephaly-associated transcription factor, for NSC reactivation and brain development. F-actin polymerization is regulated by a signaling cascade composed of G-protein-coupled receptor (GPCR) Smog, G-protein aq subunit, Rho1 GTPase, and Diaphanous (Dia)/Formin during NSC reactivation. Further, astrocytes secrete a Smog ligand Fog to regulate Gaq-Rho1-Dia-mediated NSC reactivation. Together, we establish that the Smog-Gaq-Rho1 signaling axis derived from astrocytes, a NSC niche, regulates Dia-mediated F-actin dynamics in NSC reactivation.

Keywords: neural stem cell, quiescence, actin dynamics



4:45 PM – 5:45 PM

540

EFFICACY AND SAFETY OF GENE-CORRECTED INDUCED NEURAL PRECURSOR CELLS FOR THE TREATMENT OF HUNTINGTON'S DISEASE

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Huntington's disease (HD) is an inherited neurodegenerative disease characterized by chorea, depression and dementia caused by progressive nerve cell degeneration. Since there is no effective therapeutic agent for this disease, the cell-based therapies that replace lost neurons can be a new therapeutic approach. The induced neural precursor cells were directly generated from HD patient fibroblasts(HD-iNPCs), and the expanded CAG repeats within the genome of the HD-iNPCs were corrected by a gene editing system, CRISPR/Cas9. The aim of this study is to investigate the efficacy and safety of the corrected neural stem cells in Huntington's disease rodent animal models such as Quinolinic Acid(QA)-induced rats and R6/2 transgenic(Tg) mice. The degree of recovery was measured through the rotarod, balance beam, grip strength and apomorphine-induced tests. In the groups transplanted with the gene corrected iNPCs, the rats were stayed in the rotarod for a longer time, and passed the Balance beam faster, and significantly decreased the number of rotations in apomorphin-induced test compared to the control group injected with HBSS. The R6/2 Tg mice showed a superior ability of motor function recovery and a longer life span than control group. In our immunostaining assays of model animal brain slices, the gene corrected iNPCs were differentiated into GABAergic neurons and astrocytes, which were positive against Tuj1, MAP2, GABA and GFAP antibodies. Safety tests such as tumorigenicity test, toxicity test, distribution test, were performed on nude mice. No tumors were found in safety tests for up to 6 months, and toxicity was also rarely identified. The transplanted iNPCs were only present in the brain at the site of injected. All together, these results demonstrated that transplantation of the gene-corrected iNPCs improve the motor functions without any serious side effects in the Huntington's disease model animals, which suggests that the gene-corrected iNPCs could be used as a promising therapeutic cell source for the treatments of Huntington's disease.

Funding Source: This work was supported by the Ministry of Science and ICT (2019M3E5D5065399), and the Ministry of Health and Welfare (RS-2022-00060247) of the government of the Republic of Korea.

Keywords: efficacy, safety, cell transplantation, induced neural stem cells

4:45 PM – 5:45 PM

542

DECONSTRUCTING IN VIVO GLIA-TO-NEURON CONVERSION IN THE POSTNATAL MOUSE CORTEX

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Direct lineage reprogramming in the brain offers the prospect of remodelling diseased brain circuits. Substantial evidence has emerged, supporting the possibility of converting astrocytes and oligodendrocyte precursor cells (OPCs) into functional neurons in vivo. Yet, the process by which glia cells give up their identity and adopt a neuronal fate remains enigmatic. In our work, we addressed questions regarding how specific neurogenic factors, such as Ascl1 and its phospho-deficient counterpart SA6 differentially remodel gene expression programs as glial cells convert into neurons in the mouse cortex. We also explored the contribution of glial cell heterogeneity to the variability in reprogramming success and aimed to identify potential candidate molecules that act as roadblocks for successful reprogramming. To achieve this, we injected retroviruses encoding either Ascl1 or SA6, along with the cell death regulator Bcl2 or the transcription factor involved in the differentiation of interneurons, Dlx2, into the somatosensory cortex of postnatal mice, transducing primarily proliferating astrocytes and OPCs. Subsequently, we performed single-cell RNA sequencing of cells undergoing conversion in vivo and investigated the molecular trajectories to uncover the transcriptomic changes driving this process. Bioinformatical analysis of these cells reveal that Ascl1 and SA6 both induce neurogenic programmes in astrocytes; however, they both give rise to distinct neuronal cell populations characterised by specific transcription factors, which may orchestrate the respective reprogramming outcomes. Interestingly, Ascl1 did not seem to initiate neurogenic programs in OPCs, suggesting that cell type-specific molecular properties play a crucial role in reprogramming success. On the other hand, both astrocytes and OPCs initiated neurogenesis when overexpressing SA6, resulting in distinct neuronal cell fates despite expressing the same factors. We demonstrate that the phosphorylation state of Ascl1 plays a pivotal role in initiating differential gene expression programs and determining differential neuronal cell fates in cortical astrocytes. Overall, this study may offer essential cues to further manipulate these reprogramming pathways and refine the efficacy of cell fate conversion.

Funding Source: Marie Skłodowska-Curie Actions

Keywords: direct lineage reprogramming, glia-to-neuron conversion, Ascl1

4:45 PM – 5:45 PM

544

DIRECT LINEAGE REPROGRAMMING OF HUMAN ASTROCYTES INTO INDUCED NEURONS

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The advent of direct lineage reprogramming has opened an unprecedented perspective for the replacement of lost neurons following injury and neurodegenerative diseases. Successful murine astroglia reprogramming has shown in vitro and in vivo. However, it remains unknown whether such lineage reprogramming can also be achieved in the case of human astrocytes which differ markedly from their rodent counterparts. To tackle this question, we have differentiated human astrocytes from human induced pluripotent stem cells (hiPSCs) using a genome-edited hiPSCs line. Astrocyte differentiated during two weeks have been challenged in vitro to the overexpression of the proneural transcription factor Neurog2. After Neurog2 overexpression, cells displaying neuronal morphology and expressing neuronal marker βIIItubulin were detected. In order to assess the physiology of the human astrocyte-derived induced neurons, we carried out patch clamp recordings. Neurog2-derived induced neurons were able to fire action potentials confirming their neuronal identity. To further investigate human astrocyte reprogramming in a human tissue-like context, we have generated human cerebral organoids. Taking advantage of the genome-edited hiPSCs line, we have targeted human astrocytes in cerebral organoids to overexpress Neurog2. After overexpression of Neurog2, we found cells expressing the neuronal marker βIIItubulin that could be traced back to cells of astroglia origin. Altogether, our preliminary results show that Neurog2 is able to directly reprogram human astrocytes in 2D and 3D context into induced neurons.

Keywords: direct reprogramming, human astrocyte-to-neuron conversion, Neurogenin-2

4:45 PM – 5:45 PM

546

MACROPHAGE-MEDIATED REGULATION OF RETINAL STEM CELLS IS NECESSARY FOR PROPER RETINAL DEVELOPMENT

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Anamniotes such as the teleost medaka, have a tremendous ability for lifelong postembryonic growth. This is possible due to tissue-specific postembryonic stem cells that are actively maintaining the growth of the tissue. The continuous growth poses a challenge to sustain an equilibrium between proliferation and differentiation to avoid hyperplasia. This equilibrium is crucially shaped by the stem cell niche environment to ensure proper growth and functionality of the tissue. In this study, we address how the growth of the retina is influenced by interaction of stem cells with their environment. Unpublished data from previous in-house studies suggests that retinal stem cells (RSCs) are surveyed by macrophages in the developing retina. In a homeostatic environment, macrophages phagocytose RSCs triggered by chemokine signaling. However, the influence of this interaction on RSC behavior and mechanisms to initiate the active removal remain elusive. In order to evaluate this, clonal analysis of RSCs in mutants lacking macrophages was performed. In absence of macrophages, RSCs reduce the ability to produce differentiated retinal cell types. Thus, increasing the number of retinal stem cells occupying the stem cell niche, which leads to disruption of overall tissue growth. These results indicate that macrophages might be involved in regulating the proliferative capacity of RSC in a way that cell density is maintained which eventually affects its differentiation ability. Further research into the mechanisms by which the macrophages regulate stem cells will provide an insight into the capability of immune cells to control overly proliferating cells that eventually lead to hyperplasia. This study provides to an emerging field addressing the regulation of stem cells by immune cells and highlighting a perspective other than the conventionally described roles of immune cells concerning pathogen defense mechanisms. Uncovering the significance of this process and how fundamental it might be across different stem cell systems is of importance for understanding tissue development and maintenance in health and disease.

Keywords: tissue homeostasis, retinal development, macrophage and stem cell interaction

4:45 PM – 5:45 PM

548

ELEVATED INTRACELLULAR ZINC ION LEVELS AND CHANGES IN GENE EXPRESSION OF ZINC TRANSPORTERS AFTER DIFFERENTIATION OF MOUSE NEURAL STEM/PROGENITOR CELLS

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Zinc ions play a variety of regulatory roles in mammalian neurodevelopment. Zinc deficiency has recently been reported to impair the formation of the embryonic and postnatal neurodevelopment of the central nervous system (CNS), leading to impaired CNS function and psychiatric disorders such as autism spectrum disorders (ASD) and depression. However, the role of intracellular zinc ions before and after differentiation of neural stem cells (NSCs), which play a central role in the development of the central nervous system (CNS), and their regulatory mechanisms have not been fully elucidated. In this study, we aimed to investigate how intracellular zinc ion levels change before and after the differentiation of neural stem/progenitor cells and how the expression of zinc transporter family gene changes. We used cultured mouse embryonic neural stem/progenitor cells (NSPCs) to measure changes in intracellular zinc ion concentrations before and after their differentiation. NSPCs cultured in 1.5 μM Zn2+ grew slightly faster than NSPCs cultured in zinc-depleted medium. 1.5 μM Zn2+-cultured NSPCs and their differentiated cells (DCs) had 1.34- and 2.00-fold higher

intracellular zinc concentrations than those in zinc-depleted medium, respectively. 14 Zips (transporters that take zinc ions from extracellular or organelles into the cytoplasm) and 9 ZnTs (transporters that pump zinc ions from the cytoplasm to extracellular or organelles) are known as members of the zinc transporter family The zinc transporter genes that showed more than 3.5-fold changes after neural stem cell differentiation were Zip1, Zip4, Zip12, Zip13, ZnT1, ZnT8, and ZnT10. We further investigated whether the expression trends of these zinc transporters are affected by extracellular oxygen and carbon dioxide concentrations. The cell morphology of NSPCs and DCs in hypoxic cultures with 2% oxygen and 5% CO2, in high CO2 cultures with 21% oxygen and 10% CO2, and in normal cultures with 21% oxygen and 5% CO2 was essentially the same. In addition, the expression of Zip4, Zip8, Zip12, and Zip14 did not change significantly with oxygen and carbon dioxide concentrations. In conclusion, the results indicate that neural stem/progenitor cells tend to increase the expression of many Zips and ZnTs and increase intracellular zinc concentrations as they differentiate.

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Keywords: zinc transporter, neural stem cell, neurogenesis

4:45 PM – 5:45 PM

550

RECIPROCAL COMMUNICATION BETWEEN HUMAN NEURAL STEM CELLS AND HUMAN ENDOTHELIAL CELLS DRIVES VESSEL FORMATION AND NEURAL STEM CELL TYPE B PHENOTYPE

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Neural stem and progenitor cell (NSPC) and vessel-forming endothelial cell communication throughout development and adulthood is vital for normal brain function. Despite this, much remains unclear regarding coordinated regulation of these cells, particularly in humans. NSPCs regulate vascularization of the developing central nervous system and we found human NSPCs (hNSPCs) promote human endothelial cell (hEC) vessel formation in a three-dimensional human neurovascular model. Here, we investigate the mechanisms driving the pro-vasculogenic effect of hNSPCs. Comparison of human and mouse NPSC bulk RNA-sequencing data revealed several vessel-promoting factors were more highly expressed by human cells, highlighting the importance of studying human NSPC-EC communication. Analysis by single cell RNA sequencing (scRNAseq) showed that hNPSCs co-cultured with hECs up-regulated vessel forming genes. Many factors identified by RNAseq were secreted proteins, and hNSPC conditioned media was sufficient to induce hEC vasculogenesis. ScRNAseq data for hNSPCs co-cultured with hECs identified up-regulation of genes associated with extracellular vesicles (EVs), including exosomes. EVs isolated from hNSPCs promoted hEC vasculogenesis, and blocking hNSPC exosomes abrogated the effect of hNSPC conditioned media on vessel formation. As further evidence of the reciprocal communication between hNSPCs and hECs, scRNAseq and immunostaining showed hEC contact increased hNSPC type B cells, which are GFAP-expressing adult NSPCs in the brain subventricular zone. Induction of the type B phenotype was driven by Notch signaling, and both blocking hNSPC Notch signaling and reducing hEC expression of the Notch ligand DLL4 abrogated the effect of hECs on type B hNSPCs. Thus, hNSPCs increase hEC vessel formation via secreted material, including EVs/exosomes, and hEC contact promotes human type B cells via Notch signaling. These data reveal critical reciprocal communication governing function of these important cell types.

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Keywords: exosomes, notch, extracellular vesicles

4:45 PM – 5:45 PM

552

UTILIZING 3D HYDROGELS TO PROMOTE THE SURVIVAL AND MATURATION OF DIRECTLY REPROGRAMMED HUMAN-INDUCED OLIGODENDROCYTE PRECURSOR CELLS

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Multiple Sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system characterized by the destruction of oligodendrocytes (OLs) and the myelin sheath, impairing neuronal signal transduction. Transplantation of oligodendrocyte precursor cells (OPCs) has emerged as a potential therapy to replace lost OLs, repair the myelin sheath and restore signal transduction through cell-based remyelination. However, sourcing clinically safe and viable OPCs for transplantation has been a rate-limiting step, with majority of cells sourced from human embryonic or induced pluripotent stem cells which are associated with ethical issues, lengthy protocols, and lack of OL lineage specificity. To overcome this, utilizing stable, non-integrating chemically modified mRNA, we have developed a direct cell reprogramming protocol that converts adult human fibroblasts to human induced OPCs (hiOPCs) that express key oligodendroglial markers A2B5, NG2, PDGFRa, NKX6.2 and OLIG1 and can be differentiated in vitro to express mature OL markers OSP, O1, MOG, PLP1 and MBP. What is more, when transplanted onto a demyelinated brain slice culture, hiOPCs generate MBP+ and MOG+ cells, contributing to remyelination of the brain slices ex vivo.This work highlights the potential of hiOPCs as a cell-based remyelination therapy. However, within the literature, cell transplantation studies report issues with long-term survival, and integration of transplanted cells. To address this, we propose the encapsulation of hiOPCs into three-dimensional gelatin methacryloyl (GelMA) hydrogels to provide a more permissible environment for survival, maturation, and functional integration post-transplantation. We show that when encapsulated in GelMA for up to 2 weeks, hiOPCs retain viability comparable to cells seeded in culture plates, and express key oligodendroglial markers A2B5 and

O1. GelMA-encapsulated hiOPCs give rise to OLs expressing a higher percentage of MOG+ cells throughout the course of culture that wasnt seen when cultured in 2D. This study demonstrates that directly reprogrammed hiOPCs can survive and mature to OLs within 3D hydrogels. The ability for encapsulated hiOPCs to remyelinate the demyelinated brain will next be assessed using (1) ex vivo brain slice cultures and (2) the cuprizone mouse model.

Funding Source: Neurological Foundation of New Zealand New Zealand Multiple Sclerosis Research Trust

Keywords: cell reprogramming, oligodendrocyte precursor cells, Multiple Sclerosis

4:45 PM – 5:45 PM

556

NOVEL REPROGRAMMING STRATEGY FOR THE GENERATION OF HUMAN OLIGODENDROCYTES FOR AXONAL REMYELINATION

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Axonal demyelination is a common feature in many neurodegenerative disorders, especially in multiple sclerosis (MS). In this context, astrocytes play an important role in lesion formation, being the main component of the glial scar as well as helping peripheral blood immune cells to reach the central nervous system. Here, I propose a new approach to converting astrocytes into functional oligodendrocytes. Previously, in vivo conversion of mouse astrocytes to oligodendrocytes has been achieved by overexpression of the Sox10 gene. Regarding cells of human origin, only one study has demonstrated that a human astrocyte cell line could be reprogrammed into oligodendrocytes by treatment with epigenetic modifiers. However, their myelination capacity has yet to be reported. I aim to optimize a direct conversion protocol through the overexpression of Olig2 and Sox10, transcription factors involved in oligodendrocyte generation, under astrocyte-specific promotor using the following viral vectors: LV-EGFP-GFAP>hOLIG2 and LV-mCherry-GFAP>hSOX10. Astrocytes conversion into oligodendrocytes will be done directly in human organotypic culture to analyze the capacity of the newly-generated oligodendrocytes to myelinate human axons in a 3D cell culture system with similar tissue architecture and cellular composition as in a human brain. GFP- and mCherry-expressing astrocytes will be tracked over time to study changes in their morphology. The expression of astrocyte- and oligodendrocyte-specific markers will be examined using immunohistochemistry, and axonal myelination of hACtx neurons by newly generated oligodendrocytes will be assessed using immuno-EM. Moreover, the effect of remyelination by reprogrammed oligodendrocytes on neuronal function and circuitry connectivity will be studied by whole-cell patch clamp recording and MEA, respectively. This study will allow the generation of a new strategy to diminish MS-related pathogenesis by promoting de novo axonal remyelination.

Funding Source: Region Skåne, Rut och Erik Hardebos, Neurofonden, Åke Wibergs Stiftelse, Sven och Dagmar Saléns Stiftelse

Keywords: remyelination, oligodendrocyte, Multiple Sclerosis

4:45 PM – 5:45 PM

558

DIRECT NEURONAL REPROGRAMMING OF NDUFS4 PATIENT CELLS IDENTIFIES THE UPR AS A NOVEL GENERAL REPROGRAMMING HURDLE

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Mitochondrial dysfunction has been associated with the pathogenesis of many age-related and neurodegenerative diseases. Mitochondrial stress and excessive production of reactive oxygen species not only negatively impact endogenous neurons but have also been reported to hinder the direct neuronal conversion of murine glial cells. However, little is known about the impact of mitochondrial dysfunction on the direct neuronal reprogramming of human astrocytes. Therefore, we first differentiated human induced pluripotent stem cells (hiPSCs), from either control or patient fibroblasts carrying mutations in the NDUFS4 gene, essential for mitochondrial Complex I function, into astrocytes; then, we reprogrammed them into neuronal cells. NDUFS4-hiPSC-derived astrocytes showed a significantly reduced conversion efficiency compared to controls. Remarkably, pharmacological treatments aimed at overcoming mitochondria-related hurdles led us to identify the unfolded protein response (UPR) as a novel, general hurdle in direct neuronal reprogramming, as its transient inhibition improves the conversion rate of both control and patient-derived astrocytes to up to 70%. To investigate which UPR branches are induced, and at which point during direct neuronal reprogramming, we co-expressed the reprogramming factor with genetic sensors for ATF4 and ATF6, the endoplasmic reticulum (ER) transcription factors downstream PERK and ATF6 activation, respectively. Remarkably, we observed a significant increase in ATF4 activation at the early stages of the conversion process and further corroborated these findings by performing RT-qPCR of downstream targets. Considering that the UPR regulates global translation rates and protein folding, we also investigated these mechanisms during direct reprogramming. Indeed, we observed an increase in translation rates coupled with an increase in protein misfolding, suggesting that the accumulation of misfolded proteins in the ER acts as the trigger for UPR activation during neuronal reprogramming. Overall, our data identifies the UPR as a key hurdle in the direct neuronal reprogramming of human astrocytes: its inhibition not only significantly improves the conversion but highlights the key role of proteostasis mechanisms in direct neuronal reprogramming for the first time.

Keywords: direct neuronal reprogramming, unfolded protein response, mitochondrial dysfunction

4:45 PM – 5:45 PM

560

SPINAL CORD TISSUEOID TRANSPLANTATION COMBINED WITH TAIL NERVE ELECTRICAL STIMULATION PROMOTES THE VOLUNTARY MOVEMENT OF PARALYZED HINDLIMBS IN RATS WITH TRANSECTED SPINAL CORD INJURY

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The recovery of voluntary movement of paralyzed limbs after completely transected spinal cord injury (SCI) still faces the challenge of reconstructing spinal cord neural pathways and restoring the excitatory/inhibitory balance of motor neural circuit. To address this question, a spinal cord tissueoid (SCToid) that mimicked natural spinal cord tissue was transplanted to injured spinal cord to reconstruct neural pathway in the present study, and simultaneously, a tail nerve electrical stimulation (TNES) was combined to activate the motor neural circuit. The results showed that the regenerated corticospinal tract nerve fibers and sensory afferent nerve fibers efficiently constructed synaptic connectivitys with SCToid neurons under the TNES treatment, which could play a role of neuronal relayer. Afterwards, using mono-synaptic tracers, the axons from SCToid neurons were detected to directly innervate the lumbar spinal central pattern generator (CPG) interneurons, and some CPG interneuronal axons and sensory afferent nerve fibers also constructed synaptic connectivitys efficiently with the motor neurons. Compared to the control, SCToid transplantation combined with TNES brought the ratio of excitatory/inhibitory synaptic terminals on the surface of CPG interneurons and motor neurons closer to that of normal spinal cord, and finally enhanced the excitability of motor neural circuit, and recovered the weight-bearing walking ability of hindlimbs. Importantly, SCToid transplantation combinated with TNES indicate that neural pathway reconstruction and the balance between excitation and inhibition of CPG-controlled motor neural circuit are necessary and sufficient for recovering coordinative locomotion. The synergistic mechanism revealed in this study will provide the theoretical and clinical translation bases for further treatment of complete SCI via the strategies of biology combined with physics.

Funding Source: The National Natural Science Foundation of China (Grant No. 81891003)

Keywords: spinal cord tissueoid, tissue-engineered neuron relayer, complete spinal cord injury

4:45 PM – 5:45 PM

562

UNFOLDED PROTEIN RESPONSE LIMITS DIRECT NEURONAL REPROGRAMMING OF HUMAN CELLS

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Neurodegenerative diseases are often associated with mitochondrial deficts. Direct neuronal reprogramming aims at replacing neurons lost upon injury or neurodegeneration by converting local, endogenous non-neuronal cells into functional neurons. Astrocytes are a suitable starter cell for direct conversion, as they are widespread in the CNS and developmentally related to neurons. However, little is known about the direct conversion of human astrocytes into neurons and the impact of mitochondrial dysfunction on the process. To tackle these questions, we obtained IPSCs from fibroblasts of control individuals or patients carrying mutations in NDUFS4 gene, required for ETC Complex I assembly and associated to Leigh syndrome. Control of patient iPSCs-derived astrocytes were transduced with various reprogramming factors: remarkably, patient astrocytes were reprogrammed less efficiently than control counterparts. As the expression of plant-derived Complex-I analogue only partially improved the conversion efficiency of NDUFS4-derived astrocytes, we transiently modulated various aspects of mitochondria (turnover, electron transport, redox state) or endoplasmic reticulum (UPR, ISR) during astrocyte-to-neuron direct neuronal reprogramming. Surprisingly, inhibiting the UPR branches PERK and IRE1 increased the conversion rate not only of patient but also of control astrocytes. Molecular analysis revealed the presence of cells undergoing a successful conversion, but also cells failing the conversion either because a lack of metabolic shift or because of a higher expression of ferroptosis markers, suggesting the activation of a cell death pathway. Remarkably, induced neurons become more mature when cells undergoing reprogramming were treated with the PERK inhibitor. Finally, direct neuronal reprogramming of human fibroblasts was boosted following UPR inhibition. In summary, this work suggests UPR as a key hurdle in direct reprogramming, leading to slow and aborted reprogramming. PERK pathway seems particularly relevant, as its activation slows translation, thus preventing the transcriptional changes manifesting at the protein level, indeed reducing the execution of the newly imposed neuronal program.

Keywords: human astrocytes, direct neuronal reprogramming, unfolded protein response

4:45 PM – 5:45 PM

564

CHARACTERIZATION OF THERAPEUTICALLY RESISTANT GBM STEM-LIKE CELLS

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Glioblastoma (GBM) is a lethal brain tumor almost invariably relapsing within 15 months after a standard treatment based on surgery, chemotherapy with temozolomide (TMZ) and radiotherapy (IR). GBM is known to arise from stem-like cells (GSCs), which possess inherent therapeutic resistance and can evolve under therapeutic pressure, thereby driving tumor recurrence. Notably, in individual treatment-naïve GBMs, multiple GSC subpopulations can coexist, displaying different genetic and phenotypic features, which may underlie varying intrinsic resistance to treatments. These features may be selected or modulated by therapy, fostering the therapeutic refractoriness of the relapsing GBM. The mechanisms supporting GSC therapeutic resistance need to be investigated to discover vulnerabilities exploitable for treating recurrences. As recurrent GBMs are seldom removed, making their GSCs rarely available, we have developed a protocol for ex vivo selecting GSCs that are resistant to conventional therapies, ideally representing the GSC subclones driving tumor recurrence. Briefly, starting from treatment-naïve GBMs removed with ultrasonic aspirator, we virtually collected the whole tumor population and cultured it in a medium that allows maintaining GSC subclone heterogeneity. These cultures were concomitantly subjected to conventional chemo- or radio- therapies, mimicking patient treatment. From each GBM, we obtained long-term propagating cultures enriched in GSCs (neurospheres, NS), referred to as 'NS families', each including at least one member selected by TMZ or IR (TMZ-NS and IR-NS respectively), in addition to an untreated control (CTRL-NS). NS families were extensively characterized by biological, genomic and transcriptomic comparative analyses, highlighting properties associated with the emergence of therapeutic resistance. These properties include increased stemness features and tumor-initiating ability, genetic alterations related to DNA damage repair, and major cytogenetic changes. In addition, therapeutically resistant GSCs display adaptive but inheritable transcriptional modulation of genes fostering a prosurvival signaling profile, which can be pharmacologically exploited to eradicate GSCs in recurrent GBMs or prevent their emergence in primary GBMs.

Keywords: cancer stem cells, heterogeneity, therapeutic resistance

4:45 PM – 5:45 PM

TOPIC: NO TISSUE SPECIFICITY

566

TARGETED DNA-METHYLATION AT AGE-ASSOCIATED CPG-SITES TRIGGERS GENOME-WIDE RESPONSE OF AGING CLOCKS

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Aging is reflected in highly reproducible DNA methylation changes, but it is largely unclear how these epigenetic modifications are regulated and whether they are functionally relevant. Despite the large number of age-associated CG dinucleotides (CpGs) it might be possible to interfere with epigenetic clocks by direct modulating the DNA methylation pattern. To explore this possibility, we used CRISPR-guided approaches to manipulate DNA methylation at specific CpGs, which become either hyper- or hypomethylated during aging. Analyses using Illumina BeadChip technology, pyrosequencing, and bisulfite amplicon sequencing confirmed distinct and targeted DNA methylation at the specified regions. Particularly hypermethylated aging CpGs exhibited persistent modifications for up to 100 days, while neighboring CpGs showed dynamic changes in patterns. Notably, targeted DNA methylation had significant and highly reproducible "off-target" effects that were markedly enriched in other genomic regions that gain DNA methylation during aging. To investigate if this co-regulation at age-associated CpGs is related to chromatin conformation, we performed a 4C analysis for a hypermethylated region in PDE4C. The results showed a significant enrichment of the interaction with other regions that gain methylation with age. Next, we sought to reverse the epigenetic clock

by multiplexed epigenome editing at five regions that lose methylation during aging. Targeted DNA methylation at these hypomethylated CpGs appears less stable and off-target effects were also enriched hypermethylated CpGs. Conversely, epigenetic clocks tend to be accelerated by this treatment, too, which was observed in HEK cells and primary T cells. Our study demonstrates that age-related DNA methylation changes are co-regulated in a dynamic network possibly supported by chromatin conformation. While rejuvenation does not appear to be possible with targeted DNA methylation, there is evidence that it can accelerate epigenetic aging clocks.

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Keywords: epigenetic clock, aging, DNA-methylation

4:45 PM – 5:45 PM

568

REGULATION OF ADRENOCORTICAL PROGENITOR CELLS DURING STRESS ADAPTATION

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Chronic stress is a pervasive concern in the modern society. The prolonged hyper-activation of the HPA axis in chronic stress is tightly linked with changes in the total functional mass of the adrenal gland and elevated amounts of stress hormones, resulting in a variety of clinical implications and psychiatric disorders. Emerging key players that contribute to the gland's great capacity to respond to changes are adrenocortical progenitor cells. In this study, we aim to unravel the mechanisms involved in the regulation of adrenocortical progenitors during stress adaptation that are still elusive. To that aim, we used a tamoxifen inducible Gli1:CreERT2/R26R:eYFP mouse line to lineage trace and monitor GLI1 adrenocortical progenitors in immobilization stress in the adult adrenal cortex in vivo. Preliminary results revealed that clusters of GLI1+ and NR2F2+ progenitor cells slowly migrate towards zona glomerulosa (zG) in the adrenal cortex of stressed mice, whereas in the non-stressed male mice they are found only in the capsular area. These results are in line with the increased expression of RSPO3 in the cortex of these mice, a signalling molecule involved in the control of cell renewal in the tissue. Additionally, to investigate if ACTH has a role in cell proliferation, we incorporated an in vitro system of non-adherent adrenocortical spheroids from primary mouse cells that were stimulated with ACTH. ACTH treatment resulted in increased growth of the spheroids compared to unstimulated controls as wells as increased steroidogenesis. This indicates a potential role of the hormone in cell proliferation and stem cell behaviour. By combining our in vivo results along with single-cell RNA-seq technology, we aim to shed light into molecular factors involved in the adult adrenal cortex regeneration and remodelling.

Keywords: adrenal cortex, stress, progenitors

4:45 PM – 5:45 PM

570

DE NOVO DESIGN OF TRANSCRIPTION FACTORS FOR EFFICIENT DIRECT CELLULAR REPROGRAMMING

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Transdifferentiation, or direct conversion of cells of one somatic lineage to another, is a promising new avenue for regenerative medicine. However, current methods utilizing native TF cocktails are limited by a low efficiency caused by the emergence of refractory populations, presumably caused by the non-specific regulation of genomic elements by these TFs. The goal of this study was to design de novo TFs; we hypothesize that designing binders against a tunable set of lineage-specific regulatory elements will result in more efficient reprogramming. Our lab has previously created a computational model for the design of small DNA-binding proteins (DBPs) with affinities in the nM range that recognize specific target sequences through interactions with bases in the major groove. We have experimentally validated that DBPs exhibit high specificity for their target sequences and modulate transcriptional outcomes in mammalian cells when fused with VP64 native effector domains. Now, we will leverage the ability of DBPs to avoid off-target binding in order to modulate a tunable set of motifs that commonly occur in lineage-specific enhancers. Currently, the precise regulatory elements that span the entire genomic landscape critical for a specific lineage conversion are unknown. We will perform high-throughput RNA and ATAC sequencing to screen combinations of DBPs targeting lineage-specific conversion elements in two well-characterized transdifferentiation processes: 1) Fibroblast conversion to skeletal muscle via MYOD, and 2) Fibroblast conversion to cardiomyocytes via Gata4, Mef2c, Tbx5 (GMT). These screens will reveal combinations of regulatory elements that are critical for efficient reprogramming, and this information combined with knowledge of elements affected by native TF cocktails will guide design of subsequent generations of DBPs. We propose that a scalable and efficient method for designing synthetic TFs will facilitate precise investigations into the global changes in the epigenetic landscape underlying somatic reprogramming processes, allow for foundational studies into chromatin dynamics and transcriptional regulation; and overcome current limitations in regenerative medicine by generating a novel set of therapeutic molecules that increase the efficiency of cellular reprogramming.

Keywords: de novo protein design, direct reprogramming, synthetic transcription factors

4:45 PM – 5:45 PM

572

REGENERATION OF HAIR CELLS IN ZEBRAFISH NEUROMAST REGARDING TETRASPANIN1, KEY MARKER FOR PLANARIAN REGENERATION

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Besides the difference in regeneration between mammalian and other lower animals, the detailed mechanism of damage-induced regeneration in animals is still primarily elusive. As a vertebrate model, zebrafish share similarities with mammals, making it a valuable animal model for regenerative biology. In this study, we investigated the role of Tetraspanin1 (TSPAN1), a key marker for planarian neoblasts, a pluripotent stem cell contributing to regeneration, in zebrafish. We utilized CRISPR/Cas9 to knockout tspan1 in zebrafish and found that tspan1 is related to regeneration capacity in zebrafish. In neuromast, a sensory organ consisting of differentiated hair cells, supporting cells with stem cell-like properties, and mantle cells, tspan1 is exclusively expressed in mantle cells that potentially amplify supporting cells. We observed that the tspan1 knockout mutant had fewer Sox2-positive cells, a marker for cells with stem cell-like functions. Additionally, the tspan1 knockout mutants showed impaired regeneration in hair cells, confirmed by a reduced number of hair cells and supporting cells. Also, we observed a decrease in the expression of Numb, a known notch inhibitor, and an increase in the expression of notch3 in tspan1 knockout mutants, indicating that tspan1 is involved in notch signaling. Although the exact mechanism underlying these effects is still unclear and requires further study, considering the role of tspan1 during regeneration in planarian and zebrafish, our findings suggest that the mechanism of maintaining stem cell population during regeneration might be evolutionarily conserved.

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Keywords: regeneration, Tetraspanin1, stem cells population

4:45 PM – 5:45 PM

574

UNBIASED NICHE IDENTIFICATION OF METASTATIC COLORECTAL CANCER CELLS

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Metastatic spread is a highly inefficient process, as the vast majority of disseminated tumor cells fail to colonize distant organs. Nevertheless, the successful engraftment of a miniscule fraction of circulating tumor cells is responsible for more than 90% of cancer-related deaths. Metastatic success of a disseminated tumor cell depends on a combination of cell-intrinsic and cell-extrinsic features. In particular, the rare microenvironments that allow for initial metastatic engraftment are poorly understood. Likewise, the dynamic changes that these microenvironments undergo as a micro-metastasis progresses from survival to growth are unknown. Identifying these early metastatic niches and understanding the remodeling processes that are necessary for disease progression may pave the road for new therapeutic approaches. Therefore, we developed a new, highly sensitive and rapid niche labeling system based on receptor-mediated uptake of locally produced fluorophores (EnviroTag), which enables detection and analysis of niches even at the micro-metastatic stage. We applied the EnviroTag system to study niche requirements of early colorectal-cancer metastasis. We introduced the EnviroTag system into mouse colon cancer organoids and transplanted them into receiver mice, to simulate the metastatic cascade. EnviroTag tumor organoids metastasized to the liver and labelled their paracrine environment even at the stage of micro-metastasis. Based on the EnviroTag signal we isolated tumor cells and their metastatic niches from the timepoint of engraftment to the point of active metastatic growth and analyzed changes in tumor cells and their immediate microenvironment via single-cell sequencing. Our system efficiently identified liver-resident and recruited immune cell populations, fibroblasts, endothelial cells and cells of the liver parenchyme. Based on EnviroTag labeling we identified the particular changes that these cells undergo in proximity to the metastasis at different stages of metastatic progression. This dataset enabled us to reconstruct dynamic cellular and molecular changes in the metastatic niche during the engraftment process of colorectal cancer cells and identify key players in the early metastatic communication network.

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Keywords: metastatic niche, metastatic colorectal cancer, niche labelling system

4:45 PM – 5:45 PM

576

ROLE OF CONDENSIN I IN CHROMOSOME CONDENSATION OF PLURIPOTENT STEM CELLS

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Pluripotent stem cells (PSCs) hold great promise in both basic and translational research, as well as regenerative medicine. However, when in culture, these cells present genomic instability that can lead to aneuploidy, which compromises further applications. The molecular mechanisms underlying this genomic instability were not fully described yet. Here, we explore possible reasons for compromised mitotic segregation, building on known differences in the chromatin state of PSCs. We have previously shown that PSCs have weaker centromeres. Comparative analysis of the type and frequency of mitotic defects in PSCs and their somatic counterparts revealed a high frequency of lagging chromosomes during mitosis. These findings suggest that defects in centromere/kinetochore assembly, and consequently chromosome attachments may be a major mechanism underlying the observed high frequency of chromosome segregation errors. In addition to changes in the centromeres, PSCs have less compacted DNA during interphase, and we and others have observed a high frequency of condensation alterations during mitosis of PCSs. However, we did not observe a high frequency of DNA bridges, indicating that the anticipated problems in chromosome condensation and/or resolution of chromosome entanglements are efficiently resolved. These findings suggest that PSCs may have compensatory mechanisms to overcome the hypocompacted state of their chromatin. To explore this possibility, we have analysed the levels of key proteins involved in mitotic chromosome organization, the condensin complexes. We observed that the chromatin bound levels of Condensin I are increased in PSCs when compared to somatic cells. Ongoing work is aiming to test if the molecular mechanisms underlying the increased loading of Condensin I; and how the excess of Condensin I can work as a putative compensation mechanism to boost mitotic fidelity in PSCs. Understanding PSCs mitotic´s physiology will open questions to better comprehend mitosis during development. In parallel, unveiling the mechanisms responsible for a compromised mitotic fidelity will allow to devise strategies to overcome this problem in PSCs and push forward their remarkable applicability.

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Keywords: chromatin condensation, Condensin I, mitotic defects

4:45 PM – 5:45 PM

TOPIC: PANCREAS

578

HARNESSING THE POTENTIAL OF PANCREATIC STELLATE CELLS TO ENHANCE STEM CELL-DERIVED ISLET TRANSPLANTATION FOR TYPE 1 DIABETES

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Type 1 Diabetes (T1D) is an autoimmune disease affecting over 8 million people globally. It is characterized by the destruction of insulin producing β-cells in the islets of Langerhans and the subsequent dysregulation of blood glucose. Current treatment with exogenous insulin only manages symptoms, and patients remain at high risk of complications. Stem cell-derived β-cells that secrete insulin in response to changing blood glucose levels hold great promise for treating and curing T1D. Our laboratory has developed α-, β-, and δ-cell differentiation protocols and is able to model the three main cell types of the islet. While we and others have demonstrated success in transplanting stem cell derived islet cells in mice, long-term survival and function of these grafts is limited by inflammation and graft destruction. There is an urgent need to develop cytoprotective and immunomodulatory approaches that can prolong islet cell survival and function. Pancreatic stellate cells (PStCs) are an understudied stromal cell population in the pancreas. While their precise role remains unknown, they are thought to be involved in stress responses, injury, and ECM turnover. We demonstrate the presence of PStCs in stem cell (SC)-derived β cell clusters by scRNAseq and immunofluorescence, with PStCs comprising 12.2% of total cells. This allows for a detailed examination of PStCs, to understand their role in differentiations and maintaining SCβ cells. By culturing SCβ cells on PStCs we show a 2-fold prolongation of an insulin secreting phenotype in β cells as compared to SCβ cells alone. This suggests a beneficial interaction between β cells and PStCs. Our analysis of cell-cell communication from scRNAseq data obtained from SCβ and human islet cells implies the involvement of the MK, PTN, NOTCH and CADM signalling pathways, pathways related to cell survival, differentiation, and cytokine expression among others. Lastly, we show expression of immunomodulatory and M2 macrophage polarizing molecules such as TGFβ1, CCL2 and PD-L1 in SCβ-derived PStCs. These results support the potential of PStCs to impact β cell survival by providing cytoprotection and immunomodulation. Future research will aim at further characterizing PStCs and quantitatively assessing their interactions with β-cells and immune cells in vitro and in vivo.

Funding Source: Mayo Clinic Center for Regenerative Biotherapeutics J. W. Kieckhefer Foundation Khalifa Bin Zayed Al Nahyan Foundation

Keywords: stem cell therapy, stellate cells, immunomodulation

TRACK: NEW TECHNOLOGIES (NT)

4:45 PM – 5:45 PM

TOPIC: NO TISSUE SPECIFICITY

580

TRANSLATIONAL CAR-T CELL POTENCY ASSAYS USING TWO ORTHOGONAL PLATFORMS

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Chimeric Antigen Receptor (CAR)-T Cell characterization by potency includes both direct methods via cytotoxicity and indirect surrogate methods such as determining the release of critical cytokines. Both measurements involve the co-culture of effector CAR-T cells with target cells containing the target antigen and engineered with reporters such as GFP and/or Luciferase. Decentralized manufacturing facilities require robust and scalable methods that can be tech-transferred between instruments. In this study, NALM-6 target cells, a CD19 expressing acute lymphoblastic leukemia cell line engineered with GFP and Luciferase, were co-cultured with CD19 CAR-T effector cells. CD19 CAR-T cells were generated from either viral or non-viral methods (CAR+ % range: 7.5% to 25.9%) and further phenotyped to account for variability across 3 donors. Direct methods of cytotoxicity included measurement of reporter GFP and Luciferase expression, in addition to Lactate Dehydrogenase (LDH) as a third readout. Various effector CAR-T cells to target ratios (E:T) were examined and cell killing was measured either through the decrease in GFP and Luciferase signal or an increase in LDH. Sensitivity ranges of the measurement for each instrument platform and assay were established. Based on the measurement readout, GFP detection by the image-based Incucyte platform demonstrated a higher sensitivity and broader range of quantification than the Varioskan LUX plate reader platform. The dynamic range of effector-to-target cell ratios 20:1 to 0.5:1 E:T produced cytotoxicity from 0.5% – 95% depending on CAR-T cell potency. Based on assay output, cell killing by GFP was consistent across both orthogonal platforms for effector-to-target cell ratios of 5:1, 2:1, and 1:1 E:T. Results suggest that GFP assay measurement would be the most translatable across broader instrument and analysis platforms. Additionally, luciferase-based reporter analysis was comparable to GFP measurement on the plate reader platform and could serve as a suitable scalable method. The established CAR-T potency assays across platforms demonstrate utility in a variety of measurement systems for smooth integration in manufacturing applications

Keywords: CAR-T, cell therapy, potency assays

POSTER SESSIONS