Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures

Alexander Plossa,b,1, Salman R. Khetani2,1, Christopher T. Jonesa,b, Andrew J. Sydera,b,3, Kartik Trehanb, Valeriya A. Gaysinskayaa,b, Kathy Muab, Kimberly Ritolaa,b, Charles M. Ricea,b,4, and Sangeeta N. Bhatiac,d,e,4

Polarized Cell Layers and Support HCV Glycoprotein-Mediated Entry.

Recent advances have allowed HCV to be propagated in human hepatoma cells (HCV cell culture system, HCVcc). These cell lines, however, display abnormal proliferation, deregulated gene expression, as well as aberrant signaling and endocytic functions (1–4). Consequently, neither the perturbation of normal hepatocyte biology by infection, nor authentic host responses to HCV, can be studied accurately in culture (1).

Primary hepatocytes are considered a more physiologically relevant system, but are notoriously difficult to maintain in culture as they precipitously decline in viability and phenotype upon isolation from their in vivo microenvironment (6). This rapid deterioration, as well as the lack of HCV detection methods with high specificity and sensitivity, has made it difficult to assess viral replication in primary human cell cultures (5, 7–11). Over the last few decades, investigators have employed a plethora of different strategies to preserve liver-specific functions in vitro and to extend the lifetime of the model systems (12). These strategies typically include extracellular matrix manipulations, defined culture media, fluid flow using bioreactors, or alteration of cell–cell interactions by forming 3D spheroidal aggregates or cocultivation with nonparenchymal cell types (6, 12, 13, 14). Although some of these models provide necessary extracellular matrix cues, they lack crucial heterogeneous cell–cell interactions or control over tissue architecture, known to affect liver-specific functions (6, 12). In culture techniques using fragile extracellular matrix gels, 3D aggregates, and/or continuous perfusion, scaling down to 96-well and smaller formats appropriate for drug screening remains challenging. Most importantly, it is unclear whether any of these model systems supports persistent HCV infection.

Results

Primary Human Hepatocytes in Micropatterned Cocultures Form Polarized Cell Layers and Support HCV Glycoprotein-Mediated Entry.

We have recently developed a miniaturized, multifwell model of human liver tissue with optimized microscale architecture that maintains phenotypic functions for several weeks in vitro (12). In this cell culture system, primary adult human hepatocytes do not seem to proliferate. Our system is comprised of primary hepatocytes organized in micropatterned colonies of empirically optimized dimensions and subsequently surrounded by supportive stroma (micro-patterned cocultures, MPCC; Fig. 1A and B). Here, we show that primary human hepatocytes form polarized cell layers in MPCCs. Multidrug resistant protein 2 (MRP2), zona occludens protein 1 (ZO1), and HCV entry factors claudin-1 (CLDN1) (15) and occludin (OCLN) (16, 17), were located in tight junction (TJ)-like structures (canalicular domain), whereas CD26 was localized on the basolateral domain (Fig. 1). The presence of bile canalicular structures between adjacent hepatocytes was confirmed via 3D renderings reconstructing ZO1, MR2, and nuclear staining (Fig. 1I and F). Compared to human liver tissue, primary hepatocytes in MPCCs expressed similar patterns of the other known HCV entry factors, CD81 (18), scavenger receptor class B type 1 (SCARB1) (19), and CLDN1 (15) (Fig. 1I and Fig. S1).

To test whether MPCC hepatocytes can support HCV glycoprotein-mediated entry, we infected cultures with HCV pseudoparticles (HCVp). HCVpp, which are defective lentiviral particles that display the HCV glycoproteins (E1 and E2) and encode a reporter gene (here EGFP), allow rapid quantitation of infection in the absence of replication. Approximately 1–3% of the human hepatocytes in MPCCs, but none of the supporting mouse embryonic fibroblasts (3T3-J2), could be infected with HCVpp (Fig. 1K and L). Pseudoparticles lacking glycoproteins did not infect the cultures, although MPCCs were...
readily infected by pseudoparticles displaying the pan-tropic vesicular stomatitis virus glycoprotein (VSVGpp). A blocking antibody targeting CD81 completely abrogated HCVpp, but not VSVGpp, infection (Fig. 1L).

**HCV Persistently Replicates in Primary Human Hepatocyte MPCCs**

Despite considerable effort, it has not been possible to unequivocally demonstrate HCV replication in primary hepatocyte cultures over prolonged periods of time. Previous studies have relied on quantifying HCV RNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), a technique inappropriate for detecting rare infectious events due to high background of nonspecifically bound viral RNA. We instead employed a highly sensitive HCVcc reporter virus expressing secreted Gaussia luciferase (Gluc), Jc1FLAG2(p7-nsGluc2A) (20). After inoculation, cultures were washed to remove Gluc carryover, and luciferase secretion was monitored as an indicator of viral replication. We found that several conventional culture systems (i.e., collagen gel sandwich, Matrigel overlay, and randomly distributed cocultures) could not sustain HCV replication, presumably due to a decline in liver-specific phenotype (12) (Fig. S2). In contrast, MPCCs in multiwell formats supported HCV replication for at least two weeks (Fig. 2 and Fig. S3). Treatment with HCV NS3-4A protease (ITMN191) or NS5B polymerase inhibitors (2CMA), or IFN alpha (IFN-α), reduced luciferase activity to background levels (Fig. 2A), indicating that persistent signal was indeed due to ongoing viral replication. Persistent HCV infection was achieved in MPCCs created from freshly isolated or cryopreserved human hepatocytes from several donors, reflecting the reproducibility of the optimized microscale architecture and the concomitant phenotypic stability. We next attempted to quantify HCV RNA and proteins in MPCCs by qRT-PCR, Western blot, and immunofluorescence. In contrast...
Primary hepatocytes in MPCCs produce infectious virus. To determine whether primary hepatocytes in MPCCs are capable of producing infectious virions, filtered culture supernatants were used to inoculate naïve Huh-7.5 cells, followed by staining for HCV protein (NS5A) at 72 h postinfection. Infectious virus was detected in MPCC supernatants harvested at day 4 postinfection and for all time points measured up to day 12 (Fig. S4). Supernatants from MPCCs infected in the presence of specific antiviral inhibitors did not yield NS5A-positive foci in Huh-7.5 cells, indicating that de novo virus production, rather than carryover of the inoculum, was detected. Attempts to passage MPCC-produced virus onto naïve MPCCs were unsuccessful due to the low titers produced by the primary cells. The low titers also precluded further biophysical analysis of the virus.

**Proof of Principle for Preclinical Screening of Anti-HCV Therapeutics in MPCCs.** Persistently infected MPCCs may be a viable and relevant platform for preclinical screening of anti-HCV therapeutics. Antibodies blocking HCV entry factors, in particular CDS1 and SCARB1, have been shown to be effective in vitro (23, 24) and in small animal models (25). We tested the ability of monoclonal antibodies against these cellular targets, as well as four antibodies specific for HCV E2, to inhibit HCVcc entry in MPCCs; none of these reagents had previously been tested in primary cell cultures. Anti-CDS1 (JS-81) blocked HCVcc entry very efficaciously (IC50 < 0.1 μM), whereas anti-SCARB1 (C167) did not effectively inhibit viral uptake. All antibodies against E2 were able to inhibit HCVcc infection, although with varying efficiencies (IC50 for AP33 > 3/11 > CBH5 > AR3A) (Fig. 3A). A variety of specific antivirals targeting HCV enzymes are also under preclinical development. Currently, however, in vitro platforms capable of simultaneously assessing drug toxicity and efficacy are not widely available. We have previously shown the utility of MPCCs in drug metabolism and toxicity screening via assessment of gene expression profiles, phase I/II metabolism, canalicular transport, secretion of liver-specific products, and susceptibility to hepatotoxins (12). Here, we examined the use of MPCCs in evaluating antiviral efficacy (Fig. 3B). We measured HCV replication by luciferase activity at 4 days posttreatment with protease inhibitors (BILN2061 and ITMN191), polymerase inhibitor (2’CMA), or IFN-α. These compounds inhibited HCV replication in the submicromolar range, indicating the relevance of MPCCs for monitoring HCV infection. We then evaluated the efficacy of protease inhibitors (SCH-6 and BILN2061) and polymerase inhibitor (2’CMA) in HCVcc-infected MPCCs pretreated for 3 days with compounds known to modulate drug metabolism and other cellular functions in vivo (12) (Fig. 3C). We found that the addition of certain drugs severely reduced the efficacy of SCH-6 and 2’CMA, as compared to DMSO solvent control. Although the mechanisms underlying these adverse drug interactions remain unknown, these observations demonstrate the importance of conducting drug combination studies during in vitro efficacy assessment. These studies indicate that MPCCs may be well suited as a metabolically competent in vitro model of the liver, allowing HCV replication to be studied over several days to weeks, and a variety of intervention strategies to be tested for efficacy and toxicity.

**Discussion**

Here we have described a microscale primary human hepatocyte in vitro culture platform that supports the entire HCV life cycle. Primary hepatocyte MPCCs are stable for several weeks and therefore allow monitoring of human hepatotrophic infections over extended periods of time. Although this is an important step forward, limitations remain. Entry of HCVpp and tissue culture-derived virus into MPCC hepatocytes was inefficient. Although the four critical viral entry factors are present on these cells, it is possible that differences in their spatial distribution might account for the low uptake efficiency. Indeed, antibodies block entry into primary hepatocytes at different efficacies than previously reported (26–28), possibly due to limited accessibility of the HCV entry proteins.
factors on polarized cells. Furthermore, following isolation from the liver and disruption of hepatic polarity, it may be that in some hepatocytes in MPCCs, expression levels of critical viral entry factors on polarized membranes do not reach the threshold required with proper spatial localization for efficient viral uptake.

We also did not observe any increase in the number of infected cells over time, arguing for limited spread of HCV in the cultures. Several factors could contribute to this phenomenon, including limited numbers of infectious particles, heterogeneous polarity, or an inherent or acquired refractory nature of a proportion of cells. Furthermore, certain critical host factors may be heterogeneously expressed and therefore limiting in some cells, rendering them resistant to infection or unable to sustain HCV RNA replication. Although our data demonstrate that primary hepatocytes in MPCCs can produce infectious virus, the titers are low and few infectious virions are available for spread. In those cells that do become infected, HCV is capable of interfering with innate antiviral immunity via NS3-4A-mediated cleavage of critical signaling molecules, including IPS-1, TRIF, and IRF3 (29). Although this mechanism is presumably sufficient to blunt antiviral signaling and sustain replication, IFN production may not be entirely prevented, rendering adjacent cells nonpermissive. The low permissivity of MPCC hepatocytes to HCV may also reflect the in vivo reality of chronic hepatitis. Technical challenges have traditionally made it difficult to estimate the number of infected cells in an HCV-positive liver. Recently, however, two-photon microscopy methods have been used to determine that only a low proportion (7–20%) of ex vivo patient hepatocytes express viral antigens (30).

Although further improvements in infection efficiency may be possible, our system lays the foundation for preclinical assessment of antiviral therapeutics against human hepatotropic pathogens in a more physiologically relevant microenvironment. Importantly, due to the phenotypic stability of the MPCCs, infection processes can be monitored longitudinally, potentially allowing the kinetics of viral spread and antiviral signaling to be characterized at the single cell level. The polarized nature of the MPCC hepatocytes allows HCV entry and uptake inhibitors to be studied in the context of intact tight junction structures. Furthermore, using sera from HCV-infected patients and a very sensitive fluorescent reporter system (22), we were able to detect an extremely low frequency of productive infection—suggesting that a combination of authentic virus and host cells may be achievable. Proof of principle studies reported here also demonstrate the value of MPCCs in drug studies. The high baseline activities of drug metabolism enzymes (i.e., cytochrome P450s) and their drug-mediated induction/inhibition in MPCCs (12) allows for simultaneous measurements of drug efficacy, drug–drug interactions, and drug toxicity, thereby providing critical preclinical parameters. These advantages combine to make MPCCs a highly valuable system for studies of HCV biology.

Methods

Virus Genomes and Stocks. Jc1FLAG2(p7-nsGluc2A) is a fully-infectious HCVcc reporter virus encoding Gaussia luciferase between p7 and NS2 (20). Virus stocks were created by electroporation and titered by limiting dilution as previously described (23).

Liver Sections and Hepatocytes. Primary human hepatocytes were purchased from vendors permitted to sell products derived from human organs procured in the United States by federally designated Organ Procurement Organizations. Vendors included: Celsis In vitro Technologies, BD-Gentest and CellDirect. Human hepatocytes were pelleted by centrifugation at 50–100 xg for 5 min at 4 °C, resuspended in hepatocyte culture medium, and assessed for viability using Trypan blue exclusion (typically 70–90%). Liver-derived nonparenchymal cells, as judged by size (<10 μm diameter) and morphology (nonpolygonal), were consistently found to be less than 1% in these preparations. Human liver sections were obtained from the NewYork-Presbyterian Hospital from uninfected donor tissue deemed unacceptable for liver transplantation. Tissue was processed by immediately freezing in OCT compound at -80°C or by fixation in 10% formalin solution for 24 h followed by paraffin embedding. Tissue sections were cut (~5-6 μm) on poly-L-lysine coated slides. Human serum and plasma samples were obtained at Well Cornell Medical Center. All protocols for human primary material procurement were approved by the Committee on Use of Human Experimental Subjects, MIT, or by the IRB, Rockefeller University and Weill Cornell Medical Center.

Micropatterned Co-cultures of Primary Human Hepatocytes and Supportive Stromal Cells. Off-the-shelf tissue culture polystyrene (24- and 96-) or glass bottom (24)-multiwell plates, coated homogenously with rat tail type I collagen (25–50 μg/ml), were subjected to soft-lithographic techniques (12) to pattern the collagen into micro-domains (islands of 500 μm in diameter with 1200 μm center-to-center spacing). To create MPCCs, hepatocytes were seeded on collagen-patterned plates that mediate selective cell adhesion. The cells were washed with medium 2–3 h later (~3×10^6 adherent hepatocytes in 96 collagen-coated islands in 24-well plate and ~4.5×10^6 hepatocytes in 14 islands in 96-well plate) and incubated in hepatocyte medium.
antibodies

3. \( N \) in a formaldehyde and/or 4°C overnight. Fibroblast-to-tetra- 
one hour at 4°C

mouse anti-human CD81 (clone JS-81, BD Pharmingen; 1:200), rabbit anti-

SCARB1 (C167) (24), anti-CD81 (clone JS-81; BD Pharmingen), anti-E2 3/11 (25), and antibodies kindly provided by A.H. Patel (University of Glasgow, Scotland) (AP33) (26), S.K. Fong (Stanford University, Palo Alto, CA) (CBHS) (27), and D.R. Burton (The Scripps Research Institute, La Jolla, CA) (ARSA) (28). Human IgG1 (clone MOPC-21), IgG4 (MOR5391), and rat IgG2a (MCA1124R) isotype control antibodies were purchased from AbD Serotec.

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