Expansion, in vivo–ex vivo cycling, and genetic manipulation of primary human hepatocytes


aLaboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY 10065; bLaboratory of Liver Infectious Diseases, Ghent University, 9000 Ghent, Belgium; cInstitute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02142; dDépartement de Biologie, École Normale Supérieure Paris-Saclay, Université Paris-Saclay, 9423 Cachan, France; eDivision of Gastroenterology and Hepatology, Weill Cornell Medicine, New York, NY 10065; fDepartment of Molecular Virology, University Hospital Heidelberg, 69120 Heidelberg, Germany; gBio-Imaging Resource Center, The Rockefeller University, New York, NY 10065; hDivision of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida College of Medicine, Gainesville, FL 32607; iMedical Research Council Centre for Regenerative Medicine, University of Edinburgh, Edinburgh EH16 4TJ, United Kingdom; jDepartment of Pathology, NYU Langone Health, New York, NY 10003; kHerman B. Wells Center for Pediatric Research, Indiana University, Indianapolis, IN 46202; lLaboratory Animal Research Department, Central Institute for Experimental Animals, Kawasaki 210-0821, Japan; mDepartment of Medicine D and the Liver Institute, Rabin Medical Center, Beilinson Hospital, Petach-Tikva 49100, Israel; nDepartment of Gastroenterology and Liver Disease, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel; oBroad Institute of MIT and Harvard, Cambridge, MA 02139; pHoward Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139; qKoch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139; rDepartment of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA 02139; sMarble Center for Cancer Nanomedicine, Massachusetts Institute of Technology, Cambridge, MA 02139; and tDepartment of Medicine, Brigham and Women’s Hospital, Boston, MA 02115

Contributed by Charles M. Rice, December 2, 2019 (sent for review October 31, 2019; reviewed by Karl-Dieter Bissig and Raymond T. Chung)

Primary human hepatocytes (PHHs) are an essential tool for modeling drug metabolism and liver disease. However, variable plating efficiencies, short lifespan in culture, and resistance to genetic manipulation have limited their use. Here, we show that the pyrrolizidine alkaloid retrorsine improves PHH repopulation of chimeric mice on average 10-fold and rescues the ability of even poorly plateable donor hepatocytes to provide cells for subsequent ex vivo cultures. These mouse-passaged (mp) PHH cultures overcome the marked donor-to-donor variability of cryopreserved PHH and remain functional for months as demonstrated by metabolic assays and infection with hepatitis B virus and Plasmodium falciparum. mpPHH can be efficiently genetically modified in culture, mobilized, and then recultured as spheroids or retransplanted to create highly humanized mice that carry a genetically altered hepatocyte graft. Together, these advances provide flexible tools for the study of human liver disease and evaluation of hepatocyte-targeted gene therapy approaches.

Hepatocytes, the predominant cell type in the liver, perform multiple functions and play essential roles in metabolic reactions and drug clearance (1, 2). In addition, human hepatocytes are the exclusive host for several hepatotropic pathogens, including hepatitis B virus (HBV), hepatitis C virus, and malaria-causing Plasmodium parasites (3). Therefore, hepatocytes are central players in many of the world’s leading disease etiologies.

Primary human hepatocytes (PHHs) isolated from human liver are the gold standard for studies of hepatocyte biology. However, current PHH options suffer from donor-to-donor variability, limited availability of high-quality donors, high financial cost to isolate and procure, and failure to maintain long-term function in culture. Furthermore, the inability to isolate high-quality PHH from patients with liver disease has precluded the study of possible causal genetic variants in a physiologically relevant model.

Underpinning these limitations is the inability of PHHs to proliferate once isolated, despite their capacity for regeneration and expansion in vivo in response to injury. Renewable sources of human hepatocytes have been sought, including differentiation of pluripotent stem cells into hepatocyte-like cells (HLCs). Despite the potential of this approach, including the ability to generate patient-specific hepatocytes from induced pluripotent stem cells, current HLCs resemble fetal hepatoblasts rather than mature hepatocytes (4, 5). Protocols have been developed to expand PHH, including ex vivo growth as organoids (6) or immortalization (7, 8). A more widely adopted approach involves

significance

The ability to study human liver disease is limited by available hepatocyte models. Primary human hepatocytes (PHH) and xenograft models suffer from limited availability, donor-to-donor variability, and high cost. Here we report two transformative advances. First, the alkaldoid retrorsine improves humanization of the murine liver, which allows routine production of highly humanized mice and high-quality mouse-passaged PHH. Second, the ability to genetically modify PHH cultures and retransplant to create highly humanized mice with genetically altered grafts. When combined, these two advances open new frontiers for creating disease-specific PHH models and for performing genetic and other screens in PHH.


Reviewers: K.-D.B., Duke University School of Medicine; and R.T.C., Massachusetts General Hospital.

The authors declare no competing interest.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: Datasets supporting the findings of this study are deposited in the publicly available database Gene Expression Omnibus (GEO), https://www.ncbi.nlm.nih.gov/geo (accession no. GSE130219).

1 E.M. and K.V. contributed equally to this work.
2 C.M.R. and Y.P.d.J. contributed equally to this work.
3 To whom correspondence may be addressed. Email: ricec@rockefeller.edu or ydj2001@med.cornell.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1919035117/-/DCSupplemental.

PHH transplantation into immunodeficient mice with various forms of liver injury to provide a niche and milieu that favors PHH engraftment and expansion. There are several widely used murine models, including immunodeficient Fah−/−NOD Rag1−/−Il2rgnull (FNRG) mice (9–11) in which liver injury is induced by withdrawing the protective drug 2-(2-nitro-4-(trifluoromethyl) benzoyl)cyclohexane-1,3-dione (NTBC) (12). While the specific stimuli remain unidentified, this expansion method is presumed to function via the provision of signals derived from the damaged mouse liver and by creating vacant space that is liberated as murine hepatocytes die. Collectively, this environment allows transplanted human hepatocytes to engraft within the damaged scaffold and proliferate to repopulate the mouse liver parenchyma. Once these chimeric livers are highly humanized, human hepatocytes can be reisolated. However, to date, these chimeric models have suffered from the same detractors that limit the use of unexpanded PHH: most PHH donors repopulate poorly, and the level of chimerism is variable between animals.

Here, we describe a protocol that, by improving chimeric mouse liver humanization, enables the expansion of the usable PHH donor pool. Specifically, we show that, via this protocol, mouse-passaged primary human hepatocytes (mpPHHs) can successfully establish long-term cultures that are suitable for drug metabolism studies and are also susceptible to human hepatotropic pathogen infections. Notably, we observe that even poorly plateable PHHs can be passaged via humanized mouse expansion and that this process seems to normalize the subsequent plateability of almost every mpPHH tested. Perhaps most significantly, mpPHHs can be efficiently transduced with lentiviral vectors, mobilized, and replated for use in ex vivo applications or retransplanted to generate humanized mice that carry a genetically modified human graft, opening doors for the experimental study of human liver biology at a level that has thus far been unachievable.

**Results**

**Retrorsine Improves Human Hepatocyte Repopulation in Liver Chimeric Mouse Models.** To reliably study PHH in vivo in liver chimeric mice or isolate the human graft for ex vivo studies, a minimum level of humanization is required. For most applications, the minimum level is ~10%, which corresponds to 10^9 μg/mL serum human albumin (hAlb), an established surrogate marker for liver humanization (9, 10, 13). Unfortunately, with current protocols, PHHs from most donors engraft poorly, and few mice achieve the degree of humanization required for further use. To address this limitation, we sought to establish protocols where the majority of PHH donors consistently generate mice with over 10% chimerism.

In rats, the pyrrolizidine alkaloid retrorsine enhances liver repopulation with donor hepatocytes after partial hepatectomy or carbon tetrachloride administration (14, 15). We, therefore,

![Fig 1. Retrorsine improves liver repopulation of FNRG mice with PHHs. (A) Experimental schematic of FNRG mice treated with retrorsine 4 and 2 wk prior to transplantation with cryopreserved PHHs and NTBC cycling. (B) Serial hAlb measurement in serum of transplanted mice shows higher peak hAlb values in retrorsine preconditioned (squares) than vehicle control mice (circles). Median ± SEM, t test of end points. PHH1 n = 2 per group, PHH2 n ≥4 per group, and PHH3 n = 7 per group. Estimated percentage of liver humanization is shown in gray. *P < 0.05; ***P < 0.001. (C) Livers from retrorsine preconditioned PHH1 transplanted animals contain larger human areas by FAH staining than vehicle control mice. (Scale bars: Left, 1 mm; Right, 100 μm.) (D) hAlb levels in serum reached a higher plateau in retrorsine than vehicle preconditioned FNRG mice with 12 of 13 PHH donors ranging from 2- to 30-fold. Symbols are median hAlb per group of two to five transplanted mice. Color-matched numbers indicate animal numbers that reached hAlb plateau (for vehicle, numbers are shown on the left, and for retrorsine, they are on the right). (E) Retrorsine works in a dose-dependent manner, with the first dose having the largest benefit on peak serum hAlb values in PHH2 transplanted FNRG mice. Symbols are individual mice, median ± range, t test. (F) Serum hAlb values peak higher in mice receiving retrorsine at ZT0 than ZT12 prior to PHH2 transplantation (Left), while PHH3 engrafted faster in mice that received at ZT0 than ZT12 as illustrated by day 42 hAlb values (Right). Symbols are individual mice, median ± range, t test.**

Michailidis et al.
tested if preconditioning FNRG mice with two injections of retrorsine before PHH transplantation (Fig. 1A) enhanced hAlb levels in mouse serum. Across PHH1 from three donors, retrorsine improved hAlb levels (Fig. 1B) without affecting animal survival (SI Appendix, Fig. S1A). Retrorsine increased peak hAlb levels by 23- and 30-fold in mice engrafted with poor donors PHH1 and PHH2, respectively (Fig. 1B). In contrast, retrorsine had only a minor effect (twofold increase) on mice engrafted with PHH3, an efficient donor that humanizes well even with a standard protocol (vehicle control). Consistent with hAlb levels, histological staining for fumarylacetoacetate hydrolase (FAH) demonstrated that the liver parenchyma of retrorsine-treated humanized Fah−/− NOD Rag1−/− IL2rgull (huFNRG) mice was largely replaced by human hepatocytes (Fig. 1C). We then tested 10 additional PHH donors and found that 9 of them reached higher peak hAlb levels after retrorsine preconditioning. Across 13 donors, retrorsine on average resulted in 9.5-fold higher peak serum hAlb levels than vehicle control mice (P = 0.003) (Fig. 1D). This finding confirms that retrorsine preconditioning improves engraftment across a wide range of PHH donors.

We then tested if retrorsine also improved humanization with fetal hepatoblasts, which require human oncostatin-M (hOSM) supplementation for efficient repopulation of FNRG livers (13). To supplement hOSM, we created AAV8-hOSM vectors that, at low doses, were fully toxic and improved humanization in huFNRG mice (SI Appendix, Fig. S1B). As with PHHI, we found that retrorsine preconditioning of FNRG mice improved fetal hepatoblast repopulation (SI Appendix, Fig. S1C).

The scale of retrorsine effects on PHH engraftment varied with the dose such that one dose led to a large improvement and a second dose conferred a small additional benefit (Fig. 1E). For PHH3, there was no benefit of administering a third dose (1.02-fold increase in serum hAlb, n = 11 two doses vs. n = 9 three doses, P = 0.98). In rat models, retrorsine is bioactivated by CYP450 enzymes (16, 17), many of which are under circadian regulation. We, therefore, examined whether injecting mice with retrorsine at different times of the day influences its effectiveness. Interestingly, retrorsine administered at Zeitgeber time 0 (ZT0) resulted in fivefold higher (PHH2) or faster rise in hAlb (PHH3) than at ZT12 (Fig. 1F).

We hypothesized that retrorsine impaired mouse hepatocyte proliferation, limiting restoration of the damaged mouse liver and giving PHH a repopulation advantage. In the Fah−/− model (12) at least 7 d before harvest. We perfused huFNRG livers and purified hepatocyte suspensions by Percoll and low-speed centrifugations to enrich for live hepatocytes (mpPHH) (Fig. 2A). Flow cytometry to measure the expression of human and murine markers. In 78 mice (93%), this protocol yielded mostly human cells (Fig. 2C). To obtain high yields, we perfused livers from retrorsine preconditioned highly chimeric huFNRG mice based on hAlb serum levels around 104 µg/mL. Liver perfusions of 70 huFNRG mice yielded on average 75 million mpPHH per liver, ranging from 0.4 to 240 million (Fig. 2B). These data show that our isolation/purification protocol results in consistently large numbers of predominantly human hepatocytes isolated from highly chimeric huFNRG mice.

Isolation of PHHs from Chimeric Mouse Livers. Retrorsine preconditioning greatly increased the availability of highly humanized mice. Since human hepatocytes can be isolated from chimeric livers (9, 11, 21), we established protocols to enrich for live hepatocytes from these livers. Retrorsine preconditioned huFNRG mice were taken off the protective drug NTBC (12) at least 7 d before harvest. We perfused huFNRG livers and purified hepatocyte suspensions by Percoll and low-speed centrifugations to enrich for live hepatocytes (mpPHH) (Fig. 2A). Flow cytometry to measure the expression of human and murine markers. In 78 mice (93%), this protocol yielded mostly human cells (Fig. 2C). To obtain high yields, we perfused livers from retrorsine preconditioned highly chimeric huFNRG mice based on hAlb serum levels around 104 µg/mL. Liver perfusions of 70 huFNRG mice yielded on average 75 million mpPHH per liver, ranging from 0.4 to 240 million (Fig. 2B). These data show that our isolation/purification protocol results in consistently large numbers of predominantly human hepatocytes isolated from highly chimeric huFNRG mice.

Freshly Isolated mpPHHs Overcome Donor Variability and Form Long-Term Cultures. Cryopreserved PHHs from different donors vary in their ability to engraft in mice and form cultures. We transplanted six cryopreserved PHH donors (PHH2 to PHH7) into FNRG mice without preconditioning and followed repopulation by serum hAlb. Mice transplanted with PHH3 had the highest median hAlb (Fig. 3A). Based on these findings, unless otherwise indicated for subsequent experiments, we used retrorsine preconditioned FNRG mice transplanted with PHH3. Perfusion yielded on average 150 times more mpPHH than input cryopreserved PHH. To rule out that multiple cell divisions in chimeric livers affect mpPHH quality, we compared liver repopulation following adoptive transplantation and hepatocyte stability after plating to cryopreserved PHH (Fig. 3B). To test the repopulation potential as previously shown by Azuma et al. (9), equal numbers of cryopreserved PHH3 or freshly isolated mpPHH3 were transplanted into FNRG mice. mpPHH3 exhibited superior repopulation kinetics compared with cryopreserved PHH based on faster hAlb kinetics, although both groups reached similar peak values (Fig. 3C). We concluded that mpPHH retained high repopulation potential on one round of adoptive transplantation, allowing for expansion of cryopreserved PHH by over 20,000-fold.
To determine how closely mpPHH resembled cryopreserved PHH, we compared the transcriptomes of mpPHH3 suspensions from five humanized livers with two aliquots of cryopreserved PHH3. Both groups had similar expression of genes related to Phase I and Phase II detoxification pathways as well as many other liver-specific genes (SI Appendix, Fig. S3).

We then evaluated if mpPHH could form stable cultures (22) by using hAlb in culture supernatant as a marker for culture stability. Whereas cryopreserved PHH from three donors (PHH2, PHH3, PHH4) formed cultures that showed variable and modest levels of hAlb secretion that decreased over time, passing these donors through mice yielded large numbers of hepatocytes that formed robust cultures with high and stable hAlb over time (Fig. 3D). Next, we characterized the long-term stability of mpPHH cultures. We found that denser mpPHH cultures formed confluent monolayers and were stable for at least 8 wk as shown by hAlb and 4 wk by multiple other read-outs (SI Appendix, Fig. S4). We optimized these conditions for various plating formats (Methods). Because some nonplateable donors engulfed beyond 10^5 µg/mL hAlb levels in retorsine preconditioned mice (SI Appendix, Fig. S5A), we cultured nonplateable PHH8 and plateable PHH2 before and after passing through mice. Whereas plateable PHH2 formed cultures both in vitro and ex vivo, nonplateable PHH8 only attached ex vivo (SI Appendix, Fig. S5B).

Similar to the flow cytometry data on freshly isolated mpPHH (Fig. 2B and C), the vast majority of cells after plating were human hepatocytes as assessed by human CK18 and nuclear mitotic apparatus protein (NuMA) staining (Fig. 3E).

These data show that passing cryopreserved PHH through retorsine preconditioned huFNRG mice yielded large numbers of mpPHH that could be plated, resulting in robust and stable cultures and hence, overcoming marked donor-to-donor variation in plateability of cryopreserved PHH.

Drug Metabolism and Infection Studies in Long-Term mpPHH Cultures.

To exploit the stability of mpPHH cultures, we tested their potential for preclinical drug safety studies and evaluation of drug induction potential. First, we observed that Phase I CYP450 activity is retained in mpPHH cultures as seen by the stability of CYP3A4 levels measured in two different mpPHH donors (mpPHH3 and mpPHH4) for at least 1 mo postplating (Fig. 4A).

Next, we determined whether mpPHHs maintain the ability to induce CYP450 activity after long-term culture by exposing the cells to 25 µM rifampicin, a known inducer of CYP3A4. Rifampicin treatment for 48 and 96 h induced CYP3A4 activity by 5- to 10-fold in a time-dependent manner (Fig. 4B). We then tested if enzyme activity of CYP3A4 could be modulated by RNA interference (RNAi)-mediated knockdown. To this end, mpPHHs were treated with CYP3A4-targeting small interfering RNA (siRNA) or nontargeting control for 24 h, and enzyme activity was monitored for up to 20 d posttreatment. Notably, CYP3A4 activity remained silenced during the long-term culture of mpPHH (Fig. 4C). Finally, to evaluate their use for toxicological studies, we exposed mpPHH to serial dilutions of four known hepatotoxic compounds and plotted their effect on hAlb secretion as a cell injury readout (Fig. 4D). We selected drugs that are metabolized by different enzymes (CYP3A4, CYP2E1, CYP2C9,
and CYP1A2) and observed dose-dependent hepatotoxicity on a 24-h treatment. Taken together, these experiments suggest that mpPHHs are suitable for long-term drug metabolism and drug safety studies.

Next, we tested whether mpPHH cultures could support infection and propagation of Plasmodium falciparum (Pf) and HBV, two prevalent hepatotropic pathogens that exclusively infect human hepatocytes. To determine mpPHH permissiveness to Pf, we used parasite entry assays and staining with Pf-specific antibodies with or without cell permeabilization. This allowed us to distinguish parasites that only bound to the cell surface from those that invaded the cells. On average, 11% of Pf parasites successfully invaded hepatocytes (Fig. 4E). We then monitored parasite growth over time by measuring the intracellular area occupied by individual parasites and found that parasites increased in size from days 2 to 4 postinfection (Fig. 4F). Together, these data showed that mpPHHs support efficient infection and development of Pf parasites.

The chronic phase of HBV infection cannot readily be recapitulated in cell culture due to the short lifespan of current PHH systems. We took advantage of the longevity of mpPHH cultures to monitor HBV infection parameters for up to 8 wk.
Fig. 4. mpPHH cultures are suitable for long-term drug metabolism and infection studies. (A) CYP3A4 activity was stable over time in mpPHH. Freshly isolated mpPHHs derived from two donors (PHH3 and PHH4) were seeded in 96-well plates. CYP3A4 activity was measured from day 15 until day 29 postseeding, and values (mean ± SEM, n = 3) were plotted as percent activity relative to day 15. (B) Rifampicin induces CYP3A4 activity. Cells were cultured in 96-well plates and treated with 25 μM rifampicin for 48 and 96 h on day 11 or 14 postseeding. CYP3A4 activity was measured as in A. Data (mean ± SEM, n = 3) were plotted as fold change relative to untreated samples. A representative of two independent experiments is shown. *P = 0.0457 and **P = 0.0026 for 48 and 96 h, respectively; one-way ANOVA. (C) siRNA silencing of CYP3A4. Cells were cultured in 96-well plates and treated with 50 nM siRNA (either nontargeting control or targeting CYP3A4) for 24 h. Silencing efficiency was determined every 2 to 3 d by measuring CYP3A4 activity as in A. Data (mean ± SEM, n = 3) were plotted as fold change relative to the nontargeting control. P < 0.0001; two-way ANOVA. A representative of three independent experiments is shown. (D) Drug hepatotoxicity in mpPHH cultures. Cells were cultured in 96-well plates and treated with increasing concentrations of drugs for 24 h. The supernatants were harvested, and levels of secreted hAlb were measured by ELISA. Data were normalized to drug-free controls. The graph represents the mean ± SEM of three independent experiments (n = 3). The four drugs are metabolized by different CYP450 enzymes: acetaminophen (yellow), CYP2E1 and CYP3A4; atorvastatin (blue), CYP3A4; diclofenac (red), CYP2C9; and clozapine (purple), CYP1A2. (E) Cells were seeded in 96-well plates and infected 1 wk later with 10,000 Pf sporozoites per well isolated from infected mosquitoes. The percentage of invaded sporozoites at 3 h postinfection was determined with sequential immunofluorescence staining using PfCSP antibodies. Noninvaded sporozoites are detected as yellow and invaded parasites are detected as red after cell permeabilization. Values (mean ± SEM, n = 6) represent one of two independent experiments. (F) Cells infected with 40,000 Pf sporozoites per well were monitored for several days. Media were changed every 2 d, and cells were stained for PfHSP70 (red) after 2 and 4 d postinfection. Parasite intracellular development was monitored by measuring the area occupied by parasites (each dot represents a single invading parasite); 30 to 50 parasites per well were scored (n = 3). Data shown represent one of two independent experiments. (G) Cells were seeded in 96-well plates and infected with 50 GEQ per cell HBV on day 0. Media were changed every 4 d, and HBeAg levels measured and plotted over time from day 5 until day 57 postinfection. Data from three biological replicates are represented as mean ± SEM. PEI, Paul Ehrlich Institute. (H) Cells seeded in 24-well plates were infected with 300 GEO per cell HBV. Media were changed every 2 d, and cells were fixed and stained for HbcAg after 4 wk (HbcAg: red; nuclei: blue).
postinfection. Hepatitis B virus e antigen (HBeAg) was detected in the supernatant of 96-well cultures for 8 wk (Fig. 4G). In addition, at 1 mo postinfection, robust expression of hepatitis B virus core antigen (HBcAg) illustrated widespread infection of mpPHH (Fig. 4H). In summary, mpPHH supported HBV infection for at least 2 mo.

Since mpPHH were amenable to pathogen infections and drug toxicity studies, we adapted the system to a microscale 384-well format suitable for high-throughput screens. As a proof of concept, we infected cells with HBV and treated a fraction of the wells with the HBV entry inhibitor Myrcludex B. Ten days postinfection, naïve but not Myrcludex B-treated cells were efficiently infected with HBV as evidenced by secreted HBeAg and HBcAg expression (SI Appendix, Fig. S6).

These combined data illustrate that the long-term stability of mpPHH expands research into various human hepatocyte functions that cannot accurately be studied in short-term cultures.

**Lentiviral Transduction of mpPHH Generates Transgenic Spheroids and Chimeric Mice.** One of the major limitations of PHH is their resistance to efficient genetic manipulation. This stems in part from widespread observations that two-dimensional (2D) PHH cultures cannot survive mobilization, therefore impairing subsequent applications. Given the stability of mpPHH cultures, we aimed to establish protocols to efficiently transduce hepatocytes with lentiviral vectors using red fluorescent protein (RFP) as proof of concept. In addition, we sought to overcome the much larger hurdle of mobilizing transduced mpPHH and either reculture or

---

**Fig. 5.** Cultured mpPHH can be mobilized and either recultured or retransplanted, creating a modified human graft for in vitro and in vivo applications. (A) Schematic representation of lentivirus transduction of mpPHH and subsequent detachment for reculturing or retransplantation. Transduced cells are shown in red. (B) mpPHH were cultured in 12-well plates, and media were changed every 2 d for 3 wk. Cells were then mobilized and reseeded in new plates. Media were changed every 2 d for an additional week. Representative images from cultures before and after detachment and replating are shown. (C) hAlb levels from supernatants from the initial cell culture (dark yellow) and the replated cells (light yellow) were measured and plotted over time. Data from six biological replicates are represented as mean ± SEM. (D) mpPHHs were transduced with RFP-expressing lentivirus and imaged after 3 d. (E) mpPHHs were cultured in six-well plates and transduced with RFP/puro-expressing lentiviruses. Untransduced or transduced with a G418-expressing lentivirus was used as controls. After 3 d, cells were treated with puromycin for 5 d. Cells were detached and transplanted into FNRG mice. Serum hAlb levels 10 wk after transplantation are displayed for individual mice. *P < 0.05; ****P < 0.0001. (F) mpPHHs were transduced with RFP/puro-expressing lentiviruses and after 3 d, treated with different concentrations of puromycin for 5 d. Cells were detached and transplanted into FNRG mice. Serum hAlb levels rose similarly between groups. Median ± range hAlb levels of groups of 9 to 12 transplanted mice. Three to six mice per group survived until day 132. (G) Mouse livers from E were perfused, and mpPHHs were isolated. These cells were analyzed by flow cytometry to quantify RFP expression. (Magnification: B and D, 20×.)
retransplant these modified hepatocytes to create huFNRG mice with a genetically manipulated graft, as depicted in Fig. 5A.

To this end, lentiviral-infected mpPHH cultures were monitored for 3 wk, after which cells were detached and replated. Between 60 and 80% of mpPHHs survived this process and also, successfully established healthy 2D cultures (Fig. 5B) with similar hAlb levels as before detachment (Fig. 5C). We then defined conditions at which more than 90% of mpPHHs were transduced with RFP-lentiviral vectors (Fig. 5D).

We combined these two technical advances for two applications. In the first case, we attempted to create three-dimensional (3D) spheroid hepatocyte cultures, similar to those we routinely achieve via plating mpPHH in U-bottom 96-well plates. However, we observed that lentiviral RFP transfection of preformed spheroids was consistently inefficient (SI Appendix, Fig. S7A). To overcome this problem, we first transduced mpPHH in 2D, detached the cells, and then created spheroid cultures. Following this protocol, more than 90% of the spheroid cells were RFP positive (SI Appendix, Fig. S7B and C). In addition, by titrating the seeding density, we obtained spheroids of different size (SI Appendix, Fig. S7D). As a second application, we sought to generate liver chimeric mice with a modified human graft. To this goal, we transduced mpPHH with RFP puromycin resistance (RFP-puro-lenti) or G418 resistance-conferring lentiviral vectors. After 5 d of puromycin treatment in culture, mpPHHs were mobilized into single-cell suspensions and transplanted into FNRG mice. Five weeks posttransplantation, we detected hAlb in mice transplanted with RFP-puro-lenti transduced mpPHH regardless of puromycin selection, suggesting that mpPHH tolerated the selection process. As expected, both RFP-G418-lenti transduced and untransduced mpPHH cultures did not survive puromycin treatment as indicated by minimal serum hAlb levels 5 wk after transplantation (Fig. 5E).

To further optimize repopulation of modified mpPHH in FNRG mice, cells were transduced with concentrated RFP-puro-lenti and then treated with 0, 2, and 5 μg/mL puromycin. All mice reached high serum hAlb levels irrespective of puromycin selection (Fig. 5F). Four months after transplantation, immunohistochemistry on chimeric livers showed ~50% human chimerism (SI Appendix, Fig. S8A), consistent with high serum hAlb values. Flow cytometry from perfused livers of these mice showed that the majority of human hepatocytes expressed RFP (Fig. 5G). Moreover, plated mpPHHs from these animals formed stable cultures and maintained RFP expression (SI Appendix, Fig. S8B).

These data show that mpPHH cultures can be efficiently transduced in 2D format, detached, and used to create highly transduced 3D spheroids or humanized mice with robust, persistent, genetically altered grafts.

**Discussion**

The increased demand for physiologically relevant hepatocyte systems led the field to develop methods to extend this limited resource beyond the hepatocytes isolated from human livers. Different approaches are based on in vitro expansion by transducing PHH or by differentiating stem cells to immature HLCs. In parallel, in vivo approaches rely on PHH expansion using liver injury mouse models. We show here that the latter approach efficiently expanded PHH numbers by about 150-fold per round of transplantation and reduced the donor-to-donor variability of cryopreserved PHH in terms of plating efficiency. Retrorsine enhanced this process, particularly for poor donors that otherwise cannot be studied in cell culture. We are unsure of the mechanism by which retorsine improves humanization. In untransplanted FNRG mice cycled off NTBC, retorsine enhanced mouse hepatocyte Ki67 expression after restarting NTBC and modestly increased intrahepatic FGF7 and EGFR protein levels but did not change p21 expression or cause a ductular reaction. These data are consistent with observations in wild-type mice (23) and distinct from those in rats (14), suggesting that mechanisms may differ from the impaired hepatocyte proliferation widely observed in rats.

Freshly isolated mpPHHs reliably formed cultures that could be maintained for months, which contrasts with previously reported short-term cultures (9). The combination of retorsine preconditioned huFNRG mice and Percoll enrichment resulted in mpPHH cultures composed of mostly human hepatocytes. Since we did not magnetically deplete mouse cells (21), the long-term stability may, however, depend on a small fraction of undefined mouse stromal support cells. These cultures advance research across a wide spectrum of human hepatocyte biology. For example, following HBV infection and spread for 8 wk allows for studies into the molecular mechanisms by which HBV establishes chronicity. A second application is the adaptation to a 384-well microscale mpPHH format. This will facilitate high-throughput genetic and compound screens in human hepatocytes. Additionally, because yields from chimeric mice are high and mpPHH cultures are robust, plated cells were easily shipped to collaborators who then conducted drug metabolism and *Plasmodium* infection studies. This illustrates that mpPHH can be widely used by the research community without the need to set up logistically complex chimeric mouse colonies.

The most significant advance of our systems is the ability to efficiently genetically manipulate mpPHH. Although lentiviral manipulation of hepatocytes prior to transplantation has been reported, transduction remained very inefficient (24, 25) (in our hands, 5 to 10% infected). One of the reasons for this was generally believed to resist further applications, such as expansion in chimeric mice. Our systems to transduce mpPHH, select transduced cells with puromycin, mobilize these cells, and then either create spheroid cultures or repopulate huFNRG mice to high chimerism have many implications for the study of hepatocyte biology. It allows for the efficient modifications of mpPHH in vitro by either overexpressing or knocking down genes of interest. These modified mpPHHs can then be expanded in mice for in vivo studies, or chimeric mice can be subjected to manipulations, such as infections or diets, and their mpPHHs can subsequently be harvested for in vitro applications. This versatile system will allow for disease-specific genetic manipulations linked to liver physiology and hepatocyte-specific interactions. It will allow for the creation of disease-specific human hepatocyte systems and will advance the long-term goal of therapeutic hepatocyte transplantation for human liver conditions.

**Methods**

**Cells.** Cryopreserved PHHs were purchased from Triangle Research Labs and Lonza or were isolated from surgical specimens and cryopreserved in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% dimethyl sulfoxide (DMSO) and 20% fetal bovine serum (FBS) (11). Human fetal hepatoblasts were isolated from fetal liver tissue (Advanced Bioscience Resources, Inc.) as described previously (13, 26). Briefly, fetal livers were incubated with collagenase containing digestion buffer, and large cells were enriched by density centrifugation before cryopreservation. The use of human materials was approved by the Rockefeller University Institutional Review Board; however, was exempted of informed consent under category 4.

**Mice.** F.ahu−/− mice (27) were provided by Markus Grompe, Oregon Health and Science University, Portland, OR and crossed to NOD Rag1−/− Il2rg−/− mice (Jackson Labs) to derive FNRG mice as previously described (11). After the first NTBC withdrawal cycle, we observed 67.3 ± 17.1% survival in FNRG females and 1.8 ± 3.6% survival in FNRG males in our facility. Therefore, only FNRG females were used for these experiments. Thymidine kinase transgenic mice on the NOD SCID IL2rg−/− background (TK-NOG) (19) were obtained from Taconic Biosciences. Only males were used since female TK-NOG mice do not support high humanization (19). Immunodeficient SCID mice hemizygous for the uPA transgene were bred at Ghent University as previously described (20). Male uPA−/− mice were used, although both genders support humanization. FNRG mice were maintained on an ad libitum chow diet with aminosilicon and drinking water containing 16 mg/mL NTBC (Yecuris). TK-NOG and uPA mice were maintained on regular chow. Mice were housed under a 12-h light cycle.
from 7:00 AM (ZT0) to 7:00 PM (ZT12). All experiments were conducted under animal use protocols approved by Rockefeller University and Ghent University.

**Mouse Preconditioning.** Retrorsine (catalog no. R0382; Sigma Aldrich) was dissolved at 20 mg/mL in 100% ethanol at 56 °C and stored in aliquots at −20 °C. For intraperitoneal injections, 100 μL (2 mg) retorsine or 100 μL ethanol vehicle was diluted with 400 μL phosphate buffered saline (PBS) without calcium or magnesium (PBS−/−). For Fad−/− and TK-NOG experiments, retorsine was administered at 2-wk intervals, typically the first dose at 3 to 4 wk prior to transplantation and a second dose 2 to 3 wk prior to transplantation. In uPA−/− mice, retorsine was injected when 12 to 13 d before birth at 70 mg/kg. For circadian experiments, ZT injections were administered between 7:00 AM and 8:00 AM, and ZT12 injections were between 7:00 PM and 8:00 PM. For hOSM, supplementation of an AAV8-hOSM was created by cloning hOSM into pAAV-BC vector expressing hOSM driven by the cytomegalovirus enhanced chicken β-actin promoter. Seventy-two hours after cotransfetion this construct or a control vector expressing firefly luciferase with the pDB8 helper plasmid into HEK293 cells, virus was purified after pelleting using an iodixanol density gradient protocol. Titers were determined by dot blot hybridization as described previously (29). Mice received the AAV8-HOM5 vector through tail vein injections 7 to 10 d prior to transplantation of human fetal hepatoblasts.

**Transplantations.** Female FNRG recipients were withdrawn from NTBC 4 to 5 d prior to surgery. Male TK-NOG mice 7 wk of age received 48 h of 0.1 mg/kg valganciclovir (Sigma) and 20 mg/kg sodium bicarbonate (Sigma; in drinking water) 14 d prior to transplantation. In uPA−/− mice, retorsine was injected when 12 to 13 d before birth at 70 mg/kg. For circadian experiments, ZT injections were administered between 7:00 AM and 8:00 AM, and ZT12 injections were between 7:00 PM and 8:00 PM. For hOSM, supplementation of an AAV8-hOSM was created by cloning hOSM into pAAV-BC vector expressing hOSM driven by the cytomegalovirus enhanced chicken β-actin promoter. Seventy-two hours after cotransfetion this construct or a control vector expressing firefly luciferase with the pDB8 helper plasmid into HEK293 cells, virus was purified after pelleting using an iodixanol density gradient protocol. Titers were determined by dot blot hybridization as described previously (29). Mice received the AAV8-HOM5 vector through tail vein injections 7 to 10 d prior to transplantation of human fetal hepatoblasts. 

**Hepatocyte Plating and Maintenance.** Once the cells (mpPHH) were isolated and purified with two rounds of Percoll, they were freshly seeded on collagen-coated plates (BD Biosciences) in W10 plating medium. The cells were equally distributed by shaking on a flat surface and then, left on the bench at room temperature for 45 min. Once the cells settled and evenly distributed, they were transferred to a humidified 37 °C incubator. Cryopreserved PHHs were first thawed at 37 °C and then, transferred to 50 mL W10. After centrifugation at 50 × g, the cells were resuspended in W10, counted, and seeded on plates as described for mpPHH. For each plating format, we optimized the seeding density to achieve confluent cultures. To avoid concentrating cells in the middle of the well, we seeded the cells with excess medium. The next day, the cells were washed once with WEM to remove any cell debris and serum. For maintenance medium, we used hepatocyte-defined medium (HDM; catalog no. 05449; Corning) supplemented with 1% penicillin/streptomycin, 1% 200 mM -l-glutamine, 0.1% 50 mg/mL Gentamicin, and 2% DMSO (catalog no. 4-x-5; ATCC). Unless otherwise noted, our experiments were conducted in 96-well plates normalized to different formats with seeding densities and medium volumes.

**Lentiviruses Transduction.** Confluent mpPHH cultures were transduced with RFP-expressing lentiviral pseudoparticles (lentiviral vector SCRPSY; expressing red fluorescent protein [SCRPSY-RFP]) in the presence of 4 μg/mL polybrene by spinoculation for 1 h at 1,000 × g at 37 °C. Three days posttransduction, RFP expression reached maximum levels and remained stable over time.

**Mobilization of mpPHH for Replating and Retransplantation.** Untransduced or RFP-transduced mpPHH cultures were maintained from days to weeks before a detachment protocol was applied. Specifically, after multiple washes with WEM, the cultures were incubated for 30 min with HBSS (catalog no. 14175-095; Life Technologies) with 10-min interval washes to loosen hepatocyte tight junctions. Then, the cells were incubated with TrypLE Select (10x; catalog no. 10131-017-01; Life Technologies). After 4 to 5 min of TrypLE wash, the cell suspension was passed through a 70-μm cell strainer, and the cell suspension (50 mL) was spun at 50 × g for 5 min at 4 °C using an Allegra X-14R Centrifuge (Beckman Coulter). The supernatant was gently aspirated, and the cells were washed once with PBS−/−. The cell pellet was resuspended in 10 mL PBS−/− and gently mixed with equal volume of Percoll working solution (catalog no. 17–0891-01; GE Healthcare). Percoll working solution consisted of 5.4% 10x PBS−/−, 48% Williams’ E medium (catalog no. 12551-03; Life Technologies). The cell suspension was spun at 100 × g for 5 min at 4 °C, and the pellet was washed once with PBS. After centrifugation at 50 × g, the cells were resuspended in PBS and left on ice for 45 min. Then, a second round of Percoll was used to further purify the cell suspension. The final pellet was resuspended in W10 plating medium (Williams’ E medium [WEM] supplemented with 10% FBS, 1% penicillin/streptomycin [catalog no. 15140-12; Life Technologies], 1% 200 mM -l-glutamine [catalog no. 25330-081; Life Technologies], 0.1% 50 mg/mL Gentamicin reagent solution [catalog no. 15750-060; Life Technologies], and 0.1% Corning ITS premix [catalog no. 354350; Corning]), and viable cells were counted using trypan blue. Viability was usually above 95% without clumps. If clumps were present, the cell suspension was passed through a 40-μm filter. For adoptive transplant into new FNRG recipient mice, cells were spun at 50 × g for 5 min at 4 °C and resuspended in cold PBS. For each mouse, 65 μL of cell suspension of 0.5 to 1 million cells was injected. For in vitro applications, the cells were kept on ice in W10 plating medium until seeded in various formats.

**Spheroid 3D Culture Formation.** Confluent mpPHH cultures were transduced with RFP-expressing lentiviral SVG-V pseudoparticles (SCRPSY-RFP) as described above. Ten days later, the cells were detached by brief treatment with TrypLE, washed, and plated in 100 μL W10 plating medium in Ultra-Low Attachment U-bottom 96-well plates (catalog no. 7007; Costar). Plates were centrifuged at 40 × g for 5 min to promote aggregate formation. Spheroid aggregates formed between 1 and 3 d after plating, at which time the cultures were switched to serum-free medium. Spheroids of different size were formed depending on the indicated number of plated cells after 10 d in culture. Spheroid images were acquired on an inverted Zeiss Axiovert 200 microscope fitted with a Perkin-Elmer UltraView spinning disk and Andor iXon 512 × 512 electron multiplying CCD camera; 100 image slices were acquired with 3-μm steps in both the bright-field and fluorescent channels using either transmitted light or 561-nm laser excitation (Spectral Applied) and a 10x Plan-Apochromat 0.45 numerical aperture objective. Image analysis and editing were performed using ImageJ.

<table>
<thead>
<tr>
<th>Table 1. mpPHH seeding density in various plating formats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plate format</strong></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>384 well</td>
</tr>
<tr>
<td>96 well</td>
</tr>
<tr>
<td>24 well</td>
</tr>
<tr>
<td>12 well</td>
</tr>
<tr>
<td>6 well</td>
</tr>
</tbody>
</table>

**Drug Treatments.** Acetaminophen (catalog no. A7085; Sigma), diclofenac (catalog no. D6899; Sigma), clozapine (catalog no. C6305; Michaelidis et al. 1666 | www.pnas.org/cgi/doi/10.1073/pnas.1919035117 Michailidis et al.
HBV Infection and Detection. HBV stock was prepared as previously described (30). Briefly, supernatants from confluent HepDE19 cells (31) (HBV-producing cells) were harvested every 2 d for 3 wk and concentrated using Centricon Plus-70 centrifugal filter devices (Millipore-Sigma). The concentrated stock was aliquoted and maintained at −80 °C. The stock concentration was determined as HBV genome equivalents (GEQ) per milliliter using a TaqMan-based qPCR assay. For HBV infections, 4 to 5 d after mpPHH seeding the cells were infected with the indicated concentration of HBV in hepatocyte maintenance medium (HDM with 2% DMSO) supplemented with 4% polyethylene glycol 8000 (catalog no. 81268–250G; Sigma). The plates were spinoculated for 1 h at 1,000 × g at 37 °C. After 24 h, the cells were washed 5x with MEM, and maintenance medium was added. For secreted protein analysis, stable transfectants (see below) were spinoculated 30 min after infection with the indicated concentration of HBV entry inhibitor (Clone 8B17R) (32) (HBV entry inhibitor) was incubated with the inoculum and washed out the next day. Secreted HBeAg was measured from culture supernatants every 4 d by loading 50 μl into 96-well plates of a chemiluminescence immunoassay kit according to the manufacturer’s instructions (DiaSino Laboratories Co.). A FLUOstar Omega luminometer was used to read the plates. HBeAg concentrations are expressed in Paul Ehrlich Institute units per milliliter. Hepatocyte cultures were fixed in 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and permeabilized with 0.1% Triton X-100 for 10 min. After extensive washing, cells were incubated for 1 h at room temperature with blocking solution 5% goat serum in PBS (catalog no. 005–000–121; Jackson ImmunoResearch). A rabbit polyclonal anti-HBV core antibody (catalog no. HBP–023–9; Austral Biologicals) was added to the cells at 1:500 dilution in blocking solution and incubated for 1 h at room temperature. A goat anti-rabbit AlexaFluor 594 (catalog no. A–11012; Life Technologies) at a dilution of 1:1,000 was used as a secondary antibody. Nuclei were stained with DAPI. Cells were imaged using a Nikon Eclipse TE300 fluorescent microscope and processed using ImageJ.

RNA Extraction and Library Preparation. Total RNA was isolated using TRIzol (catalog no. 15596026; Thermo Fisher) followed by cleaning with RNeasy kit (catalog no. 7410; Qiagen) coupled with on-column DNase I treatment (catalog no. 79254; Qiagen); 250 ng of the DNase-treated RNA was used to generate strand-specific sequencing libraries using Truseq Stranded mRNA Library Prep (catalog no. 20020594; Illumina). Multiplexed libraries were sequenced as 50-nt single-end reads on a HiSeq2500 system (Illumina) at the Rockefeller University Genomics core facility.

RNA-sequencing Bioinformatics Pipeline. Transcript alignment and quantification were done using Kallisto (33). Transcript indices for Kallisto were generated from Ensembl release hg38 and included all annotated complementary DNA and noncoding RNA transcripts. Differential expression analysis was then done using Sleuth (34). Gene names and attributes were queried from Ensembl using biomart. For genes containing multiple transcripts, we chose the transcript with the highest mean expression across all samples as the representative example for that gene. Heat maps were generated in R Studio.

Statistical Analyses. Groups were compared by the Student t test. Correlations were calculated using the Spearman correlation coefficient. All statistical analyses were done using Prism 8 software (Graphpad).

Data Availability. Datasets supporting the findings of this study have been deposited in a publicly available database (Gene Expression Omnibus accession no. GSE130219).

ACKNOWLEDGMENTS. This work was supported by NIH Fellowship F32DK107164 (to E.M.); fellowships from the German National Academic Foundation and the German Center for Infection Research (to P.P.); and NIH Grants R01HL131093 (to R.W.H. and Y.P.d.J.), R01AI091707 (to C.M.R.), R01DK085713 (to C.M.R.), and K08DK090576 and 1R01AA027327 (to Y.P.d.J.). K.V. was supported by a Fellowship of the Belgian American Educational Foundation. Further support for this project was provided by grants from the Robertson Foundation (to E.M.). The project was cosponsored by the Center for Basic and Translational Research on Disorders of the Digestive System through the generous support of M. and Harry A. Silverman Charitable trust (to E.M.). This work was supported in part by Bill & Melinda Gates Foundation Grant OPP1023607 (to S.N.B.), Koch Institute Support Grant P30-CA14051 (to S.N.B.) from the National Cancer Institute, and Bloomberg Philanthropies (S.N.B.), which supports the insectary and parasitology core facilities at the Johns Hopkins Bloomberg School of Public Health. S.N.B. is a Howard Hughes Medical Institute Investigator. We thank Dr. Stephan Urban (Heidelberg University) for providing Myrcludex B and members of our laboratories, including Heather Fleming, for helpful suggestions on the work and editing of the manuscript. We also thank the Rockefeller University High-Throughput, Bioimaging, Flow Cytometry and Genomics Resource Centers. The following reagent was obtained through BEI Resources, National Institute of Allergy and Infectious Diseases, NIH: Monoclonal Antibody 2A10 Anti-Plasmodium falciaparum Circumsporozoite Protein (produced in vitro), MRA-183A, contributed by Elizabeth Nardin. We thank Julie Vercauteren for graphical assistance.