Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures

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

Results

Hepatitis C virus (HCV) remains a major public health problem, affecting approximately 130 million people worldwide. HCV infection can lead to cirrhosis, hepatocellular carcinoma, and end-stage liver disease, as well as extrahepatic complications such as cryoglobulinemia and lymphoma. Preventative and therapeutic options are severely limited; there is no HCV vaccine available, and 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 coclutivation with nonparenchymal cell types (6, 12, 13, 14). Although some of these models provide necessary extracellular matrix cues, they lack crucial heterotypic 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.

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 heterotypic 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.

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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 *Gaussian* 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...
producing infectious virions, mine whether primary hepatocytes in MPCCs are capable of (C508Y) RFP-NLS-IPS HCV reporter. Twenty-four hours after transduction, were transduced with lentiviruses expressing wild-type (wt) or mutant infection. Scale bar: 20 images are shown; white arrow heads show nuclear RFP, indicative of HCV NLS, from the mitochondria to the nucleus. This redistribution of NS3-4A results in translocation of the cleavage product, RFP-β substrate was stably expressed in primary hepatocyte MPCCs substrate for the HCV NS3-4A protease. The RFP-NLS-IPS Ploss et al. PNAS - Primary Hepatocytes in MPCCs Produce Infectious Virus. Supporting MPCCs harboring a cleavage-resistant reporter (C508Y) or in infected MPCC hepatocytes transduced with wild-type RFP-α C-terminal mitochondrial-targeting domain (IPS) derived from protein (RFP), an SV40 nuclear localization sequence (NLS), and fluorescence images are shown; white arrow heads show nuclear RFP, indicative of HCV infection. Scale bar: 20 μm.

Fig. 2. Primary human hepatocyte MPCCs are susceptible to HCV. (A) Persistent infection of primary human hepatocytes with HCVcc. Primary hepatocytes in MPCC were infected with Jc1FLAG2(p7-nsGluc2A). After 24 h, virus was removed and MPCC medium containing DMSO (0.1%) or the indicated inhibitors was added. All inhibitors were used at approximately 50x IC50 (polymerase inhibitor 2’CMA = 2.16 mM, protease inhibitor ITMN191 = 0.16 mM, IFN-α = 500 U/mL). Samples were taken daily and the media replaced with washing every 48 h. Accumulated luciferase activity in the supernatants is plotted. Arrows indicate the addition of fresh inhibitor. (B) Visualization of HCV infection in primary human hepatocytes. MPCCs were transduced with lentiviruses expressing wild-type (wt) or mutant (C508Y) RFP-NLS-IPS HCV reporter. Twenty-four hours after transduction, MPCCs were infected with Jc1FLAG2(p7-nsGluc2A) or plasma from HCV-infected patients in the presence of heparin (5 IU), CaCl2 (9 mM), and MgCl2 (6 mM). Twelve hours following infection, virus was removed and MPCC medium was added. Unfixed MPCCs were imaged by wide-field fluorescence microscopy at 48 h postinfection. Representative pseudocolored fluorescent images are shown; white arrow heads show nuclear RFP, indicative of HCV infection. Scale bar: 20 μm.

to recent reports (10), we were unable to obtain specific signals above background, reminiscent of failed attempts to detect HCV proteins in infected liver biopsies - probably due to the low number of HCV RNA copies per cell (21).

To demonstrate that HCV actively replicates in the primary hepatocyte component of the MPCCs, we made use of a recently developed fluorescence-based live cell reporter (22). This system uses a reporter (RFP-NLS-IPS) composed of a red fluorescent protein (RFP), an SV40 nuclear localization sequence (NLS), and a C-terminal mitochondrial-targeting domain (IPS) derived from the IFN-β promoter stimulator 1 protein (IPS-1), a known cellular substrate for the HCV NS3-4A protease. The RFP-NLS-IPS substrate was stably expressed in primary hepatocyte MPCCs (Fig. 2B). In HCV-infected cells, RFP-NLS-IPS processing by NS3-4A results in translocation of the cleavage product, RFP-NLS, from the mitochondria to the nucleus. This redistribution of fluorescence was detected in approximately 1–5% of HCVcc-infected MPCC hepatocytes transduced with wild-type RFP-NLS-IPS; no relocation was detected after infection of MPCCs harboring a cleavage-resistant reporter (C508Y) or in supporting fibroblasts (Fig. 2B). At very low frequency (approximately 1 in 30,000 cells), we also detected RFP-NLS-IPS cleavage in MPCC cultures infected with plasma or sera from HCV-infected patients (Fig. 2B).

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 CD81 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-CD81 (JS-81) blocked HCVcc entry very efficiently (IC50 < 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, canalicul 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 pre-treated 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 hepatocytes in MPCCs are stable for several weeks and therefore allow monitoring of human hepatotropic 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
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 for 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: Celcis In vitro Technologies, BD-Gentest and CellDirect. Human hepatocytes were pelleted by centrifugation at 50–100 × g 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, M.I.T, 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) multi-well 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 (∼3×10⁶ adherent hepatocytes in 96 collagen-coated islands in 24-well plate and ∼4.5×10⁶ hepatocytes in 14 islands in 96-well plate) and incubated in hepatocyte medium...
were detected using Hoechst dye (500 ng/mL in PBS, Invitrogen). Images
(1:1000) for immuno
anti-NS5A (9E10 (23), 1:2000). Secondary antibodies were goat-anti-mouse
mouse anti-human CD81 (clone JS-81, BD Pharmingen; 1:200), rabbit anti-
X-100 in PBS, cells were incubated in primary antibody overnight at 4 °C:
lowing washing and blocking in 1% BSA/0.2% milk or 1% BSA/0.3% Triton
were generated using H77 E1E2 (residues 170–207) which, Matrigel overlay, Matrigel substratum, and randomly distributed co-
cultures of hepatocytes and murine fibroblasts were created as described
previously (12).

Pseudoparticle Generation and Infection Assays. Pseudoparticles were
generated by cotransfection of plasmids encoding (1) an EGFP-encoding provirus (CSGW) or provirus encoding transgene (pTRIP) (2) HIV gag-gag-pol and (3) envelope glycoprotein(s), as previously described (17). HCVpp were
generated using H77 E1E2 (residues 170–207) which, Matrigel overlay, Matrigel substratum, and randomly distributed co-
cultures of hepatocytes and murine fibroblasts were created as described
previously (12).

Antibodies, Immunostaining, and Blocking. For immunostaining, cells or tissue
sections were fixed in 1% paraformaldehyde and/or –20 °C methanol.
ollowing washing and blocking in 1% BSA/0.2% milk or 1% BSA/0.3% Triton
were generated using H77 E1E2 (residues 170–207) which, Matrigel overlay, Matrigel substratum, and randomly distributed co-
cultures of hepatocytes and murine fibroblasts were created as described
previously (12).

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Supporting Information

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Fig. S1. HCV entry factor staining in normal human liver. Wide-field fluorescence images of fixed sections of human liver from normal uninfected donors stained nuclei (blue in merged image) and antigen-specific staining (green in merged image) for CD81 (Upper), SCARB1 (Middle) and CLDN1 (Lower). Scale bars: 30 μm.

Fig. S2. Primary hepatocytes in MPCCs maintain HCVcc infection over longer periods of time than conventional hepatocyte systems. Conventional pure hepatocyte cultures, widely used in the pharmaceutical industry, and MPCCs were created from the same donors. Conventional cultures were infected with HCVcc within 24 h of plating, whereas MPCCs were infected once they achieved functional stability (6 days after plating). Luciferase activity in supernatants was monitored over 2 weeks postinfection. One representative time point (6-12 days postinfection) is shown. Luciferase activity is expressed as percent of mock control.

Fig. S3. Miniaturized 96-well primary hepatocyte MPCCs. (A) MPCCs were created in off-the-shelf tissue culture polystyrene plates in formats up to 96-well plates using soft lithographic techniques. (B) Each well of a 96-well plate contains 14–15 islands of hepatocytes that are 500 μm in diameter and spaced 1200 μm apart (center-to-center), and (C) surrounded by 3T3-J2 murine embryonic fibroblasts to create MPCCs. Scale bars: 2 cm (a), 4 mm (b), 100 μm (c).
Fig. S4. Primary human hepatocytes in MPCCs produce infectious virus. (A) HCV infection kinetics in primary hepatocyte MPCCs. Primary hepatocytes in MPCCs were infected with Jc1FLAG2(p7-nsGluc2A) (circles) or mock infected (triangles). After 24 h, virus was removed and MPCC medium added; samples were taken daily and media replaced with washing three times every 48 h. (B) Schematic of the experimental set up. Supernatants collected pre- and postwash at days 4, 6, 8, 10, and 12 following infection were used to infect naïve Huh-7.5 cells. Twenty-four hours postinfection, media were replaced and NS5A staining was performed 72 h postinfection to visualize HCV infection. (C) HCV infection of Huh-7.5 cells was visualized by immunohistochemical staining for NS5A. Days indicate the time points when supernatants were taken from the infected MPCC cultures.