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*Persistent hepatitis C virus infection in
microscale primary human hepatocyte cultures*

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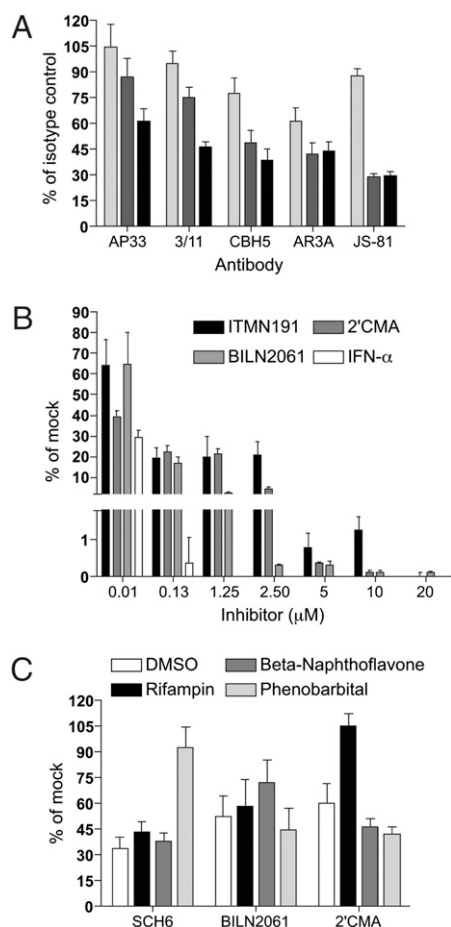


Fig. 3. Utility of primary human hepatocyte MPCCs in antibody and small molecule screening. (A) Dose-dependent inhibition of HCVcc replication in MPCCs treated with antibodies against HCV glycoproteins (AP33, 3/11, CBH5, AR3A) or cellular CD81 (JS-81). Antibody concentrations are 0.1 (light gray), 1 (dark gray), and 10 (black) μg/mL. (B) Dose-dependent inhibition of HCVcc replication in MPCCs treated with IFN-α (up to 0.13 μM) or small molecules (NS3-4A protease inhibitors, BILN2061 and ITMN191, or polymerase inhibitor, 2'CMA). HCVcc-infected MPCCs were pulse-treated for 2 days with compounds and supernatants were collected at days 2 and 4 (shown) post-inhibitor treatment. (C) Drug-drug interactions lead to reduced efficacy of small molecules in HCVcc-infected MPCCs. Infected MPCCs were treated for 3 days with prototypical inducers of drug metabolism enzymes (12, 32), followed by treatment of cultures with small molecules for 2 days. In all experiments, HCVcc replication was monitored by luciferase secretion into the supernatants. Mean and standard error of the mean are shown.

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 inter-

fering 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 \times g for 5–10 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 Weill 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-) multi-well plates, coated homogeneously 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 \times 10⁴ adherent hepatocytes in 96 collagen-coated islands in 24-well plate and ~4.5 \times 10³ hepatocytes in 14 islands in 96-well plate) and incubated in hepatocyte medium

overnight. Hepatocyte culture medium was DMEM with high glucose, 10% FBS, 0.5 U/ml insulin, 7 ng/ml glucagon, 7.5 μ g/ml hydrocortisone and 1% penicillin-streptomycin. 3T3-J2 murine embryonic fibroblasts were seeded (9×10^4 cells in each well of 24-well plate and 1.4×10^4 cells in each well of 96-well plate) in fibroblast medium (DMEM with high glucose, 10% bovine serum and 1% penicillin-streptomycin) 12–24 h later. Fibroblast-to-hepatocyte ratio was estimated to be 4:1, once the fibroblasts reached confluency. Fibroblast culture medium was replaced with hepatocyte culture medium 24 h after fibroblast seeding and subsequently replaced daily. Control conventionally-plated pure hepatocyte cultures (Collagen gel sandwich, Matrigel overlay, Matrigel substratum, and randomly distributed cocultures 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-pol and (3) envelope glycoprotein(s), as previously described (17). HCVpp were generated using H77 E1E2 (residues 170–746).

Antibodies, Immunostaining, and Blocking. For immunostaining, cells or tissue sections were fixed in 1% paraformaldehyde and/or -20°C methanol. Following washing and blocking in 1% BSA/0.2% milk or 1% BSA/0.3% Triton X-100 in PBS, cells were incubated in primary antibody overnight at 4°C : mouse anti-human CD81 (clone JS-81, BD Pharmingen; 1:200), rabbit anti-SCARB1 (NB110-57591, Novus Biologicals; 1:100), rabbit anti-CLDN1 (51-9000, Zymed; 1:200), rabbit anti-ZO1 (61-7300, Zymed; 1:200), mouse anti-OCLN (33-1500, Zymed; 1:200), mouse anti-MRP2 (Clone M2III-6, Kamiya Biomedical; 1:50), mouse anti-EEA1 (clone 14, BD Biosciences; 1:100), mouse anti-NS5A (9E10 (23), 1:2000). Secondary antibodies were goat-anti-mouse or goat-anti-rabbit AlexaFluor488- or AlexaFluor594-conjugates (Invitrogen; 1:1000) for immunofluorescence, or goat-anti-mouse HRP (ImmPress kit, Vector Labs) with DAB+ substrate (Dako) for immunohistochemistry. Nuclei were detected using Hoechst dye (500 ng/mL in PBS, Invitrogen). Images were captured on a Nikon inverted microscope using SPOT image analysis

software. Confocal images were captured on a Zeiss LSM 510 inverted microscope at $0.3\ \mu\text{m}$ optical slices using Zeiss software (v3.2). Z-stack files were uploaded into ImageJ64 software with images generated using a “Sum Slices” projection and 3D renderings were done using Imaris software. Final images were assembled using Adobe Photoshop CS3 software. Blocking experiments used human anti-SCARB1 antibody C167 (24), anti-CD81 (clone JS-81; BD Pharmingen), anti-E2 3/11 (31), and antibodies kindly provided by A.H. Patel (University of Glasgow, Scotland) (AP33) (26), S.K. Fong (Stanford University, Palo Alto, CA) (CBH5) (27), and D.R. Burton (The Scripps Research Institute, La Jolla, CA) (AR3A) (28). Human IgG1 (clone MOPC-21), IgG4 (MOR6391), and rat IgG2a (MCA1124R) isotype control antibodies were purchased from AbD Serotec.

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Supporting Information

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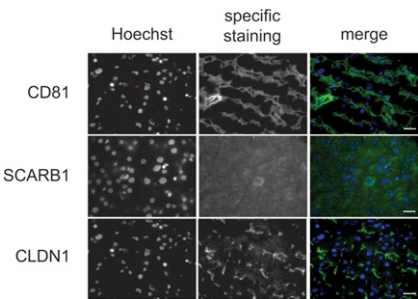


Fig. S1. HCV entry factor staining in normal human liver. Wide-field fluorescence images of fixed sections of human liver from normal uninfected donors stained nuclei (blue in merged image) and antigen-specific staining (green in merged image) for CD81 (*Upper*), SCARB1 (*Middle*) and CLDN1 (*Lower*).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

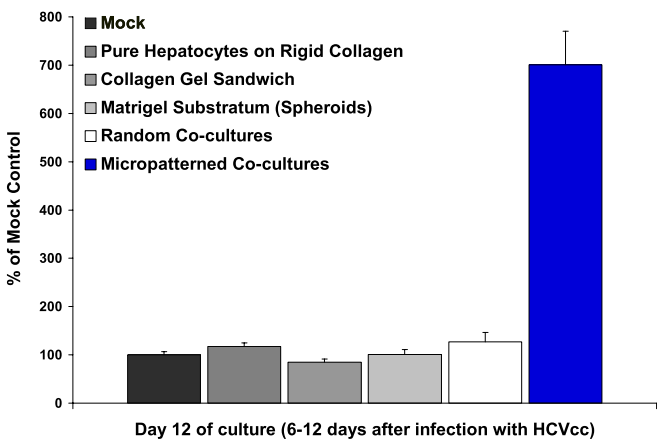


Fig. S2. Primary hepatocytes in MPCCs maintain HCVcc infection over longer periods of time than conventional hepatocyte systems. Conventional pure hepatocyte cultures, widely used in the pharmaceutical industry, and MPCCs were created from the same donors. Conventional cultures were infected with HCVcc 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.

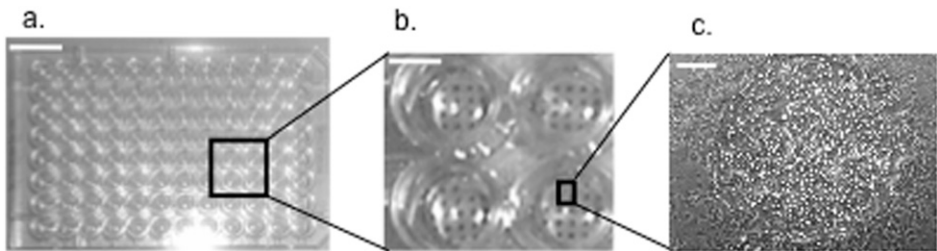


Fig. S3. Miniaturized 96-well primary hepatocyte MPCCs. (A) MPCCs were created in off-the-shelf tissue culture polystyrene plates in formats up to 96-well plates using soft lithographic techniques. (B) Each well of a 96-well plate contains 14–15 islands of hepatocytes that are 500 μ m in diameter and spaced 1200 μ m 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 μ m (c).

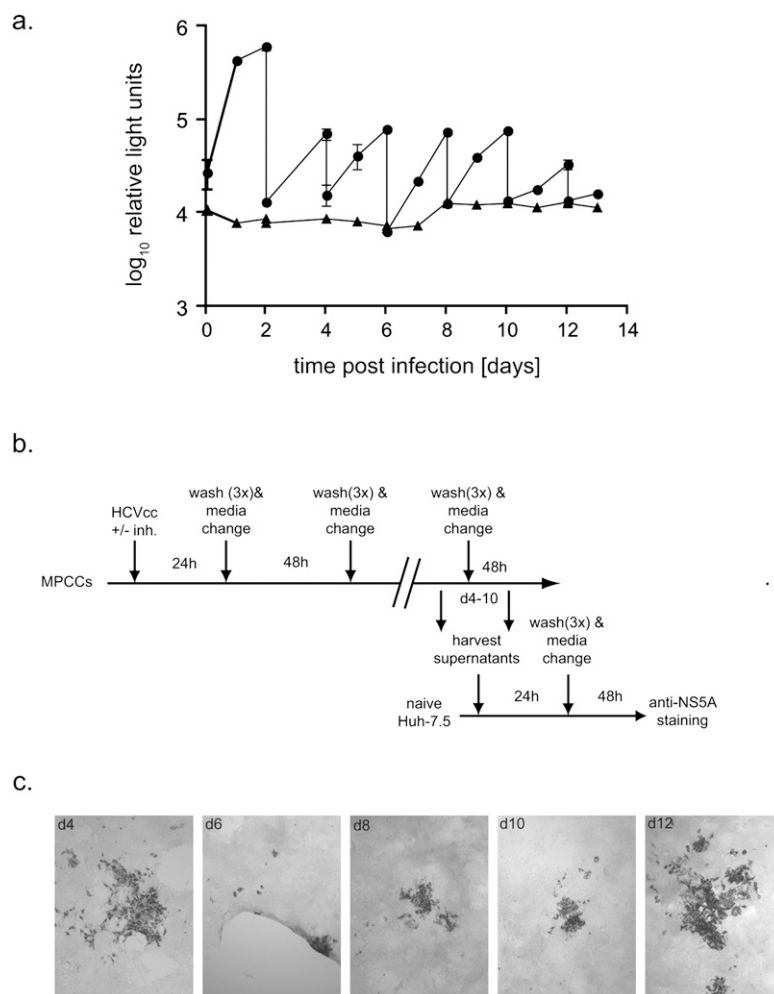


Fig. S4. Primary human hepatocytes in MPCCs produce infectious virus. (A) HCVcc infection kinetics in primary hepatocyte MPCCs. Primary hepatocytes in MPCCs were infected with Jc1FLAG2(p7-nsgluc2A) (circles) or mock infection (triangles). After 24 h, virus was removed and MPCC medium added; samples were taken daily and media replaced with fresh growth media 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.