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Citation

As Published
http://dx.doi.org/10.1073/pnas.0915130107

Publisher
National Academy of Sciences (U.S.)

Version
Final published version

Accessed
Sat Oct 27 04:44:58 EDT 2018

Citable Link
http://hdl.handle.net/1721.1/86110

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Persistent hepatitis C virus infection in microscale primary human hepatocyte cultures

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Contributed by Charles M. Rice, January 3, 2010 (sent for review December 16, 2009)

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 nonspecific, IFN-based treatments are frequently ineffective. Development of targeted antivirals has been hampered by the lack of robust HCV cell culture systems that reliably predict human responses. Here, we show the entire HCV life cycle recapitulated in micropatterned cocultures (MPCCs) of primary human hepatocytes and supportive stroma in a multiwell format. MPCCs form polarized cell layers expressing all known HCV entry factors and sustain viral replication for several weeks. When coupled with highly sensitive fluorescence- and luminescence-based reporter systems, MPCCs have potential as a high-throughput platform for simultaneous assessment of in vitro efficacy and toxicity profiles of anti-HCV therapeutics.

Hepatitis C virus in primary human hepatocyte cultures

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). 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.

Results

Primary Human Hepatocytes in Micropatterned Cocultures Form Polarized Cell Layers and Support HCV Glycoprotein-Mediated Entry. We have recently developed a miniaturized, multiwell 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, MPCCs; 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 (OCCLN) (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, MRP2, and nuclear staining (Fig. 1I and J). 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. 1 and Fig. S1).

To test whether MPC hepatocytes can support HCV glycoprotein-mediated entry, we infected cultures with HCV pseudoparticles (HCVpseud). HCVpseud, 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 murine embryonic fibroblasts (3T3-J2), could be infected with HCVpseud (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 HuVcc 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 (2'CMA), 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

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Fig. 1. Primary human hepatocytes in MPCCs form polarized cell layers, express HCV entry factors, and support HCV glycoprotein-mediated entry. Bright field images of primary hepatocytes in MPCCs (A) and in monocultures (B). Wide field fluorescence images of fixed MPCCs stained for the canalicular marker MRP2 (C), and the basolateral marker CD26 (D). Nuclear (blue) and antigen-specific staining (green) for CD81 (E), SCARB1 (near edge of hepatocyte island) (F), CLDN1 (red) (G), OCLN (H) in MPCCs. I) Merged image of primary hepatocytes stained for MRP2 (green), ZO1 (red), and nuclei (blue). J) 3D rendering of boxed area in I. K) Infection of MPCCs with retroviral pseudoparticles bearing HCV glycoproteins (HCVpp), vesicular stomatitis virus glycoprotein (VSVGpp), or no glycoproteins (Envpp) and containing an EGFP reporter gene. Representative images are shown for all experiments. L) Anti-CD81 antibody blocks entry of HCVpp (dark bars), but not VSVGpp (white bars). Concentrations of antibody (μg/mL) are noted. Mean and SD are shown. Scale bars: 100 μm (a, b, k), 50 μm (c, d), 20 μm (e-i).
producing infectious virions, we wanted to determine whether primary hepatocytes in MPCCs are capable of infecting patients in the presence of heparin (5 IU), CaCl2 (9 mM), and MgCl2. MPCCs were infected with Jc1FLAG2(p7-nsGluc2A) or plasma from HCV-(C508Y) RFP-NLS-IPS HCV reporter. Twenty-four hours after transduction, images are shown; white arrow heads show nuclear RFP, indicative of HCV infection.

To demonstrate that HCV actively replicates in the primary hepatocyte component of the MPCCs, we used 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 NS3-4A results in translocation of the cleavage product, RFP-(Fig. 2).

**Fig. 2.** Primary human hepatocyte MPCCs are susceptible to HCV. (A) Persistent infection of primary human hepatocytes with HCVcc. Primary hepatocytes in MPCCs 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 50× 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 infected MPCC hepatocytes transduced with wild-type RFP-NLS-IPS; no relocalization was detected after infection of infected MPCC hepatocytes transduced with wild-type RFP-NLS-IPS, or IFN-α. These compounds inhibited HCV replication in the submicromolar range, indicating the relevance of conducting drug combination studies during in vivo 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 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 sites.
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 discovery. 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-msGluc2A) 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-) 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 (∼3x10⁶ adherent hepatocytes in 96 collagen-coated islands in 24-well plate and ∼4.5x10⁶ 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 μg/ml hydrocortisone and 1% penicillin-streptomycin. 3T3-L2 murine embryonic fibroblasts were seeded (9x10^4 cells in each well of 24-well plate and 1.4x10^5 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. Cultures of hepatocytes on conventionally-plated pure hepatocyte cultures (Collagen gel sandwich, 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 EGP-encoding provirus (CSG01) or provirus encoding transgene (gTRIP) (2) HIV gag-pol and (3) envelope glycoprotein(s), as previously described (17). HCVpp were generated using H77 E1E2 (residues 170–1883) in the presence of high-density lipoprotein.

**Antibodies, Immunostaining, and Blocking.** For immunostaining, cells or tissue sections were fixed in 1% paraformaldehyde and/or –2°C methanol. Following washing and blocking in 1% BSA/0.2% milk or 1% BSA/0.3% Triton X-100, samples were incubated in primary antibodies either overnight at 4°C or mouse anti-human CD81 (clone JS-81, BD Pharmingen; 1:200), rabbit anti-CLDN1 (51–480, 1:100), mouse anti-human CD81 (clone JS-81, BD Pharmingen), anti-E2 3'11/31 (36), 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) (AR3A) (28). Human IgG1 (clone MOPC-21), IgG4 (MOR6391), and rat IgG2a (MCA1124R) isotype control antibodies were purchased from AbD Serotec.

**ACKNOWLEDGMENTS.** The authors thank Amelie Forest, Megan Holz, Michelle Hunter, Maryline Panis, Jodie Tassello, and Anesta Webson for excellent technical assistance, and Catherine Murray for superbly editing the manuscript. Allison North and The Rockefeller University Bioimaging Core, provided outstanding technical support. The authors also thank Ira Jacobson, Queeney Brown, Ryan Peterson, and Steve Gonzales (Weill Cornell Medical College), Luis Chiriboga, and Herman Yee (New York University) for assistance with the histological analysis, as well as Robert Brown and Raghu Varadarajan (Columbia University), who kindly provided primary liver tissue for histological analysis. This work was supported by the Greenberg Medical Research Institute, the Ellison Medical Foundation, the Starr Foundation, the Ronald A. Shelly Memorial Fund, the Richard Salomonon Family Foundation, and funded in part by a Grant from the Foundation for the National Institutes of Health through the Grand Challenges in Global Health initiative (grants to C.M.R., and the National Institutes of Health (grants R01 AI075099 to C.M.R., R01 DK56996 to S.N.B., and NRSF DK081193 to C.T.J.). C.M.R. is an Ellison Medical Foundation Senior Scholar in Global Infectious Diseases. This work was funded by the National Institutes of Health through the National Institutes of Health Roadmap for Medical Research, Grant 1 R01 DK085713-01 to C.M.R., S.N.B., and A.H. Patel. Information on this Roadmap Transformative R01 Program can be found at http://grants.nih.gov/grants/guide/rfa-files/RFA-RM-08-029.html. A.P. is a recipient of a Kimberly Lawrence-Netter Cancer Research Discovery Fund Award. S.N.B. is an HHMI investigator.

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) HCVcc 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 NSSA staining was performed 72 h postinfection to visualize HCV infection. (C) HCV infection of Huh-7.5 cells was visualized by immunohistochemical staining for NSSA. Days indicate the time points when supernatants were taken from the infected MPCC cultures.