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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.

R esults

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, MPCC; Fig. 1 A and B). Here, we show that primary human hepatocytes form polarized cell layers in MPCCs. Multidrug resistant protein 2 (MRP2), zona occludens protein 1 (ZO1), and HCV entry factors claudin-1 (CLDN1) (15) and occludin (OCLN) (16, 17), were located in tight junction (TJ)-like structures (canalicular domain), whereas CD26 was localized on the basolateral domain (Fig. 1). The presence of bile canalicular structures between adjacent hepatocytes was confirmed via 3D renderings reconstructing ZO1, MRP2, and nuclear staining (Fig. 1 I 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 A and Fig. S1).

To test whether MPCC hepatocytes can support HCV glycoprotein-mediated entry, we infected cultures with HCV pseudoparticles (HCVpps). HCVpp, which are defective lentiviral particles that display the HCV glycoproteins (E1 and E2) and encode a reporter gene (here EGFP), allow rapid quantitation of infection in the absence of replication. Approximately 1–3% of the human hepatocytes in MPCCs, but none of the supporting murine embryonic fibroblasts (3T3-J2), could be infected with HCVpp (Fig. 1 K and L). Pseudoparticles lacking glycoproteins did not infect the cultures, although MPCCs were

www.pnas.org/cgi/doi/10.1073/pnas.0915130107
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. 1).

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

Despite considerable effort, it has not been possible to unequivocally demonstrate HCV replication in primary hepatocyte cultures over prolonged periods of time. Previous studies have relied on quantifying HCV RNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), a technique inappropriate for detecting rare infectious events due to high background of nonspecifically bound viral RNA. We instead employed a highly sensitive HCVcc reporter virus expressing secreted Gaussia luciferase (Gluc), Jc1FLAG2(p7-nsGluc2A) (20). After inoculation, cultures were washed to remove Gluc carryover, and luciferase secretion was monitored as an indicator of viral replication. We found that several conventional culture systems (i.e., collagen gel sandwich, Matrigel overlay, and randomly distributed cocultures) could not sustain HCV replication, presumably due to a decline in liver-specific phenotype (12) (Fig. S2). In contrast, MPCCs in multiwell formats supported HCV replication for at least two weeks (Fig. 2 and Fig. S3). Treatment with HCV NS3-4A protease (ITMN191) or NS5B polymerase inhibitors (2CMA), or IFN alpha (IFN-α), reduced luciferase activity to background levels (Fig. 2A), indicating that persistent signal was indeed due to ongoing viral replication. Persistent HCV infection was achieved in MPCCs created from freshly isolated or cryopreserved human hepatocytes from several donors, reflecting the reproducibility of the optimized microscale architecture and the concomitant phenotypic stability.

We next attempted to quantify HCV RNA and proteins in MPCCs by qRT-PCR, Western blot, and immunofluorescence. In contrast...
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 relocalization was detected after infection of MPCCs harboring a cleavage-resistant reporter (C50BY) or 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 μg/mL), whereas anti-SCARB1 (C167) did not effectively inhibit viral uptake. All antibodies against E2 were able to inhibit HCVcc infection, although with varying efficiencies (IC50 for AP33 > 3/11 > CBH5 > AR3A) (Fig. 3A). A variety of specific antivirals targeting HCV enzymes are also under preclinical development. Currently, however, in vitro platforms capable of simultaneously assessing drug toxicity and efficacy are not widely available. We have previously shown the utility of MPCCs in drug metabolism and toxicity screening via assessment of gene expression profiles, phase I/II metabolism, canalicular transport, secretion of liver-specific products, and susceptibility to hepatotoxins (12). Here, we examined the use of MPCCs in evaluating antiviral efficacy (Fig. 3B). We measured HCV replication by luciferase activity at 4 days posttreatment with protease inhibitors (BILN2061 and ITMN191), polymerase inhibitor (2’CMA), or IFN-α. These compounds inhibited HCV replication in the submicromolar range, indicating the relevance of MPCCs for monitoring HCV inhibition. We then evaluated the efficacy of protease inhibitors (SCH-6 and BILN2061) and polymerase inhibitor (2’CMA) in HCVcc-infected MPCCs pretreated for 3 days with compounds known to modulate drug metabolism and other cellular functions in vivo (12) (Fig. 3C). We found that the addition of certain drugs severely reduced the efficacy of SCH-6 and 2’CMA, as compared to DMSO solvent control. Although the mechanisms underlying these adverse drug interactions remain unknown, these observations demonstrate the importance of conducting drug combination studies during in vitro efficacy assessment. These studies indicate that MPCCs may be well suited as a metabolically competent in vitro model of the liver, allowing HCV replication to be studied over several days to weeks, and a variety of intervention strategies to be tested for efficacy and toxicity.

Discussion

Here we have described a microscale primary human hepatocyte in vitro culture platform that supports the entire HCV life cycle. Primary hepatocyte MPCCs are stable for several weeks and therefore allow monitoring of human hepatotrophic infections over extended periods of time. Although this is an important step forward, limitations remain. Entry of HCVpp and tissue culture-derived virus into MPCC hepatocytes was inefficient. Although the four critical viral entry factors are present on these cells, it is possible that differences in their spatial distribution might account for the low uptake efficiency. Indeed, antibodies block entry into primary hepatocytes at different efficacies than previously reported (26–28), possibly due to limited accessibility of the HCV entry site.
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-
overnight. Hepatocyte culture medium was DMEM with high glucose, 10% FBS, 0.5 U/ml insulin, 7 ng/ml glarglan, 7.5 μg/ml hydrocortisone and 1% penicillin-streptomycin. 3T/12 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. Cells were 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 EGFP-encoding provirus (CSG04) 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, samples were incubated in primary antibody (at 4°C overnight) and mouse anti-NS5A (9E10 (23), 1:200) were used as negative controls. Secondary antibodies were goat-anti-mouse HRP (ImmPRESS kit, Vector Labs) with DAB substrate (Dako) for immunochemistry staining. 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 μm 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 (1:100), anti-CD81 (clone 14E9, 1:200), anti-CD63 (clone 1B5, 1:200), anti-CD14, anti-CD10, anti-CD15, anti-CD16, anti-CD19, anti-CD34, anti-CD45, anti-CD49, anti-CD56, anti-CD11c, anti-CD274, anti-CD54, anti-CD69, anti-CD80, anti-CD86, anti-CD137, anti-CD152, 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 Co. 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 Raghuv 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. Shellor Memorial Fund, the Richard Salomon 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 DK56966 to S.N.B., and NRSA 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 DK05713-01 to C.M.R., S.N.B., and information on this Roadmap Transformative R01 Program can be found at http://grants.nih.gov/grants/guide/rfa-files/RFA-RR-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 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.