Modeling hepatitis C virus infection using human induced pluripotent stem cells


Human pathogens impact patient health through a complex interplay with the host, but models to study the role of host genetics in this process are limited. Human induced pluripotent stem cells (iPSCs) offer the ability to produce host-specific differentiated cells and thus have the potential to transform the study of infectious disease; however, no iPSC models of infectious disease have been described. Here we report that hepatocyte-like cells derived from iPSCs support the entire life cycle of hepatitis C virus, including inflammatory responses to infection, enabling studies of how host genetics impact viral pathogenesis.

Results and Discussion

iPSC-Derived Hepatocyte-Like Cells Express HCV Host Factors. To test the hypothesis that iPSC-derived differentiated cells are permissive to infection, we sought to model HCV infection (Fig. 1). HCV infects human hepatocytes, and we recently demonstrated the directed differentiation of human iPSCs into hepatocyte-like cells (iHLCs) (14). iHLCs routinely demonstrate an expected cobbledstone morphology (Fig. 24, Left), and more than 80% express both albumin and hepatocyte nuclear factor 3β (HNF-3β) (Fig. 24, Right). In addition, iHLCs secrete liver-specific serum proteins such as albumin and α-1-antitrypsin at levels 15% and 50%, respectively, of those of primary human hepatocytes maintained in long-term culture models (Fig. 2B, Lower) (15). Here, we investigated whether iHLCs express host genes important for HCV infection (“host factors”), are capable of supporting the HCV life cycle, and respond to infection with an appropriate antiviral inflammatory response. We found that iHLCs express known HCV host factors, including the liver-specific microRNA-122 (mir-122) (Fig. 2B) and entry factors (CD81, SRBI, CLDN1, and occludin (OCLN)) (Fig. 2C and D); analysis of iPSC and iHLC transcriptional microarrays (14) confirmed that host factors previously identified in an shRNA screen (16) were enriched in iHLCs and were expressed to a greater extent in iHLCs than iPSCs (Fig. 2E and Dataset S1).

Although iHLCs exhibit many characteristics of adult hepatocytes, their expression of phase-1 and phase-2 enzymes [high expression of cytochrome P450, family 3, subfamily A, polypeptide 7 (CYP3A7); cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1)]] and glutathione S-transferase α4 (GSTA4) and low expression of cytochrome P450, family 2, subfamily C (CYP2C) family genes and cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4) and coexpression of α-fetoprotein and albumin is collectively more consistent with the characteristics of a fetal hepatocyte (14, 17, 18). Experimental evidence suggests that iPSCs are fully capable of differentiating into terminally differentiated adult hepatocytes, as demonstrated in mouse IPS tetraploid complementation experiments and in mouse and human iHLC transplantation experiments (14); however, culture conditions have not yet been established that allow terminal differentiation, as indicated by loss of α-fetoprotein expression and fetal cytochrome P450 expression.


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121400109/-/DCSupplemental.

PNAS | February 14, 2012 | vol. 109 | no. 7

2544–2548

www.pnas.org/cgi/doi/10.1073/pnas.1121400109

2544–2548
HCV Infection Induces an Antiviral Inflammatory Response from iHLCs. We next determined whether infection induces an antiviral inflammatory response, which is central to the natural history of clinical disease progression but defective in existing in vitro models of HCV (24). qRT-PCR on iHLC lysates 2 or 14 dpi revealed that expression of inflammatory markers was up-regulated by infection (Fig. 4A), and ELISA on culture supernatants 14 dpi verified persistent TNF-α secretion as a result of infection (Fig. 4B). These results are characteristic of an ongoing inflammatory response in cells with an intact innate immune axis. Notably, IL-28B, whose gene variation predicts response to hepatitis C treatment in genome-wide association studies (8), was expressed in response to viral infection 2 dpi but declined over 2 wk, underscoring the potential for such a platform to provide clinically relevant biological insights.

Existing model systems to study host genetics, such as polymorphisms in IL-28B, are limited to needle biopsies, surgical resection, organ donation, and hepatoma cell lines of a single background. We believe that this study lays the foundation for personalized in vitro models that can capture genetic variation of both host and pathogen, whereby iPSCs can be generated from identified patients with known or unknown genetic defects that impact infection. In this study we report that hepatocyte-like cells derived from iPSCs support the entire life cycle of HCV, including inflammatory responses to infection, but we believe that in the future patient-derived iHLCs will serve as a model system to probe the basis of hepatitis viral pathogenesis and that the model ultimately can be extended to other pathogens and tissue systems. Such models will advance our understanding of host-pathogen interactions and help realize the potential of personalized medicine.

Materials and Methods
IPSC Culture and Hepatocyte-Like Cell Generation. Undifferentiated iPSC were maintained and differentiated into iHLCs as described (14). In brief, iPSCs were cultured in monolayer on Matrigel (Becton Dickinson), and directed differentiation was achieved by sequential exposure to Activin A, bone morphogenic protein 4, basic FGF, HGF, and oncostatin M (OSM).

HCV Cell-Culture Preparation, Infection, Luciferase Assay, Antiviral Drugs, and Nonstructural Protein 3/4A Activity Imaging. As described (19), Gluc-expressing reporter virus Jc1FLAG2(p7-nsGluc2A) stocks were prepared by electroporation in vitro-transcribed RNA into Huh-7.5 cells, collection of supernatant, and filter concentration. The 50% tissue culture infectious dose (TCID50) was determined by titrating on Huh-7.5s to be 10^7 TCID50/mL. These stocks were diluted 10x in serum-free, OSM-containing medium and were used to inoculate iHLCs for 24 h. Cultures were washed with serum-free medium and propagated in OSM-containing medium. Supernatants were collected daily and frozen at −80 °C for luciferase quantification. To demonstrate drug-sensitive HCV infection, the HCV NS5B polymerase inhibitor CMA (EC50 = 27 nM on Huh7.5) (21) and the NS3/4A protease inhibitor VX-950 (telaprevir) (EC50 = 400 nM on Huh7.5) (21) were added to culture medium at 50°EC50 and 25°EC50, respectively, at final 0.1% DMSO. 2°CMA was the gift of D. Olsen and S. Carroll (Merck Research Laboratories, West Point, PA) and also was obtained from Carboysnht Limited. VX-950 was obtained from Asemic Limited. Real-time fluorescence reporter of HCV infection by monitoring NS3/4A protease activity was performed as described (20). Briefly, lentivirus carrying the reporter was used to infect iHLCs carried infection to Huh7.5s. Thus, iHLCs support the complete HCV life cycle, including replication and release of infectious virions. Therefore, iHLCs sustain the entire HCV viral life cycle of at least genotype 2a, in accordance with prior reports showing that human fetal hepatocytes are capable of sustaining the HCV life cycle (22, 23). Future work toward a fully personalized in vitro model of HCV infection would incorporate both personalized hepatocyte-like cells and isolates from HCV patients, including the most common HCV genotype in the United States, genotype 1a.

HLCs Support the Entire Life Cycle of HCV. To assess HCV replication in iHLCs, we used a genotype 2a HCV reporter virus expressing secreted Gaussia luciferase (GLuc) (19). GLuc signal was persistently higher in infected cultures than in uninfected (mock) controls (Fig. 3A), but not in undifferentiated iPSCs (Fig. S1); further, initiating daily treatment with either the HCV nonstructural protein 5B (NS5B) replicase inhibitor 2′-C-methyladenosine (2′CMA) or the nonstructural protein 3/4A (NS3/4A) protease inhibitor VX-950 (telaprevir) 7 d post-infection (dpi) rapidly abolished GLuc production (Fig. 3A, Upper). Furthermore, consistent with the GLuc assay, quantitative RT-PCR (qRT-PCR) on iHLC lysates 14 dpi showed that HCV genomes were significantly more abundant in the absence of antivirals (Fig. 3B). In addition, using a real-time fluorescence reporter of infection (20), we confirmed HCV protease activity in infected iHLCs (Fig. 3C). Together, these results indicate ongoing HCV replication in infected iHLCs. To verify that infected iHLCs produce infectious virions and thus recapitulate the entire viral life cycle, culture supernatants were passaged 13 dpi onto uninfected Huh-7.5 cells, which are highly permissive to HCV and thus allow sensitive detection of infectious virions (21).

As shown by GLuc production and HCV nonstructural protein 5A (NS5A) staining (Fig. 3A, Lower), supernatants from infected iHLCs carried infection to Huh7.5s. Thus, iHLCs support the complete HCV life cycle, including replication and release of infectious virions. Therefore, iHLCs sustain the entire HCV viral life cycle of at least genotype 2a, in accordance with prior reports showing that human fetal hepatocytes are capable of sustaining the HCV life cycle (22, 23). Future work toward a fully personalized in vitro model of HCV infection would incorporate both personalized hepatocyte-like cells and isolates from HCV patients, including the most common HCV genotype in the United States, genotype 1a.
transduce iPSCs. Infection was carried out 5 d later, and protease activity was assayed 7 dpi.

**Huh-7.5 Culture and Infection Transmission Assay.** Huh-7.5 cells were propagated in a DMEM with L-glutamine (Cellgro)-based medium containing 100 U/mL penicillin and 100 µg/mL streptomycin (Cellgro) and 10% (vol/vol) FBS ( Gibco). To test if infected iHLCs produced infectious virions, iHLCs were placed in OSM-containing medium without supplementation with antivirals. Supernatants collected 1 d later were used to inoculate Huh-7.5 cells. After overnight incubation, cells were washed and placed in Huh-7.5 medium for 48 h before being washed again. On day 5 postinoculation, supernatants were assayed for luciferase as described (19). To assess NS5A antigen expression, Huh-7.5 cells were fixed in methanol, counterstained with Hoechst (Invitrogen), and immunostained with mouse anti-NSSA (9E10) and goat anti-mouse Alexa Fluor 594 (Invitrogen).

**RT-PCR for Detection of Cytokines and HCV RNA.** Total RNA was isolated with the RNAsesy Plus Mini Kit (Qiagen). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Bio-Rad). Quantitative PCR for cytokines was carried out with Taq polymerase and SYBR Green in the supplier’s reaction buffer containing 1.5 mM MgCl₂ (Bio-Rad). Oligonucleotide primer sequences are available by request (25). Amplicons were analyzed by 2% (wt/vol) agarose gel electrophoresis. Quantitative PCR on HCV genomes was performed as described (19).

**Immunofluorescence Analysis for Hepatic Gene Expression and Host Factor Expression.** iHLCs were fixed in 4% (wt/vol) paraformaldehyde and/or –20 °C methanol. After washing and blocking in 0.1% donkey serum/0.1% Triton X-100 in PBS, cells were incubated in primary antibodies overnight at 4 °C: mouse anti-human albumin (Sigma-Aldrich), rabbit anti–α-1-antitrypsin (A1AT) secretion by iHLCs. Error bars show SD. (C) Liver-specific factors in iHLCs. (Upper Right) MIRNA-122 expression blot and quantification (Lower Right) for two typical batches of iHLCs (iHLC-A and iHLC-B). Adult human hepatocytes (heps) (15) are included as a reference. (Lower Left) Quantification of albumin (Alb) and α-1-antitrypsin (A1AT) secretion by iHLCs. Error bars show SD. (C) Liver-specific factors in iHLCs for HCV entry factors SRBI (red) and CD81 (green), with DAPI costaining (blue). (Scale bar: 40 µm.) (Right) Immunofluorescence imaging of iHLCs for HCV entry factors OCLN (red) and CLDN1 (green), with DAPI costaining (blue). (Scale bar: 40 µm.) (D) Western blot for HCV entry receptors CD81, SRBI, CLDN1, and OCLN in two typical batches of iHLCs (iHLC-A and iHLC-B) in duplicate samples. (E) Relative expression of HCV host factors (16) by three batches of iPSCs (iPSC-A, iPSC-B, and iPSC-C) and iHLCs (14). [Host factors are organized by GO biological process terms, including repeats for genes associated with multiple terms.]

Western Blot for Entry Receptors. Total protein was extracted with radioimmunoprecipitation assay lysis buffer and samples were separated by electrophoresis on 12% (wt/vol) polyacrylamide gels and electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories). Blots were probed with mouse anti-human CD81 (Millipore), rabbit anti-SCARB1 (Novus Biologicals), and mouse anti-human OCLN (Invitrogen). Secondary antibodies were donkey anti-mouse DyLight 594, donkey anti-rabbit DyLight 488, and donkey anti-rabbit DyLight 594 conjugates. Cells were counterstained with Hoechst dye (Invitrogen).

**Fig. 2.** iHLCs express known HCV host factors. (A) (Left) Phase image of iHLCs. (Scale bar: 100 µm.) (Right) Immunofluorescence imaging of iHLCs for albumin (red), HNF-3β (green), and DAPI (blue). (Scale bar: 90 µm.) (B) Liver-specific factors in iHLCs. (Upper Right) MIRNA-122 expression blot and quantification (Lower Right) for two typical batches of iHLCs (iHLC-A and iHLC-B). Adult human hepatocytes (heps) (15) are included as a reference. (Lower Left) Quantification of albumin (Alb) and α-1-antitrypsin (A1AT) secretion by iHLCs. Error bars show SD. (C) Liver-specific factors in iHLCs for HCV entry factors SRBI (red) and CD81 (green), with DAPI costaining (blue). (Scale bar: 40 µm.) (Right) Immunofluorescence imaging of iHLCs for HCV entry factors OCLN (red) and CLDN1 (green), with DAPI costaining (blue). (Scale bar: 40 µm.) (D) Western blot for HCV entry receptors CD81, SRBI, CLDN1, and OCLN in two typical batches of iHLCs (iHLC-A and iHLC-B) in duplicate samples. (E) Relative expression of HCV host factors (16) by three batches of iPSCs (iPSC-A, iPSC-B, and iPSC-C) and iHLCs (14). [Host factors are organized by GO biological process terms, including repeats for genes associated with multiple terms.]
antibodies, and were developed by SuperSignal West Pico substrate (Thermo Scientific).

miR-122 Analysis. Total RNA was isolated with the miRNeasy Mini Kit (Qiagen). MiRNAs were polyadenylated by poly(A) polymerase, and cDNA was synthesized using miScript PCR kit (Qiagen). Quantitative real-time PCR on miR-122 then was performed using Homo sapiens (hsa) miR-122–specific primer (Qiagen) and normalized to RNA, U6B small nuclear (RNU6B) (Qiagen). Standard curves were performed to obtain absolute levels with synthetic miR-122 (Thermo Scientific).

Albumin and α-1-Antitrypsin ELISA. Spent medium was stored at −20 °C. α-1-Antitrypsin and albumin media concentrations were measured using sandwich ELISA technique with HRP detection (Bethyl Laboratories) and 3,3′,5,5′-tetramethylbenzidine (Thermo Scientific) as a substrate.

Microarray Analysis and Host Factor Expression. Microarray analysis was performed as described (14). Microarray profiles on iHLCs (http://www.ncbi.nlm.nih.gov/geo/, accession no. GSE14897) were analyzed using gene set enrichment analysis v. 2.0 with a list of previously identified HCV host factors (16). Enriched genes were determined by random permutation of gene sets and a P value < 0.05. Gene ontology (GO) terms and gene associations were obtained using Gene Set Analysis Toolkit v. 2. Statistical analysis was performed using a hypergeometric distribution to identify terms enriched with two genes and a P value < 0.05 and then connected in a tree hierarchy (26).

ACKNOWLEDGMENTS. We thank Prof. Maria Mota, Dr. Alice Chen, Dr. Margaret Scull, Dr. Maria Teresa Catanese, Dr. Gabriel Kwong, Dr. Salil Desai, Cheri Li, Justin Lo, Nathan Reticker-Flynn, Kevin Lin, and Charlie Whittaker for insightful discussions. This work was supported by National Institutes of Allergy and Infectious Diseases Grant R01 AI072613, National Institutes of Health (NIH) through the NIH Roadmap for Medical Research Grant 1 R01 DK085713-01, The Greenberg Medical Research Institute, The Starr Foundation, and Howard Hughes Medical Institute.


Fig. S1. Induced pluripotent stem cells (iPSCs) do not support hepatitis C virus (HCV) infection. iPSCs and iPSC-derived hepatocyte-like cells were infected concurrently with HCV reporter virus expressing secreted Gaussia luciferase (HCVcc; n = 6) or were mock infected (mock; n = 6). Cells subsequently were sampled and washed daily. After 5 d supernatants were collected and assayed for luciferase activity. Mock- and HCV-infected iPSCs showed no statistically significant difference by two-tailed t test. RLU, relative light units.