

Modeling hepatitis C virus infection using human induced pluripotent stem cells

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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.

host variation in disease | infectious disease model | personalized medicine | hepatotropic infection | viral hepatitis

Human pathogens impact patient well-being through complex host–pathogen interactions. Despite the importance of host genetics in this interplay, *in vitro* model systems for studying the role of host genetic variation in infection often are unavailable because of tissue scarcity and challenges in primary culture. The discovery of cellular reprogramming and the ability to generate host- and tissue-specific cells from induced pluripotent stem cells (iPSCs) have the potential to transform the study of development, infectious disease, and degenerative disorders (1, 2). For example, iPSCs have been used for the mechanistic study of a variety of cells types implicated in a wide diversity of disease (e.g., Friedreich ataxia, long-QT syndrome, Leopard syndrome, Rett syndrome, and α -1-antitrypsin disease) (3–7). However, no iPSC models of any infectious disease have been reported to date. In this study, we describe the use of iPSC-derived cells as a model system for studying host–pathogen interactions for the hepatitis C virus (HCV).

Afflicting more than 170 million people worldwide, HCV is a prototypic pathogen for which host genetic factors have been implicated in modulating disease natural history and treatment response but whose functions remain poorly understood because of the lack of robust experimental systems. For example, genome-wide association studies have identified host polymorphisms in the *IL-28B* locus that correlate with spontaneous HCV clearance and viral response to IFN-based therapy (8). However, the functional consequences of these well-described polymorphisms remain elusive. Additionally, individuals with mutations in genes that are critical for HCV entry [e.g., low-density lipoprotein receptor, CD81, scavenger receptor, class B, type 1 (SRBI), occludin (OCLN), claudin 1 (CLDN1)], assembly (apolipoprotein E or apolipoprotein B), or immune response (signal transducers and activators of transcription 1) have been described (8–13). Despite our awareness that host genetics impacts viral pathogenesis in such individuals, the mechanistic basis for these correlations remains unclear largely because of the lack of a robust experimental system incorporating host cells with these genetic backgrounds. The development of an iPSC-derived HCV model has the potential to elucidate further the role of these host factors in disease 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 cobblestone morphology (Fig. 2*A, Left*), and more than 80% express both albumin and hepatocyte nuclear factor 3 β (HNF-3 β) (Fig. 2*A, 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. 2*B, 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. 2*B*) and entry factors [CD81, SRBI, CLDN1, and occludin (OCLN)] (Fig. 2*C 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. 2*E* 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 α 4 (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.

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The authors declare no conflict of interest.

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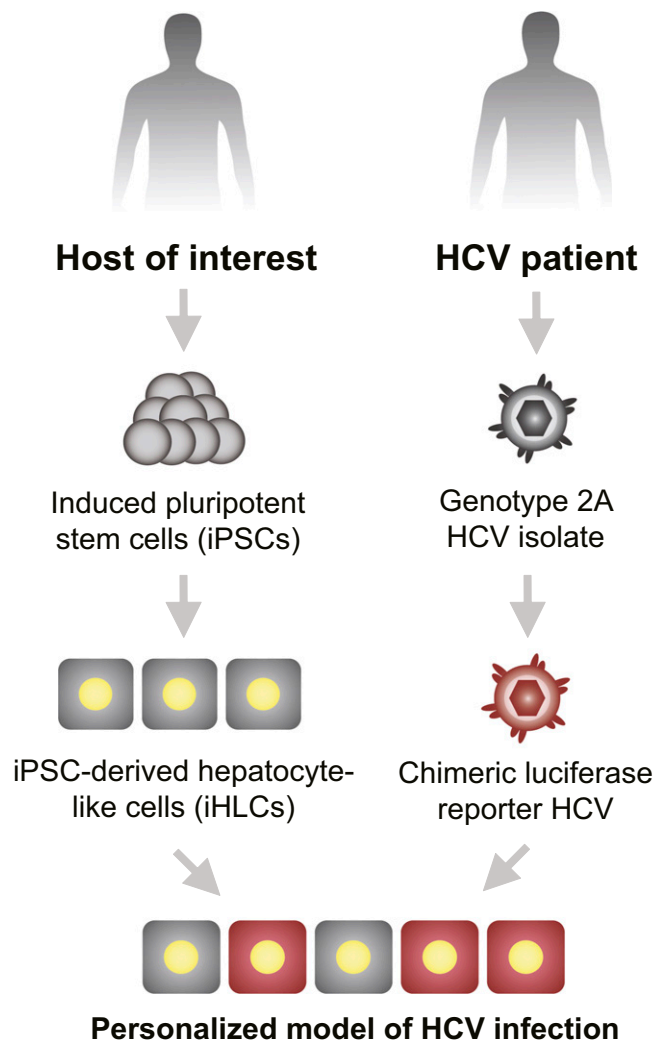


Fig. 1. Personalized model of HCV infection obtained by infecting iHLCs from one donor with HCV from another donor.

iHLCs 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, Upper) 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.

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 electroporating 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 10⁷ 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 2' 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 Carboxynth Limited. VX-950 was obtained from Alembic 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

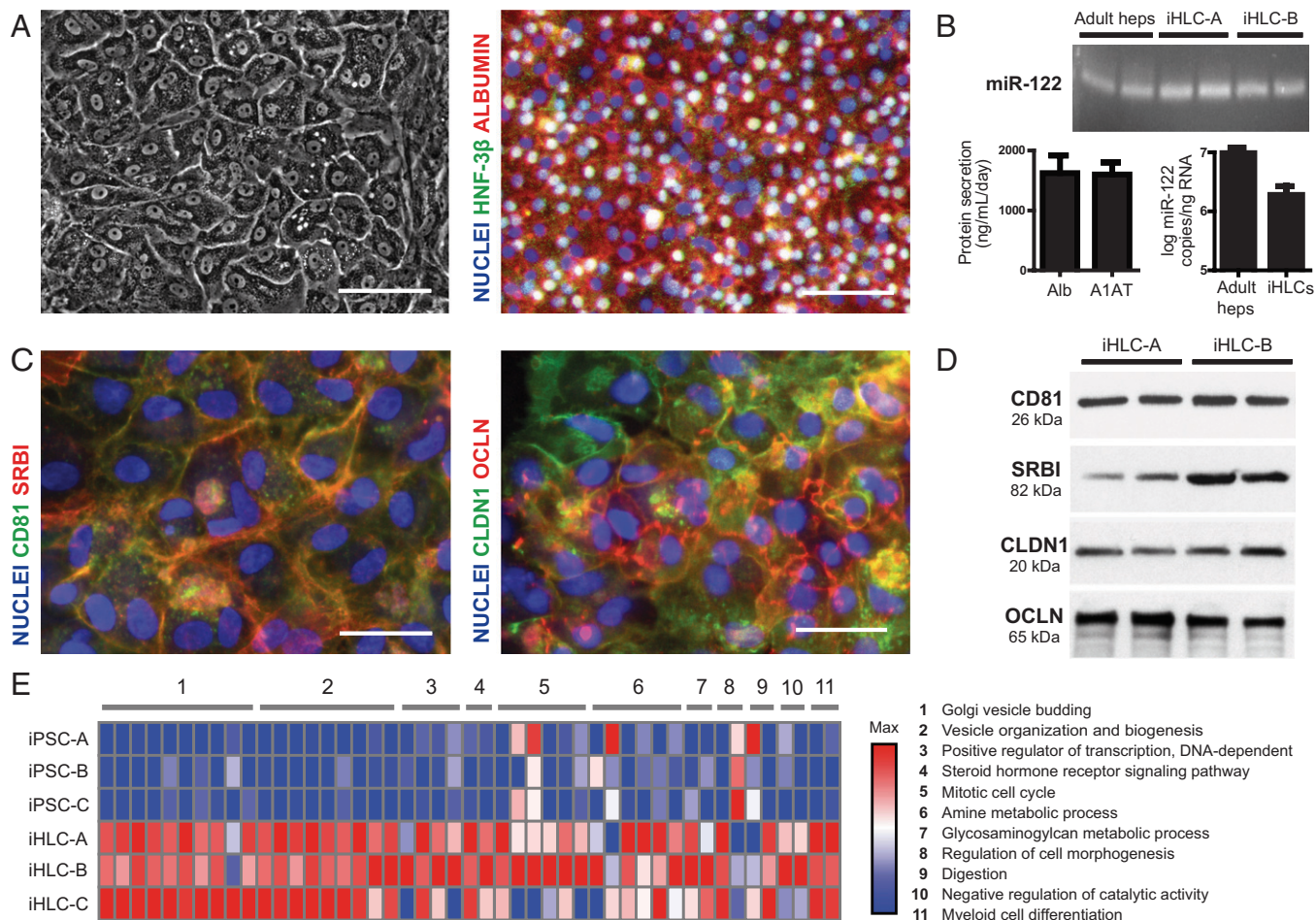


Fig. 2. iHLCs express known HCV host factors. (A) (Left) Phase image of iHLCs. (Scale bar: 100 μm .) (Right) Immunofluorescence imaging of iHLCs for albumin (red), HNF-3 β (green), and DAPI (blue). (Scale bar: 90 μm .) (B) Liver-specific factors in iHLCs. (Upper) MiRNA-122 expression blot and quantification (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) (Left) Immunofluorescence imaging of 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 (iPAC-A, iPAC-B, and iPAC-C) and iHLCs (14). [Host factors are organized by GO biological process terms, including repeats for genes associated with multiple terms.]

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 $\mu\text{g}/\text{mL}$ 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-NS5A (9E10) and goat anti-mouse Alexa Fluor 594 (Invitrogen).

RT-PCR for Detection of Cytokines and HCV RNA. Total RNA was isolated with the RNeasy 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-HNF-3 β (Santa Cruz Biotechnology), mouse anti-human CD81 (Becton Dickinson), rabbit anti-CLDN1 (Invitrogen), rabbit anti-SCARB1 (Novus Biologicals), and mouse anti-human OCLN (Invitrogen). Secondary antibodies were donkey anti-mouse DyLight 594, donkey anti-rabbit DyLight 488, donkey anti-mouse DyLight 488-, and donkey anti-rabbit DyLight 594 conjugates. Cells were counterstained with Hoechst dye (Invitrogen).

Western Blot for Entry Receptors. Total protein was extracted with radio-immunoprecipitation 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 (NB110-57591; Novus Biologicals), rabbit anti-CLDN1 (51-9000; Invitrogen), and rabbit anti-OCLN (40-4700; Invitrogen), followed by HRP-conjugated secondary

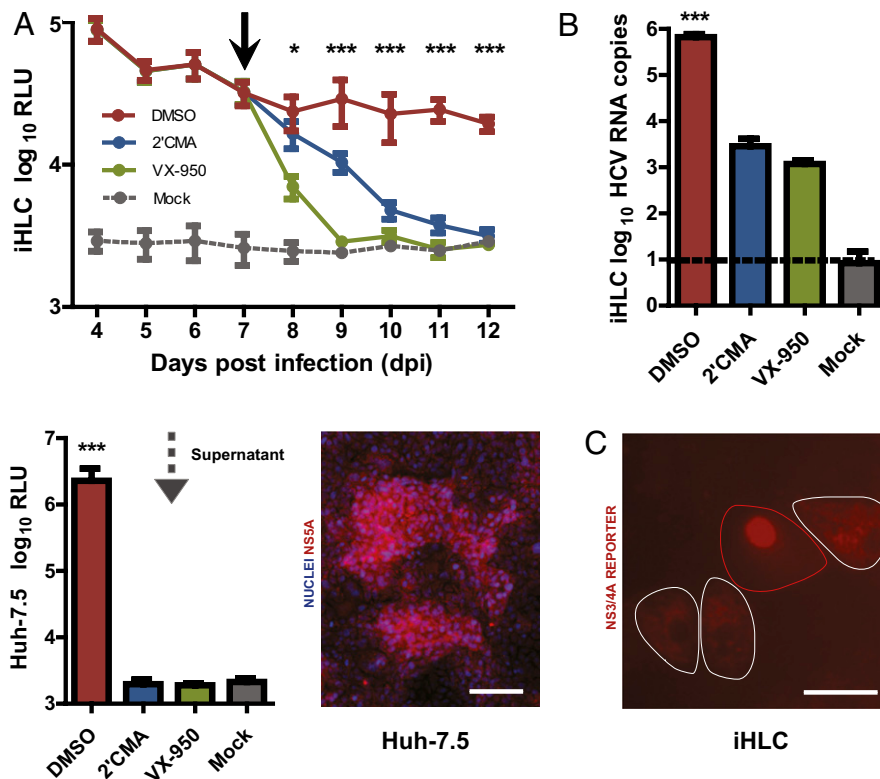


Fig. 3. iPSC-derived iHLCs as a model for hepatitis C. (A) iHLC cultures were infected with HCV reporter virus expressing secreted GLuc ($n = 18$) or were mock infected ($n = 6$) and subsequently were sampled and washed daily. After 7 d (solid gray arrow), infected iHLCs were treated with the NS5B polymerase inhibitor 2'CMA ($n = 6$), NS3/4A protease inhibitor VX-950 ($n = 6$), or vehicle DMSO ($n = 6$). Drug treatment was discontinued 12 dpi, and supernatants collected after an additional day of culture were assayed for the presence of infectious virus by passage onto Huh-7.5 cells. Medium from Huh-7.5 cells was harvested 5 d after passage for GLuc assay. (Upper) GLuc secretion by iHLCs. The difference between DMSO- vs. 2'CMA-treated cultures was statistically significant: $*P < 0.05$, $***P < 0.001$ (one-way ANOVA with Tukey's post test). RLU, relative light units. (Lower Left) GLuc secretion by Huh-7.5s after passage of iHLC supernatants. DMSO vs. mock was statistically significant: $***P < 0.001$ (one-way ANOVA with Tukey's post test). (Lower Right) NS5A staining of infected Huh-7.5 cells post passage. (Scale bar: 50 μm .) (B) iHLCs were lysed 14 dpi. Copies of HCV RNA in lysates were quantified by qRT-PCR. DMSO vs. 2'CMA was statistically significant: $***P < 0.001$ (one-way ANOVA after log transformation with Tukey's post test). (C) NS3/4A activity imaging of HCV-infected iHLCs (20). White lines surround uninfected cells; red line surrounds an infected cell. (Scale bar, 25 μm .) Data in A–C are means; error bars show SD.

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 (Dharmacon).

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).

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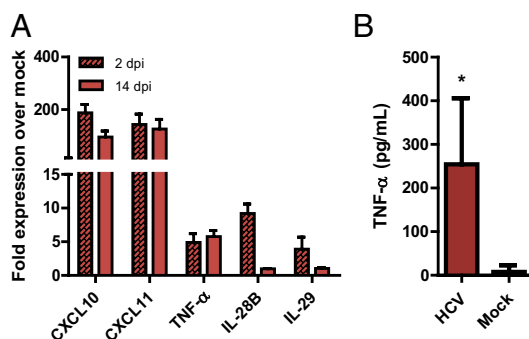


Fig. 4. iHLCs demonstrate an inflammatory response to HCV infection. (A) mRNA expression of innate immune/inflammatory markers in lysates of infected, DMSO-treated iHLCs relative to mock-infected cells at 2 and 14 dpi. (B) TNF- α secretion by HCV- and mock-infected iHLCs 14 dpi. The difference was statistically significant: $*P < 0.05$. Data in A and B are means; error bars show SD.

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Supporting Information

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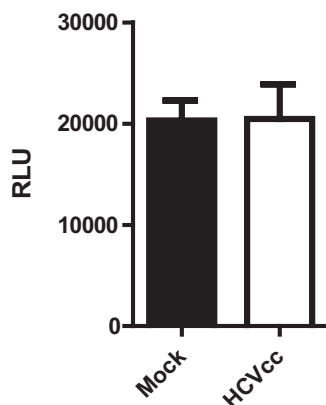


Fig. S1. Induced pluripotent stem cells (iPSCs) do not support hepatitis C virus (HCV) infection. iPSCs and iPSC-derived hepatocyte-like cells were infected concurrently with HCV reporter virus expressing secreted *Gaussia* luciferase (HCVcc; $n = 6$) or were mock infected (mock; $n = 6$). Cells subsequently 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.

Other Supporting Information Files

[Dataset S1 \(XLSX\)](#)