Published in final edited form as:

Gastroenterology. 2018 February ; 154(3): 663–674.e7. doi:10.1053/j.gastro.2017.10.041.

Pan-Genotype Hepatitis E Virus Replication in Stem Cell-derived Hepatocellular Systems

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Abstract

Background & Aims—The 4 genotypes of hepatitis E virus (HEV) that infect humans (genotypes 1–4) vary in geographical distribution, transmission, and pathogenesis. Little is known about the properties of HEV or its hosts that contribute to these variations. Primary isolates grow poorly in cell culture; most studies have relied on variants adapted to cancer cell lines, which likely alter virus biology. We investigated the infection and replication of primary isolates of HEV in hepatocyte-like cells (HLCs) derived from human embryonic and induced pluripotent stem cells.

Methods—Using a cell culture-adapted genotype 3 strain and primary isolates of genotypes 1–4, we compared viral replication kinetics, sensitivity to drugs, and ability of HEV to activate the innate immune response. We studied HLCs using quantitative reverse-transcriptase PCR and immunofluorescence assay and ELISAs. We used an embryonic stem cell line that can be induced to express the CRISPR-Cas9 machinery to disrupt the peptidylprolyl isomerase A gene (PPIA), encoding cyclophilin A (CYPA)—a protein reported to inhibit replication of cell culture-adapted

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Author contributions: XW, VLDT, JG, DM and CMR conceived and designed the experiments. XW, VLDT, PL, CNT, KX and LA performed the experiments. XW, VLDT, JG, DM and CMR analyzed the data. XW, VLDT, CNT, JG, DM and CMR interpreted the data and wrote the manuscript.

Conflict of interest: The authors have declared that no conflict of interest exists.

HEV. We further modified this line to rescue expression of CYPA before terminal differentiation to HLCs and performed HEV infection studies.

Results—HLCs were permissive for infection by non-adapted, primary isolates of HEV genotypes 1–4. HEV infection of HLCs induced a replication-dependent type III interferon response. Replication of primary HEV isolates, unlike the cell culture-adapted strain, was not affected by disruption of *PPIA* or exposure to the CYPA inhibitor cyclosporine A.

Conclusions—Cell culture adaptations alter the replicative capacities of HEV. HLCs offer an improved, physiologically relevant, and genetically tractable system for studying the replication of primary HEV isolates. HLCs could provide a model to aid development of HEV drugs and a system to guide personalized regimens, especially for patients with chronic hepatitis E who have developed resistance to ribavirin.

Keywords

HLCs; antiviral; primary isolates; personalized medicine

Introduction

Hepatitis E virus (HEV) is among the most common causes of acute hepatitis in the world¹. It is a positive-strand RNA virus and a member of the *Orthohepevirus* genus within the *Hepeviridae* family. Four major genotypes (gt), gt1–4, can infect humans. Gt1 and gt2 are restricted to humans and lead to large outbreaks in developing regions. Gt3 and gt4 can be transmitted zoonotically through the ingestion of infected meat and cause infections also in the developed world (reviewed in²). A single case study suggests that camelid HEV gt7 can be transmitted to humans³. The severity of HEV-associated hepatitis seems to correlate with the status of the host's immune system; but viral factors may also play important roles in the pathogenesis of the disease⁴. Indeed, gt1 and gt2 have vastly different epidemiological patterns from gt3 and gt4. Gt1 and gt2 lead to infections and illness in young healthy individuals, with high fatality rates of pregnant women. Gt3 and gt4 mostly infect the middle-aged and elderly, with males being the most likely to experience severe disease (reviewed in⁵). Altogether, additional studies of HEV genotypes are needed in order to better understand viral transmission and pathogenesis.

The HEV 7.2-kb positive-strand RNA genome harbors at least three open reading frames (ORFs). ORF1 encodes the replicase, ORF2 the capsid protein and ORF3 a small protein involved in virus secretion (reviewed in²). Recently ORF4 has been identified in gt1 isolates which is necessary for RNA replication under conditions of endoplasmic reticulum stress⁶.

A detailed understanding of HEV molecular biology has been hampered by the absence of efficient cell culture systems. Only recently, major advances were made by passaging primary gt3 and gt4 HEV isolates and selecting variants that replicate in carcinoma cells. These infectious, cell culture-adapted viruses have made the study of the entire HEV life cycle possible. Similar efforts have not been as successful for gt1 or gt2 isolates. Emerson and colleagues developed an HEV gt1 cell culture system that supports the full replication cycle of the Sar55 strain, however, this system produces only low virus titers⁷.

The mutations found in the gt3 and gt4 HEV cell culture-adapted viruses are not limited to single base changes. Some also include insertion of sequences derived from human host genes or the viral genome itself into the hypervariable region (HVR) of ORF1^{8–10}. These insertions appear to confer a replicative advantage in cancer cells as they become the dominant viral species upon passaging; however, by doing so they likely alter HEV biology. In this regard, HEV mimics other human hepatotropic viruses such as hepatitis A virus and hepatitis C virus (HCV), which usually also require adaptive mutations to replicate efficiently in cell culture^{11,12}.

Another limitation of current hepatotropic virus cell-culture systems is their heavy reliance on cancer-derived cell lines. These cell lines are typically de-differentiated and have altered metabolic, innate immune, and apoptotic responses. Primary human hepatocytes (PHHs) present an attractive alternative for studying hepatotropic viruses. They are certainly more physiologically relevant. However, PHHs have drawbacks. They are highly variable, expensive, and difficult to maintain and manipulate genetically. There is, however, another alternative: human embryonic (hESC) and induced pluripotent stem cells (iPSC). Unlike PHHs, these cells provide a renewable resource and can be genetically manipulated to create patient-specific disease models. Further, they can be differentiated into many cell types, including hepatocytes. We previously found that hESC- or iPSC-derived hepatocyte-like cells (HLCs) are permissive for hepatitis B virus (HBV) and HCV^{13–15} infection. We and others^{16,17} showed that HLCs are permissive for virus derived from an infectious HEV gt3 Kernow-C1 cDNA clone that was selected after six serial passages (P6) in HepG2 cells. Here we show that HLCs, in contrast to hepatoma cells, are not only permissive for the P6 variant but also for virus derived from an early passage (P1) of the same isolate in which the recombinant genome is only a minor species. Moreover, we found that HLCs are fully permissive for infection and replication of HEV primary isolates of gt1–4 derived from infected animals. Thus, for the first time, HLCs allow *in vitro* studies of non-adapted HEV of all genotypes that infect humans.

Methods

Reagents and antibodies

The following antibodies were used for immunofluorescence staining: anti-OCT3/4 (Stemgent, Cambridge, MA), anti-SEE44 (Stemcell Technologies, Vancouver, Canada), anti-GATA4 (Cell Signaling, Danvers, MA), anti-HNF4 α (Cell Signaling), anti-AFP (Sigma-Aldrich, St. Louis, MO), anti-ALB (Cedarlane, Burlington, Canada), and anti-ORF2 (a kind gift from Suzanne U. Emerson, NIH). The following antibodies were used for Western blot (WB) analysis: anti-HCV NS5A clone 9E10¹⁸, anti-CypA (Santa Cruz, Dallas, TX), and anti-actin HRP (Sigma-Aldrich). IFN- β was purchased from pBL Assay Science (Piscataway, NJ), Sofosbuvir from Acme Bioscience (Palo Alto, CA), and Cyclosporin A, Ribavirin and BX795 from Sigma-Aldrich. Activin-A, Wnt-3A and oncostatin-M were purchased from R&D Systems (Minneapolis, MN), bFGF from Life Technologies, and BMP-4, EGF and HGF were purchased from Peprotech (Rocky Hill, NJ).

Plasmids and cells

Using standard cloning techniques and site-directed mutagenesis, sequences encoding for PPIA-wt and PPIA-R55A were cloned into the pRetroX-TRE3G (Takara Bio, Mountain View, CA). Plasmids encoding the HEV genotype 3 Kernow-C1 p6 strain and S10-3 cells were a kind gift from Suzanne U. Emerson. HepG2/C3A cells were purchased from ATCC. S10-3 and HepG2/C3A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fischer Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fischer Scientific). Human hPSC lines RUES2, HUES8-iCas9 and iPSC3A were a kind gift from Ali Brivanlou (The Rockefeller University), Danwei Huangfu (Memorial Sloan Kettering Cancer Center), and Stephen Duncan (Medical University of South Carolina), respectively. All hPSC lines were cultured in defined, feeder-free maintenance medium mTeSR (Stemcell Technologies) and used between passages 30 and 40.

HEVcc RNA preparation and transfection

Cell culture grown HEV (HEVcc-P6) was generated as previously described¹⁶.

HEV primary isolates

Primary isolates gt1 strain Sar-55 (1.21×10^8 viral RNA copies/ml) and gt2 Mexico-14 (1.65×10^6 viral RNA copies/ml) in 10% fecal suspension from infected macaques and Kernow-C1 P1 supernatant from HepG2 cells (2.3×10^6 viral RNA copies/ml), were a kind gift from Suzanne U. Emerson¹⁹. Primary isolates gt3 strain US-2 (1.6×10^4 viral RNA copies/ml) and gt4 strain TW6196E (9.39×10^3 viral RNA copies/ml) in 10% fecal suspension from infected pigs were a kind gift from Xiang-Jin Meng (Virginia Tech)²⁰.

Generation of human pluripotent stem cell-derived hepatocyte-like cells and HEV infection

ESC or iPSC were differentiated into HLCs as previously described¹⁶ in basal medium (BM) consisting of RPMI 1640, 1% B27 serum-free supplement, and 0.5% non-essential amino acids (all from Life Technologies). First, 2×10^5 cells/cm² cells were differentiated to definitive endoderm by harvesting them with gentle cell dissociation reagent (Stemcell Technologies) and plating into Matrigel-coated culture dishes (Corning, New York, NY) in mTeSR1 medium (Stemcell Technologies). The next day, culture medium was changed to medium A (BM/activin-A/basic fibroblast growth factor [bFGF]/Wnt-3A) for 24h, followed by 72h of culture in medium B (BM/activin-A/bFGF). To induce hepatic differentiation, definitive endoderm cells were re-seeded and cultured in the presence of medium C (BM/bone morphogenic protein 4 [BMP-4]/bFGF) for five days and then in the presence of medium D (BM/epidermal growth factor [EGF]/hepatocyte growth factor [HGF]) for five more days. Cells were further matured in Hepatocyte Culture Medium (HCM, Lonza, Basel, Switzerland) supplemented with oncostatin-M for five to seven days. Cultures with at least 70% of ALB positive cells were used for infection assay. HLCs differentiated in 24-well plates ($\sim 8 \times 10^4$ cells/well) were infected with either cell culture grown or isolate virus at a 1:10 dilution in HCM for 12–16h, followed by removal of the inoculum by thorough but gentle washes with HCM. HLCs were cultured for 7d with medium changes every second

day, prior to harvesting for downstream assays. Inhibitors or drugs were replenished with every medium change at the indicated concentrations.

Generation of PPIA^{KO}-29, iPPIA-WT and iPPIA-R55A cell lines

Guide RNA sequences targeting exon 2 and 5 of *PPIA* locus (Supplemental Table 2) were cloned into the pX458 vector (Addgene, Cambridge, MA), *in vitro* transcribed using MEGAshortscript Kit, and purified using MEGAclear Kit (Thermo Fisher Scientific). The two guide RNAs were transfected into HUES8-iCas9wt cells, which inducibly expressed CRISPR-Cas9²¹ upon treatment with 3 µg/ml Dox. Dox was added to transfected cells for 48h, which were then seeded in clonal limiting dilution and grown on mouse embryonic fibroblasts. WB analysis of 15 out of 36 cell clone lysates did not show any detectable PPIA expression, indicating a homozygote PPIA knockout. Several of these clones were chosen for further analysis of their genomes.

For the generation of Dox-inducible iPPIA-WT and iPPIA-R55A cells, PPIA^{KO}-29 cells were transduced with retroviral particles encoding either PPIA-WT or PPIA-R55A. PPIA-WT or PPIA-R55A expression was controlled by a tetracycline response element and inducibly expressed by Dox-treatment due to the previously knocked-in reverse tetracycline-controlled transactivator element in HUES8-iCas9wt cells²¹. Cell lines iPPIA-WT and iPPIA-R55A were generated upon transduction and selection. For HEV infection studies, cell lines were first differentiated into HLCs in the absence of Dox. Two days prior to HEV infection, 3µg/ml Dox was added to the differentiation media. Dox was then maintained in the media throughout the infection experiment (Figure 5C).

Statistics

Statistical differences were determined using the unpaired two-tailed Student's *t* test using GraphPad PRISM5. A *p* value of <0.05 was considered significant, and *p* values of <0.01 and <0.001 were considered highly significant. Data are presented as means ± standard deviations (SD) of values from three independent experiments, or as indicated in the figure legends.

Human subjects

All protocols involving the use of human tissue were reviewed and exempted by the Rockefeller University Institutional Review Board.

Accession Numbers

All data have been deposited to the Sequence Read Archive (SRA) under accession number GSE97987.

Results

Differentiation of human embryonic and induced pluripotent stem cells to hepatocyte-like cells

A protocol based on chemically defined culture media was used to differentiate the human ESC line RUES2 and the iPSC line iPSC.C3A into HLCs^{15,16}. Briefly, either human

pluripotent cell line (hPSC) was first differentiated to definitive endoderm (DE), which became positive for endodermal markers GATA4 and FoxA2 by day 5 of the differentiation protocol (Figure 1A). DE cells were then differentiated towards the hepatocyte lineage. By day 10, expression of the nuclear hormone receptor HNF4a became detectable, which is essential for the specification of human hepatic progenitor (HepProg) cells²². Further maturation yielded immature hepatocytes (ImHep) as evidenced by expression of the fetal liver marker alpha-fetoprotein (AFP). AFP expression decreased upon further differentiation into HLCs, which became highly positive for the adult hepatocyte marker albumin (ALB) and displayed typical polygonal hepatocyte-like morphology. Further validation included the quantification of mRNA levels of specific markers at the distinct stages (Figure 1B). Throughout the differentiation, albumin and urea secretion (Figure 1C) as well as expression of cytochrome P450 family members, key enzymes responsible for hepatocyte drug metabolism, increased. CYP3A7 is predominantly expressed in the fetal liver. Together with the elevated AFP expression in HLCs and in agreement with previous studies (reviewed in²³), this suggests that HLCs mimic fetal rather than adult hepatocytes. By day 20 of the protocol, HLCs synthesized glycogen as evidenced by periodic acid-Schiff staining (PAS) (Figure 1D) and metabolized indocyanine green (ICG) (Figure 1E).

Acquisition of permissiveness to cell culture-grown HEV infection during hepatic differentiation

We and others recently showed that HLCs are permissive for cell culture grown HEV (HEVcc) P6^{16,17}. In the present study we exposed the cells with HEVcc-P6 at the different stages during HLC differentiation (Figure 2A). In order to control for active replication, we treated the cells with sofosbuvir (SOF) at its 3-fold inhibitory concentration (IC₅₀). SOF is an HCV RNA-dependent RNA polymerase (RdRp) inhibitor for which we recently described an inhibitory effect on HEV replication *in vitro*¹⁶. SOF has a lower IC₅₀ (IC₅₀=1.2μM) than ribavirin (RBV, IC₅₀=8μM), the treatment of choice for chronic HEV patients. SOF acts likely on HEV replication directly by competing with nucleotides for incorporation into the nascent RNA by the HEV polymerase, whereas RBV is a broad antiviral that may act also on the cell by depleting intracellular GTP-pools²⁴.

As the HEVcc-P6 inoculum was prepared from lysates of HEV P6 RNA-transfected S10-3 cells, we also used, as negative control, lysates obtained from S10-3 cells transfected with HEVcc-P6 RNA bearing a GDD-GAD mutation ablating the RdRp active site. As expected, the HEVcc-P6 GAD mutant did not lead to productive infection in HLCs. (Supplemental Figure 1).

Undifferentiated stem cells and DEs were not permissive for HEVcc-P6 virus entry and/or replication (Figure 2A). HepProg cells also appeared to be non-permissive. However, we detected up to 2×10^5 HEV copies per μg of total intracellular RNA in both untreated and SOF-treated infected cells. Whole transcriptome analysis on the cellular stages during HLC differentiation revealed that cathepsin A, an enzyme involved in SOF-activation, is expressed less in HepProgs compared to ImHeps and HLCs (data not shown) explaining the absence of sensitivity HEV to SOF in those cells. Altogether, these results could indicate that HepProgs may support HEV entry and a productive RNA replication, but without more

specific and sensitive assays for HEV entry, other explanations—like non-specific sticking—are possible. In agreement, we did not observe ORF2 positive events in HepProg cells incubated with HEVcc.

Similar to HCV¹⁵, ImHeps supported HEV infection and replication at levels comparable to HLCs (Figure 2A&B). Productive infection was assessed by immunofluorescence staining of intracellular capsid protein ORF2 in both iPSC- or ESC-derived ImHeps and HLCs. HEV infection of either iPSC- or ESC-derived HLCs led to similar numbers of highly ORF2-positive events (0.5–1%). The percentage was equal in both cell types at both stages, further confirming that ImHeps are already permissive for HEV infection. In line with these results, we also observed productive HEVcc-P6 infection of human fetal liver cells (HFLCs; Supplemental Figure 2).

We are fully aware that stem cells and their differentiated progeny from different genetic backgrounds can yield differences in HEV permissiveness, as has been previously observed¹⁷. Yet, in this study we observed similar results for the hESC and iPSC lines tested. For the rest of the study, we therefore continued to use iPSC-derived HLCs (iPSC-HLCs). Supernatants of iPSC-HLCs infected with HEVcc-P6 were used to re-infect naïve iPSC-HLCs. As shown in Figure 2D, naïve iPSC-HLCs were productively infected with progeny virus recovered from the primary iPSC-HLCs infection, albeit at low levels. These data show that infectious HEV particles can be assembled and released by iPSC-HLCs, thus completing the entire virus life cycle.

HEV replication induces an antiviral innate immune response in iPSC-HLCs

We first assessed the replication dynamics of HEV in iPSC-HLCs. HEV RNA levels were monitored in lysates of infected iPSC-HLCs treated with DMSO or SOF up to 9 days p.i. (Figure 3A). HEVcc-P6 replication became detectable at day 3 and plateaued at day 7 p.i. SOF treatment decreased HEV replication in HEVcc-infected iPSC-HLCs until day 9 p.i., when the SOF-treated cells reached similar HEV RNA levels compared to DMSO treated cells. Sequencing of the polymerase region of HEVcc-P6 virus after 9 d SOF treatment did not reveal any dominant mutations. While this does not rule out the emergence of minor resistant species, the low replication levels and the short duration of the experiment argue against the emergence of resistance.

Previous reports based on the use of cancer cells suggest that HEV has developed mechanisms to counteract Interferon (IFN)-mediated host defenses. HEV infected cells could not be cured by even high doses of IFN treatment^{16,24,25} and interferon-stimulated gene (ISG) expression was decreased in HEV replicating cells^{25,26}. HLCs are believed to have a fully functional innate immune response^{13,14}. Therefore, we next determined whether HEVcc-P6 infection and/or replication induced an innate immune response in iPSC-HLCs. We quantified IFN- α , - β , - $\lambda 1$ and - $\lambda 3$ gene expression in HEVcc-P6 infected iPSC-HLC lysates at early (6h) and late (day 9) time points p.i. (Figure 3B–E). Whereas IFN- α expression remained undetectable, IFN- β , - $\lambda 1$ and - $\lambda 3$ expression sharply increased from day 5 p.i. Interestingly, lower IFN- β , - $\lambda 1$ and - $\lambda 3$ levels were induced in the presence of SOF compared to the vehicle control, suggesting an HEV replication-dependent induction. This was further confirmed by the observation that none of the IFNs were induced in

infected cells treated with BX795, an inhibitor of TANK-binding kinase 1 (TBK1) which is an upstream activator of the IFN response pathway²⁸. For further validation, we quantified secreted IFNs in the supernatant of infected iPSC-HLCs. Whereas neither IFN- α nor - β were detected (Supplemental Figure 3), we detected high levels of type III IFN- λ 1 and - λ 3 at days 7 and 9 p.i. (Figure 3F). The low levels of IFN- β and - λ 1/3 measured in the supernatant at 6–12h p.i. did not correlate with an increase in intracellular mRNA levels and likely originated from the S10-3 lysates from which virus was recovered for infection. With the increase in virus replication and concomitant IFN induction, we observed an induction of ISGs such as OAS2, ISG15, and IFITM1 but not MX1 or IFITM3 (Figure 3 G–I and data not shown).

Altogether our data support a model in which HEVcc-P6 replication induces a robust innate immune response in iPSC-HLCs, which does not clear the infection but potentially contains it. This was further corroborated by our observation that treatment with BX795 did not significantly enhance HEV replication at any time point (Figure 3A), but, as expected and consistent with a previous study²⁷, BX795 enhanced HCV replication (Supplemental Figure 8C). We also found that immune competent HFLCs are highly permissive for HEVcc-P6 infection (Supplemental Figure 4), while infection with other viruses, such as HCV, requires inhibition of IFN induction and signaling²⁷.

iPSC-HLCs are permissive for non-cell culture-adapted HEV

The HEVcc-P6 virus used in this study was generated from an infectious cDNA clone, which contains multiple point mutations as well as a 58-amino-acid sequence of the human S17 ribosomal protein inserted in the ORF1 HVR^{8,29}. To examine whether iPSC-HLCs could support replication of non-adapted HEV, we used an early passage (P1) of the gt3 Kernow-C1 isolate²⁹. In the P1 population, the recombinant genome is only a minor species; a P1-derived infectious clone replicated to only low levels in hepatoma cells²⁹, which we further confirmed in HepG2/C3A cells (Supplemental Figure 5). Interestingly, insertion of the HEV P6 S17 sequence into this P1 backbone enhanced replication 5-fold²⁹.

We then compared HEVcc-P6 with Kernow-C1 P1 virus replication kinetics in iPSC-HLCs. As shown in Figure 4B, Kernow-C1 P1 increased sharply by day 5 reaching slightly higher levels than HEVcc-P6 (Figure 4A). Productive Kernow-C1 P1 infection of iPSC-HLCs, was also confirmed at the protein level (Supplemental Figure 6). Next, we compared the permissiveness of iPSC-HLCs and HepG2/C3A cells for non-adapted HEV isolates (Supplemental Figure 5). We used the following: HEV gt1 Sar-55 and gt2 Mexico-14 strains from infected macaques¹⁹, and gt3 US-2 and gt4 TW6196E strains from infected pigs²⁰. As the antibody used in this study does not react with ORF2 protein from all HEV genotypes, a multiplicity of infection for these isolates could not be determined. Moreover, as co-purified fecal components could be either inhibitory or beneficial for HEV infection, we used equal volumes of the isolates to infect iPSC-HLCs or HepG2/C3A cells. Whereas the gt1 isolate replicated in both cell types, the gt2 and gt3 isolates replicated only in iPSC-HLCs and not HepG2/C3A cells (Supplemental Figure 5). Similar results were obtained for S10-3 cells, in which neither the P1 virus, nor the gt2–4 isolates replicated (data not shown). We then assessed the replication kinetics of gt1–4 isolates in infected iPSC-HLCs in the

presence or absence of SOF (Figure 4C–F) to distinguish between productive replication versus RNA carry-over from the inoculum¹⁶. Replication initiated for all viruses at day 3 p.i. and increased sharply by day 5. In contrast to gt2–4 isolates, gt1 replication decreased by day 7. All four genotypes were sensitive to inhibition by SOF. However, unlike gt1 and gt2, replication of gt3 and gt4 was delayed but eventually plateaued at the same level as the DMSO control (Figure 3A and 4C–F). Given the short treatment duration, it seems unlikely that resistance mutations have developed. Analysis of additional isolates will be required to determine if these are true genotype-specific differences, with possible implications for the use of SOF or SOF-related compounds for HEV treatment.

Interestingly, upon iPSC-HLCs infection with the four primary isolates, we observed a differential, most likely replication-dependent, ISG induction (Supplemental Figure 7). Previous attempts to infect HLCs with a serum derived clinical HEV isolate were not successful¹⁷. In agreement with our results, highly infectious HEV particles are shed into feces³⁰ and may therefore represent a better inoculum for infection studies.

Cyclophilin A (PPIA) restricts HEVcc but not primary isolate replication

Recently, cyclosporine A (CsA), an immunosuppressant widely used in organ transplantation to prevent rejection, has been shown to enhance HEVcc-P6 RNA replication in hepatoma cells³¹. CsA binds to cytosolic and ubiquitously expressed cyclophilins to inhibit their activities³²; downregulation of cyclophilin A (PPIA) and B promoted HEVcc-P6 replication in hepatoma cells³¹. In order to assess the effect of CsA on pan-genotype replication of HEV isolates, we used HUES8-iCas9wt cells²¹, a parental stem cell line designed to inducibly express CRISPR-Cas9 in the presence of doxycycline (Dox) to create PPIA knockout cell clones. Cell clone 29 (PPIA^{KO}-29), which was used in further experiments, harbored a 625bp deletion resulting in complete removal of PPIA exons 3 and 4 (Figure 5A). The knockout did not affect the pluripotency of PPIA^{KO}-29 cells, as verified by staining of pluripotent markers Oct-4 and SSEA4 (Figure 5B).

First, the permissiveness of PPIA^{KO}-29 cells for HCV (Supplemental Figure 8) was assessed. HCV replication requires PPIA activity and CsA inhibits HCV replication³³. HUES8-iCas9wt-derived HLCs, but not at their stem cell level, were susceptible to HCV infection (Supplemental Figure 8A). HCV infection in PPIA^{KO}-29-derived HLCs was compromised but could be rescued with ectopic expression of wild-type but not mutant PPIA³⁴. These results confirmed the loss of PPIA activity in PPIA^{KO}-29 cells and their effective differentiation into functional HLCs in the absence of PPIA. This demonstrates that the observed phenotype is due the absence of CypA rather than off-target effects of the guide RNA used to generate PPIA^{KO}-29 cells.

In order to provide regulated rescue of PPIA expression, we generated Dox-inducible iPPIA-WT and iPPIA-R55A cell lines on the PPIA^{KO}-29 cell background (Figure 5C). All hPSC lines exhibited similar hepatocyte differentiation efficiency in the absence or presence of Dox, as evidenced by comparable expression of ALB and HNF4 α (Figure 5D), as well as ALB secretion (Figure 5E). We then used iPPIA-WT- and iPPIA-R55A-derived HLCs for HEV infections. Consistent with previous observations³¹, rescue of PPIA expression inhibited HEVcc-P6 replication by almost one order of magnitude (Figure 5F). In contrast,

Dox-mediated expression of mutant PPIA did not have any effect on HEVcc-P6 replication, suggesting that PPIA isomerase activity may be required. We next examined non-adapted HEV isolates replication. As shown in Figure 5F, the presence of either PPIA-WT or PPIA-R55A did not affect the replication of any isolate tested, despite different replication levels. In order to confirm this observation, we also treated iPSC-derived HLCs with DMSO or CsA and infected them with the different viruses (Figure 5G). In agreement with previous observations³¹, CsA treatment enhanced HEVcc-P6 replication by more than two fold, while it had no or even slightly inhibitory effects on isolate replication. These findings underscore at least one striking difference in virus-host biology between primary and cell culture adapted HEV.

Sofosbuvir enhances ribavirin's inhibition of pan-genotype HEV replication

Given these unexpected observations with CsA, we examined the inhibitory potency of RBV, alone or in combination with SOF, on primary isolate replication in iPSC-HLCs. We infected iPSC-HLCs with HEVcc-P6, Kernow-C1 P1, or gt1–4 isolates and treated them with RBV, with or without SOF at their IC₅₀. Neither of the drugs at the given concentration had cytotoxic effects on iPSC-HLCs (data not shown). As shown in Figure 6, RBV inhibited the replication of all viruses. The gt2 isolate was the most sensitive to the treatment (up to 75% inhibition), while the gt1 isolate was the least sensitive (up to 45% inhibition). Replication of all viruses except gt2 decreased with addition of 1 μ M SOF, confirming and extending our previous observations with HEVcc-P6 virus¹⁶.

Discussion

Studies of human hepatotropic viruses are limited by the availability of physiologically relevant hepatic cell culture systems. Numerous studies have demonstrated the utility of hPSC-derived HLCs for HCV and HBV^{13–15,35–38} investigations. Here, we outline the importance of using this attractive cellular system to investigate HEV. The present study constitutes an important step forward from recent work by Helsen *et al.*¹⁷ and our own study¹⁶ which were both limited to the use of cell culture adapted HEVcc-P6 virus.

HLCs for the studies of pan-genotype, non-cell culture adapted HEV isolates

Fundamental aspects of the four human HEV genotypes and their biology remain unknown, such as those regarding their zoonotic transmission, species and cell tropism, as well as differences in their mechanisms of pathogenesis. Here we show that, in contrast to hepatoma cells, HLCs are permissive for replication of a low passage virus of the Kernow-C1 HEV gt3 strain, as well as for non-adapted HEV isolates of gt1–4. Of note, replication of HEVcc-P6 virus was lower compared to its parent virus Kernow-C1 P1, indicating that cell culture adaptations may impair virus replication in more physiologically relevant hepatocellular systems. This has been seen before for HCV³⁹. Despite infection with different doses, replication of all isolates except gt4 led to accumulation of similar amounts of RNA by day 5. Future studies using more strains and launching infection *via* HEV RNA transfection instead of infection with difficult to standardize fecal samples should allow better comparison of replication kinetics and cellular requirements. We are aware that the strains used in this study represent only a subset of the HEV genotypes found in nature. However,

given the striking success so far with these divergent types it is likely that HLCs will support pan-genotype HEV replication. HLCs therefore provide a useful platform for dissecting the mechanisms by which cell culture-adaptive mutations act. It also provides a foundation for the development of *in vitro* replication systems for all HEV genotypes, in particular for gt2, which is currently not available. This enables molecular studies of genotypes that are prevalent in patients but understudied in cell culture to define and study genotype-specific differences in HEV biology.

HLCs for the studies of innate immune response to HEV infection

Previous studies describing an innate immune response of HEV infection employed cancer cells^{25,40}, which have altered and defective innate immune capabilities. The present manuscript is the first report showing that HEV can evade a functional cellular innate immune response. HEV genome replication, but not simple exposure to virus, stimulated type III IFN expression and triggered ISG induction. Remarkably, HEVcc-P6 virus was not cleared by this response (Figure 3). Treatment with TBK1 inhibitor had no effect on HEV replication, dissimilar to what has been observed for HBV or HCV. These findings agree with previous studies suggesting that HEV has developed mechanism(s) to counteract IFN-mediated host defenses^{26,40} given its inability to be cured by high doses of IFN^{16,24,25} and its persistence in PHH despite inducing a type III IFN response⁴¹.

The cellular innate immune response may influence genotype-dependent replication in cell culture⁴² given the differences observed in replication-dependent ISG induction among the four isolates (Supplemental Figure 7). Of immediate interest will be a more comprehensive examination of gt1 and gt2 strains, since they usually lead to self-limited infections in patients. On the other hand, gt3 and gt4, which can persist in immunocompromised patients, may have an inherent ability to persist despite the induction of a cellular innate immune response. HLCs represent an attractive cellular system for these studies given their innate immune competence and their ability to support replication of non-adapted HEV.

HLCs for personalized HEV disease modeling and pre-clinical evaluation of anti-HEV treatments

Cellular host factors of interest can be altered using CRISPR-Cas9 genome editing, as we did here to knockout PPIA. Rescue experiments with either wild-type or mutated PPIA showed that its isomerase activity may be involved in restricting HEVcc-P6 replication. Surprisingly, we found that replication of the non-adapted viruses was not modulated by the presence of PPIA. Consistent with this finding, treatment with CsA enhanced only HEVcc-P6 replication but not replication of the non-adapted isolates. SOF sensitivity provides another example of differences between primary and cell culture adapted HEV and cellular background. We previously found that a chimeric Sar55/S17 replicon construct was not significantly inhibited by SOF treatment¹⁶. In contrast, in the current study we found that SOF can inhibit primary Sar55 replication.

The ability to genetically modify hPSCs before terminal differentiation to HLCs, together with their permissiveness for virus isolates, will allow patient-specific disease modeling. For example, the $\epsilon 3$ and $\epsilon 4$ alleles of the *apolipoprotein E* (*ApoE*) gene are associated with

protection against HEV infection in non-Hispanic blacks⁴³. HLCs can be used to probe the molecular basis of this protection by engineering *ApoE* polymorphisms into wild-type hPSC backgrounds, followed by HLC differentiation and HEV infection studies. Alternatively, patient-specific HLCs can be produced from clinically interesting cohorts. The HLC system also provides an attractive platform identifying and refining new anti-HEV drugs. Personalized disease modeling and the possibility of testing new drugs on primary isolates may eventually help guide therapy choices for patients with chronic hepatitis E who develop RBV resistance^{10,44,45}. Thus, HLCs constitute a new resource for studying the HEV life cycle and probing host-virus interactions. Our results also underscore the importance of validating findings made with cell culture-adapted HEV strains in more physiologically relevant cell models with non-adapted HEV isolates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge Suzanne U. Emerson and Xiang-Jin Meng for generously sharing reagents and providing invaluable advice. We thank Ali Brivanlou for RUES2 cells, Danwei Huangfu for HUES8-iCas9 cells and Stephen Duncan for iPS.C3A cells. We thank Ype de Jong and Koen Vercauteren for help with the albumin ELISAs and William Schneider for helpful discussions.

Funding: This work was funded by the National Institutes of Health (Grant T-R01 DK085713), the Starr Foundation, anonymous donors and the Robertson Therapeutic Development Fund (to CMR). XW was supported by a postdoctoral fellowship from Bristol-Myers Squibb at The Rockefeller University. VLDT was supported by a postdoctoral fellowship from the German Research Council (Deutsche Forschungsgemeinschaft). PL and KX were supported by the China School Council (CSC). JG was supported by the Gilead Sciences International Research Scholars Program in Liver Disease (Award 2015) and DM by the Swiss National Science Foundation (grant 31003A-156030).

Abbreviations

ALB	albumin
AFP	alpha-fetoprotein
CsA	cyclosporine A
ESC	embryonic stem cells
Dox	doxycycline
iPSC	induced pluripotent stem cells
gt	genotype
HBV	hepatitis B virus
HCV	hepatitis C virus
HEV	hepatitis E virus
HEVcc	cell culture grown HEV

HFLCs	human fetal liver cells
HLCs	hepatocyte-like cells
hPSC	human pluripotent stem cells
HVR	hypervariable region
IFN	interferon
ISG	interferon-stimulated gene
ORF	open reading frame
P1	passage 1
P6	passage 6
PHH	primary human hepatocytes
p.i	post-infection
PPIA	cyclophilin A
p.t	post-transduction
RdRp	RNA-dependent RNA polymerase
RBV	ribavirin
SOF	sofosbuvir
TBK1	TANK-binding kinase 1

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Author names in bold designate shared co-first authorship

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Short summary

Stem cell-derived hepatocyte-like cells are permissive for pan-genotype hepatitis E virus (HEV) isolate infection. Our results reinforce the importance of using physiologically more relevant culture systems for HEV studies.

Figure 1. Differentiation of human pluripotent stem cells to hepatocyte-like cells

(A and B) Immunofluorescence staining and relative mRNA levels of markers at the following stages during HLC-differentiation: induced pluripotent stem cell (iPSCs), definitive endoderm (DE), hepatic progenitor (HepProg), immature (ImHep) and hepatocyte-like cells (HLCs). Scale bars = 100μm. (C and D) Albumin and urea secretion as well as relative mRNA levels of cytochrome P450 genes during HLC differentiation. (E and F) Periodic-Acid-Schiff and indocyanine green staining of iPSC-HLCs compared to iPSCs. Shown are representative results of iPSCs differentiation to HLCs, ESC differentiation yielded similar results.

Figure 2. ImHeps and iPSC-HLCs are permissive for HEV infection

(A) Susceptibility of cells to HEVcc-P6 infection along terminal HLC differentiation. Cells at the indicated stages were infected in the presence or absence of 4μM SOF. Infection was assessed by RT-qPCR 7d p.i. (B) Immunofluorescence staining of RUES2- or iPS.C3A-derived ImHeps or HLCs infected with HEVcc 7d p.i. Cells were stained for AFP (green) and ORF2 (magenta). (C) HEVcc-P6 particles harvested in the supernatant of infected iPSC-HLCs (DMSO treated) 7d p.i. were used for secondary infection of iPSC-HLCs in the presence or absence of 4μM SOF. Secondary HEV infection was assessed by measuring viral RNA genomes in infected cell lysates 7 days p.i. Results represent the mean of six independent experiments ± SD. Statistical analysis was performed by an unpaired Student's *t* test *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Scale bars = 100μm.

Figure 3. HEVcc replication persists in iPSC-HLCs despite a robust interferon induction. (A) HEVcc-P6 RNA replication, (B–E) interferon α – λ gene expression, (F) secreted IFN- λ 1 and - λ 3 and (G–I) ISG gene expression in HEVcc-P6 infected iPSC-HLCs treated with DMSO (solid black line), 4 μ M SOF (red line) or 1 μ M TBK-inhibitor (TBKi, dashed black line). Results represent the mean of four independent experiments $\pm$ SD. Statistical analysis was performed by an unpaired Student's *t* test *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 4. iPSC-HLCs are permissive for non-cell culture adapted HEV and primary virus isolates

Equal volumes of (A) HEVcc-P6, (B) Kernow-C1 P1 virus and (C-F) HEV primary isolates gt1-4 were used to infect iPSC-HLCs treated with DMSO (black line) or 4μM SOF (red line). HEV genome copies were quantified in lysates of infected cells at indicated time points p.i. by qRT-PCR. Results represent the mean of four independent experiments ± SD. Statistical analysis was performed by an unpaired Student's *t* test *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 5. Cyclophilin A (PPIA) isomerase activity restricts HEVcc but not primary isolate replication

(A) PPIA knock-out strategy to generate PPIA^{KO}-29 cells. (B) Immunofluorescence staining of pluripotent markers OCT4 (magenta) and SSEA4 (green) in HUES8-iCas9wt cells and PPIA^{KO}-29 cells. (C) Rescue strategy and WB analysis of Dox-inducible expression of wild-type (iPPIA-WT) or mutated PPIA (iPPIA-R55A) in PPIA^{KO}-29 cells. (D and E) Immunofluorescence staining of ALB (green) and HNF4α (magenta) and ALB secretion during 48hrs in the supernatant of HLCs derived from indicated cell line treated with or without 3μg/ml Dox. (F) Infection with HEVcc-P6 and primary isolates in iPPIA-WT (black

bars) or iPPIA-R55A (grey bars) -derived HLCs treated with (open bars) or without (full bars) 3µg/ml Dox. (G) Infection of wild-type iPSC-HLCs treated with DMSO (black bars) or 5µg/ml cyclosporine (orange bars) with indicated virus. HEV RNA in cell lysates was quantified at 7d p.i. Results represent the mean of four independent experiments $\pm$ SD. Statistical analysis was performed by an unpaired Student's *t* test *: $p < 0.05$; **: $p < 0.01$. Scale bars = 100µm.

Figure 6. Sofosbuvir potentiates ribavirin inhibition of primary isolate replication

Infection of HEVcc-P6 and primary isolates in iPSC-HLCs treated with 10 μ M RBV +/- 1 μ M SOF. Drugs were added 24hrs p.i. and replaced with medium changes every second day. HEV replication was measured in intracellular lysates at 7d p.i. by qRT-PCR and shown as relative to DMSO treated infected iPSC-HLCs. Results represent the mean of four independent experiments $\pm$ SD. Statistical analysis was performed by an unpaired Student's *t* test *: $p < 0.05$; **: $p < 0.01$.

Table-1

Primers used for qRT-PCR

Gene	Forward primer (5'—3')	Reverse primer (5'—3')
HEV	GGTGGTTTCTGGGGTGAC	AGGGGTGGTTGGATGAA
RPS11	GCCGAGACTATCTGCACTAC	ATGTCCAGCCTCAGAACTTC
IFN α	GCCTCGCCCTTTGCTTTACT	CTGTGGGTCTCAGGGAGATCA
IFN β	ATGACCAACAAGTGCTCCTCC	GGAATCCAAGCAAGTTGTAGCTC
IL28B	TAAGAGGGCCAAAGATGCCTT	CTGGTCCAAGACATCCCCC
IL29	GTGACTTTGGTGCTAGGCTTG	GCCTCAGGTCCCAATTCCC
MX1	GGATTGAAGGATGCTGTCTT	CAAGGTGGAGCGATTCTGA
OAS2	ACAGCGAGGGTAAATCCTTGA	CAGTCTGGTGAGTTTGCAGT
IFITM1	CCAAGGTCCACCGTGATTAAC	ACCAGTTCAAGAAGAGGGTGTT
IFITM3	TGTCGTCTGGTCCCTGTTCAAC	ACCTTCACGGAGTAGGCGAATG
ISG15	CTGTTCTGGCTGACCTTCG	GGCTTGAGGCCGTACTCC
CXCL10	AGGAACCTCCAGTCTCAGCA	ATTTTGCTCCCCTCTGGTTT

Table-2 Guide RNA sequence used for generating PPIA^{KO}

guide RNA	Forward primer (5'—3')	Reverse primer (5'—3')
Exon-2	CACCGACAAGGTCCCAAAGACAGC	AAACGCTGTCTTTGGGACCTTGTC
Exon-5	CACCGCAACACTCTTAAC TCAAACG	AAACCGTTTGAGTTAAGAGTGTGC