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Stem cell-derived hepatocytes: A novel model for hepatitis E virus replication

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Background & Aims: Yearly, approximately 20 million people become infected with the hepatitis E virus (HEV) resulting in over 3 million cases of acute hepatitis. Although HEV-mediated hepatitis is usually self-limiting, severe cases of fulminant hepatitis as well as chronic infections have been reported, resulting annually in an estimated 60,000 deaths. We studied whether pluripotent stem cell (PSC)-derived hepatocytes, mesodermal and/or neuroprogenitor cells support HEV replication.

Methods: Human PSC were differentiated towards hepatocyte-like cells, mesodermal cells and neuroprogenitors and subsequently infected with HEV. Infection and replication of HEV was analyzed by qRT-PCR, RNA *in situ* hybridization, negative strand RT-PCR, production of infectious virions and transfection with a transient HEV reporter replicon.

Results: PSC-derived hepatocytes supported the complete replication cycle of HEV, as demonstrated by the intracellular presence of positive and negative strand HEV RNA and the production of infectious virions. The replication of the virus in these cells was inhibited by the antiviral drugs ribavirin and interferon-α2b. In contrast to PSC-derived hepatocytes, PSC-derived mesodermal cells and neuroprogenitors only supported HEV replication upon transfection with a HEV subgenomic replicon.

Conclusion: We demonstrate that PSC can be used to study the hepatotropism of HEV infection. The complete replication cycle of HEV can be recapitulated in infected PSC-derived hepatocytes. By contrast other germ layer cells support intracellular replication but are not infectable with HEV. Thus the early steps in the viral cycle are the main determinant governing HEV tissue tropism. PSC-hepatocytes offer a physiological relevant tool to study the biology of HEV infection and replication and may aid in the design of therapeutic strategies.

Introduction

In 1978, a novel non-A, non-B hepatitis virus was discovered which was identified in 1983 as the hepatitis E virus (HEV). HEV is an important and emerging cause of acute self-limiting hepatitis [1,2]. However, fulminant cases of hepatitis may occur particular in pregnant women with mortality rates up to 20–30%. In immunocompromised solid-organ transplant recipients and HIV-infected patients the virus may result in chronic hepatitis, which evolves, in some patients, rapidly to cirrhosis, graft loss and death [3–8].

In vitro HEV culture systems have only recently been established. Therefore relatively little information is available on the biology of HEV infection and replication, for example the mechanism by which HEV enters the host cell remains elusive. It is believed that following entry in the cell, HEV replicates in the cytoplasm through a negative strand RNA ((−)ssRNA) intermediate synthesized by the viral RNA-dependent RNA polymerase (RdRp) [9]. Although HEV is a hepatotropic virus, there is evidence that it may also replicate in extrahepatic sites; for instance, HEV RNA has been detected in cerebrospinal fluid, possibly linking HEV infection to neurological conditions that are occasionally observed in patients with (chronic) HEV infections [10,11].

In recent years, HEV strains have been isolated from fecal specimens of patients with fulminant (JE03-1760F strain) and

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Abbreviations: (+)ssRNA, positive strand RNA; (−)ssRNA, negative strand RNA; αSMA, alpha smooth muscle actin; AAT, alpha 1-antitrypsin; AFP, alpha fetoprotein; BLBP, brain lipid-binding protein; BMP4, bone morphogenetic protein 4; COL1A1, collagen type 1 alpha 1; DLX2, distal-less homeobox 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGF, fibroblast growth factor; FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hESC, human embryonic stem cells; HEV, hepatitis E virus; hiPSC, human induced pluripotent stem cells; HNF4α, hepatocyte nuclear factor 4; IFN, interferon alpha; iMEF, inactivated mouse embryonic mouse fibroblasts; LOX1, leech homeobox 1; NIM, neural induction medium; NMM, neural maintenance medium; ORF, open reading frame; PAX6, PDGFRβ, beta-type platelet-derived growth factor receptor; PSC, pluripotent stem cells; RBV, ribavirin; RdRp, RNA-dependent RNA polymerase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SEM, standard error of the mean; UTR, untranslated region.

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chronic hepatitis (Kernow-C1 strain) that replicated efficiently in cell culture [12–14]. Cell lines that are used to study the molecular biology of HEV are the hepatoma cell lines HuH7, HepG2/C3A, PLC/PRF/5, HepaRG and surprisingly the lung-derived A549 adenocarcinoma cell line [12–15]. These transformed cell lines are physiologically less relevant to study HEV replication than primary hepatocytes, the latter are however not readily available. In general hepatoma cell lines poorly express drug metabolizing enzymes and might lack certain host factors that are important to study infection with hepatotropic viruses. For instance, hepatoma cell lines can only be infected with the hepatitis B virus when they overexpress the sodium taurocholate co-transporting polypeptide [16–19]. Although human primary hepatocytes would be the best cell source to study HEV, they are short in supply.

Human embryonic (hESC)- and induced pluripotent stem cell (hiPSC)-derived hepatocytes are a valuable alternative to primary hepatocytes. Compared to primary hepatocytes, hESC and hiPSC have numerous advantages, including their capacity to self-renew long-term without loss of differentiation potential, their potential to differentiate to any given cell type and their ability to generate patient-specific disease models [20–23]. Although stem cell-derived hepatocytes mimic fetal but not adult hepatocytes [24], numerous studies have demonstrated the use of differentiated hepatocytes to study drug-induced liver toxicity [25–28]. Moreover, hiPSC-derived hepatocytes make it possible to model liver diseases *in vitro* and to assess patient-specific drug responses [29,30]. hESC- and hiPSC-derived hepatocyte-like cells (hPSC-hepatocytes) may also be valuable cell culture models to study the infection of hepatocytes with hepatotropic viruses and parasites [31]. Others and we previously demonstrated that hESC/hiPSC- hepatocytes can be infected with the hepatitis C virus [32–34] and that such cultures offer a model to study virus-host interactions [35]. Furthermore, PSC-hepatocytes have been shown to be susceptible to infection with the hepatitis B virus [36].

Here, we demonstrate that PSC-hepatocytes support the complete HEV replication cycle, infection, replication and generation of infectious virions, making this an attractive and relevant *in vitro* model system (in non-cancerous hepatocytes) to study the biology of HEV replication that and to aid in the design of therapeutic strategies against the virus. Because PSC can not only differentiate into hepatocytes, but also to mesodermal and neuroprogenitors (NPCs), this PSC-derived model also allowed to demonstrate that non-endodermal germ-line progeny do not allow HEV entry, even if they do support HEV replication upon transfection with a subgenomic HEV replicon.

Materials and methods

Virus

Wild-type and 1634R infectious HEV stocks (Kernow-C1 p6, genotype 3, GenBank accession number JQ679013) [13] were derived from plasmid DNA as described [37–39].

Virus inoculation

Day 20 PSC-derived hepatocyte progeny was infected with 300 µl HEV stock diluted to 3×10^7 viral RNA copies/ml per well and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. After 24 h, the inoculum was removed and cells were washed 5 times with 500 µl of DMEM, before addition of 500 µl of hepatocyte differentiation medium. Medium was changed every other day by collecting 300 µl and replacing it with 350 µl fresh hepatocyte differentiation medium. Infection of mesodermal and neural progenitor cell differentiations was performed similarly. Infection experiments were ended 12 days post-infection. Supernatant was collected every other day during medium changes while cells were lysed with 350 µl RLT buffer (Qiagen, Hilden) 4, 8, 10 and 12 days post-infection. An identical protocol was used for a clinical plasma sample from an acutely HEV genotype 3-infected patient (a kind gift from Heiner Wedemeyer, Hannover, Germany). The plasma sample was diluted 1:3 in hepatocyte differentiation medium and subsequently used for inoculation.

Viral infection inhibition experiments

Ribavirin (ICN Pharmaceuticals, Quebec) and interferon alpha 2b (IFN; Intron-A®, Schering-Plough, Kenilworth, NJ) were used to inhibit viral replication. Ribavirin (100 µM) and interferon (1000 U/ml) were added to the differentiation medium starting at the time of the inoculation until the end of the infection experiment.

Reinfection assays

HepG2/C3A cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24 h at 37 °C. Day 8, 10 and day 12 culture medium samples from HEV-infected stem cell-derived hepatocytes (a fixed volume of 400 µl for each sample) were diluted with 500 µl of DMEM supplemented with 10% FBS and inoculated on HepG2/C3A cultures. Infection was allowed to proceed for 4 h at 35 °C. Afterwards, inoculum was removed, cell layers were washed three times with 2 ml of phosphate-buffered saline, 2.5 ml of DMEM with 10% FBS and 1% pen/strep was added and cultures were incubated at 35 °C for 20 days with regular changing of the medium as described [37]. After 20 days, cellular lysates were prepared as described.

QuantiGene ViewRNA fluorescence in situ hybridization (FISH)

RNA FISH was performed using the QuantiGene ViewRNA protocol. Briefly, infected or uninfected stem cell-derived hepatocytes or HepG2/C3A cells were fixed with 4% formaldehyde for 30 min at room temperature. After fixation, cells were permeabilized with detergent solution for 5 min (Affymetrix, Santa Clara, CA) and treated with proteinase K (Affymetrix) for 10 min. Cells were hybridized for 3 h at 40 °C with a Quantigene ViewRNA designed probe covering the region 858–1791 of ORF1 of the HEV clone Kernow-C1 p6 (Accession number HQ389543). After hybridization the signal was amplified by sequential reaction of the PreAmplifier and the Amplifier mix (Affymetrix) followed by conjugation with the fluorescent dye-conjugated label probe (Affymetrix). Cells were counterstained with DAPI (Affymetrix). Images were taken by the AxioImagerZ.1 fluorescence microscope.

HEV replicon replication

Genotype 3 reporter replicon viral RNA was derived from a plasmid encoding Kernow-C1 p6/luc, (kind gift from Suzanne U. Emerson) [13]. Viral RNA was *in vitro* transcribed from MluI-linearized plasmid DNA with the RiboMAX Large Scale RNA Production System-T7 (Promega) and capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). HuH7 cells were seeded into 24-well plates at 4×10^4 cells per well, mesoderm cells and neuroprogenitors were seeded at a density of 2×10^5 cells per 24 well. Cells were transfected with capped RNA transcripts (200 ng per well) 24 h later using Lipofectin (Life Technologies) according to the manufacturer's instructions. Transfected cells were incubated at 37 °C and 30 µl of cell culture medium was removed from each well and stored at −80 °C every day. After 3 days, media were thawed and Gaussia luciferase activity was measured in 20 µl culture medium with the Renilla luciferase assay system (Promega). For mesodermal cell differentiations, luminescence signal was normalized for the approximate number of seeded cells where necessary.

Statistics

Data values represent average ± standard error of the mean (SEM) and were analyzed by the two-tailed Student's *t* test. p values <0.05 (*), p <0.01 (**), p <0.001 (***) and p <0.0001 (****) were considered statistically significant.

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Results

Stem cell-derived hepatocyte cultures support the full HEV replication cycle

The pluripotency of hESC (H9) and hiPSC (BJ1) lines was first confirmed (Supplementary Fig. 2). PSCs were differentiated towards hepatocyte-like cells (hPSC-hepatocytes) during a 20-day differentiation protocol. Immunofluorescence staining, flow cytometry and gene expression analysis demonstrated that PSC-hepatocytes were positive for AFP, HNF4α, AAT and albumin, and secreted albumin and AAT (Supplementary Fig. 3).

PSC-hepatocytes were inoculated with Kernow-C1 p6 genotype 3 HEV that carries a G1634R mutation in the C-terminal region of the HEV polymerase. We recently reported that this mutation increases the replication capacity of genotype 3 HEV *in vitro* [38]. HEV RNA was detected (by qRT-PCR) in the culture medium of infected hESC-hepatocytes on day 2 after infection and increased overtime from $1.5 \pm 0.4 \times 10^5$ to $5.1 \pm 1 \times 10^5$ viral RNA copies ml^{-1} on day 12 after infection (Fig. 1A). It should be noted that the copy numbers were not corrected for culture medium replacement. Indeed, every other day, half of the culture medium was replaced, which accounts for a final dilution factor of about 40. Viral RNA was also detected on day 4, 8, 10 and 12 after infection in lysates of the infected hESC-hepatocytes and the viral RNA copy number remained constant during this time-span (Fig. 1A). The number of RNA copies in the supernatant of hiPSC-hepatocytes increased from $6 \pm 2 \times 10^4$ to $2.9 \pm 0.5 \times 10^5$ RNA copies ml^{-1} between day 2 and 12 after infection (Fig. 1A), and remained stable at the intracellular level (Fig. 1B). Although the differentiation capacity of hESC and hiPSC was comparable (Supplementary Fig. 3), intracellular HEV RNA copy numbers in hiPSC-hepatocytes were significantly lower (Fig. 1A). This might be explained by the presence of a stronger innate immune response in hiPSC-hepatocytes. Indeed we detected a higher level of transcripts for the IFN response genes, *EIF2AK2*, *MX1*, *ISG15* and *IFNβ* in hiPSC-hepatocytes compared with hESC-hepatocytes (10 days post-infection) (Fig. 1B).

As control, hPSC-hepatocytes were inoculated with a UV-inactivated virus stock. No HEV RNA copies were detected intracellularly or in the culture medium of the infected hPSC-hepatocytes (data not shown). The characteristics of the infection of hPSC-hepatocytes by HEV was directly compared with that of the established HEV model in HepG2/C3A cells [13]. HepG2/C3A cells were infected under the same conditions as hPSC-hepatocytes (Fig. 1A). At the intracellular level there was only a slight difference between the viral RNA levels detected in infected hPSC-derived hepatocytes and the infected HepG2/C3A cells. Between day 2 and day 6 post-infection, HEV RNA copy numbers in supernatants of HepG2/C3A cells were comparable with those in PSC-hepatocyte supernatants. However, the number of RNA copies continued to increase in supernatants of infected HepG2/C3A cells whereas levels reached a plateau in infected hPSC-hepatocytes from day 4–6 onwards. This might be explained by the continued proliferation of HepG2/C3A cells during the infection process whereas hPSC-hepatocytes are mostly non-dividing. Alternatively, the fact that the Kernow-C1 p6 strain has been adapted to the specific environment of HepG2/C3A cells through six consecutive passages might explain this difference [13].

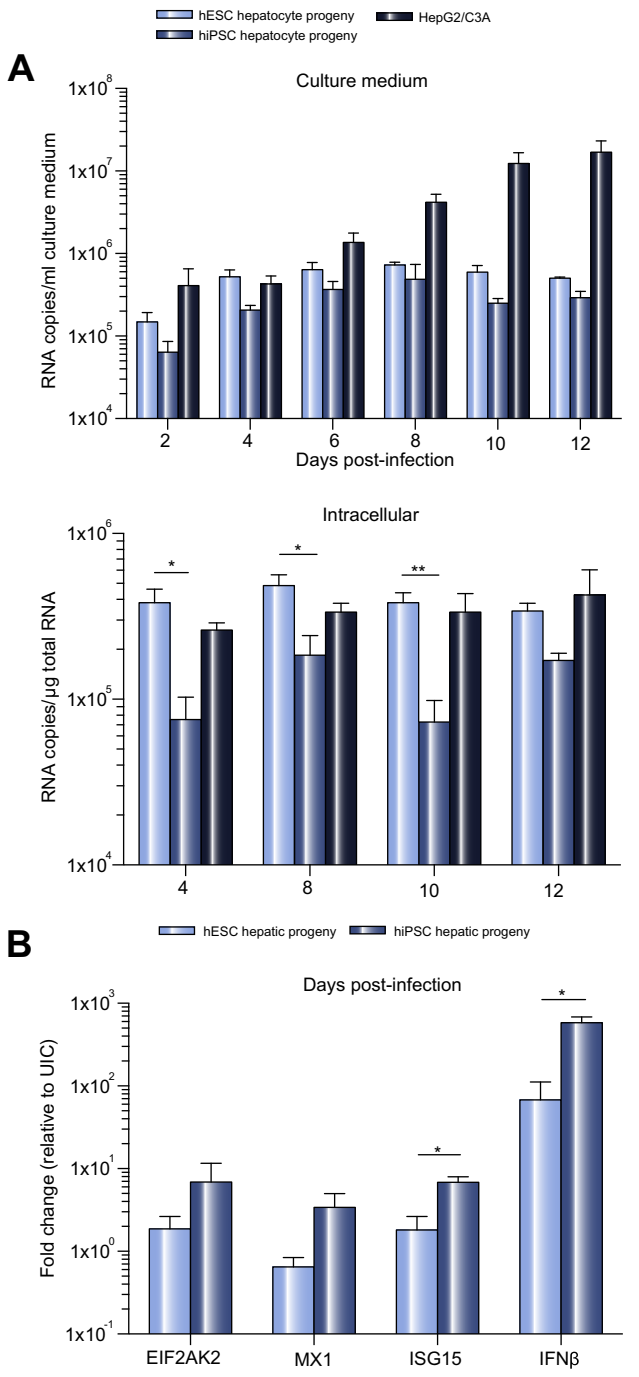

Fig. 1. HEV infection of hESC- and hiPSC-derived hepatocyte cultures. (A) After inoculation of day 20 hESC (n = 6), hiPSC (n = 3)-hepatocytes and HepG2/C3A cells (n = 3), HEV RNA was detected in the culture supernatant on the indicated time points and quantified by qRT-PCR. HEV RNA was quantified in intracellular lysates 4, 8, 10 and 12 days after infection. Results were expressed as the mean of six (hESC), three (hiPSC) and three (hepG2/C3A) independent experiments ± SEM. (B) qRT-PCR results 10 days after infection for innate immune response genes demonstrated higher expression of interferon response genes in hiPSC-hepatocytes compared to hESC-hepatocytes. Results represent the mean of three independent experiments ± SEM relative to uninfected control cells (UIC).

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Finally, we inoculated hPSC-hepatocytes with serum from an acutely infected genotype 3 HEV patient. However, no robust HEV replication was observed, which is not unexpected, since *in vitro* culturing of clinical isolates of HEV has proven to be extremely difficult, even in primary hepatocytes [45,46]

Both (+)ss and (−)ss HEV RNA is detected in the infected hepatic progeny

Next RNA *in situ* hybridization was used to localize (+)ss viral RNA in hPSC-hepatocytes. This technique specifically detects the presence of HEV RNA by hybridization of an RNA probe to the positive-sense viral RNA. Small clusters of positive cells were detected 10 days post-infection in infected hPSC-hepatocytes and HepG2/C3A cells, but not in mock-infected control cells or infected cultures that had been stained without the HEV probe (Fig. 2A; Supplementary Fig. 4).

Because HEV replicates, akin to other (+)ssRNA viruses, through a (−)ssRNA intermediate, the presence of negative strand RNA is the ultimate proof of active viral replication [47]. To this end, we used a strand-specific RT-PCR protocol. No (+)ssRNA or (−)ssRNA was detected in non-infected lysates and culture medium collected from mock-infected cells (Fig. 2B). (+)ssRNA was detected in the lysates and the supernatant of HEV-infected hESC- and hiPSC-hepatocytes whereas (−)ssRNA was only detected in the lysates, but not in the supernatant of infected hESC- and hiPSC-hepatocytes. This confirms that the hPSC-hepatocytes support HEV replication (Fig. 2B).

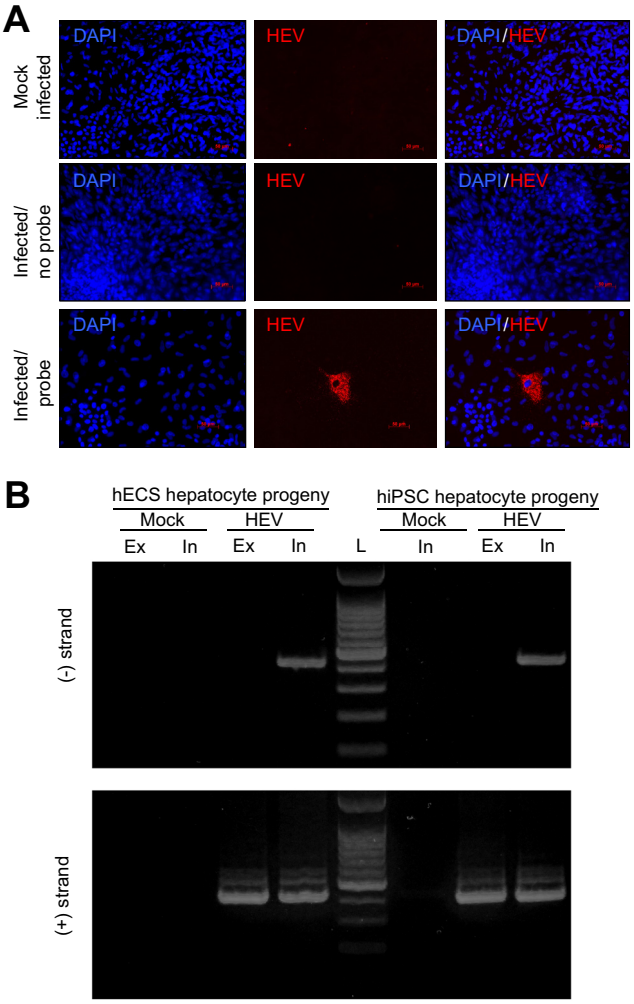

Fig. 2. Detection of both (+)ss and (−)ss HEV RNA in hepatocyte progeny. (A) Ten days after infecting ESC-derived hepatocytes, cells were fixed and stained for HEV RNA by *in situ* RNA hybridization. Mock-infected and infected cells stained without HEV RNA probe were used as negative controls. Images are representative of three independent experiments (Scale bar = 50 μm). (B) Strand-specific RT-PCR demonstrated that negative strand HEV RNA was detected in the intracellular lysates of ESC- and iPSC-hepatocytes while positive-sense RNA was detected in all inoculated cultures in both culture medium samples and lysates. Images are representative of three independent experiments. Ex, RNA extracted from culture medium samples; In, RNA extracted from lysates; L, 100 basepair DNA ladder (Promega).

Inhibition of HEV replication

To further corroborate the observation that HEV replicates in hPSC-derived hepatocyte progeny, the effect of the replication inhibitors ribavirin (RBV) and IFN was studied [37]. Treatment of HEV-infected hepatocyte progeny with 100 μM ribavirin or 1000 U/ml IFN markedly and significantly reduced HEV RNA copies number both intra- and extracellularly in infected hESC- (Fig. 3A) and hiPSC-hepatocytes (Fig. 3B). The observed lag time in the antiviral effects of IFN and RBV in hPSC-hepatocytes at day 4 post-infection could be due to the detection of residual viral RNA remaining from the inoculum that has attached to the cell, but has not been internalized. By day 8 post-infection, these remnants are probably degraded or released, explaining the strong antiviral effect observed at this point. Differences between hESC- and hiPSC-hepatocytes could be due to subtle differences in the cell membrane composition of these cells, resulting in differences in virus attachment.

Comparison of the replication efficiency of wild-type and 1634R mutant HEV in hESC-derived hepatocyte progeny

We recently demonstrated that a G1634R mutation in the HEV polymerase enhances the *in vitro* HEV replication in HepG2/C3A and HuH7 cells [38]. Because of this replication advantage, initial studies were performed with HEV genotype 3 containing the 1634R mutation. In line with these results, the HEV RNA copy number in hPSC-hepatocytes or supernatant following infection with wild-type HEV was consistently lower than in cultures infected with mutant HEV, although this did not reach statistical significance (Supplementary Fig. 5).

hESC-derived hepatocyte progeny infected with 1634R mutant HEV produces infectious virus capable of re-infecting hepatoma cells

Detection of infectious virus in culture supernatant of infected hPSC-hepatocytes provides evidence that these cells support the full replication cycle of HEV. Additional confirmation was obtained by assessing whether HEV infected PSC-hepatocyte supernatant contained infectious virions. When day 8, 10 or 12 culture medium of wild-type HEV-infected hESC-hepatocytes (three independent experiments) was used for reinfection of HepG2/C3A, in only one of the 9 samples detectable levels of intracellular HEV RNA were measured. By contrast, culture

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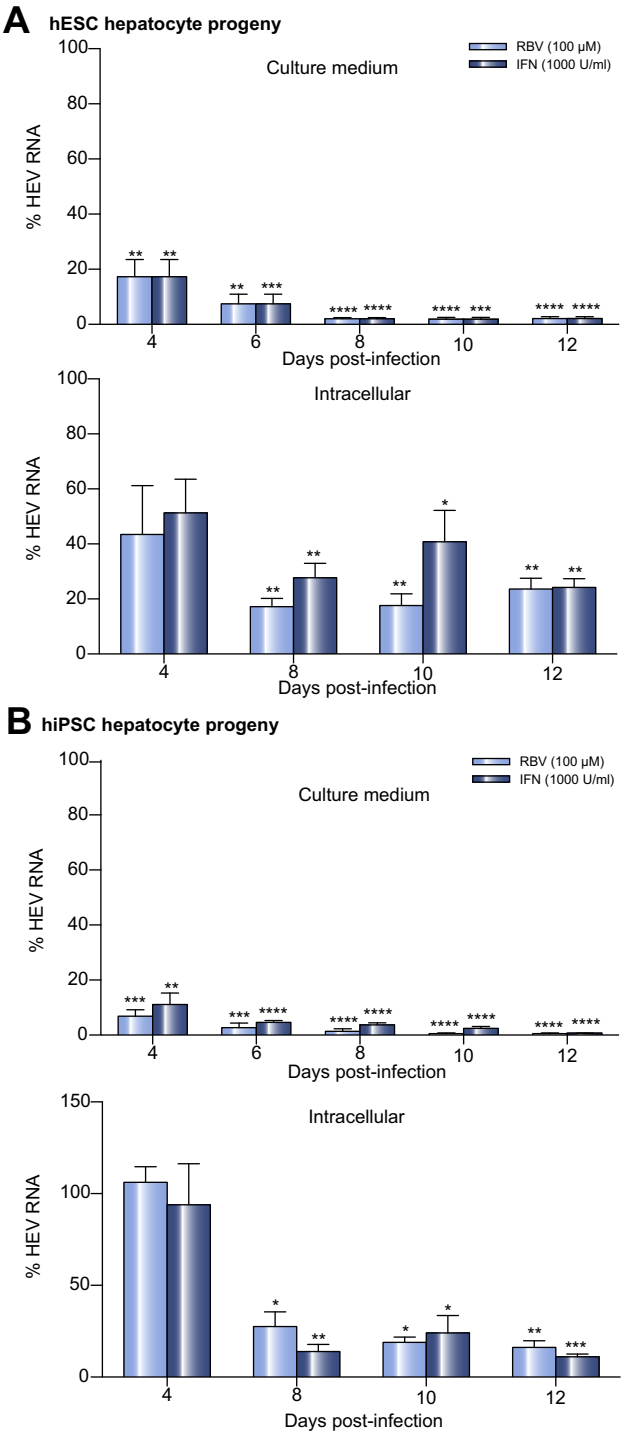

Fig. 3. Inhibition of HEV replication. (A) HEV replication was inhibited hESC-hepatocytes cells by 100 μM ribavirin (RBV) and 1000 U/ml interferon alpha (IFN) added from the inoculation until the end of the infection experiment. (B) Similarly, infected hiPSC-hepatocytes treated with 100 μM RBV and 1000 U/ml IFN displayed strongly inhibited HEV replication as analyzed by qRT-PCR. The percentage HEV replication represents the number of RNA copies in the treated condition relative to the number in the untreated condition. Results represent the mean of three independent experiments ± SEM.

supernatant collected from medium of hESC-hepatocytes that had been infected with mutant R1634 HEV, resulted in infection in 4 of 9 HepG2/C3A cultures. The moderate number of successful re-infections may be explained by the rather low viral titer in the culture medium combined with the relatively low efficiency of infection of HepG2/C3A cells [13,14]. Nevertheless, the infectivity of the culture medium collected from mutant 1634R HEV-infected hESC-hepatocytes provides further evidence that these cells support the complete HEV replication cycle, including the production of infectious viral particles.

hPSC-derived mesoderm and neuroprogenitor cells do not support complete HEV replication

To assess whether HEV infection and replication occurs specifically in hPSC-derived hepatocytes and not in other cell types, hESC-derived mesodermal cells (hESC-mesoderm) and differentiating NPCs (hESC-NPCs) were generated. Flow cytometry analysis of the hESC-mesoderm demonstrated that >95% of cells stained positive for PDGFRβ, a marker expressed by mesenchymal cells (Supplementary Fig. 6A). qRT-PCR analysis a 10–100 fold increase in levels of mesoderm specific transcripts (alpha smooth muscle actin (*αSMA*), leech homeobox 1 (*LOX1*) and collagen type 1 alpha 1 (*COL1A1*) (Supplementary Fig. 6B)). Immunofluorescence staining confirmed the presence of αSMA while there was no detectable expression of AAT, HNF4α, albumin or AFP (Supplementary Fig. 6C). qRT-PCR analysis of the hESC-NPCs demonstrated a 10–1000 fold increase in transcripts for NPC-specific transcripts (distal-less homeobox 2 (*DLX2*), *PAX6* (paired box 6) and brain lipid-binding protein (*BLBP*)) (Supplementary Fig. 6D). In addition, hESC-NPCs stained positive for nestin and PAX6 but negative for AAT, HNF4α, albumin or AFP (Supplementary Fig. 6E).

hESC-mesoderm and -NPCs were infected under the same conditions that were used to infect hPSC-hepatocytes. On day 2 post-infection, viral HEV RNA was detectable in the culture supernatant of hESC-mesoderm and -NPCs, however from day 4 onwards the number of RNA copies in both cultures decreased significantly, which was in contrast to the situation in culture medium of hESC-hepatocytes (Fig. 4). Similarly, HEV RNA copy numbers in cell extracts of hESC-mesoderm and -NPCs decreased progressively (Fig. 4). Neither RBV nor IFN had a significant effect on the viral RNA levels, suggesting that the viral RNA detected was not the result of HEV replication but rather a remnant of the inoculum (Fig. 5A). Moreover, no negative strand RNA was detected in the lysates of infected hESC-mesoderm and -NPCs, confirming that these non-endodermal lineages do not support replication of the HEV (Fig. 5B).

The entry of HEV is restricted to hPSC-derived hepatocytes

To examine whether the apparent failure to infect hESC-mesoderm and -NPCs with HEV occurs at the level of RNA replication or rather at an early step of the viral life cycle, both cell populations were transfected with the genotype 3 subgenomic p6/luc HEV replicon to examine whether these non-endodermal progeny cells support intracellular HEV replication. Interestingly, we demonstrated that both hESC-mesoderm and -NPCs do support replication of the HEV replicon, which for the hESC-NPCs,

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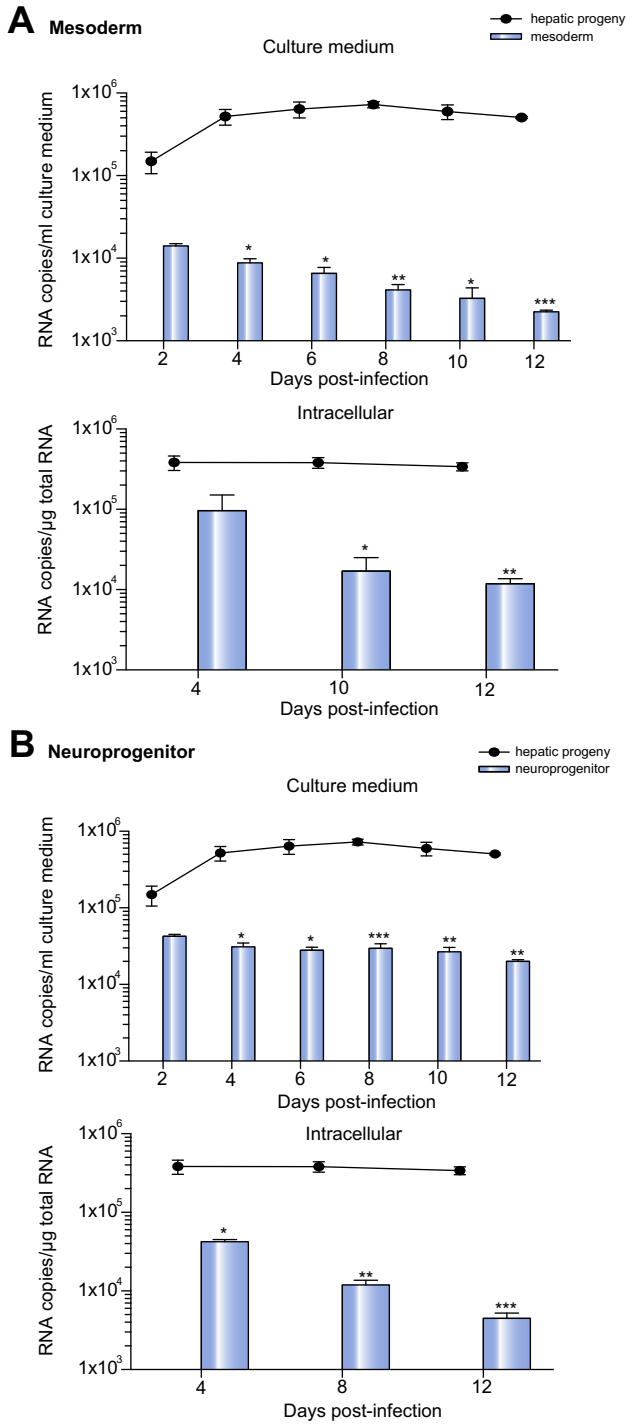

Fig. 4. HEV specifically infects stem cell-derived hepatocytes. (A and B) Viral HEV RNA was detected in the culture medium and intracellular lysates hPSC-mesoderm and NPCs on the indicated time points by qRT-PCR. Results were expressed as the mean of two (mesoderm) and three (NPC) independent experiments ± SEM. Also shown (line above the bar graphs), is the level of HEV RNA copies in the supernatant of hPSC-hepatocytes and in the cellular lysate of hPSC-hepatocytes, as reference. The line graph represents the data also shown in Fig. 1.

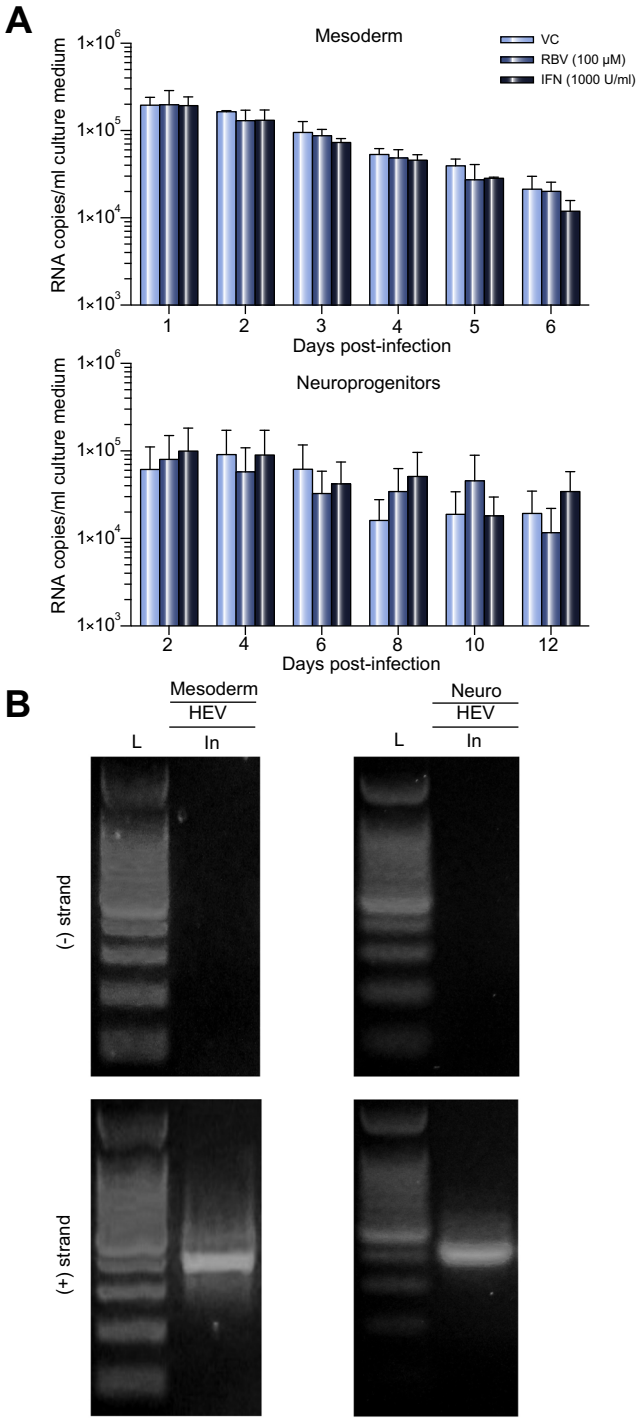

Fig. 5. Mesoderm and neuroprogenitors cells do not support HEV replication. (A) Neither ribavirin (RBV) nor interferon alpha (IFN) had an effect on the detected viral HEV viral RNA levels in hPSC-mesoderm and NPCs. Results represent the mean of three independent experiments ± SEM. (B) No negative strand RNA was detected in the lysates of hPSC-mesoderm and NPCs. Positive strand RNA was however detected in lysates of both cell types. Images are representative of three independent experiments. Ex, RNA extracted from culture medium samples; In, RNA extracted from lysates; L, 100 basepair DNA ladder (Promega).

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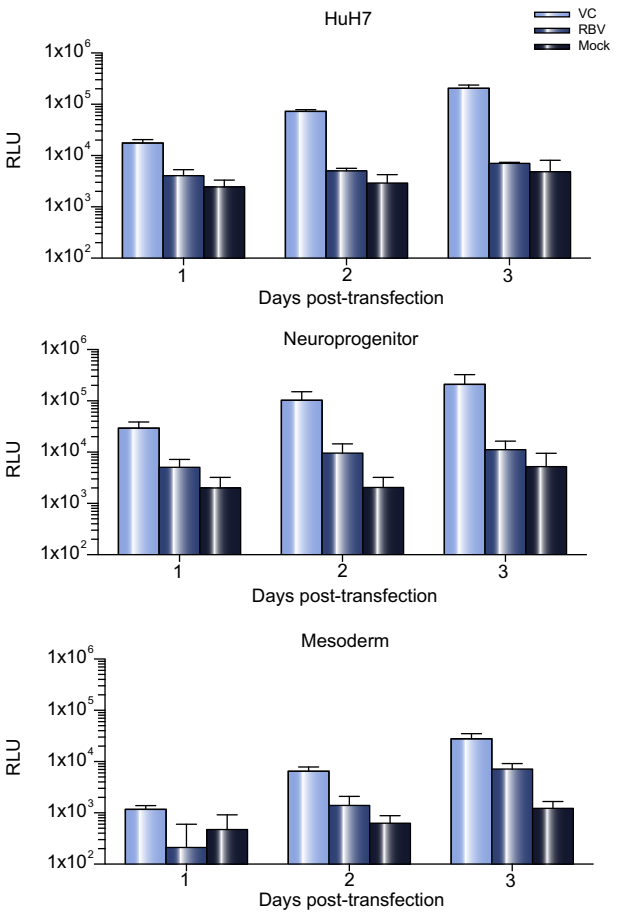

Fig. 6. Mesoderm and neuroprogenitors cells do support HEV replication upon transfection. HuH7 hepatoma, hPSC-mesoderm and NPCs were transfected with capped HEV p6/luc replicon RNA and Gaussia luciferase activity was measured in the culture medium at 1, 2 or 3 days post transfection in the presence or absence of ribavirin (RBV). Solid intracellular replication can be observed in all three cell types. Results represent the mean of three independent experiments ± SEM. VC, virus control; RBV, ribavirin; RLU, relative light units.

approached levels that were also observed in the HuH7 hepatoma control cell line (Fig. 6). Furthermore, HEV replicon replication was in all three cell lines efficiently inhibited by RBV treatment. This provides compelling evidence that the inability of HEV to enter into hESC-mesoderm and -NPCs is the main reason why these cells are not supporting the complete replication cycle of the HEV.

Discussion

Hepatitis E is an emerging disease in developing and industrialized countries that typically results in acute self-limiting hepatitis, but severe cases of both chronic and acute hepatitis have been reported [48]. Here, we demonstrate that differentiated hepatocyte progeny from PSC support the complete replication of HEV and thus forms a physiologically relevant model system to study the biology of HEV replication and strategies to inhibit viral replication. Moreover, the pluripotent nature of these stem cells, allowed us to generate mesodermal and ectodermal cell types. We demonstrate that these cannot be infected although they support intracellular replication of the virus. This cell culture model (s) thus provides an elegant model to study the mechanism underlying the hepatotropism of HEV.

Interest in HEV research is growing due to the increasing medical importance of HEV infections, yet currently available cell culture models rely mostly on the use of hepatoma cells. These are however transformed cell lines that may not completely recapitulate the biology of HEV replication in the infected hepatocyte. Therefore, other cell sources are being explored for their ability to support HEV infection and replication.

In 2012, a porcine ESC line was differentiated into hepatocytes and successfully infected with swine fecal samples containing HEV of genotype 3 [15]. Viral HEV RNA was detected until 35 days after infection in the supernatant and started to increase from day 8 onwards. Similarly to our findings, intracellular viral RNA levels remained constant in the swine ESC-derived hepatocytes, but were lower compared to what we observed in hPSC-hepatocytes. Negative strand RNA was also detected in porcine-derived hepatocytes, but because the low viral titer in the culture medium, reinfection experiments failed. By contrast, we detected in the supernatant of almost half of infected hESC-hepatocytes infectious viral progeny capable of re-infecting HepG2/C3A cells. Thus human PSC-hepatocytes support the entire HEV replication cycle and thus allow the study of the complete biology of HEV infection and replication in a physiologically relevant context. In line with this, a recently published paper employed hPSC-hepatocytes to study the effect of sofosbuvir on HEV replication [49].

The number of HEV RNA copies in the culture medium was lower in primary HEV-infected hPSC-hepatocytes compared to HepG2/C3A cells that had been infected under the same conditions. Noteworthy, intracellularly and during the initial 4–6 days after infection RNA copy levels were comparable in both systems. We hypothesize that the difference in viral kinetics may in part be due to the fact that hepatoma cells continue to proliferate in culture, whereas hPSC-hepatocytes are chiefly quiescent. An alternative explanation might be that that the Kernow-C1 p6 strain, used in this study, was passaged six times in HepG2/C3A cells and may thus have been adapted to the HepG2/C3A cellular environment [13]. The advantage of using PSCs is that they can easily be obtained from different donors whereas hepatoma cell lines are derived from a single donor. Availability of multiple donor hPSC-hepatocytes should allow further examination of specific virus-host interactions. Indeed, we found that there were differences in innate immune response following HEV infection of two PSC-derived hepatocyte progeny. Use of additional patient-specific iPSC lines should permit to further elaborate on this observation to fully understand virus-host interactions. Unfortunately, we were unable to cultivate HEV from a clinical plasma sample in our system, which confirms the challenging nature of HEV propagation in tissue culture.

In the hPSC-hepatocytes, HEV replication was efficiently inhibited by RBV and IFN, the only drugs currently available to treat HEV infections. Both drugs decreased (both intra- and extracellular) levels of viral RNA but did not fully eliminate the virus from the hPSC-hepatocytes. Complete eradication may require either longer treatment, more potent drugs, or may only be possible in the context of an intact immune response in the infected host.

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Initially, infection studies were performed with genotype 3 HEV carrying an additional G1634R mutation. Comparison of the replication efficiency between wild-type and mutant HEV in hESC-hepatocytes revealed the 1634R mutant might have some replication advantage in these cells as well, although the differences were not statistically significant. However, only supernatant collected from hESC-hepatocytes that had been infected with the mutant HEV was able to re-infect HepG2/C3A cells, which is consistent with the notion that 1634R mutant results in an increased viral fitness. hESC-hepatocytes might therefore also serve as a valuable tool to study the importance of mutations identified in HEV-infected patients that became non-responsive to ribavirin treatment.

In hPSC-mesoderm and NPCs, (−)ssRNA was not detectable and levels of (+)ssRNA decreased over time, suggesting that these cells do not support *in vitro* the full cycle of HEV infection and replication, which would be consistent with the tropism seen *in vivo*. However, as successful infection of a culture system depends not only on viral replication but also on the ability of the virus to enter the cell, i.e. infect the cell, we explored whether hPSC-mesoderm and NPCs support HEV subgenomic replication following transfection of the replicon in the host cells. hPSC-mesoderm and NPCs could support intracellular HEV replication, and therefore strongly suggest that entry is the limiting factor to allow the complete replication cycle of the HEV and may be the key determinant of HEV tissue tropism. Although some studies have demonstrated that HEV infection can be associated with neurological symptoms such as Guillain-Barré syndrome [50–52], it is not clear whether such neurological symptoms are a direct or indirect consequence of HEV infection. In one study, HEV viral RNA was detected in cerebrospinal fluid of some patients and viral sequences found in the cerebrospinal fluid were different from those detected in serum [53]. This may suggest that HEV variants with neurotropic capacity may indeed exist. The HEV infection model in PSC (from which we can generate many different mature cell types) will allow studying cell/tissue tropisms of HEV as well as the underlying mechanism.

In conclusion, PSCs have the unique capacity to differentiate into any given cell type, which allows to study HEV infection not only in hPSC-hepatocytes but also to discover possible extrahepatic sites of HEV infection and replication. We here demonstrate that hPSC-mesoderm and NPCs support HEV replication, but only upon transfection of viral (replicon) RNA, suggesting that the *in vivo* hepatotropism seen is likely due to the inability of HEV to enter non-hepatic cells. On the contrary, hepatocyte-like cells generated from hPSCs are a valuable alternative for hepatoma cell lines and human primary hepatocytes in the study of the viral biology of the HEV.

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Conflict of interest

The authors who have taken part in this study declared that they do not have any conflict of interest with respect to this manuscript.

Authors' contributions

N.H. and Y.D. conceived the study, generated the data and wrote the paper. J.P. contributed to the infection experiments. K.D was involved in study design and experimental set-up. R.B., M.C., P.S.B. contributed to the mesoderm differentiations. C.C. contributed to the differentiation of neural progenitor cells. J.N. and C.M.V. designed the study and wrote the paper. All the authors have read and edited the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.11.013.

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