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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.

Keywords: Pluripotent stem cells; Hepatitis E virus; Cell culture model; Hepatocytes.

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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.

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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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.