Stem Cell–Derived Culture Models of Hepatitis E Virus Infection

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Similar to other hepatotropic viruses, hepatitis E virus (HEV) has been notoriously difficult to propagate in cell culture, limiting studies to unravel its biology. Recently, major advances have been made by passaging primary HEV isolates and selecting variants that replicate efficiently in carcinoma cells. These adaptations, however, can alter HEV biology. We have explored human embryonic or induced pluripotent stem cell (hESC/iPSC)-derived hepatocyte-like cells (HLCs) as an alternative to conventional hepatoma and hepatocyte cell culture systems for HEV studies. HLCs are permissive for nonadapted HEV isolate genotypes (gt)1–4 replication and can be readily genetically manipulated. HLCs, therefore, enable studies of pan-genotype HEV biology and will serve as a platform for testing anti-HEV treatments. Finally, we discuss how hepatocyte polarity is likely an important factor in the maturation and spread of infectious HEV particles.

Compared with the other hepatotropic viruses, hepatitis E virus (HEV) infections show dramatic heterogeneity and little is known about the underlying viral or host determinants that affect disease severity and outcome. Four major genotypes (gt), 1–4, can infect humans. gt1 and gt2 are transmitted fecal–orally and lead to large outbreaks in developing countries. In gt1- and gt2-endemic areas, most infections and illness occur in young healthy individuals, with high fatality rates in pregnant women. gt3 and gt4 infect animals and can be transmitted to humans zoonotically through the ingestion of infected meat. These genotypes can cause acute or chronic infections in the developing and developed world, with middle-aged to elderly males the most likely to experience severe disease (reviewed in Hartl et al. 2016). A single case study

suggests that camelid HEV gt7 may also be zoonotic and transmitted to humans (Lee et al. 2016). Of the human hepatitis viruses, HEV is the only one in which animal reservoirs can seed human infections. HEV infections primarily cause hepatitis but can also feature extrahepatic disorders, such as neurological syndromes, renal injury, pancreatitis, and hematological problems (Kamar et al. 2014). This parallels observations in HEV-infected animals in which replicative RNA intermediates are found in numerous organs such as lymph node, colon, small intestine, brain, and spleen (Williams et al. 2001; Liu et al. 2013), and in cell culture in which different lung, intestinal, and neuronal tissue cell lines are permissive for HEV infection (reviewed in Okamoto 2011). To date, the determinants of species and tissue tropism of HEV are unknown.

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Similar to other hepatotropic viruses, HEV is notoriously difficult to propagate in conventional cell cultures. Although this has limited studies of its biology in the past, recent advances in cell culture systems, including in particular the use of human embryonic or induced pluripotent stem cell (hESC/iPSC)-derived hepatocyte-like cells (HLCs), offer substantial promise for unraveling how HEV interacts with and replicates within the hepatocyte.

HETEROLOGOUS EXPRESSION SYSTEMS FOR THE STUDY OF HEV

HEV has a 7.2-kb, positive-strand RNA genome that harbors three partially overlapping open reading frames (ORFs). ORF1 encodes all the functions required for viral RNA replication and is encoded by the full-length genome RNA (Agrawal et al. 2001; Magden et al. 2001; Karpe and Lole 2010). In addition, viral replication leads to the generation of a shorter, subgenomic RNA of 2.2 kb that encodes the capsid protein, ORF2 (Surjit et al. 2004; Xing et al. 2010), and the small phosphoprotein, ORF3, which is involved in virus secretion (Yamada et al. 2009; Nagashima et al. 2011; Ding et al. 2017). Recently, a novel ORF (ORF4) was identified within the ORF1 sequence, which is largely conserved among gt1 isolates (Nair et al. 2016; also see Kenney and Meng 2018).

As discussed in the next paragraph, HEV replication systems for studies in cell culture have been developed only recently. Before that, a range of different heterologous expression systems was used to study the HEV proteins and life cycle in cell culture, with disparate results. For example, HEV ORF1 has been expressed in *Escherichia coli*, insect, or mammalian cells using a variety of different systems, including vaccinia virus-, baculovirus- and T7-, CMV-, or SV40-driven plasmid expression. In these studies, the ORF1 translation product was either found non-processed as a large 185 kDa polypeptide or processed with varying cleavage products, depending on the system used (Ansari et al. 2000; Ropp et al. 2000; Suppiah et al. 2011; Perttinen et al. 2013). Likewise, the predicted HEV ORF2 product contains three putative *N*-glycosylation sites

and a putative signal peptide at its carboxyl terminus. Yet, reports describing its glycosylation status, and the importance of these features of ORF2 during infectious particle formation using different mammalian expression systems, are inconsistent (Jameel et al. 1996; Torresi et al. 1999; Zafrullah et al. 1999; Surjit et al. 2007; Graff et al. 2008; de Oya et al. 2012). Further, protein interaction studies with ORF2 and ORF3 are largely based on yeast two-hybrid systems (Tyagi et al. 2001; Roy et al. 2004; Osterman et al. 2015; Tian et al. 2017). Altogether, the description of basic steps of the HEV life cycle in authentic replication systems and relevant cell culture models is missing.

CELL CULTURE ADAPTATIONS ENABLING MOLECULAR STUDIES OF HEV BIOLOGY IN CARCINOMA CELLS

Only recently, major advances have been made by passaging primary gt3 and gt4 HEV isolates in human hepatoma cells and selecting for variants that replicate efficiently in culture. These cell culture-adapted variants have enabled studies of the entire HEV life cycle and triggered a wealth of new molecular studies that have advanced knowledge on HEV entry (Yin et al. 2016), replication (Emerson et al. 2013; Zhou et al. 2014; Kenney and Meng 2015), assembly (Kenney et al. 2015; Ding et al. 2017), release (Nagashima et al. 2011, 2014; Ding et al. 2017), cellular tropism (Shukla et al. 2011; Nguyen et al. 2014; Drave et al. 2016; Helsen et al. 2016; Zhou et al. 2017), innate immune evasion (Dong et al. 2012; Nan et al. 2014; Todt et al. 2016a; Sooryanarain et al. 2017), and preclinical drug evaluation (Debing et al. 2014; Wang et al. 2014; Zhou et al. 2014; Dao Thi et al. 2016).

HEV is therefore similar to other human hepatotropic viruses like hepatitis A virus (HAV) and hepatitis C virus (HCV) that usually require adaptive mutations to replicate efficiently in cell culture (Emerson et al. 1991; Lohmann et al. 2001). However, in contrast to HAV and HCV, the HEV cell culture-adapted variants include not only single base changes (Tanaka et al. 2007, 2009; Okamoto 2011), but also insertions of human messenger RNA (mRNA)

sequences or portions of the HEV genome (Shukla et al. 2011; Nguyen et al. 2012; John et al. 2014; Debing et al. 2016). These inserted sequences appear to confer a replication advantage in hepatoma cells. For example, Emerson and colleagues recently developed an infectious HEV gt3 Kernow-C1 complementary DNA (cDNA) clone that was derived from a chronically infected HEV patient and selected after six serial passages (P6) in HepG2/C3A cells (Shukla et al. 2011, 2012). This adapted virus contains multiple point mutations as well as a 58-amino-acid segment of the human S17 ribosomal protein fragment inserted in frame in the ORF1-encoded hypervariable region. The early passage (P1) of the Kernow-C1 isolate replicates to only low levels in human hepatoma cells and the recombinant genome is only a minor species (Shukla et al. 2012). Insertion of the S17 sequence into the P1 backbone (Shukla et al. 2012) or into the low replicative gt1 Sar55 strain (Nguyen et al. 2014) increases their ability to replicate in hepatoma cells. Conversely, removal of the S17 sequence from the HEV P6 cDNA clone decreases its replicative ability (Shukla et al. 2012).

These rather dramatic alterations of the viral genome likely alter HEV biology. To our knowledge, similar efforts to adapt gt1 or gt2 isolates to grow in cell culture have not been successful. Emerson and colleagues (2010) developed an HEV gt1 cell culture system that supports the full replication cycle of the Sar55 strain, but, as mentioned above, this system produces only low virus titers. The apparent ability of HEV gt3 and gt4 to adapt to a new cellular environment through relatively simple genetic changes may be an important factor in the ability of these genotypes to cross species barriers.

IMPROVED HEPATOCELLULAR SYSTEMS TO STUDY NONADAPTED HEV BIOLOGY

A major concern of hepatotropic virus studies in cell culture is the widespread use of cancer-derived cell lines. There is no question that these cells have been essential for advancing our basic understanding of hepatotropic virus biology. However, because of their dedifferentiated cancerous nature, they do not necessarily recapitu-

late the proliferative, metabolic, apoptotic, and innate immune pathways found in primary hepatocytes (PHHs). Thus, important cellular factors and pathways influencing authentic virus infection, RNA replication, and progeny virus release may be missing. Indeed, the identification of such factors has proven instrumental in developing improved hepatitis B virus (HBV) and HCV cell culture systems. For example, ectopic expression of the lipid-binding protein SEC14L2 in hepatoma cells renders them permissive for replication of nonadapted clinical HCV isolates (Saeed et al. 2015). HBV can only infect human hepatoma cells when the HBV entry receptor sodium taurocholate cotransporting polypeptide (NTCP) was identified and then ectopically expressed (Yan et al. 2012; Ni et al. 2014).

Recently, endoplasmic reticulum (ER) stress was reported to enhance HEV gt1 replication in hepatoma cells by inducing the translation of HEV ORF4 (Nair et al. 2016). However, it is unclear whether ER stress is also relevant in primary cells or whether this is merely a function of metabolically altered cancer cells. It is likely that the use of cell culture systems that more closely mimic hepatocyte biology *in vivo* will yield more reliable insight into HEV-host biology.

PHHs isolated from adult or fetal livers represent more physiologically relevant HEV cell culture systems. Mature PHHs, as well as human fetal liver cells (HFLCs), support infection of the cell culture–adapted Kernow-C1 P6 strain (Shukla et al. 2012; Wu et al. 2017; Yin et al. 2017). However, donor-to-donor and batch-to-batch variability, limited accessibility, as well as the restricted ability to perform genetic modifications limit the use of PHHs for many research applications. In addition, mature PHHs often dedifferentiate quickly on plating and lose their hepatic phenotype and key functions (Elaut et al. 2006; Khetani and Bhatia 2008). Therefore, the field would benefit from more reliable, reproducible, and renewable hepatocellular systems.

Embryonic stem cells (ESCs) or iPSCs coupled with highly efficient and reproducible differentiation protocols to generate HLCs provide an attractive alternative to both hepatoma cells and PHHs (reviewed in Schwartz et al. 2014). We and others have shown the usefulness of stem

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cell-derived culture systems to study HCV (Yoshida et al. 2011; Roelandt et al. 2012; Schwartz et al. 2012; Wu et al. 2012; Carpentier et al. 2014), HBV (Paganelli et al. 2013; Shlomai et al. 2014; Kaneko et al. 2016; Xia et al. 2017), and HEV (Dao Thi et al. 2016; Helsen et al. 2016; Wu et al. 2017). Although HLCs resemble fetal hepatocytes, they recapitulate more hepatic functions than hepatoma cells. For example, unlike hepatoma cells, HLCs support infection of wild-type HCV (Wu et al. 2012) and, without further genetic manipulation, HBV (Shlomai et al. 2014; Xia et al. 2017). Similarly, we found that HLCs could be infected with natural isolates of HEV gt1–4 (Fig. 1) (Wu et al. 2017). HLCs, therefore, provide a reproducible and genetically tractable platform to study nonadapted HEV, particularly gt2 for which currently no cell culture system exists. These studies should help understand genotype-specific differences in HEV biology and

may provide insights into the mechanism by which cell culture-adaptive mutations act.

In addition, the intrinsic ability of stem cells to give rise to cells of all three germ layers will allow a better understanding of HEV tissue tropism. Helsen et al. (2016) showed that mesodermal and neural progenitor cells do not support HEV replication but some neuronal cell lines (Drave et al. 2016) and iPSC-derived neurons (Zhou et al. 2017) are permissive. Using stem cells to generate terminally differentiated primary cell mimics of various lineages and challenging these cells with HEV may help define and understand the determinants of HEV tissue tropism.

CELL CULTURE ADAPTATIONS ALTER HEV BIOLOGY

Using HLCs, we identified striking differences in virus–host biology between primary and cell

Figure 1. Stem cell culture models of hepatitis E virus (HEV) infection. Cells collected from identified HEV-positive individuals can be reprogrammed to generate induced pluripotent stem cells (iPSCs). Relevant single nucleotide polymorphisms (SNPs) can be repaired or introduced into established human embryonic or induced pluripotent stem cell (hESC)/iPSC lines using CRISPR-Cas9, followed by differentiation to the cell type of interest. Differentiated cells can be directly infected with HEV isolates from patients or animals for modeling virus–host interactions in a dish.

culture–adapted HEV (Wu et al. 2017). In HLCs, the replication of the cell culture–adapted gt3 Kernow-C1 P6 virus was attenuated compared with its parental P1 virus, indicating that cell culture adaptations may impair virus replication in more physiologically relevant hepatocellular systems. This is similar to what has been described for HAV and HCV, in which cell culture–adaptive, replication-enhancing mutations are deleterious for infection in vivo (Funkhouser et al. 1996; Bukh et al. 2002). We also found that cell culture adaptations can alter host factor dependence (Wu et al. 2017). Cyclophilin A (CypA) has been shown to inhibit Kernow-C1 P6 replication in human hepatoma cells (Wang et al. 2014). We confirmed these observations in HLCs. However, when analyzing nonadapted virus, we found that primary isolates as well as the Kernow-C1 P1 virus were not inhibited by CypA nor enhanced by treatment with the calcineurin inhibitor (CNI) cyclosporine A. Other discrepancies are also observed between effects of molecules on HEV replication in vitro and in vivo. Similar to CNIs, mechanistic target of rapamycin (mTOR) inhibitors were reported to promote (Zhou et al. 2014), and mycophenolic acid to inhibit HEV Kernow-C1 P6 replication in vitro (Wang et al. 2014). Yet, in vivo, the time to clear HEV was similar in chronic patients receiving CNIs or mTOR inhibitors, and the use of CNIs or mTOR inhibitors did not influence sustained virologic response rate (SVR) (Kamar et al. 2015). These differences in host factor dependence or drug responsiveness will be important for evaluating future anti-HEV treatments, which can now be validated using cellular systems that allow replication of non-adapted HEV strains, such as HLCs.

A mechanism for the inhibitory role of CypA in cell culture–adapted HEV biology remains to be determined. CypA may interact directly with the HEV proteins, similar to HCV nonstructural protein NS5A (Yang et al. 2010) and be modulated by the presence of the S17 sequence. Future studies might compare CypA levels in hepatoma cells to PHHs. An example for HCV is the study by Harak et al. (2017), who found that adaptive mutations were actually loss-of-function mutations preventing phos-

phatidylinositol 4-kinase III α overactivation, an enzyme whose expression is high in hepatoma cells compared with PHHs. Moreover, CypA prolyl isomerase activity is a critical positive regulator of RIG-I-mediated innate antiviral immune responses (Liu et al. 2017). Whether the cell culture–adapted strain is better or differentially sensed by the innate immune system would be an interesting subject for future studies. Given their immune competence, HLCs represent an attractive cellular system to study the role of innate immunity in genotype-dependent persistence and/or pathogenesis.

PERSONALIZED HEV MODELS USING STEM CELL TECHNOLOGY

It is clear that host genetics play a pivotal role in determining susceptibility to infectious diseases. Many virus infection–related Mendelian immune deficiencies inherited as monogenic traits have been uncovered in recent years (Casanova 2015). The ability to generate patient-specific iPSCs (Takahashi et al. 2007) to create personalized cell culture models has the potential to broaden our ability to model these phenotypes in vitro to understand the mechanisms by which they act. The spectrum of genetic polymorphisms and their impact on infectious disease outcome is likely vast and only partially explored.

A striking feature of HEV infections is their varied and unpredictable outcome, which poses challenges in the clinic. HEV variants found in patients have been described to impact disease pathogenesis, clinical outcome, and antiviral resistance (reviewed in van Tong et al. 2016). For example, drug-resistant, high-fitness variants have been identified in ribavirin-treated chronic HEV patients, leading to treatment failure (Dalton and Kamar 2016; Debing et al. 2016; Todt et al. 2016b). HEV quasispecies diversity is associated with persistent infection and progression to liver fibrosis (Lhomme et al. 2012). Pregnant women in South Asia, but not in high-income countries such as France (Renou et al. 2014), experience more severe disease on HEV infection, leading to increased maternal and fetal morbidity and mortality (reviewed in

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Navaneethan et al. 2008). Nutritional and immunological factors may be important but genetic polymorphisms also may be at play. Future human genetic studies on hepatitis E patient groups may help reveal whether there is a genetic basis for these dramatic differences.

Known examples are the $\epsilon 3$ and $\epsilon 4$ alleles of the apolipoprotein E gene (*APOE*), which are associated with protection against HEV infection in non-Hispanic blacks (Zhang et al. 2015). ApoE protein is also up-regulated in acutely infected pigs, suggesting a role in HEV infection and/or pathogenesis (Rogee et al. 2015). However, the introduction of *APOE* isoforms into the human hepatoma cell line Huh-7.5 does not affect HEV RNA replication or virus production (Weller et al. 2016). Perhaps different results will unfold in a more physiologically relevant cellular environment. This could be achieved by engineering *APOE* polymorphisms into wild-type hESC/iPSCs using CRISPR-Cas9 editing or by generating patient-specific iPSC followed by HLC differentiation and HEV infection (Fig. 1). As iPSCs can be differentiated to many cell types, such studies will not be restricted to characterizing the impact of single nucleotide polymorphisms (SNPs) in HLCs but can be extended to other cell types such as placental or immune cells. The ability to use a patient's own HEV isolate and study its replication without further cell culture adaptation in autologous iPSC-derived HLCs and other cell types will provide a platform for modeling personalized HEV infection, with application not only for understanding HEV-host biology but also evaluating therapeutic options.

HOW DOES CELLULAR POLARITY AFFECT HEV BIOLOGY?

In the liver, hepatocytes form a cell layer that separates sinusoidal blood from the canalicular bile. This way, hepatocytes are engaged in two counter-current flow systems: On one hand, they take up, process, and secrete the sinusoidal blood components and, on the other hand, synthesize and secrete bile (Musch 2014). To mediate these functions, hepatocytes have a unique polarization with multiple basal membranes

facing the sinusoids and multiple apical membranes forming bile canaliculi (Fig. 2A).

Similar to HAV, HEV is an enterically transmitted virus that replicates predominantly in hepatocytes before excretion into feces via bile (Feng et al. 2014). It is assumed that both HEV and HAV enter hepatocytes through their basolateral surface and exit through the apical surface (reviewed in Feng et al. 2014). To support this notion, HEV structural proteins ORF2 and ORF3 are localized at the apical membrane of hepatocytes in chronic HEV patients (Lenggenhager et al. 2017) and infected humanized mice (Allweiss et al. 2016; Sayed et al. 2017). Despite the absence of viral glycoproteins, both viruses acquire a cell host-derived lipid envelope during virus morphogenesis, allowing the virus to bud in a noncytolytic fashion into multivesicular bodies (Emerson et al. 2010; Takahashi et al. 2010; Feng et al. 2013, 2014; Nagashima et al. 2014; Chapuy-Regaud et al. 2017). As a result, cell culture-grown HEV particles released in the cellular supernatant (Nagashima et al. 2011, 2014; Qi et al. 2015; Yin et al. 2016; Chapuy-Regaud et al. 2017) and those circulating in the blood of infected patients (Takahashi et al. 2010) and humanized mice (Allweiss et al. 2016; Sayed et al. 2017) are quasi-enveloped. This envelope decreases cell attachment and thus entry (Yin et al. 2016) but protects the particles from neutralizing antibodies (Takahashi et al. 2010; Chapuy-Regaud et al. 2017) to most likely ensure spread within the infected liver or to other tissues in within the host. In contrast, HEV particles inside infected cells and those excreted into feces are nonenveloped and highly infectious (reviewed in Feng et al. 2014; summarized in Fig. 2), destined to infect the next host. In vitro, treatment with detergent converts quasi-enveloped virions into naked virions (Emerson et al. 2010; Takahashi et al. 2010). In vivo, it is assumed that bile salts of the gastrointestinal tract remove the envelope. However, to date, it remains unknown whether progeny HEV particles bud from the apical membrane as nonenveloped or quasi-enveloped virions.

A subclone of the hepatoma cell line HepG2, when seeded on semipermeable membranes in

Figure 2. Hepatocyte polarity and hepatitis E virus (HEV) secretion. HEV particle release from hepatocytes with (A) hepatic, (B) columnar, or (C) no polarity. Blue boxes in A and B are tight junctions separating apical and basal membranes.

transwells, acquires an epithelial, columnar type of polarization (Fig. 2B). In this configuration, the cells grow as confluent monolayers and develop tight junctions separating the cell's apical membrane facing the top compartment from the basal membrane facing the bottom compartment of the transwell. This system has been used for HAV (Snooks et al. 2008; Hirai-Yuki et al. 2016), HBV (Bhat et al. 2011), and HCV (Belouzard et al. 2017) entry, secretion, and release studies. HAV release from polarized HepG2 cells is vectorial, with a majority of the virus particles released from the basolateral membrane (Hirai-Yuki et al. 2016). This is in contrast to intestinal, polarized Caco-2 cells, from which HAV buds mainly from the apical membrane (Blank et al. 2000). Whether released from the apical or basolateral side, HAV always exits the cells as enveloped virions, which can be converted into nonenveloped particles by mixing with high concentrations of exogenous human bile salt (Hirai-Yuki et al. 2016). A noncancerous, polarized hepatocyte system that can support HEV replication and at the same time apically release bile salts would be ideal, as HepG2 cells are deficient in bile salt synthesis (Everson and Polokoff 1986).

PHH can be grown as sandwich cultures, which allows them to retain their hepatocyte polarization (Fig. 2A); however, in this configuration, the apical membrane and cargo is not accessible because of the closed bile canaliculi. Some evidence suggests that hepatocyte differentiation involves columnar intermediates (Treyer and Musch 2013). Therefore, a significant advance will be achieved if hESC/iPSCs can be differentiated on transwells to generate columnar polarized HLCs (Fig. 2B). Similar to polarized HepG2 cells, this system would enable studies of HEV vectorial release to delineate the differential trafficking routes of HEV to apical and basolateral cell surfaces. It will further allow studies on the role of bile salts in the maturation of virus particles. This system could be also used to determine whether HEV spreads to the neighboring cells through their apical surfaces, which may occur in the proximity of the bile canaliculi *in vivo*. The comparison with noncancerous polarized intestinal epithelial cells such as hESC/iPSC- or adult stem cell-derived intestinal organoids (reviewed in Fatehullah et al. 2016) would also be highly desirable. In contrast to hepatocytes, the virus is believed to bud primarily from intestinal cells through their

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basal membrane to reach the sinusoidal bloodstream and eventually the liver. Yet, similar to HAV, HEV particles bud from HEV-replicating Caco-2 cells primarily from the apical membrane (Emerson et al. 2010). Therefore, more physiologically relevant cellular systems will likely enable an improved and comprehensive modeling of enteric transmission pathways of viruses such as HAV and HEV.

The possibility to differentiate stem cells to HLCs in transwells or other micropatterned configurations (Berger et al. 2015) will allow co-cultures with other stem cell-derived liver resident cell types and pave the way for the development of complex isogenic systems that better model the liver environment. This will enable improved drug development and drug-drug interaction predictions, especially for patients who have already progressed to severe liver disease and who have only limited treatment options.

CONCLUDING REMARKS

Despite the increasing awareness that hepatitis E is an important public health issue, HEV remains an understudied virus. The recent development of infectious clones has opened up new avenues for HEV studies in cell culture. However, basic life cycle steps, from HEV cell entry, over genome replication to particle assembly and release, remain poorly characterized. A pan-genotype, comprehensive characterization of the HEV replicase, including its subcellular localization and cellular cofactors, will likely be necessary for a better understanding of HEV host and tissue tropism as well as for the development of specific anti-HEV therapies. Stem cells providing renewable, genetically tractable, and noncancerous cellular systems hold the promise for advancing these studies. These systems may also aid in answering other fundamental questions, such as the identification of the HEV cell entry receptor(s) and its interaction with naked versus quasi-enveloped particles, genotype-dependent innate immune induction or evasion, which may be at play for the development of chronic hepatitis E, extrahepatic HEV replication and its clinical manifestations, and the role of insertions into the ORF1 hypervari-

able region (HVR), which facilitate replication in hepatoma cells. In addition, the capability to study replication of nonadapted HEV isolates in tandem with autologous, patient-derived iPSCs further enables personalized models of HEV infection. The use of stem cell-derived culture models for studying HEV biology will lead to a better understanding of virus-host interactions and disease pathogenesis and may help develop novel, specific treatments.

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