

Hepatitis E Virus Infection: Pathogenesis and Therapy

Xinying Zhou

The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands.

The research was funded by:

Netherlands Organization for Scientific Research (NWO); Dutch Digestive Foundation (MLDS); Daniel den Hoed Foundation.

Financial support for printing of this thesis was provided by:

Erasmus Postgraduate School Molecular Medicine

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Cover design and layout: Optima Grafische Communicatie

Printed by: Optima Grafische Communicatie

ISBN: 978-94-6169-902-2

Hepatitis E Virus Infection: Pathogenesis and Therapy

Hepatitis E Virus Infectie:
Pathogenese en Therapie

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.A.P. Pols

and in accordance with the decision of the Doctorate Board
The public defense shall be held on

Tuesday 27th September 2016 at 15:30

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Xinying Zhou

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Promoter:

Prof.dr. M. P. Peppelenbosch

Inner Committee:

Prof.dr. R.A. de Man

Prof.dr. P.J. Coffe

Prof.dr. B.C. Jacobs

Copromoter:

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Chapter 1

**General introduction and
outline of the thesis**

Hepatitis E virus (HEV) infection

Hepatitis E virus (HEV), first identified in 1983 ¹, is the causative agent of hepatitis E. It infects about 20 million people and causes 70,000 deaths every year, and HEV infection has emerged as an important public health issue ². Although only one serotype of HEV exists, there are four genotypes (HEV1-4) ³ infecting humans on the basis of classification of the nucleotide sequences of genome (Figure 1) ⁴. HEV Genotypes 1 and 2, obligate human pathogens, are found mainly in developing countries, and commonly transmit through contaminated water in areas with poor sanitary infrastructure. Although they predominately affects young adults, infection with HEV genotype 1 are particularly severe among pregnant women, with resulting high maternal, fetal and neonatal morbidity and mortality rates as high as 25% ⁵.

HEV genotype 3 and 4, that lead to a porcine zoonosis and mostly cause asymptomatic infections (> 90%), are prevalent in industrialized countries, where they are spread mainly through eating undercooked pork or game products ⁷. Since various animal species can serve as reservoirs, including swine, wild boar, deer, mongooses, chickens, rabbits, trout, cattle, camel, and ferrets, its zoonotic potential has raised considerable public health concerns ⁸. Patients of middle-aged or elderly men are particularly prone to develop symptomatic and self-limiting hepatitis ⁹. Although it was initially thought to cause acute infection only, chronic infection by HEV3 has been well documented in immunocompromised individuals, including those with HIV infection, or undergoing chemotherapy for cancer, but most commonly in organ transplant recipients ⁴. A total of 65% of HEV-associated organ transplant recipients fail to clear the viral infection and rapidly develop chronic infection ⁴. In addition, HEV has been found with high frequency in the human blood supply in numerous developed countries, and several cases of transfusion-transmitted infection have been reported ⁹. Thus, HEV infection is a prominent health concern worldwide.

Molecular biology and life-cycle

As the commonest cause of acute viral hepatitis worldwide, HEV is classified in the genus *Orthohepevirus* within the family *Hepeviridae* ¹⁰, containing a positive-sense single-stranded

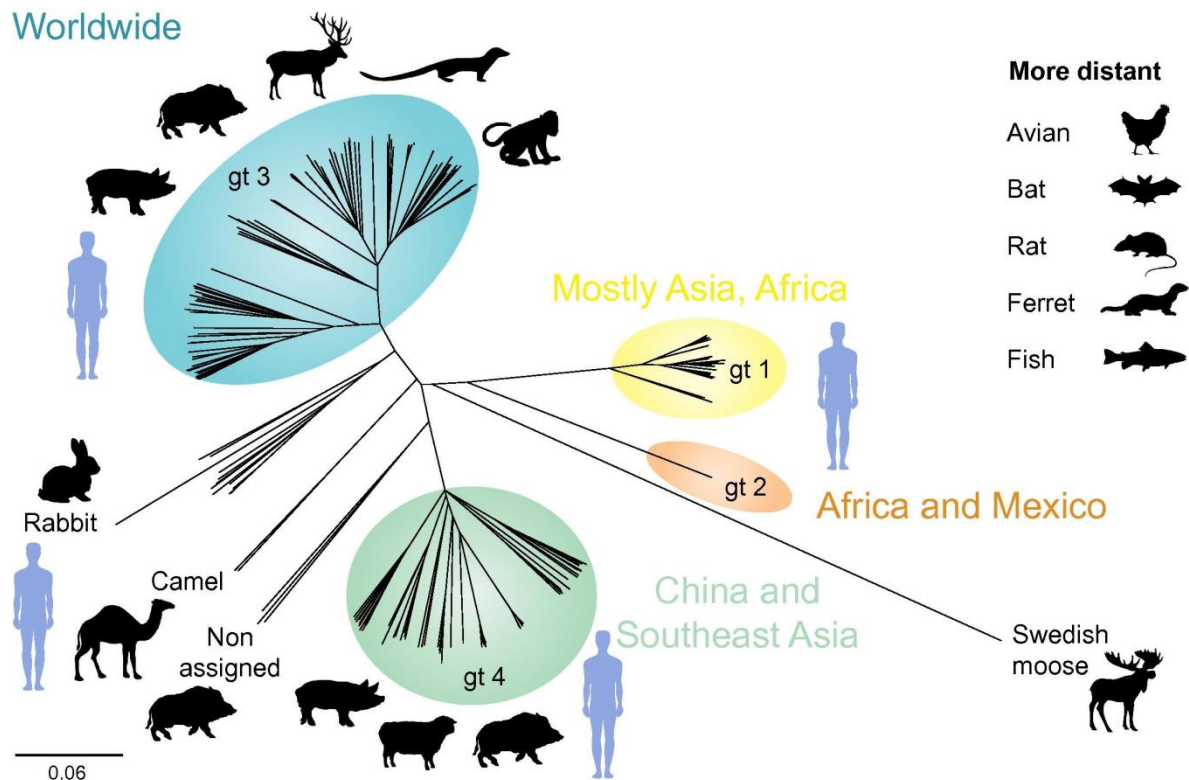


Figure 1. Phylogenetic relationship of hepeviruses identified in various hosts. Genotypes 1 and 2 (gt 1 and 2; yellow and orange, respectively) are restricted to humans and to endemic regions such as Asia, Africa and Mexico, genotypes 3 and 4 (gt 3 and 4; blue and green, respectively) are also found in a wide range of animal species. (Adapted from Yannick Debing, et al. Journal of Hepatology, Review 2016.⁶)

RNA genome of approximate 7,200 nucleotides in length¹¹, and with a cap and poly-(A) tail at its 5' and 3' ends, respectively⁵. It was observed to be a naked 28-34 nm virus-like particle with spikes and inundations¹². The HEV genome contains three open reading frames (ORFs) designated ORF1, ORF2, and ORF3¹³ that encode nonstructural proteins including RNA-dependent RNA polymerase, a capsid protein, and a small protein, respectively (Figure 2)¹⁴. These structures and the junction region between them containing regulatory elements are important for replication of the HEV genome¹¹. The ORF1 nonstructural polyprotein contains domains (starting from the N-terminal end) with methyltransferase (MeT), putative papain-like cysteine protease (PCP), RNA helicase (Hel), and RNA-dependent RNA polymerase (RdRp) activities, which are important for viral replication¹¹. The ORF2 protein consists of three linear domains and forms homodimers, which act as capsomeres and form the viral capsid (Figure 2)¹⁵. The ORF3 protein is required for HEV replication in the host, but not *in vitro*; in addition, it has pleiotropic effects on host cell pathways and plays a role in

viral egress from infected cells ¹⁶. As many other viruses, the life cycle of HEV consists of binding and entry host cells, viral genome uncoating and release, translation of the viral replicase by the host translation machinery, polyprotein processing, viral replication, particle assembly and release ⁶. All these steps might constitute potential avenues for the development of antiviral therapy.

Entry of the virus into the host is believed to be primarily by the oral route via contaminated water or food. Virus first replicates in the intestinal tract from where it reaches the liver presumably via the portal vein. It then replicates in the cytoplasm of hepatocytes and is released into the bile and the blood by an unknown mechanism. The pathogenesis of HEV infection and particularly the severe forms of it remains obscure ¹⁴. Prompting for this research, at first contact with the target cell, HEV, similar to many other viruses, attaches to heparin sulfate proteoglycans followed by entry through as yet unidentified receptor(s) ¹⁸. After clathrin mediated endocytosis, viral positive-strand RNA genome is released into the cytosol. This is followed by translation of ORF1 protein with the help of host translation machinery. Then full-length and a 2.2 kb subgenomic RNAs replicate through a negative-strand RNA intermediate, and ORF2 and ORF3 proteins are translated from the subgenomic RNA ¹⁹. The last step of life-cycle is packaging, assembly and release of newly formed virus. ORF3 protein is likely associated with intracellular membranes and may trigger virion release via the endosomal sorting complexes required for transport (ESCRT) pathway. Recent studies suggest that virus secreted into the bloodstream is associated with the ORF3 protein and wrapped by cellular membranes while virus secreted into the bile is nonenveloped ⁶. However, it is fair to say that many aspects of HEV biology and pathogenesis are still required clarification.

HEV studies have been long hindered because of its difficulty of cultivation and the lack of an efficient cell culture system ^{20, 21}. In 2007, an efficient culture system with PLC/PRF/5 hepatic carcinoma cell lines was established, and A549 lung carcinoma cell lines was appeared proficient for HEV3, isolated from faecal samples of a patient with acute hepatitis in Japan, and sustained a very high titre of HEV RNA ²². These cell lines also permitted the propagation of HEV4 from a faecal extract ²¹ as well as HEV1, HEV3, and HEV4 from serum samples ²³. Subsequently, the Kernow C1 strain of genotype 3 virus, isolated from a chronically infected patient, was adapted to grow in cultured HepG2/C3A cells ²¹. As a state-of-the-art culture model, efficient replication has also been performed in human

hepatoma 7 (HuH7) cell lines²⁰. There are two kinds of models for HEV infection in HuH7 cells: a full-length infectious model and subgenomic replication model. These culture systems are essential for a better understanding of the biology and pathogenesis of HEV and potential targets for the development of antiviral drugs, and have been used for many research studies. Besides, several animal models have been developed for subsequent molecular and pathogenetic studies for HEV infection. The specific pathogen-free pigs have been identified as an alternative animal model for swine HEV3 and 4 infection²⁴. The recent discovery of HEV infection has been observed in numerous animal models including mouse²⁵, rabbit²⁶, three shrew²⁷, mongolian gerbils²⁸, as well as nonhuman primates, such as chimpanzees²⁹ and various macaque species^{30,31}. The advent of these models would now allow further investigation into the biology and pathogenesis of HEV infection, with revealing of its mechanistic details.

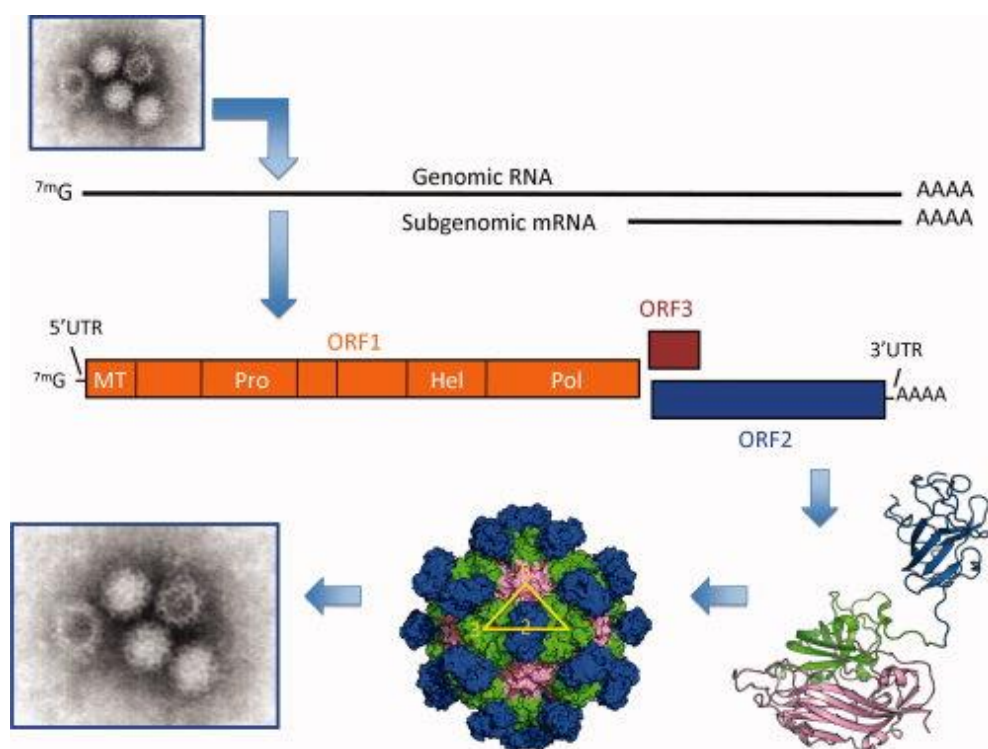


Figure 2. HEV and its genome. HEV particles contain a positive-sense genomic RNA of 7.2 kb (kilobases) that is capped and polyadenylated and carries short 50 and 30 untranslated regions (UTRs). During genome replication, a subgenomic RNA of 2 kb is also produced. The genomic RNA carries three open reading frames (orfs) that encode the nonstructural ORF1 (orange), capsid ORF2 (blue), and regulatory ORF3 (brown) proteins. The ORF1 polyprotein carries various biochemical domains: methyltransferase (MT), protease (Pro), helicase (Hel), and RNA-dependent RNA polymerase (Pol). The ORF2 protein monomer contains three domains (shown in pink, green, and blue) that make up different structural elements on the HEV particles. The icosahedral 2-, 3-, and 5-fold symmetry axes are indicated. (The HEV particle structure is adapted from Tetsuo Yamashita, *et al.*¹⁵. This figure is adapted from Rakesh Aggarwal and Shahid Jameel. *Hepatology*, Review 2011.¹⁷)

Extra-hepatic manifestations

HEV is a primarily hepatotropic virus, which was thought to exclusively infect hepatocytes resulting in hepatocellular necrosis and hepatitis. However, HEV can replicate to some extent in other tissues and HEV infection is associated with a broad range of idiopathic extrahepatic manifestations, including renal injury ³², acute pancreatitis ³³, hematological diseases ³⁴ and a variety of neurological disorders (Figure 3) ³⁵.

Kidney injury. Impaired renal function has been noted in both acute and chronic HEV infections ^{32, 34}. Glomerular disease can be caused by HEV1 and 3 infection, as with other hepatotropic viruses ³². Membrano-proliferative and membranous glomerulonephritis of two different histological patterns of glomerular disease have been observed ³². The pathophysiological mechanisms of HEV associated renal injury are uncertain, but cryoglobulinemia may be an important cause for this and cryoglobulinemia has been documented for patients with chronic infection ^{32, 36}.

Neurological disorders. Among these, neurological disorders have been described as a relatively common, but under-recognized, extrahepatic manifestation related to HEV infection ⁹. An increasing number of central or peripheral nervous system diseases have been documented in patients associated with acute or chronic HEV infection ^{9, 37}, including Guillain-Barré syndrome (GBS) and neuralgic amyotrophy (NA) ^{37, 38}. To date, 91 cases of HEV-associated neurological injury have been documented, from both developed and developing countries (HEV 1 and 3, respectively). Interestingly, HEV RNA could be detected in the cerebrospinal fluid (CSF) of some patients with neurological disorders ³⁵, but the exact incidence and underlying pathogenic mechanisms are not clear yet. Hitherto, the most widely held neuropathogenic hypothesis posits that HEV causes neurological injury by immune mechanisms related to molecular mimicry, as has been well described for GBS associated with a variety of infectious triggers ^{37, 39}. In addition, clonal sequences were detected in the CSF and serum of a kidney transplant recipient with HEV associated neurological symptoms, and the quasispecies compartmentalization has been demonstrated, suggesting the possible link between the emergence of neurotropic variants and HEV-associated neurological injury ⁴⁰. The pathogenesis of these neurological disorders remains obscure and constitutes an important question in HEV infection.

Other complications associated with HEV. Acute pancreatitis in the setting of non-fulminant HEV infection, as well as several musculoskeletal manifestations associated with the acute phase of HEV infection have been reported ³⁴. Furthermore, hematological disorders including thrombocytopenia and aplastic anemia have been reported for acute HEV infection ⁴⁰.

In conjunction, the broad scope of extra-hepatic manifestations raises doubts as to the exact level of hepatotropism of HEV.

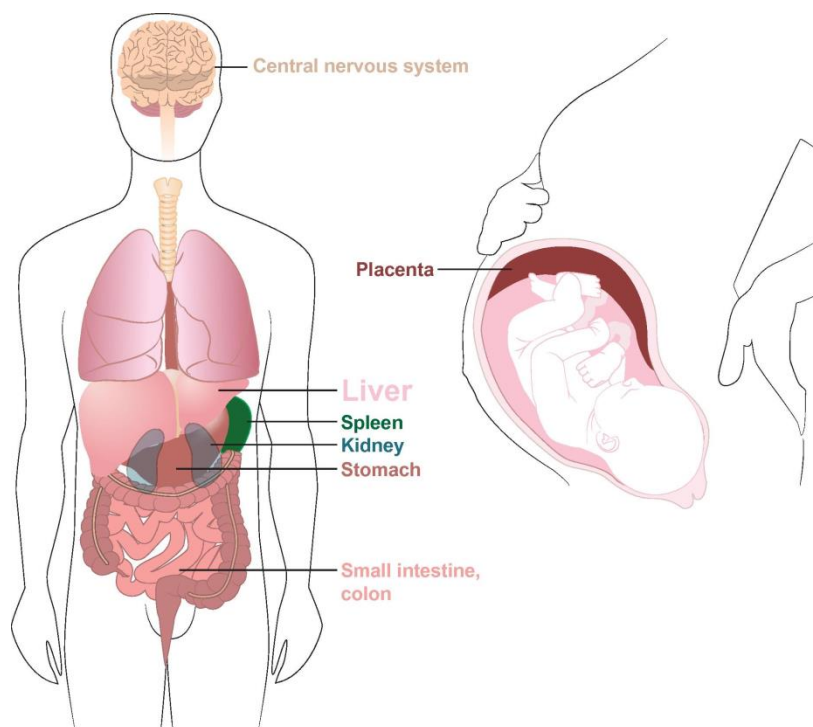


Figure 3. Reported sites of HEV replication. HEV infects and replicates primarily in the liver. However, studies performed in animal models reported HEV replication also in the small intestine, colon and lymph nodes as well as kidney, spleen and stomach. Furthermore, replication in the kidney has been recently suggested by the presence of HEV in the urine of patients with acute and chronic HEV as well as experimentally infected monkeys. Among extrahepatic manifestations, neurological complications are the most frequent. HEV RNA has been found in the cerebrospinal fluid of some patients with such complications and evidence for intrathecal antibody production has been provided in one case, suggesting possible infection of the central nervous system. The most severe symptoms are observed in pregnant women, possibly related to the reported infection of placental tissue. (Adapted from Yannick Debing, et al. Journal of Hepatology, Review 2016.⁶)

Therapy and potential targets

Although most cases of acute HEV infection are self-limiting and require no treatment, some patients with acute HEV1 or HEV3 infection have been treated with antiviral therapy.

Particularly in patients with either pre-existing chronic liver disease or severe or fulminant hepatitis, treatment of ribavirin monotherapy has been used to produce rapid clearance of HEV and avoid liver transplantation in some patients ^{41, 42}. Effective treatment of pregnant women with acute HEV1 infection is not established and in demand. Ribavirin therapy is contraindicated in pregnancy due to teratogenicity ⁴³, which hampers its usefulness.

Chronic HEV infection is common in immunocompromised patients, in particular organ transplant recipients. These kind of patients represent the majority of chronic HEV patients. Reduction of immunosuppressive therapy in solid organ transplant (SOT) patients, especially of agents that target T cells ⁴⁴, is often the first line of therapeutic intervention ⁴⁵, and results in viral clearance in nearly one-third of patients ^{44, 46}. Antiviral therapy is considered for patients for whom immunosuppressive therapy cannot be reduced and for those who fail to achieve viral clearance after reducing immunosuppressants. Although no approved HEV medication is available, ribavirin, interferon- α (IFN- α), or a combination have been successfully used as off-label treatment for some cases, in particular chronically infected patients ⁵.

IFN signaling plays an important role in host defense system of innate immunity ⁴⁷ and several host factors ⁴⁸⁻⁵⁰ can be potential targets for the development of antiviral drugs. Furthermore, both viral functional structures and elements which serve for various steps in the HEV life cycle, including replication and translation, can also be good drug targets ⁵¹. In addition, interference with HEV RNA replication has been reported by using ribozymes and small interfering RNAs ^{52, 53}. Never the less, it is evident that novel pharmaceutical agents to combat HEV infection are urgently needed.

Scope of the thesis

As outlined above, many immunocompromised patients in particular recipients of organ transplants, who receive various kinds of immunosuppressants to prevent rejection, have a high risk of developing chronic hepatitis, due to HEV infection ⁶. Although chronic HEV infection is generally associated with immunosuppressive therapies, little is known about how different immunosuppressants affect HEV infection. In part one of the thesis, we focus on evaluating the clinical impact of HEV in SOT patients and investigating the specific effects

and mechanisms of different immunosuppressive medication on the outcomes of HEV infection in hepatocytes. Apart from hepatitis, an increasing number of extra-hepatic manifestations such as renal injury and neurological disorders, have been reported associated with HEV infection. However, the etiology of HEV-associated renal and neurological injury is unknown. In part two, we investigate the infection biology and pathogenesis of HEV into the urine and nervous system. For anti-HEV therapy, although pegylated IFN- α , ribavirin or the combination have already been used to treat individual cases or small case series of HEV infection as off-label drugs, their mechanism-of-action in the setting of HEV remain poorly investigated. In addition, the specific new HEV antivirals either targeting the virus or host are with emergence to develop. In part three, we explore the anti-HEV potential of IFN- α , as well as several important host cell machinery and factors, to help with the management of HEV patients and future therapeutic development. It is hoped that these lines of investigation would contribute to the quest for better understanding and treatment for HEV infection.

Outline of the thesis

Part I: In **Chapter 2** we comprehensively reviewed the prevalence, infection course, and management of HEV infection after solid organ transplantation, based on a series of published cohort studies, to evaluate the clinical impact of HEV in these patients. In **Chapter 3** we investigate the different impacts of commonly used immunosuppressants, including steroids, calcineurin inhibitors (tacrolimus [FK506] and cyclosporin A [CsA]), and phenolic acid (MPA), on HEV infection in cell culture. In **Chapter 4** we focus our attention on the effects and mechanism-of-actions of rapalogs (rapamycin and everolimus), another group of important immunosuppressants, and its related PI3K-PKB-mTOR pathway, on the HEV replication by using two state-of-the-art cell culture models. In conjunction, these studies provided an important insight in the interaction of immunosuppressive regimens and the antiviral immunity of host cell.

Part II: In **Chapter 5** we establish a monkey model for persistent HEV infection and investigate whether HEV can be disseminated into urine and lead to pathological changes within two monkeys at 272 and 650 days post infection. In **Chapter 6** neurotropism of HEV is explored in various human neural cell lines, embryonic stem cell-derived neural lineage cells,

induced pluripotent stem (iPS) cell-derived human neurons and primary mouse neurons, as well as mouse and monkey animal models. We retrospectively analyze three cohorts of 18 HEV-associated neurological patients to investigate whether HEV RNA is present in cerebrospinal fluid (CSF). These studies provide a rational explanation as to the HEV-associated extra-hepatic manifestations.

Part III: In **Chapter 7** we characterize the role of cytokines in regulating HEV infection and investigate virus-host interactions in HEV infection with focusing on the IFN signaling, in comparison with HCV infection. In **Chapter 8** we further investigate how interferon stimulated genes (ISGs), important downstream elements of IFN signaling, counteract with HEV infection, focusing on the action of IRF1 on HEV replication. This bears significant implications in management of HEV patients and future therapeutic development. In **Chapter 9** we reasoned that since HEV exploits elements from host cell biochemistry, the role of three subunits of the eukaryotic translation initiation factor 4F (eIF4F) complex in HEV replication has been investigated. This provides important clues for the development of novel antiviral therapy against HEV. In **Chapter 10** we explore whether the inhibition of HEV by well-known proteasome inhibitor, MG132, is specific.

The novel insights obtained in this thesis will be summarized and discussed in **Chapter 11**, providing some clues to determine rational immunosuppressive therapy for HEV-associated immunocompromised patients, to understand the pathogenesis of extrahepatic manifestations, and to develop future strategies for HEV therapy.

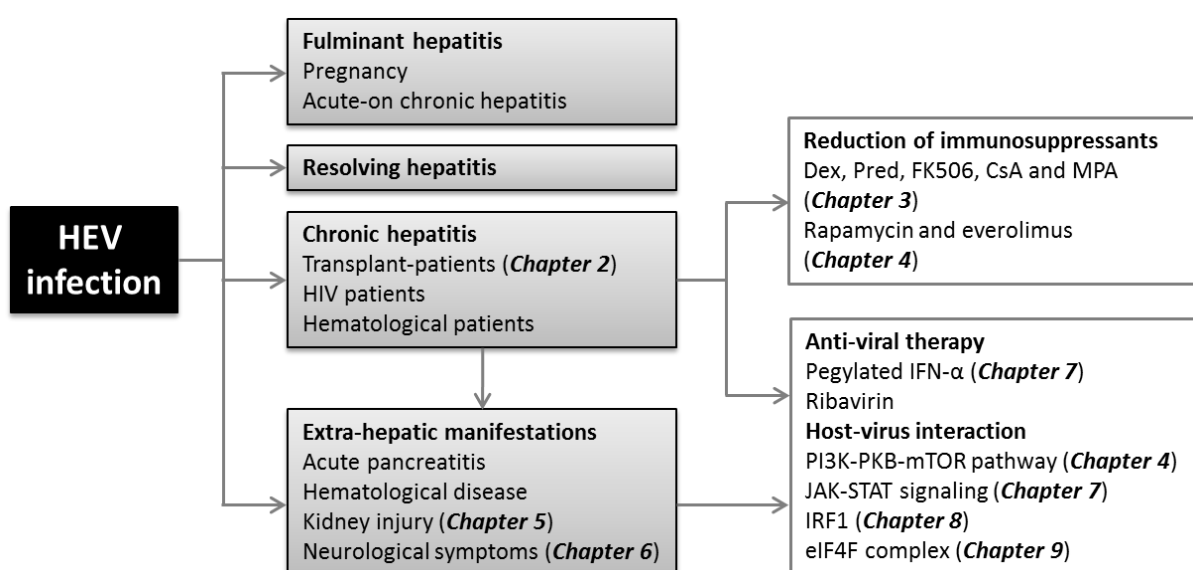


Figure 4. Different patterns of hepatitis E virus infection and outline of the thesis.

References

1. Balayan MS, *et al.* Evidence for a virus in non-A, non-B hepatitis transmitted via the fecal-oral route. *Intervirology* **20**, 23-31 (1983).
2. Park SB. Hepatitis E vaccine debuts. *Nature* **491**, 21-22 (2012).
3. Okamoto H. Genetic variability and evolution of hepatitis E virus. *Virus Res* **127**, 216-228 (2007).
4. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
5. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
6. Debing Y, Moradpour D, Neyts J & Gouttenoire J. Update on Hepatitis E Virology: Implications for Clinical Practice. *J Hepatol*, (2016).
7. Dalton HR, Bendall R, Ijaz S & Banks M. Hepatitis E: an emerging infection in developed countries. *Lancet Infect Dis* **8**, 698-709 (2008).
8. Meng XJ. Hepatitis E virus: animal reservoirs and zoonotic risk. *Vet Microbiol* **140**, 256-265 (2010).
9. Dalton HR, *et al.* Hepatitis E virus and neurological injury. *Nat Rev Neurol* **12**, 77-85 (2016).
10. Smith DB, *et al.* Consensus proposals for classification of the family Hepeviridae. *J Gen Virol* **96**, 1191-1192 (2015).
11. Chandra V, Taneja S, Kalia M & Jameel S. Molecular biology and pathogenesis of hepatitis E virus. *J Biosci* **33**, 451-464 (2008).
12. Panda SK, Datta R, Kaur J, Zuckerman AJ & Nayak NC. Enterically transmitted non-A, non-B hepatitis: recovery of virus-like particles from an epidemic in south Delhi and transmission studies in rhesus monkeys. *Hepatology* **10**, 466-472 (1989).
13. Tam AW, *et al.* Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* **185**, 120-131 (1991).
14. Panda SK & Varma SP. Hepatitis e: molecular virology and pathogenesis. *J Clin Exp Hepatol* **3**, 114-124 (2013).
15. Yamashita T, *et al.* Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. *Proc Natl Acad Sci U S A* **106**, 12986-12991 (2009).
16. Ahmad I, Holla RP & Jameel S. Molecular virology of hepatitis E virus. *Virus Res* **161**, 47-58 (2011).
17. Aggarwal R & Jameel S. Hepatitis E. *Hepatology* **54**, 2218-2226 (2011).
18. Holla P, Ahmad I, Ahmed Z & Jameel S. Hepatitis E virus enters liver cells through a dynamin-2, clathrin and membrane cholesterol-dependent pathway. *Traffic* **16**, 398-416 (2015).
19. Mori Y & Matsuura Y. Structure of hepatitis E viral particle. *Virus Res* **161**, 59-64 (2011).
20. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
21. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).

22. Tanaka T, Takahashi M, Kusano E & Okamoto H. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. *J Gen Virol* **88**, 903-911 (2007).
23. Takahashi M, *et al.* Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. *J Clin Microbiol* **48**, 1112-1125 (2010).
24. Purcell RH & Emerson SU. Animal models of hepatitis A and E. *ILAR J* **42**, 161-177 (2001).
25. Huang F, *et al.* Experimental infection of Balb/c nude mice with Hepatitis E virus. *BMC Infect Dis* **9**, 93 (2009).
26. Han J, *et al.* SPF rabbits infected with rabbit hepatitis E virus isolate experimentally showing the chronicity of hepatitis. *PLoS One* **9**, e99861 (2014).
27. Yu W, *et al.* Characterization of hepatitis E virus infection in tree shrew (*Tupaia belangeri chinensis*). *BMC Infect Dis* **16**, 80 (2016).
28. Li W, *et al.* Experimental infection of Mongolian gerbils by a genotype 4 strain of swine hepatitis E virus. *J Med Virol* **81**, 1591-1596 (2009).
29. Yu C, *et al.* Pathogenesis of hepatitis E virus and hepatitis C virus in chimpanzees: similarities and differences. *J Virol* **84**, 11264-11278 (2010).
30. Ji Y, *et al.* Swine hepatitis E virus in rural southern China: genetic characterization and experimental infection in rhesus monkeys (*Macaca mulatta*). *J Gastroenterol* **43**, 565-570 (2008).
31. Huang F, *et al.* Seroepidemiology and molecular characterization of hepatitis E Virus in *Macaca mulatta* from a village in Yunnan, China, where infection with this virus is endemic. *Hepat Mon* **11**, 745-749 (2011).
32. Kamar N, *et al.* Hepatitis E virus and the kidney in solid-organ transplant patients. *Transplantation* **93**, 617-623 (2012).
33. Mishra A, Saigal S, Gupta R & Sarin SK. Acute pancreatitis associated with viral hepatitis: a report of six cases with review of literature. *Am J Gastroenterol* **94**, 2292-2295 (1999).
34. Bazerbachi F, Haffar S, Garg SK & Lake JR. Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. *Gastroenterol Rep (Oxf)* **4**, 1-15 (2016).
35. Kamar N, Marion O, Abravanel F, Izopet J & Dalton HR. Extrahepatic manifestations of hepatitis E virus. *Liver Int* **36**, 467-472 (2016).
36. Del Bello A, *et al.* Successful treatment of hepatitis E virus-associated cryoglobulinemic membranoproliferative glomerulonephritis with ribavirin. *Transpl Infect Dis* **17**, 279-283 (2015).
37. Kamar N, *et al.* Hepatitis E virus and neurologic disorders. *Emerg Infect Dis* **17**, 173-179 (2011).
38. van Eijk JJ, *et al.* Neuralgic amyotrophy and hepatitis E virus infection. *Neurology* **82**, 498-503 (2014).
39. Willison HJ, Jacobs BC & van Doorn PA. Guillain-Barre syndrome. *Lancet*, (2016).
40. Kamar N, Dalton HR, Abravanel F & Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* **27**, 116-138 (2014).

41. Peron JM, Dalton H, Izopet J & Kamar N. Acute autochthonous hepatitis E in western patients with underlying chronic liver disease: a role for ribavirin? *J Hepatol* **54**, 1323-1324; author reply 1324-1325 (2011).
42. Gerolami R, *et al.* Treatment of severe acute hepatitis E by ribavirin. *J Clin Virol* **52**, 60-62 (2011).
43. Abbas Z & Afzal R. Hepatitis E: when to treat and how to treat. *Antivir Ther* **19**, 125-131 (2014).
44. Kamar N, *et al.* Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology* **140**, 1481-1489 (2011).
45. Wang Y, Metselaar HJ, Peppelenbosch MP & Pan Q. Chronic hepatitis E in solid-organ transplantation: the key implications of immunosuppressants. *Curr Opin Infect Dis* **27**, 303-308 (2014).
46. Kamar N, *et al.* Influence of immunosuppressive therapy on the natural history of genotype 3 hepatitis-E virus infection after organ transplantation. *Transplantation* **89**, 353-360 (2010).
47. Sen GC. Viruses and interferons. *Annu Rev Microbiol* **55**, 255-281 (2001).
48. Morikawa K, Suda G & Sakamoto N. Viral life cycle of hepatitis B virus : host factors and druggable targets. *Hepatol Res*, (2016).
49. Montero H, Garcia-Roman R & Mora SI. eIF4E as a control target for viruses. *Viruses* **7**, 739-750 (2015).
50. Huang F, *et al.* Hepatitis E virus infection activates signal regulator protein alpha to down-regulate type I interferon. *Immunol Res*, (2015).
51. Kenney SP & Meng XJ. Therapeutic targets for the treatment of hepatitis E virus infection. *Expert Opin Ther Targets* **19**, 1245-1260 (2015).
52. Sriram B, Thakral D & Panda SK. Targeted cleavage of hepatitis E virus 3' end RNA mediated by hammerhead ribozymes inhibits viral RNA replication. *Virology* **312**, 350-358 (2003).
53. Huang F, *et al.* RNA interference inhibits hepatitis E virus mRNA accumulation and protein synthesis in vitro. *Vet Microbiol* **142**, 261-267 (2010).

Part I.

Hepatitis in immunocompromised patients

Chapter 2

Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review

Xinying Zhou, Robert A. de Man, Robert J. de Knecht, Herold J. Metselaar,
Maikel P. Peppelenbosch and Qiuwei Pan

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center,
Rotterdam, Netherlands

Abstract

Hepatitis E virus (HEV) infection has emerged as a global public health issue. Although it often causes an acute and self-limiting infection with low mortality rates in the western world, it bears a high risk of developing chronic hepatitis in immunocompromised patients with substantial mortality rates. Organ transplant recipients who receive immunosuppressive medication to prevent rejection are thought to be the main population at risk for chronic hepatitis E. Therefore, there is an urgent need to properly evaluate the clinical impact of HEV in these patients. This article aims to review the prevalence, infection course, and management of HEV infection after solid organ transplantation by performing a comprehensive literature review. In addition, an in-depth emphasis of this clinical issue and a discussion of future development are also presented.

Introduction

HEV infection is an emerging public health problem worldwide ¹. It infects about 20 million people and causes 70,000 deaths every year ². In fact, about a third of the world population lives in areas endemic for HEV and is at risk for infection ³. HEV is mainly an enterically transmitted pathogen that causes waterborne outbreaks in developing countries and sporadic cases of acute hepatitis in industrialized countries. There are four documented routes of transmission, including waterborne, zoonotic food borne, blood borne and perinatal mother-to-child transmission ⁴. Although only one serotype of HEV exists, there are four genotypes infecting humans based on classification of the nucleotide sequences of the genome ⁵. Genotypes 1 and 2 are found mainly in underdeveloped countries, where they are spread via contaminated water. In contrast, genotypes 3 and 4 are prevalent in industrialized countries, where they are spread mainly through eating undercooked pork or game products. These two genotypes are zoonotic, and animals serve as a reservoir ^{1,6}. In addition to pigs, several other animal species including deer, rats, mongoose, chickens, rabbits, trout and ferrets also harbor HEV strains, and thus its zoonotic potential has raised considerable public health concerns ⁷.

Hepatitis E typically causes an acute and self-limiting infection in immune-competent individuals with low mortality rates in general. However, fulminant hepatitis and high mortality are described, reaching 25% in cases of pregnant women infected with genotype 1 in developing countries and 70% in cases involving underlying liver disease infected with genotype 3 ⁸. In contrast with immune-competent persons, HEV infection in immunocompromised patients, in particular organ transplant recipients who receive immunosuppressants to prevent rejection ⁹, have an increased risk of developing chronic hepatitis with substantial graft loss and mortality rates. No specific licensed treatment is available at the moment, and only its symptoms are treatable. The overall management of HEV during pregnancy is not different from managing jaundice due to other causes of viral hepatitis. However, having learned from managing HCV infection after liver transplantation ^{10, 11}, much effort is currently ongoing to optimize immunosuppressive medication and to apply potential anti-HEV regimens, in order to better manage HEV infection post-organ transplantation. This article aims to provide an overall view of the prevalence, disease

course and management of HEV infection after solid organ transplantation by performing a comprehensive literature review.

Epidemiology and clinical manifestations

Epidemics of hepatitis E occur periodically throughout the developing world, which are mainly caused by HEV genotype 1 (HEV1) in Asia and Africa, and HEV2 in Africa and Mexico. Outbreaks affecting thousands of people have occurred in India, China, Somalia and Uganda ⁶. In 2007, an outbreak in Uganda infected more than 10,000 people and killed 160. By the end of September 2012, more than 200 cases of jaundice caused by hepatitis E had been reported in refugee camps in Kenya since August, and three refugee camps in South Sudan had seen 16 deaths and 400 cases of hepatitis E infection since July ². This epidemic of hepatitis E is escalating across refugee camps in Maban County, South Sudan. So far, MSF has treated 3,991 patients (for symptoms only) in its health facilities in the camps and has recorded 88 deaths, including 15 pregnant women (www.msf.org, reported on 6 Feb 2013).

The disease often affects young adults and is particularly severe among pregnant women and persons with preexisting liver diseases. It occurs as occasional sporadic cases, most often among elderly men with pre-existing illnesses ¹². HEV-infected mothers can transmit the infection to the fetus, leading to premature birth, increased fetal loss and hypoglycaemia, hypothermia, and anicteric or icteric acute hepatitis in the newborn ¹³. The overall death rate among young adults and pregnant women is 0.5%-3% and 15%-25%, respectively ^{4,8}. In the Kashmir outbreak (1978-1979), 8.8%, 19.4% and 18.6% of pregnant women in the first, second and third trimesters, respectively, had clinical disease. In contrast, only 2.1% of non-pregnant women and 2.8% of men were affected ¹⁴. Occasional cases with atypical non-hepatic manifestations, such as acute pancreatitis, hematological abnormalities, autoimmune phenomena, and neurological syndromes have been reported ¹³, although the pathogenesis of these manifestations remains unclear.

Although the clinical issue of HEV infection initially was only recognized in developing countries, recent reports have raised awareness of this virus in western countries as well. In recent years, HEV3 infections have been reported in Europe, New Zealand, and North America. Both HEV3 and HEV4 are found in Japan ^{6,15}. In the developing world, HEV infection

is recently recognized as a potential cause of decompensation in patients with chronic liver disease and can result in a very high mortality ^{1,16}. In addition, HEV infection can easily be misdiagnosed as drug-induced hepatitis ¹. However, the most serious clinical issue of HEV infection in the western countries exists in HEV-infected organ transplant recipients ¹⁷. Although this virus was initially thought to resemble hepatitis A with acute infection only, chronic infection was discovered later and exclusively among immunocompromised patients, including organ transplant recipients ⁹, HIV patients ¹⁸ and cancer patients receiving chemotherapy ¹⁹. This unique infection course in organ transplant patients urges further investigation.

Prevalence of HEV infection in solid organ transplant patients

Prevalence of anti-HEV antibodies

Antibodies to HEV are indicative of exposure to HEV infections. Anti-HEV antibodies have been detected in 5%-30% of the general population in developing countries where the disease is endemic ²⁰. In industrialized countries, prevalence of anti-HEV antibodies was reported to be 2.5% in the USA ²¹ and 0.4%-3% in Western Europe ^{22,23}, although the rates vary between different reports ²⁴. IgG and IgM antibodies appear at the time of clinical onset, whereas IgG titers are increased and IgM decreased during recovery phase. In general, IgM antibody is detectable for only 3-12 months, whereas IgG antibody persists for many years ¹⁵. Thus, detection of IgG is commonly used for assessing seroprevalence of HEV. Of note, the earlier serological assays were developed for genotype 1; while these tests recently have been adapted to genotype 3 showing a much higher incidence. Thus, the interpretation of seroprevalence data needs to be cautious.

To specifically investigate the seroprevalence of HEV in solid organ transplantation patients, we have searched the literature and identified 10 cohort studies (table 1) ^{9, 25-33}, which performed EIA to detect anti-HEV IgG antibodies. Four studies are from France, three from Germany, one from the Netherlands, one from Canada and one from Iran. Out of the total 2202 solid organ transplant recipients, 256 (11.6%) patients were positive for anti-HEV IgG. Except for one study ²⁷, all reported the IgG prevalence according to the types of organ

transplantation. Thus, we further sub-analyzed the prevalence in kidney (71/510; 13.9%), liver (53/718; 7.4%) and heart (31/274; 11.3%) transplantation (Figure 1A).

Table 1. Prevalence of anti-HEV IgG antibody in solid organ transplantation patients

Reference	Patients	Type of TX	HEV test	Seropositive	Region
9	327	KTX 241 LTX 86	ELISA: IgG	35 (14.5%) 9 (10.5%)	France
25	49	KTX	ELISA: IgG	3 (6.1%)	France
26	46	KTX	ELISA: IgG	3 (6.5%)	France
27	700	KTX 529 LTX 171	ELISA: IgG	101 (14.4%)	France
28	226	LTX	ELISA: IgG	10 (4.4%)	Germany
29	274	HTX	ELISA: IgG	31 (11.3%)	Germany
30	124	KTX 83 LTX 41	ELISA: IgG	2 (2.4%) 2 (4.9%)	Germany
31	285	LTX	ELISA: IgG	10 (3.5%)	Netherlands
32	80	LTX	ELISA: IgG	22 (27.5%)	Canada
33	91	KTX	ELISA: IgG	28 (30.8%)	Iran
Total	2202			256 (11.6%)	

HTX, heart transplant; LTX, liver transplant; KTX, kidney transplant.

Prevalence of HEV genomic RNA

HEV is a non-enveloped virus classified as a *Hepevirus* in the family *Hepeviridae*. It has a positive-sense and single-stranded RNA genome, ranging from about 6.6-7.2 kb in length. The genome encodes three ORFs flanked by a capped 5' end and a poly A tail at the 3' end ⁷. ORF1 encodes a non-structural protein. ORF2 encodes the viral capsid protein involved in virion assembly, interaction with host cells and immunogenicity. ORF3, which overlaps ORF2, encodes a small protein involved in virion morphogenesis and release ⁶. Human HEV are classified into four genotypes with five, two, ten and seven subtypes for genotype 1, 2, 3 and 4, respectively. However, with the increasing number of newly discovered strains, in particular from animals, the classification of HEV variants is currently still in transition without agreed definitions for genotypes and subtypes ³⁴.

Nevertheless, detection of HEV genomic RNA by RT-PCR remains a rigorous approach for diagnosis, confirmation and monitoring HEV infection. A World Health Organization standard is available, which makes reliable quantification possible ³⁵. To assess the prevalence of HEV RNA in solid organ transplantation patients, we performed a literature search and identified 12 cohort studies ^{9, 26-32, 36-39} that have performed RT-PCR to detect

HEV genomic RNA (Table 2). Among those, eight studies also performed EIA to detect IgG that have already been included in Table 1.

In total, 99 HEV RNA positive patients were identified out of 5050 (2%) organ transplant recipients (Table 2). Sub-analysis showed HEV RNA positivity in 43 out of 2676 (1.6%) kidney, 20 out of 1089 (1.8%), 9 out of 533 (1.7%) heart, 11 out of 521 (2.1%) lung and two out of 14 (14.3%) multiple organ transplant patients (Figure 1B).

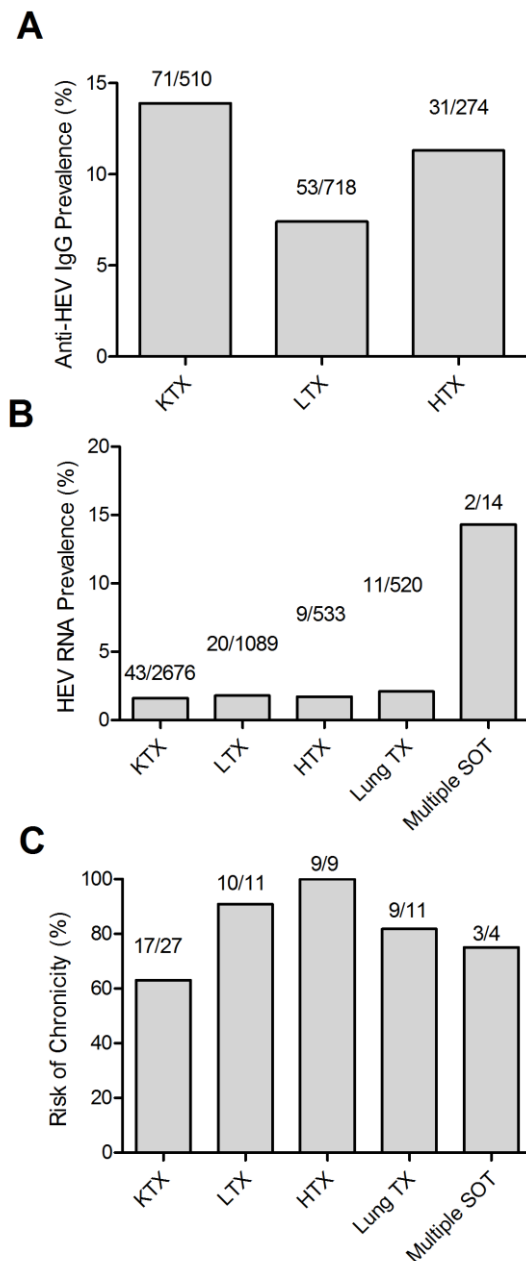


Figure 1. Sub-analysis of anti-HEV IgG prevalence (A), HEV RNA prevalence (B), and risk of chronic development (C), according to the types of solid organ transplantation. KTX, kidney transplant; LTX, liver transplant; HTX, heart transplant; SOT, solid organ transplant.

Risk of developing chronic hepatitis

To assess the overall risk of developing chronic hepatitis in solid organ transplant recipients who are positive for HEV RNA, data from the studies listed in Table 2 were analyzed^{9, 26-32, 36-39}. Chronic infections developed in 64 out of 99 (64.6%) infected transplant recipients. Sub-analysis showed that the risks of developing chronic hepatitis are higher than 60% in all types of transplant groups, although the included patient numbers are rather small (Figure 1C). This is consistent with a large retrospective multicenter study (some centers overlap with the studies listed in Table 2) reporting 56 of 85 (65.9%) HEV infected organ transplant recipients developed chronic infection⁴⁰.

Table 2. Prevalence of HEV genomic RNA in solid organ transplantation patients

Reference	Patients	Type of TX	HEV test	HEV RNA positive	Chronic	Region
9	217	KTX	RT-PCR	9	3	France
		LTX		3	3	
		Multiple SOT		2	2	
26	44	KTX	RT-PCR	0	0	France
27	700	LTX 171; KTX 529	RT-PCR	12 22	16	France
36	1,350	KTX	RT-PCR	16	12	France
28	226	LTX	RT-PCR	3	2	Germany
29	274	HTX	RT-PCR	4	4	Germany
30	124	KTX 83 LTX 41	RT-PCR	1 0	1	Germany
31	285	LTX	RT-PCR	1	1	Netherlands
37	1,200	HTX 259 Lung TX 53 LTX 300 KTX 574 Multiple SOT 14	RT-PCR	5(1.9%) 1 (1.9%) 3 (1.0%) 1 (0.2%) 2 (14.3%)	5 1 3 1 1	Netherlands
38	468	Lung TX	RT-PCR	10	8	Netherlands
32	66	LTX	RT-PCR	1	1	Canada
39	96	KTX	RT-PCR	3	unknown	Brazil
Total	5050			99 (1.96%)	64	

SOT: solid organ transplant; HTX: heart transplant; lung TX: lung transplant; LTX: liver transplant; KTX: kidney transplant.

In fact, chronic infection with HEV could result in rapid progression to liver fibrosis and cirrhosis in organ transplant recipients. This has been observed in kidney^{41, 42}, liver⁴³ as well as heart⁴⁴ transplantation, although the exact risk rate is still unclear. It was observed

that four out of six heart transplant recipients developed advanced fibrosis within only 2 years after HEV infection ⁴⁴. In the large retrospective multicenter study ⁴⁰, eight out of the 56 infected patients developed cirrhosis, whereas two liver transplant patients required a second liver transplant, and two died because of decompensated cirrhosis. Similarly, HCV re-infection after liver transplantation often results in accelerated recurrence of liver fibrosis and the early development of cirrhosis ⁴⁵. Thus, an intriguing question regarding the main cause of rapid progression of advanced fibrosis and cirrhosis by HEV in organ transplant recipients is whether this is because of the virus itself, the suppressed immune system, particular immunosuppressants or a combination of factors.

Management of HEV infection post-transplantation

Manipulation of immunosuppression

Immunosuppressants are used life-long in organ transplant patients in order to prevent rejection. Various types of immunosuppressive drugs with distinct mechanism of action to suppress the immune system are currently used in the clinic, including corticosteroids, calcineurin inhibitors, mTOR inhibitors, selective antiproliferative agents, and anti-lymphocyte antibodies. Withdrawal or even dose reduction bears a high risk of acute rejection. The strong link between immunocompromising conditions and development of chronic HEV infection probably inspired the early exploration of manipulating immunosuppressive regimens in HEV-infected organ transplant patients.

A potential beneficial effect of withdrawal immunosuppression was initially observed in a patient who developed acute hepatitis 1 month after kidney transplantation. When immunosuppressants were stopped because of septic shock, serum HEV RNA surprisingly became undetectable a few days later. When immunosuppression was re-introduced, viral RNA was again detected in serum. At its discontinuation, a few days later, HEV RNA became definitively undetectable ⁴⁶. Reducing dosage of the calcineurin inhibitor tacrolimus was assumed to be associated with clearance of HEV in other cases of renal transplantation with acute infection ⁴⁷.

More evidence to support the potential benefits of reduction or withdrawal of immunosuppression comes from chronic patients ^{36, 40, 44, 46, 48}, although the rates of viral

clearance after reduction of immunosuppression differ a lot among different studies. Eight of 15 kidney transplant ³⁶, whereas only one of six heart transplant recipients ⁴⁴ and three of 15 different organ transplants ⁴⁹ who could clear the virus after reduction of immunosuppression have been reported. In a large retrospective study, 18 of 56 transplant patients were reported to clear the virus after immunosuppression reduction ⁴⁰. The use of tacrolimus has been associated with higher risk of developing chronic hepatitis, compared with another calcineurin inhibitor, cyclosporin A (CsA) ⁴⁰, although the number of CsA users was small. In contrast, clearance of HEV after heart transplantation was found to be more frequent in patients with immunosuppressive medication containing mycophenolate mofetil (MMF) ²⁹. However, this may be biased by a reduced dose of CsA or tacrolimus in these cases.

Experience, learned from these case series and small cohort studies, suggests that dose reduction of immunosuppression should be the first intervention strategy to achieve viral clearance in HEV-infected immunocompromised patients ⁴⁴. Nevertheless, long-term follow-up is required to assess the eventual outcome and this strategy should be used with caution in patients at higher risk of rejection or in those who are more difficult to monitor for rejection (e.g. in heart and lung transplantation).

Antiviral therapy

Although no proven antiviral therapy for HEV infection exists so far, interferon, ribavirin or a combination as off-label drugs have been used to treat individual cases or small case series. Apparently, this is based on the current standard therapy of chronic hepatitis C, which is a combination of peg-interferon-alpha with ribavirin. The therapeutic success depends on the genotype of HCV and the viral load at the start of therapy and during treatment. Overall, about half of the patients can develop a sustained virologic response (SVR, completely eradicate the virus) ⁵⁰. However, in HCV recurrence after liver transplantation SVR rates of only 20% are observed ⁵¹. The combination of interferon and ribavirin has been reported to completely clear the virus in a chronic hepatitis E patient with HIV infection ⁵². Combination therapy was also occasionally used in incidental organ transplant recipients with chronic hepatitis E ⁴⁰. Substantial success of peg-interferon monotherapy has been reported in treating chronic hepatitis E in liver transplant recipients ^{53, 54}. However, interferon therapy is

contraindicated in kidney transplant patients ⁵⁵, which has been associated with a high risk of acute rejection and subsequent kidney failure ^{56, 57}.

The use of ribavirin monotherapy is gaining acceptance for treating hepatitis E although this is not based on evidence. This is very different from the setting of chronic hepatitis C. Ribavirin, although showing anti-HCV activity *in vitro* ⁵⁸, is generally considered to have little or no detectable anti-viral activity as monotherapy in HCV patients ⁵⁹. By analyzing the early viral kinetics during ribavirin monotherapy, only minor and transient effects with 0.5-1 log reduction of HCV viral load were observed ^{60, 61}. Only when combined with interferon-alpha, ribavirin doubles the response rate, compared with interferon alone ⁶². However, ribavirin monotherapy seems sufficient to completely clear HEV in many reported uncontrolled cases. In a patient with severe acute hepatitis E patient infected with genotype 3 HEV, treatment with ribavirin for 21 days resulted in undetectable viral RNA in serum with normalization of alanine aminotransferase level ⁶³. In four patients with genotype 1 HEV-induced acute on chronic liver failure, treatment with ribavirin resulted in clearance of the virus without serious adverse effects ⁶⁴. More evidence to support ribavirin monotherapy comes from the organ transplantation setting. Success of HEV clearance by ribavirin has been claimed in various types of organ transplantation, including kidney ^{55, 65}, heart ^{29, 44, 66}, lung ³⁸, and multi-organ ⁶⁷ recipients. In a recent prospective case series, 10 of 11 acute HEV patients spontaneously recovered and the one with severe acute infection was cured after ribavirin therapy. Of 11 organ transplant recipients with prolonged HEV viraemia, nine patients achieved viral clearance after ribavirin treatment ⁴⁹.

The overall response rate to interferon, ribavirin or combination therapy is far from conclusive, because only limited number of patients have been treated so far with considerable variations of viral clearance rates and no randomized controls. In the large retrospective study, among 20 patients who received antiviral therapy (interferon = 5; ribavirin = 14 and combination = 1), 14 patients achieved sustained viral clearance and six were still viremic and still receiving therapy ⁴⁰. Clearly, a proper randomized controlled trial of the efficacy of anti-HEV therapies is urgently called for and such an endeavor should also result in the definition of standard guidelines for treating hepatitis E.

Discussion

The current diagnosis of HEV is based on detection of anti-HEV antibodies, PCR amplification of the viral RNA genome, or combination of both techniques, but none have formal Food and Drug Administration approval¹⁵. Diagnosis of acute infection relies on the detection of specific IgM antibodies; whereas the determination of previous exposure to HEV is based on detection of IgG antibodies. In organ transplant patients, it is more common to evaluate the prevalence of IgG antibodies. Currently, a few enzyme immunoassays are available either developed by individual laboratories or from commercial sources. However, these assays use different HEV antigens and their sensitivity and specificity vary widely^{68, 69}. Thus, the detection of HEV genomic RNA from blood or fecal samples by RT-PCR serves as an important line for the diagnosis, confirmation and monitoring of the infection. Despite only one serotype, there are four genotypes as well as other less defined strains discovered from various infected animals³⁴, the genome of HEV thus can vary dramatically. Therefore, the design of specific primers and particular protocols for PCR amplification by different laboratories can result in huge bias and variation. A proper positive control, for instance produced by the World Health Organization, is recommended to be included in every PCR assay. In the organ transplant setting, the current assumption is that these patients are only affected by genotype 3 HEV. The published studies almost exclusively focused on the diagnosis of genotype 3. An intriguing question is whether other genotypes can also infect these patients. It will be of great interest, for instance, to evaluate the prevalence of genotype 1 in transplant patients in Asia. Although a combination of serologic and molecular assays were used for many of the studies in the transplant setting, there is still a possibility of positivity for only antibody or only viral RNA^{37, 39}. Thus, there is an urgent need for developing robust and standardized diagnostic assays, possibly combining both serology and molecular techniques and including properly produced control materials.

Other than immunosuppressive medication, whether genotype is also responsible for chronic progression could be an interesting question because only genotype 3 is reported in organ transplant patients. Regarding immunosuppression in general, clinical evidence showed that lymphocyte subset counts, mainly cluster of differentiated 4 T-cells, were found to be significantly lower in HEV patients who then evolved to chronicity compared with those who cleared the virus within 6 months⁹. It is not surprising that a healthy immune

system can control and even eliminate the infection. Thus, dose reduction or even withdrawal (if possible) of immunosuppression is the first intervention strategy to achieve viral clearance in HEV-infected transplant patients ⁴⁴, although bearing the risk of graft rejection. Another aspect learned from HCV recurrence in liver transplantation is that different types of immunosuppressants could interact positively or negatively with the host immune system as well as with the virus directly ¹¹. In fact, there is evidence in HEV-transplant patients that tacrolimus but not CsA has been found to be more frequently associated with persistent infection ⁴⁰, whereas MMF may help to clear the virus ²⁹. However, the current clinical studies are not able to conclusively address the impact of different immunosuppressants, because of limited patient numbers. Thus, there is substantial room for fundamental and translational research to further investigate these interesting questions by using state-of-art HEV cell culture ⁷⁰ and possibly animal models ^{71, 72}.

Despite a clear benefit of manipulating immunosuppressive regimens, a substantial proportion of patients is still not able to clear the virus and rapidly progresses towards chronic hepatitis ⁴⁰. Antiviral therapy appears to be the only option for managing those patients. Although no proven medication is available for HEV, transplant hepatologists have apparently learned from the standard treatment of chronic hepatitis C. Strikingly, ribavirin monotherapy appears sufficient to achieve HEV clearance in some reported patients, whereas ribavirin is only effective when combined with interferon for treating chronic hepatitis C. Its anti-HCV mechanism has been proposed as direct inhibition of viral replication by depletion of intracellular nucleotide pool, “error catastrophe” or immunomodulation ⁷³, although much is still not proven and controversial ⁷⁴. A joint effort from the bed and the bench is thus required to solidly evaluate the anti-HEV efficacy and the working mechanism of ribavirin. A potential limitation of ribavirin is side effects, in particular for transplant patients already under immunosuppression. One death has been reported after experiencing a virological breakthrough associated with ribavirin dose reduction because of severe anaemia ⁴⁹. Another cautionary note is that drug-drug interactions may occur between ribavirin and particular immunosuppressants. Development of new antiviral therapy would be another scenario, although there is apparent lack of interest from pharmaceutical companies because of low rate of financial return for this disease. Thus, the hope and responsibility of developing new anti-HEV remedies now rest on academia.

Conclusion

On the basis of a series of published cohort studies, the prevalence of anti-HEV IgG is approximately 11.6% and genomic viral RNA is 2% in solid organ transplant patients. A total of 65% of patients who were positive for HEV RNA developed chronic infection. Reduction of immunosuppression is the first intervention strategy to achieve viral clearance, otherwise treatment with off-label antivirals, in particular ribavirin, should be considered. Because of limited patient numbers and no randomized trials, the exact efficacy and safety of either immunosuppression reduction or antiviral therapy are inconclusive. However, the clinical evidence is encouraging for these two approaches.

Nevertheless, HEV remains largely under diagnosed in the general population as well as in transplant patients. Because only a few transplant centers are performing the screening primarily for research purposes, with technical limitations and possible patient selection bias, the data represented by this study may not be able to fully reflect the real clinical status. However, this is a moment to call attention to this emerging clinical issue in the transplant setting, hopefully leading to proper positioning of its clinical impact soon and finally solving the problems.

References

1. Dalton HR. Hepatitis: hepatitis E and decompensated chronic liver disease. *Nat Rev Gastroenterol Hepatol* **9**, 430-432 (2012).
2. Park SB. Hepatitis E vaccine debuts. *Nature* **491**, 21-22 (2012).
3. Ahmad I, Holla RP & Jameel S. Molecular virology of hepatitis E virus. *Virus Res* **161**, 47-58 (2011).
4. Mushahwar IK. Hepatitis E virus: molecular virology, clinical features, diagnosis, transmission, epidemiology, and prevention. *J Med Virol* **80**, 646-658 (2008).
5. Purdy MA & Khudyakov YE. The molecular epidemiology of hepatitis E virus infection. *Virus Res* **161**, 31-39 (2011).
6. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
7. Raj VS, *et al.* Novel hepatitis E virus in ferrets, the Netherlands. *Emerg Infect Dis* **18**, 1369-1370 (2012).
8. Moal V, Zandotti C & Colson P. Emerging viral diseases in kidney transplant recipients. *Rev Med Virol* **23**, 50-69 (2013).
9. Kamar N, *et al.* Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* **358**, 811-817 (2008).
10. Ciesek S & Wedemeyer H. Immunosuppression, liver injury and post-transplant HCV recurrence. *J Viral Hepat* **19**, 1-8 (2012).
11. Pan Q, Tilanus HW, Metselaar HJ, Janssen HL & van der Laan LJ. Virus-drug interactions--molecular insight into immunosuppression and HCV. *Nat Rev Gastroenterol Hepatol* **9**, 355-362 (2012).
12. Borgen K, *et al.* Non-travel related Hepatitis E virus genotype 3 infections in the Netherlands; a case series 2004 - 2006. *BMC Infect Dis* **8**, 61 (2008).
13. Aggarwal R & Jameel S. Hepatitis E. *Hepatology* **54**, 2218-2226 (2011).
14. Khuroo MS, Teli MR, Skidmore S, Sofi MA & Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med* **70**, 252-255 (1981).
15. Hoofnagle JH, Nelson KE & Purcell RH. Hepatitis E. *N Engl J Med* **367**, 1237-1244 (2012).
16. De Silva S, Hassan-Ibrahim MO, Austin M, Newport M & Verma S. Hepatitis E infection is an under recognized cause of acute decompensation in patients with chronic liver disease. *Dig Liver Dis* **44**, 930-934 (2012).
17. Kamar N, Legrand-Abravanel F, Izopet J & Rostaing L. Hepatitis E virus: what transplant physicians should know. *Am J Transplant* **12**, 2281-2287 (2012).
18. Dalton HR, Bendall RP, Keane FE, Tedder RS & Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. *N Engl J Med* **361**, 1025-1027 (2009).
19. Ollier L, *et al.* Chronic hepatitis after hepatitis E virus infection in a patient with non-Hodgkin lymphoma taking rituximab. *Ann Intern Med* **150**, 430-431 (2009).
20. Aggarwal R & Naik S. Epidemiology of hepatitis E: current status. *J Gastroenterol Hepatol* **24**, 1484-1493 (2009).
21. Tsang TH, *et al.* Acute hepatitis E infection acquired in California. *Clin Infect Dis* **30**, 618-619 (2000).

22. Ijaz S, *et al.* Non-travel-associated hepatitis E in England and Wales: demographic, clinical, and molecular epidemiological characteristics. *J Infect Dis* **192**, 1166-1172 (2005).
23. Mansuy JM, *et al.* Hepatitis E in the south west of France in individuals who have never visited an endemic area. *J Med Virol* **74**, 419-424 (2004).
24. Scotto G, Martinelli D, Giammario A, Prato R & Fazio V. Prevalence of antibodies to hepatitis e virus in immigrants: a seroepidemiological survey in the district of foggia (Apulia-southern Italy). *J Med Virol* **85**, 261-265 (2013).
25. Buffet C, *et al.* A high hepatitis E virus seroprevalence among renal transplantation and haemophilia patient populations. *J Hepatol* **24**, 122-125 (1996).
26. Maylin S, *et al.* Prevalence of antibodies and RNA genome of hepatitis E virus in a cohort of French immunocompromised. *J Clin Virol* **53**, 346-349 (2012).
27. Legrand-Abravanel F, *et al.* Hepatitis E virus infection without reactivation in solid-organ transplant recipients, France. *Emerg Infect Dis* **17**, 30-37 (2011).
28. Pischke S, *et al.* Hepatitis E virus infection as a cause of graft hepatitis in liver transplant recipients. *Liver Transpl* **16**, 74-82 (2010).
29. Pischke S, *et al.* Chronic hepatitis e in heart transplant recipients. *Am J Transplant* **12**, 3128-3133 (2012).
30. Hoerning A, *et al.* Prevalence of hepatitis E virus infection in pediatric solid organ transplant recipients--a single-center experience. *Pediatr Transplant* **16**, 742-747 (2012).
31. Haagsma EB, *et al.* Prevalence of hepatitis E virus infection in liver transplant recipients. *Liver Transpl* **15**, 1225-1228 (2009).
32. Halac U, *et al.* Chronic hepatitis E infection in children with liver transplantation. *Gut* **61**, 597-603 (2012).
33. Rostamzadeh Khameneh Z, Sepehrvand N & Masudi S. Seroprevalence of hepatitis E among Iranian renal transplant recipients. *Hepat Mon* **11**, 646-651 (2011).
34. Smith DB, Purdy MA & Simmonds P. Genetic variability and the classification of hepatitis E virus. *J Virol* **87**, 4161-4169 (2013).
35. Baylis SA, Hanschmann KM, Blumel J, Nubling CM & Group HEVCS. Standardization of hepatitis E virus (HEV) nucleic acid amplification technique-based assays: an initial study to evaluate a panel of HEV strains and investigate laboratory performance. *J Clin Microbiol* **49**, 1234-1239 (2011).
36. Moal V, *et al.* Infection with hepatitis E virus in kidney transplant recipients in southeastern France. *J Med Virol* **85**, 462-471 (2013).
37. Pas SD, *et al.* Hepatitis E virus infection among solid organ transplant recipients, the Netherlands. *Emerg Infect Dis* **18**, 869-872 (2012).
38. Riezebos-Brilman A, *et al.* Chronic hepatitis E infection in lung transplant recipients. *J Heart Lung Transplant* **32**, 341-346 (2013).
39. Passos AM, Heringer TP, Medina-Pestana JO, Ferraz ML & Granato CF. First report and molecular characterization of hepatitis E virus infection in renal transplant recipients in Brazil. *J Med Virol* **85**, 615-619 (2013).
40. Kamar N, *et al.* Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology* **140**, 1481-1489 (2011).

41. Kamar N, *et al.* Hepatitis E virus-related cirrhosis in kidney- and kidney-pancreas-transplant recipients. *Am J Transplant* **8**, 1744-1748 (2008).
42. Gerolami R, Moal V & Colson P. Chronic hepatitis E with cirrhosis in a kidney-transplant recipient. *N Engl J Med* **358**, 859-860 (2008).
43. Haagsma EB, *et al.* Chronic hepatitis E virus infection in liver transplant recipients. *Liver Transpl* **14**, 547-553 (2008).
44. Koning L, *et al.* Clinical implications of chronic hepatitis E virus infection in heart transplant recipients. *J Heart Lung Transplant* **32**, 78-85 (2013).
45. van der Laan LJ, *et al.* Results of a two-center study comparing hepatic fibrosis progression in HCV-positive liver transplant patients receiving cyclosporine or tacrolimus. *Transplant Proc* **42**, 4573-4577 (2010).
46. Kamar N, *et al.* Influence of immunosuppressive therapy on the natural history of genotype 3 hepatitis-E virus infection after organ transplantation. *Transplantation* **89**, 353-360 (2010).
47. Kamar N, *et al.* Hepatitis E virus and the kidney in solid-organ transplant patients. *Transplantation* **93**, 617-623 (2012).
48. Halleux D, Kanaan N, Kabamba B, Thomas I & Hassoun Z. Hepatitis E virus: an underdiagnosed cause of chronic hepatitis in renal transplant recipients. *Transpl Infect Dis* **14**, 99-102 (2012).
49. Pischke S, *et al.* Ribavirin treatment of acute and chronic hepatitis E: a single-centre experience. *Liver Int*, **33**, 722-726 (2013).
50. Pan Q, Peppelenbosch MP, Janssen HL & de Knecht RJ. Telaprevir/boceprevir era: from bench to bed and back. *World J Gastroenterol* **18**, 6183-6188 (2012).
51. Samuel D, *et al.* Interferon-alpha 2b plus ribavirin in patients with chronic hepatitis C after liver transplantation: a randomized study. *Gastroenterology* **124**, 642-650 (2003).
52. Dalton HR, Keane FE, Bendall R, Mathew J & Ijaz S. Treatment of chronic hepatitis E in a patient with HIV infection. *Ann Intern Med* **155**, 479-480 (2011).
53. Haagsma EB, Riezebos-Brilman A, van den Berg AP, Porte RJ & Niesters HG. Treatment of chronic hepatitis E in liver transplant recipients with pegylated interferon alpha-2b. *Liver Transpl* **16**, 474-477 (2010).
54. Kamar N, *et al.* Pegylated interferon-alpha for treating chronic hepatitis E virus infection after liver transplantation. *Clin Infect Dis* **50**, e30-33 (2010).
55. Kamar N, *et al.* Ribavirin therapy inhibits viral replication on patients with chronic hepatitis e virus infection. *Gastroenterology* **139**, 1612-1618 (2010).
56. Weimar W, *et al.* The incidence of cytomegalo- and herpes simplex virus infections in renal allograft recipients treated with high dose recombinant leucocyte interferon: a controlled study. *Scand J Urol Nephrol Suppl* **92**, 37-39 (1985).
57. Rostaing L, *et al.* Treatment of chronic hepatitis C with recombinant interferon alpha in kidney transplant recipients. *Transplantation* **59**, 1426-1431 (1995).
58. Pan Q, Tilanus HW, Janssen HL & van der Laan LJ. Ribavirin enhances interferon-stimulated gene transcription by activation of the interferon-stimulated response element. *Hepatology* **53**, 1400-1401; author reply 1402 (2011).
59. Lee JH, *et al.* Effect of ribavirin on virus load and quasispecies distribution in patients infected with hepatitis C virus. *J Hepatol* **29**, 29-35 (1998).

60. Pawlotsky JM, *et al.* Antiviral action of ribavirin in chronic hepatitis C. *Gastroenterology* **126**, 703-714 (2004).
61. Rotman Y, *et al.* Effect of ribavirin on viral kinetics and liver gene expression in chronic hepatitis C. *Gut*, **63**, 161-169 (2014).
62. Poynard T, *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* **352**, 1426-1432 (1998).
63. Gerolami R, *et al.* Treatment of severe acute hepatitis E by ribavirin. *J Clin Virol* **52**, 60-62 (2011).
64. Goyal R, Kumar A, Panda SK, Paul SB & Acharya SK. Ribavirin therapy for hepatitis E virus-induced acute on chronic liver failure: a preliminary report. *Antivir Ther* **17**, 1091-1096 (2012).
65. de Niet A, *et al.* Chronic hepatitis E after solid organ transplantation. *Neth J Med* **70**, 261-266 (2012).
66. Chaillon A, *et al.* Sustained virologic response with ribavirin in chronic hepatitis E virus infection in heart transplantation. *J Heart Lung Transplant* **30**, 841-843 (2011).
67. Mallet V, *et al.* Brief communication: case reports of ribavirin treatment for chronic hepatitis E. *Ann Intern Med* **153**, 85-89 (2010).
68. Bendall R, Ellis V, Ijaz S, Ali R & Dalton H. A comparison of two commercially available anti-HEV IgG kits and a re-evaluation of anti-HEV IgG seroprevalence data in developed countries. *J Med Virol* **82**, 799-805.
69. Ghabrah TM, *et al.* Comparison of tests for antibody to hepatitis E virus. *J Med Virol* **55**, 134-137 (1998).
70. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
71. Purcell RH & Emerson SU. Animal models of hepatitis A and E. *ILAR J* **42**, 161-177 (2001).
72. Boonstra A, van der Laan LJ, Vanwolleghem T & Janssen HL. Experimental models for hepatitis C viral infection. *Hepatology* **50**, 1646-1655 (2009).
73. Feld JJ & Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **436**, 967-972 (2005).
74. Paeshuyse J, Dallmeier K & Neyts J. Ribavirin for the treatment of chronic hepatitis C virus infection: a review of the proposed mechanisms of action. *Curr Opin Virol* **1**, 590-598 (2011).

Chapter 3

Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus

YijinWang¹, Xinying Zhou¹, Yannick Debing², Kan Chen^{1,3}, Luc J.W. van der Laan⁴,
Johan Neyts², Harry L. A. Janssen^{1,5}, Herold J. Metselaar¹,
Maikel P. Peppelenbosch¹, and Qiuwei Pan¹

¹Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands; ²Department of Microbiology and Immunology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium; ³Bio-X Center, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou, China; ⁴Department of Surgery, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands; and ⁵Division of Gastroenterology, University Health Network, Toronto, Canada

Abstract

Background and aims: Many recipients of organ transplants develop chronic hepatitis, due to infection with the hepatitis E virus (HEV). Although chronic HEV infection is generally associated with immunosuppressive therapies, little is known about how different immunosuppressants affect HEV infection. **Methods:** A subgenomic HEV replication model, in which expression of a luciferase reporter gene is measured, and a full-length infection model were used. We studied the effects of different immunosuppressants, including steroids, calcineurin inhibitors (tacrolimus [FK506] and cyclosporine A), and mycophenolic acid (MPA, an inhibitor of inosine monophosphate dehydrogenase) on HEV replication in human hepatoma cell line Huh7. Expression of cyclophilins A and B (the targets of cyclosporine A) were knocked down using small hairpin RNAs. **Results:** Steroids had no significant effect on HEV replication. Cyclosporine A promoted replication of HEV in the subgenomic and infectious models. Knockdown of cyclophilin A and B increased levels of HEV genomic RNA by 4.0 ± 0.6 -fold and 7.2 ± 1.9 -fold, respectively ($n = 6$, $P < 0.05$). A high dose of FK506 promoted infection of liver cells with HEV. In contrast, MPA inhibited HEV replication. Incubation of cells with guanosine blocked the antiviral activity of MPA, indicating that the antiviral effects of this drug involve nucleotide depletion. The combination of MPA and ribavirin had a greater ability to inhibit HEV replication than MPA or ribavirin alone. **Conclusions:** Cyclophilins A and B inhibit replication of HEV; this might explain the ability of cyclosporine A to promote HEV infection. On the other hand, the immunosuppressant MPA inhibits HEV replication. These findings should be considered when physicians select immunosuppressive therapies for recipients of organ transplants who are infected with HEV.

Introduction

Hepatitis E virus (HEV) is one of the most common causes of acute hepatitis worldwide. It is a single-stranded positive-sense RNA virus, which mainly infects the liver hepatocytes. Although only a single HEV serotype is recognized, at least four different genotypes of human HEV exist ¹. Genotypes 1 and 2 are found mainly in developing countries and are transmitted via contaminated water sources. In contrast, genotypes 3 and 4 are prevalent in industrialized countries, and are zoonotic nature and spread mainly through eating undercooked pork or game products ². In general, HEV infection is a self-limiting disease and associated with low mortality, but fulminant hepatitis and high mortality have been described, reaching 25% in cases of pregnant women infected with genotype 1 in developing countries ³. In the Western world, the main clinical challenge is posed by HEV genotype 3 infection in patients receiving orthotopic organ transplantation ⁴. More than 60% of organ recipients infected with HEV will develop chronic hepatitis with rapid progression to cirrhosis ^{5, 6}. Which factors determine outcome in these patients remains obscure at best, hampering efforts to develop rational therapy and to address the increasing challenge of HEV infection in organ transplantation recipients.

Organ transplant patients take immunosuppressants for life-long, in order to prevent graft rejection. The resulting immunosuppression, however, also affects host immunity against viral challenges, and the use of immunosuppressive drugs has been proposed to be a key factor for developing chronic hepatitis after HEV infection ⁴. Consequently, dose reduction of immunosuppression is often used as the first intervention strategy to achieve viral clearance in HEV-infected organ recipients ⁷. Interestingly, however, clinical evidence suggests that different immunosuppressive regimens can differentially affect the infection course of HEV. The calcineurin inhibitor tacrolimus, but not cyclosporin A (CsA), has been found to be more frequently associated with persistent infection ⁶, and mycophenolate mofetil (MMF), the pre-drug form of mycophenolic acid (MPA) can help to clear the virus ⁸. However, the current clinical studies are not able to conclusively address the impact of different immunosuppressants because of limited patient numbers and lack of mechanistic insight as to how differences in immunosuppressive medication might be linked with an altered clinical course of HEV infection.

The observation that different immunosuppressive medication seems to have specific effects on the outcome of HEV infection suggests that such medication can have direct effects on viral replication, apart from influencing antiviral immunity. This consideration prompted us to test whether different immunosuppressive medication affects HEV replication in hepatocytes directly. The recent development of a genotype 3-based cell culture system^{9,10} makes it possible to study such questions in a highly detailed fashion. We show that different commonly used immunosuppressants have very specific effects on viral replication and that especially calcineurin inhibitors strongly facilitate HEV replication, and MPA suppresses viral replication. Thus, these results will serve as an important reference about the choice of particular immunosuppressive medication for HEV-infected orthotopic organ transplant recipients.

Materials and methods

Immunosuppressants

CsA and tacrolimus (FK506) were purchased from Abcam (Cambridge, MA). Dexamethasone (Dex), prednisolone (Pred) and MPA were purchased from sigma (St Louis, MO). All the reagents were dissolved in dimethylsulfoxide (DMSO), except MPA, which was dissolved in methanol. The effects of these immunosuppressants on host cell viability were determined by MTT assay (Supplementary Figure 1).

Cell culture

Human hepatoma cell line Huh7 and human embryonic kidney epithelial cell line 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 IU/mL streptomycin.

HEV cell culture models

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) and a construct containing subgenomic HEV sequence coupled with a gaussia luciferase reporter gene (p6-Luc) were used to generate HEV genomic RNA by

using the Ambion mMESSAGE mMACHINE® *in vitro* RNA transcription Kit (Life Technologies Corporation)^{9, 10}. Huh7 cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or replication models, respectively¹⁰.

Quantification of HEV infection

For the HEV replication model (p6-Luc), the activity of secreted gaussia luciferase in the cell culture medium was measured using BioLux® *Gaussia* Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA), as quantification of viral replication. To further determine the specific effects on viral replication-related luciferase activity, Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used as household luciferase activity for normalization¹¹. For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 minutes at 37°C. Both gaussia and firefly Luciferase activity were quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

For the p6 infectious HEV model, SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify genomic RNA. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), and the primers of housekeeping gene GAPDH were 5'-TGTCCCCACCCCAATGTATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense).

Gene knockdown by lentiviral vector delivered short hairpin RNA

Lentiviral vectors, targeting Cyclophilin A (CypA), Cyclophilin B (CypB) or GFP, were produced in 293T cells as previously described¹². After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. The shRNA sequences were: CypA, 5'-CCGGTGGTGACTTCACACGCCATAACTCGAGTTATGGCGTGTGAAGTCACCATTTTTG-3', and CypB, 5'-CCGGGCCTTAGCTACAGGAGAGAACTCGAGTTTCTCTCCTGTAGCTAAGGCTTTTTG-3'.

To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 µg/ml puromycin (Sigma) in the cell culture medium. For the infectious model, HEV particles were incubated with knockdown and control Huh7

cells. For the subgenomic model, p6-Luc cells were directly transduced with lentiviral shRNA vectors and selected by adding 2.5 µg/ml puromycin.

Western blot

For Western blot, commercial antibodies against CypA and CypB (Rabbit polyclonal; Abcam, Cambridge, UK) were used. Proteins in cell lysates were heated 5 min at 95°C followed by loading onto a 15% sodium dodecyl sulfate-polyacrylamide SDS gel and separating by electrophoresis (SDS-PAGE). After 90 minutes running in 115 V voltage, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 hour with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 mL blocking buffer and 2.5 mL PBS containing 0.05% Tween 20 (PBS-T). It was followed by incubation with rabbit anti-CypA (1:5000) or anti-CypB (1:7500) antibody overnight at -4°C. Membrane was washed 3 times followed by incubation for 1.5h with an anti-rabbit peroxidase conjugated secondary antibody (1:5,000). After 3 times washing, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

Statistical analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann–Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). P values less than 0.05 were considered statistically significant.

Results

Glucocorticosteroids did not affect HEV replication

Prednisolone (Pred) and its close analogue dexamethasone (Dex) remain important drugs in the clinical management of patients receiving orthotopic organ transplantation¹³. To study the possible effects of these drugs on HEV replication, we used a model in which cells were transfected with a subgenomic construct of HEV coding sequence in which the 5' portion of ORF2 was replaced with the in-frame secreted form of luciferase derived from the marine copepod *Gaussia princeps*. Accumulation of luciferase thus serves as reporter for HEV RNA synthesis (p6-Luc), and the loss of the capsid protein precludes the formation of novel viral

particles. In parallel, Huh7 cells constitutively expressing a non-secreted firefly luciferase were used for normalization of non-specific effects on luciferase signals. However, as shown in Figure 1, neither Pred nor Dex significantly affected HEV replication. We conclude that steroids have no direct effects on HEV replication.

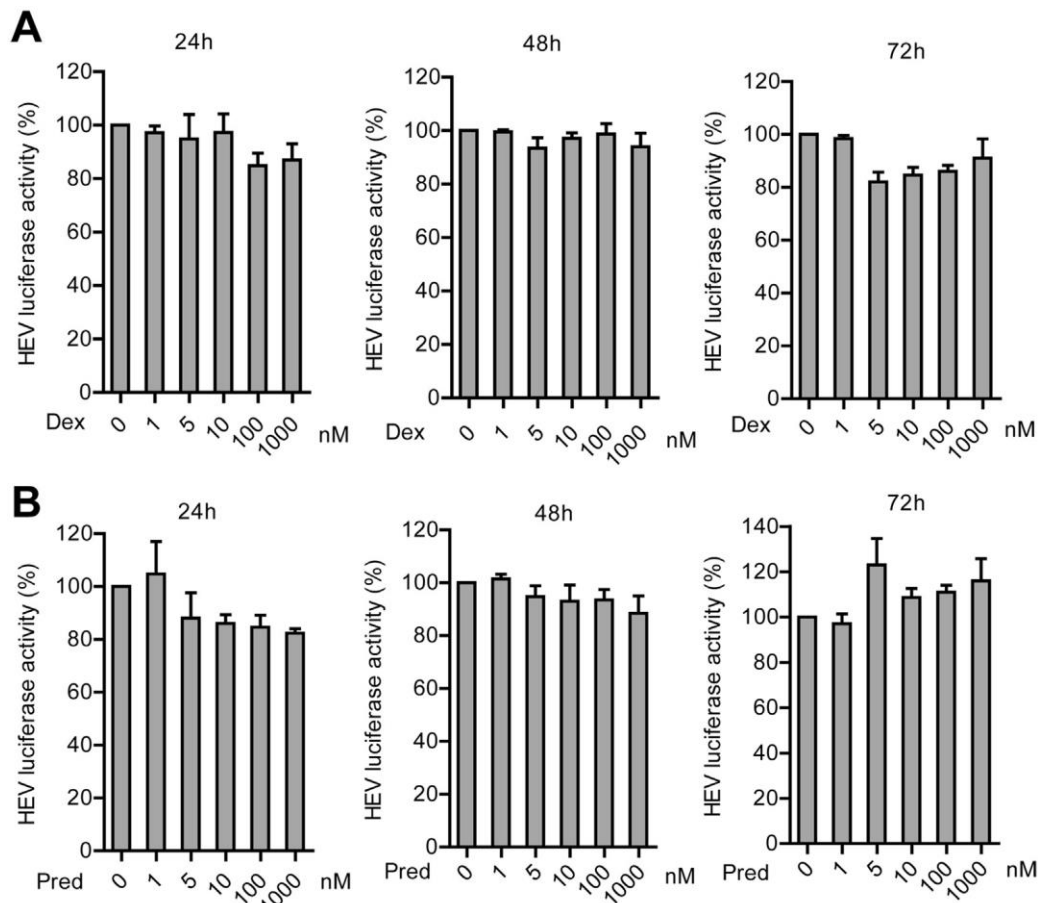


Figure 1. The effects of steroids on HEV replication in subgenomic cell culture model. Huh7 cells-based subgenomic HEV replicon containing the luciferase reporter gene was treated for 24h, 48h and 72h with a dose-range of Dex and Pred. (A) Dex and Pred (B) didn't significantly affect luciferase activity. Data represent as Mean \pm SD of three independent experiments.

CsA dose-dependently enhanced HEV replication

CsA, a calcineurin inhibitor, is an important drug for prevention of graft rejection. To examine the effects of CsA on HEV replication, We tested the effects of 0.1, 0.5 and 5 $\mu\text{g/mL}$ CsA on viral replication using the subgenomic p6-Luc model as a read-out. It appeared that CsA dose-dependently increased HEV replication-related luciferase activity (Figure 2A). Consistently, CsA also dose-dependently increased HEV infection in the full-length (p6) infectious model (Figure 2B). 48 hours treatment with CsA (5 $\mu\text{g/mL}$) resulted in 2.67 ± 0.7 -

fold (Mean \pm SD, $n = 5$, $P < 0.01$) increase of HEV genomic RNA level (determined by qRT-PCR), compared with the control (Figure 2B). CsA directly promotes viral replication in a hepatocyte-like cells and experimentation was initiated to establish the molecular basis of this effect.

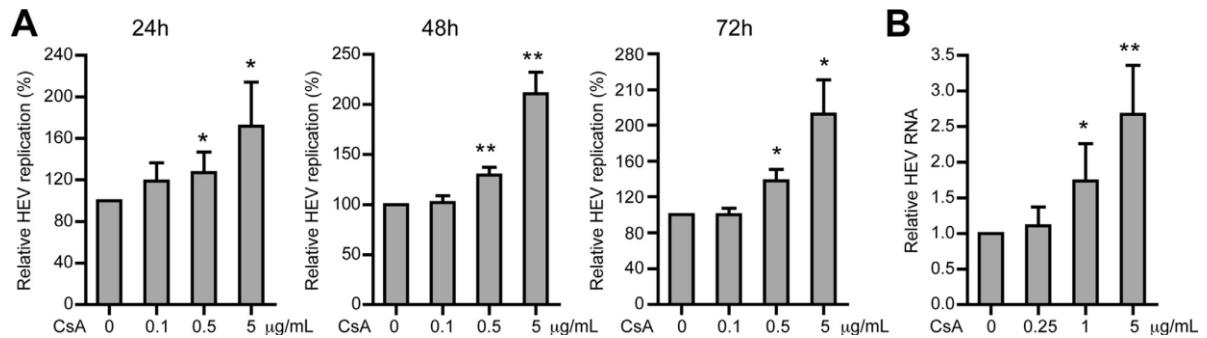


Figure 2. CsA promoted HEV infection. (A) The subgenomic HEV replicon containing the luciferase reporter gene was treated for 24 ($n = 5$), 48 ($n = 7$) and 72 hours ($n = 7$) with different doses of CsA. Treatment with CsA (0.5 or 5 $\mu\text{g/mL}$) significantly increased HEV luciferase activity. (B) The Huh7 cells-based infectious HEV model was treated with CsA for 48 hours. CsA significantly increased HEV RNA at 0.5 and 5 $\mu\text{g/mL}$ concentrations ($n = 5$). Data represent as Mean \pm SD of multiple experiments. * $P < 0.05$; ** $P < 0.01$.

Silencing the cellular targets of CsA, cyclophilin A and B, enhanced HEV replication

CsA acts through binding and inhibition of the cyclophilin A/B complex. The effects of CsA on HEV replication could be potentially mediated through cyclophilins. Cyclophilin A (CypA)¹⁴ and B (CypB)¹⁵ have been implicated in the anti-hepatitis C virus (HCV) mechanism of CsA. Therefore, lentiviral-mediated RNA interference was used for knockdown of these two genes, as to allow investigation of their potential function in the effects of CsA on HEV replication. To this end, Huh7 cells were transduced with integrating lentiviral vectors expressing both shRNA and puromycin. Cells stably transduced with the vector were selected and expanded by adding puromycin to the relevant cell cultures. The shRNA clones with most potent efficacy of CypA and CypB knockdown were selected for follow-up experimentation (Figure 3A). Cells stably integrated with shRNA targeting GFP (as control), CypA or CypB were inoculated with infectious HEV viruses (p6). The level of infection was quantified by qRT-PCR of genomic viral RNA in the cells 3 days post-inoculation. As shown in Figure 3B, knockdown of CypA has resulted in 4.0 ± 0.6 -fold (Mean \pm SEM, $n = 6$, $P < 0.01$) increase of HEV RNA;

whereas knockdown of CypB has resulted in 7.4 ± 1.9 -fold (Mean \pm SEM, $n = 6$, $P < 0.05$) increase of viral genomic RNA. Consistently, silencing of CypA and CypB in HEV subgenomic model significantly increased viral replication-related luciferase activity by $350.4 \pm 11.7\%$ (Mean \pm SEM, $n = 12$, $P < 0.0001$) and $406 \pm 14.5\%$ (Mean \pm SEM, $n = 12$, $P < 0.0001$), respectively (Figure 3C). The most straightforward explanation of these results is that Csa through cyclophilin binding and inhibition facilitates HEV infection (Figure 2).

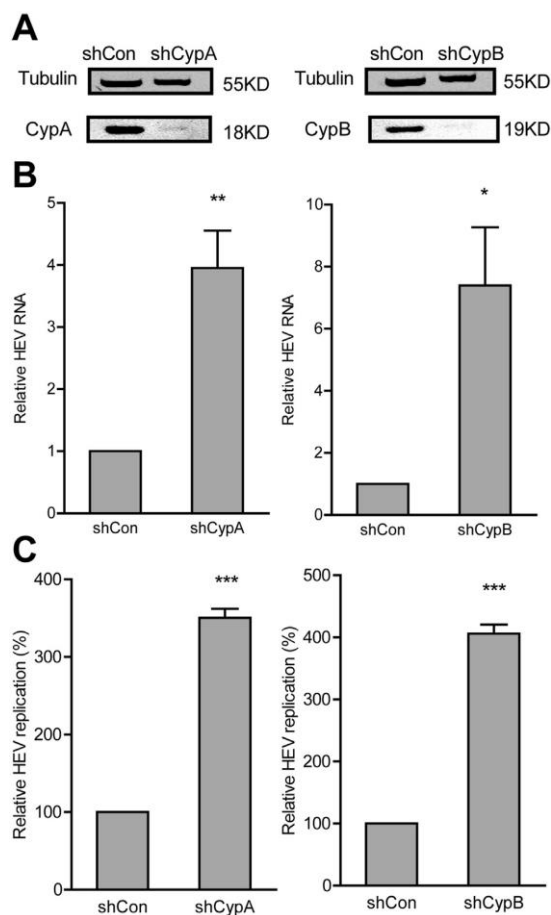


Figure 3. Gene silencing of CypA or CypB facilitated HEV infection. (A) Western blotting showed dramatic down-regulation of CypA and CypB protein by lentiviral RNAi mediated gene knockdown. (B) Silencing of CypA or CypB resulted in a significant increase of cellular HEV RNA. Data represent as Mean \pm SEM of six independent experiments. * $P < 0.05$. (C) Silencing of CypA or CypB significantly increased viral replication-related luciferase activity in the HEV subgenomic model (Mean \pm SEM, $n = 12$ replicates of three experiments in total). *** $P < 0.001$.

High dose of FK506 promoted HEV replication

FK506 is another type of calcineurin inhibitor that binds to FK binding proteins. To determine the effects of FK506 on HEV replication, p6-Luc cells were treated with FK506 at concentrations of 0.1, 0.5 and 5 $\mu\text{g/mL}$. As shown in Figure 4A, only high dose (5 $\mu\text{g/mL}$) of FK506 significantly increased HEV replication, seen at 24, 48 and 72 hours post-treatment. This was also further confirmed in the p6 infectious model that HEV genomic RNA was increased by $35 \pm 9.6\%$ (Mean \pm SD, $n = 4$, $P < 0.01$) by treatment with 5 $\mu\text{g/mL}$ FK506 for 48 hours (Figure 4B).

Because the immunosuppressive mechanisms of calcineurin inhibitors are mediated via the Ca^{2+} -NFAT signal transduction, we tested the effects of N,N-Dimethyl-D-erythro-sphingosine (DMS), a compound that can efficiently increase cellular Ca^{2+} levels,¹⁶ on HEV infection. As shown in Supplementary Figure 2A, DMS (1-4 $\mu\text{g}/\text{ml}$) triggered clear induction of Ca^{2+} levels in Huh7 cells visualized with a fluorescent dye, Fluo-4/AM. However, no clear effects were observed on HEV infection in either the subgenomic (Supplementary Figure 2B) or the infectious (Supplementary Figure 2C) model. Thus, the proviral effects of calcineurin inhibitors on HEV infection appear to be independent of Ca^{2+} levels.

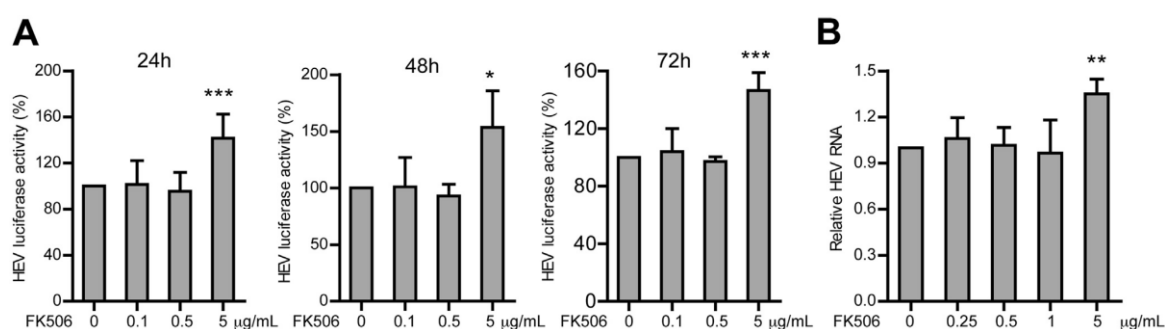


Figure 4. High dose of FK506 enhanced HEV infection. (A) Treatment with 5 $\mu\text{g}/\text{mL}$ (but not 0.5 and 1 $\mu\text{g}/\text{mL}$) resulted in significant increase of luciferase activity in the HEV subgenomic model (Mean \pm SD, $n = 5-8$) and (B) significant increase of HEV RNA in the infectious model (Mean \pm SD, $n = 4$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

MPA inhibited HEV replication by depletion of cellular nucleotide pool

MPA, an inhibitor of inosine monophosphate dehydrogenase (IMPDH) (the biosynthesis of guanine), is an immunosuppressive drug often used in organ transplantation, but also has a broad antiviral activity against a spectrum of viruses¹⁷. We investigated whether MPA could also be able to inhibit HEV infection. Treatment with MPA (0.1-10 $\mu\text{g}/\text{mL}$) has resulted in a significant reduction of HEV replication-related luciferase activity in the subgenomic replicon. For example, with 10 $\mu\text{g}/\text{mL}$ MPA treatment, the luciferase activity were $42.8 \pm 2.3\%$ (Mean \pm SEM, $n = 9$, $P < 0.001$), $32.8 \pm 5.3\%$ (Mean \pm SEM, $n = 10$, $P < 0.001$) and $39.5 \pm 4.6\%$ (Mean \pm SEM, $n = 12$, $P < 0.001$) of the control group at day 1, 2 and 3, respectively (Figure 5A). Consistently, MPA also dose-dependently inhibited cellular viral RNA in the infectious HEV model. 48 hours treatment with MPA (10 $\mu\text{g}/\text{mL}$) resulted in $65 \pm 9\%$ (Mean \pm SD, $n = 5$, $P < 0.01$) inhibition of HEV genomic RNA level (determined by qRT-PCR), compared with the control (Figure 5B).

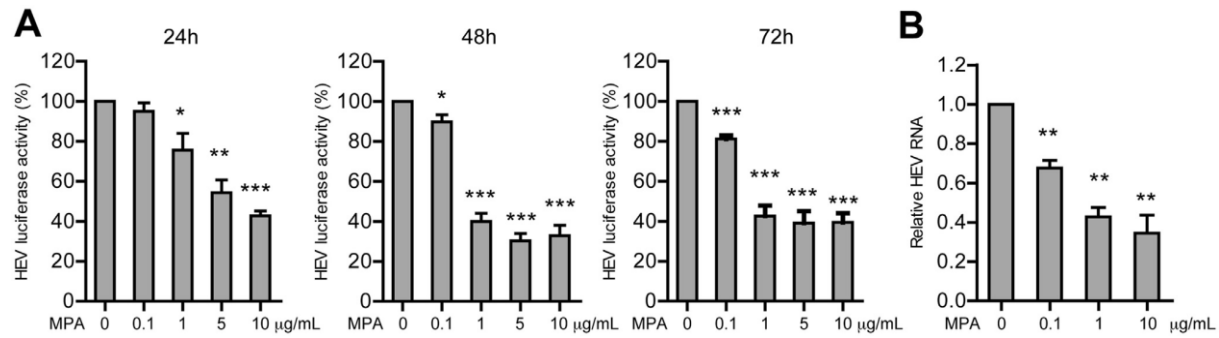


Figure 5. Potent anti-HEV activity of MPA. (A) Treatment of MPA for 24, 48 or 72 hours has resulted in significant reduction of HEV luciferase activity in the subgenomic model (Mean \pm SEM, $n = 9-12$). (B) In the infectious model, treatment with 0.1, 1 and 10 $\mu\text{g/mL}$ of MPA for 48 hours has significantly inhibited HEV RNA by 32%, 57% and 65%, respectively (Mean \pm SD, $n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

To further investigate whether the effects of MPA are via depletion of cellular nucleotides, additional guanosine was added to the MPA treatment. As shown in Figure 6, supplement of exogenous guanosine completely abrogated the antiviral activity of MPA in both subgenomic and infectious HEV models, suggesting that the action of MPA is exclusively via nucleotide depletion. Immunosuppressive drugs have highly diverse effects on HEV replication, calcineurin inhibitors stimulating viral replication but MPA exerting direct inhibition of HEV replication

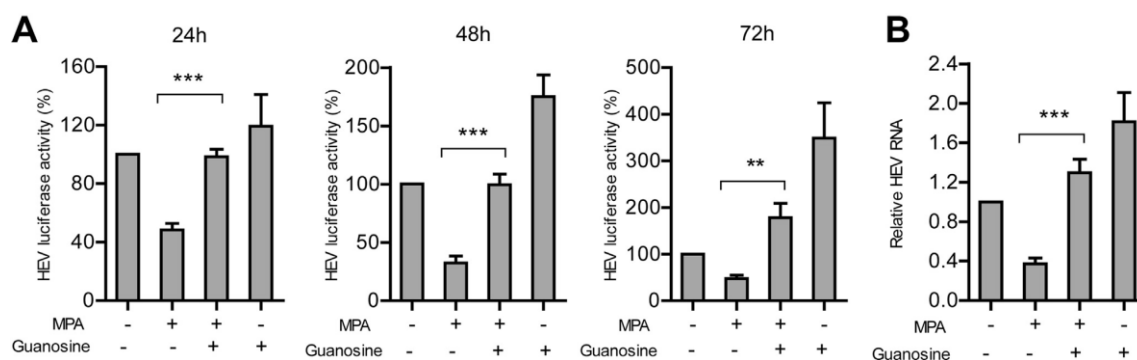


Figure 6. Supplement of exogenous guanosine completely abrogated the anti-HEV effects of MPA. (A) In the subgenomic HEV replicon, the antiviral effects by treatment of MPA at concentration of 10 $\mu\text{g/mL}$ for 24, 48 and 72 hours were abrogated by adding exogenous guanosine (100 $\mu\text{g/mL}$) (Mean \pm SEM, $n = 7-10$). (B) Similarly, the antiviral effects by treatment of MPA at concentration of 10 $\mu\text{g/mL}$ for 48h was also abrogated by adding 100 $\mu\text{g/mL}$ exogenous guanosine in the infectious model (Mean \pm SEM, $n = 8$). ** $P < 0.01$; *** $P < 0.001$.

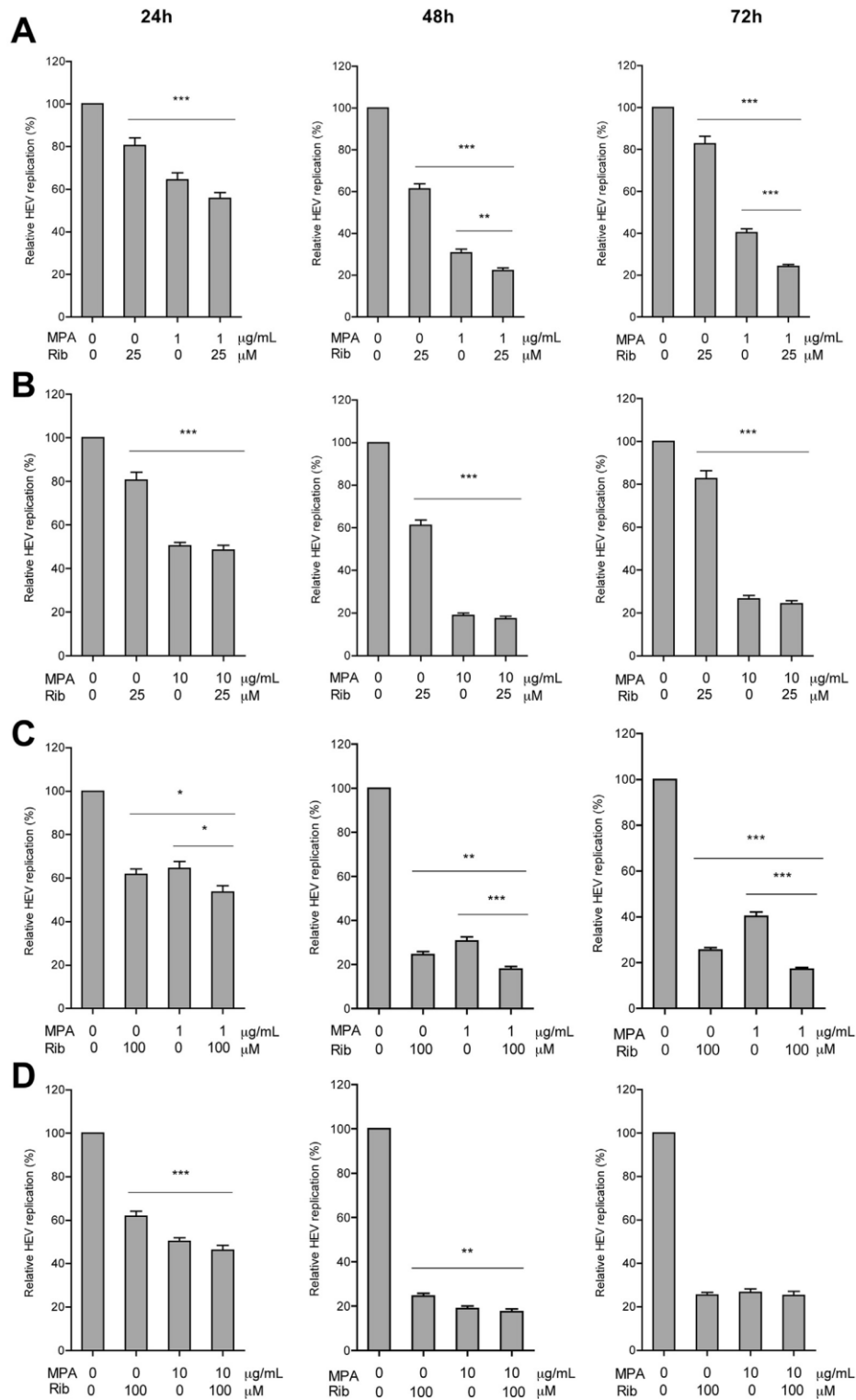


Figure 7. Combination of MPA with ribavirin extended their antiviral activity. Treatment with ribavirin alone has showed significant anti-HEV effects (Mean \pm SEM, n = 16 replicates in total) and a combination of MPA with ribavirin demonstrated an additional antiviral potency in particular combination groups; MPA doses: 1 μ g/ml; 10 μ g/ml; ribavirin doses: 25 μ M; 100 μ M. (A) 1 μ g/ml MPA combined with 25 μ M ribavirin. (B) 10 μ g/ml MPA combined with 25 μ M ribavirin. (C) 10 μ g/ml MPA combined with 100 μ M ribavirin. (D) 1 μ g/ml MPA combined with 25 μ M ribavirin * P < 0.05; ** P < 0.01; *** P < 0.001.

Combination of MPA with ribavirin extended their antiviral activity

Because the use of ribavirin monotherapy as off-label drug is gaining favor for treating hepatitis E ¹⁸, we also investigated the antiviral effects of combining MPA with ribavirin. As shown in Figure 7, a serial of combination groups have demonstrated a general beneficial effect and no negative drug-drug interference was observed. For instance, combining 1 µg/ml MPA with 25 µm ribavirin resulted in $76 \pm 1\%$ inhibition of HEV luciferase, whereas MPA alone resulted in $60 \pm 2\%$ and ribavirin alone resulted in $17 \pm 3\%$ inhibition (Mean \pm SEM, n = 16, P < 0.001) after 72 hours treatment (Figure 7A). Therefore, a combination of ribavirin with MPA appears compatible against HEV infection and constitutes an attractive clinical option for preventing rejection in HEV-infected patients.

Discussion

Immunosuppressive medication has been proposed to be a key factor for developing chronic hepatitis E in organ transplantation recipients ⁴ and is often solely attributed to diminished antiviral immunity. Clinical evidence, however, suggests that different immunosuppressive regimens can differentially affect the infection course of HEV ^{6, 8}. By testing different immunosuppressants in two HEV replication models, we have consistently demonstrated that steroids (Pred and Dex) did not affect viral replication, calcineurin inhibitors (CsA and FK506) promoted HEV infection, and MPA suppressed viral infection *in vitro*. The concentrations of these immunosuppressants used in this study are in general covering the achievable blood concentrations in patients ¹⁹⁻²¹. Of note, animal studies have indicated that certain immunosuppressant even accelerates in the liver and drug levels in hepatocytes will exceed those observed in serum ²². Therefore, we propose that the results of this *in vitro* study will be a valuable reference regarding the choice of particular immunosuppressant for orthotopic organ transplantation patients who are infected with HEV.

Steroids have been used since the early years of organ transplantation. Pred and its close analogue Dex are potent suppressors of the immune system, as they modulate cellular and inflammatory responses via stimulation or inhibition of gene transcription ²³. In the setting of liver transplantation for HCV patients, evidence suggested that steroid boluses used to treat acute rejection are associated with an increase in viral load and the severity of

HCV recurrence^{24, 25}. Using subgenomic cell culture model of HCV replicon, a study demonstrated that both Pred and Dex have no stimulatory effect on viral RNA levels, but rather have minor inhibitory effects¹³. As to infectious HCV model, however, Pred was reported to promote HCV infection by enhancing virus entry, including up-regulation of two essential HCV entry factors: occludin and scavenger receptor class B type I²⁶. In both subgenomic and infectious models of HEV, we did not observe clear effect on HEV infection by either Pred or Dex. Although limited studies have reported the impact of steroids in HEV patients, one case report has documented a good clinical and biochemical response to steroid therapy in a patient of acute hepatitis E with autoimmune hepatitis, who maintained health with low dose of steroids²⁷.

The first *in vitro* evidence that CsA but not FK506 can inhibit HCV replication²⁸ sparked the clinical debate on the possible differential effects of these two drugs on HCV recurrence after liver transplantation²⁹. Several follow-up studies have demonstrated that the targets of CsA, CypA and CypB, are host factors supporting HCV infection^{14, 15}. CsA exerts anti-HCV effects by inhibition of these cellular factors³⁰. Interestingly, we observed a proviral effect of CsA in HEV cell culture models. Using RNA interference gene silencing approach, we further demonstrated that knockdown of either CypA or CypB enhanced HEV infection, suggesting that both factors could restrict HEV infection. This convincingly explained why CsA could facilitate HEV infection. Although a number of reports have demonstrated a supportive role of CypA in infections of HIV³¹, HCV¹⁴, or HBV³², recent studies also reported that CypA possesses a repressive effect on the replication of some viruses including Influenza A virus³³ and rotavirus³⁴, similar to what we have observed for HEV. Because the mechanistic insight is still largely missing for the antiviral action of cyclophilins, it deserves further investigation. In addition, we also observed a proviral effects of FK506, but only at high dose. To our knowledge, there is no evidence of FK506 affecting HCV infection in cell culture^{28, 35}. In fact, compared with CsA, dose reduction of FK506 was assumed to be more associated with clearance of HEV in cases of renal transplantation with acute infection³⁶. In a large retrospective study (although only 85 patients included), the use of FK506 was the main predictive factor for chronic hepatitis E in organ recipients⁶. Our *in vitro* results have indicated that both FK506 and CsA can promote HEV infection. However, these data do not necessarily contradict to the clinical observation, because the number of patients currently investigated in the clinic is rather too small to draw solid conclusion. In

addition, besides the direct effects we observed in cell culture, drugs can also have indirect influence on the infection.

The antiviral effects of MPA/MMF have been demonstrated against a broad spectrum of viruses, including dengue virus, West Nile, yellow fever virus, Chikungunya virus, HBV and HCV³⁶⁻³⁹. This is consistent with our finding that MPA also potently inhibited HEV replication. For several viruses, MPA exerts antiviral effects by targeting IMPDH to deplete cellular nucleotide pools³⁶. In case of HCV, the IMPDH-dependent pathway only partially contributed to its antiviral activity¹¹. In contrast, supplementation of exogenous guanosine completely abrogated the anti-HEV activity of MPA, suggesting a crucial role of IMPDH inhibition leading to depletion of cellular nucleotides. Interestingly, clearance of HEV after heart transplantation was found to be more frequent in patients with immunosuppressive medication containing MMF⁸, although this may be biased by a reduced dose of CsA or FK506 in these cases.

Despite of a clear benefit of manipulating immunosuppressive regimens, a substantial proportion of patients is still not able to clear the virus and rapidly progresses towards chronic hepatitis⁶. Although no proven medication is available, the use of ribavirin monotherapy as off-label drug is gaining acceptance for treating hepatitis E¹⁸. An intriguing question is whether immunosuppressants can interfere with or promote the anti-HEV efficacy of ribavirin. In this study, we have finally demonstrated a beneficial effect of combining ribavirin with MPA (Figure 7). This does provide a proof-of-concept that it is important to choose the right immunosuppressive medication while under antiviral therapy of HEV in organ transplant recipients.

In conclusion, this study has profiled differential effects of different immunosuppressants on HEV infection in cell culture. Steroids did not affect genotype 3 HEV replication *in vitro*, but high dose of FK506 promoted HEV infection. CsA dose-dependently facilitated HEV infection by targeting cellular factors CypA and CypB. In contract, MPA potently suppressed HEV infection by depletion of cellular nucleotide pools. In addition, a clear beneficial effect was observed when MPA combined with another antiviral regimen ribavirin. Although experimental research alone will not be able to clarify these complicated but important clinical issues, the knowledge gained from this study is for sure a valuable reference for the management of immunosuppression in organ transplantation recipients

infected with HEV. Hopefully, it will also promote the initiation of randomized controlled clinical studies to address these issues in the near future.

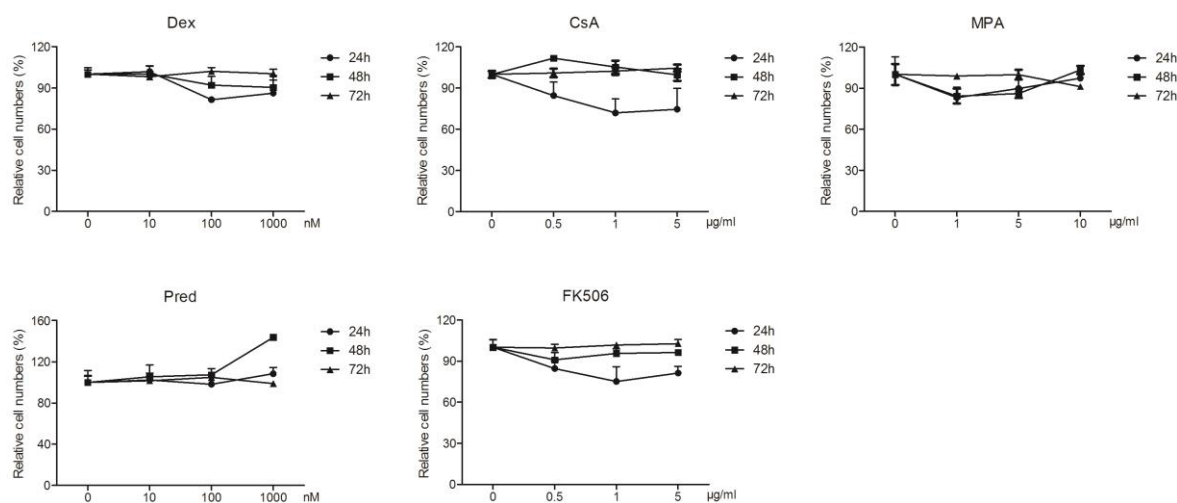
Supplementary methods and Figures

MTT assay

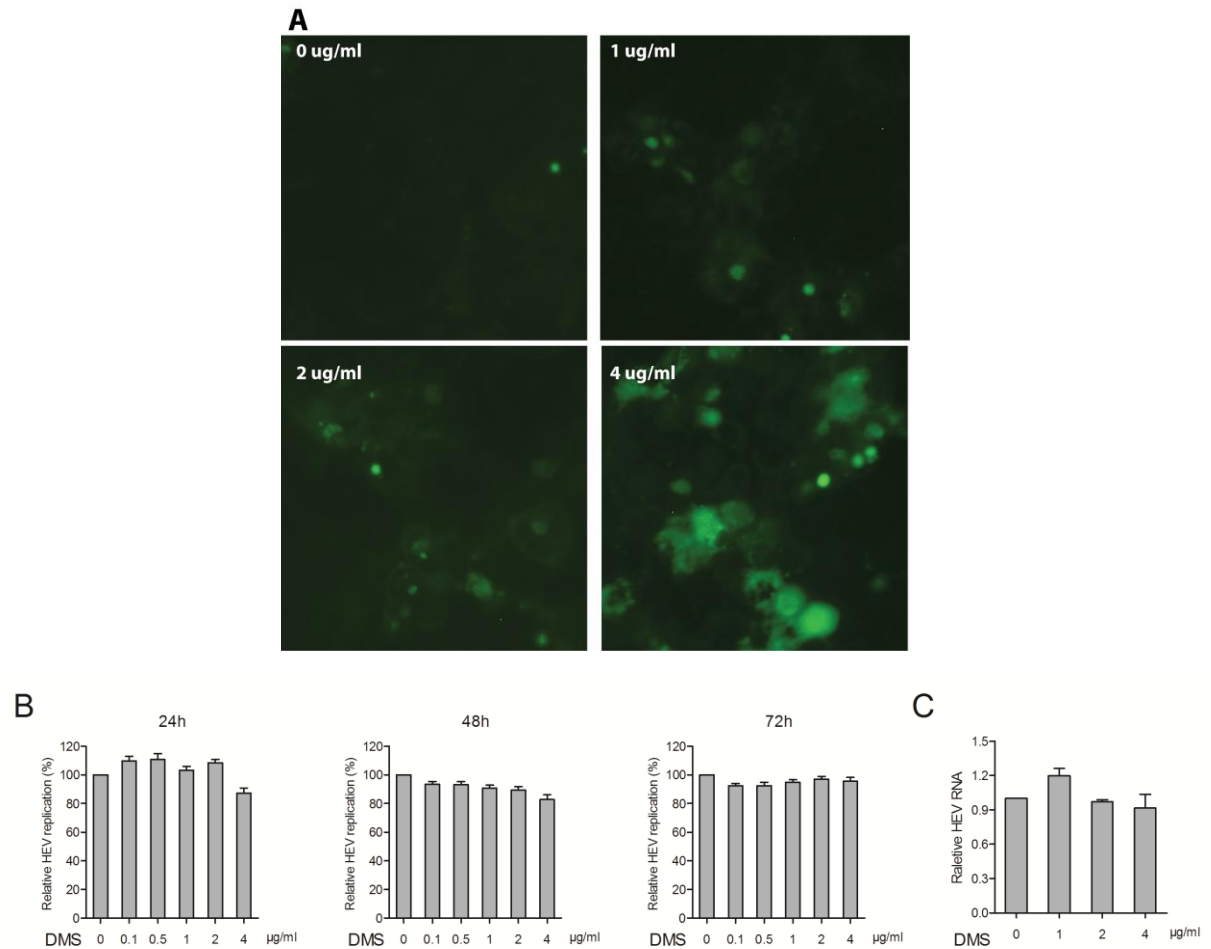
Huh7 cells were plated in 96-well plates and treated with immunosuppressants. At the indicated times, the number of metabolically active cells was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) assay.

Measurement of $[Ca^{2+}]_i$ concentration

The intracellular $[Ca^{2+}]_i$ concentration was measured using the fluorescent dye, Fluo-4/AM (Dojindo Laboratories, Kumamoto, Japan). The Huh7 cells were treated with serial dilutions of DMS for 48 hours, then resuspended in PBS containing 1% bovine serum and incubated for 30 min with 5 μ M Fluo-4/AM in the dark. After being washed with PBS, the Fluo-4/AM-labeled cells were observed under an inverted fluorescence microscope.



Supplementary Figure1. The effects of immunosuppressants on viability of Huh-7 cells. Cells were treated with immunosupprants at different concentrations for 24, 48 and 72 hours. Cell viability was assayed by the MTT test. Shown is Mean \pm SD from at least 3 independent experiments.



Supplementary Figure 2. Induction of cellular Ca^{2+} concentration by N,N-Dimethyl-D-erythro-sphingosine (DMS) did not affect HEV infection. (A) DMS (1-4 $\mu\text{g/ml}$) triggered clear induction of Ca^{2+} levels in Huh7 cells visualized with a fluorescent dye, Fluo-4/AM. (B) HEV replication in the subgenomic model was not affected by DMS treatment for 24, 48 and 72 hours, respectively. (C) HEV infection was also not affected by DMS treatment for 48 hours in the infectious model quantified by qRT-PCR. Shown is Mean \pm SD from 3 independent experiments.

References

1. Purdy MA & Khudyakov YE. The molecular epidemiology of hepatitis E virus infection. *Virus Res* **161**, 31-39 (2011).
2. Hoofnagle JH, Nelson KE & Purcell RH. Hepatitis E. *N Engl J Med* **367**, 1237-1244 (2012).
3. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
4. Kamar N, *et al.* Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* **358**, 811-817 (2008).
5. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
6. Kamar N, *et al.* Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology* **140**, 1481-1489 (2011).
7. Koning L, *et al.* Clinical implications of chronic hepatitis E virus infection in heart transplant recipients. *J Heart Lung Transplant* **32**, 78-85 (2013).
8. Pischke S, *et al.* Chronic hepatitis e in heart transplant recipients. *Am J Transplant* **12**, 3128-3133 (2012).
9. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
10. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
11. Pan Q, *et al.* Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* **55**, 1673-1683 (2012).
12. Pan Q, *et al.* Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection. *FEBS Lett* **585**, 1025-1030 (2011).
13. Henry SD, Metselaar HJ, Van Dijck J, Tilanus HW & Van Der Laan LJ. Impact of steroids on hepatitis C virus replication in vivo and in vitro. *Ann N Y Acad Sci* **1110**, 439-447 (2007).
14. Ciesek S, *et al.* Cyclosporine A inhibits hepatitis C virus nonstructural protein 2 through cyclophilin A. *Hepatology* **50**, 1638-1645 (2009).
15. Watashi K, *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* **19**, 111-122 (2005).
16. Chen K, *et al.* DMS triggers apoptosis associated with the inhibition of SPHK1/NF-kappaB activation and increase in intracellular Ca²⁺ concentration in human cancer cells. *Int J Mol Med* **32**, 1345-1354 (2013).
17. Pan Q, Tilanus HW, Metselaar HJ, Janssen HL & van der Laan LJ. Virus-drug interactions--molecular insight into immunosuppression and HCV. *Nat Rev Gastroenterol Hepatol* **9**, 355-362 (2012).
18. Pischke S, *et al.* Ribavirin treatment of acute and chronic hepatitis E: a single-centre experience. *Liver Int* **33**, 722-726 (2013).

19. Kanda Y, *et al.* Effect of blood cyclosporine concentration on the outcome of hematopoietic stem cell transplantation from an HLA-matched sibling donor. *Am J Hematol* **81**, 838-844 (2006).
20. Rodrigo E, *et al.* Evolution of tacrolimus blood levels and concentration-dose ratios in patients who develop new onset diabetes mellitus after kidney transplantation. *Transpl Int* **18**, 1152-1157 (2005).
21. Borrows R, *et al.* Mycophenolic acid 12-h trough level monitoring in renal transplantation: association with acute rejection and toxicity. *Am J Transplant* **6**, 121-128 (2006).
22. Matsuzawa Y & Nakase T. Metabolic fate of ethyl O-[N-(p-carboxyphenyl)-carbamoyl] mycophenolate (CAM), a new antitumor agent, in experimental animals. *J Pharmacobiodyn* **7**, 776-783 (1984).
23. Lowenberg M, Verhaar AP, van den Brink GR & Hommes DW. Glucocorticoid signaling: a nongenomic mechanism for T-cell immunosuppression. *Trends Mol Med* **13**, 158-163 (2007).
24. Gane EJ, *et al.* A longitudinal analysis of hepatitis C virus replication following liver transplantation. *Gastroenterology* **110**, 167-177 (1996).
25. Gane EJ, *et al.* Long-term outcome of hepatitis C infection after liver transplantation. *N Engl J Med* **334**, 815-820 (1996).
26. Ciesek S, *et al.* Glucocorticosteroids increase cell entry by hepatitis C virus. *Gastroenterology* **138**, 1875-1884 (2010).
27. Desai HG & Naik AS. Acute viral E hepatitis with chronic liver disease (autoimmune hepatitis). *Indian J Med Sci* **59**, 113-115 (2005).
28. Watashi K, Hijikata M, Hosaka M, Yamaji M & Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* **38**, 1282-1288 (2003).
29. Berenguer M. Viral hepatitis: Cyclosporin versus tacrolimus for HCV transplant recipients. *Nat Rev Gastroenterol Hepatol* **8**, 422-424 (2011).
30. Nakagawa M, *et al.* Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. *Gastroenterology* **129**, 1031-1041 (2005).
31. Franke EK, Yuan HE & Luban J. Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* **372**, 359-362 (1994).
32. Tian X, *et al.* Hepatitis B virus (HBV) surface antigen interacts with and promotes cyclophilin a secretion: possible link to pathogenesis of HBV infection. *J Virol* **84**, 3373-3381 (2010).
33. Liu X, *et al.* Cyclophilin A interacts with influenza A virus M1 protein and impairs the early stage of the viral replication. *Cell Microbiol* **11**, 730-741 (2009).
34. Zhou D, Mei Q, Li J & He H. Cyclophilin A and viral infections. *Biochem Biophys Res Commun* **424**, 647-650 (2012).
35. Pan Q, *et al.* Calcineurin inhibitor tacrolimus does not interfere with the suppression of hepatitis C virus infection by interferon-alpha. *Liver Transpl* **16**, 520-526 (2010).
36. Kamar N, *et al.* Hepatitis E virus and the kidney in solid-organ transplant patients. *Transplantation* **93**, 617-623 (2012).
37. Diamond MS, Zachariah M & Harris E. Mycophenolic acid inhibits dengue virus infection by preventing replication of viral RNA. *Virology* **304**, 211-221 (2002).

38. Henry SD, *et al.* Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporin A and interferon-alpha. *Gastroenterology* **131**, 1452-1462 (2006).
39. Ying C, Colonno R, De Clercq E & Neyts J. Ribavirin and mycophenolic acid markedly potentiate the anti-hepatitis B virus activity of entecavir. *Antiviral Res* **73**, 192-196 (2007).

Chapter 4

Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway

Xinying Zhou¹, Yijin Wang¹, Herold J. Metselaar¹, Harry L. A. Janssen^{1, 2},
Maikel P. Peppelenbosch¹ and Qiuwei Pan¹

¹Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands. ²Division of Gastroenterology, University Health Network, Toronto, Canada

Abstract

Background and aims: Humans are frequently exposed to hepatitis E virus (HEV). Nevertheless, the disease mainly affects pregnant women and immunocompromised individuals. Organ recipients receiving immunosuppressants, such as rapalogs, to prevent rejection have a high risk for developing chronic hepatitis following HEV infection. Rapalogs constitute potent inhibitors of mTOR including rapamycin and everolimus. As a master kinase, the mechanism-of-action of mTOR is not only associated with the immunosuppressive capacity of rapalogs but is also tightly regulated during pregnancy because of increased nutritional demands. **Methods:** We thus investigated the role of mTOR in HEV infection by using two state-of-the-art cell culture models: a subgenomic HEV containing luciferase reporter and a full-length HEV infectious cell culture system. **Results:** In both subgenomic and full-length HEV models, HEV infection was aggressively escalated by treatment of rapamycin or everolimus. Inhibition of mTOR was confirmed by Western blot showing the inhibition of its down-stream target, S6 phosphorylation. Consistently, stable silencing of mTOR by lentiviral RNAi resulted in a significant increase in intracellular HEV RNA, suggesting an antiviral function of mTOR in HEV infection. By targeting a series of other up- and down-stream elements of mTOR signaling, we further revealed an effective basal defense mechanism of PI3K-PKB-mTOR pathway against HEV, which is through the phosphorylated eIF4E-binding protein 1 (4E-BP1), however independent of autophagy formation. **Conclusions:** The discovery that PI3K-PKB-mTOR pathway limits HEV infection through 4E-BP1 and acts as a gate-keeper in human HEV target cells bears significant implications in managing immunosuppression in HEV-infected organ transplantation recipients.

Introduction

Although hepatitis E virus (HEV) infection is underdiagnosed, it is clear that the virus represents one of the most abundant infectious challenges to humans ¹. In Western countries, HEV infection of healthy individuals almost exclusively remains subclinical and otherwise causes an acute and self-limiting infection in immune-competent individuals with low mortality rates ². In contrast, patients with HEV infection in immunocompromised individuals that include organ transplantation recipients ³, HIV patients ⁴ and cancer patients receiving chemotherapy ⁵ have substantially high risk of developing chronic hepatitis. The use of immunosuppressants, such as rapalogs, in organ transplant recipients to prevent rejection is associated with substantial pathology and in particular an increased risk of developing chronic hepatitis with substantial graft loss and mortality rates ⁶.

However, in undernourished populations in the developing world, fulminant hepatitis and high mortality are described, reaching 25% in case of pregnant women ⁶. In the current (2012-2013) hepatitis E outbreak among refugees in South Sudan, a total of 5,080 acute jaundice syndrome cases had been reported from all four Maban County refugee camps, as of January 27, 2013. An acute jaundice syndrome case-fatality rate of 10.4% was observed among pregnant women across all camps ⁷. Humans appear to have powerful HEV combating mechanisms, but that apparently require good nutritional and host defense status for optimal functionality ⁸. The nature of these mechanisms has not been characterized, due to the lack of robust HEV cell culture models. The advent of new technology that mimics the HEV infectious process *in vitro*, in particular the development of *in vitro* adapted infectious clones and subgenomic HEV reporters, has led to hopes that the mechanisms that control HEV infection in normal physiology can now be identified ^{9,10}.

Rapalogs comprise, amongst others rapamycin (RAPA, rapamune, sirolimus; originally isolated from *Streptomyces hygroscopicus*) and everolimus (the 40-O-[2-hydroxyethyl] derivative of rapamycin). This immunosuppressive medication is gaining increasing popularity in the transplantation context, mainly because of its low nephrotoxicity ¹¹. Their molecular mode of action is well characterized and involves inhibition of the mammalian target of rapamycin (mTOR) pathway. mTOR is a central element within the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB)-mTOR signaling ¹² and integrates nutritional information and receptor tyrosine kinase signaling to control cellular growth via a

variety of cellular effectors, including activation of p70 S6 kinase and subsequent protein synthesis as well as inhibition of autophagy. Activation of PI3K-PKB-mTOR signaling following viral infection of liver cells has been reported and linked to both viral supportive functions (*e.g.* prevention of apoptosis in hepatitis C-infected cells)¹³, but also to the induction of the production of antiviral interferons¹⁴. Thus, generally speaking the role of this signaling cascade in combating viral infection of the liver remains unclear, prompting further research.

Given the important and increasing role of rapalogs implications in both clinical practice and the lack of insight into the mechanisms employed by the body to constrain HEV infection, we investigated the role of the PI3K-PKB-mTOR signaling cascade in HEV infection using state-of-the-art cell culture models. These results show that mTOR inhibition drastically promotes HEV replication in a autophagy-independent fashion but through phosphorylated 4E-BP1 in infected hepatocytes.

Materials and methods

Reagents

Stocks of rapamycin (Merck, Schiphol-Rijk, Netherlands) and everolimus (Sigma-Aldrich, St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) with final concentration of 2mM. Stocks of LY294022, an inhibitor of PI3K-PKB (Sigma-Aldrich), BEZ 235, a dual inhibitor of PI3K-PKB and mTOR (Selleck Chemicals), FG-4592, an inhibitor of HIF-1 α (Selleck Chemicals) and PF-478671, an inhibitor of p70 S6 kinase (Selleck Chemicals) were dissolved in DMSO. All agents were stored in 15 μ l aliquots and frozen at -20°C. Antibodies including LC3-I/II (Cell signalling technology, Netherland), S6, phosphor S6, p70 S6 kinase, phosphor PKB, 4E-BP1 and β -actin (Santa Cruz Biotech, Santa Cruz, CA); anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were used for Western blot. Lentiviral particles of GFP-LC3-II (Millipore, Billerica, MA, USA), expressing GFP-LC3 fusion protein, were used for visualization of autophagy formation. Other reagents including EBSS medium (Lonza), E-64-d (Santa Cruz Biotech, Santa Cruz, CA), pepstatin A (Santa Cruz Biotech, Santa Cruz, CA) and chloroquine (Sigma–Aldrich) were also used.

HEV cell culture models

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a gaussia luciferase reporter gene (p6-Luc), using the Ambion mMESSAGE mMACHINE® in vitro RNA transcription Kit (Life Technologies Corporation) ^{9, 10}. The human hepatoma Huh7 cells were collected and centrifuge for 5 minutes, 1500 rpm, 4°C. Supernatant was removed and washed with 4 mL Optimem by centrifuging for 5 minutes, 1500 rpm, 4°C. Cell pellet was re-suspended in 100 ul Optimem and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 volt, pulse length 0.5, number 1 and cuvette 4 mm) ⁹. The supernatant of the long term cultured p6 full-length HEV RNA cells were collected and used as secondary genuine infection.

Cell culture

Naïve or vector transduced Huh7 cells (human hepatoma cell line) and HEK293T cells (human fetal kidney epithelial cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). Stable firefly luciferase expressing cells were generated by transducing naïve Huh7 cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter (LV-PGK-Luc). For visualization of autophagy formation, Huh7 cells were transduced with lentiviral vector expressing the GFP-LC3 fusion protein.

Gene knockdown by lentiviral vector delivered short hairpin RNA (shRNA)

Lentiviral vectors (Sigma–Aldrich), targeting mTOR, 4E-BP1 or GFP (shCon), were obtained from the Erasmus Center for Biomimics and produced in HEK 293T cells as previously described ¹⁵. After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences were described in Supplementary Table 1.

To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 µg/ml puromycin (Sigma) in the cell culture medium. For the infectious model, HEV particles were incubated with knockdown and control Huh7 cells.

Measurement of luciferase activity

For gaussia luciferase, the activity of secreted luciferase in the cell culture medium was measured using BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 minutes at 37°C. Both gaussia and firefly Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to the cells seeded in 96-well plate and the cells grow at 37°C with 5% CO₂ for 3 hrs. The medium was removed and 100 µL of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm. All measurements were performed in triplicates.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of HEV and GAPDH were amplified by 40 cycles and quantified with SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to manufacturer's instructions. GAPDH was considered as reference gene to normalize gene expression. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), and the primers of housekeeping gene GAPDH were 5'-TGTCCCCACCCCAATGTATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense).

Western blot assay

Proteins in cell lysates were heated 5 min at 95°C followed by loading onto a 10-15% sodium dodecyl sulfate-polyacrylamide SDS gel and separated by electrophoresis (SDS-PAGE). After 90 minutes running in 120 voltage, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 hrs with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 mL blocking buffer and 2.5 mL PBS containing 0.05% Tween 20 (PBS-T). It was followed by incubation with rabbit LC3-I/II, p-PKB, p-mTOR, mTOR 4E-BP1, p-4E-BP1 or p-S6 (1:1000) antibody overnight at 4°C. Membrane was washed 3 times followed by incubation for 1.5 hrs with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Li-cor, Lincoln, USA) (1:5,000) at room temperature. Blots were assayed for actin content as standardization of sample loading, and scanned and quantified by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE, USA). Results were visualized with Odyssey 3.0 software.

Confocal laser electroscope assay

Lipidated LC3 (LC3-II) is a robust marker of autophagic membranes. Autophagosomes were visualized as bright green fluorescent protein GFP-LC3-II puncta by fluorescence microscopy. For nutrient starvation, cells were incubated in EBSS medium with 1 mM pepstatin A and E-64-d solution for overnight prior to fix for confocal laser electroscope analysis. The cells were fixed with 70% ethanol and GFP-LC3-II puncta was detected using confocal electroscope.

Statistical analysis

All results were presented as mean \pm SD. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a P value less than 0.05.

Results

mTOR inhibition by rapalogs facilitates HEV replication

The 7.2-kb genome of HEV is a single strand positive-sense of RNA containing three overlapping reading frames (ORFs). We employed a model in which human hepatoma cells

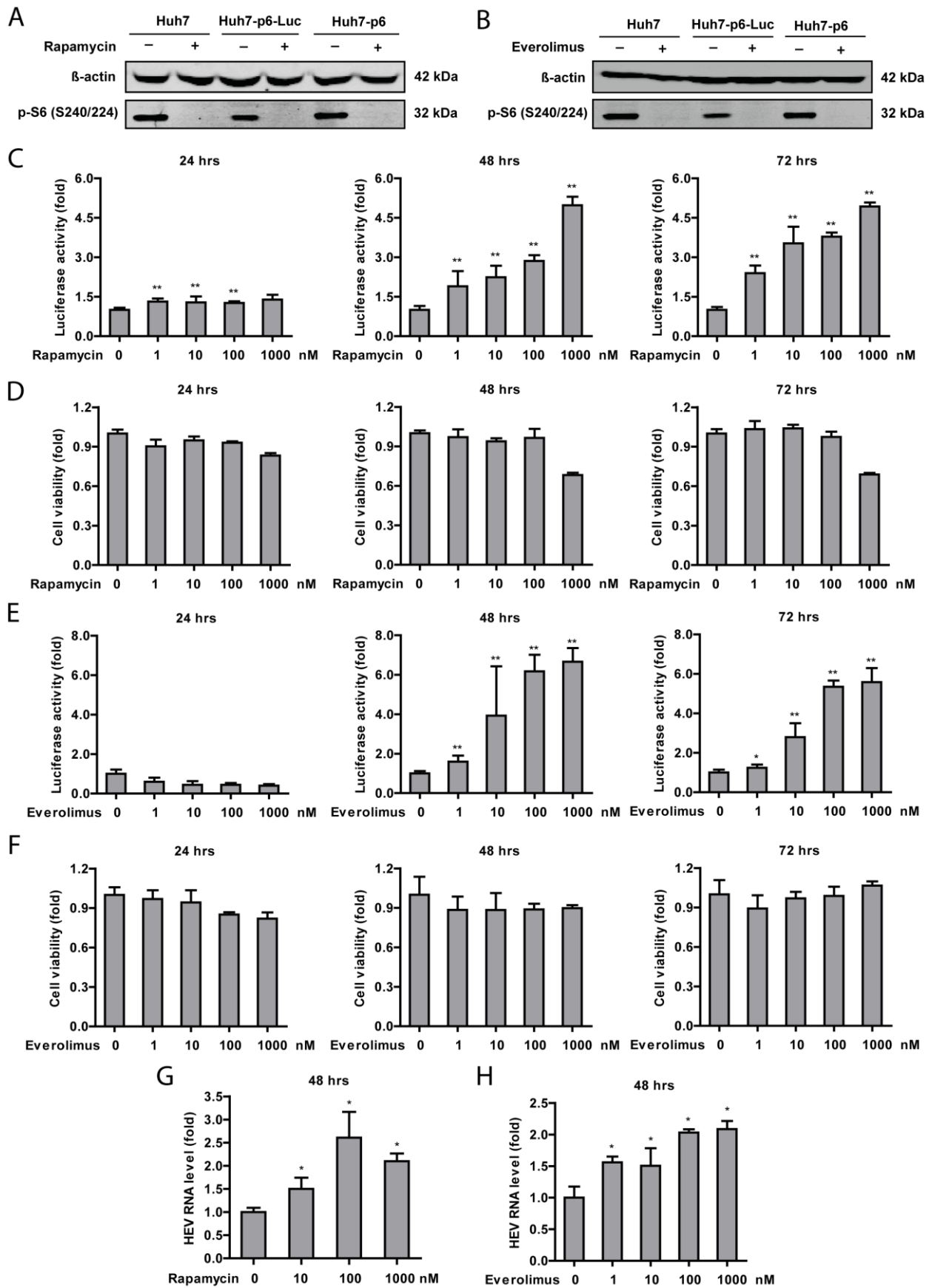


Figure 1. mTOR inhibition by rapalogs facilitate HEV replication. (A) Western blot showed inhibition of S6 phosphorylation by treatment of 500 nM rapamycin for 48 hrs. β -actin served as an internal reference. (B) Western blot showed inhibition of S6 phosphorylation by treatment of 500 nM everolimus for 48 hrs. β -actin served as an internal reference. (C) In the Huh7 cell-based subgenomic HEV replicon, treatment with rapamycin dose-dependently increased viral replication-related luciferase activity (Mean \pm SD, n = 3 independent experiments with each 2-3 replicates). (D) Rapamycin did not increase cell proliferation determined by MTT assay (OD₄₉₀ value) (Mean \pm SD, n = 5). (E) In the Huh7 cell-based subgenomic HEV replicon, treatment with everolimus dose-dependently increased viral replication-related luciferase activity (Mean \pm SD, n = 3 independent experiments with each 2-3 replicates). (F) Everolimus did not increase cell proliferation determined by MTT assay (OD₄₉₀ value) (Mean \pm SD, n = 5). (G) In the HEV infectious model, rapamycin significantly increased cellular viral RNA determined by qRT-PCR (Mean \pm SD, n = 5). (H) everolimus also significantly increased cellular HEV RNA in the infectious model (Mean \pm SD, n = 3-6). Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.

(Huh7) were transfected with a 3' subgenomic construct of HEV coding sequence in which the 5' portion of ORF2 was replaced with the in-frame secreted form of luciferase derived from the marine copepod *Gaussia princeps* (p6-Luc) (Supplementary Figure 1). Accumulation of luciferase in Huh7 cells thus serves as reporter for HEV RNA synthesis, whereas the loss of the capsid protein in the model system precludes the formation of novel viral particles^{9,10}. In parallel, Huh7 cells constitutively expressing a non-secreted firefly luciferase are used for normalization of non-specific effects on luciferase signals. In addition, a Huh7 based full-length infectious HEV model (p6) was also employed (Supplementary Figure 1)^{9,10}.

Direct investigation of the phosphorylation status of phospho-Ser-240/224 S6 and phospho-Ser-473 PKB showed that Huh7 cells represent a PI3K-PKB-mTOR-proficient model system (Figure 1A and B). Importantly, inhibiting mTOR rendered this system sensitive to HEV infection as evident from higher levels of *G. princeps* luciferase and that increased over the time domain. Treatment with 100 and 1000 ng/ml of rapamycin for 48 hrs resulted in 1.9 ± 0.4 (Mean \pm SD, n = 3, $P < 0.05$) and 2.7 ± 0.6 (Mean \pm SD, n = 3, $P < 0.01$) -fold increase of HEV luciferase activity (Figure 1C) and corresponds to a concomitant decrease in mTOR activity as assessed by phospho-Ser-240/224 S6 levels (Figure 1A). At 72 hrs, HEV luciferase activity was further increased up to 3.8 ± 0.5 (Mean \pm SD, n = 3, $P < 0.01$) and 4.9 ± 0.5 (Mean \pm SD, n = 3, $P < 0.01$) -fold, respectively (Figure 1C). A possible artifact here would direct growth-promoting effects of rapamycin, but MTT assay showed that rapamycin did not promote cell growth (Figure 1D).

Next to rapamycin, everolimus is often used for clinical mTOR inhibition following orthotopic organ transplantation. Like rapamycin, everolimus also remarkably permitted

HEV replication. In the p6-Luc model, treatment with 1ng/ml of everolimus has already significantly increased HEV luciferase activity. Treatment with 100 and 1000 ng/ml of everolimus resulted in 7.0 ± 2.2 (Mean \pm SD, $n = 3$, $P < 0.01$) and 6.7 ± 1.4 (Mean \pm SD, $n = 3$, $P < 0.01$) -fold increase at 48 hrs, 5.3 ± 0.8 (Mean \pm SD, $n = 3$, $P < 0.01$) and 5.6 ± 1.9 (Mean \pm SD, $n = 3$, $P < 0.05$) -fold increase of HEV luciferase activity at 72 hrs (Figure 1E). Everolimus also did not promote cell proliferation determined by MTT assay (Figure 1F). Dephosphorylation of S6 was also confirmed (Figure 1B)

To exclude that this effect is an consequence of the loss of the capsid protein and ORF3 protein in our luciferase model, we repeated experiments with the full-length infectious HEV model. Again, HEV infection was facilitated under mTOR-deficient conditions. For instance, treating with 100 or 1000 ng/ml rapamycin has increased viral RNA levels up to 2.6 ± 0.6 (Mean \pm SD, $n = 5$, $P < 0.01$) or 2.1 ± 0.4 (Mean \pm SD, $n = 5$, $P < 0.01$)-fold, respectively (Figure 1G). Treatment with 1, 10, 100 and 1000 ng/ml of everolimus for 48 hrs resulted in increase of cellular viral RNA up to 1.6 ± 0.1 (Mean \pm SD, $n = 3$, $P < 0.01$), 1.5 ± 0.3 (Mean \pm SD, $n = 6$, $P < 0.05$), 2.0 ± 0.1 (Mean \pm SD, $n = 3$, $P < 0.01$) and 2.1 ± 0.2 (Mean \pm SD, $n = 3$, $P < 0.01$) (Figure 1G). Hence, both major drugs used for clinical mTOR inhibition provoke an altered cellular state in hepatocyte-like cells that allows efficient HEV replication to proceed.

Gene silencing of mTOR by RNAi enhances HEV replication

To evaluate the direct effects of mTOR on HEV, Huh7 cells were transduced with integrating lentiviral vectors expressing short hairpin RNA (shRNA) specifically targeting mTOR or a control shRNA (shCon). As shown in Figure 2A, three of the tested four shRNA vectors targeting mTOR exert potent gene silencing capacity, resulting in a profound down-regulation of mTOR protein level but an elevation of PKB expression (probably due to a feedback activation). Correspondingly, mTOR silencing resulted in significant increase of cellular HEV RNA level, which were measured by qRT-PCR after inoculation of HEV particles which indicating genuine infection for 72 hrs. For instance, knockdown of mTOR by the shmTOR clone 2 led to 2.6 ± 0.8 -fold (Mean \pm SD, $n = 3$, $P < 0.05$) increase of HEV RNA (Figure 2A). These data provide direct and strong evidence that mTOR plays an important role in restricting HEV infection.

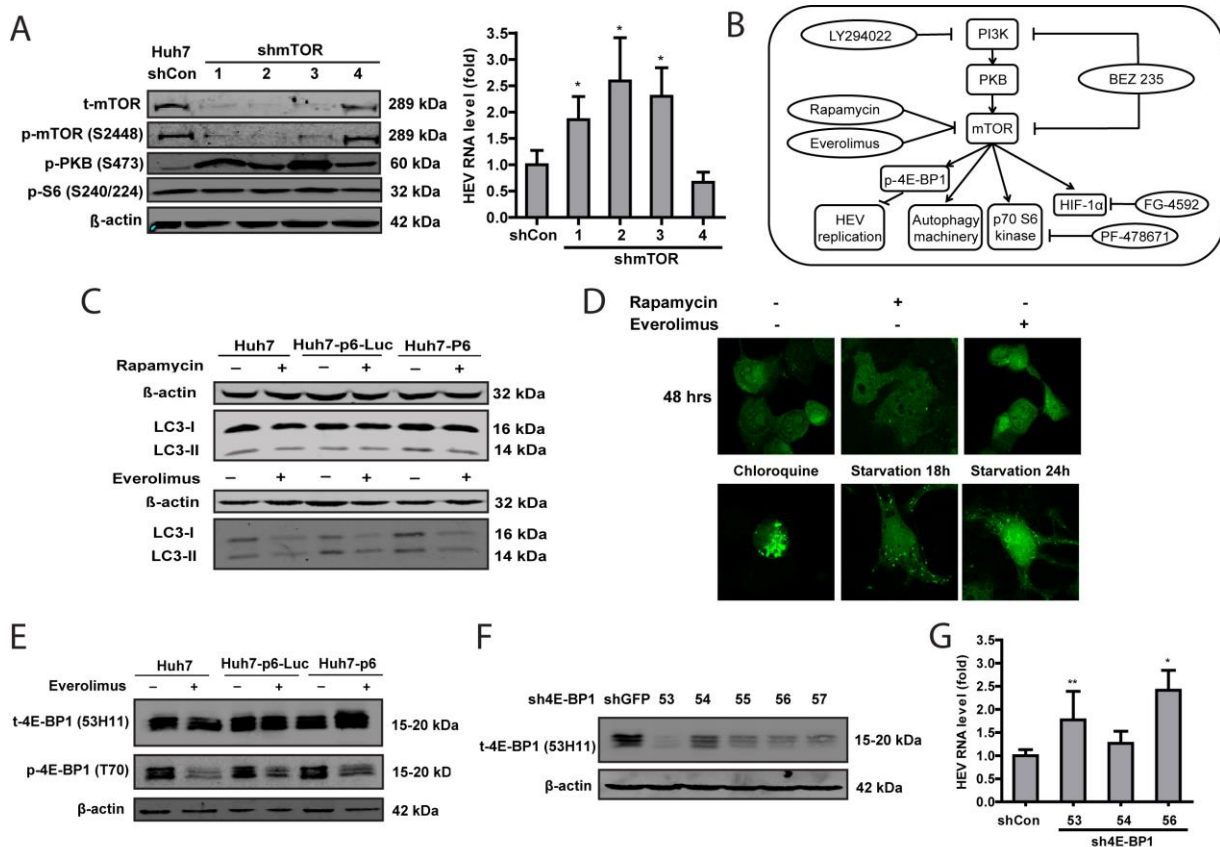


Figure 2. Gene silencing of mTOR and 4E-BP1 by lentiviral RNAi enhances HEV replication independent of autophagy machinery. (A) Knockdown of mTOR by lentiviral shRNA vectors. Compared with the control vector transduced cells, the shmTOR clone 1, 2 and 3 but not 4 exert potent silencing capability shown at protein levels of both total- (t-mTOR) and phospho-mTOR (p-mTOR), which also resulted in dramatic elevation of phospho-PKB (p-PKB). S6 phosphorylation (p-S6) was also determined by Western blot and β -actin served as an internal reference. Correspondingly, knockdown of mTOR resulted in significant increase of cellular HEV RNA level (Mean \pm SD, $n = 3$), which were measured by qRT-PCR after inoculation of HEV particles which indicating genuine infection for 72 hrs. * $P < 0.05$; ** $P < 0.01$. (B) Illustration of the effects on HEV infection by inhibiting different components of the PI3K-PKB-mTOR pathway. Rapamycin/everolimus, inhibitors of mTOR; LY294022, an inhibitor of PI3K-PKB; BEZ-235, a dual inhibitor of PI3K-PKB and mTOR; PF-4708671, an inhibitor of p70 S6 kinase and FG-4592, an inhibitor of HIF-1 α were used. (C) Naïve Huh7, subgenomic HEV replicon and HEV infected Huh7 cells were treated with rapamycin and everolimus for 48 hrs. The accumulation of LC3-II, a hallmark of autophagy formation, was not observed by Western blot analysis. β -actin was served as an internal reference. (D) Consistently, green puncta formation, an indication of autophagosome formation, was not observed in Huh7 cells expressing GFP-LC3-II fusion protein, by treatment of rapamycin and everolimus for 24, 48 and 72 hrs. In contrast, autophagosome formation was observed in the positive control groups treated with 30 μ M chloroquine for 48 hrs or at the circumstance of starvation in BESS media with 1 μ M pepstatin A and E-64-d for either 18 or 24 hrs. Oil-lenses (40 \times) was used (1024 \times 1024 image). (E) Western blot showed inhibition of 4E-BP1 phosphorylation by treatment of 500 nM everolimus for 48 hrs. β -actin served as an internal reference. (F) Knockdown of 4E-BP1 by lentiviral shRNA vectors. Compared with the control vector transduced cells, the sh4E-BP1 clone 53, 55, 56 and 57 but not 54 exert potent silencing efficacy shown at protein levels of total 4E-BP1 (t-4E-BP1), β -actin served as an internal reference. (G) Correspondingly, knockdown of 4E-BP1 resulted in significant increase of cellular HEV RNA level (Mean \pm SD, $n = 5$). * $P < 0.05$; ** $P < 0.01$.

mTOR limits HEV replication via 4E-BP1

mTOR is a key kinase controlling cellular behavior. Its most important effector pathways include induction of protein transcription via p70 S6 kinase pathway (Figure 2B)¹⁴. However, this pathway does not seem a major effector mechanism as inhibition of p70 S6 kinase by its inhibitor PF-478671 did not affect HEV infection (Supplementary Figure 2). Inhibition of another downstream target of mTOR, hypoxia-inducible factor-1 α (HIF-1 α) by FG-4592 (Supplementary Figure 3) also did not affect HEV infection. Notably, mTOR is also the main inhibitor of autophagy in cellular metabolism and it is possible that HEV replication requires autophagosome formation. However, inhibition of mTOR did not change the levels of microtubule-associated protein 1 light chain 3 β (LC3-II) in our model system (Figure 2C), a hallmark of autophagosome formation. Furthermore, Huh7 cells stably intergraded with a lentiviral vector expressing GFP-LC3-II were used to visualize autophagosome formation. In

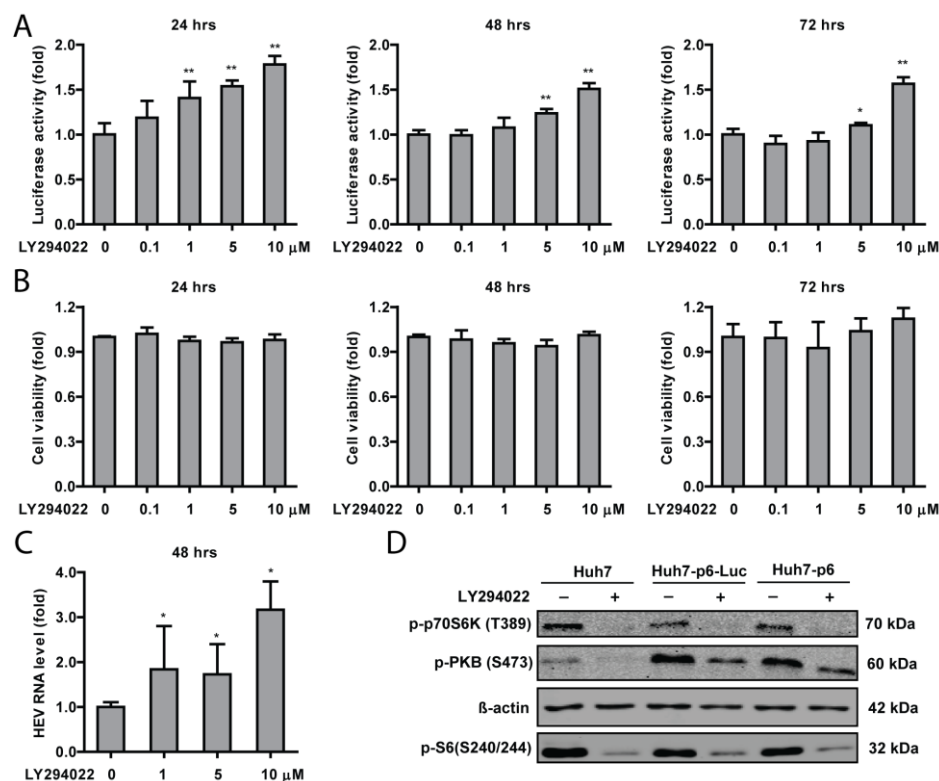


Figure 3. Inhibition of PI3K-PKB promotes viral replication. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with LY294022, a PI3K inhibitor, dose-dependently increased viral replication-related luciferase activity (Mean \pm SD, $n = 3$). (B) LY294022 did not affect cell proliferation determined by MTT assay (OD_{490} value) (Mean \pm SD, $n = 4$). (C) In the HEV infectious model, LY294022 significantly increased cellular viral RNA determined by qRT-PCR (Mean \pm SD, $n = 3$). (D) Western blot showed inhibition of PKB, S6 and p70 S6 kinase phosphorylation by treatment of 5 μ M LY294022 for 48 hrs. β -actin served as an internal reference. Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.

the positive control groups, cells were either treated with 30 μ M chloroquine or under condition of starvation in EBSS medium. As expected, green puncta of LC3-II was clearly emerging, indicating the formation of autophagosome (Figure 2D). In contrast, no changes of the autophagy machinery were observed with treatment of rapamycin or everolimus (Figure 2D), which was consistent with the results of Western blot (Figure 2C). Thus, these findings exclude the possibility that the proviral effect of rapamycin/everolimus is via the autophagy machinery.

4E-BP1 is another important element induced by mTOR for cellular cap-dependent translation⁷⁵. Treatment of mTOR inhibitor everolimus (500 nM) for 48 hrs resulted in clear dephosphorylation of 4E-BP1 as shown by Western blot (Figure 2E). To further confirm the regulation of HEV replication by 4E-BP1, Huh7 cells were transduced with integrating lentiviral vectors expressing shRNA specifically targeting 4E-BP1 or a control shRNA (shCon). Cells stably transduced with the vector were also selected and expanded by adding puromycin to the relevant cell cultures. Four out of five shRNA vectors targeting 4E-BP1 exert gene silencing capacity, resulting in a various level down-regulation of total 4E-BP1 protein (Figure 4F). Correspondingly, we selected two 4E-BP1 silencing cell-lines with optimal gene silencing potency that resulted in a significant increase of cellular HEV RNA level, which was measured by qRT-PCR of HEV RNA. For instance, knockdown of 4E-BP1 by the sh4E-BP1 clone 53 led to 1.7 ± 0.6 -fold (Mean \pm SD, $n = 5$, $P < 0.01$) increase of HEV RNA and clone 56 led to 2.4 ± 0.9 -fold (Mean \pm SD, $n = 4$, $P < 0.05$) increase (Figure 4G). Consistently, the clone 54 with minimal gene silencing efficacy only exert minor effect (1.3 ± 0.3 -fold, Mean \pm SD, $n = 4$, $P > 0.05$) on HEV replication (Figure 4G). These data indicated that the antiviral effect of mTOR is via its downstream target, 4E-BP1.

Inhibition of PI3K-PKB promotes viral replication

Although distinct molecules, rapamycin and everolimus share important structural characteristics. To exclude the possibility that the effects of these compounds on HEV replication represent a mTOR-independent off-target effect, independent confirmation of the role of PI3K/PKB/mTOR signaling cascade in preventing HEV replication was sought through experiments in which more upstream elements of this signaling cascade were targeted (Figure 2B). When Huh7 p6-Luc cells were treated with different concentrations (0.1-10 μ M) of the well-established PI3K inhibitor LY294002, enhancement of HEV

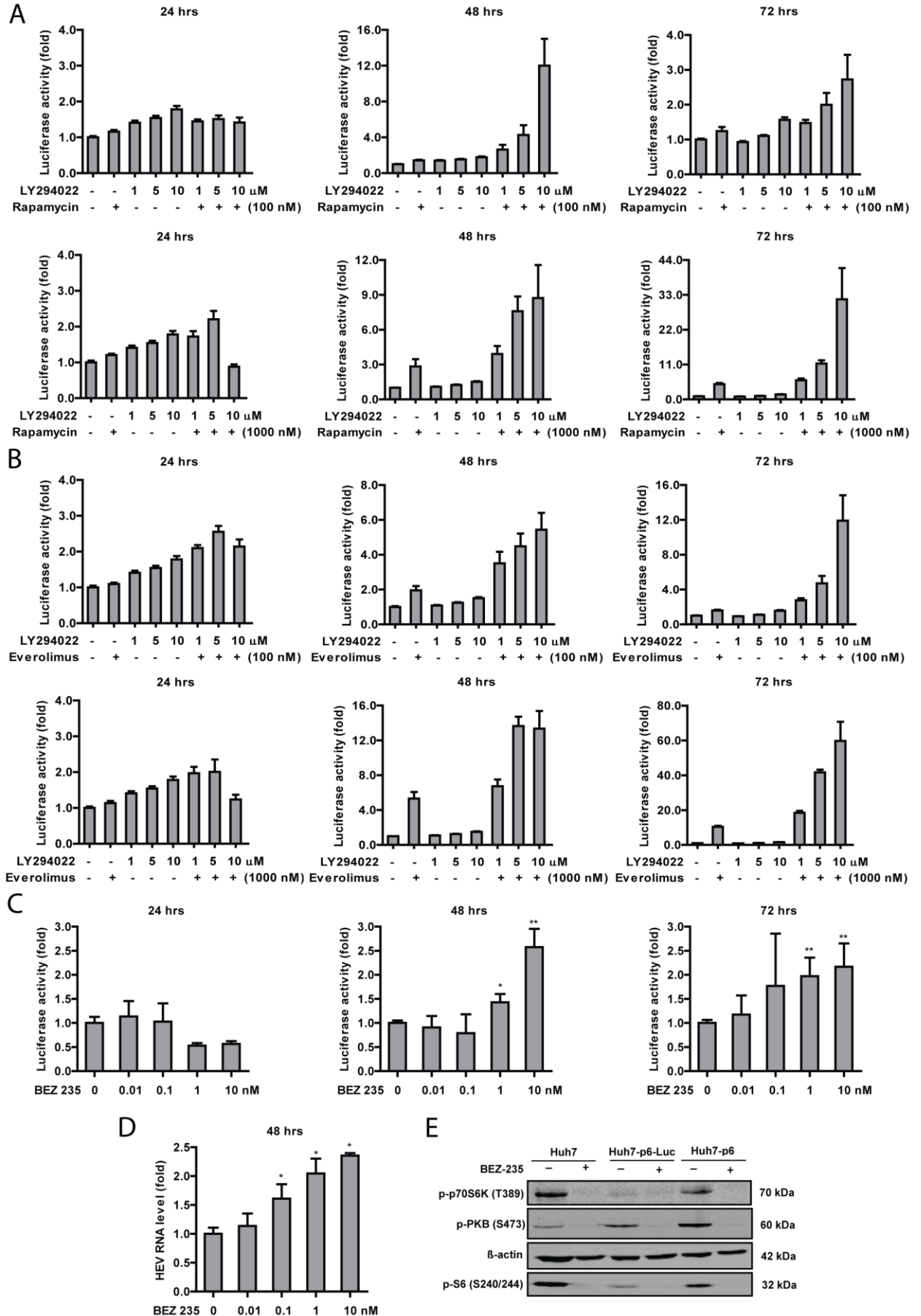


Figure 4. Simultaneous inhibition of PI3K and mTOR further increased viral replication. In the HEV subgenomic replicon, viral replication-related luciferase activity was presented when 1, 5 or 10 μ M LY294022 was combined with 100/1000 nM of rapamycin (A) or everolimus (B). Treatment time was indicated as 24, 48 or 72 hrs. Data was presented as Mean \pm SD, n = 11 replicates in total. (C) BEZ-235 is a dual inhibitor of PI3K-PKB and mTOR. In the Huh7 cell-based subgenomic HEV replicon, treatment with BEZ-235 significantly increased viral replication-related luciferase activity (Mean \pm SD, n = 5). (D) In the HEV infectious model, BEZ-235 significantly increased cellular viral RNA determined by qRT-PCR (Mean \pm SD, n = 3). (E) Western blot showed inhibition of PKB, S6 and p70 S6 kinase phosphorylation by treatment of 1 nM BEZ-235. β -actin served as an internal reference. Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$

replication became apparently similar to that observed with mTOR inhibitors (Figure 3A), which was also not related to enhanced cell proliferation (Figure 3B). Consistently, LY294002 also significantly increased cellular HEV RNA in the infectious model up to 3.2 ± 1.1 -fold (Mean \pm SD, n = 3, $P < 0.05$) at dose of 10 μ M (Figure 3C). These effects corresponded with the observed inhibition of the biological target (Figure 3D).

Simultaneous inhibition of PI3K and mTOR further enhanced viral replication

Simultaneous treatment with rapamycin/everolimus and LY294002 apparently had stronger effects than rapamycin or LY294002 alone. The strongest effect on HEV replication was observed with the combination of 100 ng/ml rapamycin and 10 μ M LY294002 at 48 hrs (up to 12.1 ± 3.1 -fold, Mean \pm SD, n = 11, $P < 0.01$ Vs untreated; $P < 0.01$, Vs rapamycin; $P < 0.01$, Vs LY294002) (Figure 4A), and at 72 hrs with the combination of 1000 ng/ml rapamycin and 10 μ M LY294002 (up to 31.7 ± 9.9 -fold, Mean \pm SD, n = 11, $P < 0.01$ Vs untreated; $P < 0.05$, Vs rapamycin; $P < 0.01$, Vs LY294002) (Figure 4A). Similar effects were observed when everolimus was combined with LY294002 (Figure 4B). Furthermore, these results were found not to be related to enhancement of cell proliferation either with rapamycin (Supplementary Figure 4A) or everolimus (Supplementary Figure 4B). BEZ-235 is a dual inhibitor of mTOR and PI3K signalling, which is at the stage of clinical development for treating cancer patients (NCT00620594, ClinicalTrials.gov) (Figure 2B). We further investigated the effect of simultaneously inhibiting PI3K-PKB and mTOR by a single compound BEZ-235. As shown in Figure 4C and 4D, BEZ-235 significantly promoted HEV infection in both models. Furthermore, results corresponded to inhibition of biological targets of this pathway (Figure 4E). The most straightforward interpretation of these data is that HEV can efficiently replicate in the context of deficient signaling through the PI3K-PKB-mTOR cascade.

Discussion

Large zoonotic reservoirs of hepatitis E exist in cattle and poultry and it is generally accepted that humans are frequently infected with the virus ⁶. Almost invariably, however, the disease remains subclinical ². Here we present evidence that the inability of HEV to effectively replicate in humans is linked to constitutive mTOR activation. This novel action of mTOR in directly counteracting viral replication in liver cell themselves rather as acting through the adapted immune system, represents a highly novel non-canonical action of this kinase in a new adapted immune system-independent antiviral mechanism and thus our results are highly unexpected.

Patients after orthotropic organ transplantation when receiving immunosuppressants, such as rapalogs, to prevent rejection are well known to be at extremely high risk of developing chronic hepatitis with persistence of infection ^{3, 17, 18}. It is known that the clinical symptoms of this hepatitis reacts very well to reducing dose of immunosuppression ¹⁸. Hitherto, this beneficial effect of decreasing immunosuppressive therapy was attributed to increased immunity ¹⁹. However, different types of immunosuppressants can also have direct effects on HEV replication in the target cells of the virus ²⁰. In transplantation patients, the blood concentrations of rapalogs can reach by approx. 15 ng/ml ^{21, 22}, whereas the levels in cancer patients can be up to approx. 100 ng/ml ²³. We have demonstrated that 1 ng/ml everolimus has sufficient to trigger a significant stimulatory effects on HEV replication *in vitro*, which thus clearly bears important clinical relevance.

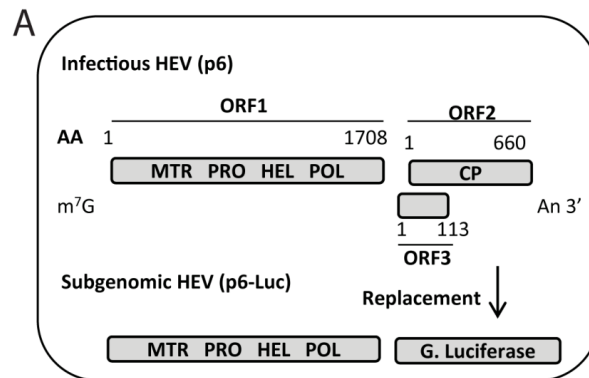
In fact, more evidence supporting the potential proviral effects of rapalogs have come from hepatitis B virus (HBV) infected patients. In a randomized clinical trial comparing two everolimus dosing schedules in patients with advanced hepatocellular carcinoma, four patients were hepatitis B surface antigen (HBsAg)-seropositive. During treatment of everolimus, all these patients experienced episodes of HBV flare with > 1-log increase in the serum HBV DNA level accompanied by alanine transaminase elevations ²⁴. Similarly, a patient with renal cell carcinoma also experienced a HBV flare during everolimus treatment ²⁵. These observations could be explained by affecting the adaptive immune system but may also by direct effects on viral replication. The current experimental study has firmly demonstrated the proviral effects of both rapamycin and everolimus in two state-of-the-art

HEV cell culture models. Further detailed mechanistic investigation has revealed an antiviral function of the PI3K-PKB-mTOR pathway, which appears support the recent clinical observations in viral hepatitis patients^{17, 24, 25}.

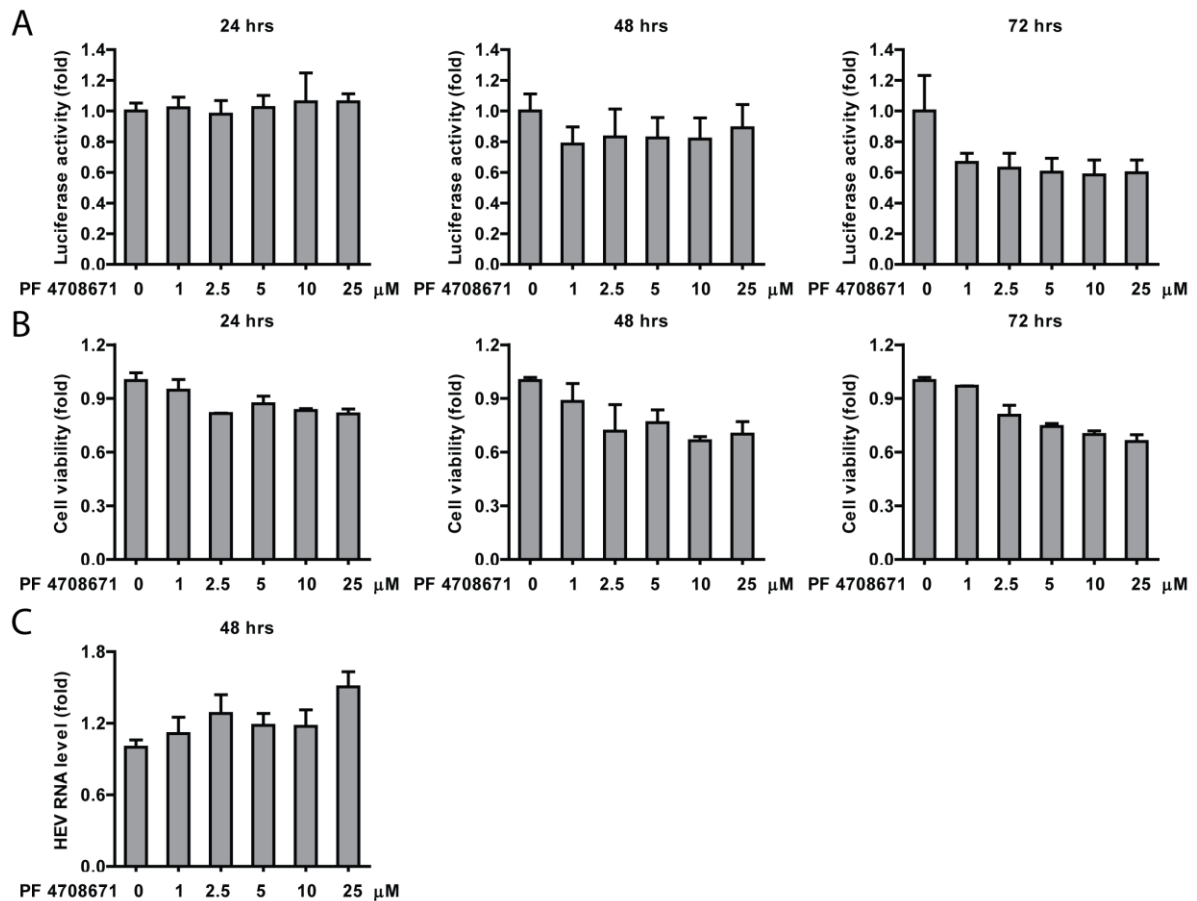
Another group of patients at high risk for HEV caused death constitute pregnant women²⁶. Although this effect is in literature generally linked to diminished immunity²⁶, immune suppression during pregnancy is relatively moderate²⁷. Interestingly, the increased nutritional demands of pregnancy²⁸ provoke a powerful activation of the ATP/ADP-sensitive kinase AMPK²⁹. In turn, this kinase is potent inhibitor of mTOR³⁰ and indeed pregnancy is associated with a significant down regulation of mTOR³¹. It is tempting to speculate that pregnancy-specific down regulation of mTOR may help to understand why this group is specifically sensitive to HEV infection. In apparent agreement, malnutrition in general is also associated with susceptibility to HEV⁷. We thus speculate that HEV may preferentially affect the human population when hepatic mTOR activity is below its constitutive level.

Because of its favorable side effect-profile, rapalog therapy is quickly gaining popularity for treating a variety of clinical syndromes, especially in oncological disease, in congenital diseases like Peutz-Jeghers syndrome and Tuberous sclerosis complex, in transplantation medicine and autoimmunity. Therefore, recognition of the anti-HEV function of PI3K-PKB-mTOR pathway bears magnificent implications in clinical practice regarding the choice of particular immunosuppressant for HEV-infected organ transplant recipients. In particular, the use of mTOR inhibitors in these patients should be taken caution. In addition, these results may also help to understand the underline mechanism that why pregnant women are more susceptible to HEV infection with devastating outcome.

Supplementary Figures and Tables

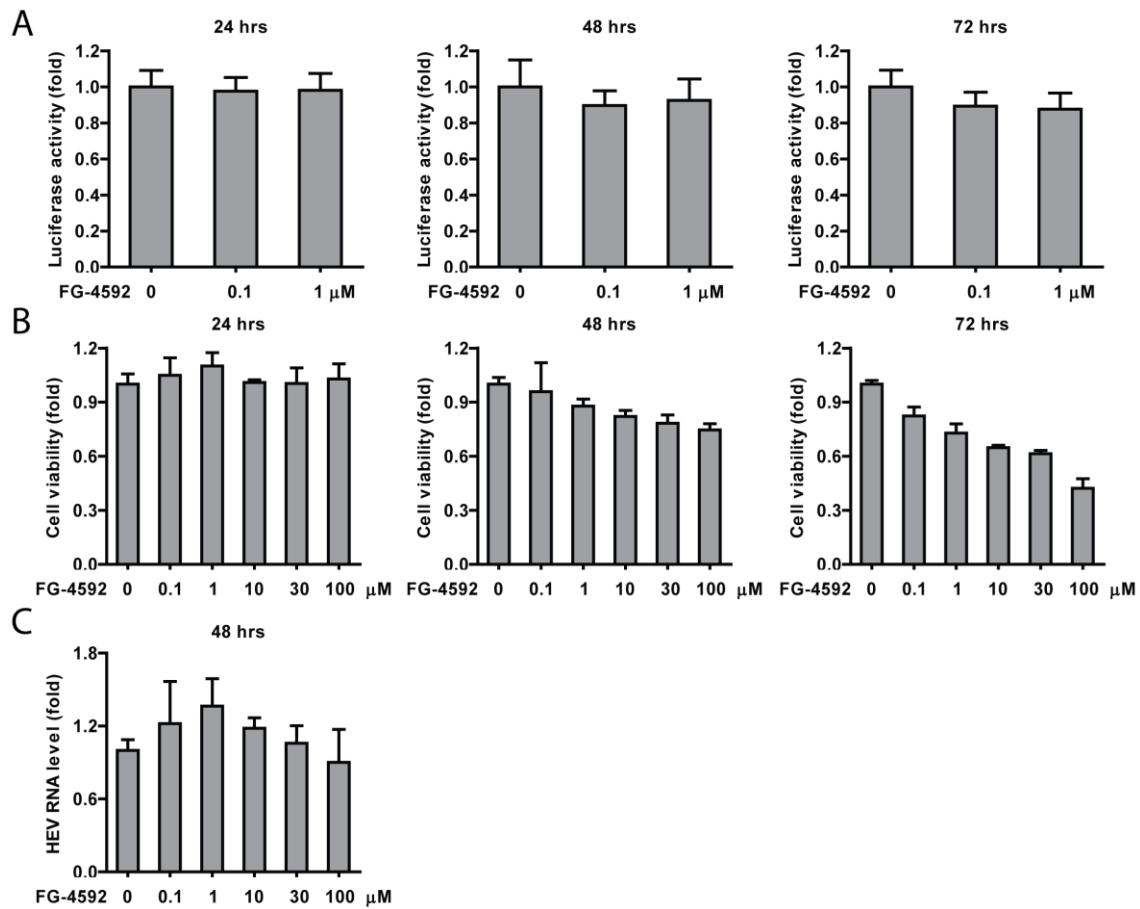


Supplementary Figure 1. Genomic structure of the infectious HEV (p6) and subgenomic HEV replicon (p6-Luc) models.

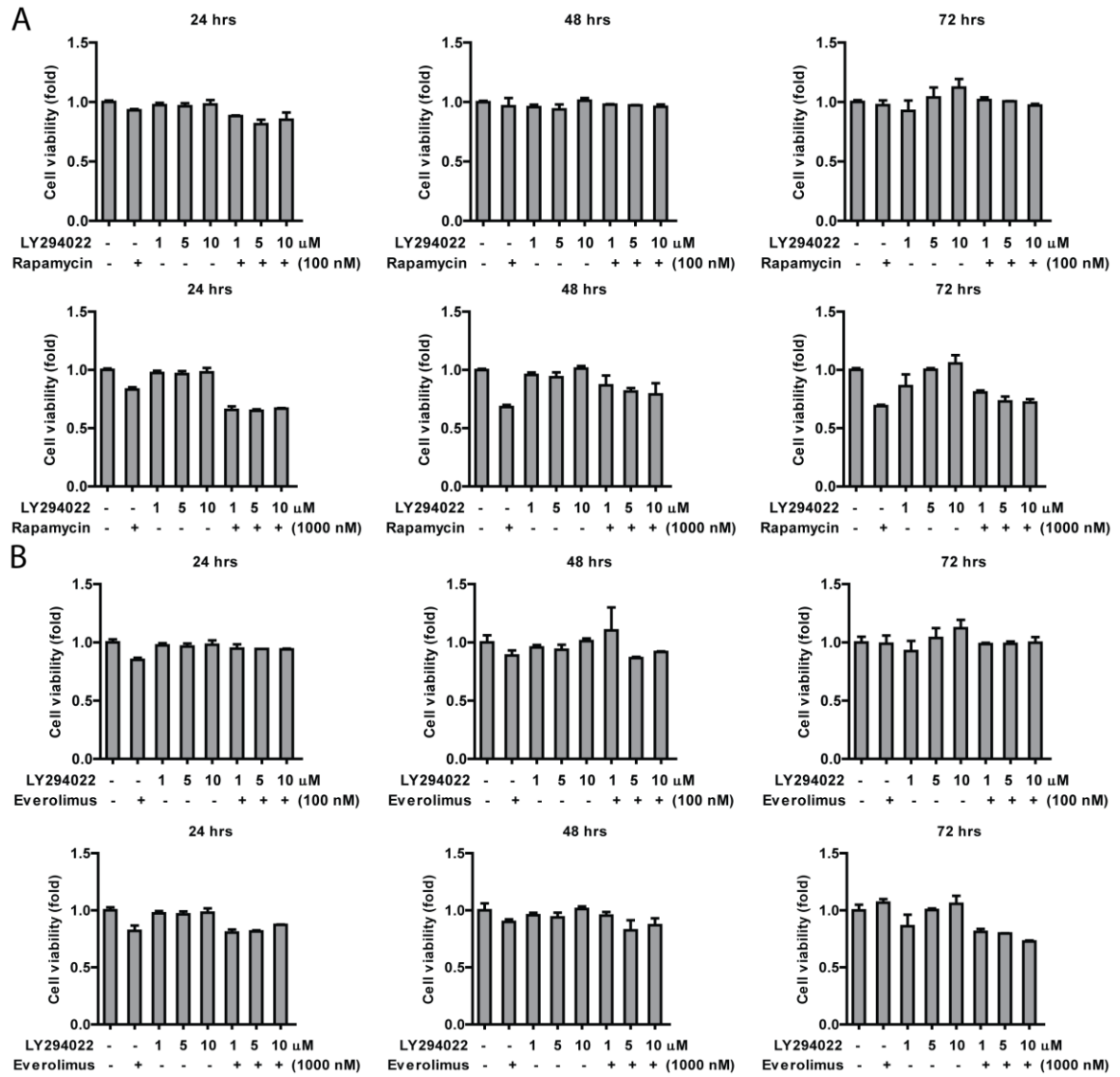


Supplementary Figure 2. PF-478671, a p70 S6 kinase inhibitor has no effect on HEV replication. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with PF-478671 for 24, 48 and 72 hrs did not have clear effects on viral replication-related luciferase activity. (B) PF-478671 did not increase cell proliferation determined by MTT assay (relative OD₄₉₀)

value). (C) In the HEV infectious model, treatment with PF-478671 also did not have clear effects on HEV cellular RNA.



Supplementary Figure 3. FG-4592, a HIF-1 α inhibitor has no effect on HEV infection. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with FG-4592 for 24, 48 and 72 hrs did not have clear effects on viral replication-related luciferase activity. (B) FG-4592 did not increase cell proliferation determined by MTT assay (relative OD₄₉₀ value). (C) In the HEV infectious model, treatment FG-4592 also did not have clear effects on HEV cellular RNA.



Supplementary Figure 4. Simultaneous inhibition of PI3K and mTOR did not affect cell proliferation. In the HEV subgenomic replicon, viral replication-related luciferase activity was presented when 1, 5 or 10 μ M LY294022 was combined with 100/1000 nM of rapamycin (A) or everolimus (B). Treatment time was indicated as 24, 48 or 72 hrs. Cell proliferation determined by MTT assay (relative OD₄₉₀ value).

Supplementary Table 1. shRNA sequences

shmTOR-1	CCGGGCCAGAATCTATTCACTTTCTCGAGAAAGAATGAATAGATTCTGGCTTTTTG
shmTOR-2	CCGGGCCTTGTTTGTGGCTCTGAATCTCGAGATTCAGAGCCACAAACAAGGCTTTTTG
shmTOR-3	CCGGGAACCAATTATACCCGTTCTTCTCGAGAAGAACGGGTATAATTGGTTCTTTTTG
shmTOR-4	CCGGGCTGTGCTACACTACAAACATCTCGAGATGTTTGTAGTGTAGCACAGCTTTTTG
sh4E-BP1-53	CCGGGCCAGGCCTTATGAAAGTGATCTCGAGATCACTTTCATAAGGCCTGGCTTTTTG
sh4E-BP1-54	CCGGAGGATCATCTATGACCGGAAACTCGAGTTTCCGGTCATAGATGATCCTTTTTTG
sh4E-BP1-55	CCGGACAGTTTGAGATGGACATTTACTCGAGTAAATGTCCATCTCAAAGTGTTTTTG
sh4E-BP1-56	CCGGCGGTGAAGAGTCACAGTTTGACTCGAGTCAAAGTGTGACTCTTCACCGTTTTTG
sh4E-BP1-57	CCGGGCGCAATAGCCCAGAAGATAACTCGAGTTATCTTCTGGGCTATTGCGCTTTTTG

References

1. Dalton HR. Hepatitis: hepatitis E and decompensated chronic liver disease. *Nat Rev Gastroenterol Hepatol* **9**, 430-432 (2012).
2. Hoofnagle JH, Nelson KE & Purcell RH. Hepatitis E. *N Engl J Med* **367**, 1237-1244 (2012).
3. Kamar N, *et al.* Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* **358**, 811-817 (2008).
4. Dalton HR, Bendall RP, Keane FE, Tedder RS & Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. *N Engl J Med* **361**, 1025-1027 (2009).
5. Ollier L, *et al.* Chronic hepatitis after hepatitis E virus infection in a patient with non-Hodgkin lymphoma taking rituximab. *Ann Intern Med* **150**, 430-431 (2009).
6. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
7. Centers for Disease C & Prevention. Investigation of hepatitis e outbreak among refugees - upper Nile, South Sudan, 2012-2013. *MMWR Morb Mortal Wkly Rep* **62**, 581-586 (2013).
8. Lisy K. Integration of HIV/AIDS services with maternal, neonatal and child health, nutrition, and family planning services. *Public Health Nurs* **30**, 451-453 (2013).
9. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
10. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
11. Rostaing L & Kamar N. mTOR inhibitor/proliferation signal inhibitors: entering or leaving the field? *J Nephrol* **23**, 133-142 (2010).
12. van Veelen W, Korsse SE, van de Laar L & Peppelenbosch MP. The long and winding road to rational treatment of cancer associated with LKB1/AMPK/TSC/mTORC1 signaling. *Oncogene* **30**, 2289-2303 (2011).
13. Ishida H, Li K, Yi M & Lemon SM. p21-activated kinase 1 is activated through the mammalian target of rapamycin/p70 S6 kinase pathway and regulates the replication of hepatitis C virus in human hepatoma cells. *J Biol Chem* **282**, 11836-11848 (2007).
14. Cao W, *et al.* Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* **9**, 1157-1164 (2008).
15. Pan Q, *et al.* Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* **87**, 713-722 (2009).
16. Licursi M, Komatsu Y, Pongnopparat T & Hirasawa K. Promotion of viral internal ribosomal entry site-mediated translation under amino acid starvation. *J Gen Virol* **93**, 951-962 (2012).
17. Koning L, *et al.* Clinical implications of chronic hepatitis E virus infection in heart transplant recipients. *J Heart Lung Transplant* **32**, 78-85 (2013).
18. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
19. Kamar N, *et al.* Influence of immunosuppressive therapy on the natural history of genotype 3 hepatitis-E virus infection after organ transplantation. *Transplantation* **89**, 353-360 (2010).

20. Wang Y, *et al.* Calcineurin Inhibitors Stimulate and Mycophenolic Acid Inhibits Replication of Hepatitis E Virus. *Gastroenterology* **146**, 1775-1783 (2014).
21. Kovarik JM, *et al.* Everolimus therapeutic concentration range defined from a prospective trial with reduced-exposure cyclosporine in de novo kidney transplantation. *Ther Drug Monit* **26**, 499-505 (2004).
22. Leogrande D, *et al.* Monitoring biological action of rapamycin in renal transplantation. *Am J Kidney Dis* **50**, 314-325 (2007).
23. Cloughesy TF, *et al.* Antitumor activity of rapamycin in a Phase I trial for patients with recurrent PTEN-deficient glioblastoma. *PLoS Med* **5**, e8 (2008).
24. Shiah HS, *et al.* Randomised clinical trial: comparison of two everolimus dosing schedules in patients with advanced hepatocellular carcinoma. *Aliment Pharmacol Ther* **37**, 62-73 (2013).
25. Sezgin Goksu S, Bilal S & Coskun HS. Hepatitis B reactivation related to everolimus. *World J Hepatol* **5**, 43-45 (2013).
26. Khuroo MS, Teli MR, Skidmore S, Sofi MA & Khuroo MI. Incidence and severity of viral hepatitis in pregnancy. *Am J Med* **70**, 252-255 (1981).
27. Mor G, Cardenas I, Abrahams V & Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Ann N Y Acad Sci* **1221**, 80-87 (2011).
28. Brenseke B, Prater MR, Bahamonde J & Gutierrez JC. Current thoughts on maternal nutrition and fetal programming of the metabolic syndrome. *J Pregnancy* **2013**, 368461 (2013).
29. Pimentel GD, Ropelle ER, Rocha GZ & Carvalheira JB. The role of neuronal AMPK as a mediator of nutritional regulation of food intake and energy homeostasis. *Metabolism* **62**, 171-178 (2013).
30. Korsse SE, Peppelenbosch MP & van Veelen W. Targeting LKB1 signaling in cancer. *Biochim Biophys Acta* **1835**, 194-210 (2013).
31. Framarino-dei-Malatesta M, *et al.* Impact of mTOR-I on fertility and pregnancy: state of the art and review of the literature. *Expert Rev Clin Immunol* **9**, 781-789 (2013).

Part II.

Extra-hepatic manifestations

Chapter 5

Rhesus macaques persistently infected with hepatitis E shed virus into urine

Fen Huang^{1, 2}, Chenchen Yang¹, Xinying Zhou³, Wenhai Yu¹, Qiuwei Pan³

¹Medical Faculty, Kunming University of Science and Technology, 727 Jingming Road, Kunming, China; ²Kunming General Hospital of Chengdu Military Region, Kunming, China; ³Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, Netherlands.

Hepatitis E virus (HEV) is the most common cause of acute hepatitis worldwide. Recently, it is also recognized to cause chronic hepatitis (persistent infection for longer than 6 months) in immunocompromised patients ¹. Because HEV has a fecal-oral transmission route, patient fecal samples in addition to their blood have been widely used for diagnosis and monitoring the infection. Surprisingly, a recent study by Geng *et al.* has discovered dissemination of HEV into urine ². This intriguing finding bears important implications in understanding the infection biology and pathogenesis of HEV, as well as diagnosis and management of patients with hepatitis E.

The authors have described the presence of HEV RNA in the urine samples from one chronic case and 3 out of 8 acute hepatitis E patients. They further demonstrated the dissemination of HEV RNA into the urine of two experimentally infected monkeys (inoculated with human genotype 1 or 4 HEV) up to 4 weeks post-inoculation. However, the virus was subsequently cleared from these two infected monkeys. Thus, this model appears to represent acute and self-limiting infection. More interestingly, freshly collected urine from an infected monkey was infectious and resulted in acute hepatitis after inoculation into a naïve monkey.

Since we are interested in chronic hepatitis E ³, we have attempted to establish a monkey model for persistent HEV infection. Two 2-year-old male rhesus macaques (#1 and #2) were intravenously injected with 2 mL of fecal supernatant from swine infected with HEV (genotype 4, KM01 strain, 2×10^4 copies). One macaque (#3) without inoculation served as control. Surprisingly, HEV RNA was constantly detected in both serum (Figure 1A) and fecal (Figure 1B) samples from both monkeys up to 650 (#1) and 272 (#2) days post-inoculation (dpi), as we have followed up to date. The successful establishment of this model can potentially mimic chronic HEV infection in patients.

Although it remains unclear what are the exact factors contributing to the persistent infection, this particular swine HEV strain that we have used could be one of the important factors. Upon inoculation, both macaques had only very weak and transient humoral immune response, as shown by the low serum levels of anti-HEV IgM and IgG (Figure 1C). At day 7, minor elevation of IgM (Figure 1C) was observed accompanied by slight increase of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Figure 1D), but it was normalized shortly. HEV IgG was hardly detectable throughout the infection course (Figure 1C). Since HEV is a single-standard RNA virus, it is prone to mutagenesis

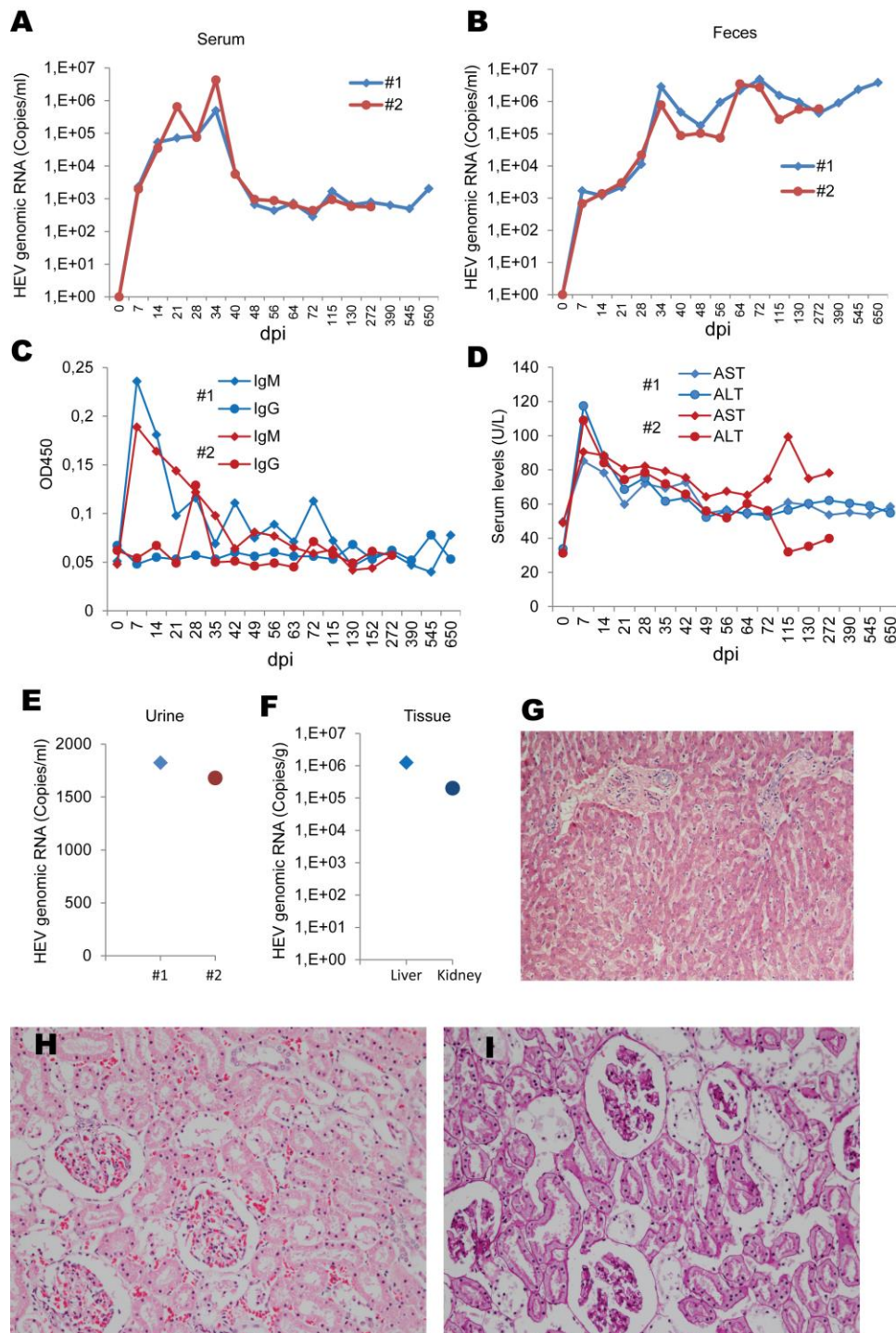


Figure 1. Rhesus macaques persistently infected with HEV shed virus into urine. Quantification of HEV genomic RNA in serum (A) and stool samples (B) from #1 and #2 monkeys was determined by qRT-PCR. (C) Measurement of serum HEV IgM and IgG levels by ELISA kit. (D) Measurement of serum ALT and AST levels. (E) Quantification of HEV genomic RNA in urine samples from #1 and #2 monkeys at 650 and 272 days-post-inoculation (dpi), respectively. (F) Quantification of HEV genomic RNA in liver and kidney tissues of #1 monkey. (G) Histology of the liver tissue of #1 monkey with haematoxylin and eosin (HE) staining; 100x. Histology of the kidney tissue of #1 monkey with HE (H) and Periodic acid-Schiff (PAS) (I) staining, 200x. Details of methodology were provided in Supplementary Information file.

during viral replication and thereby could possibly evolve to better adapt to the host. Nevertheless, further investigations are still required to determine the exact factors causing persistent infection.

Consistent with the study by Geng *et al.*², we also detected HEV RNA in the urine of both monkeys at 272 and 650 dpi, respectively (Figure 1E), although the titers in urine are lower than in feces (Figure 1B) but comparable to the serum levels (Figure 1A). One intriguing question is how HEV can be disseminated into urine? Geng *et al.*² have proposed that HEV may enter the urine from the liver through viremia or following replication in the kidney. The authors have assessed the impact of HEV infection on kidney in an infected monkey. Although renal function appears normal, viral protein was detected and pathological changes were observed at 4 weeks post-inoculation. Because our #1 macaque persistently infected with HEV died unexpectedly, we were able to perform both virological and pathological evaluation for both liver and kidney tissues. As expected, HEV is positive in the liver as shown by qRT-PCR of the viral genome (Figure 1F), but no clear histological change was observed (Figure 1G). Interestingly, HEV was also positive in the kidney (Figure 1F) and clear pathological changes were observed (Figure 1H and 1I). Renal cortical tissue showed signs of congestion as seen in the glomeruli and in the peritubular capillaries (Figure 1H). In addition, there is ischemia as denoted by an increased space of Bowman in the glomeruli and mild to moderate acute tubular necrosis (Figure 1I). This may indicate that HEV could replicate in kidney and subsequently cause tissue injury, which may facilitate the release of the virus into urine, although detailed investigation is certainly required to further explore the role of HEV infection in renal physiology and pathology. Since HEV infection has recently been reported to be associated with renal manifestations in patients⁴, it would be interesting for Geng *et al.* to also look into kidney functions and potential renal manifestations in their patients who are positive for HEV in their urine².

In summary, it is clear that HEV could be released into urine in acute and chronically infected HEV patients and monkeys. Because monkeys are capable of modelling both acute and chronic HEV infection, they are particularly useful for further mechanistic study towards understanding how HEV was disseminated into urine, as well as for HEV research in general.

Supplementary materials and methods

Quantification of HEV RNA by Real-Time qPCR

Total RNA was extracted from serum, feces or tissues (liver and kidney) by Trizol (Invitrogen, America), according to the instructions of the manufacturer. Reverse transcription was performed using an MLV Reverse Transcriptase for RT-nPCR (Takara, Japan) according to the instructions of the manufacturer. The copy number of HEV in serum, feces or tissues was analyzed using SYBR green-based qPCR assays with HEV specific primers as described previously ⁵. In brief, the synthesized first-strand cDNA (2 µL) was added as a template. Real-time qPCR was performed under the following conditions: 95°C for 30 s, followed by 39 cycles of 95°C for 5 s and 60 C for 31 s. The housekeeping gene (GAPDH) served as a loading control. Real-time qPCR was performed using an ABI PRISM 7300 Real-Time PCR System.

Measurement of HEV IgG and IgM by ELISA

HEV IgG and IgM antibodies were determined using a commercial ELISA kit (KHB, China) based on recombinant HEV fusion proteins according to the instructions of the manufacturer.

Profile liver biochemistry in serum

The serum levels of ALT were measured with an automated biochemistry analyzer (Olympus 2700, Japan).

Histopathology

Tissues for histologic examination were fixed in 10% neutral buffered formalin, routinely processed, sectioned at a thickness of 4 µm, and then stained with hematoxylin and eosin (HE), or Periodic acid–Schiff (PAS).

References

1. Huang FF, *et al.* Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol* **40**, 1326-1332 (2002).
2. Geng Y, *et al.* Detection and assessment of infectivity of hepatitis E virus in urine. *J Hepatol* **64**, 37-43 (2016).
3. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
4. Bazerbachi F, Haffar S, Garg SK & Lake JR. Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. *Gastroenterol Rep (Oxf)* **4**, 1-15 (2016).
5. Huang FF, *et al.* Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *J Clin Microbiol* **40**, 1326-1332 (2002).

Chapter 6

Hepatitis E virus infects neurons and brains

Xinying Zhou^{1#}, Fen Huang^{2#*}, Lei Xu¹, Zhanmin Lin³, Femke M. S. de Vrij⁴, Ane C. Ayo-Martin⁴, Mark van der Kroeg⁴, Manzhi Zhao¹, Yuebang Yin¹, Wenshi Wang¹, Wanlu Cao¹, Yijin Wang¹, Anouk H. G. Wolters⁵, Steven A. Kushner⁴, Jean Marie Peron^{6,7}, Laurent Alric^{7,8}, Robert A. de Man¹, Ben N. G. Giepmans⁵, Bart C. Jacobs^{9,10}, Jeroen J. van Eijk¹¹, Eleonora M. A. Aronica¹², Dave Sprengers¹, Herold J. Metselaar¹, Chris I. de Zeeuw^{3,13}, Harry R. Dalton^{14,15}, Nassim Kamar^{7,16,17}, Maikel P. Peppelenbosch¹, Qiuwei Pan¹

¹Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands. ²Medical Faculty, Kunming University of Science and Technology, Kunming, China. ³Department of Neuroscience, Erasmus MC-University Medical Center, Rotterdam, Netherlands. ⁴Department of Psychiatry, Erasmus MC-University Medical Center, Rotterdam, Netherlands. ⁵Department of Cell Biology, University Medical Center Groningen, University of Groningen, A. Deusinglaan 1, 9713 AV, Groningen, Netherlands. ⁶Service d'Hépatogastro-Entérologie, Hopital Purpan, CHU Toulouse, France. ⁷Université Paul Sabatier, Toulouse, France. ⁸MR 152 IRD-Toulouse 3 University, France Internal Medicine-Digestive Department, CHU Purpan, France. ⁹Department of Neurology, Erasmus MC-University Medical Center, Rotterdam, Netherlands. ¹⁰Department of Immunology, Erasmus MC-University Medical Center, Rotterdam, Netherlands. ¹¹Department of Neurology, Jeroen Bosch Ziekenhuis (JBZ), Henri Dunantstraat 1, 5223 ¹²Department of (Neuro)Pathology, Academisch Medisch Centrum, Amsterdam, Netherlands. ¹³Netherlands Institute for Neuroscience, Royal Dutch Academy for Arts & Science, Amsterdam, Netherlands. ¹⁴Royal Cornwall Hospital, University of Exeter, Penventinnie Lane, Truro TR1 3LJ, UK. ¹⁵European Centre for Environment & Human Health, University of Exeter, Penventinnie Lane, Truro TR1 3LJ, UK. ¹⁶Department of Nephrology and Organ Transplantation, CHU Rangueil, TSA 50032, 31059 Toulouse cedex 9, France. ¹⁷INSERM U1043, IFR-BMT, CHU Purpan, Toulouse, France.

Both authors contributed equally.

Abstract

Although hepatitis E virus (HEV), as a hepatotropic virus, is supposed to exclusively infect the liver and only cause hepatitis, a broad range of extrahepatic manifestations, including in particular idiopathic neurological disorders, have been reported in association with its infection. In this study, we demonstrated that various human neural cell lines and embryonic stem cell-derived neural lineage cells were susceptible to HEV infection. Induced pluripotent stem (iPS) cell-derived human neurons and primary mouse neurons were more permissive for HEV infection compared to human liver cells. In mice and monkeys peripherally inoculated with HEV particles, viral RNA and protein were detected in brain tissues. Finally, patients with HEV-associated neurological disorders shed virus into cerebrospinal fluid (CSF), indicating direct infection of their nervous system. These results challenge the dogma of HEV as a pure hepatotropic virus and suggest that HEV infection should be considered in the differential diagnosis of idiopathic neurological disorders.

Introduction

Hepatitis viruses, classified as A, B, C, D and E, share the common feature of infecting human liver resulting in hepatocellular inflammation. Hepatitis E virus (HEV) containing a positive-sense, single-stranded RNA genome belongs to *Hepeviridae* family and is the most common cause of acute hepatitis worldwide ¹. Although it was initially thought to cause acute infection only, chronic infection has been well documented in immunocompromised individuals, including those with HIV infection, or undergoing chemotherapy for cancer, but most commonly in organ transplantation recipients ². In contrast to other hepatotropic viruses, HEV genotypes 3 and 4 are now recognized as zoonotic in developed countries with a broad spectrum of animals serving as reservoirs, with the true primary host being the pig ³. HEV infection is surprisingly common in developed countries ⁴. Infections are normally asymptomatic or unrecognized and as a result HEV has found its way into the human blood supply. For example, recent data shows that 1 in 600 blood donors in the Netherlands are viraemic at the time of donation ⁵. Notably however, the health risks following viraemic blood transfusion remain unclear.

Previously, HEV was thought to exclusively infect hepatocytes resulting in hepatocellular necrosis and hepatitis. However, a number of recent studies have shown that HEV infection is associated with a broad range of idiopathic extrahepatic manifestations, including renal injury, acute pancreatitis, hematological diseases and a variety of neurological disorders ⁶. Among these, neurological disorders have been described as a relatively common, but under-recognized, extrahepatic manifestation related to HEV infection ^{4, 7, 8}. An increasing number of central or peripheral nervous system diseases have been documented in patients associated with acute or chronic HEV infection ^{4, 7, 9-11}, including Guillain-Barre syndrome (GBS) and neuralgic amyotrophy (NA) ^{4, 9, 12}.

The etiology of HEV-associated neurological injury is unknown. In the existing paradigm, HEV is considered to be primarily a hepatotropic virus. Hitherto, the most widely held neuropathogenic hypothesis posits that HEV causes neurological injury by immune mechanisms related to molecular mimicry, as has been well described for GBS associated with a variety of infectious triggers ^{9, 13}. The alternative hypothesis, however, that HEV causes direct injury via neurotropism has not been sufficiently evaluated. The aim of the present study was to investigate if HEV can directly infect the nervous system. Our results

demonstrate that HEV is capable of central nervous system infection in multiple species and therefore should be considered as a neurotropic virus.

Materials and methods

HEV plasmids.

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (genotype 3 Kernow-C1 p6 clone, GenBank accession number JQ679013) or a construct containing subgenomic HEV sequence coupled with a *Gaussia* luciferase reporter gene (p6-Luc), using the Ambionm MESSAGE MACHINE *in vitro* RNA transcription Kit (Life Technologies Corporation)^{33, 36}.

Reagents and cell culture.

Human IFN- α (Thermo Scientific, the Netherlands) was dissolved in PBS. Ribavirin (Sigma-Aldrich, St Louis, MO) and rapamycin (Bio-Connect, TE Huissen, the Netherlands) were dissolved in DMSO (Sigma). Mycophenolic acid (MPA) (Sigma) was dissolved in methanol. Human hepatoma cell line HuH7 was cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, the Netherlands) supplemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 lg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). All the neural cell lines were kind gifts: glioblastoma U87 cell line was from Department of Viroscience, Erasmus Medical Centre, glioblastoma U343 cell line was from Dr. Elly Hol (Department of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht), neuroepithelioma SK-N-MC cell line was from Dr. Thomas Pietschmann (Institute for Experimental Virology, Twincore, Centre for Experimental and Clinical Infection Research), and neuroblastoma SH-SH5Y cell line was from Dr. Marcel E. Meima (Department of Internal Medicine, Erasmus Medical Center). They were cultured in DMEM supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 lg/ml streptomycin and 2 mM L-glutamine. Human embryonic stem cell (H9) derived-neural progenitor cells (hES-NPCs) were cultured and differentiated as previously described³⁷. iPS cell-derived iCell Neurons of highly pure population of human neurons (Cellular Dynamics International, Inc., CDI, WI, USA.) were cultured in the complete iCell Neurons maintenance

medium (CDI) as the guild line described (https://cellulardynamics.com/assets/CDI_iCellNeuronsUsersGuide.pdf). All cells were cultured in an incubator at 37°C with 5% CO₂.

Electroporation of HEV RNA.

The glioblastoma U87 cells were collected and centrifuged for 5 min, 1500 rpm, 4°C. The supernatant was removed and the cells were washed with 4 ml Opti-MEM (Thermo Scientific, the Netherlands) by centrifuging for 5 min, 1500 rpm, 4°C. The cell pellet was re-suspended in 100 µl Opti-MEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (400 volt, pulse length 0.5, number 1 and cuvette 4 mm)³⁶. The supernatant of cultured p6 full-length HEV RNA electroporated cells were collected to perform secondary infection.

Isolation and culture of primary mouse neurons.

The primary mouse cerebellum and hippocampus were isolated from E17-E19 mouse embryos. In brief, the cerebellum and hippocampus were dissected in ice-cold HBSS supplemented with 20 µg/ml Gentamicin (both from Life Technologies), then incubated with 10 U/ml Papain (Sigma), 2.5 U/ml DNase I (Roche) and 4 mM MgCl₂ (Sigma) at 33°C for 30 min. The cerebella were titrated in HBSS with 2.5 U/ml DNase I and 4 mM MgCl₂, and were filtered with 200 µm Nylon mesh. After washing in HBSS twice, the cells were plated on poly-ornithine (500 µg/mL, Sigma) coated 1.5H glass-bottomed slide (ibidi) at a density of 1.2×10^6 cells/cm². For electroporation, these primary mouse neurons were transfected with p6 full-length HEV RNA using a Nucleofector 4D (Lonza) according to manufacturer's protocol before plating. The culture medium contains PNGM neural basal medium (Lonza), GS-21 supplement (Globalstem), 5 µg/ml Gentamicin and 2 mM Glutamax (Life Technologies). Half of the medium was changed once a week, and 2 µM Ara-C (Sigma) was added to prevent glia growth.

Quantitative real-time polymerase chain reaction (qRT-PCR).

RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, the Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of HEV, human and mouse GAPDH were amplified by 40 cycles and quantified with a SYBR Green-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH was considered as a reference gene to normalize gene expression. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), the primers of human housekeeping gene Human GAPDH were 5'-TGTCCCCACCCCAATGTATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense), and the primers of mouse GAPDH were 5'-CATCACTGCCACCCAGAAGACTG-3' (sense) and 5'-ATGCCAGTGAGCTTCCCGTTCAG-3' (antisense).

Measurement of luciferase activity.

For *gaussia* luciferase, the activity of secreted luciferase in the cell culture medium was measured by the BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs) and quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

MTT assay.

Cells seeded in 96-well plates were cultured with 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO) at 37 °C with 5% CO₂ for 3 h. After removing the medium, 100 µl of DMSO was added to each well for 50 min incubation. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at a wavelength of 490 nm. All measurements were performed in triplicate.

Western blot assay.

The supernatant of HEV RNA electroporated U87 cells with lysate buffer and DTT were heated 5 min at 95°C followed by loading onto a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis. After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF)

membrane for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 ml blocking buffer and 2.5 ml PBS containing 0.05% Tween 20 (PBS-T). It was followed by incubation with primary HEV ORF2 (1:1000) antibodies (aa 434-457, clone 1E6, Millipore, Amsterdam-Zuidoost, the Netherlands) overnight at 4°C. The membrane was washed 3 times followed by incubation for 1.5 h with anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were scanned by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantitated with Odyssey 3.0 software.

Animal models of HEV infection.

The Animal Care and Use Committee of the Kunming University of Science and Technology approved the study protocol and provided the guidelines for this study. Specific-pathogen-free (SPF) male Balb/c nude mice were purchased from the National Rodent Laboratory Animal Resources, Shanghai Branch (China) and maintained in a pathogen-free animal facility. Prior to their inoculation with HEV, all the mice were tested negative for HEV IgG and IgM antibodies and HEV antigens in both their sera and feces. Swine HEV (Genotype 4, KM01 strain) isolated from the feces was intravenously injected into mouse with a viral load of 1×10^5 copies/ml, as calculated by the viral genomic titer determined by qRT-PCR. Mice were humanely euthanized, at 14 days post-inoculation following the guidelines of the Care and Use of Laboratory Animals.

Rhesus macaque were from Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. Prior to their inoculation with HEV, they were tested negative for HEV IgG and IgM antibodies and HEV antigens in both of sera and feces. Two 2-year-old male rhesus macaques were intravenously injected with 2 ml of fecal supernatant from swine infected with HEV (genotype 4, KM01, 2×10^4 copies/ml) as previously established²⁰. One macaque without inoculation served as a control. One monkey that died unexpectedly after over 600 days of chronic infection was subjected for analysis. For the mice and monkey, serum samples, liver tissues and brain tissues were collected for qRT-PCR quantification of HEV RNA. The brain tissues were collected for immunohistochemistry staining of ORF2 viral protein.

Immunohistochemistry.

For immunofluorescence, cell lines and primary mouse brain cells grown on coverslips were fixed with 4% (w/v) paraformaldehyde (PFA) for 10 min at RT. After three washes with PBS buffer, cells were permeabilized with 0.1% (v/v) Triton X-100 for 10 min and washed three times with PBS. Cells were then incubated with primary anti-HEV ORF2 antibody (1:200) at 4°C overnight and washed three times with PBS, followed by incubation with Anti-mouse-Alexa Fluor® 488-Conjugate antibody (Cell Signaling Technology) (1:1000) covalently linked to fluorophores and DAPI for 1 h. Confocal images were visualized with an inverted LSM 510 confocal microscope (Zeiss LSM510, Jena, Germany) using a 40x oil immersion objective. All images were analyzed with a Zeiss LSM Image Browser (version 4.2).

For immunohistochemistry assay of cell cultures and brain tissues of mouse and monkey, slides were wash by PBS-T after deparaffinization. Antigen retrieval were performed in citric acid buffer (pH = 6) and PBS with 1.5% H₂O₂ were added at RT for 10 min to remove background. Rinse with PBS-T followed by blocking with 5% nonfat milk. Slides were incubated with primary anti-HEV ORF2 antibody (1:200) at 4°C overnight. After being rinsed in PBS-T, the slides were incubated for 30 min in Dako EnVision + System-HRP Labelled Polymer anti-mouse antibody (Dako, Carpinteria, USA), then rinsed with PBS-T and incubated in DAB solution (0.05% DAB, 0.0017% H₂O₂, PBS). To finish, the slides were rinsed twice with distilled water, counterstained with hematoxylin for 20 seconds, dehydrated in ethanol, cleared in xylene and embedded. Staining was visualized by a Nikon light microscope (Nikon, Amsterdam, the Netherlands).

Retrospective analysis of patient data.

Three cohorts of 18 HEV-associated neurological patients at CHU Rangueil (Toulouse, France), Erasmus MC-University Medical Center (Rotterdam, Netherlands) and Royal Cornwall Hospital (UK) were identified for analysis.

Statistical analysis.

Data were presented as mean ± SEM. Comparisons between two groups were performed with Mann-Whitney U test. Differences were considered significant at a p value less than 0.05.

Results

HEV infects neurons in cell culture.

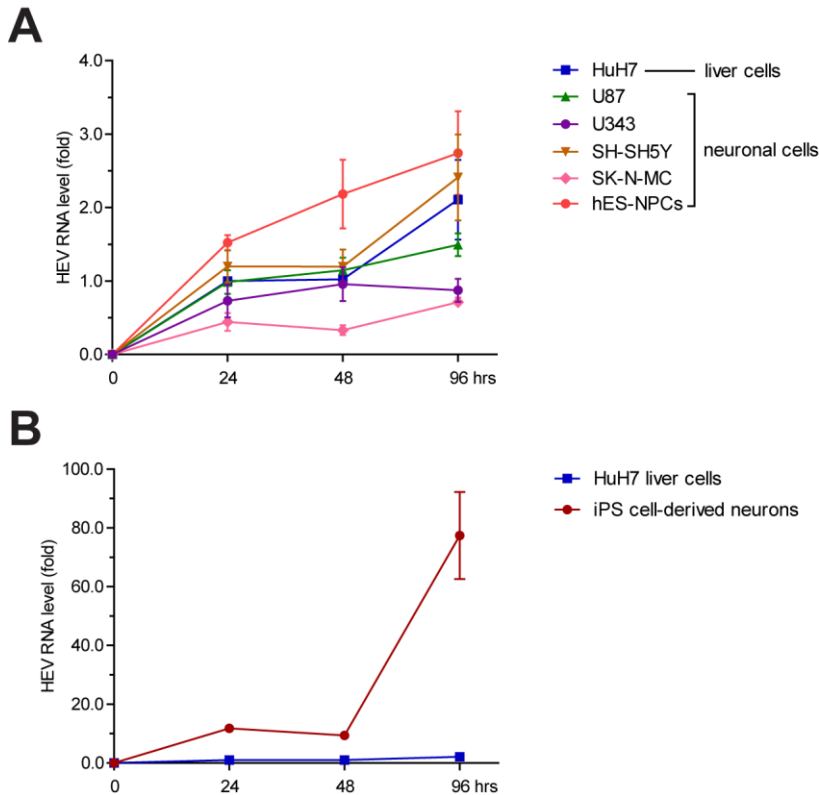


Figure 1. Neural cells are permissive for HEV infection *in vitro*. A, Cellular HEV RNA levels of various human neural cell lines and human embryonic stem cell-derived neuron progenitor cells (hES-NPCs), and B, cellular HEV RNA levels of induced pluripotent stem (iPS) cell-derived human neurons were analyzed with inoculation of cell culture-derived HEV particles for 24, 48 and 96 hrs, compared to human liver HuH7 cells, which are considered the canonical model for HEV infection (Mean \pm SD, $n = 3-4$). The level of HEV infection in HuH7 cells for 24 hrs serves as 1.

To investigate whether HEV can directly infect human neural cells, neuroblastoma SH-SH5Y, neuroepithelioma SK-N-MC, glioblastoma U87 and U343 cell lines were employed. A human liver cell line, human hepatoma 7 (HuH7), was used as a positive control because of its high permissiveness for HEV infection. After inoculation with cell culture-derived HEV particles for 24, 48 and 96 hrs, intracellular viral RNA was quantified by qRT-PCR. All of the neural cell lines were permissive to HEV infection. U87 and SH-SH5Y cells exerted a similar susceptibility compared to HuH7 cells, whereas U343 and SK-N-MC cells were less susceptible than HuH7 cells to HEV infection (Figure 1A). Moreover, human embryonic stem cell (H9) derived-neural progenitor cells (hES-NPCs) (Supplementary Figure 1A) exhibited a particularly high susceptibility to HEV infection, compared to HuH7 liver cells (Figure 1A). We investigated HEV infection of cultures in which neuronal differentiation of hES-NPC was induced. These cultures especially appeared remarkably permissive for HEV infection, as evidenced by the abundance of intracellular HEV RNA quantified four days after inoculation of HEV particles

(Supplementary Figure 1B). Subsequently, induced pluripotent stem (iPS) cell-derived human neurons with highly pure population of mixture of post-mitotic neural subtypes, as observed under the white-light microscopy analysis (Supplementary Figure 1c), strikingly exhibited the highest efficiency of HEV replication level by cellular HEV RNA levels especially at 96 hrs of virus infection, compared to HuH7 liver cells (Figure 1B). HEV copy numbers presented a time-dependent increase in the secreted medium (Supplementary Figure 1D). These results demonstrate that HEV is able to directly infect neural cells *in vitro*.

Long-term replication and production of HEV in neural cells.

Further detailed study of HEV infection was performed using the glioblastoma U87 cell line, a long-established and generally considered an appropriate model for investigating the molecular details of virus-related neuropathology¹⁴. Upon electroporation of the full-length genomic HEV RNA, viral protein ORF2 was subsequently expressed in U87-HEV cells as detected by immunofluorescent and immunohistochemical staining (Figure 2A and Supplementary Figure 2). We further investigated whether these U87-HEV cells can secrete viral particles into the supernatant. To this end, a HEV plasmid based standard curve was constructed as a reference to assess and quantify the production of infectious virion (Supplementary Figure 3A). We observed that HEV load was 1.7×10^6 copies/ml in supernatant of U87-HEV cells and 1.3×10^9 copies/ml in ultra-centrifuged medium (Supplementary Figure 3B). Viral protein was detected in the ultra-centrifuged medium by Western blot analysis (Figure 2B). Thus human glioblastoma cells appear capable of supporting the HEV life cycle.

To ascertain whether glioblastoma cells also can support long-term production of HEV, the viral load of extra-cellular U87-HEV medium was monitored over 30 consecutive days and high viral loads were consistently secreted over this entire time frame (Figure 2C). For U87-HEV-Luc cells with electroporation of subgenomic HEV RNA in which ORF2 was replaced by a *gaussia* luciferase reporter gene (p6-Luc), secreted luciferase activity directly serves as a pseudomarker for the level of HEV replication. Glioblastoma cells apparently supported long-term HEV replication as luciferase activity could be readily detected for 30 days (Figure 2D). Furthermore, comparable levels of HEV infection were detected upon inoculation of HuH7 liver cells and U87 cells with U87-produced HEV particles for 96 hrs,

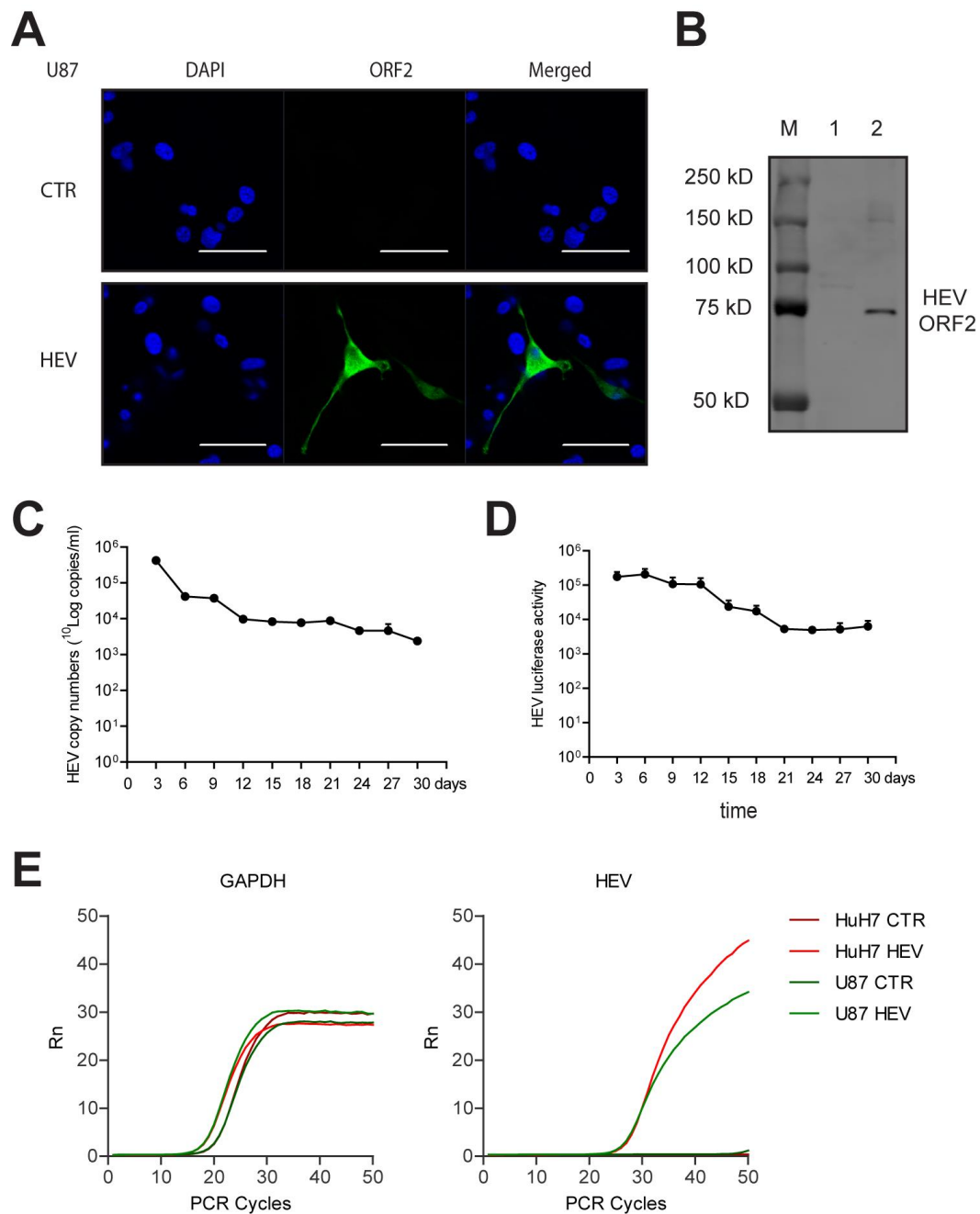


Figure 2. Neuroglial cells support long-term HEV replication and production. A, Immunofluorescence staining of viral protein ORF2 (green) in neuroglial U87 cells upon electroporation of the full-length genomic HEV RNA. DAPI (blue) was applied to visualize nuclei. Shown confocal images are representative for three independent experiments. (Scale bar, 50 μ m. 40x oil immersion objective). B, In ultra-centrifuged supernatant produced by U87-HEV cells, viral protein ORF2 is observed by Western blot assay. C, U87 supports long-term production of HEV as tested by copy numbers in the medium for 30 days. (Mean \pm SD, $n = 2$, each with two replicate experiments). D, In U87 cells upon electroporation of the sub-genomic HEV RNA with luciferase reporter, HEV luciferase activity representing virus replication level has been detected maintaining for 30 days. (Mean \pm SD, $n = 2$, each with two replicate experiments). E, RT-PCR result indicates that HuH7 and U87 cells are permissive for secondary infection with inoculation of U87-HEV produced viral particles for 96 hrs. GAPDH serves as a reference gene. One representative experiment of three is shown.

demonstrating that these cells produce infectious particles (Figure 2E), a hall mark of bona-fide infectivity. These data show that human glioblastoma cells are capable of supporting long-term replication and production of infectious HEV.

Drug treatments on HEV replication in neuroglial cells.

HEV-associated neurological manifestations are documented in both immunocompetent patients and immunocompromised patients who are in particular organ transplant recipients. If managed/treated adequately, viral load can be reduced in most of the patients and neurological symptoms can be resolved ¹⁵. Reducing the dose of immunosuppressants is often the first line of therapeutic intervention for immunosuppressed patients ¹⁶. Although no approved HEV medication is available, ribavirin or interferon- α (IFN- α) have been used as off-label treatment for some cases, in particular chronically infected patients. We previously have demonstrated the anti-viral effects of ribavirin ¹⁷ and IFN- α ¹⁸, the anti-viral effect of the immunosuppressant mycophenolic acid (MPA) ¹⁷, and the pro-viral effect of the immunosuppressant rapamycin ¹⁹ in liver cell lines infected with HEV, suggesting that such drugs have promise for combating neural HEV infection as well. Proof for this notion came from experiments in which we studied the effects of clinically relevant concentrations of these medications, in neuroglial U87 cells employing the subgenomic HEV replication model U87-HEV-Luc cells and infectious U87-HEV cells. IFN- α significantly exhibited dose-dependent anti-viral effects in U87-HEV-Luc cells and high dose (1000 IU/ml) potentially reduced the level of cellular HEV RNA level in U87-HEV cells (Figure 3A). A subset of interferon stimulated genes (ISGs) was significantly induced following treatment with 1000 IU/ml IFN- α , indicating that canonical interferon signaling mediates these anti-HEV effects in neuroglial cells (Figure 3B). Analogously, anti-HEV effects of ribavirin in these neuroglial cell cultures were observed, supporting the use for this compound for treating HEV infection complicated by neurological manifestations (Figure 3C). Interestingly, the immunosuppressive medication MPA (often used to suppress organ rejection) counteracted HEV infection, but a pro-viral effect of the immunosuppressant rapamycin (often used to suppress organ rejection) was observed (Figure 3D and E). MTT results showed no cytotoxicity treating with these drugs in U87 cells (Figure 3F). Thus our data indicates that the use of MPA may be indicated as an immunosuppressive regimen for counteracting rejection in organ transplant recipients at risk for HEV infection, whereas ribavirin appears a

rational choice for combating an established HEV infection complicated by neurological manifestations.

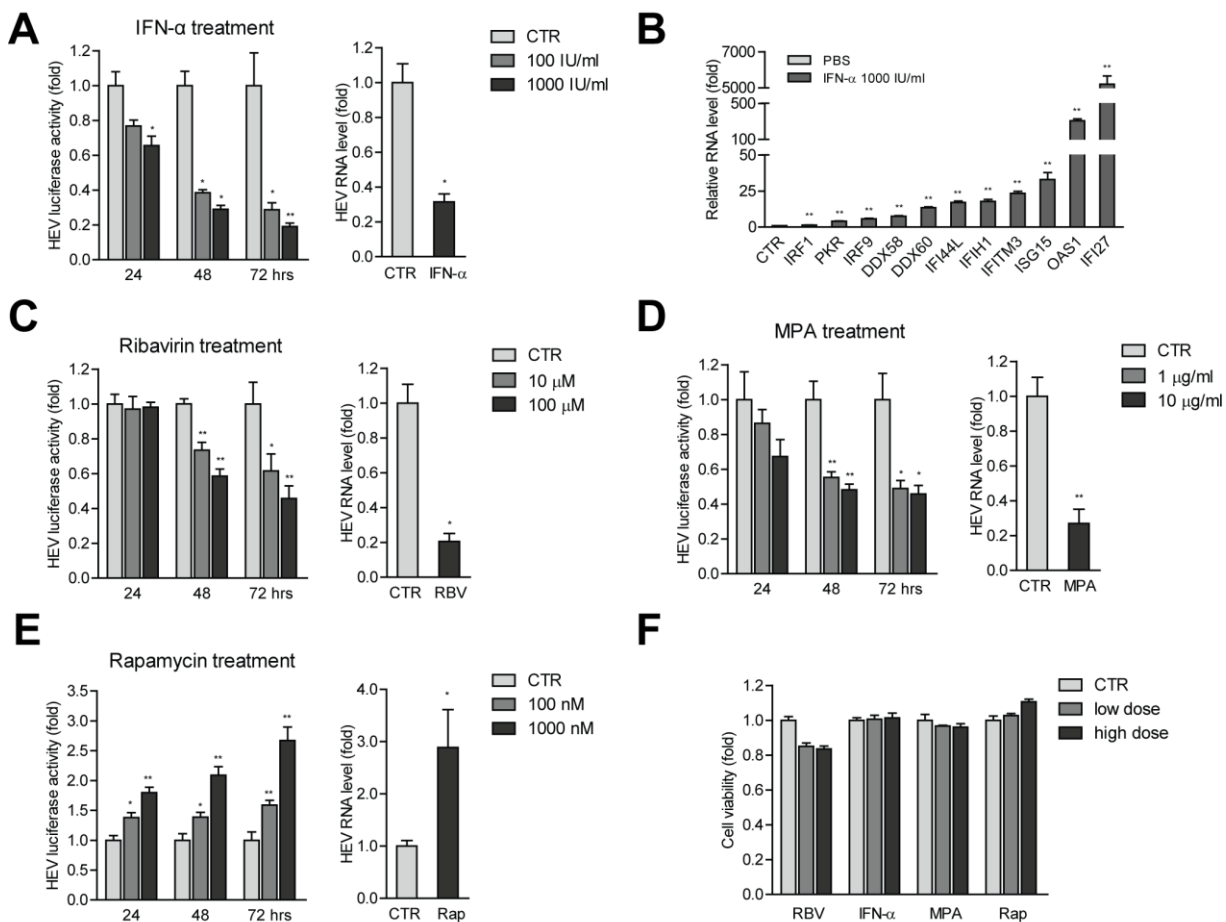


Figure 3. Drug treatments for HEV infection in neuroglial cells. (A) 100 (low)/1000 (high) IU/ml IFN- α , (C) 10 (low)/100 (high) μ M RBV, (D) 1 (low)/10 (high) μ g/ml MPA and (E) 100 (low)/1000 (high) nM rapamycin are treated in U87-HEV-Luc cells for 24, 48 and 72 hrs and in U87-HEV cells for 48 hrs. HEV luciferase activity and cellular RNA level were analyzed respectively. B, 1000 IU/ml IFN- α significantly induced a subset of interferon stimulated genes (ISGs). F, MTT assay shows no cytotoxicity of these drug treatments for three days in U87 cells.

Primary mouse neurons efficiently support HEV infection.

Further support for the results obtained with respect to the neurotropism of HEV came from experiments in which we conducted to infect primary isolated mouse neurons with HEV. Primary cerebellar and hippocampal neurons cultured from mouse embryos were incubated with cell culture-derived HEV particles (Figure 4A). HEV replicated efficiently in both neuronal cultures, as judged by cellular levels of HEV RNA. As compared to the canonical model for HEV replication, Huh7 cells, HEV infection in cerebellar cultures, was 7.09 ± 4.51

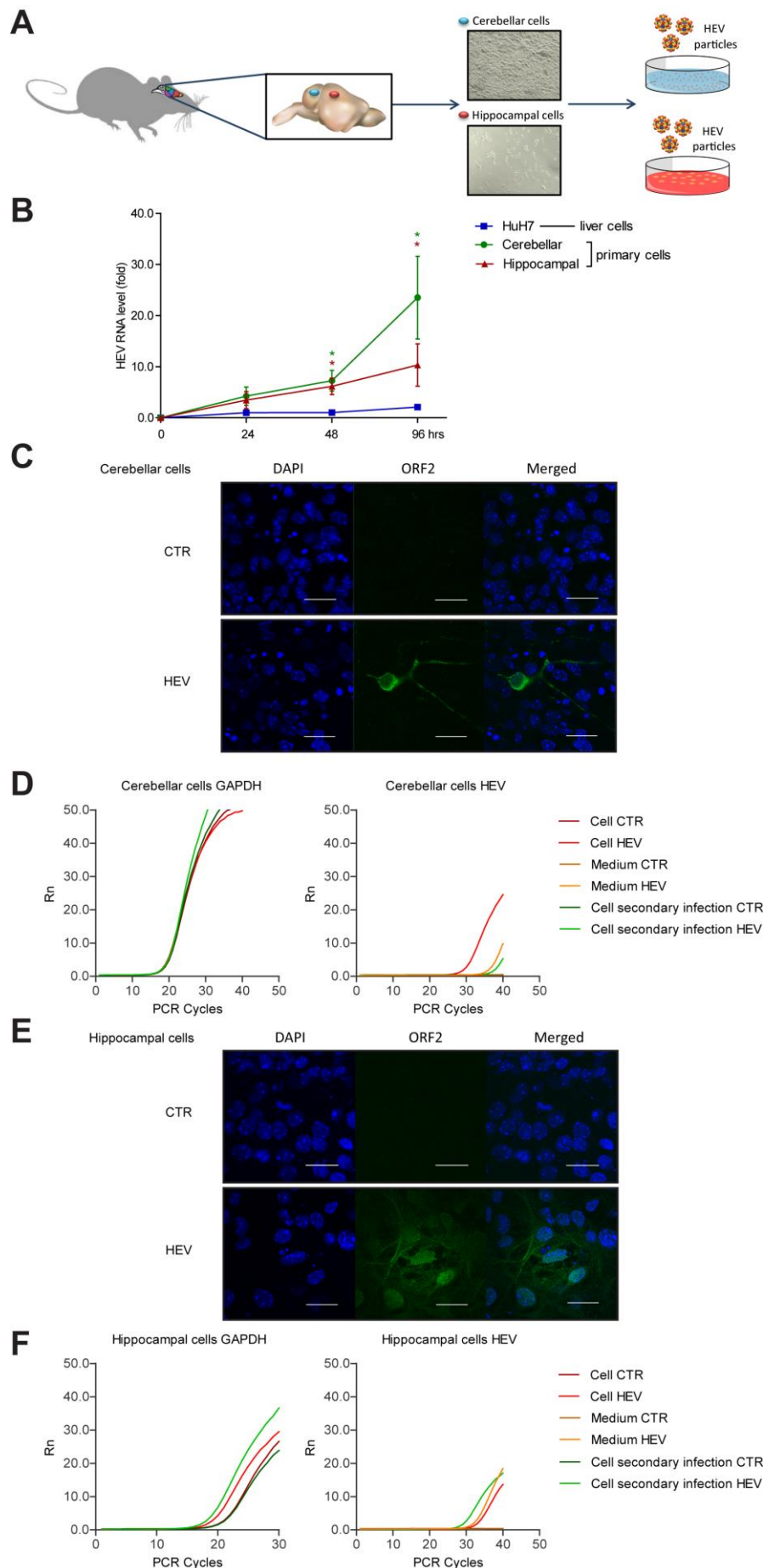


Figure 4. HEV infects primary mouse neurons.

A, Cerebellar and hippocampal neurons were cultured and inoculated with HEV particles for 96 hrs. B, RT-PCR result shows a time-dependent increase of HEV replication in both neurons (Mean \pm SEM, $n = 5$, $P < 0.05$). Immunofluorescence staining of viral protein ORF2 (green) were detected three days electroporation of HEV RNA in primary (C) cerebellar and (E) hippocampal neurons. DAPI (blue) was applied to visualize nuclei. (Scale bar, 50 μ m.) RT-PCR result indicates that primary (E) cerebellar and (F) hippocampal neurons are permissive for secondary infection with inoculation of viral particles for 96 hrs. GAPDH serves as a reference gene.

and 11.16 ± 18.09 -fold (mean \pm SEM, $n = 5$, $P < 0.05$) more efficient at 48 and 72 hrs, respectively. In hippocampal cultures, when compared with the Huh7 benchmark, HEV infection was 6.03 ± 3.92 and 4.91 ± 10.14 -fold (mean \pm SEM, $n = 5$, $P < 0.05$) more efficient at 48 and 72 hrs, respectively (Figure 4B). We next examined whether these mouse neurons can produce infectious HEV particles. For full length of HEV genome RNA was electroporated in cerebellar cells with detection of the viral ORF2 protein employing immunofluorescent staining for this protein at 72 hrs (Figure 4C). HEV RNA was present both in the cells and in the supernatant of these cultures. Inoculation of naïve cells with the supernatant resulted in HEV infection (Figure 4D). Similar results were observed in hippocampal cells (Figure 4E and F). In conjunction, these data demonstrate that both primary cerebellar and hippocampal cultures are capable of the production infectious HEV particles *in vitro* and show that primary neurons are more susceptible to HEV infection as compared to the canonical model for HEV infectivity, in human HuH7 liver cells.

HEV infects the brains of mouse and monkey.

The definitive proof for the notion that the central nervous system is a primary target for infection by HEV would be demonstration of such infection *in vivo*. Hence, mice were intravenously inoculated with fecal samples contacting HEV particles via tail vein injection (Figure 5A). As expected, high titers of HEV genomic RNA were detected in serum samples and mouse liver at 14 days post-inoculation (dpi). Importantly in parallel measurement of HEV levels in mouse brain revealed a high level of infection in this organ (Figure 5B). Consistently, in the cerebellum of the mouse brain tissue, viral protein ORF2 was observed expressing in the granule layer by immunohistochemistry analysis (Figure 5C). Analogously, in a monkey (rhesus macaque) model for persistent HEV infection²⁰ in an animal dying unexpectedly after over 600 days of chronic infection, viral RNA was detected in serum, liver and brain tissue of this monkey (Figure 5B). Again, Viral protein ORF2 was expressed in granule layer of cerebellum (Figure 5C). These data show that HEV infects the brains of mouse and monkey following experimental inoculation of HEV.

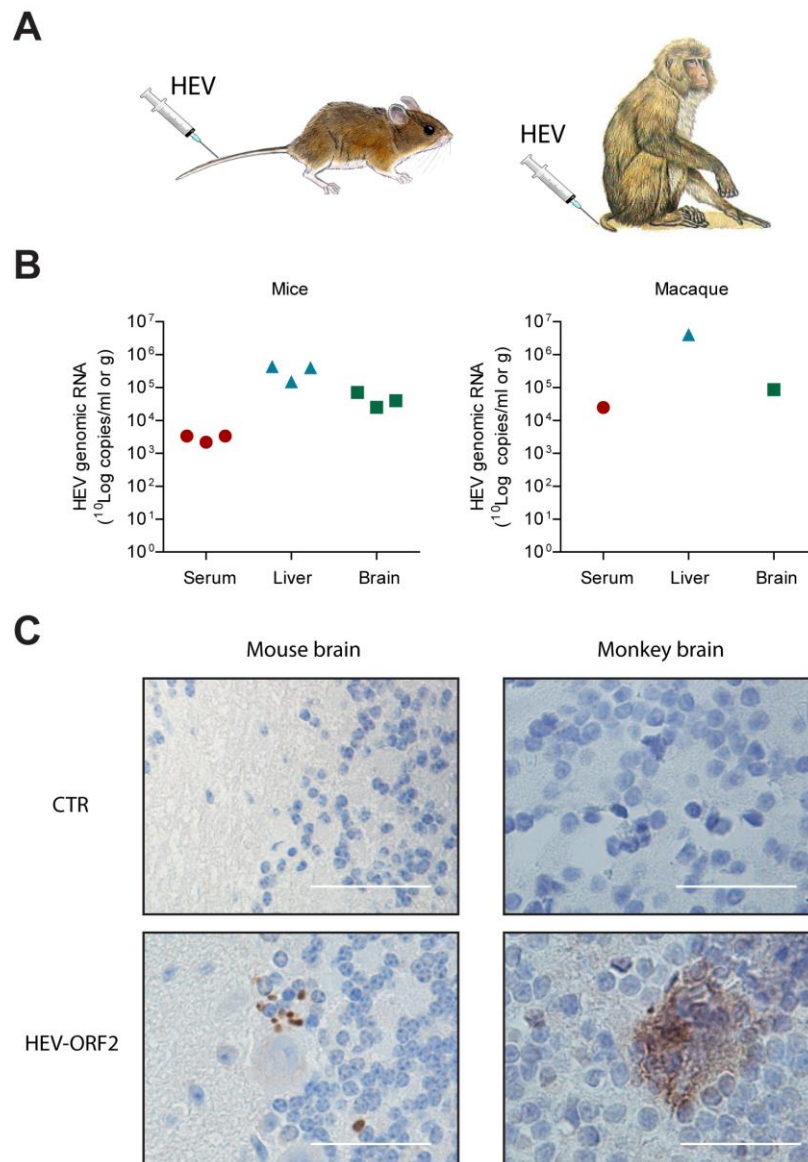


Figure 5. HEV infection in the brains of mouse and monkey. A, Three mice and one monkey have been tail intravenously injected and inoculated with HEV strains for 14 days and over 600 days with chronic infection separately. B, Viral RNA has been observed in serum samples, liver tissues and brain tissues of infected mouse and monkey. C, Viral HEV ORF2 protein are observed in brain tissues of infected mouse and chronically infected monkey by immunohistochemical assay. Brain tissues of uninfected mouse serve as a control. For monkey brain tissues, staining without inoculating viral antibody samples serve as a control. Cerebellum of the brain were observed and HEV positivity presented in the granule cell layer of the cerebellum. Hematoxylin (blue) was applied to visualize nuclei, and DAB solution was used to visualize cytoplasm (Scale bar: 50 μ m).

Human patients with HEV-associated neurological disorders shed virus into cerebrospinal fluid.

Our data that HEV can infect brain tissue suggest that the neurological manifestations associated with HEV do not necessarily relate to autoimmunity but may relate to direct HEV

infection. In order to test this hypothesis directly, cerebrospinal fluid (CSF) from patients with HEV-associated neurological disorders was collected and tested for the presence of HEV RNA by PCR, employing patients with *bona fide* acute or chronic HEV infection, as evident from testing serum for HEV antibody IgM, IgG and HEV RNA. We thus analyzed three different cohorts of patients (some patients have been described in our previous publications) with HEV-associated neurological disorders^{9, 21-23}. Over half of the patients were positive for HEV RNA in serum samples indicating active infection in these patients (Table 1). Among these patients, 42.9% (six out of 14) shed virus into CSF. These results suggest that HEV may directly infect central nervous system and/or peripheral nerve roots in humans.

Table1. Characteristics of patients with HEV-associated neurological disorders*.

Patient	Country	Gender	Age	Immunocompetence	IgG HEV	IgM HEV	PCR serum	PCR faeces	PCR CSF	Type of neurologic disorder
1	NL	M	55	+	+	+	-	NT	-	GBS
2	NL	F	60	+	+	+	-	NT	-	GBS
3	NL	M	67	+	+	+	-	NT	-	GBS
4	NL	M	44	+	+	+	-	NT	-	GBS
5	UK	M	58	+	+	+	+	NT	-	NA
6	UK	M	52	+	+	+	+	NT	-	NA
7	UK	M	49	-	+	+	+	+	+	Peripheral neuropathy
8	UK	M	34	+	+	+	+	NT	-	Peripheral neuropathy
9	UK	M	42	+	+	+	+	NT	-	GBS
10	FR	M	74	+	+	+	+	+	+	GBS
11	FR	M	33	+	-	+	+	+	-	GBS
12	FR	M	59	-	+	+	+	+	+	Ataxia
13	FR	M	32	-	+	+	+	+	+	Cognitive dysfunction
14	FR	M	44	-	+	+	+	+	+	Polyradiculoneuropathy
15	FR	M	66	-	+	+	+	+	-	GBS
16	FR	F	33	+	-	+	+	+	-	Meningitis
17	FR	M	56	+	+	+	+	+	-	NA
18	FR	M	57	-	+	+	+	NT	+	GBS

*Ig, immunoglobulin; HEV, hepatitis E virus; CSF, cerebrospinal fluid; NL, the Netherlands; UK, United Kingdom; FR, France; +, positive; -, negative; NT, not tested; GBS, Guillain-Barré syndrome, NA, neuralgic amyotrophy.

Discussion

The data in the present study show that HEV can efficiently infect neural cell types and tissues at efficacies that exceed that of canonical liver-derived models. Thus the aspect of HEV appears also neurotropic, substantially challenging current dogma. The data appear clinically relevant in that HEV can be recovered from the CSF of patients with acute HEV infection complicated by neurological manifestations. As HEV infection is often undiagnosed, they/the data suggest that HEV may be involved in at least some cases of “idiopathic” neurological disease. Taken together these findings challenge the conventional wisdom that HEV is primarily a hepatotropic virus and helps our understanding of the potential pathogenic mechanism of HEV-associated neurological injury.

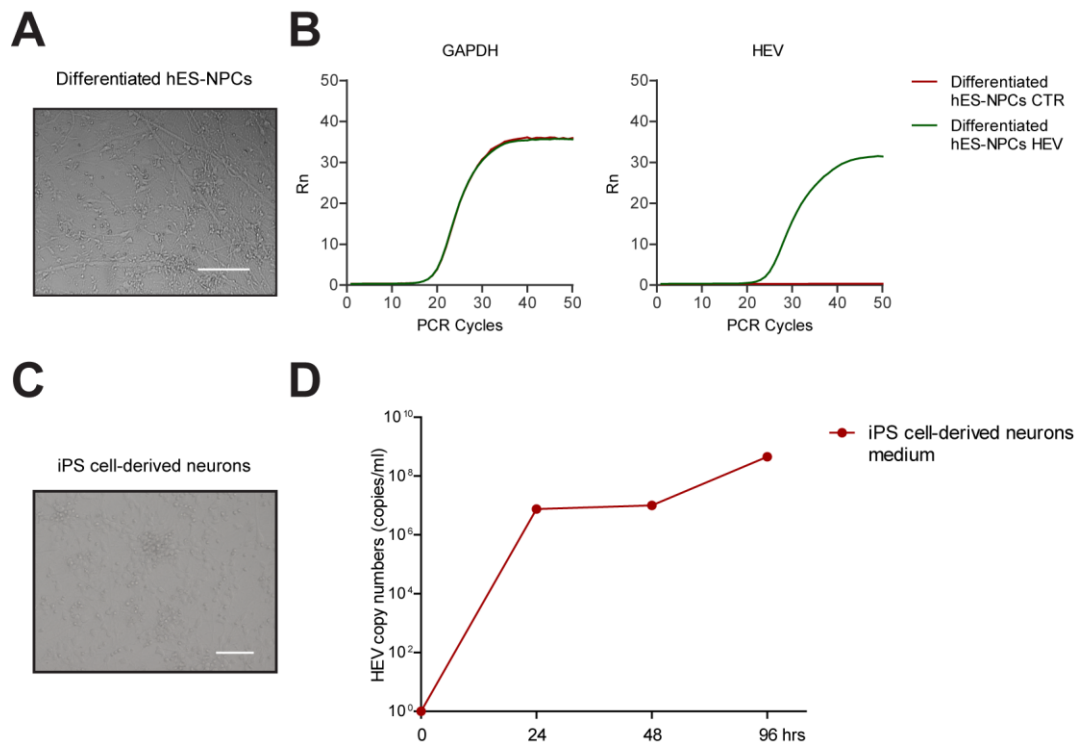
Recent studies show that HEV demonstrates tropism to a range of tissues of non-hepatological origin. Although liver cells are commonly used for modelling HEV infection *in vitro*, a human lung epithelial cell line A549²⁴ and some ectodermally-derived cell lines^{25,26} have been reported to support HEV replication in cell culture. We have recently demonstrated that mouse embryonic fibroblasts are also susceptible for HEV infection²⁷. In nude mice experimentally infected with HEV, viral RNA was detected in liver, spleen, kidney, jejunum, ileum and colon²⁸. In the tree shrew (*tupaia belangeri chinensis*), HEV capsid protein was expressed in the liver, spleen, and kidney²⁹. In specific-pathogen-free rabbits infected with rabbit HEV strain, both positive and negative-stranded viral RNA and antigen expression were detected in liver, brain, stomach, duodenum and kidney, suggesting its active replication in these tissues³⁰. In Mongolian gerbils infected with a genotype 4 strain of swine HEV, the virus could be detected in the liver, kidney, spleen and small intestine³¹. In rhesus macaques infected with human³² or swine²⁰ HEV strains, viral infection in kidney and dissemination of viruses into urine were observed. Thus our data add on a gathering momentum that HEV is not an exclusively hepatotropic virus. Furthermore, our studies also demonstrate the clinical relevance of extra-hepatic infection. Finally, our studies suggest that use of MPA for preventing rejection in organ transplant recipients might be accompanied by a lower incidence of HEV-associated neurological manifestations and that ribavirin is suitable for managing such complications.

The strain of human HEV used in the current study deserves comment. Kernow C1p6 was isolated from a UK patient with HIV-1 chronically infected with HEV genotype 3 (case 7,

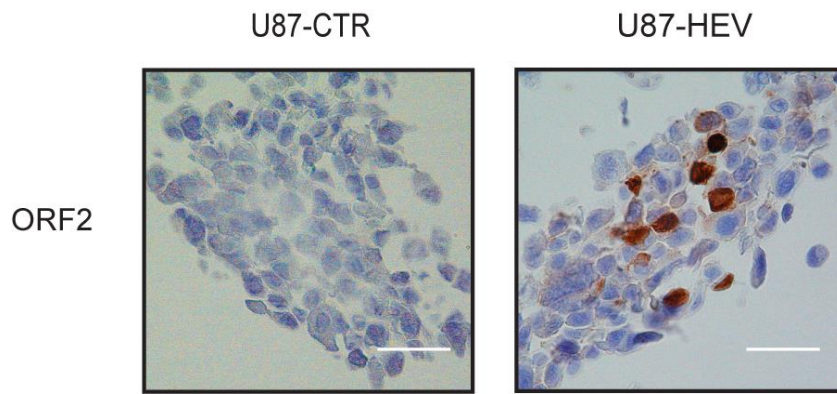
table1) ^{33, 34}. The patient had neurological symptoms in the legs which resolved with viral clearance following therapy with ribavirin/Peg-interferon ³⁵. The strain of HEV isolated from the patient was shown to have a novel virus-host recombination ³³. Thus, this strain of HEV was unusual and had documented ability to produce clinically relevant neurological injury in humans. However, for the *in vivo* mouse and monkey experiments we used HEV genotype 4 of porcine origin. This suggests that multiple HEV strains share high neurotropism. The clinical observation that HEV-associated neurological injury is found worldwide and caused by HEV of differing genotypes would support this notion⁴.

In conclusion, HEV is neurotropic *in vitro* and *in vivo* in mice, monkeys and probably human beings. These findings challenge the notion that HEV is primarily a hepatotropic virus and suggest HEV infection as a possibility in idiopathic neurological disease.

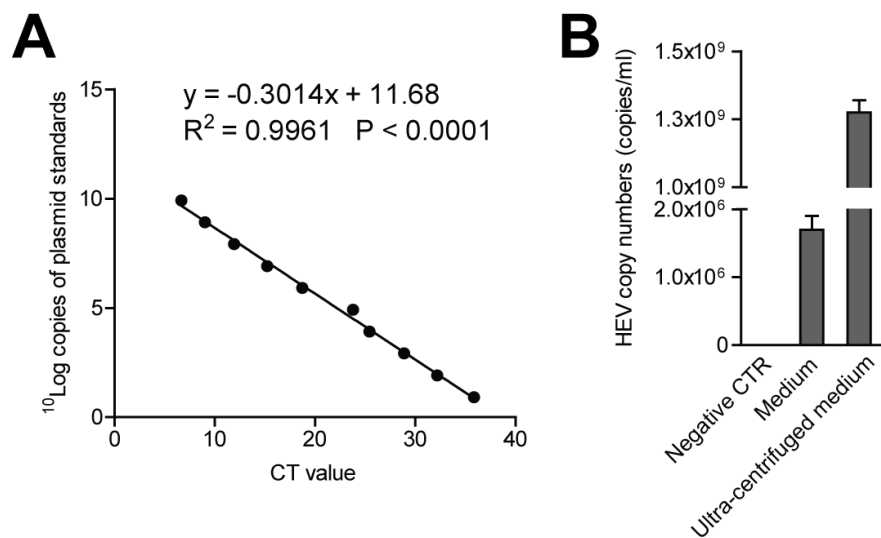
Supplementary Figures



Supplementary Figure 1. A, After differentiation, embryonic stem cell-derived neuron progenitor cells (hES-NPCs) (untransformed) as appearing under white-light microscopy. Scale bar: 100 μ m. B, After inoculation of HEV particles for 96 hrs, RT-PCR result showed that these differentiated neurons derived from hES-NPCs support HEV replication. GAPDH serves as a reference gene. One representative experiment of three is shown. C, Induced pluripotent stem (iPS) cell-derived human neurons (untransformed) were observed under white-light microscopy. Scale bar: 100 μ m. D, Time-dependent increase of HEV copy numbers secreted from the human neurons into the medium.



Supplementary Figure 2. HEV RNA electroporated to neuroglial U87 cells. Immunohistochemistry assay for HEV ORF2 in U87-HEV cells. (Scale bar, 50 μm .)



Supplementary Figure 3. HEV copy numbers of U87-HEV produced medium. A, HEV plasmid based standard curve is generated by plotting the log copy number versus the cycle threshold (CT) value. B, HEV copy numbers in supernatant of U87-HEV cells and ultra-centrifuged medium, compared with U87 cell culture medium (negative CTR).

Reference

1. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
2. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
3. Debing Y, Moradpour D, Neyts J & Gouttenoire J. Update on Hepatitis E Virology: Implications for Clinical Practice. *J Hepatol*, (2016).
4. Dalton HR, *et al.* Hepatitis E virus and neurological injury. *Nat Rev Neurol* **12**, 77-85 (2016).
5. Hewitt PE, *et al.* Hepatitis E virus in blood components: a prevalence and transmission study in southeast England. *Lancet* **384**, 1766-1773 (2014).
6. Kamar N, Marion O, Abravanel F, Izopet J & Dalton HR. Extrahepatic manifestations of hepatitis E virus. *Liver Int* **36**, 467-472 (2016).
7. Bazerbachi F, Haffar S, Garg SK & Lake JR. Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. *Gastroenterol Rep (Oxf)*, (2015).
8. Kamar N, Dalton HR, Abravanel F & Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* **27**, 116-138 (2014).
9. Kamar N, *et al.* Hepatitis E virus and neurologic disorders. *Emerg Infect Dis* **17**, 173-179 (2011).
10. Bandyopadhyay D, *et al.* Two Uncommon Causes of Guillain-Barre Syndrome: Hepatitis E and Japanese Encephalitis. *Case Rep Neurol Med* **2015**, 759495 (2015).
11. Silva M, *et al.* Hepatitis E virus infection as a direct cause of neuralgic amyotrophy. *Muscle Nerve*, (2016).
12. van Eijk JJ, *et al.* Neuralgic amyotrophy and hepatitis E virus infection. *Neurology* **82**, 498-503 (2014).
13. Willison HJ, Jacobs BC & van Doorn PA. Guillain-Barre syndrome. *Lancet*, (2016).
14. Martuza RL, Malick A, Markert JM, Ruffner KL & Coen DM. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* **252**, 854-856 (1991).
15. Kamar N, Abravanel F, Lhomme S, Rostaing L & Izopet J. Hepatitis E virus: chronic infection, extra-hepatic manifestations, and treatment. *Clin Res Hepatol Gastroenterol* **39**, 20-27 (2015).
16. Wang Y, Metselaar HJ, Peppelenbosch MP & Pan Q. Chronic hepatitis E in solid-organ transplantation: the key implications of immunosuppressants. *Curr Opin Infect Dis* **27**, 303-308 (2014).
17. Wang Y, *et al.* Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology* **146**, 1775-1783 (2014).
18. Zhou X, *et al.* Disparity of basal and therapeutically activated interferon signalling in constraining hepatitis E virus infection. *J Viral Hepat* **23**, 294-304 (2016).
19. Zhou X, *et al.* Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* **61**, 746-754 (2014).
20. Huang F, Yang C, Zhou X, Yu W & Pan Q. Rhesus macaques persistently infected with hepatitis E shed virus into urine. *J Hepatol*, (2016).
21. Woolson KL, *et al.* Extra-hepatic manifestations of autochthonous hepatitis E infection. *Aliment Pharmacol Ther* **40**, 1282-1291 (2014).

22. Kamar N, *et al.* Hepatitis E virus-induced neurological symptoms in a kidney-transplant patient with chronic hepatitis. *Am J Transplant* **10**, 1321-1324 (2010).
23. van den Berg B, *et al.* Guillain-Barre syndrome associated with preceding hepatitis E virus infection. *Neurology* **82**, 491-497 (2014).
24. Takahashi H, *et al.* A549 and PLC/PRF/5 cells can support the efficient propagation of swine and wild boar hepatitis E virus (HEV) strains: demonstration of HEV infectivity of porcine liver sold as food. *Arch Virol* **157**, 235-246 (2012).
25. Drave SA, *et al.* Extra-hepatic replication and infection of hepatitis E virus in neuronal-derived cells. *J Viral Hepat*, (2016).
26. Helsen N, *et al.* Stem cell-derived hepatocytes: A novel model for hepatitis E virus replication. *J Hepatol* **64**, 565-573 (2016).
27. Zhou X, *et al.* Requirement of the eukaryotic translation initiation factor 4F complex in hepatitis E virus replication. *Antiviral Res* **124**, 11-19 (2015).
28. Huang F, *et al.* Experimental infection of Balb/c nude mice with Hepatitis E virus. *BMC Infect Dis* **9**, 93 (2009).
29. Yu W, *et al.* Characterization of hepatitis E virus infection in tree shrew (*Tupaia belangeri chinensis*). *BMC Infect Dis* **16**, 80 (2016).
30. Han J, *et al.* SPF rabbits infected with rabbit hepatitis E virus isolate experimentally showing the chronicity of hepatitis. *PLoS One* **9**, e99861 (2014).
31. Li W, *et al.* Experimental infection of Mongolian gerbils by a genotype 4 strain of swine hepatitis E virus. *J Med Virol* **81**, 1591-1596 (2009).
32. Geng Y, *et al.* Detection and assessment of infectivity of hepatitis E virus in urine. *J Hepatol* **64**, 37-43 (2016).
33. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
34. Dalton HR, Bendall RP, Keane FE, Tedder RS & Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. *N Engl J Med* **361**, 1025-1027 (2009).
35. Dalton HR, Keane FE, Bendall R, Mathew J & Ijaz S. Treatment of chronic hepatitis E in a patient with HIV infection. *Ann Intern Med* **155**, 479-480 (2011).
36. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
37. de Esch CE, *et al.* Epigenetic characterization of the FMR1 promoter in induced pluripotent stem cells from human fibroblasts carrying an unmethylated full mutation. *Stem Cell Reports* **3**, 548-555 (2014).

Part III.

Anti-viral therapy

Chapter 7

Disparity of basal and therapeutically activated interferon signaling in constraining hepatitis E virus infection

Xinying Zhou¹, Lei Xu¹, Wenshi Wang¹, Koichi Watashi², Yijin Wang¹, Dave Sprengers¹,
Petra E. de Ruiter³, Luc J.W. van der Laan³, Herold J. Metselaar¹, Nassim Kamar^{4, 5, 6},
Maikel P. Peppelenbosch¹, and Qiuwei Pan¹

¹Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands; ²Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan; ³Department of Surgery, Erasmus MC-University Medical Center and Postgraduate School Molecular Medicine, Rotterdam, Netherlands; ⁴Department of Nephrology and Organ Transplantation, CHU Rangueil, TSA, Toulouse cedex 9, France; ⁵INSERM U1043, IFR-BMT, CHU Purpan, Toulouse, France; ⁶Université Paul Sabatier, Toulouse, France

Abstract

Hepatitis E virus (HEV) represents one of the foremost causes of acute hepatitis globally. Although there is no proven medication for hepatitis E, pegylated Interferon- α (IFN- α) has been used as off-label drug for treating HEV. However, the efficacy and molecular mechanisms of how IFN signaling interacts with HEV remain undefined. As IFN- α has been approved for treating chronic hepatitis C for decades and the role of interferon signaling has been well-studied in hepatitis C virus (HCV) infection, this study aimed to comprehensively investigate virus-host interactions in HEV infection with focusing on the IFN signaling, in comparison to HCV infection. A comprehensive screen of human cytokines and chemokines revealed that IFN- α was the sole humoral factor inhibiting HEV replication. IFN- α treatment exerted a rapid and potent antiviral activity against HCV; whereas it had moderate and delayed anti-HEV effects *in vitro* and in patients. Surprisingly, blocking the basal IFN pathway by inhibiting JAK1 to phosphorylate STAT1 has resulted in drastic facilitation of HEV, but not HCV infection. Gene silencing of the key components of JAK-STAT cascade of the IFN signaling, including JAK1, STAT1 and interferon regulatory factor 9 (IRF9) stimulated HEV infection. In conclusion, compared to HCV, HEV is less sensitive to IFN treatment. In contrast, the basal IFN cascade could effectively restrict HEV infection. This bears significant implications in management of HEV patients and future therapeutic development.

Introduction

As an emerging infectious pathogen, hepatitis E virus (HEV) represents the most common cause of acute viral hepatitis ¹. Outbreaks of hepatitis E occur periodically throughout the developing world, which often cause fulminant hepatitis with high mortality (reaching 25%) in the case of pregnant women ². In the industrialized countries, HEV usually only causes an acute and self-limiting infection, it however bears a high risk of developing chronic hepatitis in immunocompromised patients with substantial mortality rates ³. Thus, it is important to understand the underlying mechanisms of the distinct infection courses of different populations upon exposure to HEV. For severely affected patients, there is an urgent need for developing optimal therapies, because no proven antiviral medication is available for hepatitis E.

Cytokines induced by viral infection play a key role in host defense against the infection ⁴, but their roles in anti-HEV immunity remain largely obscure. HEV provokes the production of a panel of inflammatory cytokines and chemokines with considerable higher levels in experimental models and in patients ⁴⁻⁷. However, the action of these HEV-induced cytokines on the life-cycle and pathogenesis of HEV infection remain unknown. Interferons (IFNs), pleiotropic cytokines, are of vital importance for the innate defense against viral infection ⁸. They are grouped into three classes including type I, II and III, which bind to distinct receptors to stimulate their antiviral responses in host cells ⁹. Pegylated IFN- α in combination with ribavirin was considered the standard antiviral therapy for chronic hepatitis C virus (HCV) infection ¹⁰. In addition, type III IFN-lambdas (IFN- λ s), which appear to have less side effects than IFN- α , are currently clinically evaluated for the treatment of chronic hepatitis C ^{11, 12}. Although Pegylated IFN- α , ribavirin or the combination have already been used to treat individual cases or small case series of HEV infection as off-label drugs ¹³⁻¹⁵, their mechanism-of-actions in the setting of HEV remain poorly investigated.

Intriguingly, basal JAK-STAT signaling in the absence of exogenous IFN stimulation could already serve as a first line of intracellular antiviral defense ¹⁶. In fact, the seroprevalence of HEV is substantially high in both developing and developed countries; whereas majority healthy people have only asymptomatic or self-limiting acute infection. Thus, humans clearly have powerful defense mechanisms against HEV. It is of an intriguing

question whether basal IFN signaling plays a crucial role in the process of HEV resistance or self-limiting.

The absence of robust cell culture models has hampered fundamental and translational research of HEV. Fortunately, subgenomic and infectious models for HEV infection have come available ¹⁷ and such models were recently used in studying the infection biology and assessing potential antivirals ¹⁸⁻²⁰. In this study, we comprehensively characterized the role of cytokines in regulating HEV infection. IFN- α was found the sole humoral factor provoking inhibition of HEV replication. Further investigation identified the essential role of basal IFN signaling and the key components of JAK-STAT cascades in protecting against HEV replication. These results revealed distinct mode-of-actions of basal and treatment activated IFN signaling in controlling HEV infection.

Materials and methods

Patients

Chronic HEV patients treated with pegylated IFN- α monotherapy at CHU Rangueil, Toulouse, France were selected and 4 cases were identified. Patient 1, 2 and 3 were immunocompromised patients with liver transplantation and patient 4 had hematologic disease. HEV viral kinetics was analyzed in these four patients by retrieving HEV RNA titers (log copies/mL) at day 0, 1, 3, 7, 15, 21 and 30 post-treatment. The study was approved by the institutional review boards of Toulouse Hospital, and all of the patients presented their written informed consent to participate in this study.

Reagents

Cytokines and chemokines (PeproTech or R&D Systems) were used for screening. Human IFNs (Thermo Scientific, Netherlands) were dissolved in culture medium. Stocks of Jak inhibitor I (Santa Cruz Biotech, Santa Cruz, CA) was dissolved in DMSO (Sigma-Aldrich, St Louis, MO) with a final concentration of 5 mg /mL. Stocks of AG-490 and CP 690550 were dissolved in DMSO with a final concentration of 10 mg /mL. Antibodies including phospho-STAT1, total STAT1, phosphor-JAK1, interferon stimulated factor 9 (IRF9) (Cell Signalling Technology, Netherlands) and β -actin (Santa Cruz Biotech, Santa Cruz, CA); anti-rabbit or

anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were also used.

Cell culture models

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-Luc), using the Ambion MESSAGE MACHINE *in vitro* RNA transcription Kit (Life Technologies Corporation) ^{17,21}. The human hepatoma 7 (Huh7) cells were collected and centrifuged for 5 min, 1500 rpm, 4°C. Supernatant was removed and washed with 4 mL Opti-MEM by centrifuging for 5 min, 1500 rpm, 4°C. The cell pellet was re-suspended in 100 µl Opti-MEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 volt, pulse length 0.5, number 1 and cuvette 4 mm) ¹⁷. Huh7-ET replicon was based on Huh7 cells containing a subgenomic HCV bicistronic replicon (1389/NS3-3V/LucUbiNeo-ET) and maintained with 250 µg/ml G418. Huh7 cells harboring the full-length JFH1-derived genome was used as an infectious HCV model ²².

Gene knockdown and overexpression by lentiviral vector

Lentiviral vectors (Sigma-Aldrich) targeting JAK1, STAT1, IRF9 or GFP control were obtained from the Erasmus Center for Biomimics and produced in HEK 293T cells as previously described ²³. After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences were described in Supplementary Table 1. To generate gene knockdown or overexpression cells, Huh7 cells were transduced with lentiviral vectors. Since the knockdown vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 µg/ml puromycin (Sigma) to the cell culture medium.

Measurement of luciferase activity

For Gaussia luciferase, the activity of secreted luciferase in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30

min at 37 °C. Both gaussia and firefly Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates and the cells grew at 37 °C with 5% CO₂ for 3 hrs. The medium was removed and 100 µl of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm. All measurements were performed in triplicates.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA was quantified with a SYBR Green-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH was considered as reference gene to normalize gene expression. Human primer sequences were included in Supplementary Table 2.

Western blot assay

Proteins in cell lysates were heated 5 min at 95 °C followed by loading onto sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis. Proteins were electrophoretically transferred onto PVDF membrane (Invitrogen) for 1.5 hrs with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer. It was followed by incubation with rabbit p-JAK1, p-STAT1, t-STAT1, IRF9 (1:1000) antibodies overnight at 4 °C. The membrane was washed 3 times and incubated for 1 hrs with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000) at room temperature. Blots were scanned and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantitated with Odyssey 3.0 software.

Statistical analysis

All results were presented as mean \pm SEM. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a p value less than 0.05 *or 0.01 **.

Results

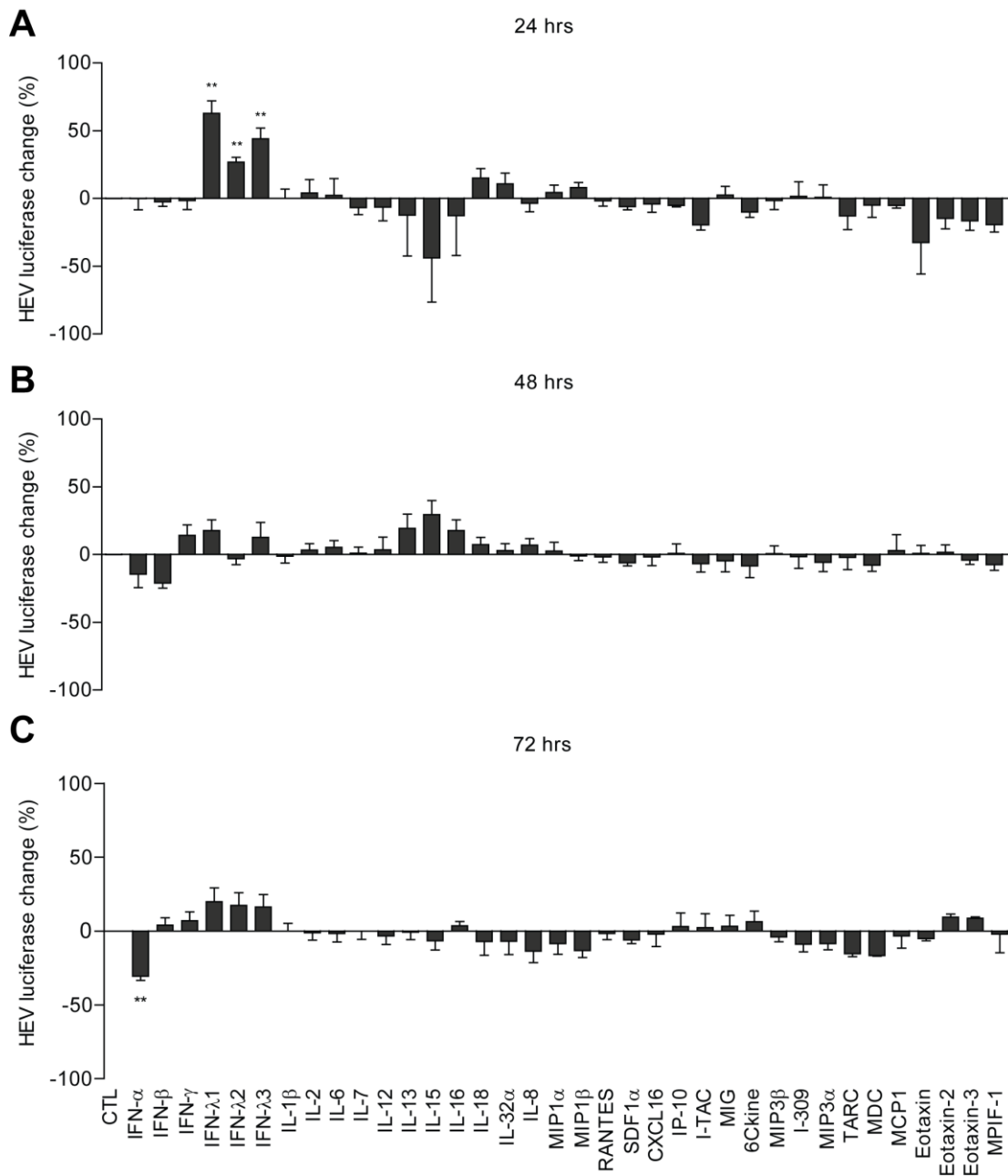


Figure 1. HEV replication was insensitive to the regulation of cytokines and chemokines. Huh7-p6-Luc cells were treated with cytokines at 100 ng/ml (except for IFN- α and IFN- β at 100 IU /mL) for 24 (A), 48 (B) and 72 hrs (C), and then luciferase activity was measured. (Mean \pm SEM, n = 4). ** $P < 0.01$.

HEV replication is insensitive to the regulation of cytokines and chemokines

Viral infections often induce various cytokines and chemokines that in turn modulate the infection course²⁴. We thus investigated the effects of a panel of cytokines and chemokines on host susceptibility to HEV infection. To this end, we employed cell culture model of human hepatoma cells (Huh7 cell line) transfected with subgenomic construct of HEV coding sequence in which the 5' portion of ORF-2 was replaced with the in-frame *gaussia* luciferase reporter (p6-Luc)¹⁷. In parallel, Huh7 cells constitutively expressing a non-secreted firefly luciferase are used for normalization of non-specific effects on luciferase signals^{18, 20}. Exploiting this experimental system, 36 cytokines and chemokines, including type I, II and III IFNs, interleukins and others, were examined in the HEV replicon, culture medium served as control. MTT assay has shown that these cytokines/chemokines in general did not exert cytotoxicity to host cells (Supplementary Figure 1). However, except for some forms of IFNs, other cytokines and chemokines had no significant effects on HEV replication (Figure 1). Hence, HEV replication is in general insensitive to the regulation of cytokines and chemokines, except for type I interferons, suggesting that antiviral immunity to HEV is mediated through a very specific branch of our immune system.

HEV, compared to HCV, is less sensitive to IFN- α treatment

Based on the results of our profiling of humoral factors involved in anti-HEV immunity, we further investigated the action of IFN- α , the archetypical type I IFN that has been successfully used to treat chronic HCV in the clinic for decades²⁵. As expected, treatment of IFN- α resulted in robust inhibition of HCV replication in the Huh7-based subgenomic replicon containing a luciferase reporter (Figure 2A), suggesting that our model system can reflect clinically relevant processes. High dose of IFN- α (10-1000 IU/mL) almost completely suppressed HCV replication as early as after 24 hrs treatment. In contrast, the action of IFN- α , although evidently present, was much less effective in suppressing HEV replication in a similar subgenomic replicon that also contains a luciferase reporter. Moderate inhibitory effects were observed only after 72 hrs treatment with relatively high concentrations (100 IU/mL: 31% \pm 8, mean \pm SEM, n = 3 independent experiments with each 2-3 replicates, P < 0.01; 1000 IU/mL: 41% \pm 3, mean \pm SEM, n = 3 independent experiments with each 2-3 replicates, P < 0.01) (Figure 2B). Consistently, in the full-length HCV and HEV infectious

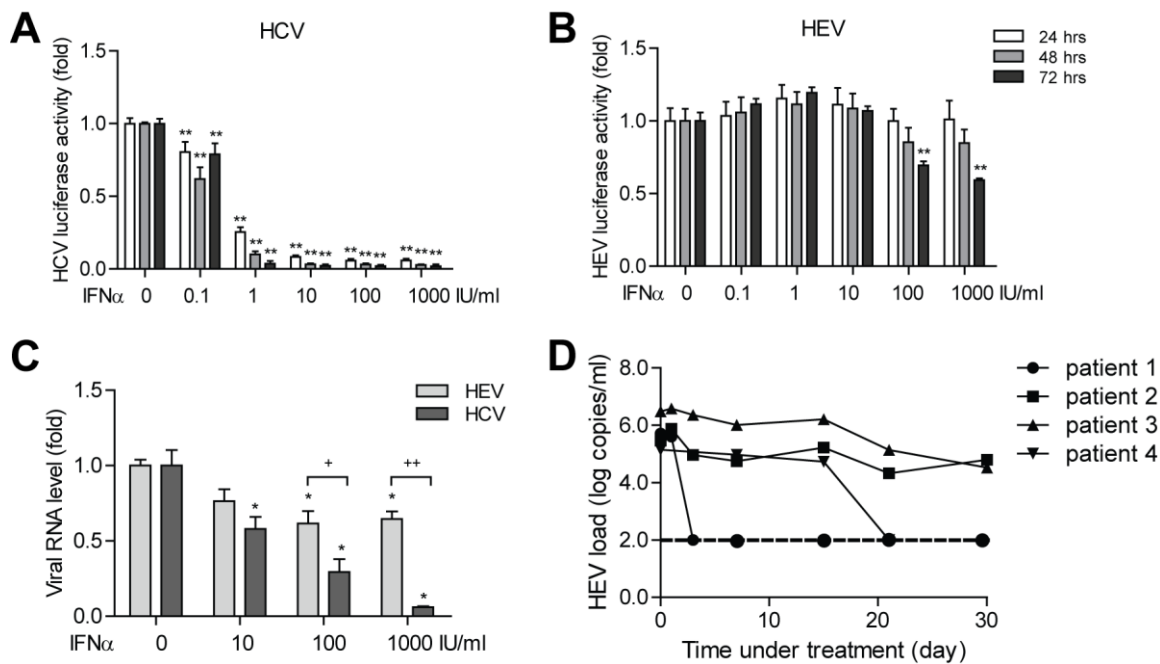


Figure 2. IFN- α exerted moderate but delayed antiviral activity against HEV. (A) In the Huh7 cell-based subgenomic HCV replicon, treatment with IFN- α dose-dependently decreased viral replication-related luciferase activity. (Mean \pm SEM, $n = 3$ independent experiments with each 2-3 replicates). (B) In the Huh7 cell-based subgenomic HEV replicon, treatment with IFN- α moderately inhibited viral replication-related luciferase activity (Mean \pm SEM, $n = 3$ independent experiments with each 2-3 replicates). (C) In the full-length HEV and HCV infectious model, IFN- α significantly decreased cellular viral RNA at 48 hrs determined by qRT-PCR (Mean \pm SEM, $n = 5$). Significant differences between level of IFN- α antiviral effects were observed. (D) Four chronic HEV patients were treated with monotherapy of IFN- α and the viral load were analyzed within 30 days. Treatment time was indicated as 24, 48 or 72 hrs * or + $P < 0.05$; ** or ++ $P < 0.01$.

models, IFN- α had significantly more potent antiviral effects against HCV than HEV (Figure 2C). In apparent agreement, whereas it is well-known that chronic HCV patients who respond to IFN- α therapy often experience a rapid and sharp reduction of viral load within the first few days upon treatment^{26, 27}, we observed that three out of four chronic HEV patients had only minor fluctuation of viral load within first two weeks of pegylated IFN- α treatment, although all the patients eventually cleared the virus (Figure 2D). These results indicate that IFN- α exerts a moderate and delayed antiviral activity against HEV, in contrast to the rapid and potent effect of this cytokine against HCV, which suggests that their underlying antiviral mechanisms differ.

The notion that anti-viral activity of IFN- α towards HEV is mechanistically different from that against HCV was further supported by experiments in which the anti-viral effect of alternative member of the family of type I, II and III IFNs were investigated. IFN- β and IFN- γ

effectively inhibits HCV replication in the replicon model, but did not exert a significant effect on HEV replication (Figure 3A and 3B). IFN- λ is currently under clinical investigation for treating chronic HCV patients and has been shown to possess good anti-HCV antiviral activity but with fewer adverse events as compared to IFN- α ¹². In HCV replicon, IFN- λ 1, IFN- λ 2 and IFN- λ 3 showed significant inhibition on viral replication. Unexpectedly, high dose of IFN- λ 1, IFN- λ 2 and IFN- λ 3 even significantly enhanced viral replication in the HEV replicon after 24 hrs treatment, although the effects were mild (Figure 3C-E). Thus, the anti-HEV activity of type I, II and III IFNs appears mechanistically distinct from that against HCV (Supplementary Figure 2).

HEV replication is sensitive to basal IFN signaling

Although humoral factors do not confer protection action against HEV replication, the only partial exception being IFN- α (Figure 2 and 3), humans appear to have powerful defence mechanisms combating HEV¹⁸, raising questions as to the nature of these mechanisms. The partial activity of IFN- α against HEV replication led us hypothesises that signaling elements, in particular JAK-STAT cascades, involved in IFN- α signal transduction might contribute to anti-HEV defence. Constitutive JAK-STAT signaling is an essential part of the innate immunity for host defence against viruses¹⁶. To investigate how the endogenous JAK-STAT signaling affects HEV replication, JAK inhibitor I that is known to predominantly inhibit JAK1 (but may also inhibit JAK2 and JAK3) was first tested. As expected, it can effectively inhibit its downstream target, the phosphorylation of STAT1, induced by treatment of IFN- α or IFN- λ (Supplementary Figure 3A). Consistently, it significantly inhibited the stimulation of the IFN response reporter, IFN-stimulated response elements (ISRE)-luciferase transcription reporter (Supplementary Figure 3B). Accordingly, IFN- α triggered induction of ISGs, as represented by four important members RSAD2, ISG15, OAS1 and PKR, were blocked by this inhibitor (Supplementary Figure 3C).

Importantly, treatment of JAK inhibitor I dramatically elevated HEV replication in both subgenomic replicon (Figure 4A) and the infectious model (Figure 4B). Treatment with 10 μ M for 72 hrs, increased HEV replication-related luciferase activity by 3.02 ± 0.50 -fold (mean \pm SEM, $n = 3$ independent experiments with each 3 replicates, $P < 0.01$) in the replicon (Figure 4A). Similarly, treatment with 10 μ M for 48 hrs, it increased viral RNA by 4.78 ± 1.13 -fold (mean \pm SEM, $n = 3$, $P < 0.05$) in the infectious model (Figure 4B). In contrast, JAK

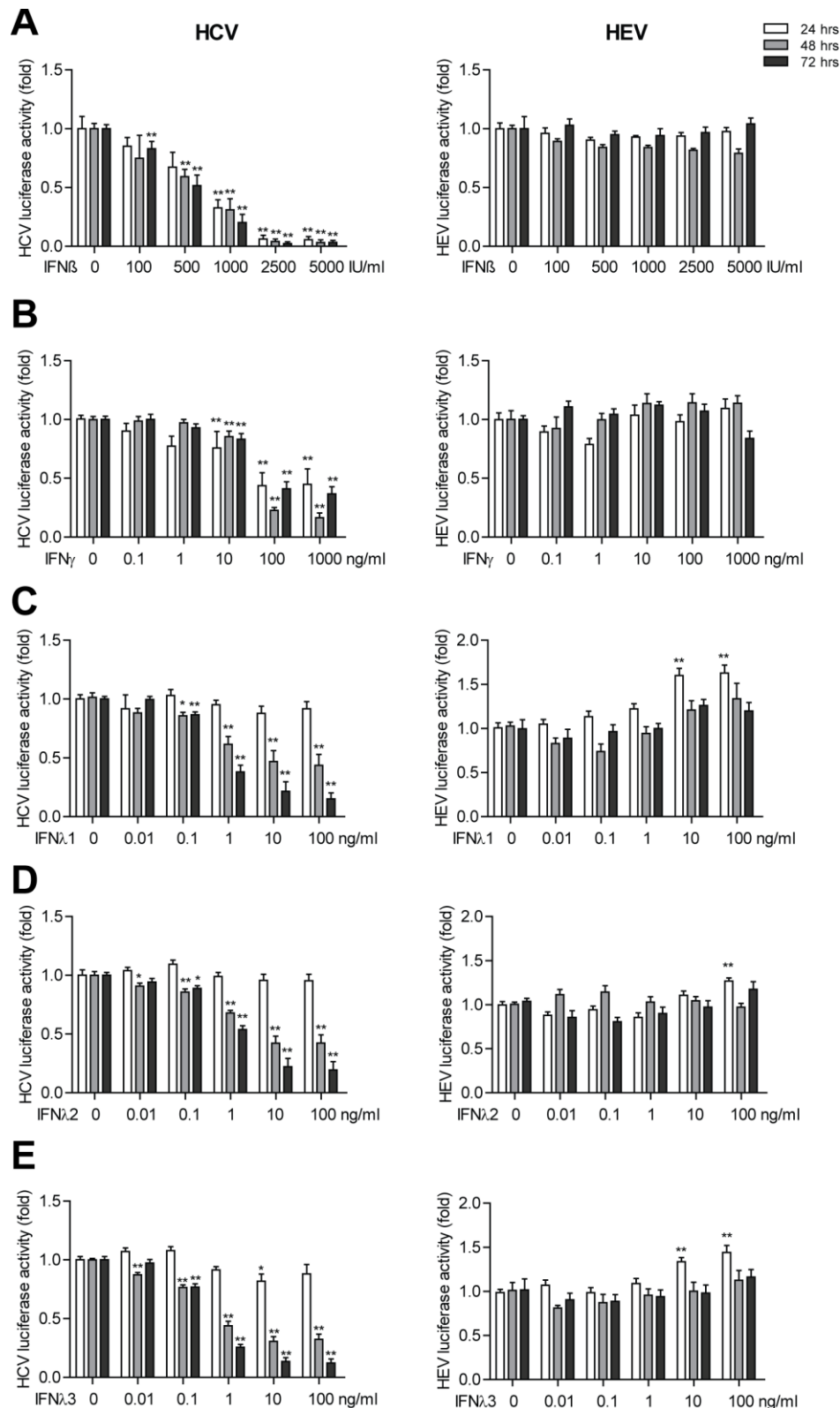


Figure 3. IFN- β , γ and λ s had no significant antiviral activity against HEV infection. In the Huh7 cell-based subgenomic HCV and HEV replicon, viral replication-related luciferase activity determined after dose-dependently treatments with IFN- β (A), IFN- γ (B), IFN- λ 1 (C), IFN- λ 2 (D) and IFN- λ 3 (E) (Mean \pm SEM, $n = 3$ independent experiments with each 3-4 replicates). Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.

did have notable effects on cell growth determined by MTT assay (Supplementary Figure 3D). Thus, the basal JAK-STAT pathway could effectively limit HEV infection, although exogenous IFN is ineffective, whereas it is *vice versa* for HCV.

Key components of JAK-STAT cascades restrict HEV infection

Upon binding of IFN- α to its receptor, JAK1 is stimulated, resulting in tyrosine phosphorylation of STAT1 and STAT2. This is followed by the formation and nuclear translocation of the STAT1-STAT2-IRF9, a transcription factor complex known as IFN-stimulated gene factor 3 (ISGF3), which in turn combines to the ISREs in the genome DNA and subsequently drives transcription of ISGs to establish an antiviral status (Figure 5A) ²⁸. Specific inhibition of the JAK/STAT signaling pathway has been reported to modulate HCV replication ²⁹.

To evaluate the direct effects of the key components of JAK-STAT pathway including JAK1, STAT1 and IRF9 on HEV infection, Huh7 cells were transduced with integrating lentiviral vectors expressing shRNA specifically targeting JAK1, STAT1, IRF9 or GFP as a control. For JAK1 knockdown cells, two of the tested three shRNA vectors targeting JAK1 exert potent gene silencing capacity, resulted in a profound down-regulation of JAK1 mRNA level (Figure 5B). Correspondingly, JAK1 silencing led to significant increase of cellular HEV RNA level, which was measured by qRT-PCR after inoculation of HEV particles for 72 hrs. For instance, knockdown of shRNA clone 2 in JAK1 led to 2.47 ± 0.66 -fold increase of HEV viral RNA (mean \pm SEM, $n = 4$, $P < 0.05$) (Figure 5B). Furthermore, STAT1, which is phosphorylated by JAK1, was successfully silenced by shRNA targeting STAT1 in Huh7 cells with a potent reduction in protein level in shSTAT1 cells (Figure 5C). Consistently, STAT1 silencing could also significantly potentiated cellular HEV RNA level to 1.6 ± 0.13 -fold (mean \pm SEM, $n = 3$, $P < 0.05$) (Figure 5C). To further determine whether event downstream of JAK-STAT cascades influences HEV replication, IRF9 was silenced with a profound down-regulation of total IRF9 protein level (Figure 5D). Similarly, silencing of IRF9 resulted in 3.70 ± 1.07 -fold (mean \pm SEM, $n = 4$, $P < 0.05$) elevation of cellular HEV RNA level. Therefore, key constituents of JAK-STAT cascades including JAK1, STAT1 and IRF9 play an intrinsic role in restricting HEV infection.

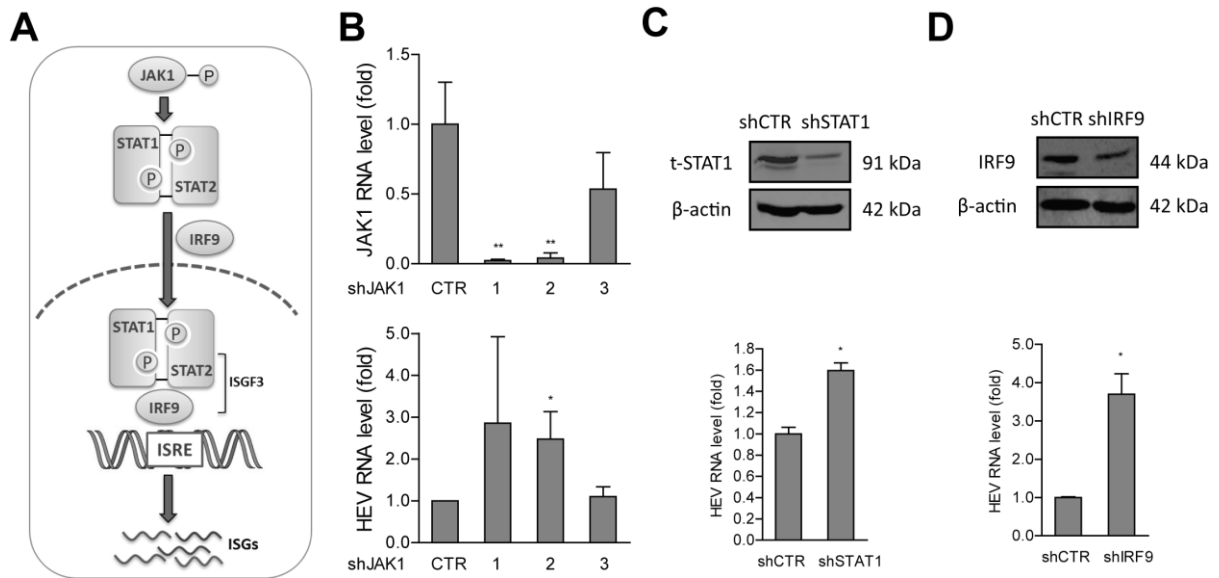


Figure 5. Key components of JAK-STAT cascades restrict HEV infection (A) Key components in interferon JAK-STAT signaling. (B) Knockdown of JAK1 by lentiviral shRNA vectors. Compared with the control vector transduced cells, the shJAK1 clone 1 and 2 exhibit potent silencing capability shown at mRNA level (Mean \pm SEM, $n = 4$). Correspondingly, knockdown of JAK1 of clone 2 resulted in significant increase of cellular HEV RNA level (Mean \pm SEM, $n = 4$). (C) Knockdown of STAT1 by lentiviral shRNA vectors. Western blot assay presented a potent decrease of total STAT1 protein level, correspondingly, silencing of STAT1 led to significant increase of cellular HEV RNA level (Mean \pm SEM, $n = 4$). (D) Knockdown of IRF9 by lentiviral shRNA vectors. Western blot assay presented a potent decrease of total IRF9 protein level, similarly, silencing of IRF9 led to significant increase of cellular HEV RNA level (Mean \pm SEM, $n = 4$). Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.

Discussion

Cytokines and chemokines are important components of the immune response for countering invading viruses³⁰. With this group of mediators, especially IFNs play a cardinal role, exerting a wide range of pleiotropic effects²⁵. Accordingly, IFN- α has become the mainstay for the treatment of chronic HCV and HBV infection, despite the considerable systemic side effects. Whereas IFN- λ shows a potent anti-viral activity as well in phase III clinical trial for treating HCV patients with much less reverse side effects, owing to the tissue-restricted expression of type III IFN receptor³¹. Hitherto, there is no approved medication for hepatitis E. Lessons have learned from standard therapy of chronic hepatitis C that IFN- α , ribavirin, or a combination as off-label drugs have been used to treat individual HEV cases or small case series³. As shown in this study (Figure 2D), four chronic hepatitis E

patients all eventually cleared the virus after monotherapy of pegylated IFN- α . However, the viral kinetics in responding to IFN- α is very different between chronic hepatitis C and E. In chronic HCV, patients who respond to the therapy often experience a rapid and sharp reduction of viral load within the first few days upon initiation of IFN- α therapy^{26, 27, 32}. In contrast, only one out of four chronic HEV patients had a rapid decline of viral load; whereas others had rather minor changes in the first two weeks of treatment. This is in line with our experimental results that HEV has a moderate and delayed responsiveness to IFN- α , compared with HCV in cell culture models. The HEV subgenomic model only mimics viral replication; whereas the HEV infectious cell culture system models the complete life-cycle of HEV infection. IFN- α probably interferes HEV infection at various steps. Thus, the infectious clone appears more sensitive to IFN- α compared to the subgenomic replicon (Figure 2A-C). Other types of IFNs, including IFN- λ , did not shown any notable antiviral activity against HEV (Figure 3).

Previous studies have provided evidence of counteracting IFN response by HEV³³. In our study, we also observed that HEV inoculation could indeed inhibit IFN- α induced phosphorylation of STAT1 and attenuate the induction of ISGs in Huh7 cells (Supplementary Figure 4C and 4D). Nevertheless, this inhibitory effect was rather minor, which may only partially contribute to the resistance of HEV to interferon treatment. Thus, the insensitivity and resistance of HEV to IFN treatments may be because of the intrinsic characteristics and pathogenesis of HEV itself. Therefore, the scenario of developing IFN-based treatment against HEV shall be carefully re-considered. Interestingly, a recent large retrospective, multicentre study showed that ribavirin as monotherapy could be very effective for treating chronic HEV infection that viral clearance was observed in the majority of patients³⁴. In contrast, ribavirin monotherapy hardly has detectable effect on HCV viral load reduction^{35, 36}. Only when combined with IFN- α , it doubles the response rate, compared with IFN- α alone³⁷.

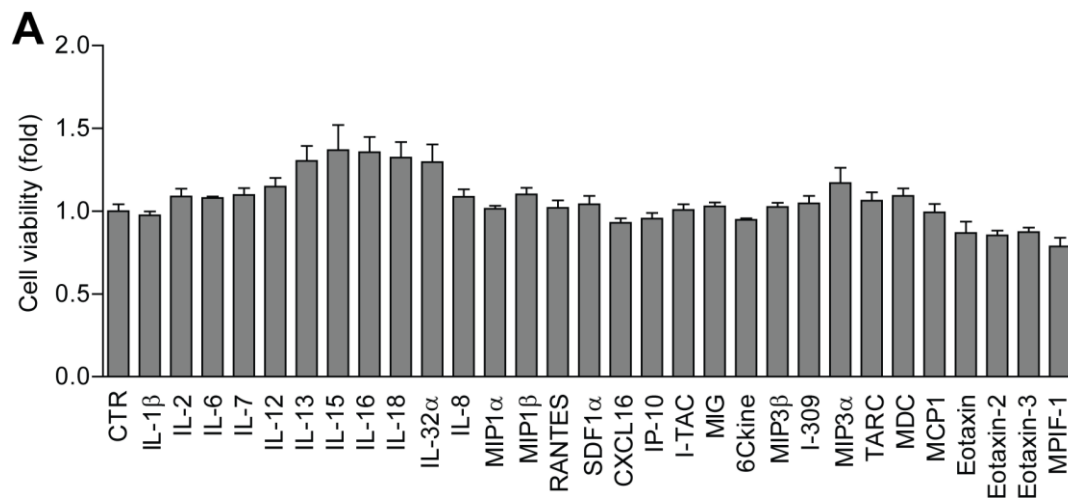
Despite the inferiority of HEV in responding to exogenous IFN treatment, we in fact demonstrated a superior function of the basal interferon signalling, which mediates IFN- α , γ and λ signalling transduction, in protecting against HEV infection. Pharmacological inhibition of JAK1, the key upstream kinase controlling JAK-STAT cascades of the IFN pathway, has resulted in drastic facilitation of HEV but not HCV infection in cell culture models (Figure 4A-C). Consistently, by gene silencing of JAK1, STAT1 and IRF9, key factors of JAK-STAT cascades,

HEV replication was efficiently potentiated (Figure 5B-D). These results highlighted the importance and mechanisms of basal IFN signaling in protecting HEV infection.

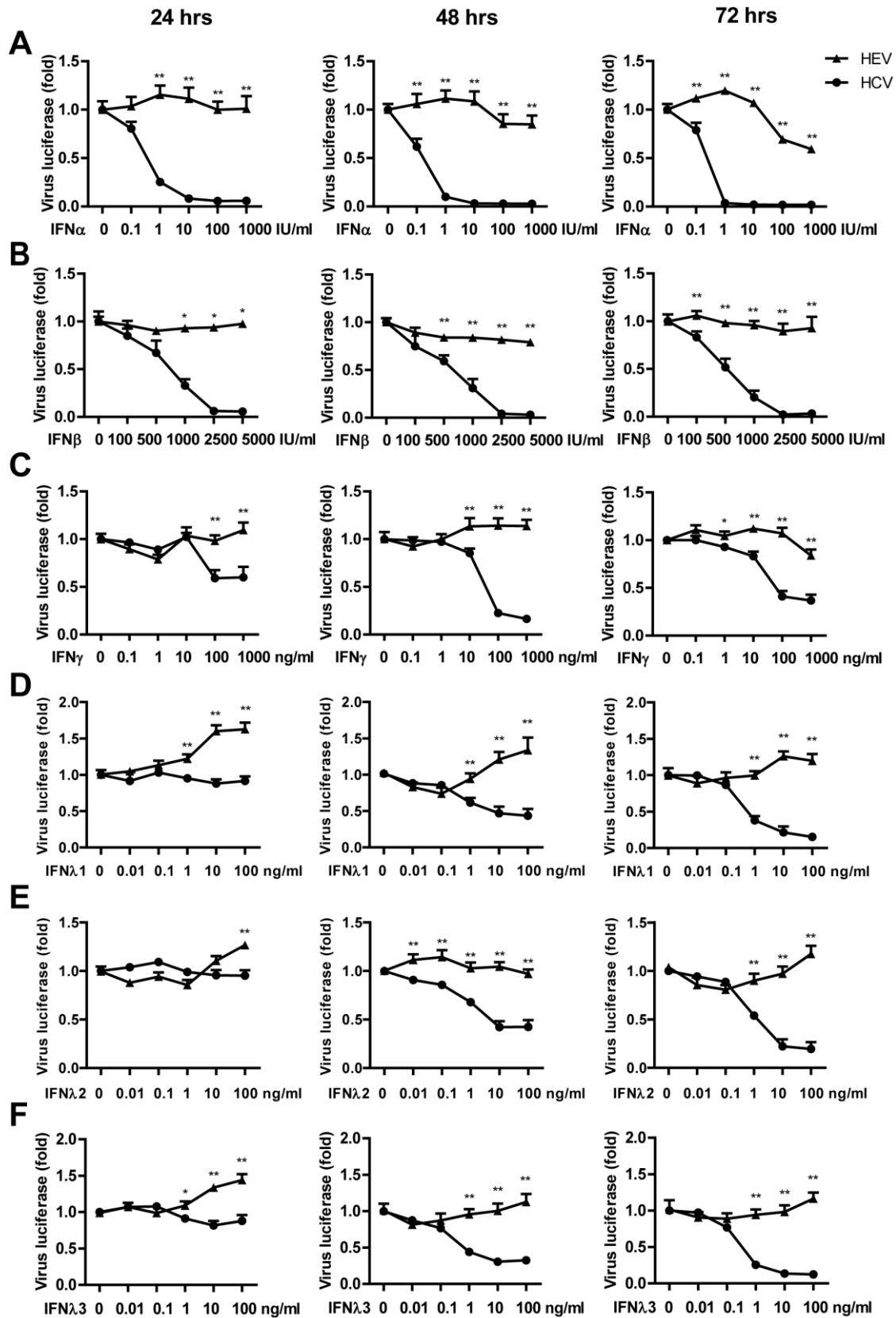
This probably at least partially explains the distinct incidence of chronic development upon exposure to HEV or HCV. In general population, HEV is mostly acute and self-limiting, except in very young children, pregnant women and immunocompromised patients that could cause severe diseases ¹. Chronic hepatitis E has so far only been reported in immunocompromised patients who are lacking of adequate immune defence power ^{20, 38}. However, 55% to 85% individuals infected with HCV are not able to clear the virus, but develop chronic hepatitis C ³⁹. Even an activated endogenous IFN response is ineffective in eradicating HCV, once chronic infection is established ⁴⁰. The only option to cure chronic HCV is antiviral treatment, including IFN-based therapies. The fact that one-third of organ transplantation patients with chronic HEV could achieve virus clearance even without any treatment, but with dose reduction of immunosuppressants ³⁸, suggests the indispensable role of endogenous innate immunity in HEV recovery. In addition, during the early stage of HEV infection, patients always remain subclinical and represent no apparent symptom at all. This rapidly turn to a self-limiting illness with spontaneously cleaning of HEV and need no specific treatment ⁴¹. The phenomenon of quick clearance of HEV infection by body itself without any treatment may be associated to the critical role of defence mechanism of basal IFN signaling in combating HEV infection. Therefore, the data in current study delineate that endogenous JAK-STAT signaling exerts much potency in protecting against HEV than HCV infection. The different sensitivities between HCV and HEV to endogenous IFN signaling may also determine the distinct clinical course and outcome between hepatitis C and hepatitis E patients.

In conclusion, we revealed that HEV is in general insensitive to the regulation of cytokines and chemokines. IFN- α treatment exerts moderate but delayed antiviral activity against HEV infection in experimental models and in patients, which suggested the ineffectiveness of interferon-based monotherapy in treating chronic hepatitis E. Interestingly, blocking the basal IFN pathway resulted in drastic facilitation of HEV infection, suggesting that basal IFN pathway can effectively protect against HEV infection. Thus, this study has shed new light on the molecular insight of HEV-host interaction, in particular the role of therapeutically activated and the basal IFN signaling. This bears significant implications in management of HEV patients and future therapeutic development.

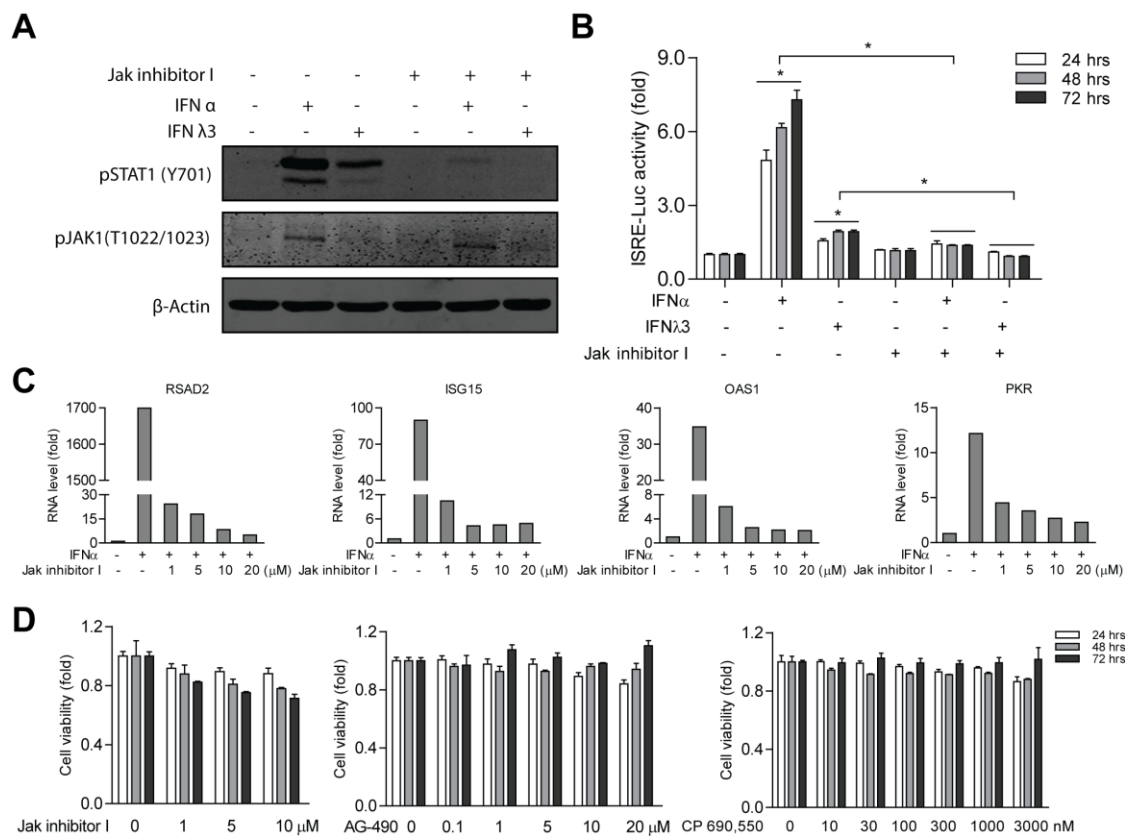
Supplementary Figures and Tables



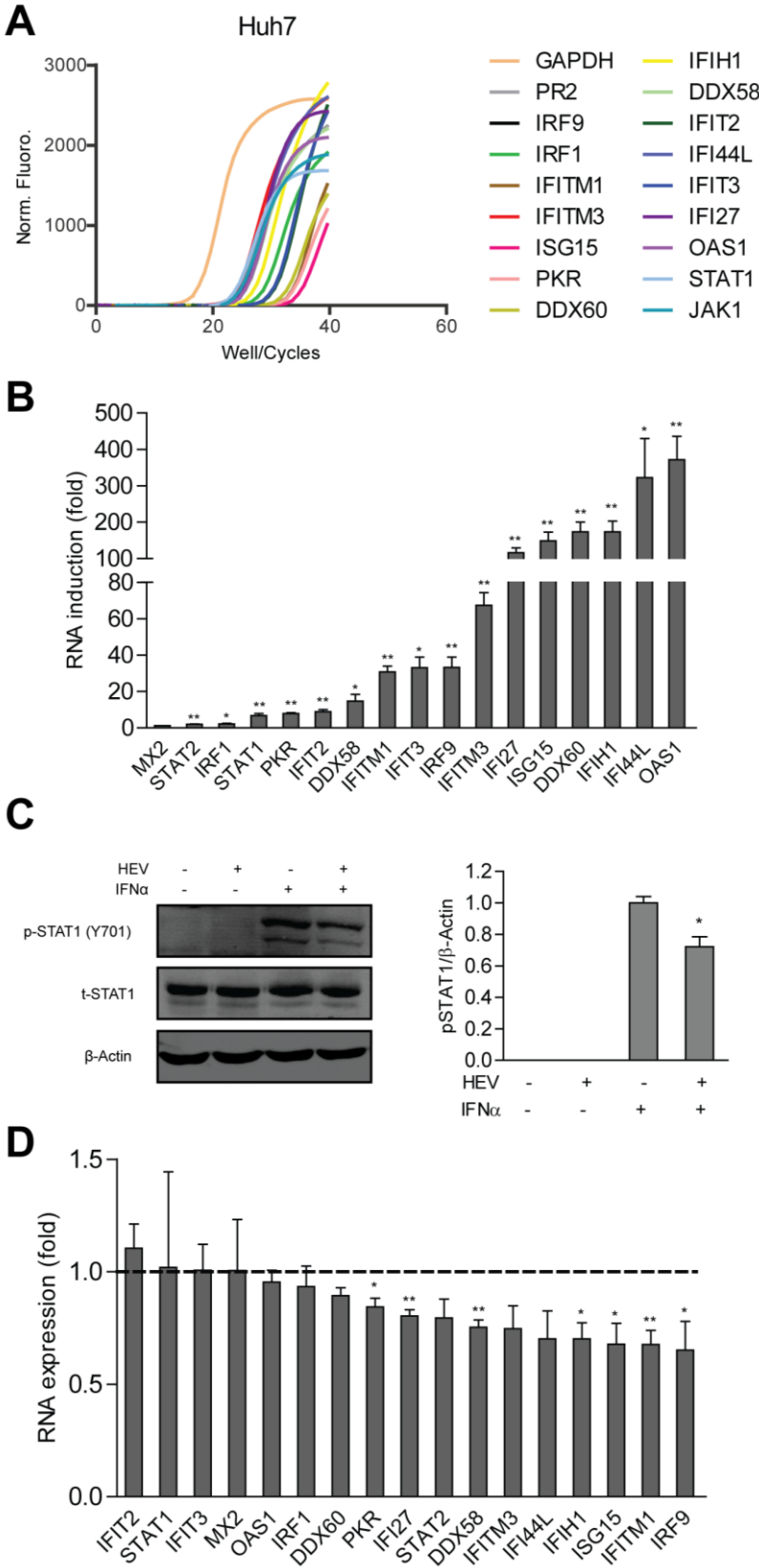
Supplementary Figure 1. Cytokines/chemokines did not affect cell proliferation at 72 hrs determined by MTT assay (OD_{490} value) (Mean \pm SD, $n = 4$).



Supplementary Figure 2. (A) IFN- α , (B) IFN- β , (C) IFN- γ , (D) IFN- λ 1, (E) IFN- λ 2 and (F) IFN- λ 3 had significantly more potent antiviral effects against HCV than HEV in the subgenomic replication models. Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.



Supplementary Figure 3. (A) Western blot showed treatment of Jak inhibitor I for 48 hrs effectively inhibited downstream target of JAK1, the phosphorylation of STAT1, induced by treatment of IFN- α or IFN- λ for 30 min. β -actin served as an internal reference. (B) Jak inhibitor I significantly inhibited the stimulation of the IFN response reporter, the ISRE-luciferase transcription reporter by IFN- α or IFN- λ for 30 min. (Mean \pm SD, $n = 4$). (C) Jak inhibitor I blocked the induction ISGs of RSAD2, ISG15, OAS1 and PKR triggered by IFN- α . (D) Jak inhibitor I, AG-490 and CP 690550 did not affect cell proliferation determined by MTT assay (OD_{490} value) (Mean \pm SD, $n = 3$). Treatment time was indicated as 24, 48 or 72 hrs. * $P < 0.05$; ** $P < 0.01$.



Supplementary Figure 4. (A) The basal expression of 16 ISGs was determined in naïve Huh7 cells by qRT-PCR. (B) The expression of 16 ISGs were significantly stimulated by treatment of 1000 IU/ml IFN- α for 24 hrs (Mean \pm SD, $n = 4$). (C) Western blot assay showed that the elevation of p-STAT1 level was significantly attenuated upon 1000 IU/ml IFN- α in HEV infected compared to naïve Huh7 cells for 48 hrs. β -actin served as an internal reference. (Mean \pm SD, $n = 3$). (D) Inoculation of HEV moderately inhibited the stimulation of most of 16 tested ISGs upon treatment with 1000 IU/ml IFN- α for 24 hrs (Mean \pm SD, $n = 4-6$). * $P < 0.05$; ** $P < 0.01$.

Supplementary Table 1. shRNA sequences.

shJAK1-1	CCGGGAGACTTCCATGTTACTGATTCTCGAGAATCAGTAACATGGAAGTCTCTTTTT
shJAK1-2	CCGGGACAGTCACAAGACTTGTGAACCTCGAGTTCACAAGTCTTGTGACTGTCTTTTTG
shJAK1-3	CCGGCGAGATCTTAAGGAACCTCTACTCGAGTAGAGGTTCTTAAGATCTCGTTTTTG
shSTAT1	CCGGGAACAGAAATACACCTACGAACTCGAGTTCGTAGGTGTATTTCTGTTCTTTTT
shIRF9	CCGGCTCAGTAGTTGTCCGTGATAACTCGAGTTATCACGGACAACACTAGTGTTTTTG
shIRF1	CCGGCGTGTGGATCTTGCCACATTTCTCGAGAAATGTGGCAAGATCCACACGTTTTT

Supplementary Table 2. Human ISG primer sequences. Sequence 5' to 3' (including modification codes if applicable).

	5' FORWARD	3' REVERSE
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
RP2	CCCATTAAACTCCAAGGCAA	AAGCTGAGGATGCTCAAAGG
IFITM1	GGCTTCATAGCATTGCGCTACTC	AGATGTTCAAGCACTTGCGCGT
ISG15	CTCTGAGCATCCTGGTGAGGAA	AAGGTCAGCCAGAACAGGTCGT
STAT2	CAGGTCACAGAGTTGCTACAGC	CGGTGAACTTGCTGCCAGTCTT
IFITM3	CTGGGCTTCATAGCATTGCGCT	AGATGTTCAAGCACTTGCGCGT
STAT1	ATGGCAGTCTGGCGGCTGAATT	CCAAACCAGGCTGGCACAATTG
IFI27	CGTCCTCCATAGCAGCCAAGAT	ACCCAATGGAGCCCAGGATGAA
PKR	GAAGTGGACCTCTACGCTTTGG	TGATGCCATCCCGTAGGTCTGT
JAK1	GAGACAGGTCTCCACAAACAC	GTGGTAAGGACATCGCTTTTCCG
IFIH1	GCTGAAGTAGGAGTCAAAGCCC	CCACTGTGGTAGCGATAAGCAG
IFIT2	GGAGCAGATTCTGAGGCTTTGC	GGATGAGGCTTCCAGACTCCAA
OAS1	AGGAAAGGTGCTTCCGAGGTAG	GGACTGAGGAAGACAACCAGGT
IFIT3	CCTGGAATGCTTACGGCAAGCT	GAGCATCTGAGAGTCTGCCCAA
IFI44L	TGCACTGAGGCAGATGCTGCG	TCATTGCGGCACACCAGTACAG
IRF1	GAGGAGGTGAAAGACCAGAGCA	TAGCATCTCGGCTGGACTTCGA
DDX58	CACCTCAGTTGCTGATGAAGGC	GTCAGAAGGAAGCACTTGCTACC
DDX60	GGTGTTCACACAGGGAGTATCG	CCAGTTTTGGCGATGAGGAGCA
IRF9	CCACCGAAGTTCCAGGTAACAC	AGTCTGCTCCAGCAAGTATCGG
MX2	AAAAGCAGCCCTGTGAGGCATG	GTGATCTCCAGGCTGATGAGCT

References

1. Kamar N, Dalton HR, Abravanel F & Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* **27**, 116-138 (2014).
2. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
3. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
4. Kumar A, *et al.* Association of cytokines in hepatitis E with pregnancy outcome. *Cytokine* **65**, 95-104 (2014).
5. Tripathy AS, Das R, Rathod SB & Arankalle VA. Cytokine profiles, CTL response and T cell frequencies in the peripheral blood of acute patients and individuals recovered from hepatitis E infection. *PLoS One* **7**, e31822 (2012).
6. Tripathy AS, Das R, Rathod SB, Gurav YK & Arankalle VA. Peripheral T regulatory cells and cytokines in hepatitis E infection. *Eur J Clin Microbiol Infect Dis* **31**, 179-184 (2012).
7. Devhare PB, Chatterjee SN, Arankalle VA & Lole KS. Analysis of antiviral response in human epithelial cells infected with hepatitis E virus. *PLoS One* **8**, e63793 (2013).
8. Heim MH. Interferons and hepatitis C virus. *Swiss Med Wkly* **142**, w13586 (2012).
9. Randall RE & Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* **89**, 1-47 (2008).
10. Ramos EL. Preclinical and clinical development of pegylated interferon-lambda 1 in chronic hepatitis C. *J Interferon Cytokine Res* **30**, 591-595 (2010).
11. Shindo H, *et al.* IL-28B (IFN-lambda3) and IFN-alpha synergistically inhibit HCV replication. *J Viral Hepat* **20**, 281-289 (2013).
12. Muir AJ, *et al.* A randomized phase 2b study of peginterferon lambda-1a for the treatment of chronic HCV infection. *J Hepatol* **61**, 1238-1246 (2014).
13. Brown LA, *et al.* Acute and chronic alcohol abuse modulate immunity. *Alcohol Clin Exp Res* **30**, 1624-1631 (2006).
14. Haagsma EB, Riezebos-Brilman A, van den Berg AP, Porte RJ & Niesters HG. Treatment of chronic hepatitis E in liver transplant recipients with pegylated interferon alpha-2b. *Liver Transpl* **16**, 474-477 (2010).
15. Kamar N, *et al.* Pegylated interferon-alpha for treating chronic hepatitis E virus infection after liver transplantation. *Clin Infect Dis* **50**, e30-33 (2010).
16. Sarasin-Filipowicz M, *et al.* Interferon signaling and treatment outcome in chronic hepatitis C. *Proc Natl Acad Sci U S A* **105**, 7034-7039 (2008).
17. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
18. Zhou X, *et al.* Rapamycin and everolimus facilitate hepatitis E virus replication: Revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* **61**, 746-754 (2014).
19. Debing Y, *et al.* Ribavirin inhibits in vitro hepatitis E virus replication through depletion of cellular GTP pools and is moderately synergistic with alpha interferon. *Antimicrob Agents Chemother* **58**, 267-273 (2014).

20. Wang Y, *et al.* Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology* **146**, 1775-1783 (2014).
21. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
22. Pan Q, *et al.* Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* **55**, 1673-1683 (2012).
23. Pan Q, *et al.* Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* **87**, 713-722 (2009).
24. Nishitsuji H, *et al.* Hepatitis C virus infection induces inflammatory cytokines and chemokines mediated by the cross talk between hepatocytes and stellate cells. *J Virol* **87**, 8169-8178 (2013).
25. Kohli A, Shaffer A, Sherman A & Kottlil S. Treatment of hepatitis C: a systematic review. *JAMA* **312**, 631-640 (2014).
26. Padmanabhan P, Garaigorta U & Dixit NM. Emergent properties of the interferon-signalling network may underlie the success of hepatitis C treatment. *Nat Commun* **5**, 3872 (2014).
27. Berenguer M, *et al.* Hepatitis C virus viral kinetics during alpha-2a or alpha-2b pegylated interferon plus ribavirin therapy in liver transplant recipients with different immunosuppression regimes. *J Clin Virol* **53**, 231-238 (2012).
28. Pan Q, Tilanus HW, Janssen HL & van der Laan LJ. Ribavirin enhances interferon-stimulated gene transcription by activation of the interferon-stimulated response element. *Hepatology* **53**, 1400-1401; author reply 1402 (2011).
29. Zhou X, *et al.* Modulating innate immunity improves hepatitis C virus infection and replication in stem cell-derived hepatocytes. *Stem Cell Reports* **3**, 204-214 (2014).
30. Zeytun A, Chaudhary A, Pardington P, Cary R & Gupta G. Induction of cytokines and chemokines by Toll-like receptor signaling: strategies for control of inflammation. *Crit Rev Immunol* **30**, 53-67 (2010).
31. Lin FC & Young HA. Interferons: Success in anti-viral immunotherapy. *Cytokine Growth Factor Rev* **25**, 369-376 (2014).
32. Melchjorsen J. Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity - clues for treatments and vaccines. *Viruses* **5**, 470-527 (2013).
33. Dong C, *et al.* Suppression of interferon-alpha signaling by hepatitis E virus. *Hepatology* **55**, 1324-1332 (2012).
34. Kamar N, *et al.* Ribavirin for chronic hepatitis E virus infection in transplant recipients. *N Engl J Med* **370**, 1111-1120 (2014).
35. Pawlotsky JM, *et al.* Antiviral action of ribavirin in chronic hepatitis C. *Gastroenterology* **126**, 703-714 (2004).
36. Rotman Y, *et al.* Effect of ribavirin on viral kinetics and liver gene expression in chronic hepatitis C. *Gut* **63**, 161-169 (2014).
37. Poynard T, *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* **352**, 1426-1432 (1998).

38. Wang Y, Metselaar HJ, Peppelenbosch MP & Pan Q. Chronic hepatitis E in solid-organ transplantation: the key implications of immunosuppressants. *Curr Opin Infect Dis* **27**, 303-308 (2014).
39. Hoofnagle JH. Course and outcome of hepatitis C. *Hepatology* **36**, S21-29 (2002).
40. Meissner EG, *et al.* Endogenous intrahepatic IFNs and association with IFN-free HCV treatment outcome. *J Clin Invest* **124**, 3352-3363 (2014).
41. Aggarwal R & Jameel S. Hepatitis E. *Hepatology* **54**, 2218-2226 (2011).

Chapter 8

Interferon regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral interferon-stimulated genes

Lei Xu¹, **Xinying Zhou**¹, Wenshi Wang¹, Yijin Wang¹, Yuebang Yin¹, Luc J. W. van der Laan²,
Dave Sprengers¹, Herold J. Metselaar¹, Maikel P. Peppelenbosch¹ and Qiuwei Pan¹

Department of ¹Gastroenterology and Hepatology and ²Surgery, Erasmus MC-University Medical Center, Rotterdam, Netherlands

Abstract

Interferon regulatory factor 1 (IRF1) is one of the most important interferon-stimulated genes (ISGs) in cellular antiviral immunity. Although hepatitis E virus (HEV) is a leading cause of acute hepatitis worldwide, how ISGs counteract with HEV infection is largely obscure. This study aims to investigate the action of IRF1 on HEV replication. Multiple cell lines were used to harbor two HEV models. In different HEV cell culture systems, IRF1 effectively inhibited HEV replication. Surprisingly, IRF1 did not trigger interferon production and Chip-seq data analysis revealed that IRF1 was able to bind to the promoter region of signal transducers and activators of transcription 1 (STAT1). Functional assay confirmed that IRF1 could drive the transcription of STAT1, resulting in elevation of total and phosphorylated STAT1 proteins. This further activated the transcription of a panel of downstream antiviral ISGs. By pharmacological inhibitors and RNAi-mediated gene-silencing approaches, we revealed that antiviral function of IRF1 was dependent on the JAK-STAT cascade. Furthermore, induction of ISGs and anti-HEV effect of IRF1 overlapped with IFN α but were potentiated by ribavirin. We demonstrated that IRF1 effectively inhibited HEV replication through the activation of JAK-STAT pathway and the subsequent transcription of antiviral ISGs, but independent of interferon production.

Introduction

Hepatitis E virus (HEV) infection is one of the most common causes of acute hepatitis, particularly in developing countries ¹. Although it is often a self-limiting disease, fulminant hepatitis and high mortality were reported in pregnant women ². In western countries, cases of chronic hepatitis E were frequently reported in immunocompromised patients with the potential cause of graft loss and even mortality ^{3,4}. However, the underlying mechanisms of how the host combats HEV infection remain largely elusive.

Upon viral infection, the host rapidly reacts by producing a panel of inflammatory cytokines and chemokines to orchestrate the immunological reaction to the pathogenic invasion ⁵. Within this group of anti-viral mediators, interferons (IFNs) are vital cytokines for innate defense against viral infection ⁶. Because of their potent antiviral activity, pegylated interferon- α (PEG-IFN α) has been used for decades to treat chronic hepatitis B virus (HBV) and C virus (HCV) infection ^{7,8}. Since no registered medication is available for HEV infection, PEG-IFN α , ribavirin, or a combination of both has been used as off-label treatments for some cases of HEV infection, although the efficacy is still inconclusive ⁹. Mechanistically, type I interferon molecules bind to cell surface receptors and subsequently initiate a signaling cascade. This binding triggers the phosphorylation of pre-associated Janus kinase 1 (JAK1). Subsequently, this leads to the recruitment and phosphorylation of signal transducers and activators of transcription 1 and 2 (STAT1 and 2), which further bind to the interferon regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3) complex. ISGF3 translocate to the nucleus to activate transcription of IFN-stimulated genes (ISGs), which are in turn regulated by the IFN-stimulated response element (ISRE). The products of ISGs are thought to be the ultimate antiviral effectors ¹⁰.

Although there are hundreds of ISGs, in fact, only a few actually have specific or broad antiviral activities ¹⁰. Interferon regulatory factor 1 (IRF1) is one of the most important ISGs that has been shown to effectively inhibit HCV, yellow fever virus, chikungunya virus and Venezuelan equine encephalitis virus infections among the 380 tested ISGs ¹¹. A further follow-up study demonstrated that IRF1 could potently inhibit the replication of 14 different viruses, representing 7 families including different DNA and RNA viruses ¹⁰. However, the exact antiviral mechanism of IRF1 remains unclear.

Given the importance of IRF1 in innate defense against viral infection but insufficient knowledge in respect to HEV, we investigated the role of IRF1 in HEV infection and the interactions with antiviral treatments by using cell culture models. We found that independent of interferon production, IRF1 could effectively restrict HEV replication through the activation of JAK-STAT cascade and the subsequent induction of a wide range of ISGs. We further demonstrated that the anti-HEV effect of IRF1 overlapped with IFN α but was augmented by ribavirin.

Materials and Methods

Reagents

Human IFN α (Thermo Scientific, the Netherlands) was dissolved in PBS. Stocks of JAK inhibitor 1 (CAS 457081-03-7, Santa Cruz Biotech, CA) were dissolved in DMSO with a final concentration of 5 mg/mL. Stocks of CP-690550 (Tofacitinib) (Santa Cruz Biotech, CA) were dissolved in DMSO with a final concentration of 10 mg/mL. Stock of ribavirin was dissolved in PBS with a final concentration of 10 mg/mL. Matched concentrations of DMSO were used as vehicle control. Phospho-STAT1 (Tyr701) (58D6, Rabbit mAb, #9167), STAT1 (Rabbit mAb, #9172), IRF1 (D5E4, Rabbit mAb, #8478) antibodies were obtained from Cell Signaling Technology. IRF9 antibody was obtained from LSBio (Rabbit Polyclonal, #LS-C155416, Life Span BioSciences, Inc.). β -actin antibodies (Mouse monoclonal, #sc-47778) were obtained from Santa Cruz Biotech (Santa Cruz, CA). Anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Li-COR Biosciences, Lincoln, USA) were also used.

Cell culture

Naïve or vector transduced human hepatoma cells (Huh7), HEK293T cells, human lung epithelial carcinoma cells (A549) and human fetal lung fibroblast cells (MRC-5)¹² were cultured in Dulbecco's modified Eagle medium (DMEM) (Lonza Biowhittaker, Verviers, Belgium) complemented with 10% (v/v) fetal calf serum (FCS) (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 μ g/ml streptomycin. HepaRG cell line was maintained in William's medium (Life Technologies) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, as well as 5 μ g/mL insulin and 5×10^{-7} M hydrocortisone hemisuccinate (Sigma Aldrich, the Netherlands)¹³. Huh7 cells containing a subgenomic HCV bicistronic replicon

(I389/NS3-3V/LucUbiNeo-ET, Huh7-ET-Luc) were maintained with 250 µg/ml G418 (Sigma, Zwijndrecht, the Netherlands) and viral replication was monitored by measuring firefly luciferase activity ¹⁴. For ISRE reporter model (Huh7-ISRE-Luc), Huh7 cells were transduced with lentiviral transcriptional reporter system which expressing the firefly luciferase gene driven by a promoter containing multiple ISRE promoter elements (SBI Systems Biosciences, Mountain View, CA) and luciferase activity represents ISRE promoter activation ¹⁵.

HEV cell culture models

In this study, multiple cell lines were employed for HEV replication, including a human hepatoma cell line (Huh7) ^{16, 17}, a human lung epithelial carcinoma cell line (A549) ¹⁸, a human fetal lung fibroblast cell line (MRC-5) as well as a hepatic cell line (HepaRG) which retains many characteristics of primary human hepatocytes. For the full-length HEV model, a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number: JQ679013) was used to generate HEV genomic RNA by using the Ambion *mMESSAGE mMACHINE*[®] *in vitro* RNA transcription Kit (Life Technologies Corporation, CA, USA) ¹⁷. Huh7 and HepaRG cells were electroporated with full-length HEV genome RNA to generate consecutive HEV infected cell models (Huh7-p6 and HepaRG-p6). For the subgenomic HEV model, a construct containing subgenomic HEV in which 5' portion of HEV ORF2 was replaced with the in-frame *Gaussia princeps* luciferase reporter gene to yield p6-Luc ¹⁷. Huh7 and A549 cells were electroporated with HEV subgenomic RNA to generate HEV subgenomic model (Huh7-p6-Luc and A549-p6-Luc) in which the accumulation of secreted luciferase serves as a reporter for HEV replication. For HEV genotype 1 replicon model, viral RNA was generated from a Sar55/S17/luc-encoding plasmid. Huh7 cells were electroporated with Sar55/S17/luc HEV RNA to generate genotype 1 replicon model as described ^{17, 19}.

Reinfection assays

Supernatant from full-length Huh7-p6 HEV model was collected and purified by ultracentrifugation. The supernatant was first filtered through 0.45 µm filter followed by centrifugation at 10000 rpm for 30 min to remove cell debris and then 22000 rpm for 2 hours to pellet HEV virus (SW 28 rotor, Beckman). The pellet was suspended and diluted to 1×10^7 HEV viral RNA copies/ml. The diluted HEV virus stock was stored at -80 °C. For HEV

infection, Huh7, HepaRG, A549 and MRC5 cells were seeded into 12-well plates at a density of 7×10^4 cells per well and incubated for 24 h. Next, different cells were incubated with 400 μ L HEV stock diluted to 1×10^7 viral RNA copies/ml per well at 37 °C for 6 h. Then, the inoculum was removed and cell layers were washed 3 times with 1 ml of PBS, and 1 ml of fresh medium was added to each well. For 6 well-plates, different cells were seeded at a density of 1.4×10^5 cells per well and incubated for 24 h. Next, different cells were incubated with 800 μ L HEV stock diluted to 1×10^7 viral RNA copies/ml per well at 37 °C for 6 h. Then, the inoculum was removed and cell layers were washed 3 times with 3 ml of PBS, and 2 ml of fresh medium was added to each well.

To investigate the anti-viral effect of IRF1 in HEV refection models, Huh7 cells were transduced with IRF1 or infected by HEV as following time point: (A) HEV infection and IRF1 transduction were start at the same time. After 6 h, the inoculum was removed, and 1 ml of medium with IRF1 lentivirus was added to each well. Intracellular HEV viral RNA was measured after 48 h. (B) Huh7 cells were first transduced with IRF1 for 48 h and then transduced cells were infected with HEV for 6 h. Next, the inoculum was removed, and 1 ml of fresh medium was added to each well. HEV viral RNA was quantified 48 h post HEV infection. (C) Huh7 cells were first infected with HEV for 6 h. 24 h post HEV infection, IRF1 was transduced and HEV RNA was quantified 48 h after transduction.

Gene knockdown and over-expression by lentiviral vectors

For gene knockdown, pLKO.1 based lentiviral vectors (Sigma-Aldrich) targeting IRF1, STAT1, IRF9 and non-targeted control vector (shCTR) were obtained from the Biomix Center in Erasmus Medical Center. Lentiviral pseudoparticles were generated in HEK293T cells following the method described previously²⁰. To generate stable gene knockdown cell line, Huh7 cells were transduced with lentiviral particles for 3 days. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 μ g/mL puromycin (Sigma-Aldrich) to the cell culture medium. After selection, cell lines shown optimal gene knockdown were chosen. pTRIP.CMV.IVSb.ISG.ires.TagRFP based IRF1 over-expression vector was a kind gift from Prof. Charles M. Rice, the Rockefeller University¹¹. Meanwhile, two vectors expressing reporter genes *Photinus pyralis* luciferase (Fluc) or GFP were used as control (also a kind gift from Prof. Charles M. Rice). Lentiviral pseudoparticles were generated as described and stored at -80 °C¹¹. Spinoculation method

was used as described for IRF1 transduction assay¹¹, target cell lines were seeded into 12-well plates at a density of 7×10^4 cells per well and transduced with transduced with lentiviral pseudoparticles at 37 °C for 24 h, 48 h or 72 h¹¹. The transduction time of each experiment was described in the figure legend of each figure. The control vector used in each experiment was also described in the figure legend of each figure.

Quantification of Hepatitis E Virus Replication

Two Huh7 based HEV models (Huh7-p6 and Huh7-p6-Luc) were well-established models which could stably harbor HEV replication for a long term. In this study, HEV viral RNA and HEV-related luciferase activity were measured around 2 months after HEV RNA electroporation when the HEV viral RNA level and luciferase activity were at stable level. For HepaRG-p6 and A549-p6-Luc model, lentivirus transduction and HEV RNA quantification was performed around 4 weeks and 2 weeks after HEV RNA electroporation, respectively. MRC5 cells were infected by stock HEV virus medium as description in Reinfection assays for 24 h. 1 week post HEV infection, IRF1 was transduced in MRC5 cells and HEV RNA was quantified 48h after transduction. For HEV-related *Gaussia* luciferase analysis (HEV-p6-Luc), the activity of secreted luciferase in the cell culture medium was measured by BioLux® *Gaussia* Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). For firefly luciferase and *Photinus pyralis* luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 10 min at 37 °C and luciferase activity was measured. For the HEV-p6 model, intracellular RNA was isolated from cellular lysates. Cells were lysed with 350 µL RA1 buffer (Bioke, Leiden, Netherlands). RNA was isolated using the Machery-NucleoSpin RNA II kit (Bioke, Leiden, Netherlands) and quantified by a Nanodrop ND-1000 Spectrophotometer (Thermo, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc.) with random hexamer primers. Intracellular HEV level and host gene expression were quantified by SYBR-Green-based (Applied Biosystems® SYBR® Green PCR Master Mix, Life technologies, CA, USA) real-time PCR with the StepOnePlus™ System (Applied BioSystems). PCR steps consisted of a 10 min holding stage (95°C) followed by 40 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C. *GAPDH* and *RP2* (Human retinitis pigmentosa 2) were used as housekeep genes and all gene expression levels (relative) were normalized to

GAPDH and RP2 using the $2^{-\Delta\Delta CT}$ method. The HEV primer sequences were 5'-ATCGGCCAGAAAGTTGGTTTTTAC-3' (sense) and 5'-CCGTGGCTATAACTGTGGTCT-3' (antisense), and the primer sequences of housekeeping gene GAPDH were 5'-TGTCCCCACCCCAATGTATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense) and the primers of housekeeping gene RP2 were 5'-CCCATTAAACTCCAAGGCAA-3' (sense) and 5'-AAGCTGAGGATGCTCAAAGG-3' (antisense).

Interferon production bioassay

Cells were seeded into 6-well plates at a density of 10×10^4 cells per well and transduced with IRF1 or control lentiviral particles at 37 °C. After 72 h, lentiviral particles were removed and cells were washed 3 times with PBS and cultured for another 72 h. The cultured supernatant was subsequently collected and filtered through a 0.45 µm-pore size membrane and added to two luciferase reporter cell lines (Huh7-HCV-Luc and Huh7-ISRE-Luc) which are sensitive to interferons.

Immunoblotting

Whole cell extracts were obtained and were heated at 95 °C for 5 min. Proteins were subjected to a 10-15 % sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated at 120 V for 100 min. Proteins were electrophoretically transferred onto a polyvinylidenedifluoride (PVDF) membrane (pore size: 0.45µM, Invitrogen) for 1.5 hour (h) with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer (LI-COR Biosciences, Lincoln, USA) in 1×PBS containing 0.1% Tween-20. Membranes were incubated with primary antibodies overnight at 4 °C. Rabbit anti-IRF1, p-STAT1, STAT1, IRF9 (1:1000) antibodies or mouse anti-β-actin (1:2000) were diluted in 5% (mass/vol) BSA in 1×PBS containing 0.1% Tween-20. The membrane was washed 3 times followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. β-actin was served as standardization of sample loading. The membrane was scanned by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). Results were visualized with Odyssey 3.0 software. Band intensity of each immunoblot data was also quantified by Odyssey Software.

MTT assay

Cells were seeded in 96-well plates and 10 mM 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Sigma, Zwijndrecht, Netherlands) was added. Plate was incubated at 37 °C with 5% CO₂ for 3 h, then the medium was removed and 100 µL of DMSO was added to each well. Incubate the microplate at 37°C for 50min. The absorbance of each well was read on the microplate absorbance reader (Bio-Rad, CA, USA) at wavelength of 490 nm.

Confocal microscopy analysis

1×10^4 cells Huh7 cells were seeded on glass slides in a 6 well-plate. Cells were transduced with IRF1 over-expression, Fluc or GFP control lentiviral viruses for 48 h. For IRF1 protein immunofluorescence staining, IRF1 antibody (1: 200, D5E4, Rabbit mAb, #8478) was used as primary antibody. Anti-rabbit-Alexa Fluor[®] 488-Conjugate antibody (1: 1000, Cell Signaling Technology) was used as secondary antibody. Cells were visualized in an inverted LSM 510 confocal microscope (Zeiss LSM510, Jena, Germany) using a 40× oil immersion objective with 1.7× zoom in. All images were analyzed with a Zeiss LSM Image Browser (version 4.2).

ChIP-seq data analysis

ChIP-seq datasets for IRF1 in K562 cells were retrieved from the ENCODE database. ChIP-seq datasets were processed and mapped to hg19 reference genome as described ²¹. ChIP-seq datasets with multiple replicates were merged. MACS 1.4.2 was used for peak calling and for the generation of binding profiles ²². The sequencing profiles of IRF1 were created in the IGV browser ²³.

Statistical analysis

All results were presented as mean ± SEM. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a p-value less than 0.05.

Results

IRF1 effectively inhibits HEV replication

Although IRF1 exerts cell-autonomous anti-viral activity toward a broad range of viruses ^{10, 11}, its capacity to combat HEV infection remains unexplored. To determine the role of IRF1 in HEV infection, we first tested the effect of forced IRF1 expression in two Huh7 cell line-based HEV models. Successful over-expression of IRF1 was visualized by the red fluorescent protein (TagRFP) expression under the confocal electroscope, since the bicistronic lentiviral vector co-expresses IRF1 and a TagRFP reporter (Supplementary Figure 1A). Consistently, IRF1 mRNA expression (Figure 1A) and protein level (Figure 1B and 1C) was increased in over-expressed cells compared to high dose IFN α (1000 IU/mL) induction. Of note, immunofluorescence staining of IRF1 showed that it predominantly located in the nucleus (Figure 1C). To exclude the non-specific effect of lentivirus transduction, two control vectors that used in previous study which expressing reporter genes *Photinus pyralis* luciferase (Fluc) or Green fluorescent protein (GFP) were also employed ¹¹. Transduction efficiency was confirmed by different methods, including visualization of TagRFP expression (data not shown), measurement of *Photinus pyralis* luciferase activity (Supplementary Figure 1B) and quantitation of TagRFP mRNA expression level (Supplementary Figure 1B) in transduced cells. As expected, Fluc and GFP control did not affect HEV replication in two Huh7-based models (Supplementary Figure 1C and 1D).

Next, we showed that over-expression of IRF1 could profoundly inhibit HCV replication in a Huh7-based HCV luciferase replicon (Huh7-ET-Luc) model (Supplementary Figure 1E) without affecting cell viability (Supplementary Figure 1F), which was consistent with the previous study ¹¹. Interestingly, after 48 h or 72 h of IRF1 transduction, HEV were significantly inhibited in both HEV Huh7-p6-Luc replicon and full-length infectious models (Figure 1D). Notably, the antiviral activity of IRF1 in both models was equivalent to high dose IFN α (1000 IU/mL) treatment (Figure 1D). Moreover, IRF1 could also effectively inhibit genotype 1 HEV-related luciferase activity in the Huh7 based genotype 1 HEV replicon model (Sar55/S17/luc) (Figure 1E). To further validate the antiviral ability of IRF1, three other different human cell lines were used. A human hepatic progenitor cells derived cell line named HepaRG which exhibits many characteristics of primary human hepatocytes ¹³, a lung epithelial carcinoma cell line A549 that widely-used for HEV propagation ¹⁸ and a human

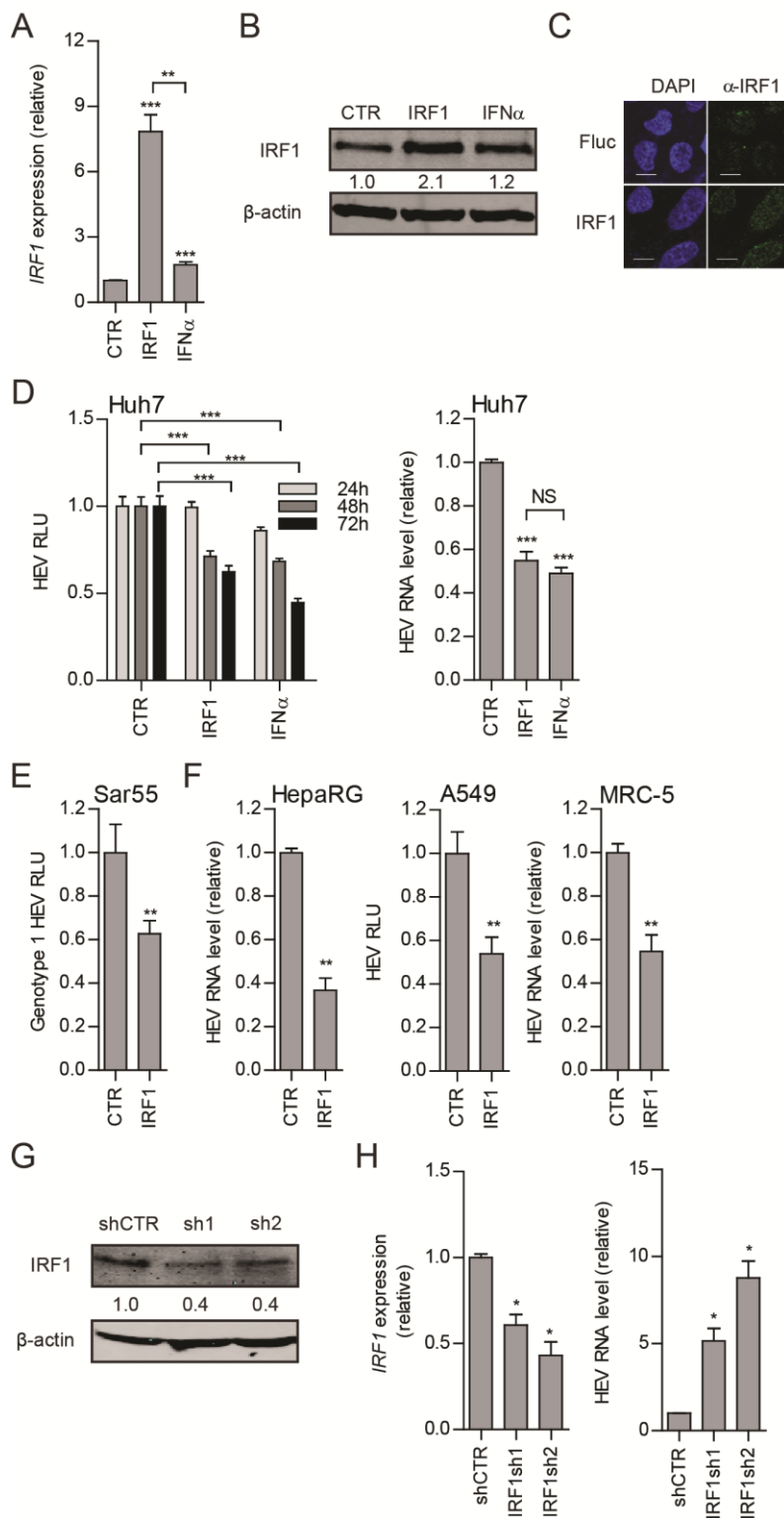


Figure 1. IRF1 effectively inhibits HEV replication.

(A) qRT-PCR analysis of *IRF1* mRNA expression in Huh7 cells transduced with IRF1, Fluc (control) vectors or treated with IFN α (1000 IU/mL) for 48 h (n = 7). Data was normalized to two housekeeping genes (*GAPDH* and *RP2*). Immunoblotting (B) and immunofluorescence (C) analysis of Huh7 cells transduced with IRF1, Fluc (CTR) vector or treated with IFN α (1000 IU/mL) for 48 h. Band intensity of each immunoblot data was quantified by Odyssey Software. All data was normalized to Actin expression and control was set as 1. Green indicates IRF1 protein and blue indicates DAPI (Scale bar: 10 μ m). (D) HEV viral replication-related *Gaussia* luciferase activity in Huh7-p6-Luc model transduced with IRF1, Fluc vector or treated with IFN α (1000 IU/mL) for 24 h, 48 h or 72 h (n = 4 independent experiments with each of 3-4 replicates) and qRT-PCR analysis of HEV viral RNA in Huh7-p6 full-length HEV model transduced with IRF1, Fluc vector or treated with IFN α (1000 IU/mL) for 48 h (n = 7). RLU: relative luciferase unit. (E) Genotype 1 HEV (Sar55/S17/luc) viral replication-related *Gaussia*

luciferase activity in Huh7-Sar55/S17/luc model transduced with IRF1 or Fluc vector for 48h (n = 4 independent experiments with each of 3-4 replicates). (F) HEV viral replication-related *Gaussia* luciferase activity in A549-p6-Luc model (n = 4 independent experiments with each of 3-4 replicates) or qRT-PCR analysis of HEV viral RNA in HepaRG-p6-HEV and MRC-5-p6 HEV model transduced with IRF1 or Fluc vector for 48 h (HepaRG: n = 4; MRC-5: n = 5). (G) Immunoblotting analysis of Huh7 cells transduced with lentiviral shRNA vectors targeting IRF1 or non-targeted control (shCTR). (H) qRT-PCR analysis of IRF1 and HEV viral RNA level relative to shCTR (n = 3-5). Data was normalized to Fluc control (CTR, set as 1) (D, E and F). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

fetal lung fibroblast cell line (MRC-5)¹² was used to further confirm the anti-HEV ability of IRF1. The over-expression of IRF1 in these cell lines was confirmed by measuring IRF1 mRNA and protein expression (Supplementary Figure 1G and 1H). Consistently, over-expression of IRF1 significantly inhibited intracellular HEV RNA level as well as HEV-related luciferase activity (Figure 1F) in these cell lines. To validate the antiviral ability of IRF1 in the reinfection HEV model, Huh7 cells were infected by HEV or transduced with IRF1 at different time point (described in Reinfection assays of Materials and Methods). The result showed over-expression of IRF1 significantly inhibited HEV infection when IRF1 was transduced with (same time, Supplementary Figure 1I) or before (pre, Supplementary Figure 1J) or after (post, Supplementary Figure 1K) HEV infection. Taken together, these results demonstrated that IRF1 over-expression could effectively inhibit HEV replication and infection in different cell culture models. Furthermore, we also investigated whether HEV infection could trigger IRF1 expression in different cell lines. Different cell lines including Huh7, HepaRG, A549 and MRC5 were infected by HEV as described. Surprisingly, we found IRF1 expression can be induced at early stage of HEV infection (2h post infection) in HepaRG and A549 cells (Supplementary Figure 2A and 2B). In Huh7 and MRC5 cells, IRF1 was just slightly induced 6 h or 2 h post infection (Supplementary Figure 2A and 2B).

Subsequently, we determined whether basal IRF1 is necessary for anti-HEV immunity. This was pursued through a loss-of-function approach involving knockdown IRF1 by lentiviral-based short hairpin RNA (shRNA) constructs. A non-targeted vector was used as a control (shCTR). Gene silencing of IRF1 was confirmed by immunoblotting and qRT-PCR (Figure 1G and 1H). Consistently, knockdown of IRF1 significantly promoted HEV replication (Figure 1H). Thus, both gain- and loss-of-function assays demonstrated that IRF1 plays an important role in restricting HEV replication.

IRF1 over-expression does not trigger interferon production

Because of the comparable anti-HEV effect of IRF1 and IFN α , we investigated whether the effect of IRF1 is merely via triggering of interferon production in our cell culture systems, since IRF1 has been reported to induce IFN β expression in particular cell types²⁴. We first investigated the effect of IRF1 on gene expression level of different interferons, including IFN α , IFN β and IFN λ in different cell lines. We found that the basal mRNA expression levels of several interferon genes including IFN α , IFN β and IFN λ were very low in Huh7 and HepaRG

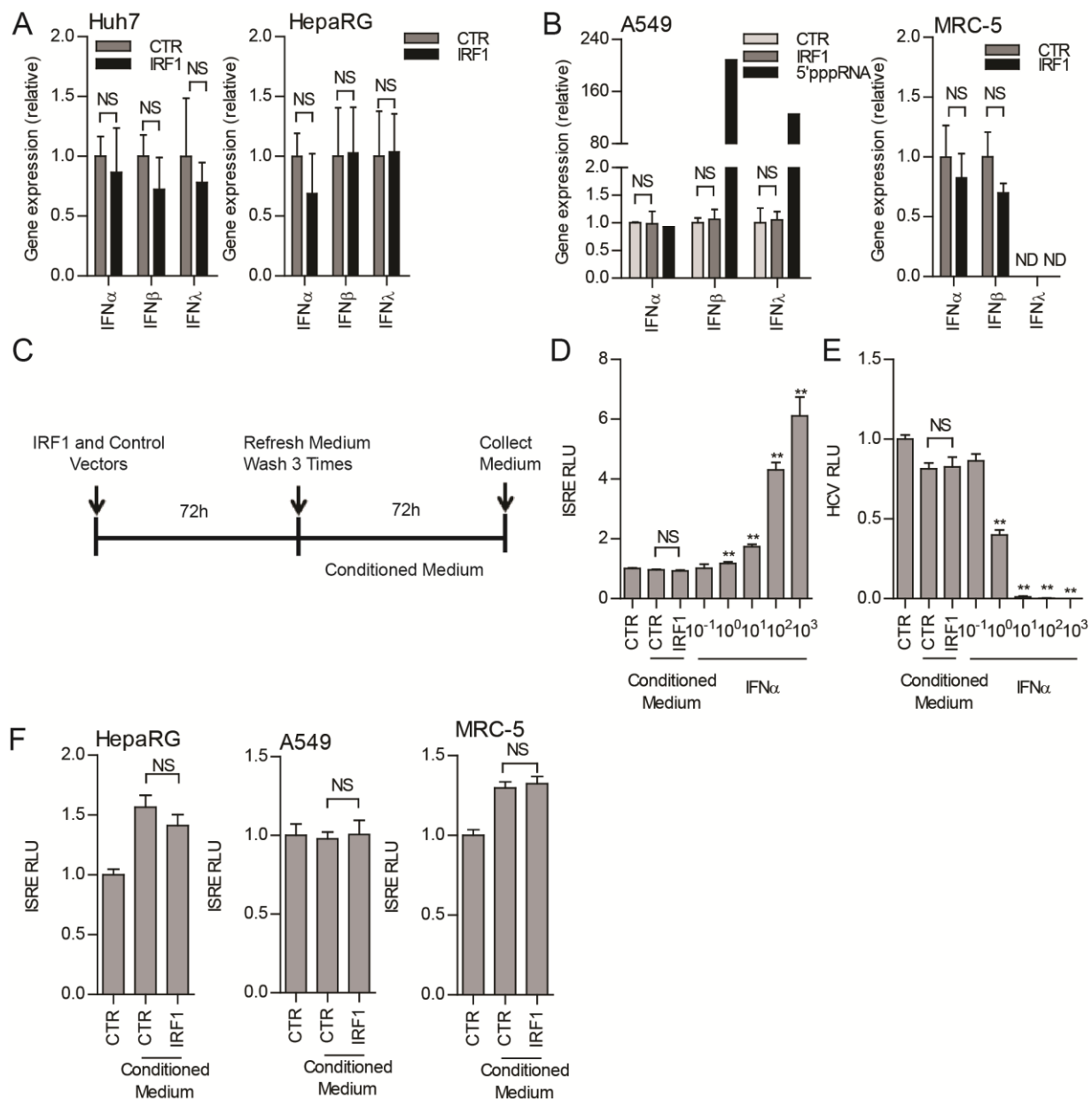


Figure 2. IRF1 over-expression does not trigger interferon production. qRT-PCR analysis of interferon gene expression in Huh7 (A), HepaRG (A), A549 (B) and MRC5 (B) cells transduced with IRF1 or Fluc vector for 48 h (n = 3). For A549 cells, 5'pppRNA (0.1 μ g/mL) was transfected as a positive control (IRF1, n = 3; 5'pppRNA, n = 1). Data was normalized to Fluc control (CTR, set as 1). (C) Schematic illustration of the production of conditioned medium (supernatant). ISRE firefly luciferase activity in Huh7-ISRE-Luc model (D) and HCV luciferase activity in Huh7-ET-Luc model (E) treated with conditioned medium from Huh7 cells or IFN α for 48 h (n = 3 independent experiments with each of 3-4 replicates). (F) ISRE firefly luciferase activity in Huh7-ISRE-Luc treated with conditioned medium from HepaRG, A549 cells or MRC-5 cells for 48 h (n = 3 independent experiments with each of 2-3 replicates). Data was normalized to GFP control (D, E and F) (CTR, set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

cells (Supplementary Figure 2C) and were not significantly affected even by IRF1 over-expression (Figure 2A). These results are consistent with the previous study that no interferon genes were affected in IRF1 over-expressed Huh7 cells ¹¹. In addition, the expression of all these three interferon genes was also not affected by IRF1 over-expression in A549 cells and MRC-5 cells (Figure 2B). As positive control, IFN β gene and IFN λ gene were effectively induced by the RIG-I activator (5'pppRNA) ²⁵ in A549 cells (Figure 2B). To further confirm the lack of interferon production in IRF1 over-expressed cells, we collected the conditioned medium from these transduced cells (supernatant) and performed an interferon functional assay as well as a highly sensitive HCV-based bioassay (Figure 2C) ²⁶. To this end, we employed a lentiviral transcriptional reporter system to mimic interferon response by expressing the firefly luciferase driven by a promoter containing multiple ISRE elements (15). As shown in Figure 2D, the supernatant from IRF1 over-expressed Huh7 cells was unable to stimulate interferon response in the ISRE reporter assay. Meanwhile, supernatants from HepaRG, A549 and MRC-5 cells were also not able to stimulate interferon response (Figure 2F) in the ISRE reporter assay. Although HCV replicon is very sensitive to interferons, the supernatant collected from IRF1 transduced cells did not affect HCV replication (Figure 2E). These data indicated that IRF1 over-expression did not trigger interferon production in Huh7, HepaRG, A549 and MRC-5 cells.

IRF1 activates STAT1 gene transcription leading to enhanced protein expression and phosphorylation

Interestingly, IRF1 was first described as a transcription factor ²⁴ and has more than 200 binding sites in the human genome ²⁷. By retrieving genome wide IRF1 ChIP-seq data from the ENCODE ChIP-seq Experiment Matrix database, we surprisingly found that IRF1 could directly bind to the promoter region of STAT1 gene (Figure 3A), a key component of the interferon signaling.

To validate whether IRF1 induces transcription of STAT1 in our system, we first tested mRNA expression of STAT1 in IRF1 over-expressed Huh7 cells. Indeed, we observed that over-expression of IRF1 potently induced mRNA expression of STAT1 (Figure 3B) but not JAK or STAT2 (Figure 3C). In addition, IRF1 could also potently induce STAT1 mRNA expression in HepaRG, A549 and MRC-5 cells (Figure 3D). Subsequently, this led to enhanced expression of

STAT1 protein and activation of STAT1 phosphorylation at the Tyr701 site, which is an indispensable marker of STAT1 activation (Figure 3E). In addition, IRF1 also led to enhanced expression of STAT1 protein and activation of STAT1 phosphorylation in HepaRG, A549 and MRC5 cells (Figure 3F). Taken together, we demonstrated that IRF1 could effectively activate STAT1 gene transcription, resulting in enhanced protein expression and phosphorylation.

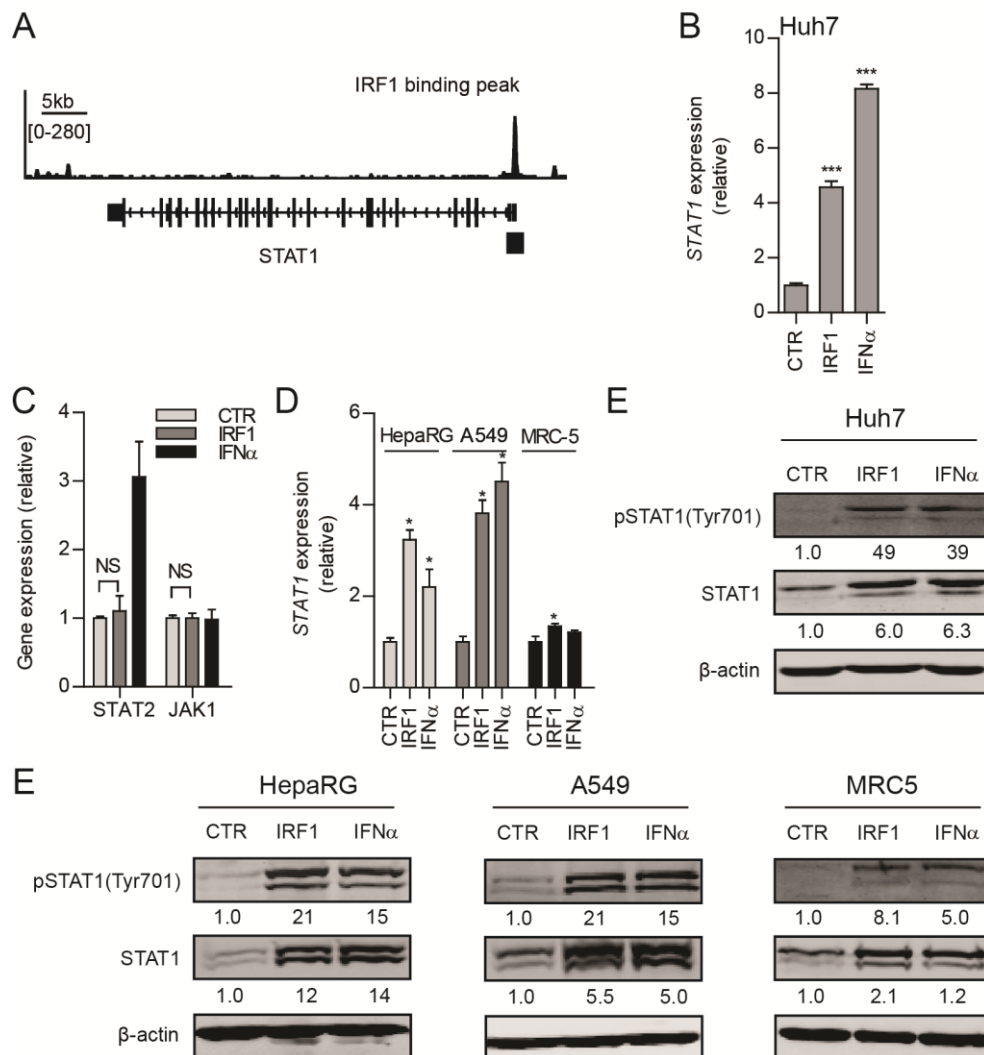


Figure 3. IRF1 activates STAT1 gene transcription leading to enhanced protein expression and phosphorylation. (A) STAT1 genes with IRF1 binding to their promoter regions. The normalized binding signals were used as the input data. Binding peak detection was performed with PeakSeq v1.01 for identifying and ranking peak regions in ChIP-Seq data analysis. The Y axis value represents the binding signaling value; the black bar in the left corner represents the scale (5k bp). qRT-PCR analysis of *STAT1* expression (B, $n = 8$) and *STAT2* and *JAK1* expression (C, $n = 3$) in Huh7 cells transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h. (D) qRT-PCR analysis of *STAT1* expression in HepaRG, A549 and MRC-5 cells transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h ($n = 5-6$). Immunoblotting analysis of Huh7 cells (E), HepaRG cell (F), A549 cells (G) and MRC5 cells (H) over-expressing IRF1 or treated with IFN α (1000 IU/mL) for 48 h. pSTAT1 (Tyr701), STAT1 phosphorylated at Tyr701 site. Data was normalized to Fluc control (B, C and D) (CTR, set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

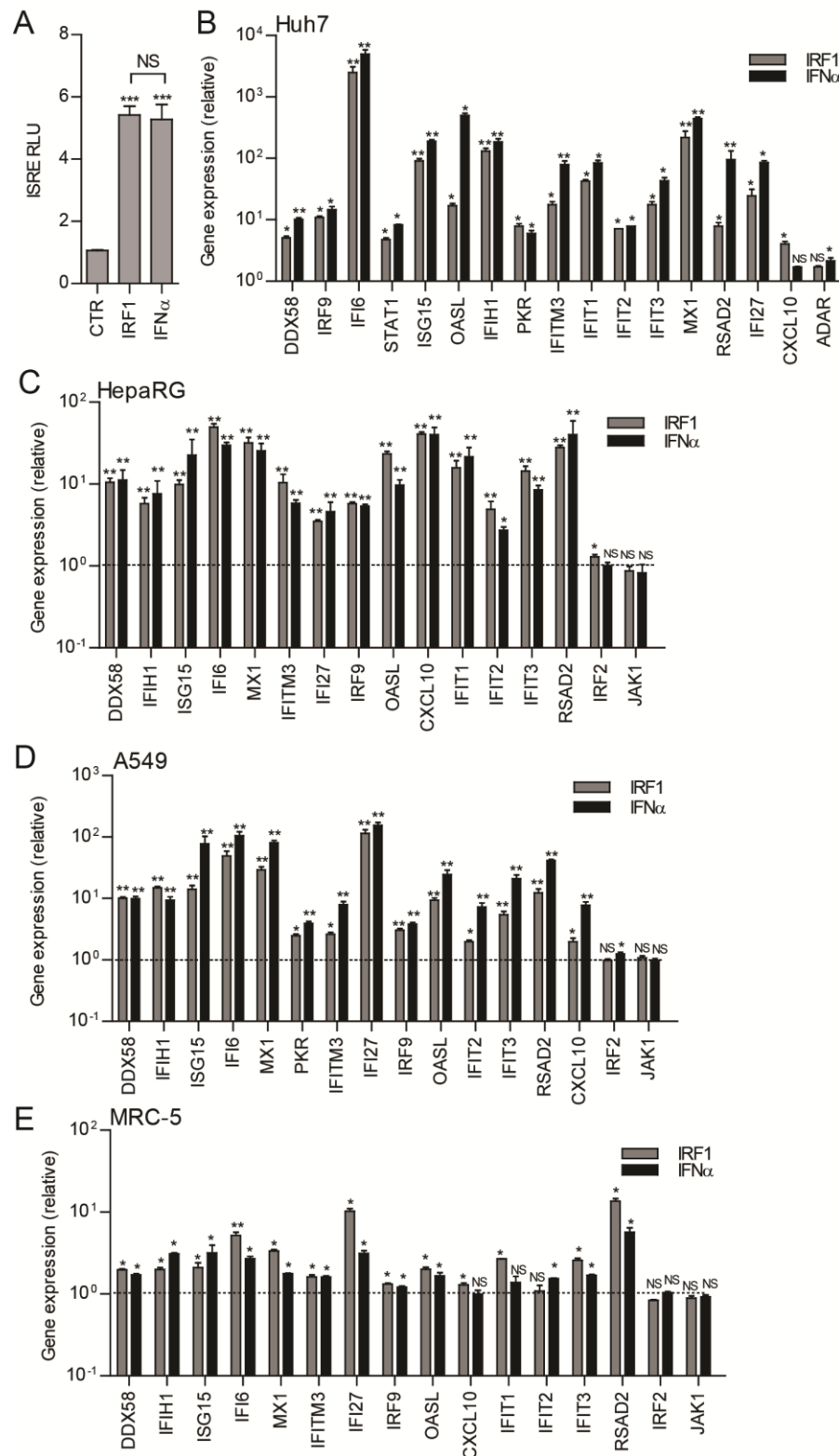


Figure 4. IRF1 activates the expression of ISGs. (A) ISRE promoter-related firefly luciferase activity in Huh7-ISRE-Luc model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h. (n = 4 independent experiments with each of 3-4 replicates). qRT-PCR analysis of gene expression in Huh7 cells (B), HepaRG cells (C), A549 (D) cells and MRC5 (E) cells transduced with IRF1, Fluc vector or treated with IFN α (1000 IU/mL) for 48 h (n = 3-5). Data was normalized to GFP (A, CTR, set as 1) or Fluc control (B, C, D and E) (set as 1, not shown). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

IRF1 activates the transcription of antiviral ISGs

In general, binding of interferons to their receptors activates JAK1, resulting in tyrosine phosphorylation of STAT1 and STAT2. This is followed by the formation and nuclear translocation of the STAT1-STAT2-IRF9 complex, a transcription factor complex known as ISGF3, which in turn binds to the ISRE motifs in the genome DNA and subsequently drives the global transcription of ISGs to establish an antiviral status²⁸. Since IRF1 activates STAT1, we further investigated whether it can also trigger functional effects of STAT1 activation including ISG transcription. Indeed, IRF1 could significantly increase ISRE-regulated luciferase activity (Figure 4A) comparable to high dose of IFN α treatment on the Huh7-ISRE-Luc model. Furthermore, IRF1 was able to stimulate the expression of 17 tested ISGs at various levels (Figure 4B and Supplementary Figure 2D), whereas the control GFP or Fluc vectors did not affect their expression (Supplementary Figure 2E). The stimulation of ISGs by IRF1 was further confirmed in HepaRG, A549 and MRC-5 cells (Figure 4C, 4D and 4E). These results triggered us to further investigate whether these actions of IRF1 are totally via the JAK-STAT pathway.

Induction of ISGs and anti-HEV of IRF1 relies on STAT1 phosphorylation

JAK1 is the key upstream kinase that dictates STAT1 phosphorylation and the activation of interferon signaling. Activation of STAT1 phosphorylation by IFN α or IRF1 over-expression could be almost completely blocked by a pharmacological JAK inhibitor, JAK inhibitor 1 (Figure 5A). Similarly, JAK inhibitor 1 could significantly diminish IRF1 and IFN α induced ISRE promoter activation (Figure 5B) and ISG transcription (Figure 5C and 5D) without affecting vector-delivered IRF1 over-expression (Figure 5E) and cell viability (Figure 5F). In line with the abrogation of ISG induction, the anti-HCV and anti-HEV ability of IRF1 was also diminished by JAK inhibitor 1 in HCV replicon model, HEV subgenomic and full-length models (Figure 5G, 5H and 5I). To further validate our observations, another JAK inhibitor, CP-690550 (Tofacitinib) was used and similar results were obtained (Supplementary Figure 3A to 3H).

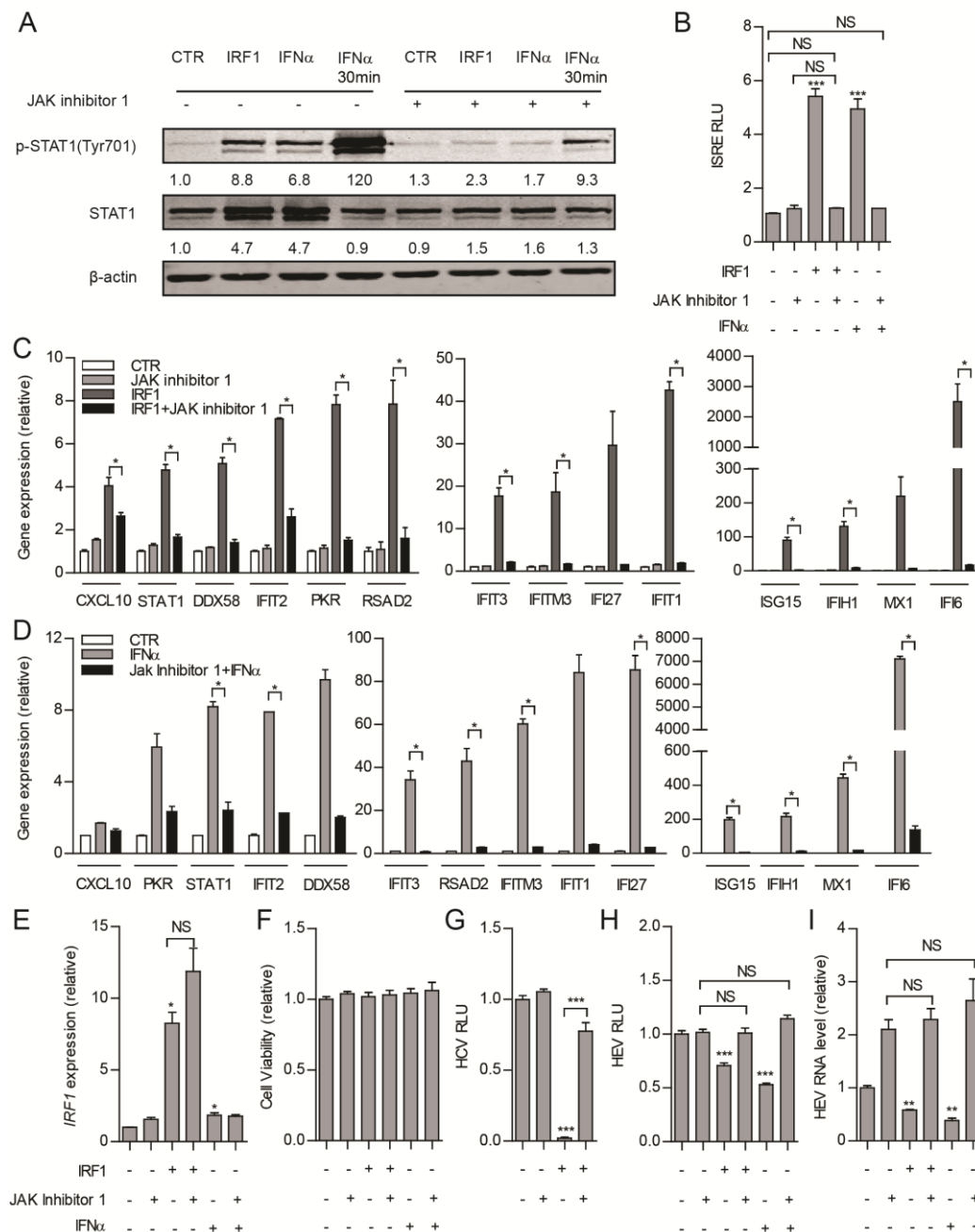


Figure 5. Inhibition of JAK1 diminishes the induction of ISG and the anti-HEV effect of IRF1. (A) Immunoblotting analysis of Huh7 cells transduced with IRF1 vector or treated with JAK inhibitor 1 (10 μ M) for 48 h or IFN α (1000 IU/mL) for 30 min or 48 h. (B) ISRE firefly luciferase activity in Huh7-ISRE-Luc model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or JAK inhibitor 1 (10 μ M) for 48 h (n = 4 independent experiments with each of 3-4 replicates). qRT-PCR analysis of ISG expression (C, n = 6; D, n = 3), *IRF1* (E) (n = 4) and MTT assay analysis of cell viability (F) (n = 3 independent experiments with each of 3-4 replicates) in Huh7 cell HEV model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or JAK inhibitor 1 (10 μ M) for 48 h. HCV viral replication-related firefly luciferase activity in Huh7-ET-Luc model (G, n = 3 independent experiments with each of 2-3 replicates) and HEV-related luciferase activity (H, n = 4 independent experiments with each of 3-4 replicates) as well as HEV viral RNA (I, n = 7) in Huh7-based cell HEV model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or JAK inhibitor 1 (10 μ M) for 48 h. Data was normalized to untreated GFP (B and G) or Fluc (C, D, E, F, H and I) control (CTR) (set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

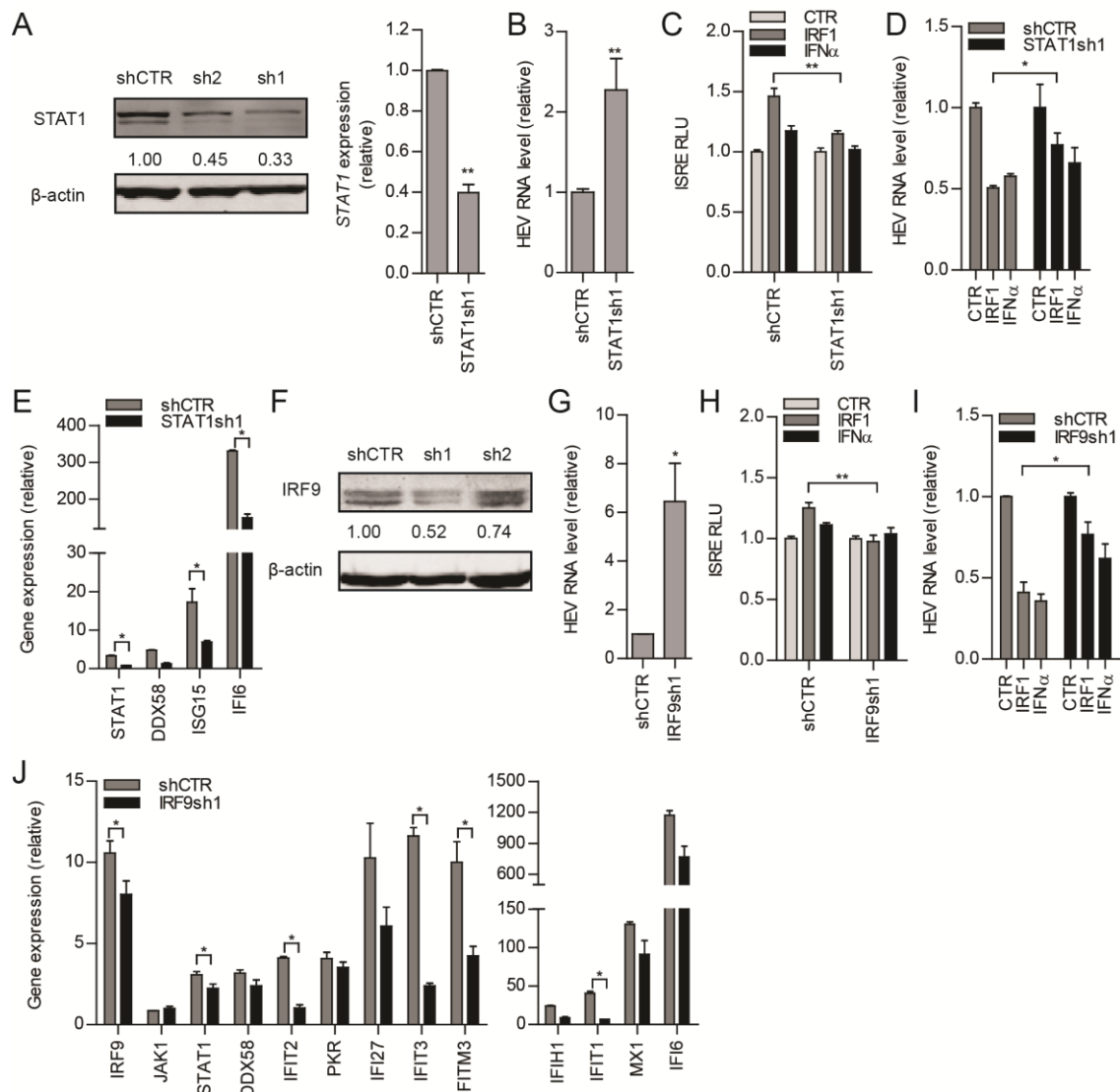


Figure 6. The antiviral function of IRF1 requires key components of the ISGF3 complex.

(A) Immunoblotting and qRT-PCR analysis ($n = 4$) of Huh7 cells transduced with lentiviral shRNA vectors that targeting STAT1 or non-targeted control vector (shCTR). (B) qRT-PCR analysis of HEV viral RNA in Huh7-p6 model with STAT1 knockdown relative to shCTR ($n = 4$). Data was normalized to shCTR control (A and B). (C) ISRE firefly luciferase activity in STAT1 knockdown Huh7-ISRE-Luc cells transduced with IRF1 vector (stock with 100 times dilution) or treated with IFN α (1 IU/mL) ($n = 3$ independent experiments with each of 4 replicates). (D) qRT-PCR analysis of HEV viral RNA in STAT1 knockdown Huh7-p6 model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h ($n = 6$). Data on (C) and (D) was normalized to untreated shCTR or STAT1sh1 cells, respectively (both set as 1). (E) qRT-PCR analysis of gene expression in STAT1 knockdown or shCTR cells transduced with IRF1 or Fluc vector for 48 h ($n = 4$). Data was normalized to shCTR cells that transduced Fluc vector (set as 1, not shown). (F) Immunoblotting analysis of Huh7 cells transduced with lentiviral shRNA vectors that targeting IRF9 or non-targeted control vector (shCTR). (G) qRT-PCR analysis of HEV viral RNA in Huh7-p6 model with IRF9 knockdown relative to shCTR ($n = 4$). (H) ISRE firefly luciferase activity in IRF9 knockdown Huh7-ISRE-Luc model transduced with IRF1 vector (stock with 100 times dilution) or treated with IFN α (1 IU/mL) ($n = 3$ independent experiments with each of 3 replicates). Data was normalized to untreated shCTR or IRF9sh1 cells, respectively (both set as 1). (I) qRT-PCR analysis of HEV viral RNA and gene expression in IRF9 knockdown Huh7-p6 model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h ($n = 5$). Data on (H, I and J) was normalized to untreated shCTR or IRF9sh1 cells, respectively (both set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

The anti-HEV ability of IRF1 also requires key components of the ISGF3 complex

To further investigate the antiviral ability of IRF1 related to JAK-STAT pathway, we studied the effects of ISGF3 complex, which is a downstream element of JAK-STAT cascade. ISGF3 complex consists of STAT1, STAT2 and IRF9 and mediates ISG transcription. Gene knockdown of STAT1 (Figure 6A) significantly promoted HEV replication (Figure 6B), suggesting a basal defense function of this pathway. Importantly, when STAT1 was silenced, IRF1 or IFN α induced ISRE promoter activation was significantly attenuated (Figure 6C). Consistently, ISG induction as well as anti-HEV effects of IRF1 were significantly attenuated in STAT1 silenced Huh7 cells (Figure 6D and 6E). Next, we silenced IRF9, another component of the ISGF3 complex (Figure 6F). Similarly, HEV replication was also significantly promoted when IRF9 was silenced (Figure 6G), also suggesting a basal anti-HEV function. Consistently, the depletion of IRF9 attenuated IRF1 induced ISRE promoter activation and ISG expression (Figure 6H and 6J). As expected, the antiviral ability of IRF1 or IFN α was also attenuated (Figure 6I). Collectively, these results suggest that ISGF3 complex is required for the induction of ISGs and the anti-HEV effect of IRF1.

The induction of ISGs and the anti-HEV effect of IRF1 overlaps with IFN α but is potentiated by ribavirin

We observed that the patterns of ISG induction by IFN α and IRF1 were highly correlated, suggesting a potential overlap of these two anti-HEV mechanisms (Figure 7A). Interestingly, gene knockdown of IRF1 did not impair IFN α mediated anti-HEV effect (Figure 7B) and ISG induction (Figure 7C and 7D). Furthermore, combination of IFN α and IRF1 over-expression did not yield additional induction of STAT1 and IRF1 expression (Figure 7E and 7F), nor the additional anti-HEV activity (Figure 7G and 7H). These results suggested that IFN α and IRF1 converged in the JAK-STAT pathway to exert anti-HEV effects.

A previous study showed that ribavirin could potentiate antiviral interferon response by augmenting ISG induction²⁹. Thus, we tested the combination effect of ribavirin and IRF1 over-expression on ISGs induction. Consistent with this study, we observed that ribavirin alone could already up-regulate several ISGs. Interestingly, in contrast to IFN α , ribavirin further promoted IRF1-induced ISG expression including IRF1 in Huh7 cells (Figure 8A).

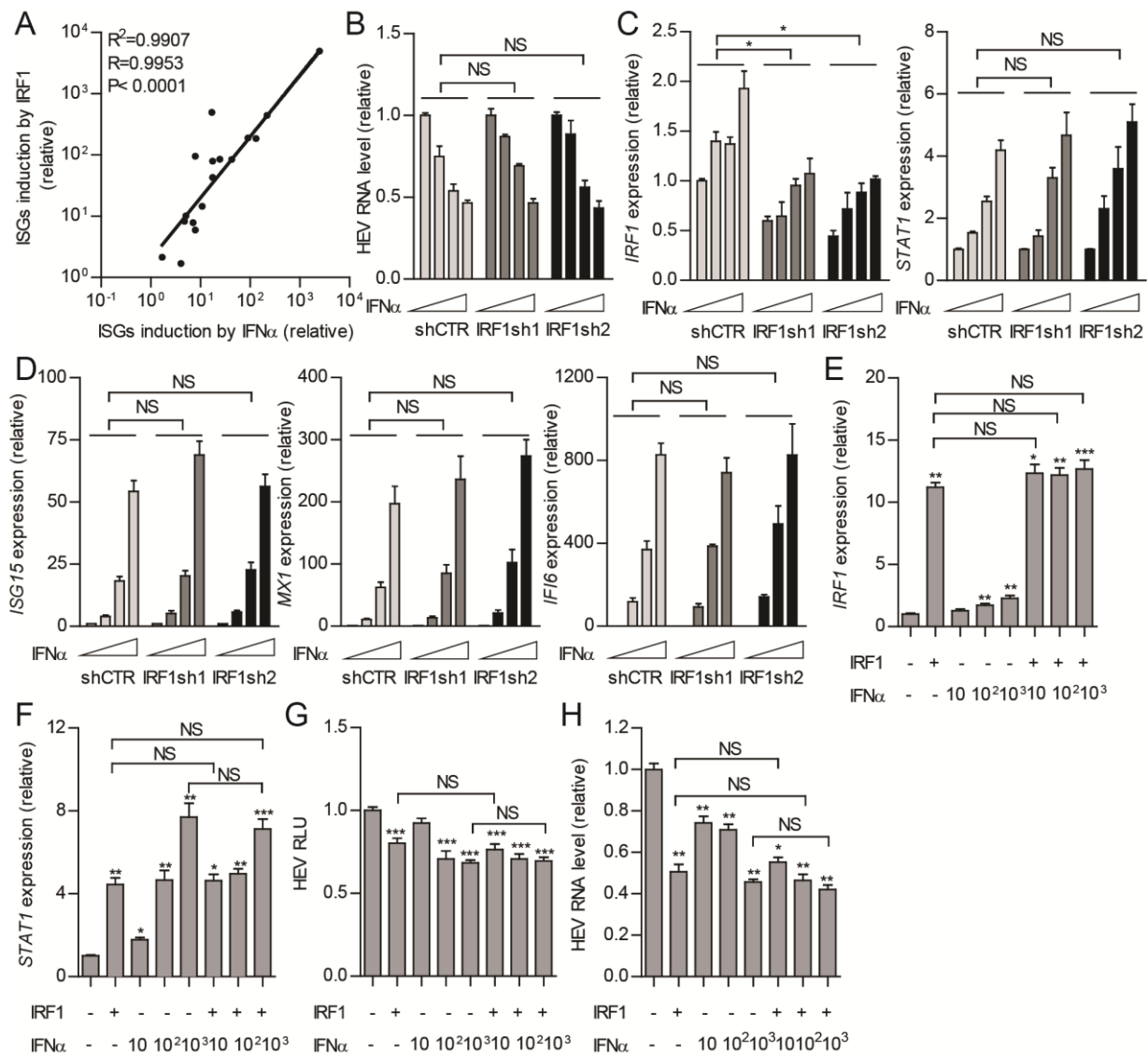


Figure 7. The induction of ISGs and the anti-HEV action of IRF1 overlaps with IFN α . (A) Correlation analysis of ISG expression in Huh7 cells transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h. Data was normalized to Fluc control (set as 1). Data was analyzed by the two-tailed Pearson correlation method. (B) qRT-PCR analysis of HEV viral RNA in IRF1 knockdown cells treated with IFN α (10, 100 or 1000 IU/mL) for 48 h (shCTR: $n = 4$; IRF1sh1 and IRF1sh2: $n = 3$). Data was normalized to untreated shCTR, IRF1sh1 or IRF1sh2 cells, respectively (all set as 1). qRT-PCR analysis of *IRF1* (C), *STAT1* (C), *ISG15* (D), *MX1* (D) and *IFI6* (D) expression in IRF1 knockdown Huh7 cells treated with IFN α (10, 100 or 1000 IU/mL) for 48 h ($n = 3$). *IRF1* expression was normalized to untreated shCTR (set as 1). *STAT1*, *ISG15*, *MX1* and *IFI6* expression was normalized to untreated shCTR, IRF1sh1 or IRF1sh2 cells, respectively (all set as 1). qRT-PCR analysis of *IRF1* (E, $n = 5$), *STAT1* (F, $n = 5$) and HEV viral RNA (H, $n = 6$) expression in Huh7-p6 model and HEV-related luciferase activity ($n = 3$ independent experiments with each of 2-4 replicates) in Huh7-p6-Luc model transduced with IRF1 vector or treated with different doses of IFN α (10, 100 and 1000 IU/mL) for 48 h. Data on (E, F, G and H) was normalized to Fluc control without IFN α treatment (set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

Furthermore, ribavirin could also enhance the ISG induction and anti-HEV ability of IRF1 in HepaRG, A549 and MRC5 cell (Supplementary Figure 4A, 4B and 4C). More importantly, the combination of ribavirin and IRF1 further augmented their anti-HCV and anti-HEV effects (Figure 8B, 8C and 8D). Overall, these results indicated that the induction of ISGs and the anti-HEV effect of IRF1 overlapped with IFN α but was potentiated by ribavirin.

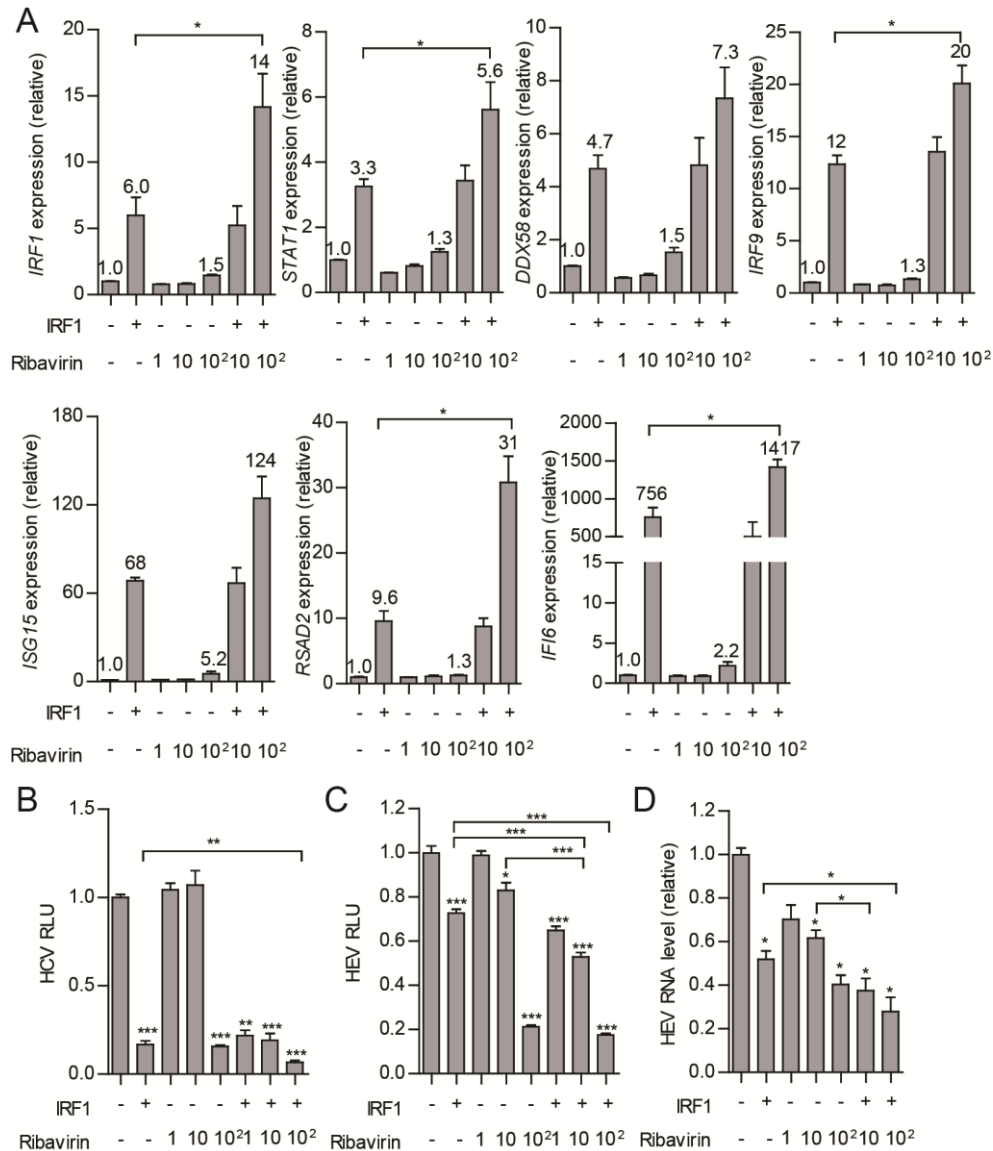


Figure 8. Ribavirin potentiates IRF1-mediated ISG induction and anti-HEV activity. (A) qRT-PCR analysis of *IRF1*, *STAT1*, *DDX58*, *RSAD2*, *ISG15*, *IRF9* and *IFI6* expression in Huh7 cells transduced with IRF1 vector or treated with ribavirin (1, 10 or 100 μ M) for 48 h (n = 5). (B) HCV-related luciferase activity in Huh7-ET-Luc model and transduced with IRF1 vector (stock with 50 times dilution) or treated with ribavirin (1, 10 or 100 μ M) for 48 h (n = 3 independent experiments with each of 4 replicates). (C) HEV-related luciferase activity in Huh7-p6-Luc model and transduced with IRF1 vector or treated with ribavirin (1, 10 or 100 μ M) for 48 h (n = 3 independent experiments with each of 4 replicates) and qRT-PCR analysis of HEV viral RNA in Huh7-p6 model transduced with IRF1 vector or treated with ribavirin for 48 h (n = 5). Data was normalized to GFP (B) or Fluc (A, C and D) control without ribavirin treatment (set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

Discussion

Innate immunity is the frontier against viral pathogens³⁰. The activation of innate immune response relies on the recognition of pathogens by specific pattern recognition receptors (e.g. Toll-like receptors, RIG-I-like receptors, NOD-like receptors)³¹, leading to the production of cytokines and chemokines such as interferons. Interferon triggers transcription of hundreds of ISGs through the JAK-STAT pathway. The products of these ISGs ultimately exert antiviral functions³⁰. Several recent studies have focused on the characterization of individual ISGs, with respect to their antiviral efficacy and potential mechanism-of-action³²⁻³⁴. Surprisingly, two large-scale screening studies found out that actually only a small subset of ISGs have antiviral abilities and some even have proviral effects on certain viruses^{10, 11}. Among more than 380 tested ISGs, IRF1 was one of the strongest antiviral ISGs against a broad spectrum of viruses^{10, 11}. In this study, we reported that IRF1 is basally expressed in liver cells and confers resistance to HEV infection. Ectopic over-expression of IRF1 effectively inhibits HEV replication as shown in multiple cellular systems.

Although IRF1 was reported could induce the expression of some ISGs similar to type I interferons^{10, 11}, the exact antiviral mechanism of IRF1 remains largely elusive. It has been reported that IRF1 could act as a transcription factor that activates either IFN β gene expression in virus-infected fibroblasts²⁴ or IFN α gene expression in un-infected cells³⁵. Therefore, we initially hypothesized that the anti-HEV effect of IRF1 might via the induction of interferon production in the cell model. However, we convincingly demonstrated that HEV cell models including human hepatoma cell line (Huh7), primary human hepatocytes-like cell line (HepaRG), human lung epithelial carcinoma cell line (A549) and human fetal lung fibroblast cell line (MRC-5) are not capable of producing interferons upon IRF1 over-expression by both gene expression and functional assays. Our observation in this respect is consistent with a previous study showing that Huh7 cell line responds to interferon but does not produce interferon³⁶. Furthermore, microarray analysis has shown that no interferon genes were up-regulated in IRF1 over-expressed Huh7 cells¹¹.

IFN α was widely used to treat chronic HBV and HCV infection²⁸. In some cases, IFN α was also used to treat chronic HEV infection⁹. In our previous study, we demonstrated that IFN α had moderate and delayed anti-HEV effects in cell culture models and IFN β / λ / γ did not

show a notable anti-viral effect on HEV replication³⁷. Similarly, Todt, D. *et al.* also reported a weak to moderate inhibition of HEV replication by different types of IFN³⁸. Interestingly, in this study we demonstrated that IRF1 could effectively inhibit HEV replication without triggering interferon production in host cells. This finding may provide new ideas for developing anti-HEV strategies. Surprisingly, both studies demonstrated that HEV could down-regulate ISG expression induced by different IFN types^{37, 38}. These observations suggest HEV have developed some strategies to subvert host antiviral defenses. Consistently, we also observed that IRF1 was induced at the early stage in most cell lines (Supplemental Figure 2A and 2B). This result indicates after the infection was established, HEV have the ability to suppress the immune response elicited by itself.

Given the fact that IRF1 is also a transcription factor and has more than 200 binding sites in the human genome²⁷, we have explored this scenario to understand its anti-HEV mechanism-of-action. Indeed, the IRF1 ChIP-seq data retrieved from database revealed that IRF1 could directly bind to the promoter region of STAT1 gene. Consistently, our functional assay demonstrated that IRF1 could drive the transcription of STAT1, resulting in protein expression and phosphorylation at Tyr701 site, which is an indispensable marker of STAT1 activation. Correspondingly, the IRF1 induced STAT1 phosphorylation leading to the transcription of a series of individual ISGs. We further showed that ISG induction and anti-HEV ability of IRF1 heavily rely on STAT1 phosphorylation. Furthermore, the integrity of the ISFG3 complex is also required. Although previous study has reported the induction of some ISGs in STAT1 deficient fibroblasts by IRF1¹¹, this may indicate that there are multiple mechanisms mediating the function of IRF1, including STAT1 dependent or independent mechanisms, probably depending on the cell type and particular circumstances. In this study, we reported a new antiviral mechanism of IRF1 by inducing the expression and phosphorylation STAT1 without triggering interferon production. This subsequently activates the JAK-STAT pathway to transcribe antiviral ISGs. It would be interesting to also address the relevance of this mechanism in IRF1-mediated effects on other viruses.

Since IRF1 and IFN α converged in the JAK-STAT cascade to drive ISG transcription, we further evaluated the combinatory effects of IFN α and IRF1. As expected, combination of IFN α and IRF1 did not further promote ISG induction and anti-HEV activities. In contrast, IFN α and ribavirin have moderately synergistic anti-HEV effects in two HEV cell culture replication models³⁹. As a broad antiviral agent⁴⁰, ribavirin can potentiate interferon by

augmenting ISG induction in HCV culture model, which is mediated by a novel mechanism different from the classical interferon or intracellular RNA sensing pathways²⁹. In this study, we also tested the combinational effects of IRF1 and ribavirin. Interestingly, ribavirin further enhanced the IRF1 induced ISGs expression. More importantly, combination of IRF1 and ribavirin could reinforce ISG induction and their anti-HEV effects, although the exact mechanism remains to be further investigated.

In conclusion, as Figure 9 shown, we characterized IRF1 as an important host factor that effectively inhibited HEV replication. Mechanistically, without triggering interferon production in host cells, IRF1 activates gene transcription of STAT1, which subsequently enhances its protein expression and phosphorylation to stimulate antiviral ISG transcription. Furthermore, the induction of ISGs and the anti-HEV effect of IRF1 overlapped with IFN α but were potentiated by ribavirin. Thus, this study has shed new light on the molecular insight into an important anti-HEV ISG, which may help to understand the complicity of HEV-host interactions and to develop new antiviral strategies.

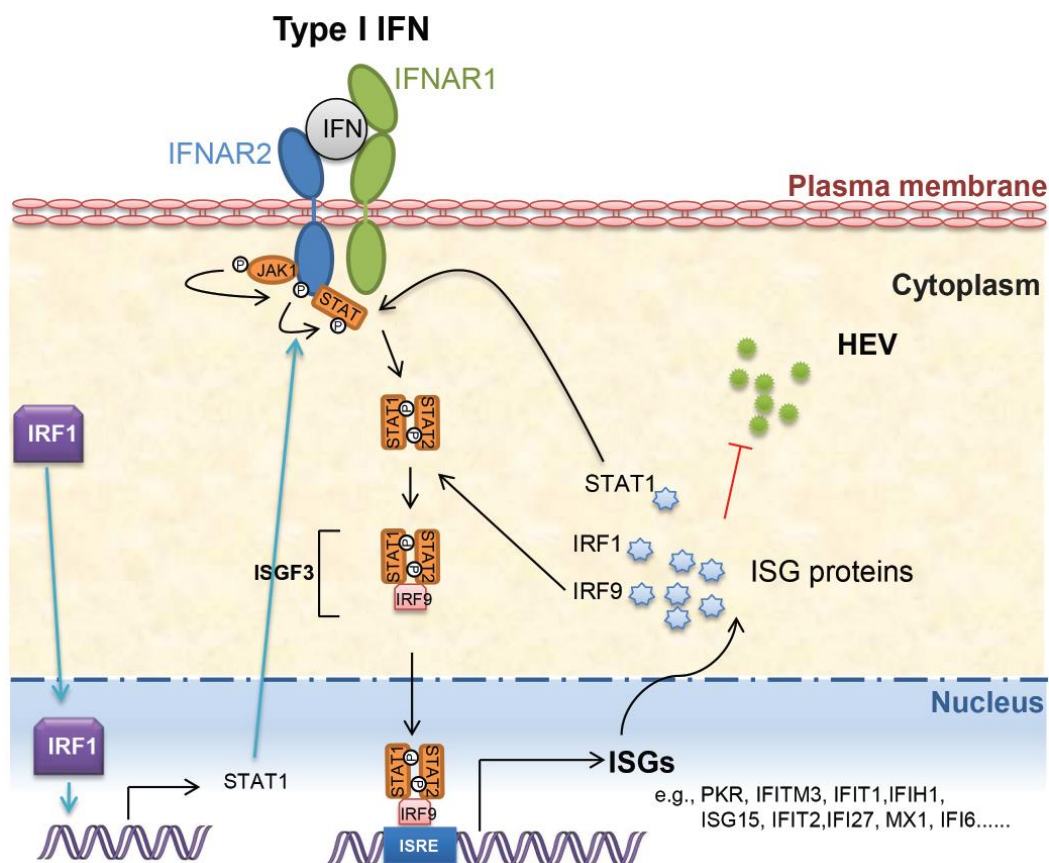
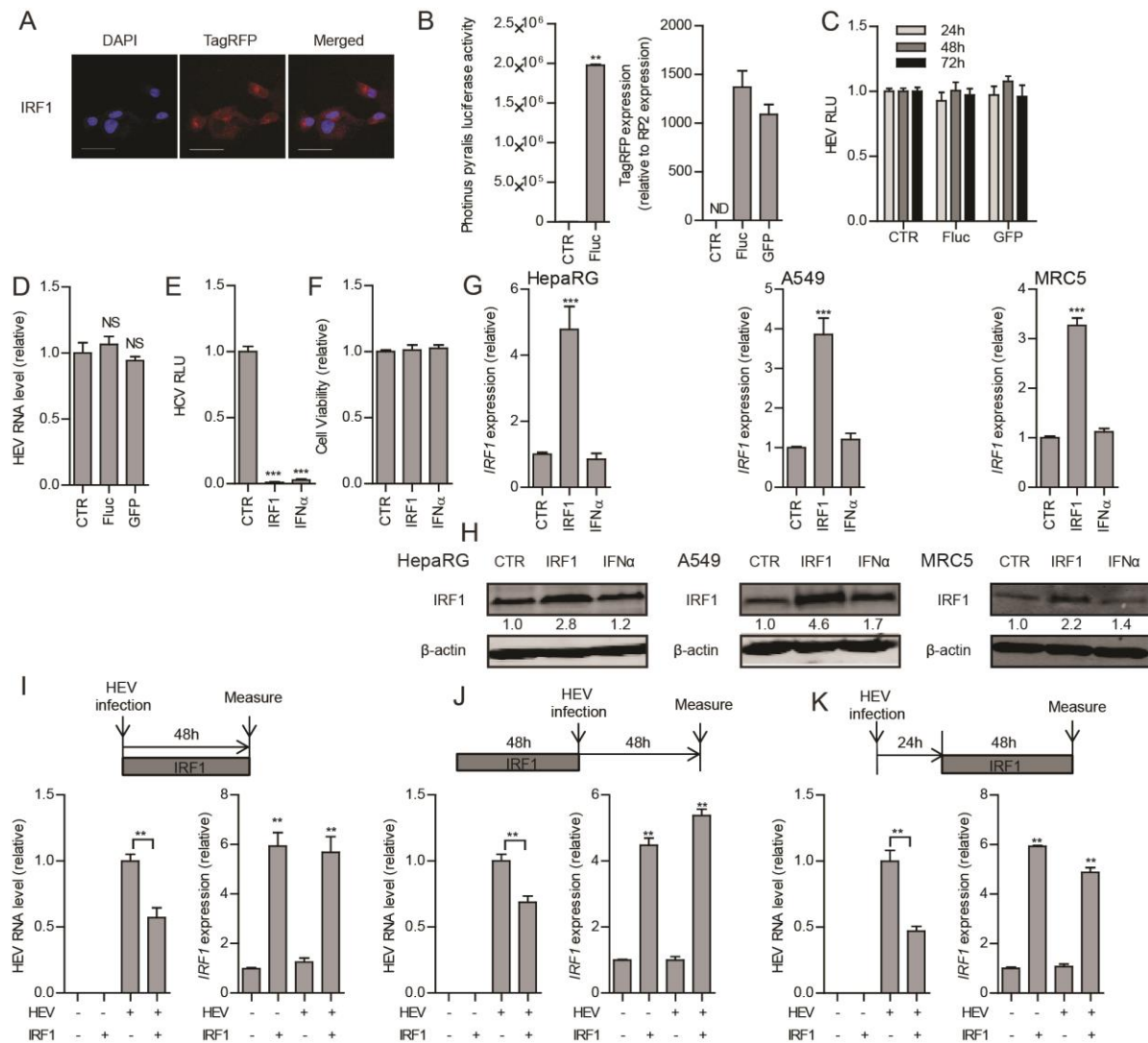
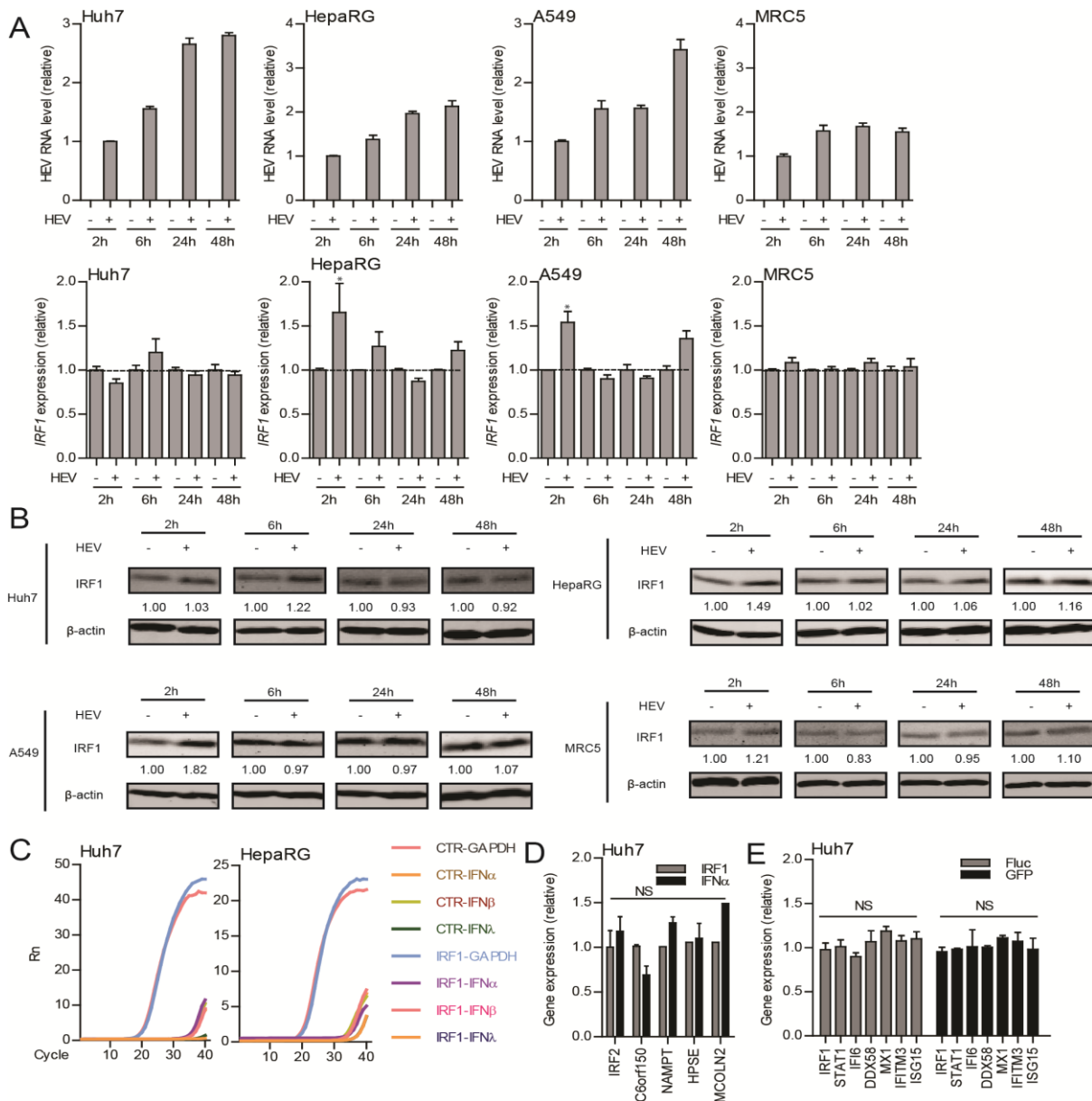


Figure 9. IRF1 restricts HEV replication by activating STAT1 to induce the expression of ISGs. IRF1 could induce the expression of STAT1. The induction of STAT1 expression further activated the transcription of a panel of downstream antiviral ISGs. The production of these ISGs could inhibit HEV replication.

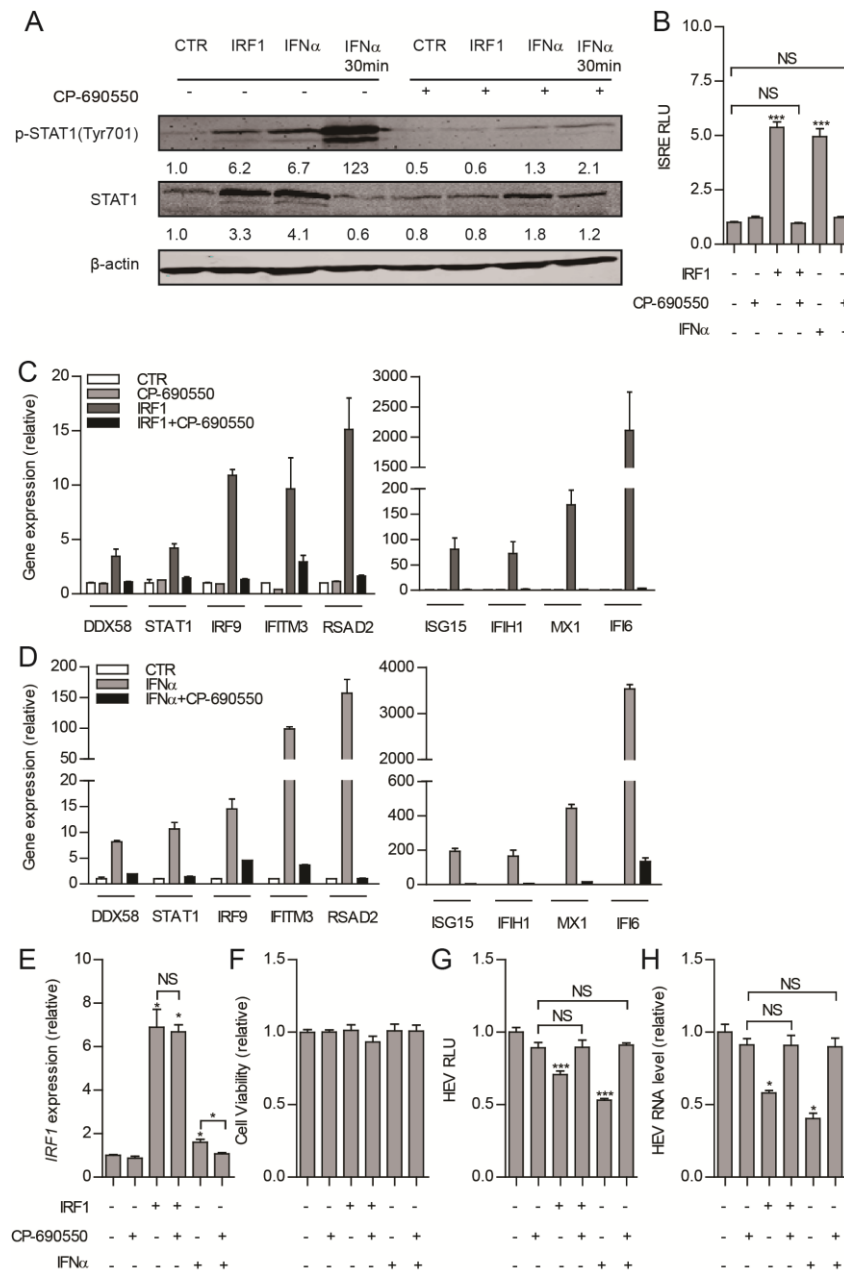
Supplementary Figures



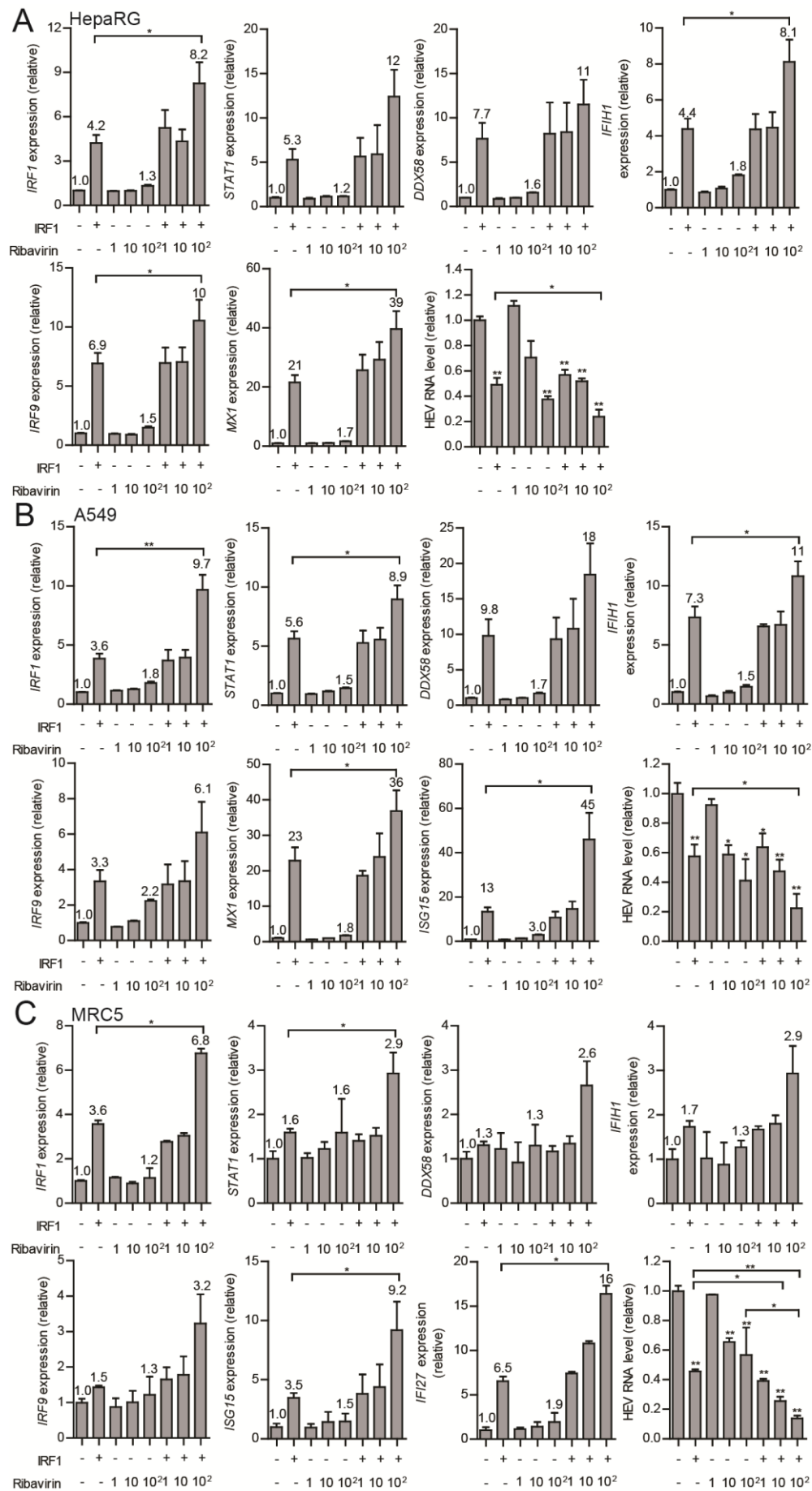
Supplementary Figure 1. Over-expression of control vectors does not affect HEV replication. (A) Fluorescent microscopic analysis of Huh7 cells transduced with TagRFP-IRF1 lentiviral vector for 48 h. Red indicates TagRFP and blue indicates DAPI (Scale bar: 50 μ m). (B) *Photinus pyralis* luciferase activity (n = 4-8) and qRT-PCR analysis of TagRFP mRNA expression (n = 3) in Huh7 cells transduced with Fluc, GFP vector or untreated (CTR) for 48 h. HEV viral replication-related *Gaussia* luciferase activity in Huh7-p6-Luc model (C) transduced with GFP, Fluc vector or untreated (CTR) for 24 h, 48 h and 72 h (n = 3 independent experiments with each of 2-3 replicates) and qRT-PCR analysis of HEV viral RNA in Huh7-p6 full-length HEV model (D) transduced for 48 h (n = 3). RLU: relative luciferase unit. HCV-related firefly luciferase activity in Huh7-ET-Luc model (E) (n = 4 independent experiments with each of 3-4 replicates) and MTT assay analysis of cell viability in Huh7 cells (F) (n = 3 independent experiments with each of 3-4 replicates) transduced with IRF1, GFP vector or treated with IFN α (1000 IU/mL) for 48 h. qRT-PCR analysis (G, n = 6) or immunoblotting analysis (H) of HepaRG cells, A549 cells and MRC5 cells transduced with IRF1, Fluc vector or treated with IFN α (1000 IU/mL) for 48 h. (I, J, K) qRT-PCR analysis of HEV and IRF1 expression in Huh7 cells transduced with IRF1 or infected by HEV at indicated time point. Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).



Supplementary Figure 2. HEV infection induces IRF1 expression in some cell lines. qRT-PCR analysis (A, n = 5) and immunoblotting analysis (B) of Huh7, HepaRG, A549 and MRC5 cells infected by HEV for 6h (For 2 h post infection, cells were incubated with HEV for 2 hours and then cells were lysed). At different time point post HEV infection (2 h, 6 h, 24 h or 48 h post infection), HEV RNA and IRF1 expression level was quantified. HEV RNA level was normalized to 2 h post infection. IRF1 mRNA level and protein level was normalized to un-infected cells at each time point. (C) Plot of qRT-PCR analysis of interferon gene expression in Huh7 and HepaRG cells transduced with IRF1 or Fluc (CTR) vector for 48 h. Rn: Fluorescence signal from the reporter dye normalized to that from the negative control. (D) qRT-PCR analysis of gene expression in Huh7 cells transduced with IRF1 vector or treated with IFN α (1000 IU/mL) for 48 h. (E) qRT-PCR analysis of gene expression in Huh7 cells transduced with GFP, Fluc vector or untreated for 48 h (n = 3). Data was normalized to untreated control (CTR, set as 1, not shown). Data presented as mean \pm SEM (*, $P < 0.05$; NS, not significant).



Supplementary Figure 3. JAK inhibitor, CP-690550 diminishes the induction of ISG and the anti-HEV effect of IRF1. (A) Immunoblotting analysis of Huh7 cells transduced with IRF1 vector or treated with CP-690550 (1000 ng/mL) for 48 h or IFN α (1000 IU/mL) for 30 min or 48 h. (B) ISRE firefly luciferase activity in Huh7-ISRE-Luc model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or CP-690550 (1000 ng/mL) for 48 h (n = 4 independent experiments with each of 3-4 replicates). qRT-PCR analysis of ISG expression (C, n = 4; D, n = 3), *IRF1* (E, n = 5) and MTT assay analysis of cell viability (F, n = 3 independent experiments with each of 3-4 replicates) in Huh7 cell HEV model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or CP-690550 (1000 ng/mL) for 48 h. HEV-related luciferase activity (G, n = 4 independent experiments with each of 4 replicates) and HEV viral RNA (H, n = 6) in Huh7-based cell HEV model transduced with IRF1 vector or treated with IFN α (1000 IU/mL) or CP-690550 (1000 ng/mL) for 48 h. Data was normalized to untreated GFP (B) or Fluc (C, D, E, F and H) control (CTR, set as 1). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).



Supplementary Figure 4. Ribavirin potentiates IRF1-mediated ISG induction and anti-HEV activity in different cell lines. qRT-PCR analysis of *IRF1*, *STAT1*, *DDX58*, *IFIH1*, *ISG15*, *IRF9*, *IFI27*, *MX1* expression and HEV viral RNA level in HepaRG-p6 model (A), A549-p6 model (B) and HEV infected MRC5 cells (C) transduced with IRF1 vector or treated with ribavirin (1, 10 or 100 μ M) for 48 h (n = 4-6). Data presented as mean \pm SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

Reference

1. Dalton HR. Hepatitis: hepatitis E and decompensated chronic liver disease. *Nat Rev Gastroenterol Hepatol* **9**, 430-432 (2012).
2. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
3. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
4. Behrendt P, Steinmann E, Manns MP & Wedemeyer H. The impact of hepatitis E in the liver transplant setting. *J Hepatol* **61**, 1418-1429 (2014).
5. Melchjorsen J. Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity - clues for treatments and vaccines. *Viruses* **5**, 470-527 (2013).
6. Sen GC. Viruses and interferons. *Annual review of microbiology* **55**, 255-281 (2001).
7. Fried MW, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**, 975-982 (2002).
8. Janssen HL, *et al.* Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* **365**, 123-129 (2005).
9. Peters van Ton AM, Gevers TJ & Drenth JP. Antiviral therapy in chronic hepatitis E: a systematic review. *Journal of viral hepatitis* **22**, 965-973 (2015).
10. Schoggins JW, *et al.* Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* **505**, 691-695 (2014).
11. Schoggins JW, *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* **472**, 481-485 (2011).
12. Xie M, *et al.* Human cytomegalovirus exploits interferon-induced transmembrane proteins to facilitate morphogenesis of the virion assembly compartment. *J Virol* **89**, 3049-3061 (2015).
13. Gripon P, *et al.* Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* **99**, 15655-15660 (2002).
14. Pan Q, *et al.* Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* **87**, 713-722 (2009).
15. Pan Q, *et al.* Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* **55**, 1673-1683 (2012).
16. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
17. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
18. Dong C, *et al.* Suppression of interferon-alpha signaling by hepatitis E virus. *Hepatology* **55**, 1324-1332 (2012).
19. Debing Y, *et al.* A mutation in the hepatitis E virus RNA polymerase promotes its replication and associates with ribavirin treatment failure in organ transplant recipients. *Gastroenterology* **147**, 1008-1011 e1007; quiz e1015-1006 (2014).

20. Zhou X, *et al.* Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* **61**, 746-754 (2014).
21. Engelen E, *et al.* Proteins that bind regulatory regions identified by histone modification chromatin immunoprecipitations and mass spectrometry. *Nature communications* **6**, 7155 (2015).
22. Zhang Y, *et al.* Model-based analysis of ChIP-Seq (MACS). *Genome biology* **9**, R137 (2008).
23. Robinson JT, *et al.* Integrative genomics viewer. *Nature biotechnology* **29**, 24-26 (2011).
24. Miyamoto M, *et al.* Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. *Cell* **54**, 903-913 (1988).
25. Hornung V, *et al.* 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994-997 (2006).
26. Frese M, *et al.* Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. *J Gen Virol* **84**, 1253-1259 (2003).
27. Frontini M, Vijayakumar M, Garvin A & Clarke N. A ChIP-chip approach reveals a novel role for transcription factor IRF1 in the DNA damage response. *Nucleic Acids Res* **37**, 1073-1085 (2009).
28. Schneider WM, Chevillotte MD & Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* **32**, 513-545 (2014).
29. Thomas E, *et al.* Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. *Hepatology* **53**, 32-41 (2011).
30. Schoggins JW & Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* **1**, 519-525 (2011).
31. O'Neill LA & Bowie AG. Sensing and signaling in antiviral innate immunity. *Curr Biol* **20**, R328-333 (2010).
32. Jiang D, *et al.* Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. *J Virol* **82**, 1665-1678 (2008).
33. Brass AL, *et al.* The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* **139**, 1243-1254 (2009).
34. Liu SY, Sanchez DJ, Aliyari R, Lu S & Cheng G. Systematic identification of type I and type II interferon-induced antiviral factors. *Proc Natl Acad Sci U S A* **109**, 4239-4244 (2012).
35. Fujita T, *et al.* Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. *Proc Natl Acad Sci U S A* **86**, 9936-9940 (1989).
36. Keskinen P, *et al.* Impaired antiviral response in human hepatoma cells. *Virology* **263**, 364-375 (1999).
37. Zhou X, *et al.* Disparity of basal and therapeutically activated interferon signalling in constraining hepatitis E virus infection. *J Viral Hepat* **23**, 294-304 (2016).
38. Todt D, *et al.* Antiviral Activities of Different Interferon Types and Subtypes against Hepatitis E Virus Replication. *Antimicrobial agents and chemotherapy* **60**, 2132-2139 (2016).
39. Debing Y, *et al.* Ribavirin inhibits in vitro hepatitis E virus replication through depletion of cellular GTP pools and is moderately synergistic with alpha interferon. *Antimicrobial agents and chemotherapy* **58**, 267-273 (2014).
40. Feld JJ & Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **436**, 967-972 (2005).

Chapter 9

Requirement of the eukaryotic translation initiation factor 4F complex in hepatitis E virus replication

Xinying Zhou, Lei Xu, Yijin Wang, Wenshi Wang, Dave Sprengers, Herold J. Metselaar,
Maikel P. Peppelenbosch and Qiuwei Pan

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center,
Rotterdam, Netherlands

Abstract

Hepatitis E virus (HEV) infection, one of the foremost causes of acute hepatitis, is becoming a health problem of increasing magnitude. As other viruses, HEV exploits elements from host cell biochemistry, but we understand little as to which components of the human hepatocellular machinery are perverted for HEV multiplication. It is, however, known that the eukaryotic translation initiation factors 4F (eIF4F) complex, the key regulator of the mRNA-ribosome recruitment phase of translation initiation, serves as an important component for the translation and replication of many viruses. Here we aim to investigate the role of three subunits of the eIF4F complex: eukaryotic translation initiation factor 4A (eIF4A), eukaryotic translation initiation factor 4G (eIF4G) and eukaryotic translation initiation factor 4E (eIF4E) in HEV replication. We found that efficient replication of HEV requires eIF4A, eIF4G and eIF4E. Consistently, the negative regulatory factors of this complex: programmed cell death 4 (PDCD4) and eIF4E-binding protein 1 (4E-BP1) exert anti-HEV activities, which further illustrates the requirement for eIF4A and eIF4E in supporting HEV replication. Notably, phosphorylation of eIF4E induced by MNK1/2 activation is not involved in HEV replication. Although ribavirin and interferon- α (IFN- α), the most often-used off-label drugs for treating hepatitis E, interact with this complex, their antiviral activities are independent of eIF4E. In contrast, eIF4E silencing provokes enhanced anti-HEV activity of these compounds. Thus, HEV replication requires eIF4F complex and targeting essential elements of this complex provides important clues for the development of novel antiviral therapy against HEV.

Introduction

Hepatitis E virus (HEV), a single-strand-positive RNA virus classified within the genus *Hepevirus* in the family *Hepeviridae*, represents the most common cause of acute viral hepatitis¹. Like all viruses, HEV is completely dependent on the translational machinery of host cells to synthesize the viral proteins essential for its productive infection². The host protein synthesis machinery commandeered by viruses has major impact on viral protein synthesis and genome replication³, but little is known regarding how HEV uses host translational machinery for its life-cycle.

As a heterotrimeric protein complex, eukaryotic translation initiation factor 4F (eIF4F) mediates recruitment of ribosomes to mRNA and is the rate-limiting step for cap-dependent translation in viruses and cells under most circumstances⁴. Functions of the constituent proteins of eIF4F include delivery of an RNA helicase eukaryotic initiation translation factor 4A (eIF4A) to the 5' region, bridging mRNA and ribosome by eukaryotic initiation translation factor 4G (eIF4G) scaffolding protein and recognition of the mRNA 5' cap structure by eukaryotic initiation translation factor 4E (eIF4E) cap-binding protein⁴. Not surprisingly, all these translation initiation factors are required for various types of viruses during their translation and replication^{2, 5-7}. In addition, eIF4E phosphorylation is induced by the eIF4G-associated kinase MNK1 to facilitate eIF4F assembly⁸. This process of translational control has been reported to be critical for the efficient viral infection^{2, 9, 10}. Furthermore, other cellular regulatory proteins of eIF4F complex such as eukaryotic translation initiation factor 4B (eIF4B)¹¹, programmed cell death 4 (PDCD4)¹² and eIF4E-binding protein 1 (4E-BP1) have been reported vital for viral protein synthesis¹³⁻¹⁵. HEV, however, has not been investigated in this context and it is currently unknown whether the virus requires eIF4F complex for efficient replication.

Interestingly, the eIF4F complex can interact with antiviral regimens, such as ribavirin or interferon- α (IFN- α), which are the classical standard therapy of chronic hepatitis C but also as off-label drugs for treating individual HEV cases or small case series^{16, 17}. Ribavirin can directly bind to eIF4E and compete for 5' cap mRNA binding^{18, 19}, whereas some regulatory factors of eIF4F complex are involved in interferon mediated antiviral immune response^{20, 21}. In absence, however, of information as to requirement of HEV for elements of the host translational machinery it is impossible to make statements whether ribavirin exerts

its anti-HEV action through inhibition of the eIF4F complex or whether alternative mechanisms are involved.

The lack of knowledge as to the requirements made by HEV on the hepatocellular host cell machinery with respect to translation of viral gene products represents a major gap in our understanding of the biology of this virus and hampers design of rational treatment. Therefore, this study has investigated the role of the eIF4F complex and its regulatory factors in HEV replication, as well as their potential involvements in the anti-HEV actions of ribavirin and IFN- α .

Materials and methods

Reagents

Compound GCP57380 as Mnk1 inhibitor (> 98% purity) was purchased from Abcam Biochemicals (UK). Ribavirin was purchased from Sigma-Aldrich (St Louis, MO). Human IFN- α (Thermo Scientific, the Netherlands) was dissolved in PBS. Doxycycline hyclate (\geq 98% TLC) was purchased from Sigma-Aldrich (St Louis, MO). Stocks of Jak inhibitor I (Santa Cruz Biotech, Santa Cruz, CA) was dissolved in DMSO (Sigma-Aldrich, St Louis, MO) with a final concentration of 5 mg/mL. Antibodies including total-eIF4E, phosphor-eIF4E, total-4E-BP1 (Cell Signalling Technology, Netherlands) and β -actin (Santa Cruz Biotech, Santa Cruz, CA); anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were also used.

Cell culture and cell models

Naïve or vector transduced HuH7 cells was established from a hepatocellular carcinoma, immortalized mouse embryonic fibroblasts (MEFs) derived from wild-type and 4E-BP1 knock-out (4E-BP1-/-) mice (kind gifts from E.N. Fish's lab), eIF4E-S209A MEFs containing an eIF4E mutation in which eIF4E cannot be phosphorylated (kind gift from Dr Sonenberg's lab, McGill University) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, Netherlands) complemented with 10% v/v fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). Authentication of cell line was performed at the Department of Pathology, Erasmus MC and

regular testing for mycoplasma contamination was performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC.

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-Luc), using the Ambion MESSAGE MACHINE in vitro RNA transcription Kit (Life Technologies Corporation)^{22,23}. The human hepatoma 7 (HuH7) cells were collected and centrifuged for 5 min, 1500 rpm, 4°C. Supernatant was removed and washed with 4 mL Opti-MEM by centrifuging for 5 min, 1500 rpm, 4°C. The cell pellet was re-suspended in 100 µl Opti-MEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 V, pulse length 0.5, number 1 and cuvette 4 mm)²². All cells were grown at 37 °C, 5% CO₂, and 100% humidity.

Gene knockdown and overexpression by lentiviral vector

Lentiviral vectors of shRNA (Sigma-Aldrich) targeting eIF4A, eIF4G, eIF4B, PDCD4, eIF4E, 4E-BP1 and controls were obtained from the Erasmus Center for Biomix and produced in HEK 293T cells as previously described²⁴. Three types of control vectors have been tested on HEV replication (CTR1: Control that will not activate the RNAi pathway because the vector does not contain an shRNA insert, CTR2: Control that will activate RISC and the RNAi pathway, but does not target any human or mouse genes. The short hairpin sequence contains 5 bp mismatches and scrambled sequences to any known human or mouse gene, CTR3: Control contains shRNA sequence that targets GFP reporter that is not expressed in our cell lines. Since no off-target effect was observed (Supplementary Figure 1), the most advanced shRNA control vector targeting GFP (GFP is not expressed in our cell lines) was used in this study as control (shCTR). After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences were described in Supplementary Table 1. To generate gene knockdown cells, HuH7 cells were transduced with lentiviral vectors. Since the knockdown vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 3 µg/mL puromycin to the cell culture medium. Overexpression of 4E-BP1 lentivector (Addgene) was a kind gift from Dr Sonenberg's lab, McGill University. To generate overexpression cells, HuH7 cells were transduced with

lentiviral vectors and doxycycline was used to add in the 4E-BP1 overexpression cell lines as the stimulation factor.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA was quantified with a SYBR Green-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH or β -actin was considered as reference gene to normalize gene expression. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense). Other human and mouse primer sequences were included in Supplementary Table 2.

Western blot assay

Proteins in cell lysates were heated 5 min at 95 °C followed by loading onto a 10-15% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis. After 90 min running at 100 V, proteins were electrophoretically transferred onto a polyvinylidenedifluoride (PVDF) membrane (Invitrogen) for 1.5 hrs with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer. It was followed by incubation with rabbit t-eIF4E, p-eIF4E, t-4E-BP1 (1:1000) antibodies overnight at 4 °C. The membrane was washed 3 times followed by incubation for 1 hrs with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for β -actin content as standardization of sample loading, scanned, and quantified by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantitated with Odyssey 3.0 software.

Measurement of luciferase activity

For Gaussia luciferase, the activity of secreted luciferase in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM, Sigma) was added to cells and incubated for 30

min at 37 °C. Both *gaussia* and firefly Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Statistical analysis

All results were presented as mean \pm SEM. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a p value less than 0.05 *or 0.01 **.

Results

Requirements of eIF4A and eIF4G for efficient HEV replication

Most of cellular and viral mRNAs rely on cap-dependent mRNA translation. The canonical mechanism of initiation commences with recognition of 5' end m⁷GpppN cap structure by the eIF4F complex formed by the DEAD-box helicase eIF4A, the scaffolding protein eIF4G and the cap recognition factor eIF4E ²⁵. Among these initiation factors, eIF4A is a subunit in charge of unwinding of secondary structure within the leader sequence of mRNA, while large scaffolding subunit eIF4G is associated with many other translation initiation factors ⁷. Because of the important roles of both eIF4A and eIF4G subunits reported in translation and replication of many viruses, we investigated their roles in HEV replication.

Firstly, we evaluated the role of the DEAD-box RNA helicase eIF4A by using RNAi-based gene loss-of-function approach. Two out of four (shEIF4A-1 and shEIF4A-2) shRNAs targeting eIF4A showed significant reduction of its mRNA level in HuH7 cells, compared with a control shRNA targeting GFP (shCTR) (Figure 1A). Consistently, their protein levels were also downregulated without affecting the expression of eIF4G and eIF4E, which suggested a successful knockdown of eIF4A (Figure 1B). No cytotoxicity has been observed in these cells as measured by MTT assay (Supplementary Figure 2A). Silencing of eIF4A resulted in significant decrease of cellular HEV RNA level by 63.1% \pm 8.6% and 57.6% \pm 13.8% (mean \pm SEM, n = 4, P < 0.05) after three days inoculation of HEV particles, respectively (Figure 1C). Next, knockdown of scaffold protein eIF4G by four shRNAs were also performed in HuH7 cells. Two clones (shEIF4G-1 and shEIF4G-4) showed efficient down-regulation of eIF4G at both mRNA level and protein level, but did not influence the protein level of eIF4A or eIF4E

(Figure 1D and 1E). No cytotoxicity was observed in these knockdown cells (Supplementary Figure 2B). Correspondingly, HEV RNA levels was significantly reduced by $36.1\% \pm 10.6\%$ and $33.8\% \pm 11.0\%$ (mean \pm SEM, $n = 4$, $P < 0.05$) in both eIF4G knockdown cells, respectively, compared to shCTR cells (Figure 1F). These results demonstrate that both eIF4A and eIF4G are required for efficient HEV replication.

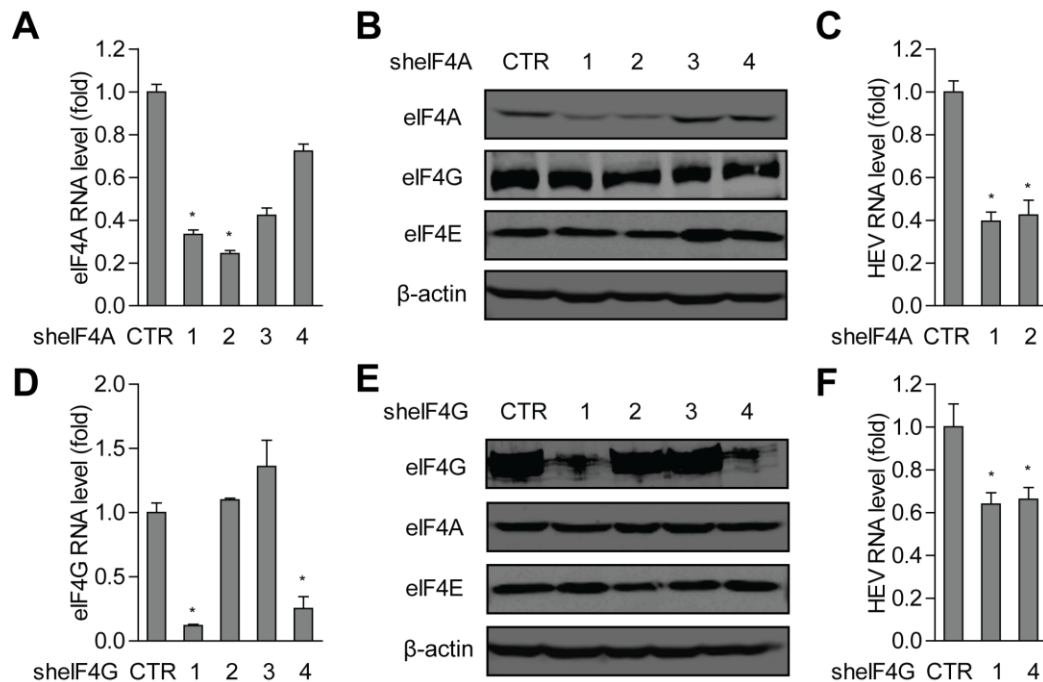


Figure 1. Requirements of eIF4A and eIF4G for HEV replication. Knockdown of eIF4A and eIF4G by lentiviral shRNA vectors were performed in Huh7 cells. Compared with the control vector transduced cells, the shelF4A clone 1 and 2 (A and B) or the shelF4G clone 1 and 4 (D and E) showed potent gene silencing at mRNA level and protein level. Correspondingly, knockdown of eIF4A (C) and eIF4G (F) resulted in significant increase of cellular HEV RNA level (Mean \pm SEM, $n = 4$). * $P < 0.05$.

PDCD4, the negative regulatory factor of eIF4A, restricts HEV replication

Given the fact that the function of eIF4A is regulated by multiple cellular factors, we first investigated the effect of its activator eIF4B that can increase the helicase activity of eIF4A. Knockdown of eIF4B by two out of four shRNA clones (shelF4B-2 and shelF4B-3) resulted in significant down-regulation of eIF4B expression (Figure 2A), but has no significant influence on cellular HEV RNA level (Figure 2B).

We next examined a negative regulatory factor of eIF4A, PDCD4, which prevents the binding of eIF4A to eIF4G and thereby inhibits the initiation of translation²⁶. To assess the effect of PDCD4 on HEV replication, lentiviral shRNA vectors were used to stably knockdown

its expression in HuH7 cells. Potent down-regulation of PDCD4 mRNA and protein expression of two clones (shPDCD4-1 and shPDCD4-3) (Figure 2C and 2D) resulted in a significant increase of HEV RNA after inoculation of HEV particles for three days (Figure 2E). No cytotoxicity was observed in knockdown cells (Supplementary Figure 2C). These results are consistent with the finding that eIF4A supports HEV replication and inhibiting the function of eIF4A by PDCD4 in turn suppresses HEV replication.

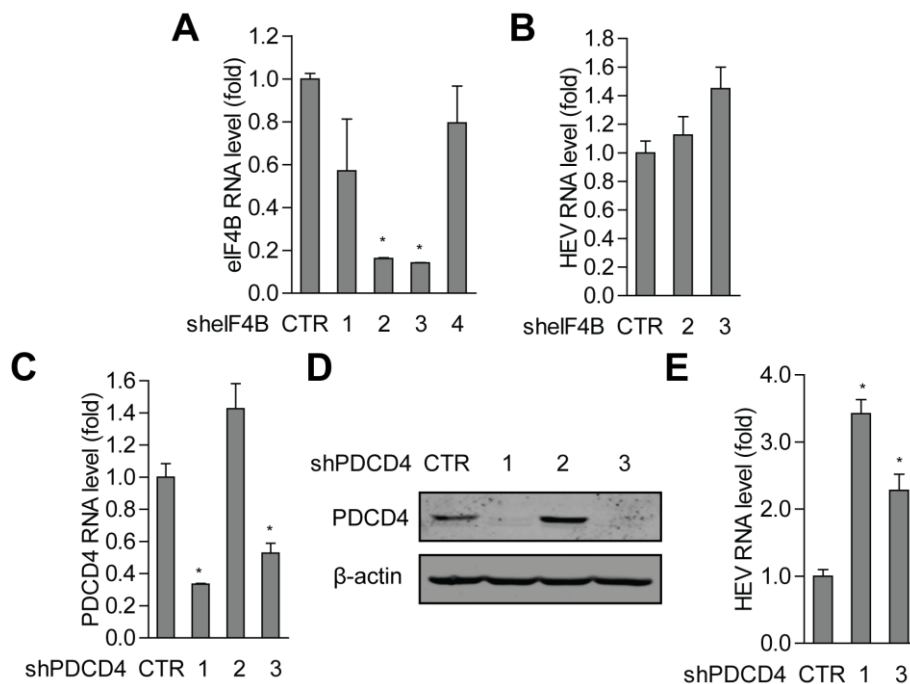


Figure 2. The regulatory factors of eIF4A, PDCD4 but not eIF4B, restricted HEV replication. (A) Clone 2 and 3 Knockdown of eIF4B by lentiviral shRNA vectors exerted significant downregulation of eIF4B at mRNA level. (B) Silencing of eIF4B did not influence HEV replication during 72 hrs inoculation of viral particles. mRNA level (C) and protein level (D) of PDCD4 were significantly reduced in clone 1 and 3. (E) HEV RNA level were dramatically increased when silencing PDCD4 in clone 1 and 3. (Mean \pm SEM, $n = 4$). * $P < 0.05$.

eIF4E is also required for efficient HEV replication

eIF4E, the least abundant component of the eIF4F complex, is a rate-limiting factor for translation²⁷. To investigate the role of eIF4E in HEV replication, its expression was silenced by RNAi. Two out of five shRNAs targeting eIF4E exerted potent knockdown at both protein (Figure 3A) and mRNA levels (Figure 3B). No off-target effect was observed on protein expression of eIF4G or eIF4A (Figure 3A), or on genes such as CyA, CyB, 4E-BP1 and mTOR, which are known to affect HEV replication as previously shown^{28,29} (Supplementary Figure 3). MTT assay showed no cytotoxicity of eIF4E silencing in cells (Supplementary Figure 2D).

Accordingly, inoculation of HEV led to reduction by $44\% \pm 12\%$ and $41\% \pm 25\%$ (mean \pm SEM, $n = 5$, $P < 0.05$) in viral RNA level in these two knockdown cells compared to shCTR cells (Figure 3C). We observed similar effect in MEFs (Figure 3D and 3E), further confirming that eIF4E plays an important role in HEV replication.

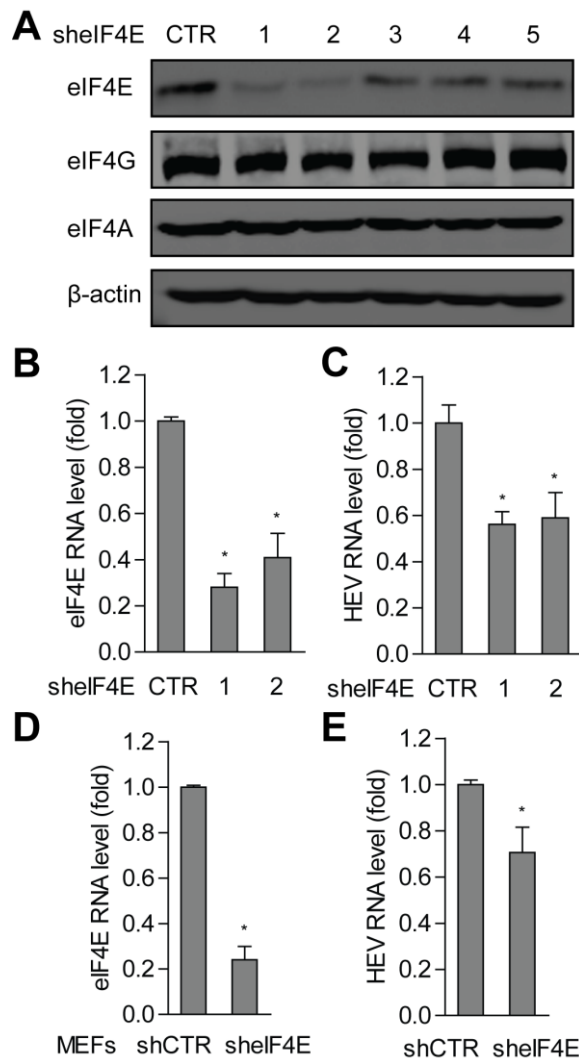


Figure 3. eIF4E supports HEV replication. Knockdown of human eIF4E by five lentiviral shRNA vectors were performed in HuH7 cells. Clone 1 and 2 exhibited a potent down-regulation of eIF4E at protein (A) and mRNA (B) levels. β-actin served as an internal reference. (C) Correspondingly, these two clones of eIF4E knockdown inhibited HEV replication. (D) Knockdown of mouse eIF4E by lentiviral shRNA vector was applied in MEFs showed a significant decrease at eIF4E mRNA level. (E) HEV RNA level was significantly reduced with eIF4E silencing in MEFs. (Mean \pm SEM, $n = 4$). * $P < 0.05$.

Phosphorylation is not required for eIF4E to support HEV replication

Ser209 phosphorylation has been shown to be required for the oncogenic potential of eIF4E³⁰. To examine whether this is also important in the context of HEV infection, HuH7 cells harboring the HEV subgenomic replicon or the full-length genome were treated with 10-100 μ M CGP57380, a well-characterized inhibitor of MNK. MNK is the only known physiologic kinase that phosphorylates eIF4E (Ser209 site)³¹. This compound potently inhibited eIF4E-S209 phosphorylation without effect on total eIF4E protein level (Figure 4A). However, CGP57380 had no effect on HEV replication in both luciferase replicon model (Figure 4B) and

infectious model (Figure 4C). To further confirm the function of phosphorylation of eIF4E, MEFs cultured from mice with S209A mutation were used. This mutation targeting the conserved phosphorylation site for MNK1/2 kinase with serine-to-alanine completely abolished phosphorylation of eIF4E at Ser209 without effect on total eIF4E (Figure 4D). Consistently, no significant effect was observed on HEV replication between mutated and wild type MEFs (Figure 4E). These data suggest that phosphorylation of eIF4E is dispensable for HEV replication.

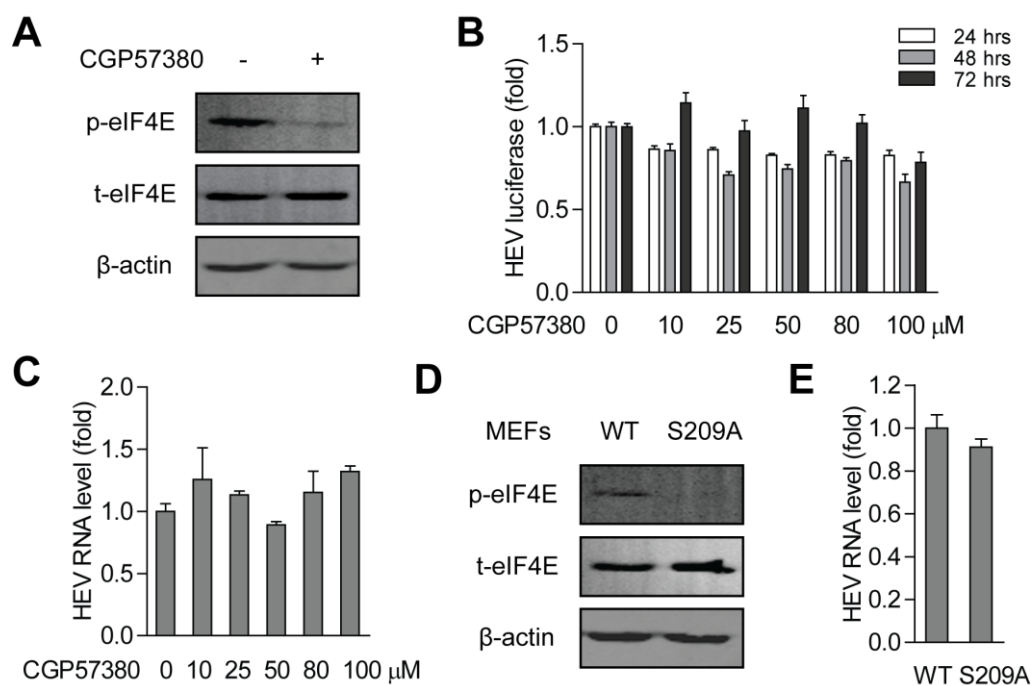


Fig. 4. eIF4E phosphorylation did not significantly affect HEV replication. (A) Treatment with MNK1/2 inhibitor CGP57380 of 100 μM dramatically decreased the phosphorylation of eIF4E, but not total eIF4E protein shown by Western blot assay. β-actin served as an internal reference. 10-100 μM CGP57380 did not significantly affected viral replication-related luciferase activity during the three days (B) and viral RNA level in HuH7-p6 infectious model for 48 hrs (C). (D) MEFs of S209A mutation abolished phosphorylation of eIF4E at Ser209 shown by Western blot assay. β-actin served as an internal reference. (E) Inhibition of phosphorylation in S209A MEFs did not significantly influence HEV replication. (Mean ± SEM, n = 4).

HEV replication is inhibited by the eIF4E suppressor, 4E-BP1

eIF4E can be released by 4E-BP1 hyperphosphorylation with elimination of translational repression²¹. For a more detailed characterization of the role of eIF4E suppressor 4E-BP1 in HEV infection, we employed both loss- and gain-of-function approaches in HuH7 cells. Using lentiviral RNAi technique, 4E-BP1 was dramatically down-regulated at both protein and mRNA levels (Figure 5A). Accordingly, 48 hrs inoculation of HEV resulted in 2.6 ± 1.2 fold

(mean \pm SEM, $n = 4$, $P < 0.05$) increase of viral RNA in 4E-BP1 knockdown HuH7 cells, compared with the mock knockdown cells (Figure 5A). In contrast, using an inducible over-expression lentiviral vector, 4E-BP1 expression was drastically up-regulated at both protein and mRNA levels with treatment of dose dependent doxycycline, which resulted in significant reduction of HEV RNA by $59\% \pm 17\%$ (mean \pm SEM, $n = 4$, $P < 0.05$) (Figure 5B).

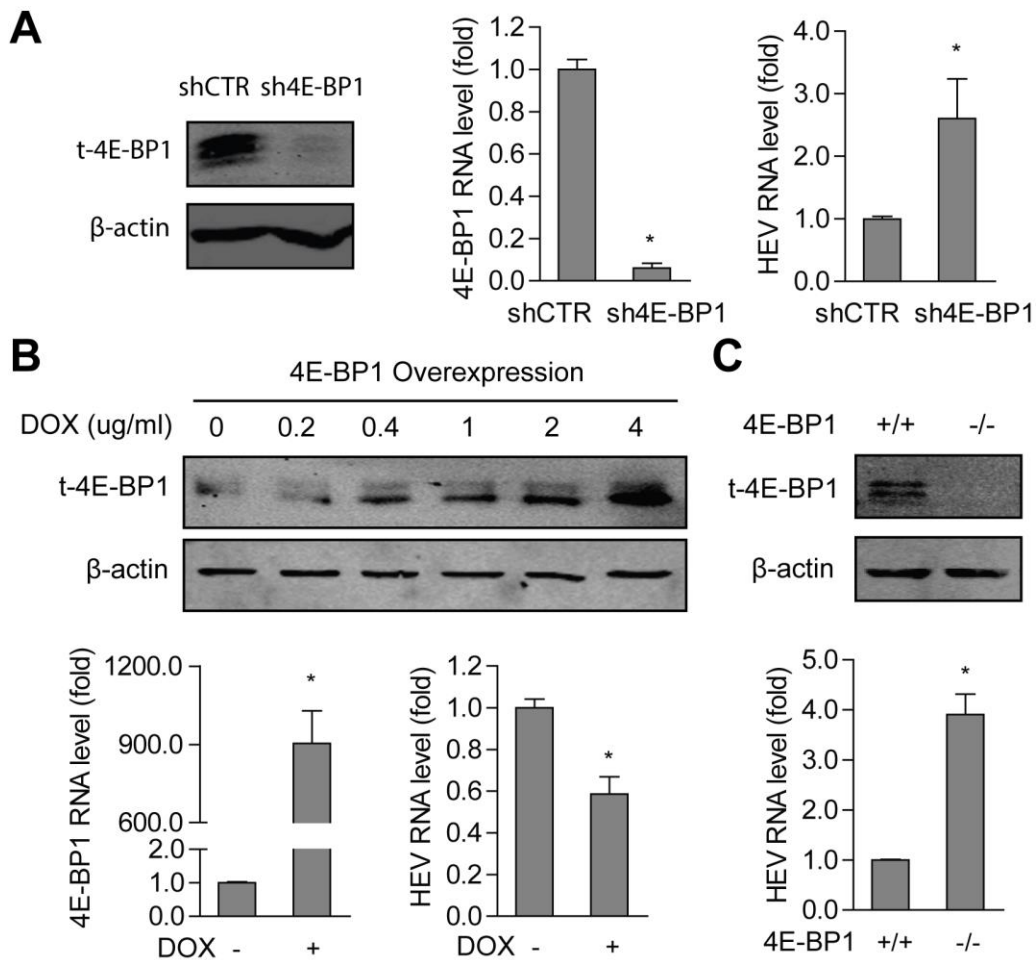


Figure 5. The eIF4E suppressor, 4E-BP1, limited HEV replication. (A) Efficient silencing of 4E-BP1 at protein level was detected by Western blot assay. β -actin served as an internal reference. Similarly, 4E-BP1 was significantly down-regulated at mRNA level (Mean \pm SEM, $n = 7$). Correspondingly, inoculation of HEV resulted in significant increase of viral RNA in 4E-BP1 knockdown Huh7 cells, compared with the mock knockdown cells (Mean \pm SEM, $n = 4$). (B) 4E-BP1 was drastically up-regulated at protein level when the over-expression cell lines were treated with different concentrations of doxycycline for 24 hrs. β -actin served as an internal reference. Similarly, mRNA levels of 4E-BP1 was dramatically increased after treatment of 4 μ g/ml doxycycline for 24 hrs. (Mean \pm SEM, $n = 5$). Correspondingly, 4E-BP1 over-expression cell line with treatment of 4 μ g/ml doxycycline for 24 hrs resulted in significant reduction of HEV RNA level (Mean \pm SEM, $n = 4$). (C). MEFs derived from 4E-BP1 knockout (4E-BP1 $^{-/-}$) mice presented an efficient silencing of 4E-BP1 at protein level compared to WT MEFs, leading to significant increase of HEV RNA level (Mean \pm SEM, $n = 4$). * $P < 0.05$.

To further validate 4E-BP1 function in HEV infection, MEFs cultured from 4E-BP1 knockout mice were studied. Western blot assay showed a complete knockout of 4E-BP1 at protein level (Figure 5C). Accordingly, 48 hrs inoculation of HEV led to 3.9 ± 0.8 fold (mean \pm SEM, $n = 4$, $P < 0.05$) increase of cellular HEV RNA level in 4E-BP1 knockout MEFs (4E-BP1^{-/-}) compared to wild type MEFs (4E-BP1^{+/+}) (Figure 5C). Hence, these data supported a role of 4E-BP1 in constraining HEV replication.

The anti-HEV activities of ribavirin and IFN- α are independent of eIF4E

Ribavirin is a well-known inhibitor of eIF4E¹⁸. To evaluate whether the anti-HEV activity of ribavirin is mediated by the inhibition of eIF4E, both shCTR and shE4E-1 cells with HEV inoculation were used for the treatments of 6.25, 12.5, 25, 50, 100 μ M ribavirin for 48 hrs. Comparable IC₅₀ in shCTR and shE4E cells were found with the range between 12.5-25 μ M (Figure 6A).

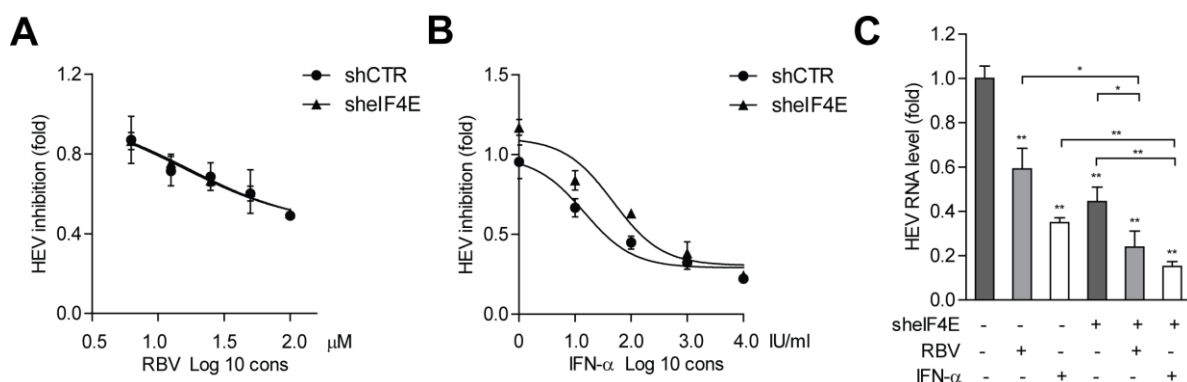


Figure 6. The anti-HEV activities of ribavirin and IFN- α is independent of eIF4E. (A) IC₅₀ of RBV was with the range between 12.5 - 25 μ M: 15.67 μ M in shCTR cells and 17.24 μ M in shE4E cells and (B) IFN- α was with the range between 10 - 50 IU/ml: 14.55 IU/ml in shCTR cells and 47.75 IU/ml in shE4E cells). (C) 25 μ M ribavirin and 1000 IU/ml IFN- α were treated in shCTR and shE4E cells. HEV RNA level was detected by qRT-PCR after 72 hrs inoculation of viral particle. (Mean \pm SEM, $n = 4 - 8$). * $P < 0.05$. ** $P < 0.01$.

The antiviral activity of IFN- α has also been associated to the regulation of translation initiation factors in particular circumstances^{20, 21}. To further assess whether eIF4E could mediate the anti-HEV activity of IFN- α , treatments of 1, 10, 100, 1000, 10000 IU/ml IFN- α on HEV in both shCTR and shE4E-1 cells for 48 hrs has been performed. Comparable IC₅₀ of IFN- α were observed with the range between 10-50 IU/ml in shCTR and shE4E cells (Figure 6B). Furthermore, as expected, HEV replication were significantly inhibited with treatment of

25 μ M ribavirin and 1000 IU/mL IFN- α for 48 hrs. However, the anti-HEV effects of ribavirin and IFN- α were further enhanced by eIF4E knockdown (Figure 6C). In addition, no clear cytotoxicity was observed in both shCTR and shEIF4E cells (Supplementary Figure 4A and 4B). These results indicated that the antiviral effects of ribavirin and IFN- α are independent of eIF4E, although silencing of eIF4E could already inhibit HEV replication.

Discussion

Most of the viruses can only encode restricted numbers of proteins and therefore they heavily rely on the host cellular machinery and their ingredients to accomplish the virus life-cycles³. Recent studies show that translation initiation mechanisms especially eIF4F complex is employed by many viruses as a primary target for cap-dependent translational control to confer advantages to generate progeny³². Three proteins: RNA helicase eIF4A, scaffolding protein eIF4G and cap binding protein eIF4E, which are components of the eIF4F complex, are related to the efficient translation and replication of various viruses^{2, 5-7}. It is, however, unknown to what extent HEV requires elements from the translation initiation complex. Our study was aimed to provide more insight in this area of HEV biology, also with the explicit goal to provide directions for the development of rational treatment of HEV-related disease. Our study demonstrated a requirement of the eIF4F complex for efficient HEV replication (Figure 1 and 3).

Among all three subunits of the eIF4F complex, eIF4E is the main regulatory nexus involved in the complex formation and has impact on many types of viral infections³, including on HEV as we showed in this study. One of the mechanisms by which eIF4E takes control of complex formation and translation initiation process is via phosphorylation on serine 209 carried out by MNK1/2³. Stimulation of eIF4E phosphorylation is correlated with facilitated translation and replication of some viruses^{2, 9, 30}. In contrast, we found that S209 phosphorylation is not required for eIF4E to support HEV replication (Figure 4). Another regulatory mechanism of eIF4E is exerted via 4E-BP1, a small-molecular-weight repressor of 5' capped mRNA translation, which has also been implicated in host defense against viral infection¹⁵. In apparent agreement, we show that 4E-BP1 can inhibit HEV replication (Figure 5). 4E-BP1 is a phosphoprotein that binds to eIF4E depending on its phosphorylation status.

4E-BP1 hyperphosphorylation results in releasing eIF4E to form the functional eIF4F complex. Conversely, 4E-BP1 hypophosphorylation allows binding of this protein to eIF4E and counteracts the formation of eIF4F complex³. Therefore, without this hijacking of eIF4E in a 4E-BP1-deficient context, eIF4E can still exert its pro-HEV activity (as we have shown in Supplementary Figure 5). Similarly, the eIF4A suppressor PDCD4 can also restrict HEV replication (Figure 2). PDCD4 sequesters eIF4A from the eIF4E-eIF4G complex, resulting in repressed translation of mRNAs¹² and thus modulates replication of various viruses^{13, 14}. Apparently this notion also holds true in the biology of HEV infection.

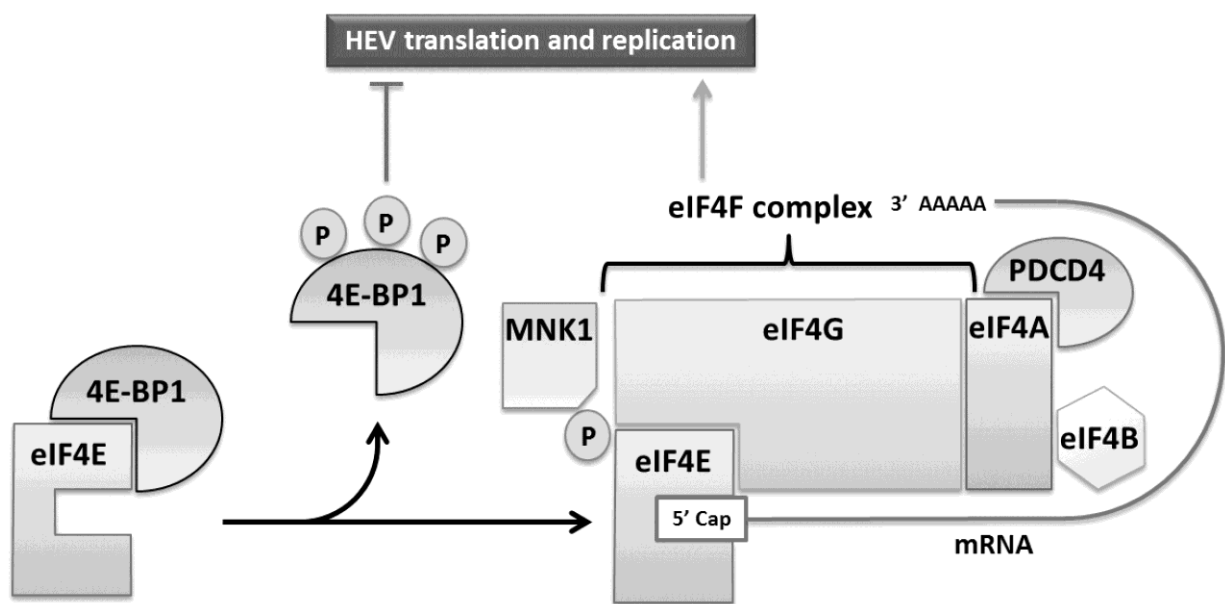


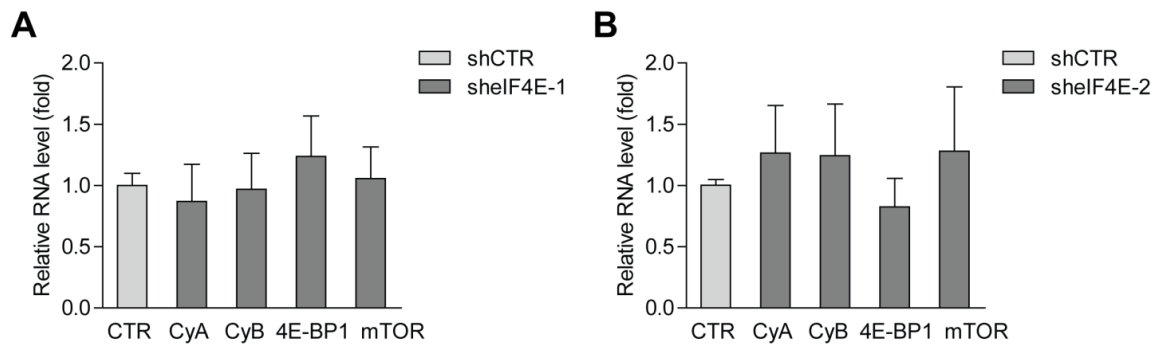
Figure 7. Schematic illustration of the involvement of the eIF4F complex in HEV replication. Three subunits of eIF4F complex: eIF4A, eIF4G and eIF4E play important roles in efficient HEV replication. Furthermore, HEV replication is limited by the cap dependent translational suppressors, PDCD4 and 4E-BP1, but is not influenced by eIF4E phosphorylation induced by MNK1/2 kinase activation.

Despite the absence of proven medications for treating HEV, ribavirin, IFN- α , or the combination have been used as off-label antiviral drugs to treat individual HEV cases or small case series¹⁶. The antiviral effect of interferons and their signaling pathways have been attributed to effects in the 4E-BP1 cascade^{15, 20}. However, loss- or gain-function of 4E-BP1 had no significant effects on the expression of IFN- α and - β (Supplementary Figure 6A) and no effect on phosphorylation of STAT1, the key element of interferon signalling transduction (Supplementary Figure 6B). Furthermore the effect of 4E-BP1 on HEV is independent of JAK-STAT cascades (Supplementary Figure 6C). Conversely, the anti-HEV effect of IFN- α is also independent of 4E-BP1 (Supplementary Figure 6D). Although ribavirin directly binds to eIF4E

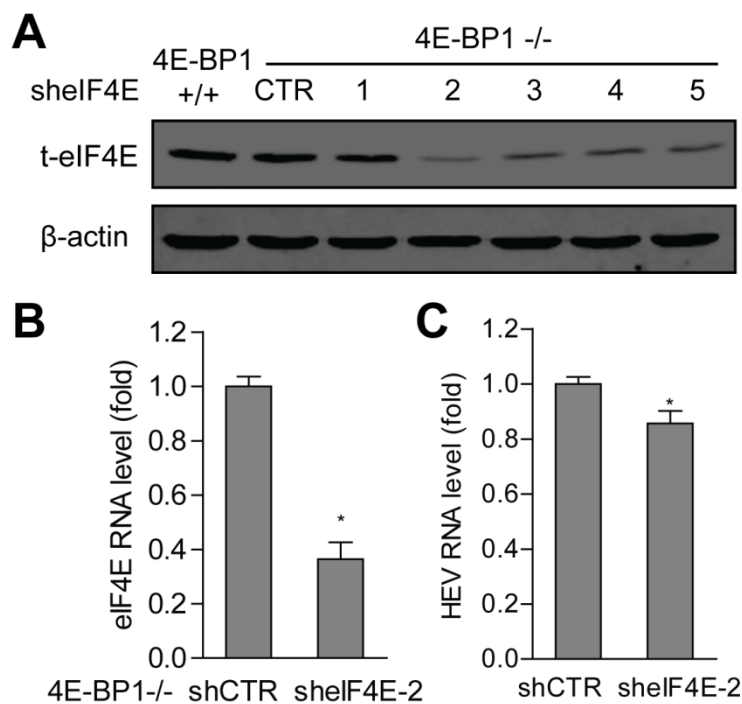
and competes for 5' cap mRNA binding^{18, 19}, the anti-HEV activity of ribavirin is also independent of eIF4E in our experimental system (Figure 6). Instead, loss of eIF4E exerts additive anti-HEV effect of IFN- α or ribavirin and suggests that treatments aimed at targeting the translation initiation complex in conjunction with IFN- α or ribavirin have significant promise.

In conclusion, we revealed that cap dependent translation machinery plays a critical role in help with efficient HEV replication. The translational suppressors PDCD4 and 4E-BP1 are important antiviral factors in restraining HEV infection (Figure 7). Thus, these results have shed new light on virus-host interactions and provided new avenue for potential antiviral drug development against HEV infection.

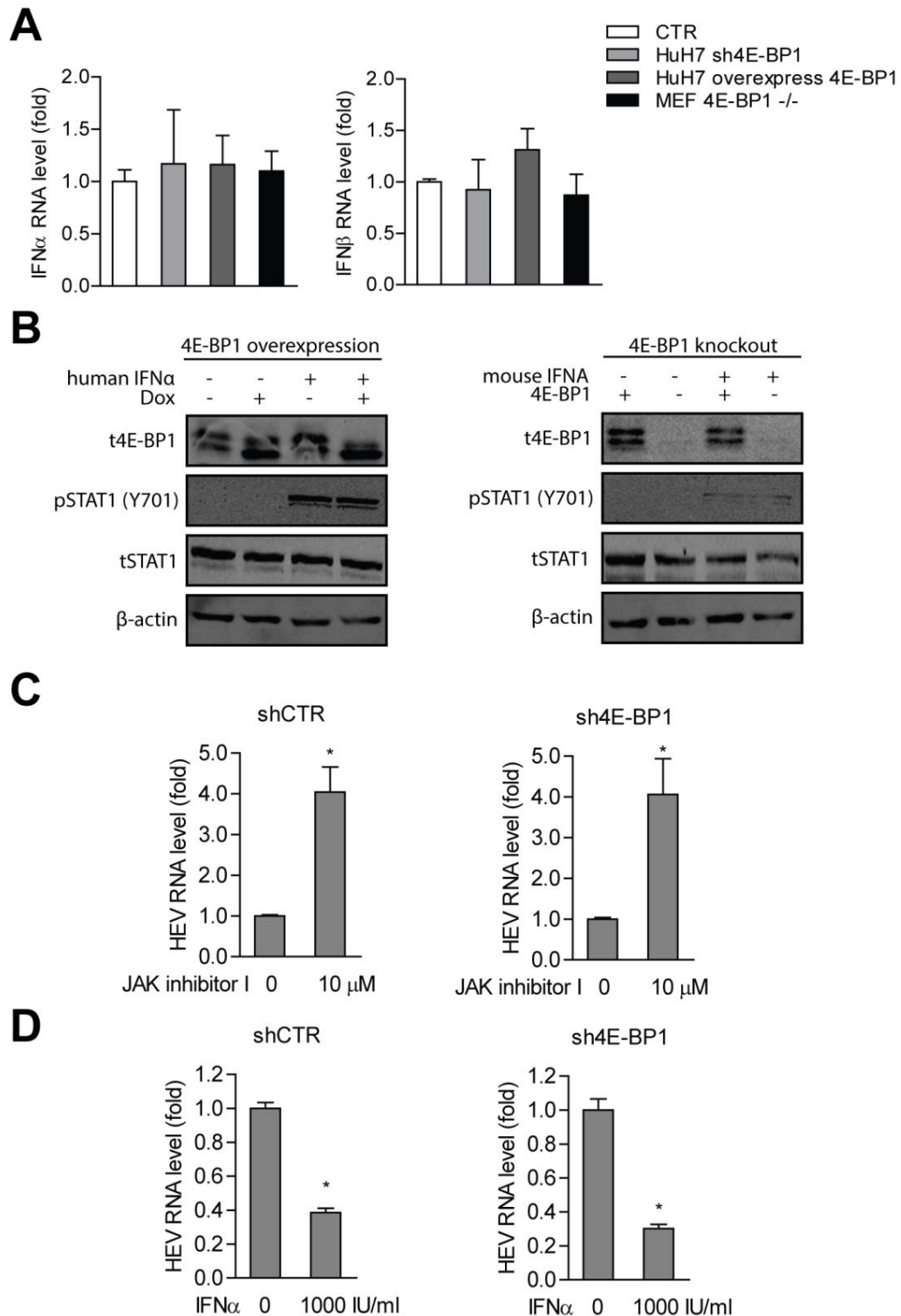
Supplementary Figures and Tables



Supplementary Figure 1. eIF4E knockdown did not significant affect off-target genes expression in Huh7 cells. CyA, CyB, 4E-BP1 and mTOR mRNA levels were not influenced in shelf4E-1 (A) and shelf4E-2 cells (B). (Mean \pm SEM, $n = 3$).



Supplementary Figure 2. eIF4E is required for efficient HEV replication in 4E-BP1-/- knockout MEFs. Clone shelf4E-2 of mouse eIF4E knockdown showed a potent eIF4E silencing at protein (A) and mRNA levels (B). β -actin served as an internal reference in Western blot assay. (C) HEV RNA level was significantly reduced in shelf4E-2 MEFs deficient of 4E-BP1. (Mean \pm SEM, $n = 4$). * $P < 0.05$.



Supplementary Figure 3. (A) IFN- α and - β production were not influenced by 4E-BP1 expression in Huh7 cells and MEFs. (B) IFN- α induced phosphorylation of STAT1, which represents key elements in antiviral JAK-STAT1 cascades, showed no difference in 4E-BP1 over-expression Huh7 cells and 4E-BP1 knockout MEFs by Western blot assay. β -actin served as an internal reference. 4E-BP1 did not mediate the pro-HEV effects of Jak inhibitor I (C) and anti-HEV effects of IFN- α (D) for 48 hrs. (Mean \pm SD, n = 4).

Supplementary Table 1. shRNA target sequences.

shelF4A-1 (human)	CCGGGCCGTGTGTTTGATATGCTTACTCGAGTAAGCATATCAAACACACGGCTTTTTG
shelF4A-2 (human)	CCGGGCCGTAAAGGTGTGGCTATTACTCGAGTAATAGCCACACCTTTACGGCTTTTTG
shelF4A-3 (human)	CCGGCCTTGATCAAGGGTTATGATCTCGAGATCATAACCCTTGATACAAGGTTTTTG
shelF4A-4 (human)	CCGGCGAAATGTAAAGCCGTGGATTCTCGAGAATCCACGGCTTAACATTTGTTTTTG
shelF4G-1 (human)	CCGGCCCTACAGAATTTGGGACCTACTCGAGTAGGTCCCAAATTCTGTAGGGTTTTTG
shelF4G-2 (human)	CCGGGCCCTTG TAGTGACCTTAGAACTCGAGTTCTAAGGTCACTACAAGGGCTTTTTG
shelF4G-3 (human)	CCGGGCAGATAGTATCCAACACGTTCTCGAGAACGTGTTGGATACTATCTGCTTTTTG
shelF4G-4 (human)	CCGGCCCAAGTAATGATGATCCCTTCTCGAGAAGGGATCATCATTACTTGGGTTTTTG
shelF4B-1 (human)	CCGGGCGGAGAAACACCTTGATCTTCTCGAGAAGATCAAGGTGTTTCTCCGCTTTTTG
shelF4B-2 (human)	CCGGGCCGTGATAGAAATCGGGATTCTCGAGAATCCCGATTCTATCACGGCTTTTTG
shelF4B-3 (human)	CCGGCGGGATGATTATAGGCGTGATCTCGAGATCACGCCTATAATCATCCCGTTTTTG
shelF4B-4 (human)	CCGGCTACCCTATGATGTTACAGAACTCGAGTTCTGTAACATCATAGGGTAGTTTTTG
shPDCD4-1 (human)	CCGGGCGGTTTGTAGAAGAATGTTTCTCGAGAAACATTCTTCTACAAACCGCTTTTTG
shPDCD4-2 (human)	CCGGCTGACCTTTGTGGGACAGTAACTCGAGTTACTGTCCACAAAGGTCAGTTTTTG
shPDCD4-3 (human)	CCGGCTACCATTACTGTAGACCAAACCTCGAGTTTGGTCTACAGTAATGGTAGTTTTTG
shelF4E-1 (human)	CCGGCCACTCTGTAATAGTTCAGTACTCGAGTACTGAACTATTACAGAGTGGTTTTTG
shelF4E-2 (human)	CCGGCCAAAGATAGTGATTGGTTATCTCGAGATAACCAATCACTATCTTTGGTTTTTG
shelF4E-3 (human)	CCGGCCGACTACAGAAGAGGAGAACTCGAGTTTCTCCTCTTCTGTAGTCGGTTTTTG
shelF4E-4 (human)	CCGGCGGCTGATCTCCAAGTTTGATCTCGAGATCAAACCTTGGAGATCAGCCGTTTTTG
shelF4E-5 (human)	CCGGCTGTTGTTAATGTTAGAGCTACTCGAGTAGCTTAACATTAACAACAGTTTTTG
shelF4E (mouse)	CCGGCCGAAGATAGTGATTGGTTATCTCGAGATAACCAATCACTATCTTCGGTTTTTG
sh4E-BP1 (human)	CCGGGCCAGGCCTTATGAAAGTGATCTCGAGATCACTTTCATAAGGCCTGGCTTTTTG

Supplementary Table 2. Human and mouse ISG primer sequences. Sequence 5' to 3' (including modification codes if applicable).

Human	
GAPDH-F	TGTCCCCACCCCAATGTATC
GAPDH-R	CTCCGATGCCTGCTTCACTACCTT
eIF4A-F	AAGCCGTGGATTCAAGGACCAG
eIF4A-R	CACCTCAAGCACATCAGAAGGC
eIF4G-F	GCCATTTCAAGAGCCCAACTTCTC
eIF4G-R	CGGAAGTTCACAGTCACTGTTGG
eIF4B-F	GGCAAAGCCTGTTGACACAGCT
eIF4B-R	TTCACCTCGCCAGCTTGGGTGT
PDCD4-F	ACTGTGCCAACCAGTCCAAAGG
PDCD4-R	CCTCCACATCATACACCTGTCC
eIF4E-F	ATGCCTGGCTGTGACTACTCAC
eIF4E-R	GAGGTCACTTCGTCTCTGCTGT
4E-BP1-F	CACCAGCCCTTCCAGTGATGAG
4E-BP1-R	CCTTGGTAGTGCTCCACACGAT
Mouse	
GAPDH-F	CATCACTGCCACCCAGAAGACTG
GAPDH-R	ATGCCAGTGAGCTTCCCGTTCAG
eIF4E-F	ATGCCTGGCTGTGACTACTCAC
eIF4E-R	GTGTCTCTAGCCAGAAGCGATC
4E-BP1-F	GGAGAGCTGCACAGCATTTCAGG
4E-BP1-R	GGAGGTATGTGCTGGTGTTTAC

References

1. Kamar N, Dalton HR, Abravanel F & Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* **27**, 116-138 (2014).
2. Walsh D & Mohr I. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. *Genes Dev* **18**, 660-672 (2004).
3. Montero H, Garcia-Roman R & Mora SI. eIF4E as a control target for viruses. *Viruses* **7**, 739-750 (2015).
4. Gingras AC, Raught B & Sonenberg N. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* **68**, 913-963 (1999).
5. George A, *et al.* Hepatitis C virus NS5A binds to the mRNA cap-binding eukaryotic translation initiation 4F (eIF4F) complex and up-regulates host translation initiation machinery through eIF4E-binding protein 1 inactivation. *J Biol Chem* **287**, 5042-5058 (2012).
6. Ventoso I, Blanco R, Perales C & Carrasco L. HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. *Proc Natl Acad Sci U S A* **98**, 12966-12971 (2001).
7. de Breyne S, *et al.* In vitro studies reveal that different modes of initiation on HIV-1 mRNA have different levels of requirement for eukaryotic initiation factor 4F. *FEBS J* **279**, 3098-3111 (2012).
8. Walsh D, Mathews MB & Mohr I. Tinkering with translation: protein synthesis in virus-infected cells. *Cold Spring Harb Perspect Biol* **5**, a012351 (2013).
9. Royall E, *et al.* Murine norovirus 1 (MNV1) replication induces translational control of the host by regulating eIF4E activity during infection. *J Biol Chem* **290**, 4748-4758 (2015).
10. Walsh D, Perez C, Notary J & Mohr I. Regulation of the translation initiation factor eIF4F by multiple mechanisms in human cytomegalovirus-infected cells. *J Virol* **79**, 8057-8064 (2005).
11. Harms U, Andreou AZ, Gubaev A & Klostermeier D. eIF4B, eIF4G and RNA regulate eIF4A activity in translation initiation by modulating the eIF4A conformational cycle. *Nucleic Acids Res* **42**, 7911-7922 (2014).
12. Dennis MD, Jefferson LS & Kimball SR. Role of p70S6K1-mediated phosphorylation of eIF4B and PDCD4 proteins in the regulation of protein synthesis. *J Biol Chem* **287**, 42890-42899 (2012).
13. Wang S, *et al.* Influenza A virus-induced degradation of eukaryotic translation initiation factor 4B contributes to viral replication by suppressing IFITM3 protein expression. *J Virol* **88**, 8375-8385 (2014).
14. Damanian P, *et al.* Hepatitis B virus induces cell proliferation via HBx-induced microRNA-21 in hepatocellular carcinoma by targeting programmed cell death protein4 (PDCD4) and phosphatase and tensin homologue (PTEN). *PLoS One* **9**, e91745 (2014).
15. Kaur S, *et al.* Regulatory effects of mammalian target of rapamycin-activated pathways in type I and II interferon signaling. *J Biol Chem* **282**, 1757-1768 (2007).
16. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
17. Kamar N, *et al.* Ribavirin for chronic hepatitis E virus infection in transplant recipients. *N Engl J Med* **370**, 1111-1120 (2014).

18. Kentsis A, Topisirovic I, Culjkovic B, Shao L & Borden KL. Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. *Proc Natl Acad Sci U S A* **101**, 18105-18110 (2004).
19. De la Cruz-Hernandez E, *et al.* Ribavirin as a tri-targeted antitumor repositioned drug. *Oncol Rep* **33**, 2384-2392 (2015).
20. Burke JD, Sonenberg N, Platanias LC & Fish EN. Antiviral effects of interferon-beta are enhanced in the absence of the translational suppressor 4E-BP1 in myocarditis induced by Coxsackievirus B3. *Antivir Ther* **16**, 577-584 (2011).
21. Nehdi A, *et al.* Deficiency in either 4E-BP1 or 4E-BP2 augments innate antiviral immune responses. *PLoS One* **9**, e114854 (2014).
22. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
23. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
24. Pan Q, *et al.* Combined antiviral activity of interferon-alpha and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing. *J Mol Med (Berl)* **87**, 713-722 (2009).
25. Sonenberg N & Hinnebusch AG. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* **136**, 731-745 (2009).
26. Pelletier J, Graff J, Ruggero D & Sonenberg N. Targeting the eIF4F translation initiation complex: a critical nexus for cancer development. *Cancer Res* **75**, 250-263 (2015).
27. Mohr I & Sonenberg N. Host translation at the nexus of infection and immunity. *Cell Host Microbe* **12**, 470-483 (2012).
28. Han J, *et al.* SPF rabbits infected with rabbit hepatitis E virus isolate experimentally showing the chronicity of hepatitis. *PLoS One* **9**, e99861 (2014).
29. Zhou X, *et al.* Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* **61**, 746-754 (2014).
30. Panda S, Vedagiri D, Viveka TS & Harshan KH. A unique phosphorylation-dependent eIF4E assembly on 40S ribosomes co-ordinated by hepatitis C virus protein NS5A that activates internal ribosome entry site translation. *Biochem J* **462**, 291-302 (2014).
31. Kyriakis JM. In the beginning, there was protein phosphorylation. *J Biol Chem* **289**, 9460-9462 (2014).
32. Bushell M & Sarnow P. Hijacking the translation apparatus by RNA viruses. *J Cell Biol* **158**, 395-399 (2002).

Chapter 10

Inhibition of hepatitis E virus replication by proteasome inhibitor is nonspecific

Lei Xu, Xinying Zhou, Maikel P. Peppelenbosch and Qiuwei Pan

Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center,
Rotterdam, Netherlands

Abstract

The ubiquitin proteasome system plays important role in virus infection. A previous study showed that the proteasome inhibitor MG132 could potentially affect hepatitis E virus (HEV) replication. In this study, we found that MG132 could inhibit HEV and hepatitis C virus (HCV) replication-related luciferase activity in subgenomic models. Furthermore, treatment with MG132 in a HEV infectious model resulted in a dramatic reduction in the intracellular level of HEV RNA. Surprisingly, MG132 concurrently inhibited the expression of a luciferase gene used as a control as well as a wide range of host genes. Consistently, the total cellular RNA and protein content was concurrently reduced by MG132 treatment, suggesting a nonspecific antiviral effect.

Introduction

The ubiquitin proteasome system (UPS), which serves as a major pathway for protein degradation and modification in eukaryotic cells, can be utilized by many types of viruses¹⁻³. Previous studies have demonstrated that UPS can regulate viral RNA-dependent RNA polymerase (RdRp), which mediates viral RNA synthesis^{1, 4-6}. In addition, UPS can also regulate ubiquitylation and degradation of some viral structural proteins⁷⁻⁹, and thus representing as a potential antiviral target.

Hepatitis E virus (HEV) is a single-strand positive-sense RNA virus that belongs to *Hepeviridae* family. It is a small non-enveloped virus with a 7.2 kb RNA genome, which is capped at the 5' termini and polyadenylated at the 3' termini¹⁰. Outbreaks of hepatitis E occur periodically throughout the developing world. It typically causes an acute and self-limiting infection, but fulminant hepatitis and high mortality (reaching 25%) are described in cases of pregnant women. In the western world, HEV mainly affects immunocompromised patients with high risk of developing chronic hepatitis¹¹. However, no proved medication is available to treat hepatitis E. A recent study reported potent antiviral effects of a well-known proteasome inhibitor MG132 against HEV¹². In a *Renilla*-luciferase-coupled HEV replication model, the author showed that treatment of MG132 resulted in dramatic reduction of HEV-related luciferase activity¹². These important findings have inspired us to further evaluate the effects of MG132 in two HEV cell culture models.

Matirials and methods

In this study, two human hepatoma cell line (Huh7)-based HEV cell culture models are employed: a subgenomic HEV replicon containing *gaussia* luciferase reporter (p6-Luc) in which the accumulation of secreted luciferase serves as a reporter for HEV replication, and a full-length infectious model (p6) in which Huh7 was electroporated with full-length HEV genome RNA (Kernow-C1 p6 clone, GenBank Accession Number JQ679013)¹³. Two firefly luciferase cell models, a cell line for normalization which stable express luciferase driven by a phosphoglycerate kinase (PGK) promoter (Huh7-PGK) and a hepatitis C virus (HCV, also a single-strand positive-sense RNA virus) subgenomic cell culture model (Huh7-ET) were also used¹⁴. The *gaussia* luciferase and firefly luciferase activity was measured as described

previously¹³ by using a Lumi Star Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). MTT assay was performed as previously described¹⁵. The absorbance of each well was read on the microplate absorbance readers (Bio-rad) at wavelength of 490 nm.

RNA was isolated with the Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Thermo, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc.). HEV, GAPDH, RP2 (Human retinitis pigmentosa 2), CyA (Cyclosporin A), CyB (Cyclosporin B), CD81 (Cluster of Differentiation 81) and IMPDH2 (Inosine-5'-monophosphate dehydrogenase 2) were quantified by SYBR-Green-based real-time PCR. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), and the primer sequences of housekeeping gene GAPDH were 5'-TGTCCCCACCCCAATGTATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense) and the primers of housekeeping gene RP2 were 5'-CCCATTAACTCCAAGGCAA-3' (sense) and 5'-AAGCTGAGGATGCTCAAAGG-3' (antisense). The primer sequences of CyA were 5'-GGCAAATGCTGGACCCAACACA-3' (antisense) and 5'-TGCTGGTCTTGCCATTCCTGGA-3' (sense) and the primers CyB were 5'-AACGCAGGCAAAGACACCAACG-3' (antisense) and 5'-TCTGTCTTGGTGTCTCCACCT-3' (sense). The primer sequences of CD81 were 5'-CTGCTTTGACCACCTCAGTGCT-3' (antisense) and 5'-TGGCAGCAATGCCGATGAGGTA-3' (sense) and the primers IMPDH2 were 5'-AGTGGCTCCATCTGCATTACGC-3' (antisense) and 5'-GGATTCCTCCATCAGCAATGACC-3' (sense).

For Western blot, cell lysates were heated 5 minutes at 95 °C followed by loading same volume of sample onto a 10% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. Mouse β -actin antibody (1:1000) was used as primary antibody. For SDS-PAGE, after 90 min running at 120 V, the gel was stained in Coomassie Brilliant Blue solution and destained.

Results

Consistent with a previous study¹², treatment with 1 μ M and 10 μ M MG132 did not significantly impair cell viability determined by MTT assay after 24 hrs (Figure 1A) and 48 hrs (Figure 1B). As expected, treatment with 1 μ M MG132 potently inhibited HEV replication-

related *gaussia* luciferase activity in the p6-Luc model (Figure 1) after 24 hrs and 48 hrs. Furthermore, we tested this proteasome inhibitor on the Huh7 based hepatitis C virus subgenomic model (Huh7-ET). Consistently, MG132 inhibited HCV coupled firefly luciferase activity (Figure 1). Surprisingly, when the Huh7-PGK cell line treated with MG132, the control firefly luciferase activity driven by the PGK promoter was also potently inhibited (Figure 1). These results raised the concern regarding the specificity of MG132 on viral replication.

To investigate further, the HEV infectious model (p6) was treated with MG132 for 48 hrs. The relative levels of HEV viral RNA and two host reference genes (GAPDH and RP2) were quantified by SYBR-based qRT-PCR. As shown in Figure 2A, treatment with 1 or 10 μ M MG132 resulted in a significant decrease in intracellular HEV viral RNA by $32 \pm 19\%$ and $76 \pm 24\%$ (mean \pm SD, $n = 6$, $p < 0.01$), respectively. Strikingly, the expression levels of two

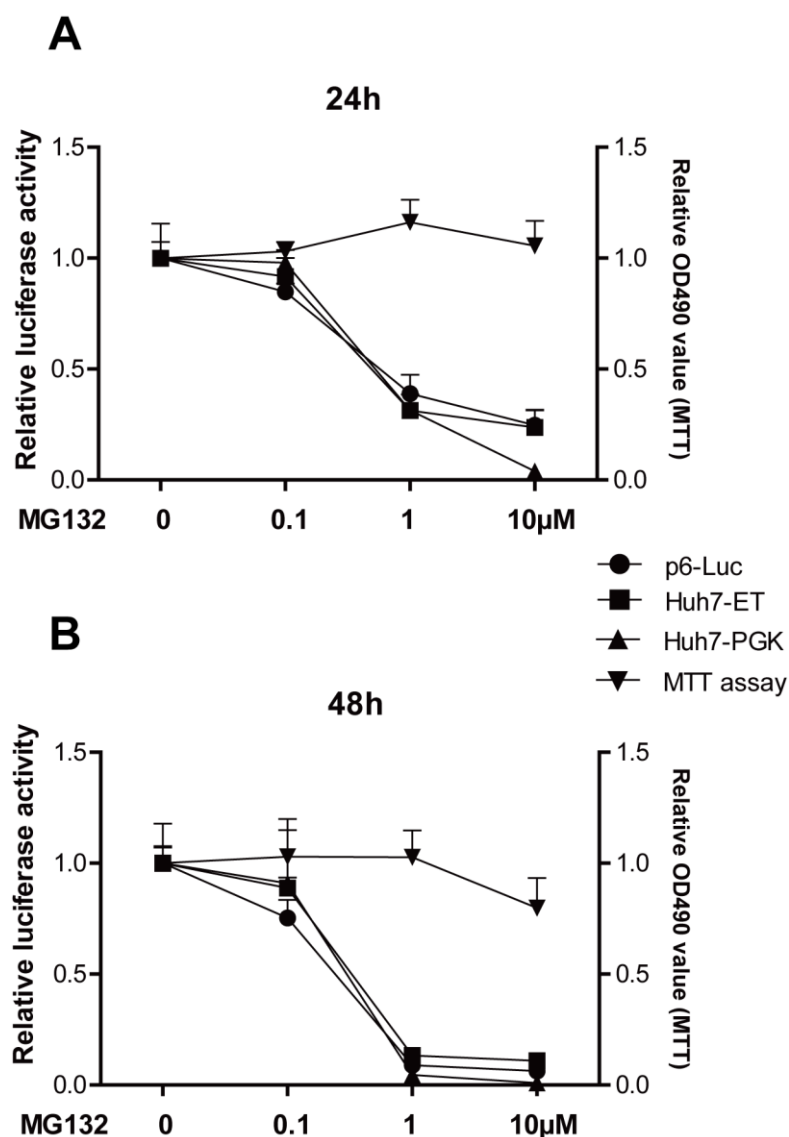


Figure 1 The proteasome inhibitor MG132 exerted non-specific effects on luciferase activity. Treatment with MG132 after 24 hrs (A) and 48 hrs (B) resulted in dramatic reduction of luciferase activity in subgenomic HEV replicon (p6-Luc) (mean \pm SD, $n = 12$), HCV replicon (Huh7-ET) (mean \pm SD, $n = 4$) and Huh7 cells constantly expressing control luciferase gene under control of the PGK promoter (Huh7-PGK) (mean \pm SD, $n = 4$). MG132 treatment did not affect cell viability determined by MTT assay (OD 490 value) (mean \pm SD, $n = 4$).

references genes, GAPDH and RP2 were concurrently decreased. In addition, the expression levels of four other host genes that we tested, including CyA, CyB, CD81 and IMPDH2, also decreased simultaneously (Figure 2B). These results confirm that the effect of MG132 is non-specific.

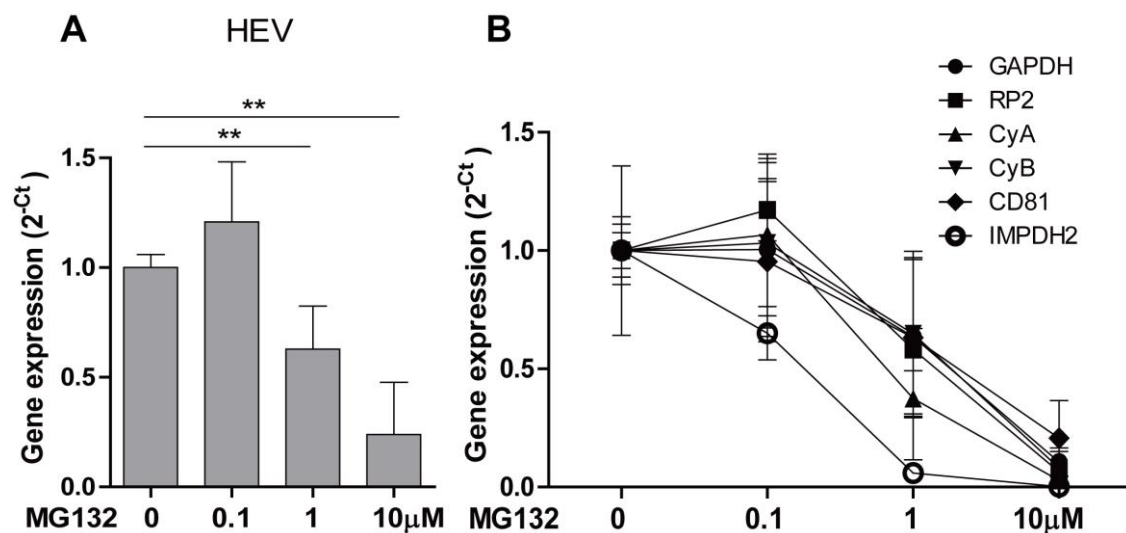


Figure 2 HEV viral RNA was significantly decreased by MG 132 treatment (A). The expression of two reference genes (GAPDH, RP2) and 4 host genes (CyA, CyB, CD81 and IMPDH2) were concurrently inhibited by treatment of MG132. Relative gene expressions was quantified by qRT-PCR. Data is presented as 2^{-Ct} and normalized to untreated control (mean ± SD, n = 6).

Next, we measured the RNA concentration and total protein content of the cells after MG132 treatment and we found that MG132 treatment (1 μM and 10 μM) drastically reduced the total cellular RNA content (Figure 3A). Furthermore, cells that were treated with MG132 and lysed showed reduced cellular protein expression. As shown in Figure 3B, the protein level of internal reference β-actin was decreased after treatment with 1 μM and 10 μM MG132, and the total protein content was also reduced (Figure 3C). However, the effects of MG132 at the protein level were less profound than that at the RNA level. These results suggest that MG132 inhibits expression and translation of a broad range of genes rather than having a specific effect on viral infection.

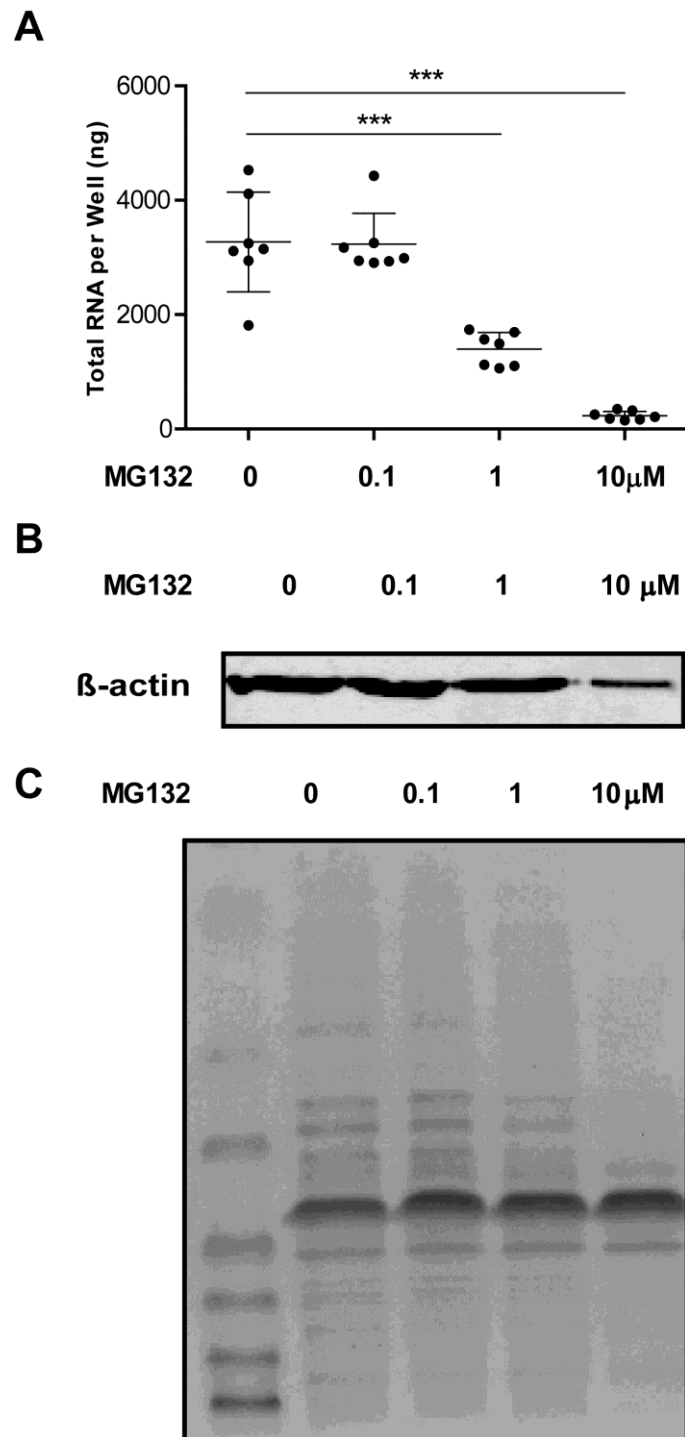


Figure 3 Internal reference β -actin was inhibited by MG132 treatment after 48 hrs. Same volume of cell lysates was loaded and the protein level was determined by Western blot (A). Total intracellular RNA in cells was dramatically reduced by MG132 treatment after 48 hrs. RNA concentration was determined by the NanoDrop 2000 spectrophotometer (mean \pm SD, n = 7) (B). Total intracellular protein was dramatically reduced by MG132 treatment after 48 hrs. Same volume of cell lysates was loaded and the gel was stained in Coomassie Brilliant Blue solution and destained (C).

Discussion

There is a substantial evidence suggest that the cellular UPS is associated with viral infection. RdRp, the essential enzyme for viral replication, can be regulated by UPS in turnip yellow mosaic virus (TYMV) ¹, sindbis virus ⁴, hepatitis A virus (HAV) ⁵ and HCV ⁶ infections. Virus-encoded proteases cleave viral polyprotein proteolytically but can also mediate the processing of many host proteins ¹⁶. Mature 3C proteases of HAV and encephalomyocarditis virus (EMCV) have been attributed to rapid, ubiquitin-mediated protein degradation ^{17,18}. As a combat strategy, some viral proteases have been shown to contain de-ubiquitinating enzyme activity. Papain-like cysteine proteases of coronavirus ¹⁹, HEV ²⁰ and foot-and-mouth disease virus (FMDV) ²¹ have the ability to hydrolyze ubiquitinating substrates. Therefore, modulating UPS represents as a potential antiviral strategy.

Treatment with proteasome inhibitor MG132 has been shown to decrease the titers of porcine circovirus type 2 (PCV2) at the early infection stage ²². Treatment with MG132 was also shown to decrease the activity of *Renilla* luciferase expressed from an HEV replicon ¹². However, our study raised the concern regarding the specificity of MG132 on HEV replication. Although we confirmed the inhibitory effects on luciferase activity of both HEV and HCV replicon models, MG132 also inhibited a constitutively expressed luciferase in control cells. Furthermore, in the full-length HEV model, MG132 treatment reduced HEV RNA levels, it also simultaneously inhibited the expression of reference genes and other host genes. We further demonstrated that MG132 dramatically decrease the total intracellular RNA and protein, which explains such a non-specific effect on viral infection.

It is not surprising that inhibition of this system could exert variety of effects on cell physiology, since UPS plays essential roles in cellular proteins processes. Proteasome could promptly degrades ubiquitylated proteins ²³, and some of these proteins are important mediators of cell-cycle progression and apoptosis ²⁴. MG132 has been shown to induce the expression of death receptor 5 (DR5), a receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), resulting in enhanced sensitivity to TRAIL-induced apoptosis in cancer cells ^{25,26}. Thus, inhibition of this major intracellular protein degradation pathway could un-specifically affect viral infection, but we do not fully exclude that UPS may also specifically modulate certain viruses ².

In summary, this study demonstrated that inhibition of HEV infection by proteasome inhibitor MG132 was non-specific. Thus, we should be careful of interpreting data regarding the effects and mechanisms of proteasome inhibitor on viral infection. Although proteasome inhibitors are emerging as anticancer agents in preclinical phase ^{24,27}, we would call for caution of developing proteasome-targeted antiviral therapies.

References

1. Camborde L, *et al.* The ubiquitin-proteasome system regulates the accumulation of Turnip yellow mosaic virus RNA-dependent RNA polymerase during viral infection. *Plant Cell* **22**, 3142-3152 (2010).
2. Choi AG, Wong J, Marchant D & Luo H. The ubiquitin-proteasome system in positive-strand RNA virus infection. *Rev Med Virol* **23**, 85-96 (2013).
3. Isaacson MK & Ploegh HL. Ubiquitination, ubiquitin-like modifiers, and deubiquitination in viral infection. *Cell Host Microbe* **5**, 559-570 (2009).
4. de Groot RJ, Rumenapf T, Kuhn RJ, Strauss EG & Strauss JH. Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc Natl Acad Sci U S A* **88**, 8967-8971 (1991).
5. Losick VP, Schlax PE, Emmons RA & Lawson TG. Signals in hepatitis A virus P3 region proteins recognized by the ubiquitin-mediated proteolytic system. *Virology* **309**, 306-319 (2003).
6. Gao G & Luo H. The ubiquitin-proteasome pathway in viral infections. *Can J Physiol Pharmacol* **84**, 5-14 (2006).
7. Yuksek K, Chen WL, Chien D & Ou JH. Ubiquitin-independent degradation of hepatitis C virus F protein. *J Virol* **83**, 612-621 (2009).
8. Shirakura M, *et al.* E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. *J Virol* **81**, 1174-1185 (2007).
9. Fan Z, *et al.* SARS-CoV nucleocapsid protein binds to hUbc9, a ubiquitin conjugating enzyme of the sumoylation system. *J Med Virol* **78**, 1365-1373 (2006).
10. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
11. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
12. Karpe YA & Meng XJ. Hepatitis E virus replication requires an active ubiquitin-proteasome system. *J Virol* **86**, 5948-5952 (2012).
13. Zhou X, *et al.* Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol* **61**, 746-754 (2014).
14. Pan Q, *et al.* Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C Virus infection in vitro and in vivo. *Hepatology* **55**, 1673-1683 (2012).
15. Wang Y, *et al.* Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology* **146**, 1775-1783 (2014).
16. Lloyd RE. Translational control by viral proteinases. *Virus Res* **119**, 76-88 (2006).
17. Gladding RL, Haas AL, Gronos DL & Lawson TG. Evaluation of the susceptibility of the 3C proteases of hepatitis A virus and poliovirus to degradation by the ubiquitin-mediated proteolytic system. *Biochem Biophys Res Commun* **238**, 119-125 (1997).
18. Lawson TG, *et al.* Identification and characterization of a protein destruction signal in the encephalomyocarditis virus 3C protease. *J Biol Chem* **274**, 9904-9980 (1999).
19. Barretto N, *et al.* The papain-like protease of severe acute respiratory syndrome coronavirus has deubiquitinating activity. *J Virol* **79**, 15189-15198 (2005).
20. Karpe YA & Lole KS. Deubiquitination activity associated with hepatitis E virus putative papain-like cysteine protease. *J Gen Virol* **92**, 2088-2092 (2011).

21. Wang D, *et al.* The leader proteinase of foot-and-mouth disease virus negatively regulates the type I interferon pathway by acting as a viral deubiquitinase. *J Virol* **85**, 3758-3766 (2011).
22. Cheng S, Yan W, Gu W & He Q. The ubiquitin-proteasome system is required for the early stages of porcine circovirus type 2 replication. *Virology* **456-457**, 198-204 (2014).
23. Rock KL, *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761-771 (1994).
24. Adams J. The proteasome: a suitable antineoplastic target. *Nat Rev Cancer* **4**, 349-360 (2004).
25. Yoshida T, *et al.* Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. *Cancer Res* **65**, 5662-5667 (2005).
26. He Q, Huang Y & Sheikh MS. Proteasome inhibitor MG132 upregulates death receptor 5 and cooperates with Apo2L/TRAIL to induce apoptosis in Bax-proficient and -deficient cells. *Oncogene* **23**, 2554-2558 (2004).
27. Elliott PJ, Zollner TM & Boehncke WH. Proteasome inhibition: a new anti-inflammatory strategy. *J Mol Med (Berl)* **81**, 235-245 (2003).

Chapter 11

Summary and discussion

Part I: Hepatitis in immunocompromised patients

HEV infection, the most frequent cause of acute hepatitis, has emerged as an important public health issue worldwide ¹. Although the majority of infections are thought to remain asymptomatic and usually cause self-limiting disease, persistent infection with chronic hepatitis may be observed in immunocompromised patients in particular organ transplant recipients ². Therefore, there is an urgent need to properly evaluate the clinical impact of HEV in these patients. In **Chapter 2** of this thesis we have performed a comprehensive review on the prevalence, infection course, and management of HEV infection after solid organ transplantation. On the basis of a series of published cohort studies, the prevalence of anti-HEV IgG is approximately 11.6%, and genomic viral RNA is 2% in SOT patients. A total of 65% of patients who were positive for HEV RNA developed chronic infection. Although a combination of serologic and molecular assays were used for many of the studies in the transplant settings, there is still a possibility of positivity for only antibody or only viral RNA ^{3, 4}. Thus, there is an urgent need for developing robust and standardized diagnostic assays, possibly combining both serology and molecular techniques and including properly produced control materials. Regarding the condition of Immunocompetence in general, significant lower lymphocyte subset counts, in particular CD4 T-cells, were found in HEV infected patients developing chronic hepatitis, compared with those who cleared the virus within six months ⁵. Hence, a healthy immune system can control and eliminate the infection, indicating dose reduction or even withdrawal (if possible) of immunosuppressants is the first intervention strategy to clear HEV infection in SOT patients.

Different types of immunosuppressants could interact either positively or negatively with the host immune system ⁴. Clinical evidence indicated that different immunosuppressive regimens can differentially affect the course of HEV infection as well ^{2, 6}. In fact, there is an evidence in HEV-transplant patients showing that tacrolimus but not CsA is more frequently associated with persistent infection ², whereas the MMF, the pre-drug form of MPA can help clearing the virus ⁶. However, current clinical studies have not been able to conclusively address the impact of different immunosuppressants, because of limited patient numbers and lack of mechanistic insight as to how differences in immunosuppressive medication might be linked with an altered clinical course of HEV infection. Thus, there is substantial room for fundamental and translational research to further investigate these

interesting questions by using state-of-the-art HEV cell culture. In **Chapter 3** and **Chapter 4** we applied recent development of a genotype 3-based cell culture system ^{7,8} to investigate the effects of different immunosuppressants, including steroids (Pred and Dex), calcineurin inhibitors (FK506 and CsA), MPA, and rapalogs (rapamycin and everolimus) on HEV replication.

In **Chapter 3** we observed no clear effect of either Pred or its close analogue Dex on HEV replication in both subgenomic and infectious models in culture system. Although limited studies have reported the impact of steroids in HEV patients, one case report has documented a good clinical and biochemical response to steroid therapy in a patient of acute hepatitis E with autoimmune hepatitis, who maintained health with a low dose of steroids ⁹. Next, CsA, a calcineurin inhibitor, directly promoted HEV replication, through inhibiting the activity of cyclophilins (CypA and CypB). Whereas another calcineurin inhibitor, FK506, only at high dose of treatment provokes a proviral effects. In fact, compared to CsA, dose reduction of FK506 appeared to be more associated with clearance of HEV in cases of renal transplantation with acute infection ¹⁰. In a large retrospective study (although only 85 patients included), the use of FK506 was the main predictive factor for chronic hepatitis E in organ recipients ². Our *in vitro* results have indicated that both FK506 and CsA can promote HEV infection. However, these data do not necessarily contradict to the clinical observation, because the number of patients currently investigated in the clinic is rather too small to draw solid conclusion. In contrast, MPA potently suppressed HEV infection by depletion of cellular nucleotide pools. In addition, a clear beneficial effect was observed when MPA combined with another antiviral regimen ribavirin.

In **Chapter 4** we dig deeper into the effects and mechanism-of-action of another type of important immunosuppressants rapalogs, including rapamycin and everolimus, on HEV infection. Both immunosuppressants are gaining increasing popularity in the transplantation context, mainly because of the low nephrotoxicity ¹¹. Their molecular mode of action is well characterized and involves inhibition of the mammalian target of rapamycin (mTOR) pathway. mTOR is a central element within the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB)-mTOR signaling ¹¹ and integrates nutritional information and receptor tyrosine kinase signaling to control cellular growth via a variety of cellular effectors, including activation of p70 S6 kinase and subsequent protein synthesis as well as inhibition of autophagy. In both subgenomic and full-length HEV models, HEV infection was

substantially elevated following treatment with rapamycin or everolimus. Consistently, stable silencing of mTOR by lentiviral RNAi resulted in a significant increase in intracellular HEV RNA, suggesting an antiviral function of mTOR in HEV infection. By targeting a series of other up- and down-stream elements of mTOR signaling, we further revealed an effective basal defense mechanism mediated by the PI3K-PKB-mTOR pathway against HEV, which involves phosphorylated eIF4E-binding protein 1 (4E-BP1), however independent of autophagy formation. This novel action of mTOR in directly counteracting viral replication in liver cell themselves rather as acting through the adapted immune system, represents a highly novel non-canonical action of this kinase and constitutes a novel adaptive immune system-independent antiviral mechanism.

Part II: Extra-hepatic manifestations

Although HEV, defined as a hepatotropic virus, is supposed to exclusively infect the liver and only causes hepatitis, a broad range of extrahepatic manifestations have been recently reported in association with its infection ¹². Most of these are case reports or small case series published during the last 5 years, reflecting increased awareness of the extra-hepatic manifestations associated with this infection in general ¹².

HEV infection has been reported to be related to renal manifestations ¹⁰ and HEV has been demonstrated to be present in the urine of acute and chronically infected individuals and experimentally acute infected monkeys ¹³. Since we are interested in chronic HEV, in **Chapter 5** we have establish a monkey model for persistent infection and detected HEV RNA in the urine of two monkeys at 272 and 650 days post infection, respectively. One macaque persistently infected with HEV died unexpectedly and we observed HEV RNA positivity in both liver and renal tissues. Furthermore, there was no clear histological change with liver tissue, but indeed there were clear pathological changes in renal tissue. This may indicate that HEV could replicate in kidney and subsequently cause tissue injury, which may facilitate the release of virus into urine. As reported, HEV-Ag EIA S/CO values were clearly higher in urine than serum, while the HEV RNA concentrations were lower in urine than serum in most patients and infected monkeys ¹³. This may suggest that HEV in urine is not solely in the form of virions; HEV-Ag without HEV RNA, as free antigen or empty capsids may be quite abundant.

Among these extra-hepatic manifestations, neurological disorders have been described as a relatively common, but under-recognized, extrahepatic manifestation related to HEV infection^{12, 14, 15}. An increasing number of central or peripheral nervous system diseases have been documented in patients associated with acute or chronic HEV infection^{12, 14}, including Guillain-Barre syndrome (GBS) and neuralgic amyotrophy (NA)^{14, 16, 17}. However, the etiology of HEV-associated neurological injury is unknown. In the existing paradigm, HEV is considered to be primarily a hepatotropic virus. Hitherto, the most widely held neuropathogenic hypothesis posits that HEV causes neurological injury by immune mechanisms related to molecular mimicry, as has been well described for GBS associated with a variety of infectious triggers^{14, 18}. The alternative hypothesis, however, that HEV causes direct injury via neurotropism has not been sufficiently evaluated. In **Chapter 6** we demonstrated that various human neural cell lines and embryonic stem cell-derived neural lineage cells were susceptible to HEV infection. Induced pluripotent stem (iPS) cell-derived human neurons and primary mouse neurons were more permissive to HEV infection compared to human liver cells. In mice and monkeys peripherally inoculated with HEV particles, viral RNA and protein were detected in brain tissues. Finally, patients with HEV-associated neurological disorders shed virus into cerebrospinal fluid (CSF), indicating direct infection of their nervous system. These results challenge the dogma of HEV as a pure hepatotropic virus and suggest that HEV infection should be considered in the differential diagnosis of idiopathic neurological disorders.

Part III: Anti-viral therapy

Since no registered medication is available for HEV infection, pegylated IFN- α , ribavirin, or a combination of both has been used as off-label treatments for some cases of HEV infection¹⁹. Because of the potent antiviral activity, pegylated interferon- α (PEG-IFN α) has been used for decades to treat chronic hepatitis B virus (HBV) and C virus (HCV) infection^{19, 20}. However, the efficacy and molecular mechanisms of how IFN signaling interacts with HEV remain undefined. In **Chapter 7** we revealed that HEV is in general insensitive to the regulation of cytokines and chemokines. IFN- α treatment exerts moderate but delayed antiviral activity against HEV infection in experimental models and in patients, which suggested the ineffectiveness of interferon-based monotherapy in treating chronic hepatitis

E. Interestingly, blocking the basal IFN pathway resulted in drastic augmentation of HEV infection, suggesting that basal IFN pathway can effectively protect against HEV infection. These results revealed distinct mode-of-actions of basal and treatment activated IFN signaling in controlling HEV infection.

Mechanistically, interferon molecules bind to cell surface receptors and subsequently initiate a signaling cascade to activate transcription of IFN-stimulated genes (ISGs), which are in turn regulated by the IFN-stimulated response element (ISRE). The products of ISGs are thought to be the ultimate antiviral effectors²¹. Interferon regulatory factor 1 (IRF1) is one of the most important ISGs which potently inhibits the replication of 14 different viruses, representing 7 families including different DNA and RNA viruses²¹. However, the exact efficacy and molecular mechanism of IRF1 on HEV remains unclear. In **Chapter 8** we investigated the role of IRF1 in HEV infection and the interactions with antiviral treatments by using cell culture models. We found that independent of interferon production, IRF1 could effectively restrict HEV replication through the activation of JAK-STAT cascade and the subsequent induction of a wide range of ISGs. We further demonstrated that the anti-HEV effect of IRF1 overlapped with IFN α but was augmented by ribavirin.

Besides the importance of host innate defense against HEV infection, other essential host elements could also be a target for the development of novel antiviral therapy against HEV. Like all viruses, HEV is completely dependent on the translational machinery of host cells to synthesize the viral proteins essential for its productive infection²². The way host protein synthesis machinery is commandeered by viruses has a major impact on viral protein synthesis and genome replication²³, but little is known regarding how HEV uses host translational machinery for its life-cycle. In **Chapter 9** we revealed that cap dependent translation machinery eIF4F complex plays a critical role in help with efficient HEV replication. The translational suppressors PDCD4 and 4E-BP1 are important antiviral factors in restraining HEV infection. Interestingly, the eIF4F complex can interact with antiviral regimens, such as ribavirin or interferon- α (IFN- α), which are the classical standard therapy of chronic hepatitis C but also as off-label drugs for treating individual HEV cases or small case series^{24, 25}. Ribavirin can directly bind to eIF4E and compete for 5' cap mRNA binding^{26, 27}, whereas some regulatory factors of eIF4F complex are involved in interferon mediated antiviral immune response^{28, 29}. In our study we observed that the antiviral effects of

ribavirin and IFN- α are independent of eIF4E, although silencing of eIF4E could already inhibit HEV replication.

Last but not least, viral functional structures which serve for various steps in the HEV life cycle can also be good drug targets³⁰. A recent study reported potent antiviral effects of a well-known proteasome inhibitor MG132 against HEV³¹. In **Chapter 10** we found that MG132 could inhibit HEV and hepatitis C virus (HCV) replication-related luciferase activity in subgenomic models. Furthermore, treatment with MG132 in a HEV infectious model resulted in a dramatic reduction in the intracellular level of HEV RNA. Surprisingly, MG132 concurrently inhibited the expression of a luciferase gene used as a control as well as a wide range of host genes. Consistently, the total cellular RNA and protein content was concurrently reduced by MG132 treatment, suggesting a nonspecific antiviral effect.

Final remarks

- Although experimental research alone will not be able to clarify these complicated but important clinical issues, our laboratory investigation of the effects of different immunosuppressants on HEV infection does provide a proof of concept for the notion that it is important to choose the right immunosuppressive medication to aid antiviral therapy for HEV infection in organ transplantation. Hopefully, it will also promote the initiation of randomized controlled clinical studies to address these issues in the near future.
- Extra-hepatic manifestations represent an important aspect of HEV infection and signs and symptoms of these conditions should be considered in the clinical management of patients with acute or chronic HEV infection. Our further investigations in pathogenesis of HEV-associated neurological disorders challenged the notion the HEV is primarily a hepatotropic virus and suggested HEV infection as a possibility in idiopathic neurologic disease. Thus large-scale testing for HEV in idiopathic neurological disease is called for.
- Our discovery of a novel antiviral mechanism has shed new light on the molecular details of HEV-host interaction, in particular the role of therapeutically-activated and the basal IFN signaling, an important anti-HEV ISG, as well as the host translation machinery, which may help to understand the complicity of HEV-host interactions and bears significant implications in management of HEV patients and future therapeutic development.

In conjunction, I hope our studies have contributed to the battle of humanity with HEV infection.

Reference

1. Kamar N, *et al.* Hepatitis E. *Lancet* **379**, 2477-2488 (2012).
2. Kamar N, *et al.* Factors associated with chronic hepatitis in patients with hepatitis E virus infection who have received solid organ transplants. *Gastroenterology* **140**, 1481-1489 (2011).
3. Pas SD, *et al.* Hepatitis E virus infection among solid organ transplant recipients, the Netherlands. *Emerg Infect Dis* **18**, 869-872 (2012).
4. Passos AM, Heringer TP, Medina-Pestana JO, Ferraz ML & Granato CF. First report and molecular characterization of hepatitis E virus infection in renal transplant recipients in Brazil. *J Med Virol* **85**, 615-619 (2013).
5. Kamar N, *et al.* Hepatitis E virus and chronic hepatitis in organ-transplant recipients. *N Engl J Med* **358**, 811-817 (2008).
6. Pischke S, *et al.* Chronic hepatitis e in heart transplant recipients. *Am J Transplant* **12**, 3128-3133 (2012).
7. Shukla P, *et al.* Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proc Natl Acad Sci U S A* **108**, 2438-2443 (2011).
8. Shukla P, *et al.* Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. *J Virol* **86**, 5697-5707 (2012).
9. Desai HG & Naik AS. Acute viral E hepatitis with chronic liver disease (autoimmune hepatitis). *Indian J Med Sci* **59**, 113-115 (2005).
10. Kamar N, *et al.* Hepatitis E virus and the kidney in solid-organ transplant patients. *Transplantation* **93**, 617-623 (2012).
11. Rostaing L & Kamar N. mTOR inhibitor/proliferation signal inhibitors: entering or leaving the field? *J Nephrol* **23**, 133-142 (2010).
12. Bazerbachi F, Haffar S, Garg SK & Lake JR. Extra-hepatic manifestations associated with hepatitis E virus infection: a comprehensive review of the literature. *Gastroenterol Rep (Oxf)* **4**, 1-15 (2016).
13. Geng Y, *et al.* Detection and assessment of infectivity of hepatitis E virus in urine. *J Hepatol* **64**, 37-43 (2016).
14. Dalton HR, *et al.* Hepatitis E virus and neurological injury. *Nat Rev Neurol* **12**, 77-85 (2016).
15. Kamar N, Dalton HR, Abravanel F & Izopet J. Hepatitis E virus infection. *Clin Microbiol Rev* **27**, 116-138 (2014).
16. Bandyopadhyay D, *et al.* Two Uncommon Causes of Guillain-Barre Syndrome: Hepatitis E and Japanese Encephalitis. *Case Rep Neurol Med* **2015**, 759495 (2015).
17. van Eijk JJ, *et al.* Neuralgic amyotrophy and hepatitis E virus infection. *Neurology* **82**, 498-503 (2014).
18. Willison HJ, Jacobs BC & van Doorn PA. Guillain-Barre syndrome. *Lancet*, (2016).
19. Peters van Ton AM, Gevers TJ & Drenth JP. Antiviral therapy in chronic hepatitis E: a systematic review. *J Viral Hepat* **22**, 965-973 (2015).

20. Janssen HL, *et al.* Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. *Lancet* **365**, 123-129 (2005).
21. Schoggins JW, *et al.* Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* **505**, 691-695 (2014).
22. Walsh D & Mohr I. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. *Genes Dev* **18**, 660-672 (2004).
23. Montero H, Garcia-Roman R & Mora SI. eIF4E as a control target for viruses. *Viruses* **7**, 739-750 (2015).
24. Zhou X, *et al.* Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol* **23**, 295-304 (2013).
25. Kamar N, *et al.* Ribavirin for chronic hepatitis E virus infection in transplant recipients. *N Engl J Med* **370**, 1111-1120 (2014).
26. Kentsis A, Topisirovic I, Culjkovic B, Shao L & Borden KL. Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap. *Proc Natl Acad Sci U S A* **101**, 18105-18110 (2004).
27. De la Cruz-Hernandez E, *et al.* Ribavirin as a tri-targeted antitumor repositioned drug. *Oncol Rep* **33**, 2384-2392 (2015).
28. Burke JD, Sonenberg N, Platanias LC & Fish EN. Antiviral effects of interferon-beta are enhanced in the absence of the translational suppressor 4E-BP1 in myocarditis induced by Coxsackievirus B3. *Antivir Ther* **16**, 577-584 (2011).
29. Nehdi A, *et al.* Deficiency in either 4E-BP1 or 4E-BP2 augments innate antiviral immune responses. *PLoS One* **9**, e114854 (2014).
30. Kenney SP & Meng XJ. Therapeutic targets for the treatment of hepatitis E virus infection. *Expert Opin Ther Targets* **19**, 1245-1260 (2015).
31. Karpe YA & Meng XJ. Hepatitis E virus replication requires an active ubiquitin-proteasome system. *J Virol* **86**, 5948-5952 (2012).

Chapter 12

Nederlandse samenvatting

Dutch summary

Samenvatting voor de leek in de Nederlandse taal.

Het hepatitis E virus (HEV) is één van de verwekkers van virale hepatitis. Het HEV komt het bloed binnen via het maag-darm kanaal en zou zich uitsluitend vermenigvuldigen in de lever. Het verloop van een acute HEV-infectie kent een aantal stadia: van subklinisch, naar acuut en uiteindelijk fulminant. Hepatitis E, dat in toenemende mate wordt gezien als een “public health concern”, heeft een mortaliteit van 0.2-1.0 %, maar kan bij zwangere vrouwen in het laatste trimester van hun zwangerschap oplopen tot een mortaliteit van 20-25%. Daarnaast is er zorg over het gevaar van het virus voor andere groepen, met name voor transplantatiepatiënten. Zulke patiënten krijgen immuunsysteem onderdrukkende medicatie voorgeschreven die potentieel de weerstand tegen HEV zouden kunnen verminderen. Inderdaad bleek uit een systematisch door mij uitgevoerd literatuuronderzoek (**hoofdstuk 2**) met betrekking tot de vatbaarheid van transplantatiepatiënten voor HEV infectie, dat zo’n twaalf procent van deze patiënten antistoffen voor HEV in haar bloed had. Bovendien was in zo’n twee procent der patiënten ook viraal RNA aanwezig. In deze laatste groep ontwikkelde 65% van de patiënten een chronische infectie. Ik concludeerde dat hepatitis E een groot probleem is bij orgaan transplantatie.

Orgaan transplantatie gaat gepaard met het gebruik van immunosuppressiva. Immers, anders wordt het getransplanteerde orgaan afgestoten. Verschillende immunosuppressiva werken mechanistisch op andere wijze; het is dus goed voorstelbaar dat sommige immunosuppressiva een directe interactie met de levenscyclus van het HEV en het virus direct remmen, terwijl andere immunosuppressiva niet zo’n interactie zouden hebben. Uiteraard zou het gebruik van een immunosuppressief regime dat tegelijkertijd ook virusrepletie remt de voorkeur moeten hebben voor patiënten met een verhoogd risico op het ontwikkelen van hepatitis E. In **hoofdstuk 3** en **hoofdstuk 4** ga ik op zoek naar zulke immunosuppressiva. Ik vond dat verschillende immunosuppressiva inderdaad een andere interactie met de HEV levens cyclus hadden. Sommigen, in het bijzonder remmers van mTOR (een moleculair element dat informatie van de voedingsstaat van de cel integreert met de informatie gegeven door immunostimulatoire hormonen) leken HEV infectie te bevorderen (mTOR lijkt dan ook een element van de verdediging van de cel tegen HEV). Andere immunosuppressiva, zoals steroïden, hadden geen effect, maar mycofenolzuur (een potent

middel om orgaanafstoting te voorkomen) remde juist HEV infectie. Dit laatste middel lijkt dan ook aangewezen bij patiënten met een verhoogd risico op hepatitis E.

Vervolgens ging ik mij op eigenschappen van het HEV richten die mogelijk de klinische verschijnselen kunnen verklaren. Met name was ik geïnteresseerd in de zogenaamde extra-hepatische manifestaties van dit virus. HEV infectie gaat vaak gepaard met neurologische verschijnselen. De reden voor deze verschijnselen is onduidelijk, maar er wordt gedacht dat de virusinfectie een auto-immuun fenomeen kan oproepen. In **hoofdstuk 5**, echter, laat ik zien dat HEV niet lever specifiek is, maar zich ook kan vermenigvuldigen in zenuwcellen. HEV infectie van zenuwcellen vormt dan ook een rationele verklaring voor de neurologische klachten geassocieerd met hepatitis E. Bovendien zou HEV infectie in sommige gevallen ook een verklaring kunnen vormen voor onbegrepen neurologische ziektes.

Het laatste gedeelte van mijn proefschrift handelt over antivirale therapie die gebruikt zou kunnen worden om hepatitis E te behandelen. Zo exploreer ik in **hoofdstuk 7** behandeling met interferon alfa. Interferon alfa wordt gebruikt bij allerlei virusziekten, maar in dit hoofdstuk stel ik vast dat dit hormoon ook een belangrijke rol kan spelen bij de behandeling van hepatitis E. Mechanistisch wordt dit verder onderzocht in **hoofdstuk 8**. Hier identificeer ik een set genen die worden geactiveerd door interferon alfa en die de effecten van interferon alfa op de levenscyclus van het HEV lijken te verklaren, met name door het aanjagen van zogenaamde JAK-STAT signalering. Bovendien laat ik in dit hoofdstuk zien dat de anti-HEV activiteit van interferon alfa verder versterkt wordt door co-applicatie van het medicijn ribavirine. De data uit deze twee hoofdstukken laten zien dat voor het behandelen van hepatitis de combinatie van interferon alfa en ribavirine een rationele keuze is.

Een venster op verbeterde behandeling van hepatitis E in de toekomst wordt geopend in **hoofdstuk 9**. Hier onderzoek ik het belang van de zogenaamde cellulaire eiwit-translerende machinerie voor de vermenigvuldiging van HEV. Ik laat zien dat het remmen van elementen uit deze machinerie een belangrijke antivirale werking heeft, met name het onderdrukken van eIF4E inhibeert virale replicatie in sterke mate. Omdat deze inhibitie onafhankelijk is van interferon alfa en ribavirine, zou het eIF4E een belangrijk nieuw doelwit kunnen worden voor nieuwe antivirale therapie. Helaas blijkt dit niet het geval voor de proteosoom inhibitor MG132. Anderen hadden eerder gerapporteerd dat deze inhibitor efficiënt was voor het bestrijden van HEV infectie. In **hoofdstuk 10** echter, laat ik zien dat

deze effecten waarschijnlijk niet specifiek zijn en dat proteosoominhibitoren waarschijnlijk dus toekomst hebben in de behandeling van hepatitis E.

In het laatste hoofdstuk (**hoofdstuk 11**) van dit proefschrift tenslotte, probeer ik alle informatie die ik heb vergaard gedurende mijn promotieonderzoek te integreren en te duiden aan de hand van de reeds bestaande biomedische literatuur. Ik concludeer dat de keuze van immunosuppressie voor transplantatiepatiënten gevolgen heeft, sommige immunosuppressiva maken de patiënt meer gevoelig voor een daaropvolgende HEV infectie, terwijl andere medicatie, in het bijzonder mycofenolzuur, juist patiënten kan beschermen tegen dit virus. Ook stel ik vast dat de extrahepatische manifestaties van hepatitis E waarschijnlijk samenhangen met directe infectie van niet-leverorganen door het virus en tenslotte doe ik uitspraken over de mechanismen die door de antivirale medicatie interferon alfa worden aangezwengeld en het virus bestrijden. Samen hoop ik dat mijn studies een nieuwe bijdrage hebben kunnen leveren in de strijd der mensheid tegen hepatitis E.

Appendix

Acknowledgements

Publications

PhD Portfolio

Curriculum Vitae

Acknowledgements (致谢)

I would like to express my gratitude to all the people who helped me with the projects and with the life, who accompanied me and supported me during my four years PhD study. This is an emotional moment for me and I would not achieve here without all your dedications.

I want sincerely gain my thanks to my co-promoter, Dr. Qiuwei (Abdullah) Pan and my promoter, Prof. Maikel Peppelenbosch.

Abdullah, thank you for your great and continuous support and supervision during four years of my entire PhD. It is my honor to be your first Chinese PhD student, and the second PhD student when you got your PhD degree. I was astonished by your amazing and excellent PhD thesis, when I came here to start my PhD. From then on, you became my idol on the way of scientific research. You are always be so nice and patient to me especially at the very beginning when I was struggling with every new technique and fresh knowledge. You gave me a lot of useful suggestions on how to raise up scientific questions, how to efficiently solve the problems during research, how to harbor a good habit when doing experiments, how to prepare and learn the right way of thinking, writing manuscript and presenting the data on seminar. I benefit a lot and all of these would give me more confidence to do a better research during my lifetime. Your powerful ambition and superior ability on doing research impressed me and I am so glad to work with you these years. Thank you for everything!

Maikel, thank you for the guidance, kindness, continuous encourage and help for the research during four years of my PhD. I am grateful to be your students. Working with you is quite an eye-opener because you know tremendous amount of various knowledge in every field, and always elaborate your proposal and suggestions on my research during the group meeting, which was held every fortnight. You taught me not to give up even when others already published similar results, and should finish what I am doing to present the best and complete story as I can. You encourage me to learn English and go to international congress to practice my skills, helped me a lot and made me more confident. All my research and paper are much more glory with the innovative ideas you made. Thank you so much for supervising me.

Prof. Robbert de Man, your pioneer on the topic of immunosuppressants on HEV recurrence has indeed paved the way of the first part of my thesis. I am honored to have you as Inner Committee for my promotion. Thank you!

Prof. Bart Jacobs, I would like thank you for all your help and your valuable and expert suggestions on the manuscript of one part of my thesis. You take a lot of time on discussing the mechanisms of HEV-associated neurological disorders and this inspired a lot. I am pleased and appreciative to have you as Inner Committee for my promotion. Thank you!

Dr. Luc van der Laan, thank you for the suggestions for my research and encouragement for me everytime when I get some good results and publications. I am so glad that you will be in my promotion.

Prof. Harry Dalton, thank you for critical and valueable suggestion on the manuscript and research work. Thank you for your efforts on my study. You encouraged me and gave me confidence when I met you in International Liver Congress before I first gave oral presentation in the Congress. You are so kind!

Prof. Nassim Kamar, thank you for valueable suggestion on the research work. You gave me confidence when we met at International Liver Congress.

Dave, thank you for your help, suggestions and discussion for most of my research during PhD. It is a precious gift for me everytime you raise up a question of “where do you want to go then?”. I think it is really important to remember this question and be ready to answer it everytime when you want to do something interesting or meaningful.

Pratika, you are the first person I know in Rotterdam and Erasmus MC, also my first friend here. Thank you for your kind help with my life especially for the first month I was here. You show me around the lab and get familiar with the rules, be patient to teach me everything I confused about. You invited me to be with your whole family to spend my first Christmas here. I really appreciate that you and your husband treated me like a family member. You will always be my older sister and hope you now have a happy time with your family, your husband, your lovely daughter: Amreta, and your cute son: Sagara. Hope we can meet each other in China or Indonesia in the near future.

Yijin, thank you for the contribution of chapters to my thesis. Thank you for your company for four years. You came here one month later than me, but I am so glad we can hold the thesis defense at the same day and graduate together. You helped me a lot in my daily life and also in lab. You take a lot of delicious food for me, I will never forget the taste of Liangpi and Roujiamo you made. I am pleased that both of us found our job before graduate. We are best friends forever and hope you can find your true love and dream house in Beijing.

Lei, many thanks for your enormous help with my lab work especially my second year of PhD. Thank you for the contribution of chapters to my thesis. You always know everything in particular how to solve software problem of PCR and so on, and this indeed helped a lot. I enjoy the time when we laughing and eating (a lot), with you and Yuebang. Wish you a success with the paper and also your job next year.

Wenhui, thank you for always inviting me have dinner with you and for your suggestions on my job and my life. You are so kind to talk to me whenever I am confused. Thank you for being my Paranimf. Hope you graduate on time and have a good life with your baby son Mengmeng.

Yuebang, many thanks for your tremendous help with my research. You are always so nice to help anyone else and be enthusiastic to everything. Thank you for the fun you made for us every lunch time. You always make delicious food for us! I enjoy the time for hotpot with you and Lei. For three years, we are “iron triangle” in one office. I really appreciate that I have a friend like you. Thank you! Wish you a success for graduation!

Wenshi, thank you for the help with my experiments and lab work. Thank you very much for introducing me to your landlord and rent the department to me. I really enjoy these two years living here. It is pleasure to attend the liver congress with you in Wien and Barcelona. You and Hongbo are so kind to invite us to your house. Hope you have a shining future in the research field.

Wanlu, thank you for the mouse brain you got for me for the experiments. We have a nice time in Barcelona. You are a young sister of Pan's group and always be simple and pure. Keep your enthusiasm to the research and hope you get a position here to continue your dream.

Kan, thank you for teaching me the knowledge of immune system. You are so kind to remind me everything might happen during my future career and I learned many things from your experience. Thank you for manage the financial in Barcelona to make us an easy trip. Hope you a success for your defense!

Wen, thank you for the help with the experiments. You are good at cooking and sharing food with us. Everytime when we have a party or go out for a trip, you always cook for us. I will never forget the delicious Ludan. Wish you a success for your paper and be happy everyday!

Manzhi, thank you for your help on my research in my last year. Thank you to company with me to do EM experiment in Groningen. You always bring happiness to everyone and also bring a lot of home-made food to us. Thank you for bring the cirtification of degree for me from China. Hope you can find your own way on research and have a happy family with Maoxing soon.

Shan, thank you for your Ganguo, the most delicious food in the world. Juan, thank you for the discussion about interesting ideas you have. Pengyu, thank you for disscuss about the research and happiness you bring for us when you give an "imitation show". Jun, thank you for bring the gift of my husband from China here. Xiaolei, thank you for encouraging me in research when I came to the lab. Yingying, thank you for inviting me home to have hotpot with your family. Zhanmin, thank you for the help with the research and everytime share the agents in lab when I need them. Guoying, thanks for inviting me to your house to have a party. Thank you for the effeorts on preparing the pubquiz party with me. Changbo and Buyun, thanks for inviting me to have dinner with you, for playing poker cards with us and thank you for be my Paranimf. Meng, thanks for the promise of going to Brugge together for the concert of Damien Rice. Ming, thank you for inviting me to Groningen and take the gift back to China for my husband. Bin, thank you for inviting me to your department to join the party.

Jan, thank you for warm smiling everytime you see me, which makes me feel like home. Patra, thank you for your help with the research work and gentler smile to me. Gwenny, thank you for your guidance for the research and valuable suggestions on my study. Wesley, thank you for discussion about mTOR pathway in my paper. I enjoy the time when we palying basket ball together. Elmer, thank you for helping me to take the staff which is too

high for me. Remember? We always can take fantastic picture together. I really enjoy the time with you in the lab and during every party. Rik, thank you for sharing the interferons for me. You are really good at palying basket ball, in an American style. Evelyn, thank you for decorating the office to give me a fancy Christmas during three years. Thank you for comfort me when my dog died. Werner, thank you for telling me lots of molecular knowledge and how to design primers by myself. Sergey, thank you for always sharing ideas and talking to me for everything. Martijn, thanks for being my desk-mate, and translating dutch letter for me, conllecting the sticks from supermarket for me. Thanks for everything. Lauke, thanks for the credir card for paying for the flight tickets for me. Rajesh, thanks for discussing the politics between India and Chaina. You are always in the office during weekend for my first year of PhD, which makes me not feel lonely. Raymond and Leonie, thank you for sloving the computer problem, ordering agents, and everything of organizing and assistance.

All MDL members whom have left and whom are present, Ron, Jaap, Andrea, Andre, Que, Jasper, Dowty, Wouter, Pauline, Vincent, Anthonie, Kim, Angela, Paula, Wendy, Alexander, Hakim, Patrick, Emmeloes, Kostas, Eelke, Renee, Henk, Marcel, Hugo, Aniek, Sonja, Gertine, Asma and so on. I want to thank you for helping me during the four years. I have indeed enjoy my time with you in the lab. Best wishes to all of you!

All the co-authors, thank you for the contribution to my thesis and to the journal publications.

For Prof. Ying Lin (supervisor of my Master research), 感谢华南理工大学生命科学与工程学院的林影教授对我读硕士研究生期间的科研指导，对学习和生活中的无限关心和鼓励。是您培养我认真对待科研的态度以及坚定追求这条道路的决心。

For Prof. Dongsong Nie (supervisor of my Bachelor's research), 感谢湖南理工学院化学化工系的聂东宋教授，对我本科期间学习和生活的指导和对科研的鼓励，让我满怀考研及继续学习生物的热情。

感谢岳阳市第一中学的黄卫东老师 (bilology teacher in high school)，您在我高中时候上的生物课让我开始对生物产生浓厚兴趣，对我的谆谆教诲让我那段时间充满信心，追求更好的未来。

感谢我的父母 (pareents), 在我学习成长期间尊重我的选择, 给我提供自由和舒适的学习环境, 默默支持我的学业, 三年在广州和四年在荷兰的时间, 很少时间陪伴在你们身边, 还好有旺仔代替我给你们作伴。希望爸爸身体一直健康, 妈妈也生活无忧无虑, 毕业以后我们可以一家团聚, 欢乐生活在一起。

感谢我的公公婆婆 (parents in law), 全力支持我出国四年继续追求我的学业, 感谢你们每次在我回家的时候都热情招待我。感谢老公的姐姐总是给我送上温暖的问候和祝福。感谢我所有的亲戚, 非常支持我出国并且以我为豪, 总是鼓励我, 帮我照顾我的父母家人, 并且常常视频来和我分享家里的一切! 谢谢你们的关心和支持!

Last but not the least, to my husband, Peilei Zhang, I really appreciate that I can know you when we doing our Master's research in the same university. You "endured" me a lot when we were together and I realized that when I was alone here. You are the person who treat me best in the world more than anyone else. It is not easy for you to wait for me for four years in China, but you did very well on your work, and on dealing with the relationship between us. We communicate with each other everyday in spite of jet leg, sometimes I call you when you are asleep at 3:00 a.m. in China, just because I am nervous about my research and feel pressured. You would never be angry with me, but ask me to calm down and not to worry. Thank you so much for what you have done to my parents, visiting and companing them when I were not by their sides. We got married during my PhD, that is a most important moment in my lifetime. I hope we can live a happy life when I go back to you for eternity! I can't waiting for that! I love you so much!

Publications

International (refereed) journals

1. **Zhou X**, Xu L, Wang W, Watashi K, Wang Y, Sprengers D, de Ruiter PE, van der Laan LJ, Metselaar HJ, Kamar N, Peppelenbosch MP, Pan Q. Disparity of basal and therapeutically activated interferon signalling in constraining hepatitis E virus infection. *J Viral Hepat.* 2016;23: 294-304.
2. Xu L, **Zhou X**, Wang W, Wang Y, Yin Y, van der Laan LJ, Metselaar HJ, Peppelenbosch MP, Pan Q. IFN regulatory factor 1 restricts hepatitis E virus replication by activating STAT1 to induce antiviral IFN-stimulated genes. *FASEB J.* 2016 Jun; Epub.
3. Huang F, Yang C, **Zhou X**, Yu W, Pan Q. Rhesus macaques persistently infected with hepatitis E shed virus into urine. *J Hepatol.* 2016;64(6):1446-7.
4. Wang Y, Wang W, Xu L, **Zhou X**, Shokrollahi E, Felczak K, van der Laan LJ, Pankiewicz KW, Sprengers D, Raat NJ, Metselaar HJ, Peppelenbosch MP, Pan Q. Cross Talk between Nucleotide Synthesis Pathways with Cellular Immunity in Constraining Hepatitis E Virus Replication. *Antimicrob Agents Chemother.* 2016;60: 2834-2848.
5. Wang W, Xu L, Brandsma JH, Wang Y, Hakim MS, **Zhou X**, Yin Y, Fuhler GM, van der Laan LJ, van der Woude CJ, Sprengers D, Metselaar HJ, Smits R, Poot RA, Peppelenbosch MP, Pan Q. Convergent Transcription of Interferon-stimulated Genes by TNF-alpha and IFN-alpha Augments Antiviral Activity against HCV and HEV. *Sci Rep.* 2016;6: 25482.
6. **Zhou X**, Xu L, Wang Y, Wang W, Sprengers D, Metselaar HJ, Peppelenbosch MP, Pan Q. Requirement of the eukaryotic translation initiation factor 4F complex in hepatitis E virus replication. *Antiviral Res.* 2015;124: 11-19.
7. Xu L, **Zhou X**, Peppelenbosch MP, Pan Q. Inhibition of hepatitis E virus replication by proteasome inhibitor is nonspecific. *Arch Virol.* 2015;160: 435-439.
8. **Zhou X**, Wang Y, Metselaar HJ, Janssen HL, Peppelenbosch MP, Pan Q. Rapamycin and everolimus facilitate hepatitis E virus replication: revealing a basal defense mechanism of PI3K-PKB-mTOR pathway. *J Hepatol.* 2014;61: 746-754.
9. Wang Y, **Zhou X**, Debing Y, Chen K, Van Der Laan LJ, Neyts J, Janssen HL, Metselaar HJ, Peppelenbosch MP, Pan Q. Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology.* 2014;146: 1775-1783.
10. **Zhou X**, de Man RA, de Knecht RJ, Metselaar HJ, Peppelenbosch MP, Pan Q. Epidemiology and management of chronic hepatitis E infection in solid organ transplantation: a comprehensive literature review. *Rev Med Virol.* 2013;23: 295-304.
11. Zhang L, Liang S, **Zhou X**, Jin Z, Jiang F, Han S, Zheng S, Lin Y. Screening for glycosylphosphatidylinositol-modified cell wall proteins in *Pichia pastoris* and their recombinant expression on the cell surface. *Appl Environ Microbiol.* 2013;79: 5519-5526.

National (refereed) journals (Abstract in English and main text in Chinese)

1. Lin Y, **Zhou X**, Han S, Zhang L, Ye Y, Zheng S. Extraction and analysis of cell wall proteins of recombinant *Pichia pastoris*. Journal of South China University of Technology (Natural Science Edition). 2012;5: 101-106.

Patent

1. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw3 of *Pichia pastoris*. 2011. Patent No. CN201110216679.4.
2. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw5 of *Pichia pastoris*. 2011. Patent No. CN201110217256.4.
3. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw12 of *Pichia pastoris*. 2011. Patent No. CN201110215840.6.
4. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw15 of *Pichia pastoris*. 2011. Patent No. CN201110215844.4.
5. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw17 of *Pichia pastoris*. 2011. Patent No. CN201110215842.5.
6. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw19 of *Pichia pastoris*. 2011. Patent No. CN201110215853.3.
7. Lin Y, **Zhou X**, Zhang L. The cell-wall surface display system and construction method of protein Gcw21 of *Pichia pastoris*. 2011. Patent No. CN201110215855.2.
8. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw28 of *Pichia pastoris*. 2011. Patent No. CN201110215823.2.
9. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw30 of *Pichia pastoris*. 2011. Patent No. CN201110215805.4.
10. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw32 of *Pichia pastoris*. 2011. Patent No. CN201110215812.4.
11. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw42 of *Pichia pastoris*. 2011. Patent No. CN201110215804.X
12. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw45 of *Pichia pastoris*. 2011. Patent No. CN201110215803.5.
13. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw49 of *Pichia pastoris*. 2011. Patent No. CN201110215813.9.
14. Lin Y, Zhang L, **Zhou X**. The cell-wall surface display system and construction method of protein Gcw51 of *Pichia pastoris*. 2011. Patent No. CN201110215811.X.

PhD Portfolio

Name PhD Student	Xinying Zhou
Erasmus MC Department	Gastroenterology and Hepatology
PhD Period	October 2012 - September 2016
Promotor	Prof. Dr. Maikel P. Peppelenbosch
Copromotor	Dr. Qiuwei (Abdullah) Pan

General Courses

- 2012, the workshop on photoshop and illustrator CS5
- 2013, the course on biomedical English writing course for MSc and PhD-students
- 2014, the virology course and symposium
- 2014, the course biomedical research techniques XIII

National and International Conferences

- 2014, 49th The International Liver Congress™ (EASL, European Association for the Study of the Liver), London, UK (Oral presentation)
- 2014, 7th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, The Netherlands (Poster presentation)
- 2014, Annual Day of the Molecular Medicine Postgraduated School, Rotterdam, the Netherlands. (Oral presentation)
- 2015, 50th The International Liver Congress™ (EASL, European Association for the Study of the Liver), Vienna, Austria (Oral presentation for Early morning workshop)
- 2015, 8th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, The Netherlands (Poster presentation)
- 2015, Annual Day of the Molecular Medicine Postgraduated School, Rotterdam, the Netherlands. (Poster presentation)
- 2016, 51th The International Liver Congress™ (EASL, European Association for the Study of the Liver), Barcelona, Spain (Poster presentation)
- 2016, 9th Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting. Veldhoven, the Netherlands (Oral presentation)
- 2016, Annual Day of the Molecular Medicine Postgraduated School, Rotterdam, the Netherlands. (Oral presentation)

Academic Awards

Young Investigator Travel Awards

- 2014, 49th The International Liver Congress™ (EASL, European Association for the Study of the Liver), London, England (€650)
- 2015, 50th The International Liver Congress™ (EASL, European Association for the Study of the Liver), Vienna, Austria (€650)
- 2016, 51th The International Liver Congress™ (EASL, European Association for the Study of the Liver), Barcelona, Spain (€650)

Scientific Awards and Grants

- 2012, China Scholarship Council (CSC) Scholarship (File No. 201206150075)

Curriculum Vitae



Xinying Zhou was born in January 4, 1987, in Aksu, Xinjiang Uygur Autonomous Region, China. She went to Yueyang, Hunan Province, the south-middle part of China when she was three months old and grew up there. She was raised by her beloved parents Zhengwu Zhou and Kehui Zhang. She attended primary, middle and high school in Yueyang City.

In 2005, she graduated from high school and started her Bachelor study in Biological Engineering at Hunan Institute of Technology. She graduated in 2009, and moved to Guangzhou, the capital of Guangdong Province, south part of China, to start her Master research in Biochemistry and molecular biology at South China University of Technology. Under supervision of Prof. Ying Lin, she started to do the research in the application of glycosylphosphatidylinositol (GPI)-anchored glycoproteins in *Pichia pastoris*. Later on, she graduated in 2012.

In 2012, she moved to the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, the Netherlands, to carry out his PhD research on hepatitis E virus (HEV) infection. Under supervision of Prof. Maikel P. Peppelenbosch and Dr. Qiuwei (Abdullah) Pan, she focused on pathogenesis and therapy for hepatitis E. Specifically, she devoted to projects, including (I) effects and mechanisms of different immunosuppressants on HEV infection; (II) the extra-hepatic manifestations related to HEV infection; (III) the therapy for hepatitis patients and potential antiviral targets of the host.

Currently, she would be a lecturer in Guangzhou, Southern Medical University and wants to continue her research.

