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

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Background & 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 downstream 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 downstream elements of mTOR signaling, we further revealed an effective basal defense mechanism of the 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.

Keywords: Hepatitis E virus; Rapamycin; Everolimus; PI3K-PKB-mTOR pathway.
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Abbreviations: mTOR, mammalian target of rapamycin; HEV, hepatitis E virus; PI3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; 4E-BP1, eIF4E-binding protein 1.

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 a 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 the case of pregnant women [7]. In the current (2012–2013) hepatitis E outbreak among refugees in South Sudan, a total of 5080 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 [8]. Humans appear to have powerful HEV combating mechanisms, but these apparently require a good nutritional and host defence status for optimal functionality [9]. The nature of these mechanisms has not been characterised, 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 [10,11].

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 [12]. Their molecular mode of action is well characterised 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 [13] 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) [14], but also to the induction of the production of antiviral interferons [15]. 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 rapalog implications in 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 an autophagy-independent fashion but through phosphorylated 4E-BP1 in infected hepatocytes.

Materials and methods

Reagents

Stocks of rapamycin (Merck, Schiphol-Rijk, The Netherlands) and everolimus (Sigma-Aldrich, St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) with a final concentration of 2 mM. Stocks of LY294022, an inhibitor of PI3K-PKB (Sigma-Aldrich), BEZ235, a dual inhibitor of PI3K-PKB and mTOR (Selleck Chemicals), FG-4592, an inhibitor of HIF-1α (Selleck Chemicals) and PF-478671, an inhibitor of p70 S6 kinase (Selleck Chemicals) were dissolved in DMSO. All agents were stored in 15 μl aliquots and frozen at −20 °C. Antibodies including LC3-I/II (Cell Signalling Technology, Netherlands), S6, phospho-S6, p70 S6 kinase, phospho-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 visualisation 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) [10,11]. The human hepatoma HuH7 cells were collected and centrifuged for 5 min, 1500 rpm, 4 °C. Supernatant was removed and washed with 4 ml Optimem by centrifuging for 5 min, 1500 rpm, 4 °C. The cell pellet was re-suspended in 100 μl 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) [10]. The supernatant of cultured p6 full-length HEV RNA electroporated cells was collected and used for secondary 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, The 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 visualisation 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 Biomics and produced in HEK 293T cells as previously described [16]. After a 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) to 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 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 grow at 37 °C with 5% CO_2 for 3 h. 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, 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 and *GAPDH* were amplified by 40 cycles and quantified with a SYBRGreen-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. The HEV primer sequences were 5′-ATTGGCCA GAAGTTGGTTTTCAC-3′ (sense) and 5′-CCGTGGCTATAATTGTGGTCT-3′ (antisense), and the primers of housekeeping gene *GAPDH* were 5′-TGTCCCCACCCCCAATGT ATC-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 sulphate-polyacrylamide gel and separated by electrophoresis (SDS-PAGE). After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) 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

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(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. The membrane was washed 3 times followed by incubation for 1.5 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for actin content as standardisation of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualised with Odyssey 3.0 software.

Confocal laser electroscope assay

Lipidated LC3 (LC3-II) is a robust marker of autophagic membranes. Autophagosomes were visualised 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 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 ± 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 (HuH7) were transfected with a 3′ subgenomic construct of the 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 Fig. 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 [10,11]. In parallel, HuH7 cells constitutively expressing a non-secreted firefly luciferase are used for normalisation of non-specific effects on luciferase signals. In addition, a HuH7 based full-length infectious HEV model (p6) was also employed (Supplementary Fig. 1) [10,11].

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 (Fig. 1A and B). Importantly, inhibiting mTOR rendered this system sensitive to HEV infection, as evident from higher levels of *G. princeps* luciferase, which increased over time. Treatment with 100 and 1000 ng/ml of rapamycin for 48 h resulted in a 1.9 ± 0.4 (mean ± SD, n = 3, p <0.05) and 2.7 ± 0.6 (mean ± SD, n = 3, p <0.01) -fold increase of HEV luciferase activity (Fig. 1C) and corresponds to a concomitant decrease in mTOR activity as assessed by phospho-Ser-240/224 S6 levels (Fig. 1A). At 72 h, HEV luciferase activity was further increased up to 3.8 ± 0.5 (mean ± SD, n = 3, p <0.01) and 4.9 ± 0.5 (mean ± SD, n = 3, p <0.01) -fold, respectively (Fig. 1C). A possible artefact here could be due to the direct growth-promoting effects of rapamycin, but the MTT assay showed that rapamycin did not promote cell growth (Fig. 1D).

Next to rapamycin, everolimus is often used for clinical mTOR inhibition following orthotropic organ transplantation. Like rapamycin, everolimus also remarkably permitted HEV replication. In the p6-Luc model, treatment with 1 ng/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 ± SD, n = 3, p <0.01) and 6.7 ± 1.4 (mean ± SD, n = 3, p <0.01) -fold increase at 48 h, 5.3 ± 0.8 (mean ± SD, n = 3, p <0.01) and 5.6 ± 1.9 (mean ± SD, n = 3, P <0.05) -fold increase of HEV luciferase activity at 72 h (Fig. 1E). Everolimus also did not promote cell proliferation determined by MTT assay (Fig. 1F). Dephosphorylation of S6 was also confirmed (Fig. 1B).

To exclude that this effect is a consequence of the loss of the capsid protein and ORF3 protein in our luciferase model, we repeated the experiments with the full-length infectious HEV model. Again, HEV infection was facilitated under mTOR-deficient conditions. For instance, treatment with 100 or 1000 ng/ml rapamycin has increased viral RNA levels up to 2.6 ± 0.6 (mean ± SD, n = 5, p <0.01) or 2.1 ± 0.4 (mean ± SD, n = 5, p <0.01) -fold, respectively (Fig. 1G). Treatment with 1, 10, 100, and 1000 ng/ml of everolimus for 48 h resulted in an increase of cellular viral RNA up to 1.6 ± 0.1 (mean ± SD, n = 3, p <0.01), 1.5 ± 0.3 (mean ± SD, n = 6, p <0.05), 2.0 ± 0.1 (mean ± SD, n = 3, p <0.01) and 2.1 ± 0.2 (mean ± SD, n = 3, p <0.01) (Fig. 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 Fig. 2A, three of the four tested shRNA vectors targeting mTOR exert potent gene silencing capacity, resulting in a profound downregulation of mTOR protein level but an elevation of PKB expression (probably due to a feedback activation). Correspondingly, mTOR silencing resulted in a significant increase of cellular HEV RNA, which was measured by qRT-PCR after inoculation of cell culture produced infectious HEV particles for 72 h. For instance, knockdown of mTOR by the sh*mTOR* clone 2 led to 2.6 ± 0.8-fold (mean ± SD, n = 3, p <0.05) increase of HEV RNA (Fig. 2A). These data provide direct and strong evidence that mTOR plays an important role in restricting HEV infection.

mTOR limits HEV replication via 4E-BP1

mTOR is a key kinase controlling cellular behaviour. Its most important effector pathways include induction of protein transcription via the p70 S6 kinase pathway (Fig. 2B) [15]. However, this pathway does not seem to be a major effector mechanism as inhibition of p70 S6 kinase by its inhibitor PF-478671 did not affect HEV infection (Supplementary Fig. 2). Inhibition of another downstream target of mTOR, hypoxia-inducible factor-1alpha (HIF-1α) by FG-4592 (Supplementary Fig. 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 (Fig. 2C), a hallmark of autophagosome formation. Furthermore, HuH7 cells stably intergraded with a lentiviral vector expressing GFP-LC3-II were used to visualise autophagosome formation. In the positive control groups, cells were either treated with 30 μM chloroquine

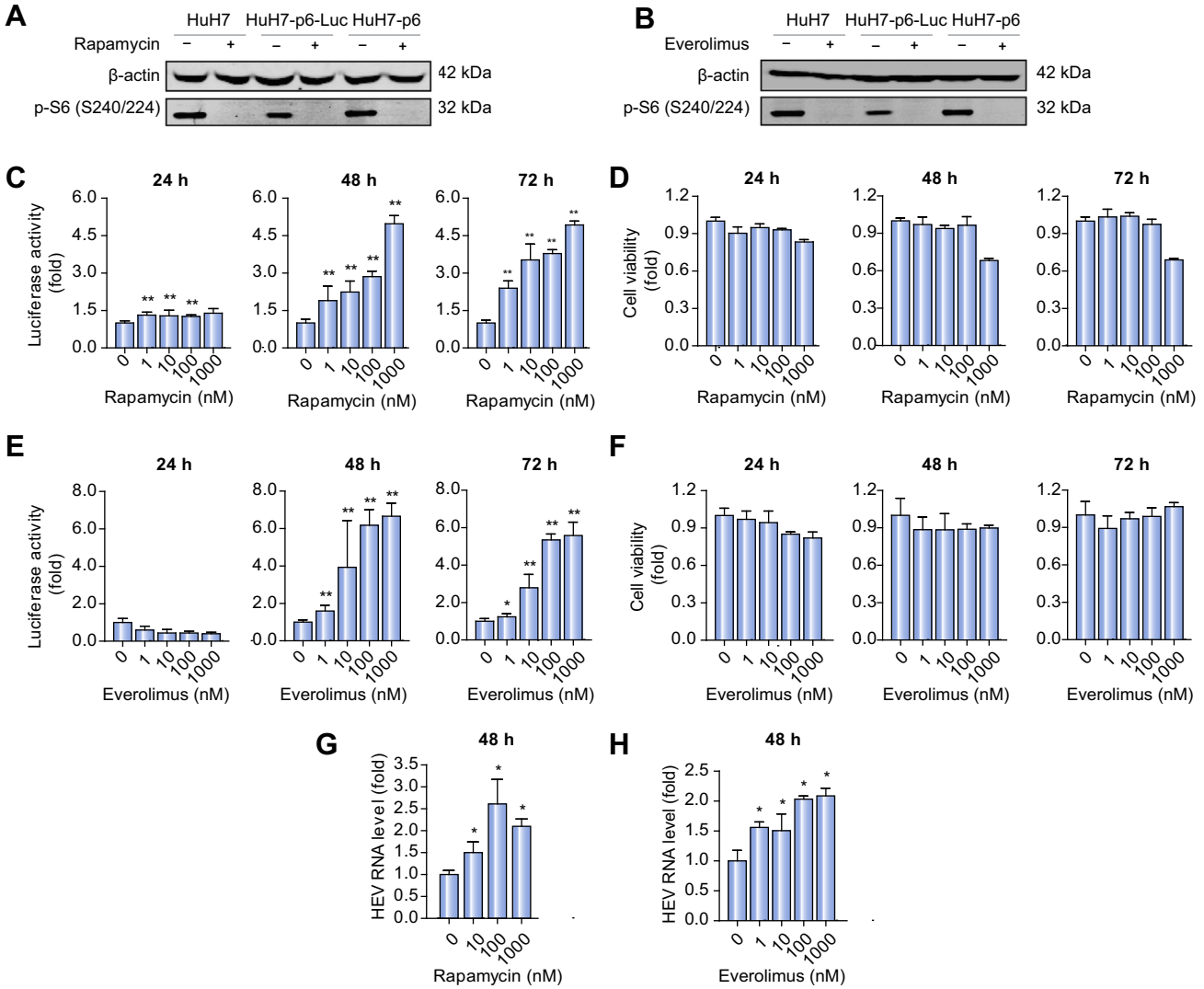

Fig. 1. mTOR inhibition by rapalogs facilitate HEV replication. (A) Western blot showing inhibition of S6 phosphorylation by treatment with 500 nM rapamycin for 48 h. β-actin served as an internal reference. (B) Western blot showing inhibition of S6 phosphorylation by treatment with 500 nM everolimus for 48 h. 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 ± SD, n = 3 independent experiments with each 2–3 replicates). (D) Rapamycin did not increase cell proliferation determined by MTT assay (OD_{490} value) (mean ± SD, n = 5). (E) In the HuH7 cell-based subgenomic HEV replicon, treatment with everolimus dose-dependently increased viral replication-related luciferase activity (mean ± SD, n = 3 independent experiments with each 2–3 replicates). (F) Everolimus did not increase cell proliferation determined by MTT assay (OD_{490} value) (mean ± SD, n = 5). (G) In the HEV infectious model, (G) rapamycin (mean ± SD, n = 5) as well as (H) everolimus (mean ± SD, n = 3–6) significantly increased cellular viral RNA determined by qRT-PCR. $^{*}p$ <0.05; $^{**}p$ <0.01.

or under condition of starvation in EBSS medium. As expected, green puncta of LC3-II were clearly emerging, indicating the formation of autophagosomes (Fig. 2D). In contrast, no changes of the autophagy machinery were observed with treatment of rapamycin or everolimus (Fig. 2D), which was consistent with the results of Western blot (Fig. 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 translation [17]. Treatment with the mTOR inhibitor everolimus (500 nM) for 48 h resulted in clear dephosphorylation of 4E-BP1 as shown by Western blot (Fig. 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 downregulation of total 4E-BP1 protein (Fig. 2F). 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 led to a 1.7 ± 0.6-fold (mean ± SD, n = 5, p <0.01) (by the sh*4E-BP1* clone 53) and 2.4 ± 0.9-fold (mean ± SD, n = 4, p <0.05) (by the clone 56) increase of HEV RNA, respectively (Fig. 2G). Consistently, clone 54, with minimal gene silencing efficacy, only exerted a minor effect (1.3 ± 0.3-fold,

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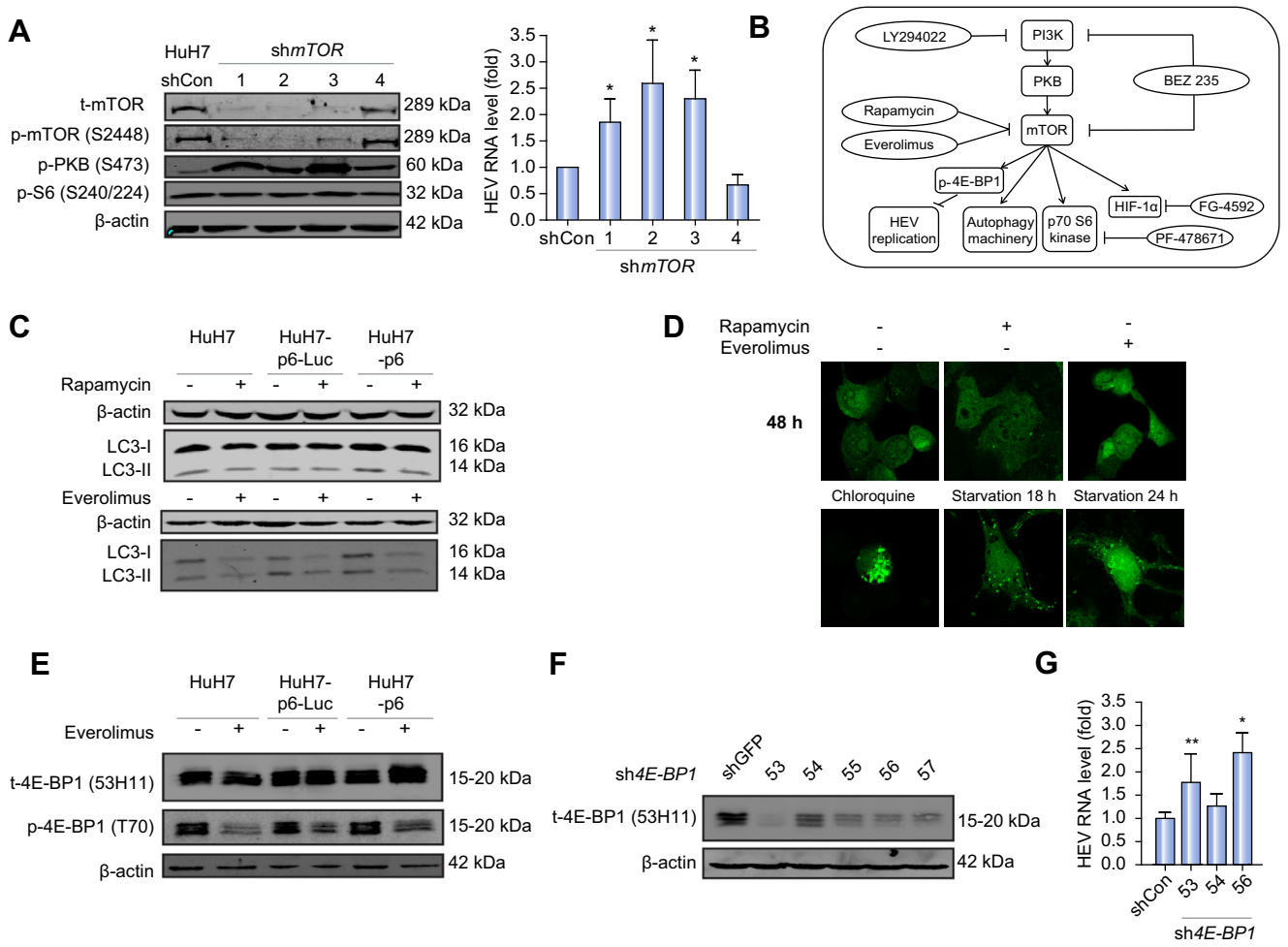

Fig. 2. Gene silencing of *mTOR* and *4E-BP1* by lentiviral RNAi enhances HEV replication independent of autophagy machinery. (A) Knockdown of *mTOR* by lentiviral shRNA vectors. Compared with the control vector transduced cells, the sh*mTOR* clones 1, 2, and 3 but not 4 exert potent silencing capability 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 ± SD, n = 3), which were measured by qRT-PCR after inoculation of cell culture produced infectious HEV particles for 72 h. $^{*}p$ <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-1α were used. (C) Naïve HuH7, subgenomic HEV replicon and HEV infected HuH7 cells were treated with rapamycin and everolimus for 48 h. 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 h. In contrast, autophagosome formation was observed in the positive control groups treated with 30 μM chloroquine for 48 h or at the circumstance of starvation in BESS media with 1 μM pepstatin A and E-64-D for either 18 or 24 h. Oil-lenses (40×) was used (1024 × 1024 image). (E) Western blot showed inhibition of 4E-BP1 phosphorylation by treatment of 500 nM everolimus for 48 h. β-actin served as an internal reference. (F) Knockdown of *4E-BP1* by lentiviral shRNA vectors. Compared with the control vector transduced cells, the sh*4E-BP1* clone 53, 55, 56, and 57 but not 54 expert 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 ± SD, n = 5). $^{*}p$ <0.05; $^{**}p$ <0.01. (This figure appears in colour on the web.)

mean ± SD, n = 4, p >0.05) on HEV replication (Fig. 2G). 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 (Fig. 2B). When HuH7 p6-Luc cells were treated with different concentrations (0.1–10 μM) of the well-established PI3K inhibitor LY294002, enhancement of HEV replication became apparently similar to that observed with mTOR inhibitors (Fig. 3A), which was also not related to enhanced cell proliferation (Fig. 3B). Consistently, LY294002 also significantly increased cellular HEV RNA in the infectious model up to 3.2 ± 1.1-fold (mean ± SD, n = 3, p <0.05) at a dose of 10 μM (Fig. 3C). These effects corresponded to the observed inhibition of the biological target (Fig. 3D).

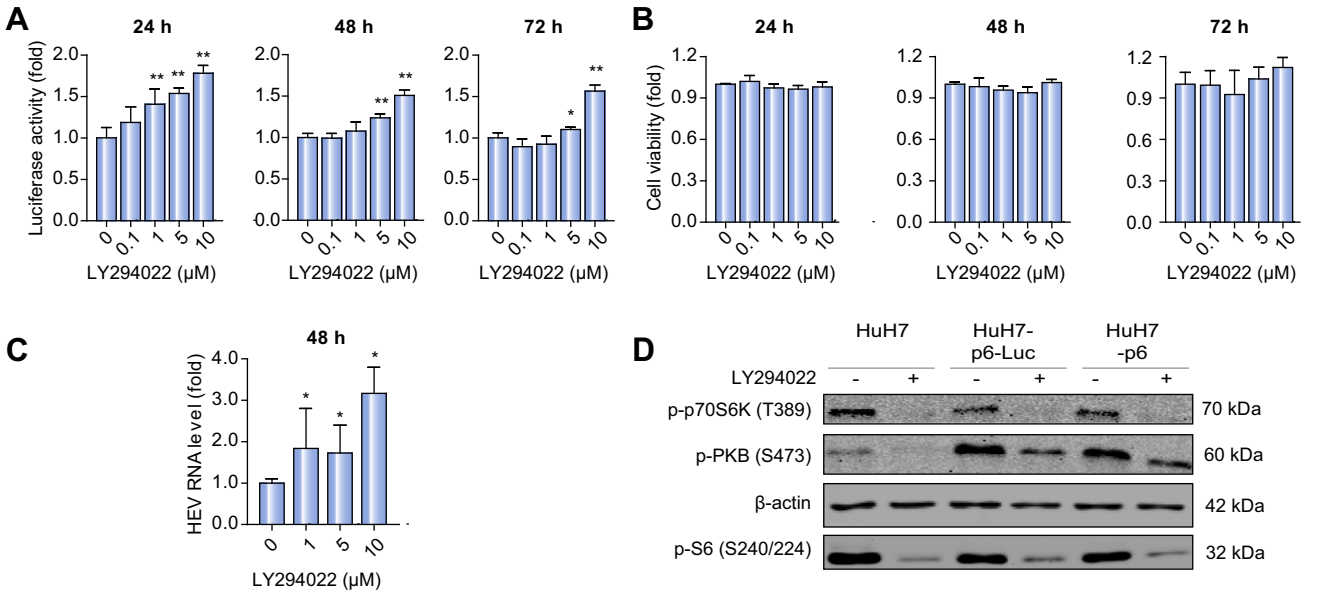

Fig. 3. Inhibition of PI3 K-PKB promotes viral replication. (A) In the HuH7 cell-based subgenomic HEV replicon, treatment with LY294022, a PI3K inhibitor, dose-dependently increased viral replication-related luciferase activity (mean ± SD, n = 3). (B) LY294022 did not affect cell proliferation determined by MTT assay (OD_{490} value) (mean ± SD, n = 4). (C) In the HEV infectious model, LY294022 significantly increased cellular viral RNA determined by qRT-PCR (mean ± SD, n = 3). (D) Western blot showed inhibition of PKB, S6 and p70 S6 kinase phosphorylation by treatment of 5 μm LY294022 for 48 h. β-actin served as an internal reference. Treatment time was indicated as 24, 48 or 72 h. $^{*}p$ <0.05; $^{**}p$ <0.01.

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 μM LY294002 at 48 h (up to 12.1 ± 3.1-fold, mean ± SD, n = 11, p <0.01 *vs.* untreated; p <0.01, *vs.* rapamycin; p <0.01, *vs.* LY294002) (Fig. 4A), and at 72 h with the combination of 1000 ng/ml rapamycin and 10 μM LY294002 (up to 31.7 ± 9.9-fold, mean ± SD, n = 11, p <0.01 *vs.* untreated; p <0.05, *vs.* rapamycin; p <0.01, *vs.* LY294002) (Fig. 4A). Similar effects were observed when everolimus was combined with LY294002 (Fig. 4B). Furthermore, these results were found not to be related to enhancement of cell proliferation either with rapamycin (Supplementary Fig. 4A) or everolimus (Supplementary Fig. 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) (Fig. 2B). We further investigated the effect of simultaneously inhibiting PI3K-PKB and mTOR by a single compound BEZ-235. As shown in Fig. 4C and D, BEZ-235 significantly promoted HEV infection in both models. Furthermore, results corresponded to inhibition of biological targets of this pathway (Fig. 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 [7]. 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 cells themselves rather than 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 [3,6,18]. It is known that the clinical symptoms of this hepatitis reacts very well to reducing dose of immunosuppression [6]. 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 approximately 15 ng/ml [21,22], whereas the levels in cancer patients can be up to approximately 100 ng/ml [23]. We have demonstrated that 1 ng/ml everolimus was sufficient to trigger 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 randomised 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.

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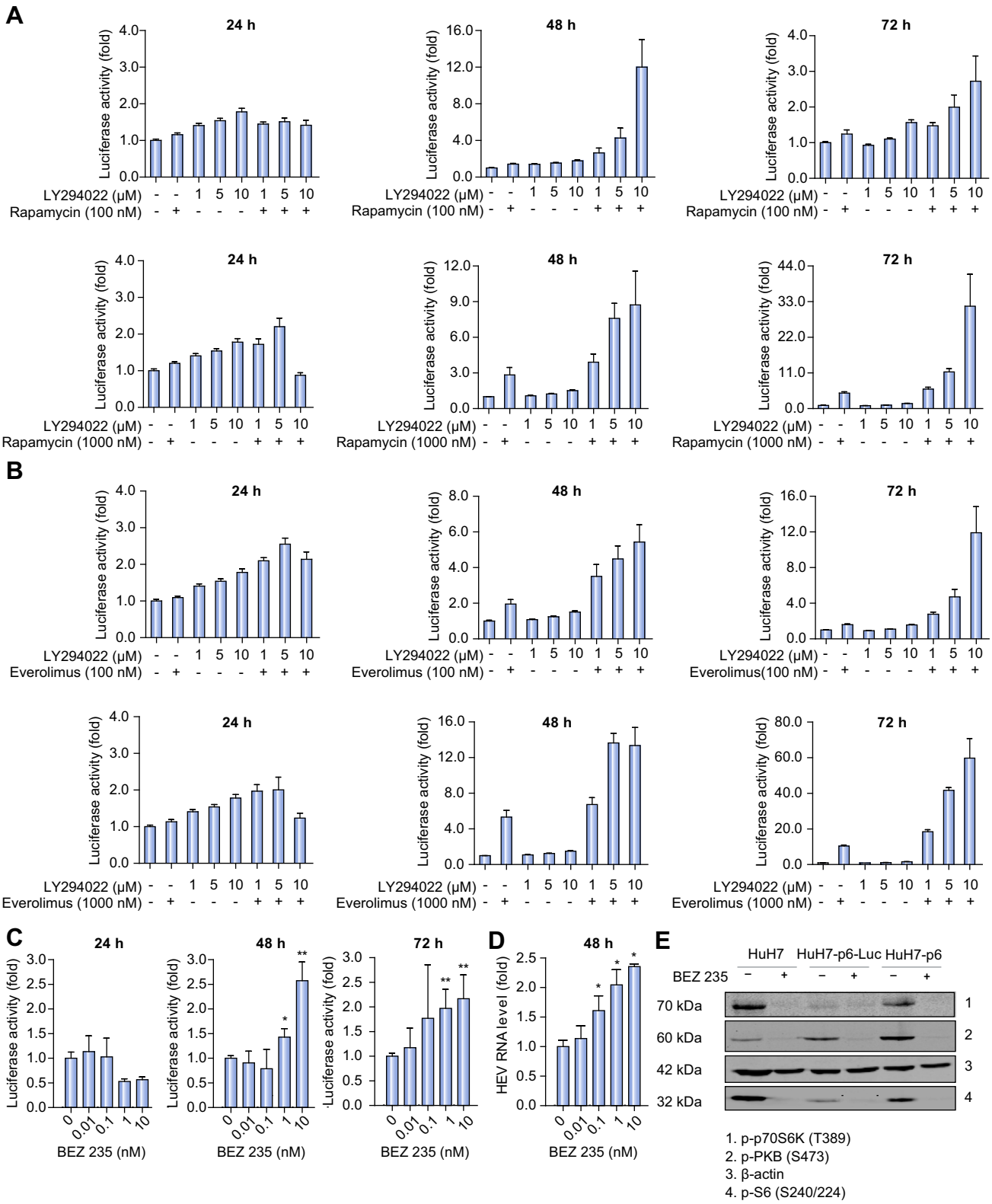

Fig. 4. Simultaneous inhibition of PI3K and mTOR further increased viral replication. In the HEV subgenomic replicon, viral replication-related luciferase activity was present when 1, 5 or 10 μM LY294022 was combined with 100/1000 nM of rapamycin (A) or everolimus (B). Treatment time was indicated as 24, 48 or 72 h. Data was presented as mean ± 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 ± SD, n = 5). (D) In the HEV infectious model, BEZ-235 significantly increased cellular viral RNA determined by qRT-PCR (mean ± 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 h. $^{*}p$ <0.05; $^{**}p$ <0.01.

The current experimental study has firmly demonstrated the pro-viral 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 to support the recent clinical observations in viral hepatitis patients [18,24,25].

Another group of patients at high risk for HEV caused death constitute pregnant women [26]. Although this effect is in literature generally linked to diminished immunity [26], immune suppression during pregnancy is relatively moderate [27]. Interestingly, the increased nutritional demands of pregnancy [28] provoke a powerful activation of the ATP/ADP-sensitive kinase AMPK [29]. In turn, this kinase is a potent inhibitor of mTOR [30] and indeed pregnancy is associated with a significant downregulation of mTOR [31]. It is tempting to speculate that pregnancy-specific downregulation 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 HEV [8]. We thus speculate that HEV may preferentially affect the human population when hepatic mTOR activity is below its constitutive level.

Because of its favourable side-effect profile, rapalog therapy is quickly gaining popularity for treating a variety of clinical syndromes, especially in oncological disease, in congenital diseases like the Peutz-Jeghers syndrome and the 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 with caution. In addition, these results may also help to understand the underlying mechanism why pregnant women are more susceptible to HEV infection with devastating outcome.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Authors' contributions

X.Z. performed the experiments, analysed data and wrote a draft of the paper. Y.W. performed some experiments. H.J.M. and H.L.J. discussed the project and edited the manuscript. M.P.P. and Q.P. conceived the ideas, designed and discussed experiments, supervised the project and extensively edited the manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.05.026.

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