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# Human hepatitis B virus surface and e antigens inhibit major vault protein signaling in interferon induction pathways

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**Background & Aims**: We previously demonstrated that major vault protein (MVP) is a novel virus-induced host factor and its expression upregulates type-I interferon production, leading to cellular antiviral response. However, it remains unclear whether the antiviral function of MVP is impaired during hepatitis B virus (HBV) infection and what mechanisms are involved. Therefore, the aim of this study was to assess whether HBV can alter MVP expression despite the lack of type-I IFN induction and shed light on the underlying mechanisms HBV utilizes to evade host innate immune response.

**Methods**: The ability of HBV surface and e antigens to inhibit MVP signaling in interferon induction pathways was evaluated by co-immunoprecipitation, immunofluorescence, quantitative RT-PCR, Western blot and reporter assays.

**Results**: In our current study, we found high levels of MVP in peripheral blood mononuclear cells, sera, and liver tissue from HBV-infected patients relative to healthy individuals. We determined that MVP intracellularly associates with MyD88, an adapter protein involved in virus-triggered induction of type-I IFN. Protein truncation analysis revealed that the middle domain of MVP (amino acid residues 310–620) was essential for MyD88 binding. Conversely, HBV inhibited MVP–induced type-I IFN production by suppressing MVP/MyD88 interaction. HBV antigens, both HBsAg and HBeAg, suppressed this interaction by competitively binding to the essential MyD88 binding region of MVP and limiting downstream IFN signaling.

**Conclusions**: MVP is a virus-induced protein capable of binding with MyD88 leading to type-I IFN production. HBV may evade an immune response by disrupting this interaction and limiting type-I IFN antiviral activity.



Keywords: Immune evasion; Viral infection; MyD88; Major vault protein.
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*Abbreviations:* PRRs, pathogen recognition receptors; TLR, Toll-like receptor; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; IFN, interferon; MVP, major vault protein; MyD88, myeloid differentiation factor 88; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; mRNA, messenger RNA; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription polymerase chain reaction; siRNAs, short interfere RNAs.

## Introduction

The hepatitis B virus (HBV), which leads to chronic hepatitis B (CHB), encodes several different viral proteins, including DNA polymerase, surface antigen (HBsAg), core antigen, and the X protein (HBx) [1]. The mechanisms for establishing and maintaining chronic infections are still unknown; however, evidence suggests that HBV has developed strategies to suppress the host immune response [2,3]. In humans, the innate immune system recognizes pathogens via pattern recognition receptors, such as Toll-like receptors (TLR), to initiate type-I interferons (IFNs) induction [4,5]. All TLRs except TLR3 recruit myeloid differentiation primary response 88 (MyD88) to their receptor complex [6]. Association of the TLRs and MyD88 into a complex in turn recruits members of interleukin-1 (IL-1) receptor–associated kinase (IRAK) family that dissociate from MyD88 and interact with tumor necrosis factor (TNF) receptor–associated factor 6 (TRAF6) [7–9]. TRAF6 forms a ubiquitin-conjugating enzyme complex, which recruits transforming growth factor beta-activated kinase 1 (TAK1) [9,10]. Activated TAK1 triggers the activation of the transcription factor, nuclear factor kappa-light-chain enhancer of activated B cells, (NF-κB) through the I kappa B kinase (IKK) complex, which is composed of IKKα, IKKβ, and NEMO, resulting in induction of IFN and inflammatory cytokines [10,11].

Several HBV proteins have been shown to interfere with the IFN-induced intracellular signal transduction pathways. HBV polymerase (Pol) is able to inhibit IFN-α–induced MyD88 induction and impair IFN-α–induced STAT activation [12]. HBeAg suppresses TLR–induced IFN-β and interferon-stimulated gene (ISG) induction in both parenchymal and nonparenchymal liver cells [3]. An additional study demonstrated that HBeAg could interact with Toll/IL-1 receptor domain-containing proteins (TIR) Mal and TRAM and disrupt homotypic TIR/TIR interaction [13]. HBsAg is able to block the TLR9-interferon regulatory factor 7 (IRF-7)-IFN-α signaling pathway through upregulation of suppressor of cytokine signaling (SOCS-1) expression [14]. Furthermore, HBx interacts with the mitochondrial antiviral signaling protein (MAVS) promoting its degradation to prevent IFN-β induction [15]. Interestingly, both the persistence of the cccDNA minichromosome along with the ability of HBV protein components to limit host immunity are responsible for HBV persistency [16].

Major vault protein (MVP) is the major constituent of vaults involved in multidrug resistance, nucleocytoplasmic transport, and cell signaling [17–19]. However, few reports have described



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the relationship between viruses, MVP, and IFN. Our previous studies showed that hepatitis C virus (HCV) infection resulted in elevated MVP expression. MVP expression, in turn, initiated a host immune response through the induction of type-I IFN mRNA expression and protein secretion [20]. However, a clear role for MVP in innate immune responses to viral infection is still not known, and the molecular mechanisms by which HBV interferes with IFN production are ill-defined.

In this study, we demonstrate that MVP interacts with MyD88 leading to activation of NF-κB and IFN-β. We further demonstrate that the hepatitis surface antigens, HBsAg and HBeAg, specifically bind virus-induced MVP, block the molecular interaction between MVP and MyD88, and suppress the MVP-induced NF-κB and IFN signaling.

## Patients and methods

### *Study subjects*

162 CHB patients and 156 healthy control subjects were recruited for this study between February 2008 and February 2014. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Institutional Review Board of the College of Life Sciences, Wuhan University in accordance with its guidelines for the protection of human subjects. All participants provided written informed consent to participate in the study. For details see Supplementary material.

### *Hydrodynamic injection of plasmid DNA in mice*

Six to seven-week-old male BALB/c mice were each inoculated with 10 μg of pAAV-HBV plasmid or 20 μg of pCMV-HBx into the tail veins in a volume of PBS equivalent to 10% of mouse body weight. The total volume of DNA was delivered into the vein with high pressure within 4–6 s (hydrodynamic *in vivo* transfection). All animal studies were approved by the Institutional Animal Care and Use Committee at Wuhan University. The methods are described in detail in the Supplementary material.

### *Virus, cell culture and transfection*

The human hepatoma cell lines HepG2 and HuH7 were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin sulfate at 37 °C in 5% $CO_2$. The influenza virus strain A/Hong Kong/498/97 was provided by the China Center Type Culture Collection. Vesicular stomatitis virus (VSV) was provided by Professor Mingzhou Chen of Wuhan University, China. The transfection methods are described in detail in the Supplementary material.

### *Luciferase reporter gene assays, quantitative RT-PCR analysis, Western blot analysis, nuclear extraction, immunofluorescence, histology and immunohistochemistry, co-immunoprecipitation*

We used classical methods of molecular biology and described in detail in the Supplementary material.

### *Peripheral blood mononuclear cell (PBMC) isolation and transfection*

The PBMCs were isolated by density centrifugation with an isolation solution of human lymphocytes (TBD Science). Cells were washed twice in saline and were cultured in RPMI 1640 medium. PBMCs were transfected with plasmid DNA by electroporation with an Amaxa Nucleofector II Device according to the manufacturers' protocols. The methods are described in detail in the Supplementary material.

### *RNA interference*

MVP shRNA and irrelevant shRNA control (shRNA-control) were purchased from GenePharma (Shanghai GenePharma) and prepared by ligation of the corresponding pairs of oligonucleotides to PGPU6/GFP/Neo. The target sequence can be found in the Supplementary data. And their efficiency of MVP shRNA was also tested in our previous study [20].

### *Statistical analysis*

All experiments were repeated at least three times. Statistical analyses were performed using paired Student's *t* tests. A value of $p < 0.05$ was considered statistically significant.

## Results

### *HBV upregulates the expression of MVP*

It has been shown that *MVP* was one of eight genes upregulated in HepG2.2.15 cells compared to HepG2 cells using human genome-wide oligonucleotide microarrays [21]. However, no further studies have investigated MVP expression in different systems and in CHB patients. To answer this question, we first investigated MVP expression during HBV infection. Peripheral blood mononuclear cells (PBMCs) were isolated from CHB patients (n = 18) and healthy individuals (n = 18). As determined by real-time RT-PCR, *MVP* mRNA levels were approximately 7-fold higher in HBV patients than in healthy individuals (Fig. 1A and Supplementary Table 1). Significant differences in serum MVP protein levels were also observed between CHB patients (n = 104) and healthy individuals (n = 98), as determined by ELISA (108.66 ± 54.85 *vs.* 58.77 ± 20.64 ng/ml) (Fig. 1B and Supplementary Table 2). To investigate whether active viral replication correlated with MVP expression, we further compared the serum MVP protein levels between the 104 HBeAg-positive and -negative CHB patients. As shown in Supplementary Table 3, higher MVP expression levels were observed in CHB HBeAg-positive patients compared with HBeAg-negative patients (130.6 ± 57.5 *vs.* 91.37 ± 46.1 ng/ml, $p < 0.01$). Furthermore, MVP expression in liver biopsy specimens from CHB patients (n = 40) and healthy individuals (n = 40) were examined using immunohistochemistry. CHB patients showed higher levels of MVP staining than healthy individuals (Fig. 1C and Supplementary Table 4).

Subsequently, we analyzed the correlation between MVP expression and the clinical-virological characteristics of CHB patients. There were linear positive correlations between MVP expression and HBV DNA level and alanine aminotransferase (ALT) in PBMCs, serum and liver biopsy specimens from CHB patients (Supplementary Fig. 1A–F). However, there were no statistical differences between MVP expression and sex, age, or HBV genotype (data not shown). In addition, CHB patients with higher inflammatory grading (G) and fibrotic staging (S) scores had more MVP positive cells ($MVP^+$ cells) in the liver biopsies than those with lower G and S scores (Supplementary Fig. 1G and H). Further analysis indicated that $MVP^+$ cell numbers were positively associated with S score and G score in these CHB patients (Supplementary Fig. 1G and H).

To test whether the expression of MVP is affected by HBV, MVP mRNA and protein expression levels in HepG2 cells were compared with those in HepG2.2.15 HBV (genotype D) positive cells. Real-time RT-PCR and Western blot assays showed that MVP mRNA and protein expression in HepG2.2.15 cells were both elevated compared with the levels in the HepG2 cells (Fig. 1D). To make sure that this was not a cell-specific event, an additional pair of human hepatoma cells, HuH7 and HuH7.37 (HuH7.37 cells



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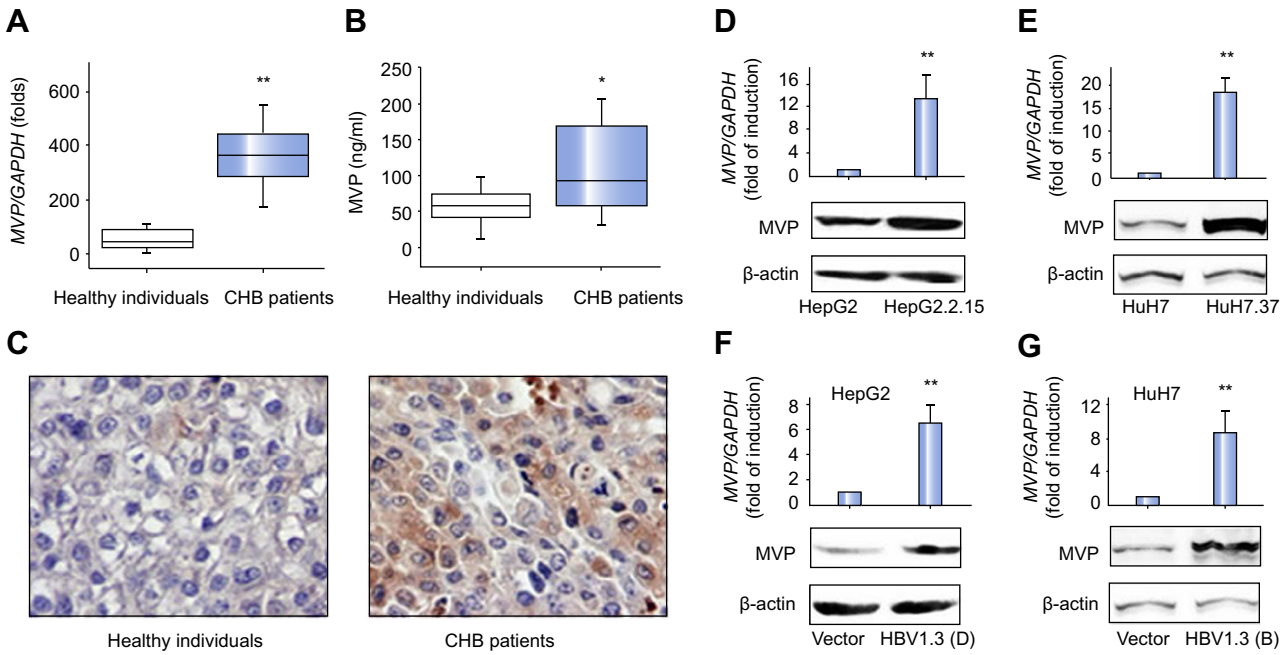


**Fig. 1. MVP is induced by HBV.** PBMCs (A), serum (B), and liver (C) MVP levels in healthy individuals and CHB patients. In panel (A), the lowest value of healthy individuals was designated as 1. MVP data are expressed as fold induction (folds) relative to the lowest value of healthy individuals. Data represent the means ± SEM. Boxplots illustrate medians with 25% and 75% and error bars for the 5% and 95% percentiles. MVP mRNA levels (upper panel) and protein levels (lower panel) in HepG2 and HepG2.2.15 cells (D) or in HuH7 and HuH7.37 cells (E). HepG2 cells were transfected with pHBV-1.3 (genotype D) (F) or HuH7 cells transfected with pHBV-1.3 (genotype B) (G) for 48 h prior to real-time RT-PCR (upper panel) and Western blot (lower panel) assays. (Data expressed as mean ± SD of three independent experiments; $^{*}p$ <0.05 and $^{**}p$ <0.01).

contain an integrated HBV genotype B and stably express HBV) [22] were also tested for MVP expression. Consistent with HepG2.2.15 cells, HuH7.37 cells expressed higher levels of MVP mRNA and protein (Fig. 1E). Next, pHBV-1.3, a 1.3-fold overlength fragment of the HBV genome (genotype B or D), which retains the ability to produce mature HBV virions, was then transfected into HepG2 cells or HuH7 cells, and MVP mRNA and protein expression levels were detected by real-time RT-PCR and Western blot assays. The results showed that pHBV-1.3 also stimulated MVP mRNA and protein expression (Fig. 1F and G). The expression of MVP was also increased in freshly isolated PBMCs after treatment with the culture supernatants from HepG2.2.15 cells, which contained HBV ($8.5 \times 10^4$ copies/ml). This elevation of MVP was subsequently reversed through mixed incubation of human hepatitis B immunoglobulin, a neutralizing antibody to HBV, at both the mRNA and protein level (Supplementary Fig. 2). These data indicate that MVP is induced by HBV in both hepatoma and immune cells.

*Transcriptional regulation of MVP by HBV X protein*

To determine the level at which HBV influences the expression of MVP, pMVP-Luc, and pHBV-1.3 (genotype B or D) were co-transfected into HepG2 or HuH7 cells. Luciferase activity reporter assays showed that both HBV subtypes activated *MVP* promoter-Luc in HepG2 and HuH7 cells (Supplementary Fig. 3A and B). To investigate which viral protein plays a role in MVP regulation, HuH7 cells were co-transfected with each of the seven HBV gene-expressing plasmids and pMVP-Luc. Luciferase activity reporter assays indicated that HBx protein stimulated MVP promoter activity; whereas the other HBV proteins had no significant effect (Supplementary Fig. 3C). RT-PCR and Western blot analyses also indicated that HBx increased MVP mRNA and protein expression (Supplementary Fig. 3D). Moreover, to further confirm the influence of HBx on MVP expression, HuH7 cells were co-transfected with pHBV-1.2 or pHBV-1.2 (ΔHBx), which is an HBV mutant that does not express HBx. Real-time RT-PCR assays indicated that wild-type HBV activated the *MVP* promoter, whereas the HBV mutant weakly induced *MVP* promoter activation (Supplementary Fig. 3E). In a hydrodynamic-based mouse model, remarkably higher levels of murine MVP were found in the group of mice with HBV infection (Supplementary Fig. 3F). Similar results were also observed from the mouse model of CMV-driven HBx expression (Supplementary Fig. 3G). Together, these results imply that HBx is the key viral component involved in MVP expression during HBV infection.

*Identification of MVP as a MyD88-associated protein*

In a previous study, we found that MVP is a novel virus-induced host factor, and its expression upregulates type-I IFN production leading to cellular antiviral responses [20]. In this study, we analyzed protein interactions with MVP that are involved in IFN signaling pathways. Results from real-time RT-PCR experiments confirmed that knockdown of MVP inhibited specific ligand-induced *IFN-β* gene expression. Specifically, using Toll-like receptor ligands, we demonstrate that knockdown of MVP inhibited signaling via TLR4 (LPS), TLR7 (R-848), and TLR9 (CpG ODN), but not TLR3 (poly (I:C))-induced IFN-β expression (Fig. 2A). Because MyD88 is an adaptor that is essential for signaling downstream of various TLRs, but not TLR3 [23], we speculated that MVP could interact with MyD88. The results of transient transfection and co-immunoprecipitation experiments indicated that Myc-tagged MVP interacted with Flag-tagged MyD88 (Fig. 2B). Reverse



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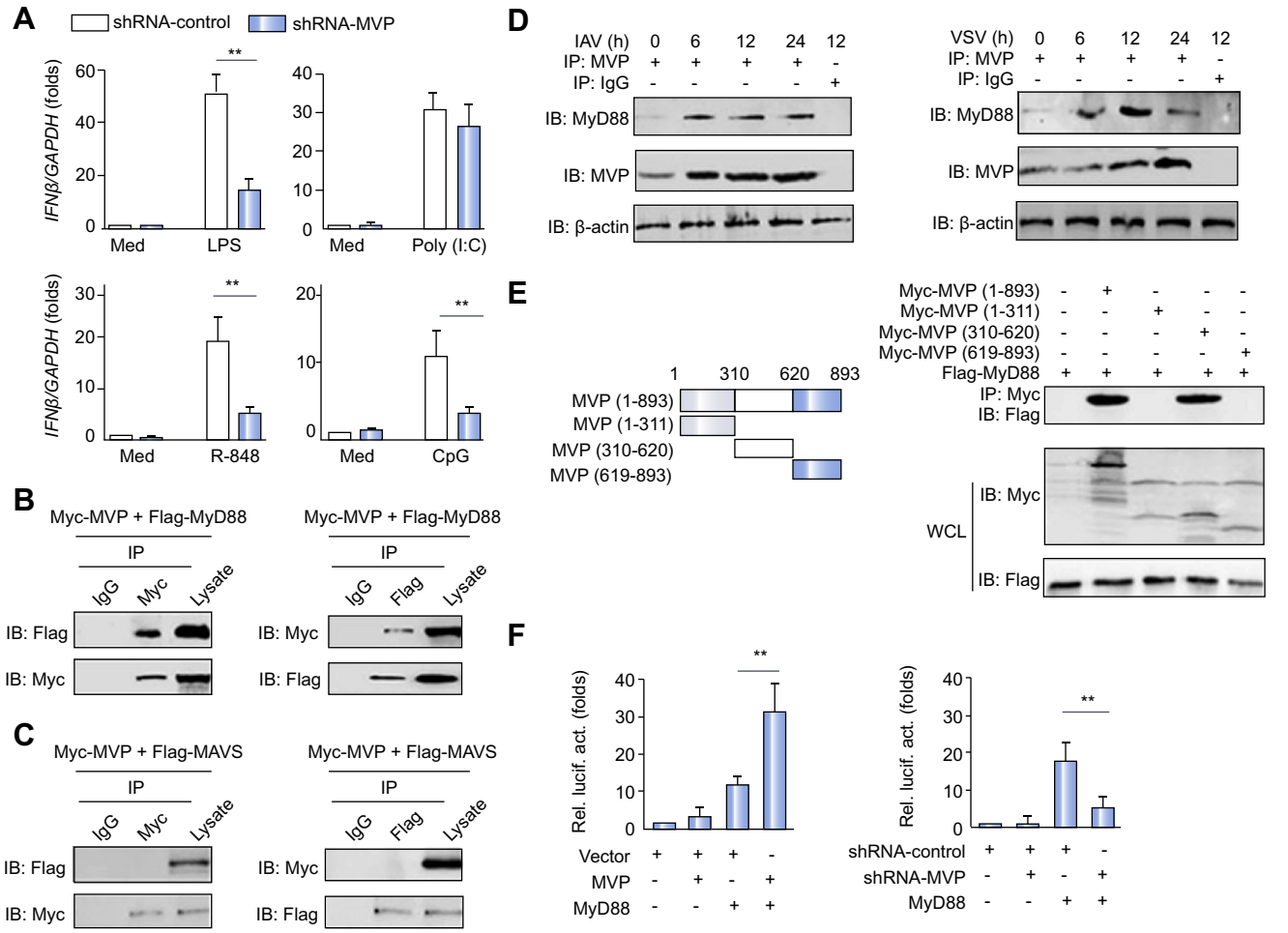


**Fig. 2. MVP associates with MyD88 to activate the type-I interferon signaling pathway.** (A) Freshly isolated PBMCs were transfected with the indicated plasmids for 36 h, stimulated or unstimulated (Med) for 12 h with LPS (100 ng/ml), poly(I:C) (50 μg/ml), R-848 (10 nM) or CpG DNA (1 μM), and subjected to real-time RT-PCR analyses for IFN-β. (B, C) 293T cells were transfected with Myc-tagged MVP (Myc-MVP) and Flag-tagged MyD88 (Flag-MyD88) (B) or Flag-tagged MAVS (Flag-MAVS) (C). Forty-eight hours post-transfection, co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (D) A549 cells were infected with IAV (left panel), and THP-1 cells were infected with VSV (right panel) for the indicated times or left uninfected. Immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (E) Schematic diagram of the full-length and truncated constructs of MVP (left panel). 293T cells were co-transfected with Flag-MyD88 and the indicated truncated MVP constructs for 48 h. Co-immunoprecipitation and immunoblot analyses were performed with the indicated antibodies (right panel). (F) 293T cells were co-transfected with NF-κB-luc and the indicated plasmids for 48 h prior to luciferase assays. All experiments were repeated at least three times with consistent results. Bar graphs represent the means ± SD, n = 3 ($^{**}p$ <0.01; $^{*}p$ <0.05).

co-immunoprecipitation experiments also indicated that Myc-tagged MVP interacted with Flag-tagged MyD88 (Fig. 2B). Similar results were obtained when we used Flag-tagged MVP and GFP-tagged MyD88 (Supplementary Fig. 4A). In contrast, another adaptor protein involved in IFN signaling, MAVS [24], did not interact with MVP (Fig. 2C). Using indirect immunofluorescence, we also showed that Myc-tagged MVP co-localized with Flag-MyD88, but not MAVS, in HuH7 cells (Supplementary Fig. 4B). We further performed endogenous co-immunoprecipitation experiments, and the results indicated that MVP was weakly associated with MyD88 in unstimulated cells, and this association increased after stimulation with influenza A virus (IAV) or vesicular stomatitis virus (VSV) (Fig. 2D).

To map the region of MVP that interacted with MyD88, we constructed a series of MVP plasmids with Myc-tagged truncation mutants (Fig. 2E left panel). We further demonstrated that the middle domain of MVP comprising of residues 310–620 was necessary for its interaction with MyD88 (Fig. 2E right panel). Because MVP is specifically associated with MyD88, we examined whether MVP is involved in the regulation of MyD88-mediated signaling. Using luciferase activity reporter assays, we showed that MVP overexpression stimulated MyD88-mediated activation of NF-κB, and MVP expression knockdown inhibited MyD88-mediated activation of NF-κB (Fig. 2F). Taken together, these data suggest that MVP is associated with MyD88, and this association positively regulates MyD88-mediated signaling pathway.

### *HBV inhibits MVP and MyD88-induced activation of NF-κB and IFN-β by disrupting MVP/MyD88 interactions*

In our previous study, we showed that MVP exhibited strong antiviral activity toward several viral infections, such as VSV, IAV, and enterovirus 71, in a type-I IFN-dependent manner [20].



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However, we recently found that MVP could not limit HBV production and replication (data not shown). We suspected that HBV has distinct strategies to block the MVP-mediated IFN signaling pathway. To confirm the influence of HBV on the MVP/MyD88-induced signaling pathway, we performed luciferase activity reporter assays, which demonstrated that MVP/VSV-induced IFN-β activation was consistently reduced in the presence of HBV (genotype D) (Supplementary Fig. 5A). We also observed that MVP/MyD88-induced NF-κB activation was reduced by HBV (genotype D) (Supplementary Fig. 5B). HBV genotype A–D also reduced MVP/VSV-induced IFN-β activation and MVP/MyD88-induced NF-κB activation (Supplementary Fig. 6A and B). We further investigated whether HBV could disrupt homotypic MVP/MyD88 interactions. In HuH7 cells, co-expressed Myc-MVP and Flag-MyD88 interact as expected, but pBlueks-HBV (genotype D) preferentially displaced the MVP/MyD88 interaction (Supplementary Fig. 5C). Moreover, an impaired MVP and MyD88 interaction was observed in HepG2.2.15 HBV positive cells compared with HepG2 cells, as determined by co-immunoprecipitation experiments (Supplementary Fig. 5D). Together, these results demonstrate that HBV suppresses MVP-mediated antiviral responses through disrupting homotypic MVP/MyD88 interactions.

*HBsAg and HBeAg inhibit MVP and MyD88-induced activation of NF-κB and IFN-β by disrupting MVP/MyD88 interactions*

To investigate which viral protein plays a role in disrupting the MVP and MyD88 interaction, we tested cells for activation of NF-κB following transfection with MVP, MyD88 and each of the five HBV genes (genotype D). The results showed that HBsAg and HBeAg were most important for disrupting the MVP and MyD88-induced activation of NF-κB (Fig. 3A). Further experiments in 293T cells indicated that both HBsAg and HBeAg inhibited MVP and MyD88-induced activation of the NF-κB in a dose-dependent manner (Fig. 3B and C). We also investigated whether HBsAg or HBeAg (genotype D) could displace MyD88 from MVP. Co-immunoprecipitation experiments in 293T cells, demonstrated that transfection with increasing amounts of plasmid encoding HBsAg or HBeAg, led to disruption of MyD88 and MVP interactions (Fig. 3D and E).

Our previous study found that MVP can promote the translocation of activated IRF7 and NF-κB from the cytosol to the nucleus, which enhances the expression of ISGs [20]. In this study, we investigated whether HBsAg or HBeAg affect MVP-induced IRF7 and NF-κB nucleocytoplasmic transport and MVP-induced ISG expression. As shown in Fig. 3F and G, HBsAg or HBeAg (genotype D) suppressed the MVP-induced translocation of IRF7 and NF-κB to the nucleus, whereas IRF3 was not affected. As expected, real-time RT-PCR and Western blot assays indicated that HBsAg or HBeAg (genotype D) suppressed the expression of conventional ISGs, such as protein kinase RNA-activated (PKR), oligoadenylate synthetase 2 (OAS2), and interferon-induced GRP-binding protein Mx1 (Mx1) (Fig. 3H). Together, these results reveal a role for HBsAg and HBeAg in mediating the inhibitory effects on MVP–induced antiviral responses.

*Both HBsAg and HBeAg interact with MVP*

To determine whether HBsAg and HBeAg are associated with MVP, we performed co-immunoprecipitation and reverse co-immunoprecipitation experiments. The results indicated that MVP interacts with HBsAg and HBeAg, but not HBx (genotype D) (Fig. 4A–C). Immunofluorescent staining experiments suggested that MVP is a cytoplasmic protein and had a similar distribution pattern as HBsAg and HBeAg (genotype D) (Fig. 4D). The interaction between MVP and HBsAg or HBeAg was confirmed by repeating the co-immunoprecipitation experiments using HBsAg or HBeAg of HBV genotype B (Supplementary Fig. 7A and B). We further examined the region of MVP that binds to HBsAg and HBeAg (genotype D). To accomplish this, Myc-tagged full-length MVP and several truncated constructs were transfected along with Flag-HBsAg or Flag-HBeAg. The co-immunoprecipitation results showed that both HBsAg and HBeAg were able to co-precipitate with the 310–620 amino acid region of MVP (Fig. 4E and F). We also aimed to determine whether MyD88 interacts with HBsAg and HBeAg in 293T cells. The co-immunoprecipitation and reverse co-immunoprecipitation experiments indicated that MyD88 could not interact with HBsAg and HBeAg (Supplementary Fig. 8A and B), which is consistent with previous reports [13]. Together, these results demonstrate that both HBsAg and HBeAg are able to bind to the middle domain of MVP suggesting a possible mechanism for HBV-mediated inhibition of type-I IFN signaling.

## Discussion

In this study, we demonstrate the presence of a unique cytoplasmic complex including the MyD88 adaptor and MVP. In addition, we identified a previously undescribed mechanism for HBV immune escape, in which HBeAg and HBsAg directly interact with MVP, but not MyD88, to dampen the interaction between MVP and MyD88. Our previous studies suggested that MVP induces type-I IFN production through translocation of IRF7 and NF-κB from the cytoplasm to the nucleus [20]. In the current study, we show that MVP protein interacts with MyD88, and MVP overexpression in combination with MyD88 activates NF-κB-luc (Fig. 2). In light of our previous and current results, we propose a model for MVP-dependent IFN signaling. According to this model, TLRs recruit and activate MyD88 during viral infection, which in turn recruit MVP. This complex signals to translocate the transcription factors, IRF7 and NF-κB, from the cytoplasm to the nucleus for subsequent production of IFN and inflammatory cytokines. Given that MyD88 recruits several proteins, such as IRAK4, IRAK1, IRF7, to form a large complex [11], MVP may also bind those proteins in a MyD88-mediated complex. Further studies are needed to verify these putative interactions.

The type-I IFN system is the first line of host defense against viral infection [4,5,25]. Previous studies have shown that TLR-induced type-I IFN and TLR2 ligand, Pam3Cys, inhibits HBV replication [26]. Importantly, a recent report has shown that IFN-α-induced MyD88 strongly inhibited HBV replication, implying the importance of MyD88 for HBV clearance [27]. However, it also has been reported that HBsAg, HBeAg, and HBV virions almost completely suppress TLR-mediated antiviral activity and cytokine induction in murine liver parenchymal cells [3], indicating that HBV can generate viral partners to hijack or degrade critical molecules in the IFN induction pathway. Although it is known that the MyD88 signaling pathway plays a critical role in the suppression of HBV replication and the induction of IFN,



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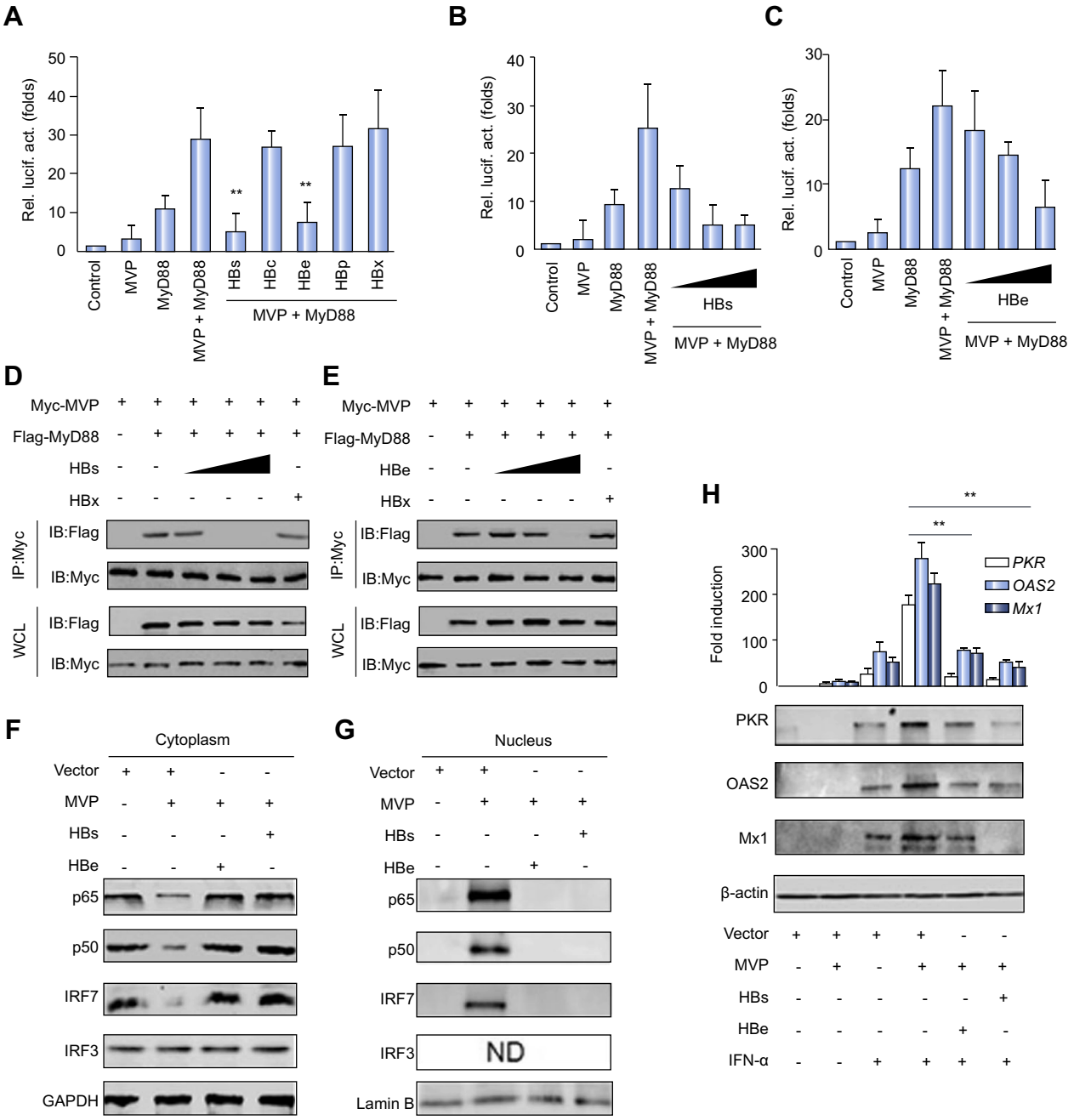


**Fig. 3. HBsAg and HBeAg interfere with the MVP/MyD88 interaction.** (A) HuH7 cells were co-transfected with NF-κB-luc and the indicated plasmids for 48 h prior to luciferase activity reporter assays. (B, C) HuH7 cells were co-transfected with NF-κB-luc (0.1 μg), MVP (0.2 μg), MyD88 (0.2 μg) or increasing amounts of HBsAg (0.1, 0.2, 0.4 μg) (B) and HBeAg (0.1, 0.2, 0.4 μg) (C). The total amount of plasmid DNA was adjusted to 0.9 μg by adding empty vector. Luciferase activity reporter assays were performed as in Fig. 1F. (D, E) 293T cells were transfected with Myc-MVP (5 μg), Flag-MyD88 (5 μg), or increasing amounts of HBsAg (0.05, 0.5, 5 μg) (D) and HBeAg (0.05, 0.5, 5 μg) (E) expression plasmids. The total amount of plasmid DNA was adjusted to 15 μg by adding empty vector. Co-immunoprecipitation and immunoblot analyses were performed as in Fig. 2B. (F, G) HuH7 cells were co-transfected with the indicated plasmids for 48 h, and cytosolic (F) and nuclear extracts (G) were subjected to Western blot analyses. Lamin A and GAPDH were used as markers for nuclear and cytosolic fractions, respectively. (H) HuH7 cells were transfected with the indicated plasmids for 24 h and treated with or without IFN-α (300 IU/ml) for 24 h prior to real-time RT-PCR (upper panel) and Western blot (lower panel) analyses. All experiments were repeated at least three times with consistent results. Bar graphs represent the means ± SD, n = 3 ($^{**}p$ <0.01; $^{*}p$ <0.05).

little is known about whether and how HBV disturbs the cytoplasmic MyD88 signaling pathway. For the first time, this study describes a mechanism for HBV suppression of the MyD88-mediated signaling pathway. We have shown here that HBeAg and HBsAg specifically interact with MVP to modulate both NF-κB and IFN activation (Fig. 3). Moreover, co-immunoprecipitation results showed that both HBeAg and HBsAg bind to MVP within the 310–620 amino acid region, which is also required for the MyD88/MVP interaction (Fig. 4E and F). Based on these results, we conclude that HBeAg and HBsAg weaken the



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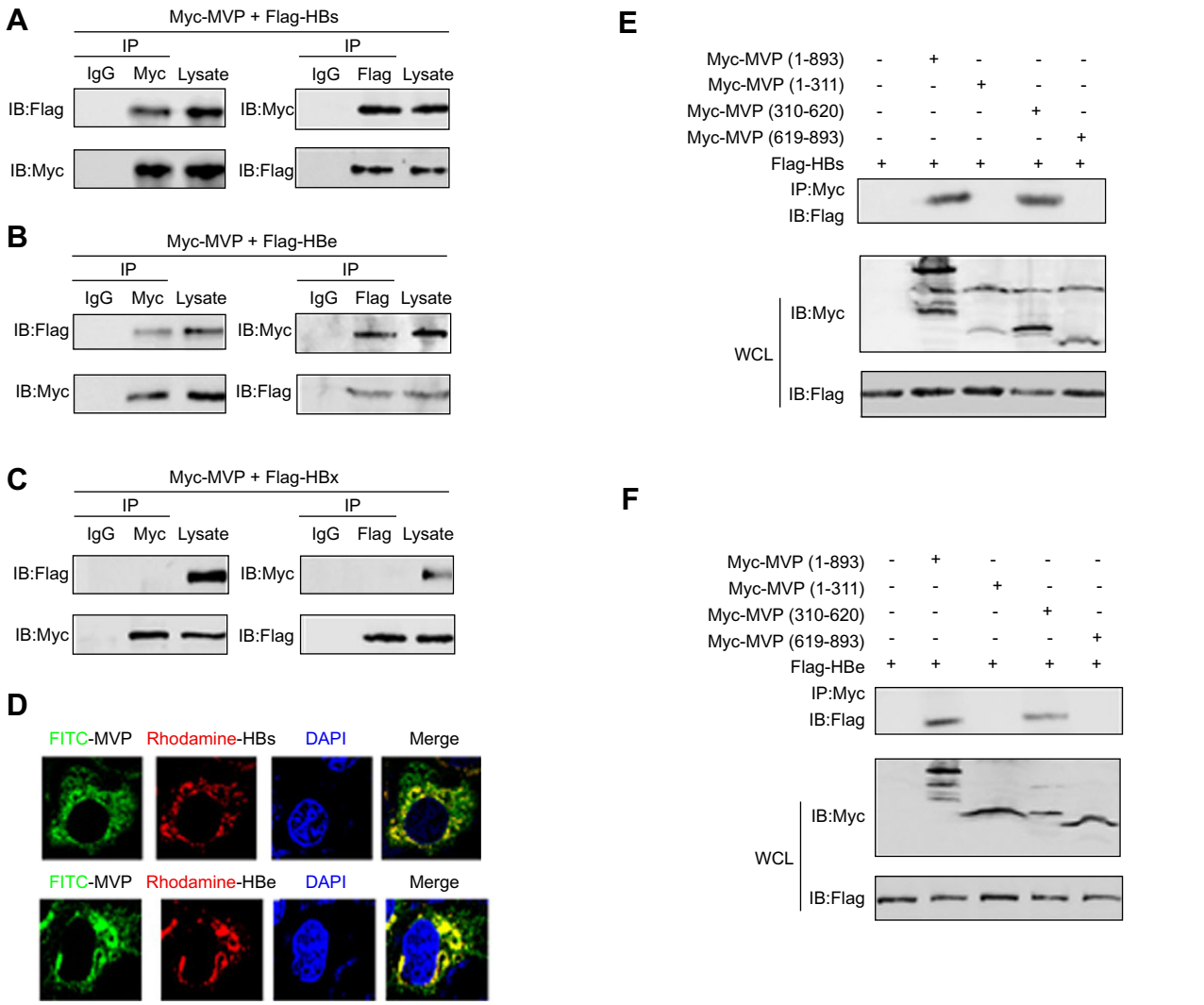


**Fig. 4. HBsAg and HBeAg interact with MVP.** (A) 293T cells were transfected with the indicated plasmids. Co-immunoprecipitation and immunoblot analyses were performed with the indicated antibodies. (B, C) Experiments were performed as described in (A) using Flag-HBe (B) or Flag-HBx (C). (D) HuH7 cells were transfected with Myc-MVP, Flag-HBs (upper panel) or Flag-HBe (lower panel) for 48 h prior to immunofluorescence assays. (E, F) Experiments were performed as described in Fig. 1E using Flag-HBs (E) or Flag-HBe (F). All experiments were repeated at least three times with consistent results.

MyD88/MVP interaction through competitive binding of the 310–620 region of MVP. This provides a potential explanation for how HBeAg and HBsAg inhibit MyD88–induced NF-κB and IFN activation.

Some infectious diseases can be a manifestation of a constant battle between the host and viruses [25]. This host–virus antagonism was vividly depicted in the current study demonstrating the interaction between MVP and HBV. MVP is capable of orchestrating a strong immune defense by interacting with MyD88; however, HBV counterattacks this activity by using HBeAg and HBsAg to bind MVP, thus crippling the immune response. In this paper, competitive co-immunoprecipitation experiments indicated that HBsAg or HBeAg disrupted the MVP/MyD88 interactions in a dose-dependent manner, and the interaction with HBsAg or HBeAg is considerably stronger than MyD88 (Fig. 3D and E). Based on these results, we propose that HBV may have won the battle against the MVP-mediated IFN response.

In general, HBV genotypes have been classified into 8 groups (A–H), and each genotype show a distinct geographic and ethnic distribution [28,29]. Several recent studies suggested that HBV genotypes may be related to clinical outcome of hepatitis B. However, due to geographical distribution, the clinical significance of HBV genotype could only be reliably compared between genotypes B and C or genotypes D and A [30]. For genotypes B and C, a large study enrolling 1734 patients reported that genotype C was more prevalent in patients with severe liver disease compared with genotype B [31,32]. For genotypes A and D, a study from Europe reported that HBsAg clearance occurred more often in patients with genotype A compared with genotype D [33], indicating that genotype D is associated with more severe diseases. In this study, we compared the influence of four HBV genotypes (A–D) on the MVP-induced signaling pathway. Luciferase activity assays indicated that genotype C and D show more effective inhibition to MVP-induced signaling than genotype A and B



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(Supplementary Fig. 6A and B). Compared with genotypes A and B, genotypes C and D can almost completely suppress MVP/MyD88 interaction, which may lead to more severe diseases as reported in prior clinical reports.

We previously demonstrated that hepatitis C virus (HCV) infection strongly induces MVP expression through the NF-κB and Sp1 pathways [20]. Interestingly, in this study, we also observed high levels of MVP expression in the sera, PBMCs, and liver tissue of patients with HBV compared to those from healthy individuals (Fig. 1). Combining all the data derived from this study, we speculate that the interaction between MVP and MyD88 rather than the amount of MVP expressed, is a key factor for MVP activity in the regulation of cellular antiviral responses. Our finding further showed that increased MVP expression was correlated with the simultaneous elevation of serum ALT levels and the induction of HBV load in the sera, PBMCs, and liver tissue of HBV patients (Supplementary Fig. 1). Serum HBV DNA level has been widely used as an indicator for active viral replication and ALT level is usually an important factor in predicting patient's prognosis [34]. Thus, we suspect that MVP induction may have other functions during HBV infection.

It has been reported that HBV enter cells by interacting with sodium taurocholate cotransporting polypeptide (NTCP), a multiple transmembrane transporter [35], but the mechanism of viral entry and infection of HBV is still not well established. Despite lack of understanding of HBV entry and infection, HBV DNA and viral components can be detected in PBMCs of most infected patients [36–38]. Interestingly, in our previous study, we electroporated freshly isolated PBMCs with pHBV-1.3 plasmid to imitate the presence of viral components [39]. The results showed that the viral RNA levels produced are about 2-fold higher than the average levels in PBMCs from 10 HBV patients [39]. Moreover, *HBx* mRNA was detected in the largest amount in PBMCs of highly viremic patients, and X protein can stimulate PBMCs of infected patients and activate the immune response [37,40]. In this study, we used clinical samples, cell and mouse models to prove that HBV induces MVP expression (Fig. 1; Supplementary Figs. 2 and 3). We further demonstrated that HBx might contribute to the expression of MVP during HBV infection (Supplementary Fig. 3). These results provide a detailed understanding of how HBV induce MVP expression. Although more studies are needed to understand the intricate regulatory mechanisms of MVP for viral replication and antiviral responses, our findings reveal a previously undescribed role for HBsAg and HBeAg in regulating MVP-mediated cellular antiviral responses.

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**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.

**Authors' contributions**

Shi Liu participated in the design of the study, carried out all the experiments, analyzed results and drafted the manuscript. Nanfang Peng and Jiajia Xie participated in animal experiment and genes clone of the study. Qian Hao collected and analyzed clinical samples of the study. Mo Zhang, Zhangchuan Xia and Gang Xu participated in genes clone of the study. Yi Zhang analyzed the data and helped to edit the manuscript. Fanpeng Zhao, Qing Wang and Tao Han contributed to acquisition of data and interpretation of the results. Ying Zhu participated in the design of the study and the critical view of manuscript writing. All authors read and approved the final manuscript.

**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2014.11.035.

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