FoxO4 inhibits HBV core promoter activity through ERK-mediated downregulation of HNF4α

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A B S T R A C T

Hepatitis B virus (HBV) infection remains a global health problem, causing nearly one million deaths annually. Forkhead box O (FoxO) transcription factors play important roles in modulating diverse physiological processes. Recent studies show that FoxOs are involved in antiviral responses. In present investigation, we found that HBV induced significant down-regulation of FoxO4 protein, while had little effect on the expression of FoxO1 and FoxO3. Further study showed that FoxO4 displayed inhibitory effect on HBV transcription and replication both *in vitro* and *in vivo*. Mechanistically, it was found that FoxO4 exerted its anti-HBV activity by targeting HBV core promoter. Further, FoxO4 was revealed to inhibit HBV core promoter activity via downregulating hepatocyte nuclear factor-4α (HNF4α), and ERK signaling was required for FoxO4-mediated suppression of HNF4α and HBV core promoter activity. Together, these data indicated that FoxO4 displayed anti-HBV activity by suppressing HNF4α expression via activation of ERK pathway, and targeting FoxO4 might present as a novel therapeutic strategy against HBV infection.

1. Introduction

Hepatitis B virus (HBV) infection remains a global health problem. Despite the availability of an effective prophylactic vaccine, about 260 million people worldwide are persistently infected with HBV, carrying a high risk of progression into cirrhosis or hepatocellular carcinoma (HCC) (Trepo et al., 2014; Tsai et al., 2018). The therapeutic efficacy of currently used anti-HBV drugs, including nucleoside analogues and interferon-α, is limited due to the short-term efficacy, drug resistance or side effects, *etc*. Thus, novel interventions against HBV infection with new action mechanisms are needed (Bertoletti and Rivino, 2014; Ward et al., 2016).

HBV contains a 3.2-kb partially double-stranded DNA genome, and replicates via reverse transcription of the 3.5-kb pregenomic RNA (pgRNA) (Seeger and Mason, 2000). The core promoter is responsible for the synthesis of pgRNA, and therefore the regulation of this promoter is important in the life cycle of HBV (Kramvis and Kew, 1999). It is now evident that a variety of liver-enriched transcription factors (LETFs), such as hepatocyte nuclear factor-4α (HNF4α), retinoid X receptor a (RXRα), peroxisome proliferator-activated receptor (PPARα), farnesoid X receptorα (FXRα) *etc*, play critical roles in modulating the

core promoter activity and contribute to the regulation of HBV transcription and replication. Targeting LETFs is therefore helpful for controlling HBV infection (Chen et al., 2012; Raney et al., 1997; Schrem et al., 2002).

Forkhead box O (FoxO) transcription factors are implicated in regulating diverse cellular processes, such as differentiation, proliferation, survival and immune responses (Accili and Arden, 2004; Becker et al., 2010; Deng et al., 2018; Eijkelenboom and Burgering, 2013). In mammals, this family consists of four members: FoxO1, FoxO3, FoxO4, and FoxO6. FoxO1, FoxO3 and FoxO4 are expressed in most tissues, while FoxO6 is mainly distributed in the brain (Accili and Arden, 2004; Huang and Tindall, 2007). As an important group of transcription factors, FoxOs may function as a positive or negative regulator of some genes by directly binding to DNA or affecting the functions of other transcription factors, coactivators or corepressors (Huang and Tindall, 2007). It is therefore not surprising that different FoxO family members may display opposite functions. For example, it is reported that FoxO1 is involved in the activation of NF-κB (Miao et al., 2012), while FoxO3 or FoxO4 is revealed to display inhibitory effect on NF-κB activity (Lin et al., 2004; Zhou et al., 2009). Our previous investigation also demonstrated that FoxO3 plays an important role in glucocorticoids-

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mediated amelioration of systemic lupus erythematosus (SLE) symptom via inhibiting the NF-κB activity (Lu et al., 2016). Accumulating evidence indicates that FoxOs may play important roles in regulating viral infection. For example, FoxO1 is reported to contribute for HBV transcription and replication via activating HBV core promoter (Shlomai and Shaul, 2009; Wang and Tian, 2017); FoxO3 acts as a negative regulator of virus-specific CD8$^+$T cells responses in chronic lymphocytic choriomeningitis virus infection, and is proposed to be a potential therapeutic target for chronic viral infection (Sullivan et al., 2012); and reports also indicate that FoxO4 may inhibit the replication of HIV-1 via acting as a transcriptional suppressor (Oteiza and Mechti, 2017).

In this study, we demonstrated that HBV induced significant downregulation of FoxO4 at the protein level, while had little effect on the expression of FoxO1 and FoxO3. FoxO4 displayed inhibitory effect on HBV transcription and replication both *in vitro* and *in vivo*. Further study showed that FoxO4 inhibited HBV core promoter activity, which was associated with its suppression of HNF4α. Furthermore, our data revealed that the activation of ERK signaling was crucial for FoxO4-mediated inhibition of HNF4α expression and the activity of HBV core promoter.

2. Materials and methods

2.1. Cell culture, transfection and infection

Huh7, HepG2 and HepG2-NTCP cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Huh7 cells were transfected with indicated plasmids by lipofectamine 2000 (Invitrogen, Carlsbad, CA), and transfected with siRNA specific for FoxO4 (5′-AGAAGCCGAUAUGUGGACC-3′) or control siRNA with HiPerFect Transfection Reagent (QIAGEN, Hilden, Germany). For the transfection of HepG2-NTCP cells, 1×10^6 cells were resuspended in 100 μl of Cell Line Nucleofector Solution C (Amaxa GmbH, Koln, Germany), and nucleofected with 2 μg of indicated plasmids or 100 nM siRNA. For HBV infection experiments, HepG2-NTCP cells were infected with HBV at 10^3 vge/cell in the presence of 4% PEG-8000. Approximately 16 h after infection, cells were washed three times with PBS, and then maintained in medium supplemented with 2.5% DMSO.

2.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from culture cells or liver tissues by TRIzol reagent and reverse-transcribed into cDNA, followed by quantitative real-time PCR using Roche Lightcycler480 II and SYBR green system (Takara, Dalian, China). The primers for real-time PCR were shown as in Supplementary Table 1. For the determination of HBV RNAs and HBV pgRNA, cDNA was synthesized from 0.5 μg of RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara), in which the gDNA Eraser was used to degrade the transfected HBV-DNA.

2.3. Western blot

Western blot was performed as described previously (Lu et al., 2016; Zhong et al., 2017). Antibodies against FoxO1 (#2880), FoxO3 (#2497), FoxO4 (#9472), p-JNK (#9251), JNK (#9252), p-p38 (#4511), p38 (#9212), p-ERK (#4370), ERK (#4695), GAPDH (#8884), Tubulin (#2128) and Lamin B1 (#15068) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against Flag (AP0007), HNF4α (BS6888) were obtained from BioWorld Biotechnology (Minneapolis, MN). Antibody against HBcAg (#8637) was obtained from Abcam (Cambridge, MA). Antibody against hexon (#MAB8052) was obtained from Chemicon, Temecula, CA).

2.4. Extraction and analysis of intracellular HBV core particles-associated HBV-DNA

The method for the extraction and analysis of intracellular core particle-associated HBV-DNA was described as previously (Parekh et al., 2003; Zhong et al., 2015, 2017). Quantitative PCR analysis of HBV-DNA was determined by real-time PCR using an HBV diagnostic kit (Kehua Biotech, Shanghai, China). For the southern hybridization, the HBV-DNA was separated by electrophoresis in 1% agarose gels, transferred onto the Hybond-N nylon membrane, and then hybridized with a digoxigenin-labeled full-length HBV probe.

2.5. Cytoplasmic/nuclear protein fractionation

Cytoplasmic/nuclear protein fractionation was performed using nuclear and cytoplasmic extraction kit (Cwbiotech, Beijing, China). The purity of the cytoplasmic extracts and the nuclear extracts was determined by western blot with anti-Tubulin and anti-Lamin B1 antibodies, respectively.

2.6. Animal study

To establish a HBV infection mouse model, 10 μg of pAAV/HBV1.2 was hydrodynamically injected into the tail veins of C57BL/6 mice in a volume of PBS equivalent to 8% of the mouse body weight as described previously (Huang et al., 2006). Four weeks after the injection, serum HBsAg was detected to confirm the successful establishment of HBV mouse model. 100 μl of the adenoviruses harboring FoxO4 (Ad-FoxO4-Flag) or the adenoviruses control (Ad-Ctrl) (3×10^9 pfu) was then intravenously injected into each HBV-infected mouse. 4 days later, the mice sera were collected and assayed for HBsAg and HBV-DNA, and liver tissues were collected for western blot or immunohistochemical staining. All animal experiments were conducted according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, P. R. China, 1998) and with the approval of the Ethic Committee of Fudan University (Shanghai, P. R. China).

2.7. Immunohistochemistry

HBV core protein in mouse liver tissues was examined by immunohistochemical staining as described previously (Gao et al., 2009). Briefly, paraffin-embedded liver tissue sections were treated with 3% hydrogen peroxide and blocked with 5% bovine serum albumin. The sections were then incubated with anti-HBcAg antibody, biotinylated secondary antibody, and avidin-biotin complex (ABC). The staining was developed with diaminobenzidine (DAB) solution and counterstained with hematoxylin.

2.8. Luciferase reporter gene assay

The plasmids CpLUC, SpLUC, XpLUC, and pS (1)pLUC, each containing one complete HBV genome in the configuration that the luciferase (LUC) gene is governed by the core, surface, X or preS promoter, respectively, were employed as reporters, as described previously (Gao et al., 2009; Raney et al., 1990). For the reporter gene assay, the HBV promoters-dependent luciferase reporter plasmids were transfected into Huh7 cells together with FoxO4 expression plasmid. 48 h post-transfection, the luciferase activity in the lysates of transfected cells was determined with the Luciferase Reporter Assay System (Promega, Madison, WI). In all transfection assays, pCMV-β-gal was co-transfected to normalize the transfection efficiency.

2.9. Statistics

At least three independent experiments were performed. Results were reported as means ± standard deviation (SD). Two-tailed t-tests
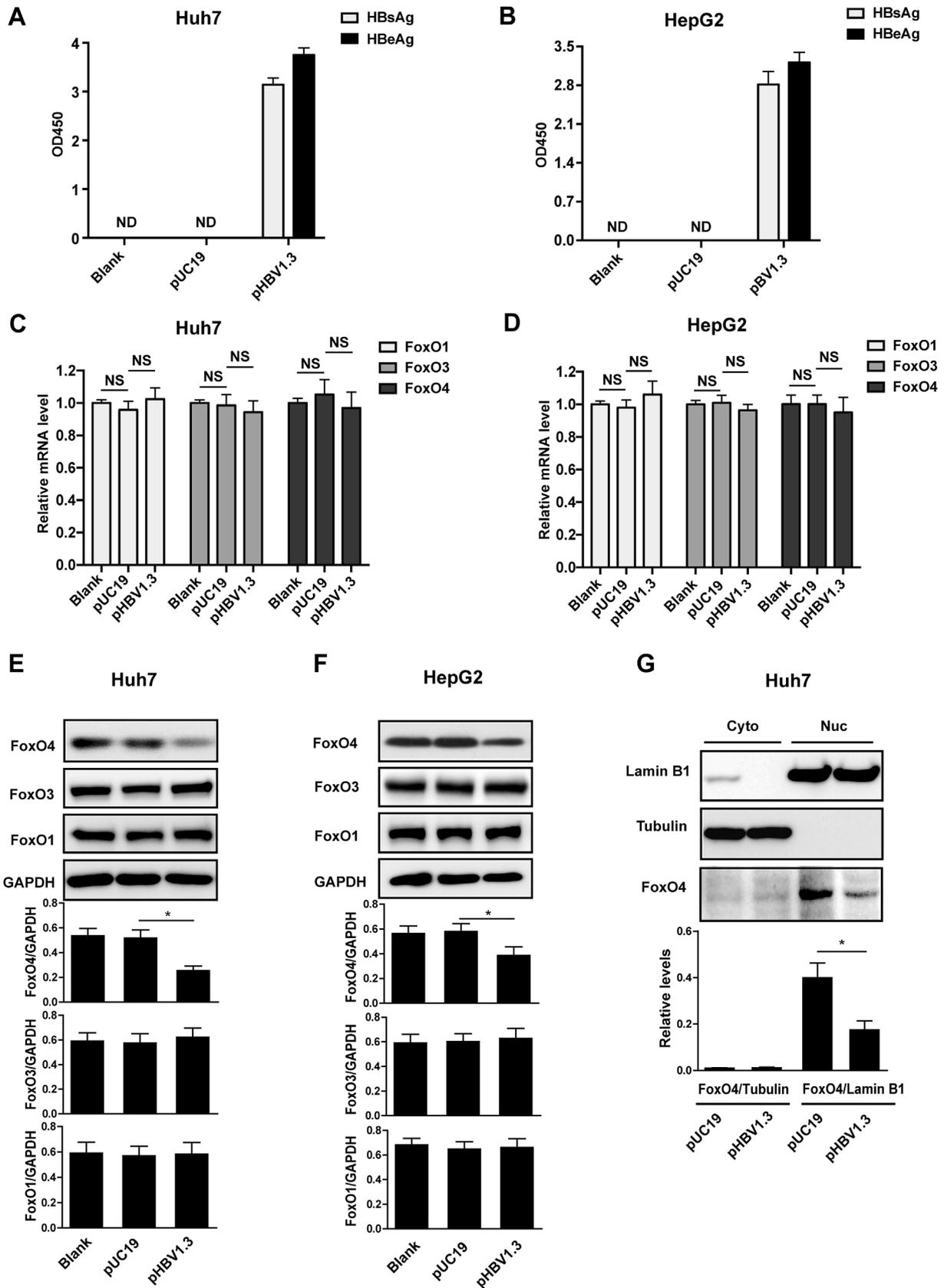
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Fig. 1. The effect of HBV on the expression level of FoxOs. (A, B) Huh7 or HepG2 cells were transfected with HBV replication-competent plasmid (pHBV1.3) or empty control vector (pUC19), or left untreated as the blank control. 48 h post-transfection, levels of HBsAg and HBeAg in culture supernatants were detected by ELISA. (C, D) Huh7 or HepG2 cells were treated as in A and B, the mRNA levels of FoxO1, FoxO3, and FoxO4 were determined by qRT-PCR. (E) Huh7 cells were treated as in A, western blot was performed using antibodies against FoxO1, FoxO3, FoxO4 or GAPDH. *Below*: the immunoblots from three independent experiments were scanned and subjected to densitometric analysis. (F) HepG2 cells were treated as in B, western blot was performed as in E. (G) Huh7 cells were transfected with pHBV1.3 or pUC19. 48 h post-transfection, nuclear and cytoplasmic extracts were examined by western blot using anti-FoxO4 antibody. Tubulin and Lamin B1 were used as fractionation controls for cytoplasmic and nuclear fractions, respectively. *Below*: the immunoblots from three independent experiments were scanned and subjected to densitometric analysis. The values were expressed as the means ± SD of three independent experiments; ND, not detected; NS, not significant (unpaired, two-tailed Student's *t* tests).

were applied to comparisons between groups. *P*-values < 0.05 was considered statistically significant.

3. Results

3.1. HBV induces significant downregulation of FoxO4 protein in hepatoma cells

To examine the effect of HBV on the FoxOs expression, we transfected HBV replicative-competent plasmid (pHBV1.3) or control empty vector (pUC19) into two hepatoma cell lines, Huh7 and HepG2. The significant production of HBsAg and HBeAg in both hepatoma cell lines confirmed the efficient transfection of pHBV1.3 (Fig. 1A and B). Next, we determined the effect of HBV on the transcriptional level of FoxO1, FoxO3 or FoxO4. Results showed that the transcription level of FoxO1, FoxO3 or FoxO4 was not significantly changed in pHBV1.3-transfected cells (Fig. 1C and D). However, further western blot analysis showed that pHBV1.3 transfection resulted in significant downregulation of FoxO4 at the protein level, while had little effect on that of FoxO1 or FoxO3 in both Huh7 and HepG2 cells (Fig. 1E and F). We and others reported that the biological functions of FoxOs were closely associated with its subcellular distribution (Brunet et al., 1999; Lu et al., 2016). We therefore investigated the effect of HBV on the subcellular distribution of FoxO4 in Huh7 cells. It was found that FoxO4 was mainly nuclear-located, and pHBV1.3 transfection significantly downregulated the protein level of FoxO4 in the nucleus (Fig. 1G).

3.2. FoxO4 inhibits HBV transcription and replication in vitro

As above data showed that HBV significantly downregulated the protein level of FoxO4, we further investigated whether FoxO4 could affect HBV replication. Firstly, we transfected pHBV1.3 into Huh7 cells together with Flag-tagged FoxO4 expression plasmid (pFoxO4-Flag) or control empty vector (pCMV). The western blot analysis confirmed the efficient expression of exogenously expressed FoxO4 in Huh7 cells (Fig. 2A). Next, we detected HBsAg and HBeAg in culture supernatants by ELISA. It was found that FoxO4 overexpression could effectively downregulate HBsAg and HBeAg levels (Fig. 2B and C). qRT-PCR analysis also revealed that FoxO4 expression significantly decreased the level of HBV RNAs (Fig. 2D) and pgRNA (Fig. 2E). Further, qPCR demonstrated that overexpression of FoxO4 significantly decreased the HBV-DNA level (Fig. 2F), and southern blot analysis confirmed the inhibitory effect of FoxO4 on HBV replication (Fig. 2G). We have also investigated the effect of FoxO4 expression on HBV transcription and replication in HepG2 cells, and similar results were obtained (Supplementary Figs. 1A–E). Furthermore, we have determined the effect of FoxO4 on HBV replication in HBV-infected NTCP-HepG2 cells. Similar to the data obtained from pHBV1.3-transfected Huh7 or HepG2 cells, overexpression of FoxO4 significantly downregulates the levels of HBV proteins (Fig. 2I), HBV transcripts (Fig. 2J) and HBV-DNA (Fig. 2K).

To further confirm the inhibitory effect of FoxO4 on HBV transcription and replication, we downregulated FoxO4 in Huh7 cells by siRNA technique, followed by the transfection of pHBV1.3. The knockdown efficiency of FoxO4 was confirmed by western blot (Fig. 3A). And siRNA-mediated FoxO4 downregulation significantly

enhanced HBV transcription and replication, as demonstrated by the increased levels of HBsAg (Fig. 3B), HBeAg (Fig. 3C), HBV RNAs (Fig. 3D), HBV pgRNA (Fig. 3E) and HBV-DNA (Fig. 3F and G). Further, we demonstrated that knockdown of FoxO4 by siRNA technique significantly enhanced HBV transcription and replication in HBV-infected NTCP-HepG2 cells (Fig. 3H–K).

In summary, these data indicated that FoxO4 displayed an inhibitory effect on HBV transcription and replication *in vitro*.

3.3. FoxO4 inhibits HBV transcription and replication in vivo

To further investigate the effect of FoxO4 on HBV replication, we established a HBV mice model by hydrodynamic injection of pAAV1.2 as described previously (Huang et al., 2006), and then treated the HBV mice with adenovirus-harboring FoxO4 (Ad-FoxO4-Flag). 4 days post Ad-FoxO4-Flag injection, the effect of FoxO4 on HBV transcription and replication was evaluated. Western blot confirmed the efficient expression of ectopic FoxO4 in the liver tissues of HBV mice (Fig. 4A). And FoxO4 expression was found to significantly downregulate levels of serum HBsAg (Fig. 4B) and HBV-DNA (Fig. 4C). Further, our data revealed that FoxO4 overexpression significantly downregulated the level of HBV RNAs (Fig. 4D) and HBV pgRNA (Fig. 4E) in the mice liver tissues. Immunohistochemical analysis also revealed that FoxO4 decreased the HBcAg expression level in the mice liver (Fig. 4F). Together, these data indicated that FoxO4 displayed inhibitory effect on HBV transcription and replication *in vivo*.

3.4. FoxO4 inhibits the activity of HBV core promoter

Our above data showed that FoxO4 was mainly nuclear-located, which is consistent with its possible role in transcriptional regulation, and FoxO4 displayed inhibitory effect on HBV transcription and replication. In mechanistic studies, we thus paid attention to the effect of FoxO4 on the activity of HBV core promoter, which is responsible for the transcription of pgRNA and the following HBV replication (Seeger and Mason, 2000). Our data showed that FoxO4 overexpression could inhibit HBV core promoter activity in a dose-dependent manner in both Huh7 cells and HepG2 cells (Fig. 5A, Supplementary Fig. S2), while the CMV promoter was not affected. And siRNA-mediated knockdown analysis further confirmed the inhibitory effect of FoxO4 on HBV core promoter activity (Fig. 5B), Additionally, we also tested the effect of FoxO4 on other HBV promoters in Huh7 cells, and it was found that FoxO4 displayed strong inhibitory effect on major surface promoter (SpII), consistent with its suppressive effect on the expression of HBsAg; while the effect of FoxO4 on the activity of two other HBV promoters (preS and X) was much less significant (Supplementary Fig. S3).

3.5. FoxO4 inhibits HBV core promoter activity via downregulating HNF4α

Evidence demonstrates that LETFs, such as FXRα, RXRα, PPARα, HNF3β and HNF4α, play a crucial role in regulating HBV core promoter activity (Tang and McLachlan, 2001; Raney et al., 1997). As above data showed that FoxO4 displayed significant inhibitory effect on HBV core promoter activity, we thus further investigated the effect of FoxO4 on the expression of LETFs. Data from qRT-PCR showed that FoxO4 overexpression significantly downregulated the mRNA level of HNF4α,
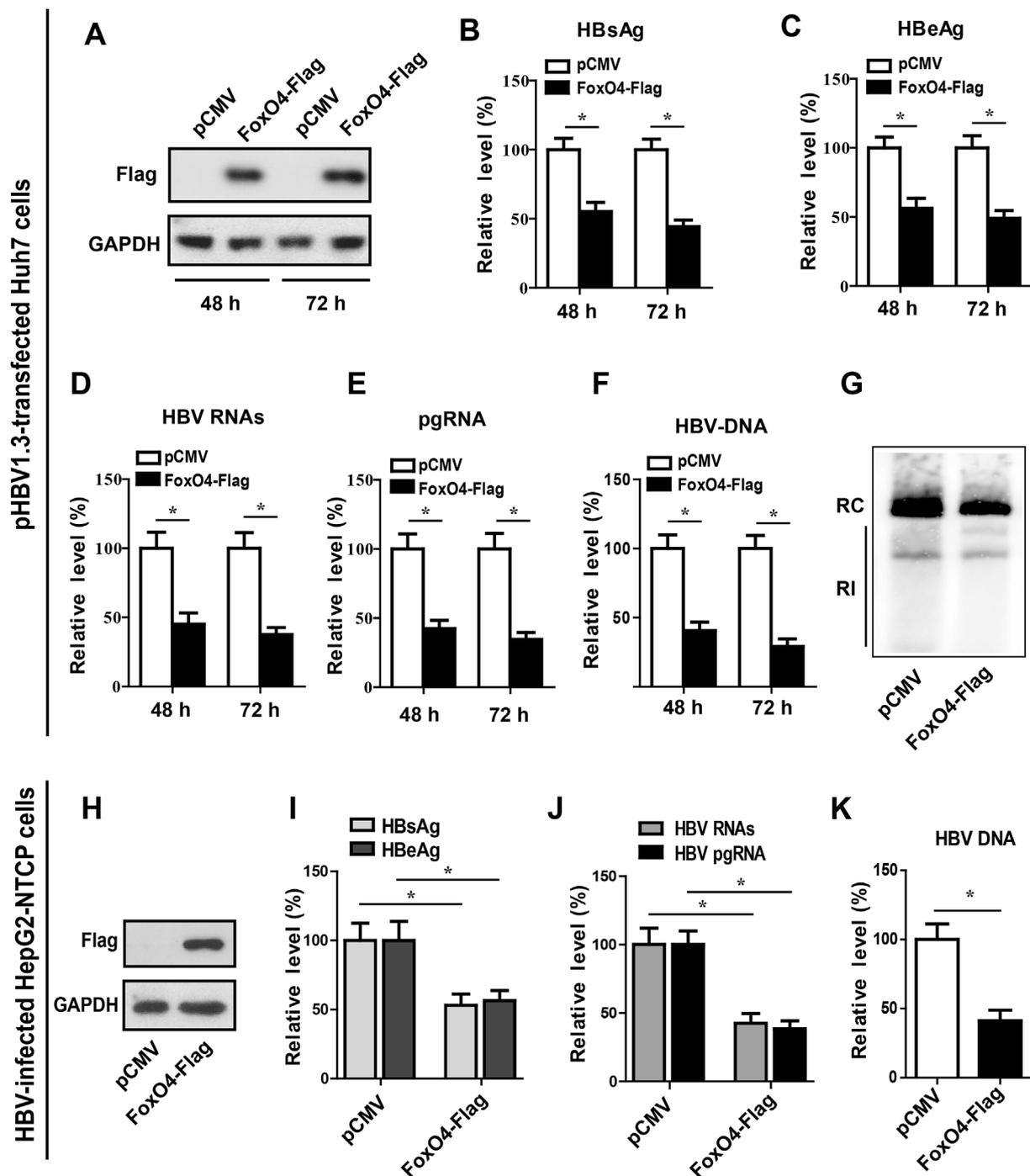
Fig. 2. FoxO4 overexpression inhibited HBV transcription and replication in hepatoma Huh7 cells. (A) pHBV1.3 was transfected into Huh7 cells together with Flag-tagged FoxO4 expression plasmid (pFoxO4-Flag) or empty pCMV vector. 48 or 72 h post-transfection, Western blot was performed using antibodies against Flag or GAPDH. (B, C) Huh7 cells were treated as in A, and levels of HBsAg and HBeAg in culture supernatants were detected by ELISA. (D, E) Huh7 cells were treated as in A, levels of HBV RNAs and pgRNA were determined by qRT-PCR. (F) Huh7 cells were co-transfected with pHBV1.3 and pFoxO4-Flag or pCMV, 72 h post-transfection, intracellular HBV-DNA levels were determined by qPCR. (G) Huh7 cells were treated as in E, the HBV-DNA level was determined by southern blot. RC, relaxed circular DNA; RI, replicative intermediate. (H–K) HepG2-NTCP cells were electro-transfected with pFoxO4-Flag or pCMV. 48 h postransfection, cells were infected with HBV at 10^3 vge/cell, and then cultured for an additional 9 days in the presence of 2.5% DMSO. The expression of exogenous FoxO4 (H) was determined by western blot; levels of HBsAg, HBeAg (I) were detected by ELISA; and levels of HBV RNAs, pgRNA (J) and HBV-DNA (K) were determined by qRT-PCR and qPCR, respectively. The values were expressed as the means ± SD of three independent experiments; *$p < 0.05$ (unpaired, two-tailed Student's t tests).

while had no significant effect on that of FXRα, RXRα, PPARα or HNF3β (Fig. 6A). Western blot analysis further confirmed the inhibitory effect of FoxO4 on HNF4α expression at the protein level (Fig. 6B). To investigate the role of HNF4α in FoxO4-mediated suppression of HBV core promoter activity, we determined the effect of FoxO4 on HBV core promoter activity in the presence of increasing amounts of HNF4α. The results showed that HNF4α expression could attenuate the FoxO4-mediated suppression of HBV core promoter activity in a dose-dependent manner (Fig. 6C and D), indicating that FoxO4 inhibited HBV core promoter via downregulating the expression of HNF4α.
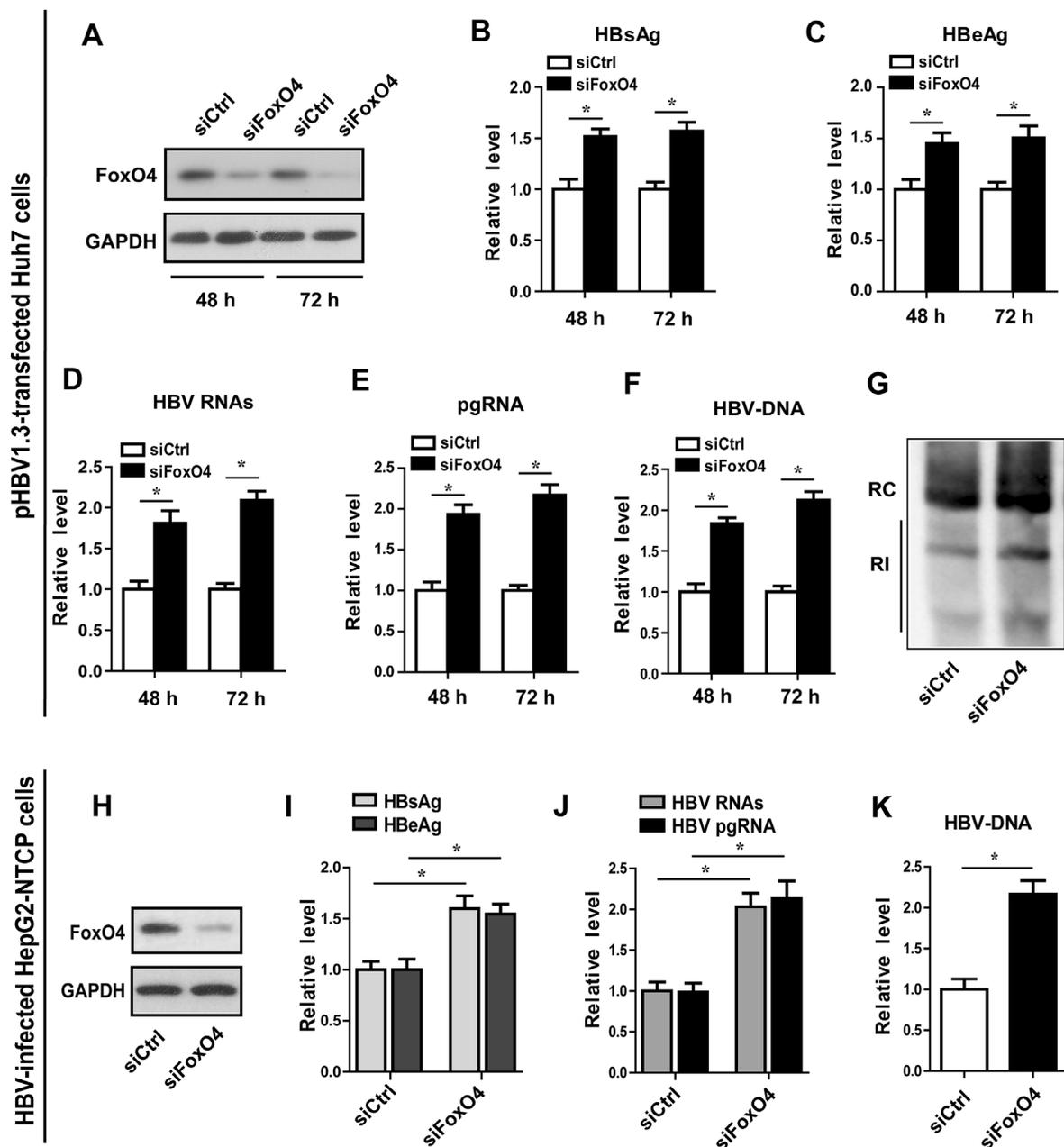
Fig. 3. FoxO4 downregulation enhanced HBV transcription and replication in hepatoma cells. Huh7 cells were transfected with control or FoxO4-specific siRNA. 20 h post-transfection, cells were further transfected with pHBV1.3 for 48 or 72 h. (A) The knockdown efficiency of FoxO4 was determined by western blot using antibodies against FoxO4 or GAPDH. (B, C) Levels of HBsAg and HBeAg in culture supernatants were detected by ELISA. (D, E) Levels of HBV RNAs and pgRNA were determined by qRT-PCR; HBV-DNA levels were determined by qPCR (F) or Southern Blot (G). (H–K) NTCP-HepG2 cells were electro-transfected with control or FoxO4-specific siRNA. 72 h post-transfection, cells were infected with HBV and cultured for an additional 9 days. The expression of exogenous FoxO4 (H) was examined by western blot; levels of HBsAg, HBeAg (I) were detected by ELISA; and levels of HBV RNAs, pgRNA (J) and HBV-DNA (K) were determined by qRT-PCR and qPCR, respectively. The values were expressed as the means ± SD of three independent experiments; *$p < 0.05$ (unpaired, two-tailed Student's t tests).

3.6. ERK signaling is required for FoxO4-mediated inhibition of HNF4α expression and HBV core promoter activity

Mitogen-activated protein kinase (MAPK) signaling pathways are reported to play an important role in controlling HNF4α expression, while evidence indicates that FoxOs are involved in the regulation of several signaling pathways (de Boussac et al., 2010; Hatzis et al., 2006; Matkar et al., 2017; Zhao et al., 2012). We therefore further investigated the effect of FoxO4 on the activation of MAPKs. As shown in Fig. 7A, FoxO4 expression significantly enhanced the phosphorylation of ERK and p38, while had little effect on that of JNK. To investigate the role of ERK and p38 signaling in FoxO4-mediated suppression of

HNF4α, we treated the Huh7 cells with ERK-specific pharmacological inhibitor (PD98059) or p38-specific pharmacological inhibitor (SB203580), and then tested the effect of FoxO4 on the protein level of HNF4α. As expected, PD98059 and SB203580 could efficiently inhibit the activation of ERK and p38, respectively (Fig. 7B). However, it was the inhibition of ERK but not p38 MAPK pathway that attenuated FoxO4-mediated suppression of HNF4α (Fig. 7C). Correspondingly, further data revealed that ERK but not p38 MAPK signaling pathway was required for FoxO4-mediated inhibition of HBV core promoter activity (Fig. 7D). We have also examined the role of these signaling pathway in FoxO4-mediated inhibition of HBV. Expectedly, inhibition of ERK, but not p38 signaling, could significantly reverse FoxO4-
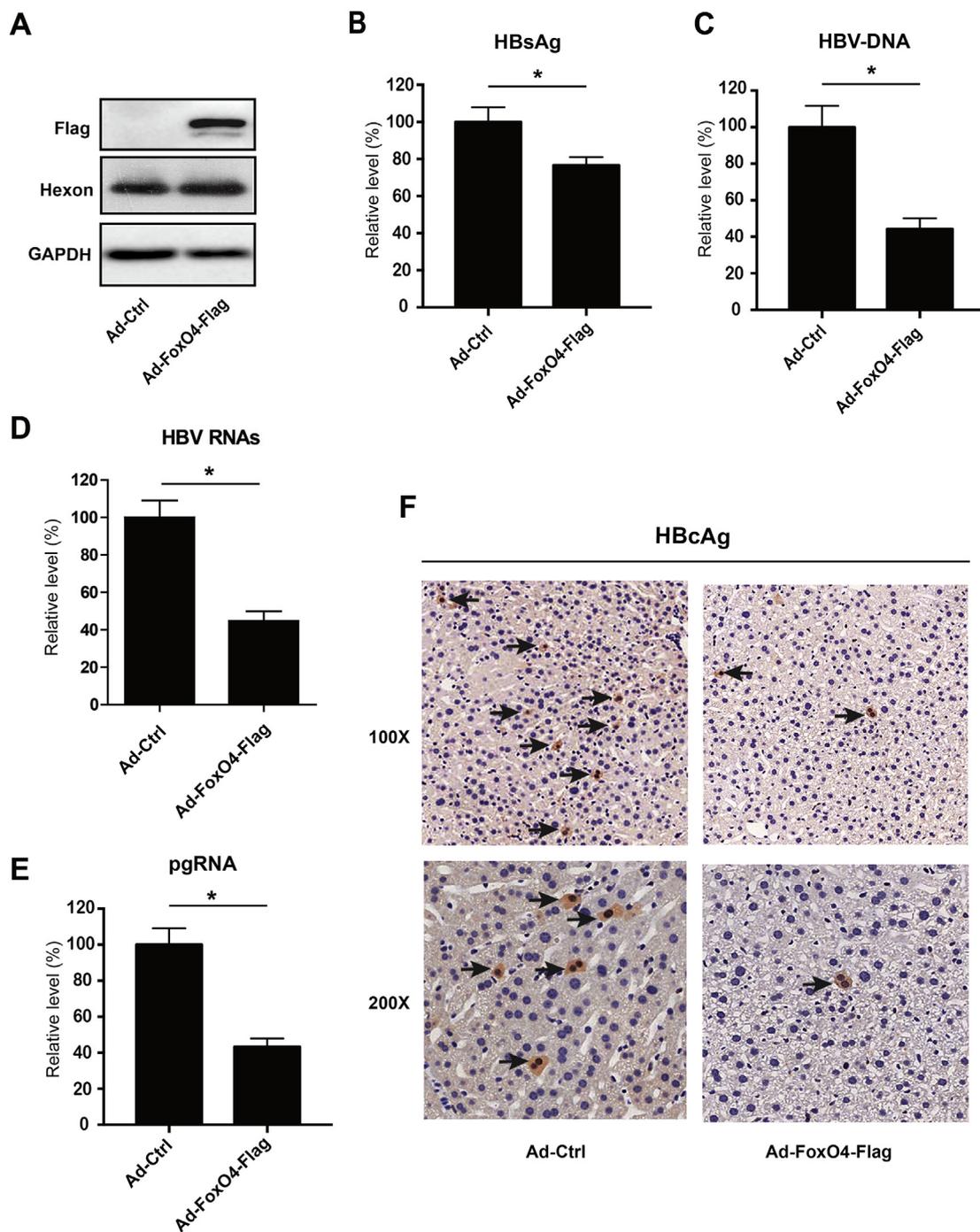
Fig. 4. FoxO4 displayed inhibitory effect on HBV transcription and replication *in vivo*. HBV-carrier mice were infected with 3 x 10⁹ Ad-FoxO4-Flag or Ad-Ctrl (n = 10). 4 days post-infection, mice liver tissues and sera were collected. (A) Mice liver tissues were subjected to western blot using antibodies against Flag, Hexon or GAPDH. Levels of HBsAg (B) and HBV-DNA (C) in mice sera were determined by ELISA or qPCR, respectively. (D, E) Levels of HBV RNAs and pgRNA in mice liver tissues were determined by qRT-PCR. (F) Levels of HBcAg in mice liver tissues were determined by immunohistochemistry. The values were expressed as the means ± SD of three independent experiments; $*p < 0.05$ (unpaired, two-tailed Student's *t* tests).

mediated inhibition of HBV replication both in pHBV1.3-tranfected Huh7 cells (Fig. 7E) and in HBV-infected HepG2-NTCP cells (Fig. 7F).

4. Discussion

Despite the well-documented importance of FoxOs in various biological processes, there is relatively little is known about FoxOs in liver disease. The most available researches on FoxOs in liver disease are for HCV infection (Deng et al., 2011; Tikhanovich et al., 2013). But the role of FoxOs in HBV infection and the precise mechanisms are still poorly

understood.

Our data showed that HBV induced the downregulation of FoxO4 protein, but had little effect on the expression level of FoxO1 or FoxO3. Further, it was found that the protein level of FoxO4 was mainly located in the nuclei of Huh7 cells, and HBV could significantly downregulate nuclear-located FoxO4 protein. However, a previous study reported that HBV x protein (HBx) overexpression upregulated the expression level of FoxO4 in hepatoma Chang cells (Srisuttee et al., 2011). The reasons for this discrepancy are unclear, which may be due to different cell lines used, or the low expression level of HBx in the context of HBV
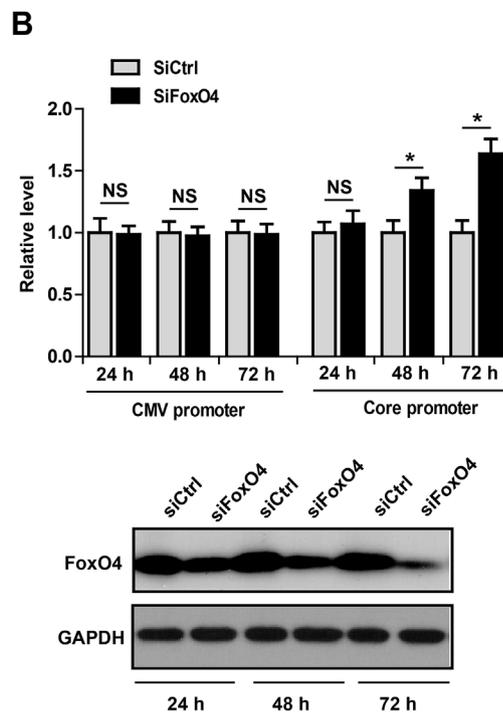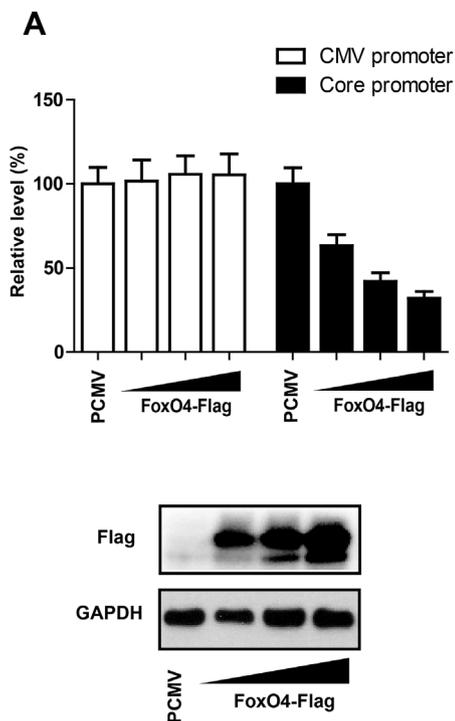
Fig. 5. FoxO4 inhibited the activity of HBV core promoter. (A) Huh7 cells were transfected with CpLUC together with increasing does of FoxO4-Flag, 48 h post-transfection, the luciferase activity in cell lysates were determined. The luciferase activity of pCMV-transfected cells was set to 100%. In all transfection assay, pCMV-β-gal was co-transfected to normalize the transfection efficiency. *Below*: the expression of ectopic FoxO4 was determined by western blot. (B) Huh7 cells were transfected with control or FoxO4-specific siRNA. 20 h post-transfection, cells were further transfected with CpLUC for 24, 48 or 72 h. The luciferase activity in cell lysates was then examined. *Below*: the expression of endogenous FoxO4 was determined by western blot. The values were expressed as the means ± SD of three independent experiments; *p < 0.05; NS, not significant (unpaired, two-tailed Student's *t* tests).

replication (Li et al., 2007). An intriguing issue about HBx-induced FoxO4 expression is that HBx as well as HBV activates PI3K/AKT pathway (Lee et al., 2001; Rawat and Bouchard, 2015; Shih et al., 2000), while we and others demonstrate that the activation of this signaling pathway may lead to the proteasomal degradation of FoxOs (Huang et al., 2005; Lu et al., 2016), and there is also evidence that AKT mutation blocks viral Tax protein-induced FoxO4 degradation (Oteiza and Mechti, 2011). As our data showed that HBV induced the down-regulation of FoxO4 at the protein level, while it didn't affect the mRNA level of FoxO4, whether PI3K/AKT signaling is involved in this process
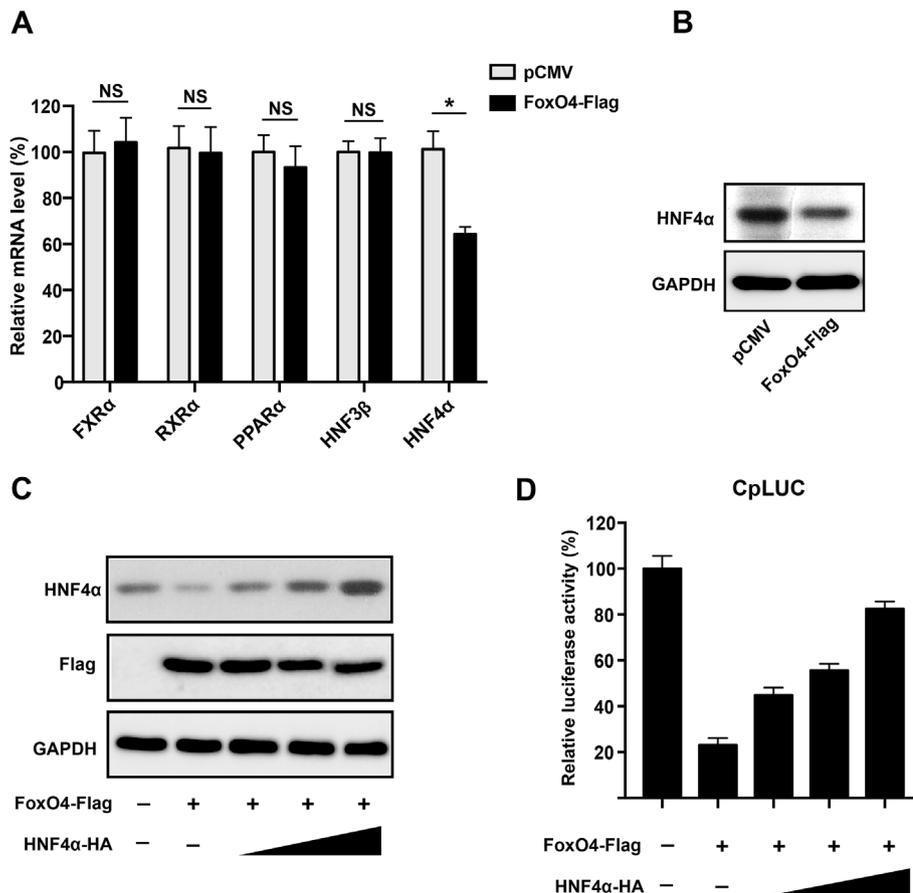

Fig. 6. Inhibitory effect of FoxO4 on HBV core promoter by suppressing HNF4α expression. (A) Huh7 cells were transfected with pCMV or pFoxO4-Flag. 48 h post-transfection, the mRNA levels of FXRα, RXRα, PPARα, HNF3β and HNF4α were determined by qRT-PCR. (B) Huh7 cells were treated as in A, the protein level was determined by western blot using antibodies against HNF4α or GAPDH. (C, D) CpLUC and pFoxO4-Flag were transfected into Huh7 cells in the absence or presence of increasing doses of HNF4α-HA. 48 h post-transfection, cells lysates were collected and subjected to western blot using antibodies against HNF4α, Flag or GAPDH (C), and the luciferase activity in the cell lysates was determined (D). The values were expressed as the means ± SD of three independent experiments; *p < 0.05 (unpaired, two-tailed Student's *t* tests).
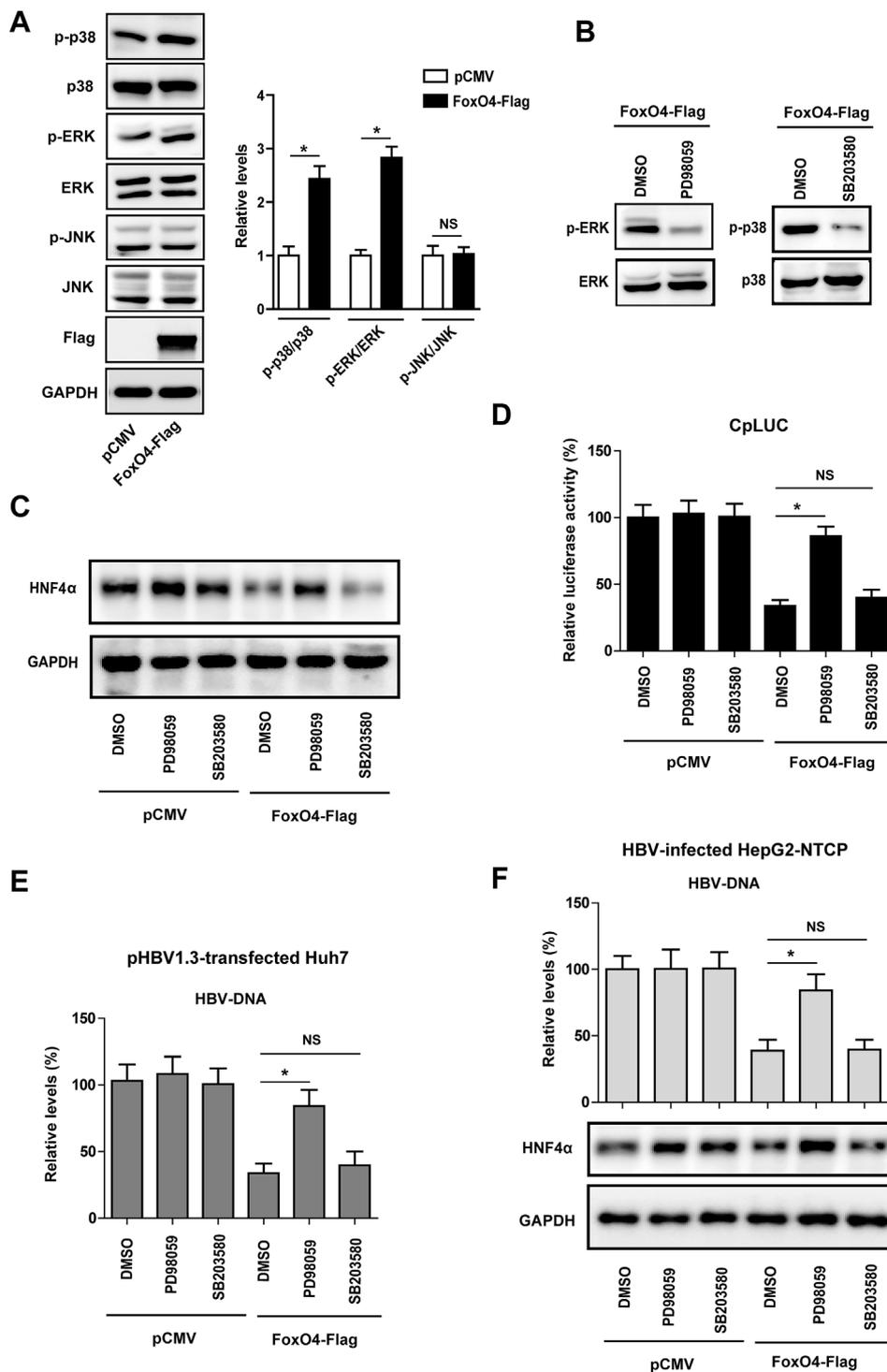
Fig. 7. ERK signaling was involved in FoxO4-mediated inhibition of HNF4α and HBV core promoter activity. (A) Huh7 cells were transfected with pFoxO4-Flag or pCMV. 48 h post-transfection, cell lysates were collected and subjected to Western Blot using antibodies against Flag, JNK, p-JNK, ERK, p-ERK, p38, p-p38 or GAPDH. *Right panel*: the immunoblots from three independent experiments were subjected to densitometric analysis. The density value from the pCMV-transfected cells was set to 1. (B) Huh7 cells were transfected with pFoxO4-Flag. 24 h post-transfection, cells were treated with ERK inhibitor PD98059 (20 μM) or p38 inhibitor SB203580 (10 μM) for another 24 h, cells were then subjected to western blot using antibodies against ERK, p-ERK, p38 or p-p38. (C, D) CpLUC was transfected into Huh7 cells together with pFoxO4-Flag or pCMV for 24 h, followed by the treatment with PD98059 or SB203580 for another 24 h. Cells were then subjected to western blot using antibodies against HNF4α or GAPDH (C), and the luciferase activity in the cell lysates was determined (D). (E) pHBV1.3 was transfected into Huh7 cells pFoxO4-Flag or pCMV. 48 h post-transfection, cells were treated with PD98059 (20 μM) or SB203580 (10 μM) for another 24 h, and HBV-DNA was then detected by qPCR. (F) HepG2-NTCP cells were electro-transfected with pFoxO4-Flag or pCMV. 48 h post-transfection, cells were infected with HBV at 10^3 vge/cell, and then cultured for an additional 7 days in the presence of 2.5% DMSO. Cells were then treated with PD98059 (20 μM) or SB203580 (10 μM) for another 48 h, and HBV-DNA was then detected by qPCR. *Below*: the effect of PD98059 or SB203580 on FoxO4-mediated downregulation of HNF4α. Values were expressed as the means ± SD of three independent experiments; *p < 0.05 (unpaired, two-tailed Student's *t* tests).

awaits further investigation.

It is well-known that there exist interplays between host and virus (Hensel et al., 2017), and HBV significantly induced the downregulation of FoxO4 protein in hepatoma cell lines, we thus focused on the effect of FoxO4 on HBV replication. It was found that FoxO4 indeed displayed inhibitory effect on HBV transcription and replication both *in vitro* and *in vivo*. Further, our data revealed that FoxO4-mediated inhibition of HBV was not due to its effect on cell proliferation and apoptosis (Supplementary Fig. S4). Recent evidence shows that FoxO4 interacts with p53 and TRIM22, and may be involved in their biological functions (Baar et al., 2017; Oteiza and Mechti, 2015). While both p53 and TRIM22 also display inhibitory effect on HBV transcription and

replication (Gao et al., 2009; Lee et al., 1998). Whether p53 or TRIM22 is involved in FoxO4-mediated inhibition of HBV awaits further investigations.

As FoxO4 was mainly nuclear-located in hepatoma cells, consistent with its role as a transcriptional regulator, and FoxO4 exhibited significant inhibitory effect on HBV transcription, we therefore paid attention to the effect of FoxO4 on the activity of HBV core promoter, which is responsible for the synthesis of 3.5 kb pgRNA, and play a central role in HBV life cycle. Our results showed that FoxO4 inhibited HBV core promoter activity significantly. Similar to our data, it is reported that FoxO4 negatively modulated the activity of HIV-1 promoter (Oteiza and Mechti, 2017), suggesting that FoxO4 may function as an

important transcriptional repressor in antiviral responses. It is also re-
ported that another FoxO family member, FoxO1, could enhance HBV
transcription via activating HBV core promoter (Shlomai and Shaul,
2009; Wang and Tian, 2017). Whether FoxO4 could interrupt FoxO1-
mediated activation of HBV core promoter deserves further investiga-
tion.

To further investigate the mechanisms whereby FoxO4 inhibited
HBV transcription and replication, special attention was paid to the
LETFs that could affect the activity of HBV core promoter and we dis-
covered that FoxO4 expression significantly downregulated the ex-
pression level of HNF4α, while had little effect on other LETFs. Further
study revealed that HNF4α expression could significantly reverse
FoxO4-mediated inhibition of HBV core promoter. Of interest, FoxO4
expression could also activate the ERK signaling pathway, which was
crucial for FoxO4-mediated suppression of HNF4α expression and the
following suppression of HBV core promoter activity and HBV re-
plication. Reports indicate that activation of TGF-β/Smad signaling also
inhibits HBV transcription through downregulating HNF4α expression
(Chou et al., 2007; Hong et al., 2012), while there might exist cross-
talking between ERK and TGF-β/Smad signaling pathway (Hayashida
et al., 2003; Talati et al., 2018). It is therefore of interest to investigate
the role of FoxO4 in TGF-β-mediated suppression of HBV transcription
and replication in future investigations.

Collectively, our data first demonstrated that FoxO4 inhibited HBV
transcription and replication through inhibiting HBV core promoter
activity. By activating ERK signaling pathway, FoxO4 downregulated
the expression of HNF4α, a well-known activator of HBV core pro-
moter. Our data also revealed that HBV *per se* could downregulate
FoxO4 expression at the protein level, indicating that HBV might has
developed strategies to escape from FoxO4-mediated antiviral effect.
Together, these data suggested that FoxO4 might present as a novel
therapeutic target against HBV infection.

Conflicts of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://
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