Hepatitis B virus X protein promotes renal epithelial-mesenchymal transition in human renal proximal tubule epithelial cells through the activation of NF-κB

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Abstract. Hepatitis B virus (HBV)-associated glomerulonephritis is the most common extra-hepatic disorder occurring with hepatitis B virus infection. In the present study, we hypothesized that HBV X protein (HBx) may play a critical role in renal interstitial fibrosis, as HBx has been shown to induce epithelial-mesenchymal transition (EMT) in renal cells. For this purpose, we successfully transfected HBx plasmid into human renal proximal tubule epithelial cells (HK-2 cells). We found that transfection with HBx plasmid significantly downregulated E-cadherin expression and upregulated α-smooth muscle actin, collagen I and fibronectin expression in a time- and concentration-dependent manner (at the lower concentrations and earlier time points). HBx also increased nuclear factor-κB (NF-κB) phosphorylation in a time- and concentration-dependent manner (again at the lower concentrations and earlier time points); however, it did not alter the phosphorylation of Smad2, Smad3, p38, phosphoinositide 3-kinase (PI3K) or extracellular signal-regulated kinase (ERK). Thus, the findings of this study demonstrate that HBx promotes EMT in renal HK-2 cells, and the potential underlying mechanisms may involve the activation of the NF-κB signaling pathway.

Introduction

Chronic infection with hepatitis B virus (HBV) not only greatly increases the risk of developing hepatic fibrosis, hepatic sclerosis and hepatocellular carcinoma, but is also associated with damage to several other extra-hepatic organs (1). Many types of extra-hepatic manifestations have been observed in patients with both acute and chronic hepatitis with HBV infection (1). Renal involvement is among the most common extra-hepatic manifestation and usually manifests in the form of immune complex-mediated nephropathy, such as membranous glomerulonephritis, membranoproliferative glomerulonephritis and immunoglobulin A nephropathy (2-4).

To date, tubular and interstitial damage has attracted much attention, although glomerular damage has been proven to be the main pathological characteristic of HBV-associated glomerulonephritis (HBV-GN). It has been reported that inflammatory cell infiltration and tubulointerstitial fibrosis are observed in patients with HBV-GN, and HBV DNA and RNA have been identified in renal tubular cells. Thus, tubulointerstitial damage may play an important role in HBV-GN (5,6).

The HBV X protein (HBx), a 17-kDa protein, is the smallest open reading frame in the HBV genome, and it is located at 1374-1838 bp of the HBV genome. The overall length is 435 to 462 bp, and the code length is of a protein containing 154 amino acids. HBx is a multifunctional protein and it activates multiple cellular signal transduction pathways and regulates apoptosis. A number of studies have suggested that HBx activates the nuclear factor-κB (NF-κB), Janus kinase/signal transducers and activators of transcription (JAK/STAT), Ras-Raf-mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K) and the Src tyrosine kinase signaling pathways (7-11) to induce host cell apoptosis.

Recently, HBx was detected in renal tissues, mainly in tubular epithelial cells (7). Wang *et al* reported that HBx induced epithelial-mesenchymal transition (EMT) and immunity disorder in renal tubule epithelial cells through the Notch1 pathway (12). However, the transforming growth factor-β (TGF-β) (13,14) pathway plays an important role in renal EMT, and its downstream factors, such as Smads, p38, extracellular signal-regulated kinase (ERK), PI3K and NF-κB also play an important role in EMT. Thus, in the present study, we aimed to determine the role of HBx in HBV-GN-induced renal EMT and to elucidate the potential underlying mechanisms.

For this purpose, we successfully transfected HBx plasmid into human renal proximal tubule epithelial cells (HK-2 cells), and determined that HBx promotes renal EMT through the activation of NF-κB phosphorylation, but not through that of other TGF-β downstream factors.

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Materials and methods

HBx plasmid construction. HBx plasmid was provided by GeneCopoeia (Guangzhou, China). Full-length HBx was PCR-amplified from the p1.2II plasmid (HBV adr genome). The forward primer was 5'-GCGGTAGGCGTGTACGGT-3 and the reverse primer was 5'-GTGGCACCTTCCAGGGTC-3'. These were synthesized and inserted into the pEZ-M09 vector GeneCopoeia). An empty pEZ-M09 was used as a control. The PCR amplification protocol consisted of an initial 4-min denaturation at 94˚C, 25 cycles of denaturation at 94˚C (1 min each), annealing at 60˚C for 1 min, extension at 72˚C for 1 min and a final extension at 72˚C for 10 min. All ligated vectors were confirmed by sequence analysis. The stable selection marker was neomycin.

Cell culture and treatment. HK-2 cells (a kind gift from Dr Huiyao Lan, Li Ka Shing Institute of Health Sciences, Department of Chemical Pathology, The Chinese University of Hong Kong, Hong Kong, China) were cultured in DMEM-Ham's medium supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Life Techologies, Grand Island, NY, USA). Cells at approximately 60% confluence were used for the *in vitro* experiments.

The HBx plasmid and empty plasmid (pEZ-M09) were mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) separately and transfected into the cells following serum starvation for 12 h at various concentrations (0, 0.5, 1.0, 1.5 and 2.0 μg/ml). The cells were cultured in medium without FBS and harvested at different time points following transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was prepared from the HK-2 cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen). The RNA concentration was calculated using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Aliquots of each RNA extraction were reverse-transcribed simultaneously into cDNA using the One-Step RT-PCR kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. Each qPCR reaction was performed in a total volume of 25 μl in duplicate using the SYBR® Premix Ex Taq™ kit (Takara) and the Fast Real-Time PCR system 7500 (Applied Biosystems Inc., Foster City, CA, USA). The sequences of the primer pairs are listed in Table I. The thermal cycling conditions comprised 30 sec at 95˚C, followed by 95˚C for 5 sec and 60˚C for 34 sec for 40 cycles with melting curve analysis. The relative quantification of each gene was calculated following normalization to GAPDH mRNA using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. Western blot analysis was performed as previously described (15). Briefly, total protein was extracted from the cells by lysis in 500 μl of buffer containing Nonidet P-40 (10%), Tris-HCl (25 mM), NaCl (150 mM), ethylenediaminetetraacetic acid (EDTA) (10 mM) and a 1:50 dilution of a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 30 min on ice. The cell lysates were centrifuged at 12,000 x g for 15 min (4˚C). Cell lysates were heated at 95˚C and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Transferred membranes were immunoblotted with the following primary antibodies, respectively: anti-E-cadherin (610181; BD Biosciences, Franklin Lakes, NJ, USA), anti-α-smooth muscle actin (α-SMA; A5228-200), anti-fibronectin (F364B, Sigma), anti-HBx (ab235, Abcam, Cambridge, NK), anti-collagen I (234167; Calbiochem, San Diego, CA, USA); anti-neomycin (myc; #2276), anti-phosphorylated NF-κB ((#3033), and anti-NF-κB (#3032); anti-p-Smad2 (#9510), anti-p-Smad3 (#9520), anti-Smad2 (#5339), anti-Smad3 (#9523), anti-p-p38 (#4511), anti-p38 (#8690), anti-p-PI3K (#4228), anti-PI3K (#4249), anti-p-ERK (#4370) and anti-ERK (#9102) (all from Cell Signaling Technology Inc., Beverly, MA, USA).

Following extensive washing, the membranes were incubated with the secondary antibodies (anti-mouse IgG, #7076 and anti-rabbit IgG, #4414; Cell Signaling Technology Inc.). Immobilized antibodies were then detected using an Odyssey detector (LI-COR Biosciences, Lincoln, NE, USA).

Table I. The sequences of the primer pairs used for RT-PCR.

Primer	Sequence
Human E-cadherin	
Forward	5'-CTCAGTGTTTGCTCGGCGTTTGC-3'
Reverse	5'-GCTCTGGGTTGGATTCAGAG-3'
Human collagen I	
Forward	5'-ACGTCCTGGTGAAGTTGGTC-3'
Reverse	5'-ACCAGGGAAGCCTCTCTCTC-3'
Human α-SMA	
Forward	5'-CATCACCAACTGGGACGACATGGAA-3'
Reverse	5'-GCATAGCCCTCATAGATGGGGACATTG-3'
Human fibronectin	
Forward	5'-TCCTTGCTGGTATCATGGCAG-3'
Reverse	5'-AGACCCAGGCTTCTCATACTTGA-3'
Human GAPDH	
Forward	5'-GCTGGCGCTGAGTACGTCGTGGAGT-3
Reverse	5'-CACAGTCTTCTGGGTGGCAGTGATGG-3'

Immunofluorescence staining. Immunofluorescence staining was performed as previously described (16). Briefly, the cells cultured on cover slips were fixed, permeabilized with 0.5% Triton X-100, and incubated with the primary antibodies overnight at 4˚C, followed by incubation with secondary antibodies (anti-mouse IgG, #7076; anti-rabbit IgG #4414; Cell Signaling Technology Inc.) conjugated to Alexa Fluor 488 or 588 (Invitrogen). The cells were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Images were acquired using a confocal microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis. Data are expressed as the means ± SD. Comparisons between 2 groups were conducted using a two-tailed t-test. Comparisons between multiple groups was made using one-way ANOVA followed by the Student-Newman-Keuls test. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

HBx gene is successfully expressed in HK-2 cells following transfection. We first examined whether the HBx plasmid was successfully transfected into the HK-2 cells. The levels of both

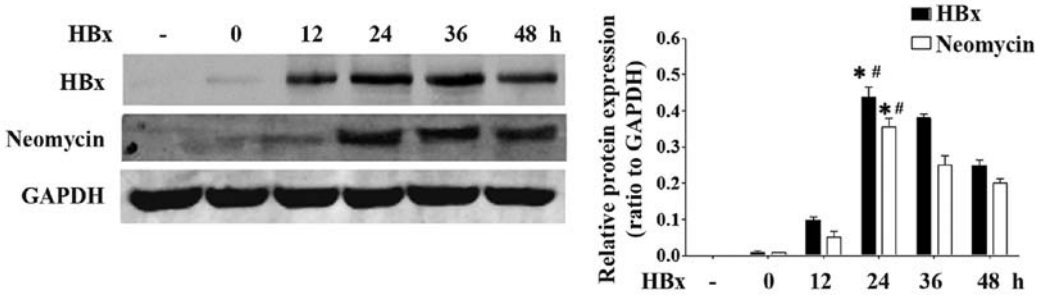

Figure 1. HBV X protein (HBx) plasmid was successfully transfected into HK2 cells. HK2 cells were co-transfected with empty pEZ-M09 or pEZ-M09-HBx plasmids. Cells were collected at 0, 12, 24, 36 and 48 h after transfection, and western blot analyses were performed with the antibodies against HBx and neomycin. Data are expressed as the means ± SD of 3 independent experiments. *p<0.05 vs. transfection with empty plasmid (HBx -); #p<0.05 vs. 0 h (HBx 0) post-transfection) (as shown by ANOVA).

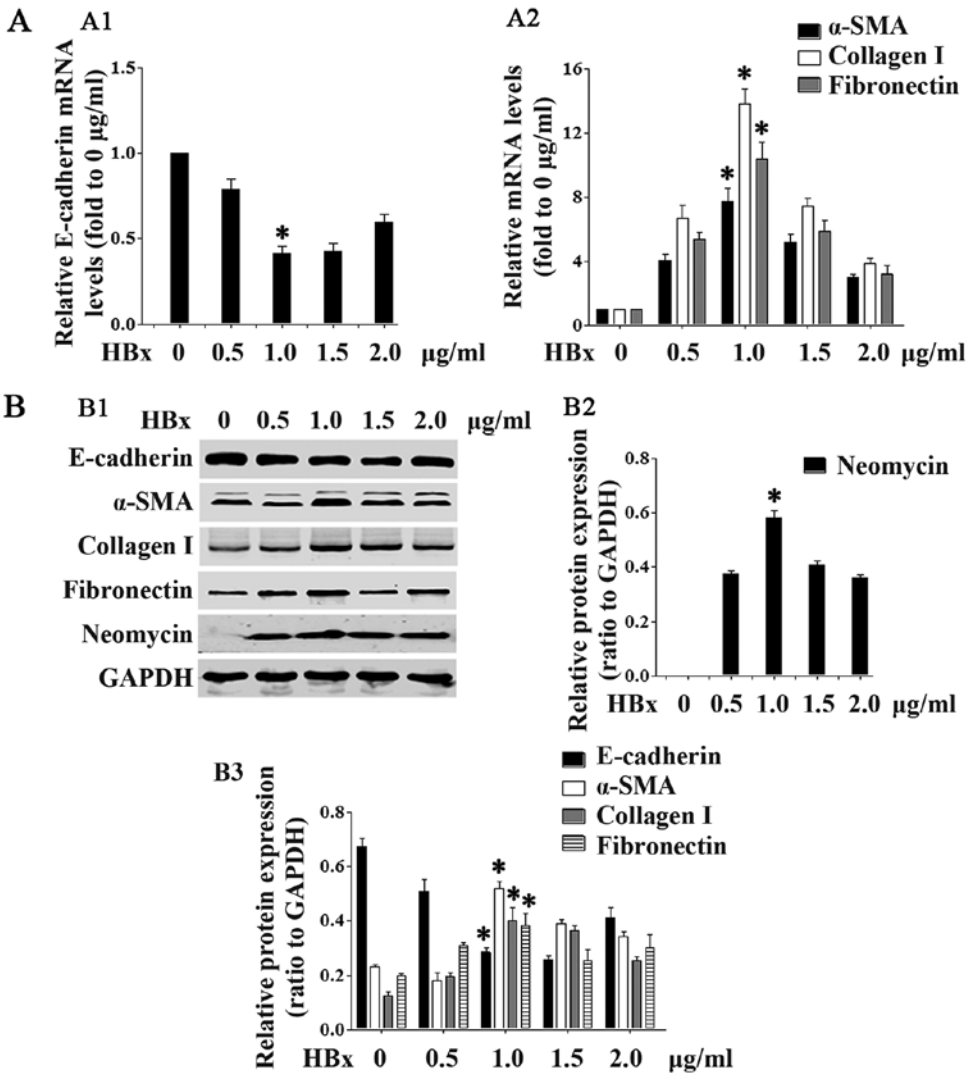

Figure 2. HBV X protein (HBx) protein promotes renal epithelial-mesenchymal transition (EMT). In concentration-dependent experiments, HK2 cells were transfected with the pEZ-M09-HBx plasmid at the concentrations of 0.5, 1.0, 1.5 or 2.0 μg/ml. (A) Cells were collected 24 h after transfection and RT-PCR was performed to detect the mRNA expression of (A1) E-cadherin, and (A2) α-smooth muscle actin (α-SMA), collagen I and fibronectin. (B) Cells were collected 36 h after transfection, and western blot analyses were performed to detect the protein expression of neomycin, E-cadherin, α-SMA, collagen I and fibronectin. (B1) Representative blots showing the expression of neomycin, E-cadherin, α-SMA, collagen I and fibronectin. (B2) Quantification of neomycin. (B3) Quantification of E-cadherin, α-SMA, collagen I and fibronectin. Data are expressed as the means ± SD of 3 independent experiments. *p<0.05 vs. 0 μg/ml groups in (A1, A2, B2 and B3), as shown by ANOVA.

HBx (17 kDa) and neomycin (17 kDa) were markedly increased at 24 h following co-transfection with the HBx plasmid (Fig. 1). These data suggested that the HBx plasmid was successfully transfected into the HK-2 cells.

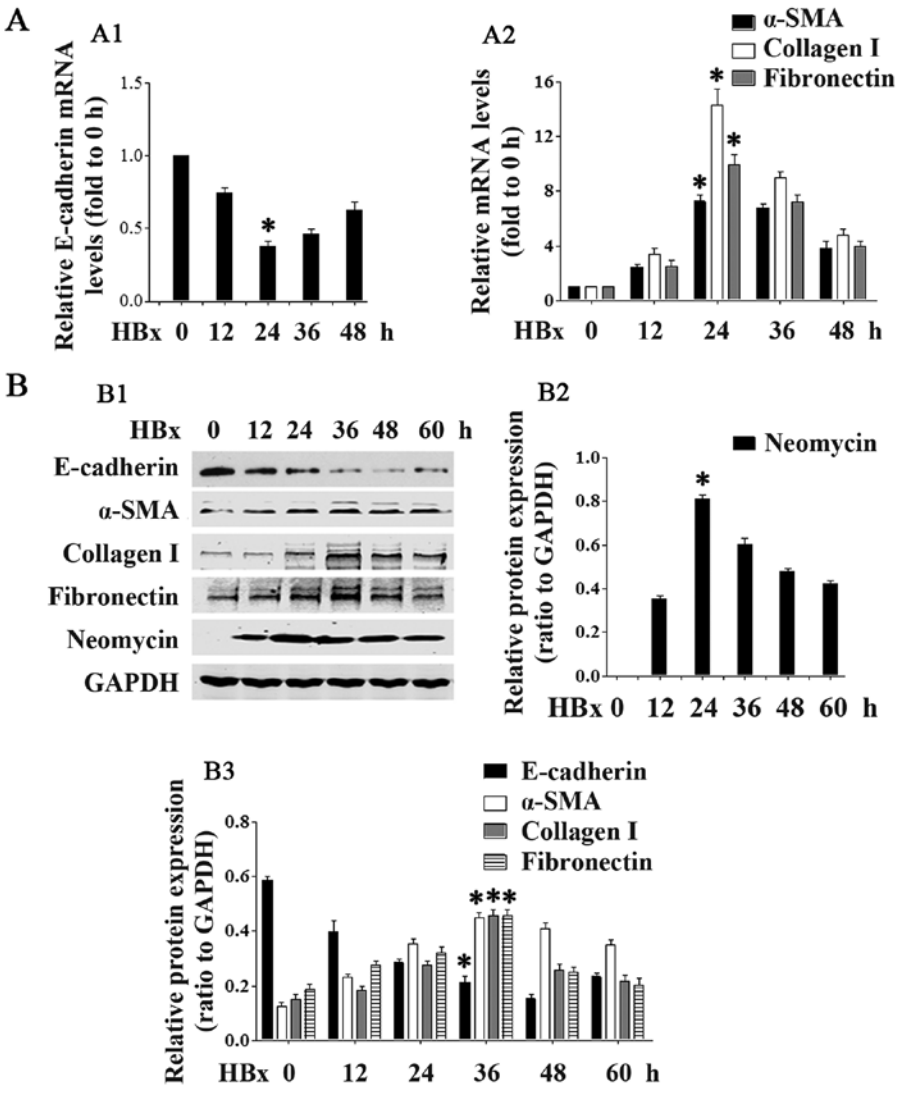

Figure 3. HBV X protein (HBx) promotes renal epithelial-mesenchymal transition (EMT). In time-dependent experiments, HK2 cells were transfected with the pEZ-M09-HBx plasmid (1.0 μg/ml) for the indicated periods of time. (A) Cells were collected and RT-PCR was performed to detect the mRNA expression of (A1) E-cadherin, and (A2) α-smooth muscle actin (α-SMA), collagen I and fibronectin. (B) Cells were collected and western blot analyses were performed to detect the protein expression of neomycin, E-cadherin, α-SMA, collagen I and fibronectin. (B1) Representative blots showing the expression of neomycin, E-cadherin, α-SMA, collagen I and fibronectin. (B2) Quantification of neomycin. (B3) Quantification of E-cadherin, α-SMA, collagen I and fibronectin. Data are expressed as the means ± SD of 3 independent experiments. *p<0.05 vs. 0 h groups in (A1, A2, B2 and B3), as shown by ANOVA.

HBx induces renal EMT. We then examined whether HBx affects EMT-related gene expression. As shown in Figs. 2 and 3, transfection with the lower concentrations (0.5-1.0 μg/ml; Fig. 2) of the HBx plasmid and at the earlier time points (12 and 24 h; Fig. 3) downregulated E-cadherin mRNA and protein expression in the HK-2 cells in a concentration- and time-dependent manner. Statistical significance was reached at the concentration of 1.0 μg/ml and at the 24-h time point. Transfection with the higher concentrations (1.5 and 2.0 μg/ml) of HBx and at the later time points (36 and 48 h) slightly increased E-cadherin expression. At the same time, transfection with the HBx plasmid upregulated the mRNA and protein expression of α-SMA, collagen I and fibronectin in a concentration- and time-dependent manner, again at the lower concentrations (Fig. 2) and earlier time points (Fig. 3). Statistical significance was reached at the concentration of 1.0 μg/ml and at the 24-h time point. At the higher concentrations and later time points, there was a slight decrease in the levels of α-SMA, collagen I and fibronectin (Figs. 2 and 3).

As shown by confocal immunofluorescence microscopy, transfection with the HBx plasmid induced complete EMT in the HK-2 cells (as evidenced by the loss of E-cadherin expression, and the strong expression of α-SMA and fibronectin) (Fig. 4).

HBx increases NF-κB phosphorylation. We then examined the potential molecular mechanisms responsible for the effects of HBx in renal fibrosis. Given the critical role of NF-κB activation in renal fibrosis, we hypothesized that HBx may be able to affect the phosphorylation of NF-κB. As shown in Fig. 5, transfection with HBx at the lower concentrations (0.5-1.0 μg/ml; Fig. 5A) and at the earlier time points (6-12 h; Fig. 5B) increased NF-κB phosphorylation in a concentration- and time-dependent manner. Statistical significance was reached at the concentration of 1.0 μg/ml and at the 12-h time point. At the higher concentra-

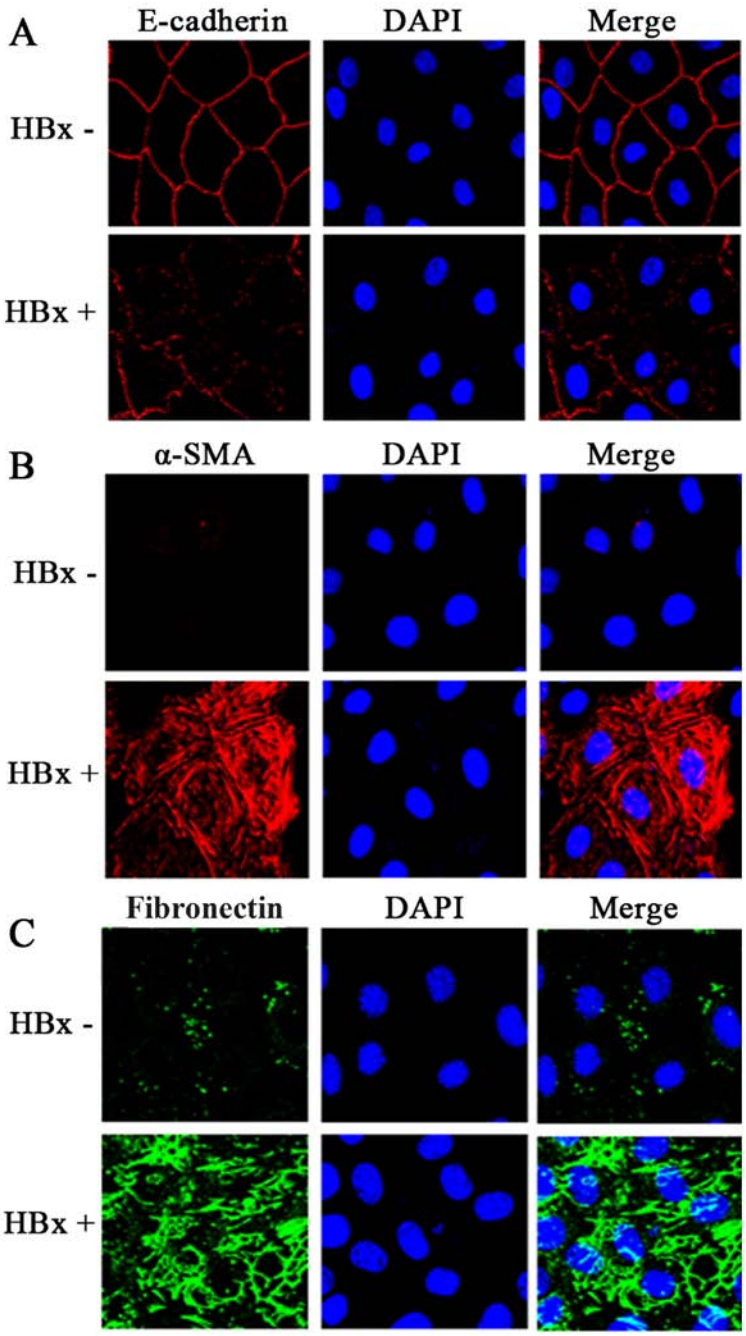

Figure 4. Immunofluorescence staining of E-cadherin, α-smooth muscle actin (α-SMA), collagen I, and fibronectin. HK2 cells were transfected with the empty pEZ-M09 (HBx -) or pEZ-M09-HBV X protein (HBx +) plasmids (1.0 μg/ml) for 36 h. Cells were collected and immunofluorescence staining was performed to detect the protein expression of (A) E-cadherin, (B) α-SMA and (C) fibronectin.

tions (1.5-2.0 μg/ml) and later time points (18-36 h), there was a slight decrease in NF-κB phosphorylation.

HBx does not affect TGF-β1-associated Smad2, Smad3, p38, PI3K or ERK phosphorylation. We further examined whether HBx affects the other typical phosphorylation-associated signaling pathways, such as Smad2, Smad3, p38, PI3K and ERK. As shown in Fig. 6, HBx did affect Smad2 or Smad3 phosphorylation in the HK-2 cells. No significant differences were observed at any of the concentrations of HBx (Fig. 6A) or at any time point (Fig. 6B). Furthermore, as shown in Fig. 7, HBx was also unable to affect the phosphorylation of p38, PI3K or ERK, at any of the concentrations of HBx (Fig. 7A) or at any time point (Fig. 7B). These data suggested that HBx did not affect the phosphorylation of associated signaling pathways.

Discussion

In the present study, we demonstrated that HBx, a 17-kDa protein, significantly promoted renal EMT in renal HK-2 cells. Furthermore, HBx may promote renal EMT by increasing the phosphorylation of NF-κB.

HBV-GN has been recognized as the most prevalent extra-hepatic manifestation caused by HBV infection (1-4). Its

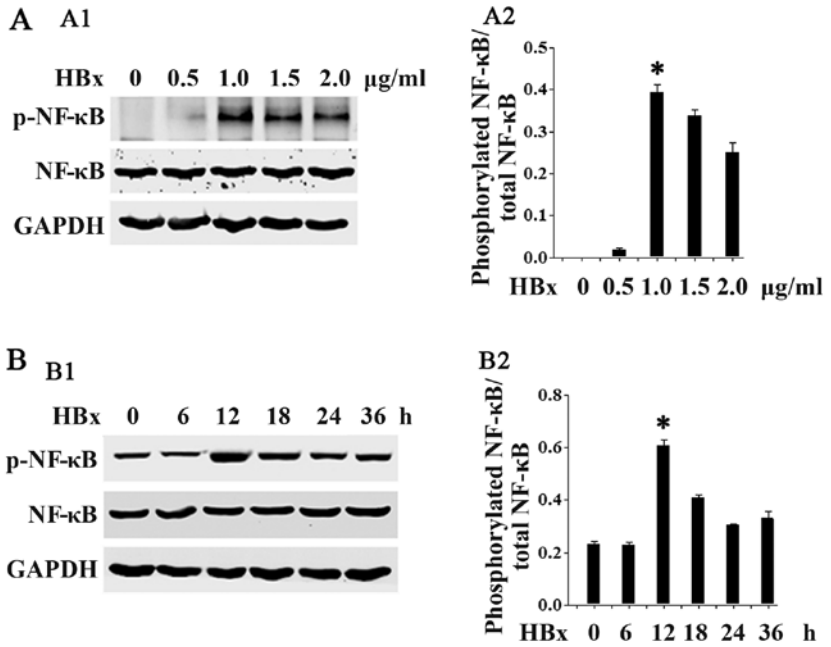

Figure 5. HBV X protein (HBx) increases nuclear factor-κB (NF-κB) phoshorylation. (A) HK2 cells were transfected with the pEZ-M09-HBx plasmid at the concentrations of 0.5, 1.0, 1.5, or 2.0 μg/ml. Cells were collected and western blot analyses were performed to detect the phosphorylation of NF-κB and total NF-κB. (B) HK2 cells were transfected with the pEZ-M09-HBx plasmid (1.0 μg/ml) for 6, 12, 18, 24 or 36 h. Cells were collected and western blot analyses were performed to detect the phosphorylation of NF-κB and total NF-κB. Data are expressed as the means ± SD of 3 independent experiments. $^{*}p<0.05$ vs. control groups in (A2 and B2), as shown by ANOVA.

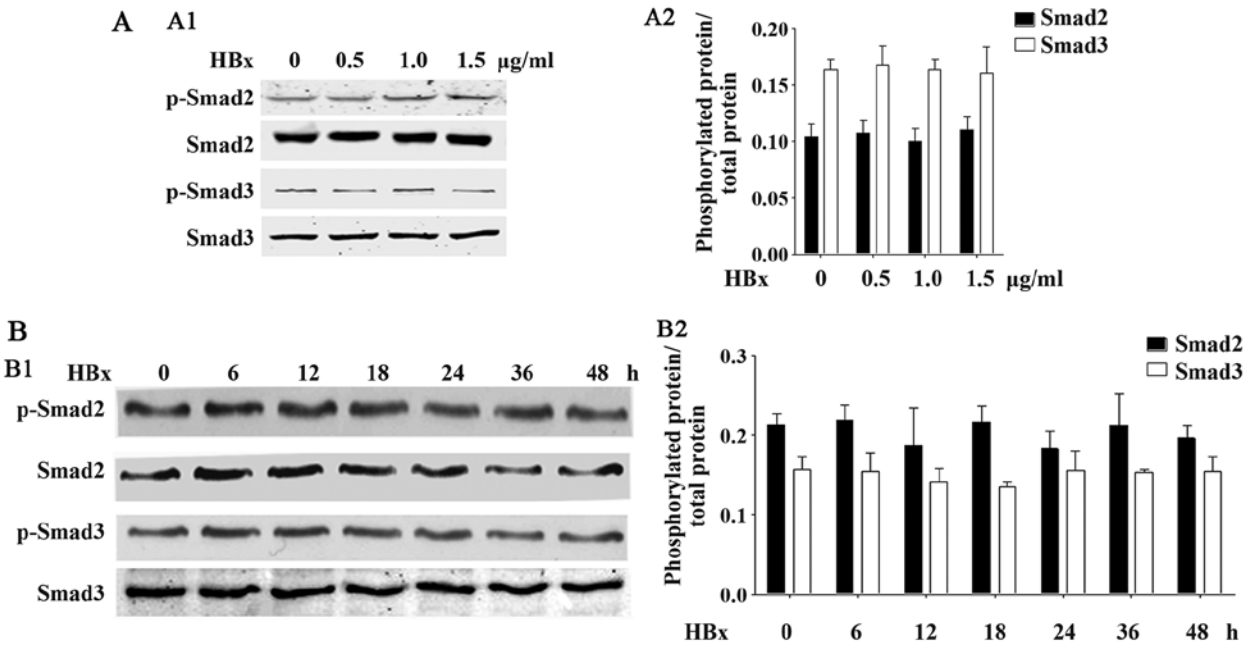

Figure 6. HBV X protein (HBx) does not affect Smad2 or Smad3 phoshorylation. (A) HK2 cells were transfected with the pEZ-M09-HBx plasmid at the concentrations of 0.5, 1.0 or 1.5 μg/ml. Cells were collected and western blot analyses were performed to detect the levels of phosphorylated Smad2, total Smad2, phosphorylated Smad3 and total Smad3. (B) HK2 cells were transfected with the pEZ-M09-HBx plasmid (1.0 μg/ml) for 6, 12, 18, 24, 36 or 48 h. Cells were collected and western blot analyses were performed to detect the levels of phosphorylated Smad2, total Smad2, phosphorylated Smad3 and total Smad3. Data are expressed as the means ± SD of 3 independent experiments.

pathogenesis has not yet been completely clarified. HBV-DNA, covalently closed circular DNA (cccDNA) and even complete viral particles (17,18) have been found in the kidneys of patients with HBV-GN, supporting the view that HBV can directly infect the kidneys and *in situ* reproduction to cause diseases. Over the years, research has focused on the existence and significance of HBV-related nucleic acid molecules in nephridial tissue, such as HBeAg, HBsAg and HBcAg (19-23). Recently, HBx was detected in renal tissues, mainly in tubular epithelial cells from patients with HBV-GN (5). In this study, we showed that HBx was highly expressed in HK-2 cells following transfection with HBx plasmid.

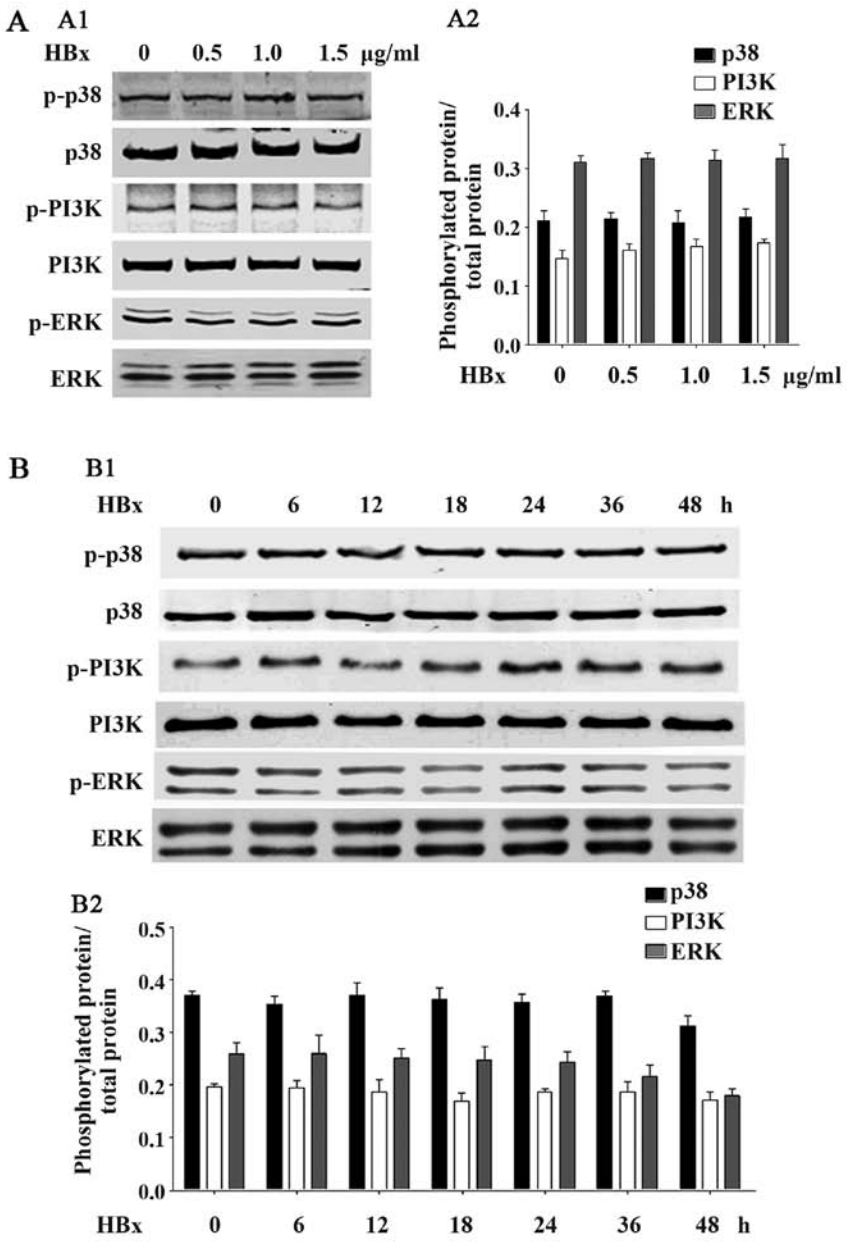

Figure 7. HBV X protein (HBx) protein does not affect p38, PI3K or ERK phoshorylation. (A) HK2 cells were transfected with the pEZ-M09-HBx plasmid at the dose of 0.5, 1.0 or 1.5 μg/ml, respectively. Cells were collected and western blot analyses were performed to detect the levels of phosphorylation of phosphorylated p38, total p38, phosphorylated PI3K, total PI3K, phosphorylated ERK and total ERK. (B) HK2 cells were transfected with the pEZ-M09-HBx plasmid (1.0 μg/ml) for 6, 12, 18, 24, 36 or 48 h. Cells were collected and western blot analyses were performed to detect the levels of phosphorylated p38, total p38, phosphorylated PI3K, total PI3K, phosphorylated ERK and total ERK. Data are expressed as the means ± SD of 3 independent experiments.

In liver cells, HBx is the most important determinant of viral pathogenesis, and it can modulate the transcriptional activation of AP-1 and NF-κB (8,24), activate the Ras/Raf/ERKs, PI3K-Akt and JAK/STAT signaling pathways (7), promote cellular proliferation (25), affect apoptosis (26) and enhance the invasive potential (27) of infected cells. However, the influence of HBx in renal cells remains far from being completely understood. Renal fibrosis, characterized by massive interstitial myofibroblast activation and excessive matrix protein accumulation, is the final common pathway of virtually all types of progressive chronic kidney disease (CKD), leading to end stage renal disease (ESRD) (28). HBV-GN plays a critical role in the progression of CKD. Thus, we were wished to determine whether HBx plays a role in renal fibrosis. The present study demonstrated that co-transfection with HBx plasmid markedly downregulated E-cadherin mRNA and protein expression, and upregulated α-SMA, collagen I, and fibronectin expression at the same time, suggesting that HBx promotes renal EMT. Although it has been demonstrated that the major cell component that produces extracellular matrix in unilateral ureteral obstruction is interstitial myofibroblasts (29), the contribution of tubular epithelial cell injury to ECM accumulation in fibrotic kidneys cannot be excluded (30).

Clippinger *et al* (8) successively researched the location of HBx protein in the mitochondrion and its influence on liver cell apoptosis. As a result, it was found that in liver cells, the function of HBx in apoptosis was mainly dependent on the NF-κB signaling pathway. Considering the imperative role

of NF-κB in mediating renal EMT (31,32), in this study, we examined the effects of HBx on NF-κB signaling. Indeed, our findings demonstrated that HBx markedly increased NF-κB phosphorylation in the HK-2 cells, in a concentration- and time-dependent manner (at the lower concentrations and earlier time points). These data suggest that HBx may promote renal EMT through the NF-κB pathway. As the TGF-β1 signaling has been recognized as a typical pathway in renal fibrosis and EMT, we also examined the effects of HBx on TGF-β1-related signaling. Of note, co-transfection with the HBx plasmid did not affect the phosphorylation of Smad2, Smad3, p38, ERK or PI3K, suggesting that HBx may selectively promote NF-κB phosphorylation.

Therefore, as there is still no well-used animal model of HBV-GN, we could not confirm these results in animal experiments. Further studies are warranted using other types of renal cells, as well as animal models.

In conclusion, this study demonstrated that the activation of the NF-κB signaling pathway and changes in the levels of EMT-associated genes are involved in the process of EMT in HK-2 cells induced by HBx. These findings demonstrate that HBx may promote renal EMT through the activation of NF-κB. Since an immortalized cell strain was used as the object of investigation in the present study, it is necessary to conduct further research in order to fully confirm our findings.

Acknowledgements

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