

# Hepatitis B virus X protein decreases nephrin expression and induces podocyte apoptosis via activating STAT3

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**Abstract.** The gene for hepatitis B virus X protein (*HBx*) comprises the smallest open reading frame in the HBV genome, and the protein product can activate various cell signaling pathways and regulate apoptosis, among other effects. However, in different cell types and under different external conditions, its mechanism of action differs. In the present study, the effect of *HBx* on the viability and apoptosis of mouse podocyte clone 5 (MPC5) cells was investigated. The cells were transfected with the *HBx* gene using pEX plasmid, and real-time quantitative PCR and western blot analysis were used to test the transfection efficiency and assess related protein expression. The highest expression of *HBx* occurred at 48 h after MPC5 cells were transfected with *HBx*. The expression of nephrin protein in the *HBx* transfection group was lower than that in blank and negative control groups. Following transfection of the *HBx* gene, podocyte viability was suppressed, while the rate of cell apoptosis was increased; moreover, the expression of signal transducer and activator of transcription 3 (STAT3) and phospho-STAT3 was increased compared with in the control groups. The present study suggests that STAT3 activation may be involved in the pathogenic mechanism of renal injuries caused by HBV

injection. Thus STAT3 is a potential molecular target in the treatment of HBV-GN.

## Introduction

Chronic hepatitis B is a worldwide epidemic disease, with China in particular representing a high hepatitis B virus (HBV) epidemic area. In 2006, an epidemiological survey of HBV in China revealed that the incidence of HBV surface antigen was 7.2% in individuals aged 1-59 years old among the general population, and that the incidence of HBV infection with glomerulonephritis was 6.8-20.0% (1). As such, HBV-associated glomerulonephritis (HBV-GN) is an important cause of chronic kidney disease (CKD) in China (2,3). While Combes *et al* (4) first reported on HBV-GN in 1971, the pathogenic mechanism is still yet to be fully elucidated. The deposits of immune complexes formed by HBV antigens and antibodies are the main causes of HBV-GN (5,6). However, some recent studies have demonstrated that HBV induced renal damage and may serve an important role in HBV-GN (7-9). The pathological presentation of HBV-GN is varied, and includes membranous nephropathy (MN), membranoproliferative glomerulonephritis, mesangial proliferative glomerulonephritis, minimal change disease and focal segmental glomerulosclerosis (FSGS), though most clinical manifestations are of those classified under nephrotic syndrome (10,11). As is well established, the glomerular endothelial cells, glomerular basal membrane and podocytes together constitute the glomerular filtration barrier, and podocyte damage is considered to be among the most critical factors resulting in proteinuria (12,13). This is due to podocytes, as a highly differentiated cell type with specific structure and biological function, being unrenovable following sustained damage (12,14). Previous research indicated that the number and density of podocytes decreased significantly in patients with HBV-MN, with this change accompanied by increases in urinary protein (15). Cell apoptosis, exfoliation and loss of proliferative capacity are considered the main mechanisms underlying podocyte reduction (15).

The hepatitis B virus X protein gene (*HBx*) comprises the smallest open reading frame in the HBV genome, and its product serves as the basic viral protein in the virus infection cycle (16). With advances in research, the X protein has been verified as a multifunctional protein, which can activate

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**Abbreviations:** HBV, hepatitis B virus; *HBx*, hepatitis B virus X protein; HBV-GN, hepatitis B virus-associated glomerulonephritis; CKD, chronic kidney disease; MPC5, mouse podocyte clone 5; MN, membranous nephropathy; FSGS, focal segmental glomerulosclerosis; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated; DMEM, Dulbecco's modified Eagle's medium; OD, optical density; TBST, Tris-buffered saline with Tween-20

**Key words:** hepatitis B virus X protein, podocyte, nephrin, apoptosis, signal transducer and activator of transcription 3

various cell signaling pathways and regulate apoptosis, among other effects (17). However, in different types of cell and under different external conditions, the regulation of these pathways by HBx is governed by differing mechanisms (18). Overexpression of HBx in extracorporeal podocytes limits the proliferative capacity of the cells through cell cycle regulation, and this mechanism may occur due to upregulation of cyclin B1 and p21 (19). However, the mechanisms underlying podocyte apoptosis induced by HBx are unclear. Therefore, the current study examined the effect of HBx on the viability and apoptosis of mouse podocyte clone 5 (MPC5) cells, and nephrin protein expression was detected in an HBx transfection group to investigate the possible mechanism involved.

## Materials and methods

**Cell culture.** MPC5 cells were obtained from CHI Scientific, Inc. (Maynard, MA, USA). After frozen MPC5 cells were recovered, they were cultured in low sugar Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% heat-inactivated fetal calf serum (HyClone; GE Healthcare, Little Chalfont, UK). The MPC5 cells were cultured and expanded in this medium also containing 10 U/ml interferon- $\gamma$  (PeproTech, Inc., Rocky Hill, NJ, USA) at 33°C and 5% CO<sub>2</sub>.

**Podocyte transfection and grouping.** MPC5 cells were inoculated on 6-well plates at  $5 \times 10^4$  cells/cm density, with each group assigned three wells. When the cells reached 70% confluence, they were divided into different groups according to transfection treatment. The cells were transfected with pEX-HBx or pEX-neo plasmids (both Shanghai GenePharma, Co., Ltd., Shanghai, China) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were divided into three groups: A HBx transfection group (pEX-HBx group; treated with 2  $\mu$ l Lipofectamine 2000 + 20 pmol HBx-plasmid), a negative control group (pEX-neo group; treated with 2  $\mu$ l Lipofectamine 2000 + 20 pmol empty plasmid) and a blank control group (MPC5 group; treated with 2  $\mu$ l Lipofectamine 2000 alone).

**RNA isolation and real-time quantitative PCR (qPCR).** The transcript levels of the HBx gene were examined using qPCR. Following transfection of MPC5 cells for 12, 24, 48 and 72 h, the cells were digested with pancreatin (0.25%), washed with phosphate-buffered saline and collected. Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed to cDNA using a high-capacity cDNA archive kit (Takara Biotechnology, Co., Ltd., Dalian, China) according to the manufacturer's instructions. qPCR was performed using an Applied Biosystems 7300 real-time PCR system (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 94°C for 4 min, followed by 40 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec as well as 82°C for 30 sec to collect fluorescence data. Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), the sequences of which are listed in Table I. Expression of the *abelson* murine leukemia viral oncogene homolog gene (ABL) was used as a control. The

relative expression of HBx was calculated by the comparative 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (20). In order to reduce the error, each group was assayed three times.

**MTT assay.** The MTT method was used to detect the viability of podocytes, using an MTT kit purchased from American Biomol (Farmingdale, NY, USA). MPC5 cells in the exponential phase of growth were trypsinized and seeded into 6-well plates at a density of  $4 \times 10^5$  cells per well, with three wells per group. After 48 h of incubation at 33°C, MTT reagent (5 mg/ml, 20  $\mu$ l) was added and the cells were incubated for another 4 h. Then, the supernatant was discarded, 150  $\mu$ l dimethyl sulfoxide (DMSO) was added to each well, and the wells were agitated for 15 min. The optical density (OD) of each well at 570 nm (OD<sub>570</sub>) was read with an ELISA plate reader, and the cell viability rate was calculated according to the following formula: Viability rate = OD<sub>570</sub><sub>treatment group</sub>/OD<sub>570</sub><sub>control group</sub>  $\times 100\%$ . The experiment was repeated three times.

**Flow cytometry.** After transfection for 48 h, MPC5 cells in the exponential phase of growth were trypsinized, centrifuged at 112  $\times$  g for 10 min at 4°C, and the cell pellet suspended in cell culture medium. Then,  $1 \times 10^6$  cells were resuspended in 200  $\mu$ l of 1X Nexin buffer (Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences, San Jose, CA, USA). A total of 50  $\mu$ l of the suspension was transferred into a tube containing 5  $\mu$ l propidium iodide and 5  $\mu$ l Annexin V stain (BD Pharmingen; BD Biosciences), and incubated for 30 min at room temperature in the dark. Following addition of 250  $\mu$ l 1X Nexin buffer into each tube, apoptotic cells were detected by flow cytometry using a fluorescence-activated cell sorter (FACSCalibur cytometer) and CELL Quest™ software (version 3.3) (both from BD Biosciences).

**Western blot analysis.** The total protein of cells in each group was extracted with radioimmunoprecipitation assay buffer and measured using bicinchoninic assay reagents (Beyotime Institute of Biotechnology, Haimen, China). Then, 60  $\mu$ g protein per lane was subjected to polyacrylamide gel electrophoresis in 6-12% gels, and the resulting bands were transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 2 h in Tris-buffered saline with Tween-20 (TBST; 0.2% Tween-20) containing 5% skimmed milk, then incubated at 4°C overnight with primary antibodies (1:1,000) against HBx, nephrin, signal transducer and activator of transcription 3 (STAT3), phosphorylated (p)-STAT3 and GAPDH (Abcam, Cambridge, UK; cat nos. ab157480, ab58968, ab119352, ab76315 and ab8245, respectively). Following washing with TBST, the appropriate horseradish-peroxidase-labeled secondary antibody (1:5,000; cat no. A0208; Beyotime Institute of Biotechnology) was added and incubated for 2 h at room temperature, after which the membranes were washed three to five times with TBST. Finally, an electrochemiluminescence kit (Sigma-Aldrich; Merck KGaA) and gel imager were used to expose and visualize the proteins, and protein grayscale values were measured with Quantity One software (version 4.52; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the actual grayscale value of the target protein = the target protein average grayscale value/the GAPDH average grayscale value.

Table I. Primer sequences and product size.

Primer	Oligonucleotide sequence, 5'-3'	Product size, bp
HBx sense	TGCGGACGACCCTTCTCGGG	195
HBx antisense	GGGCAACATTTCGGTGGGCGT	
ABL sense	TCCTCCAGCTGTTATCTGGAAGA	118
ABL antisense	TCCAACGAGCGGCTTCAC	

HBx, hepatitis B virus X protein; ABL, abelson murine leukemia viral oncogene homolog.

**Statistical analysis.** All experiments were repeated at least three times. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to generate charts and complete statistical analyses. The data are presented as the mean  $\pm$  standard deviation, and t-tests were used to compare the data between two groups. The comparisons between multiple groups were made by one-way analysis of variance followed by post-hoc Tukey's tests.  $P < 0.05$  was considered to indicate statistical significance.

## Results

**HBx is over-expression in the pEX-HBx group.** HBx mRNA in the pEX-HBx group was expressed at the highest level at 48 h after transfection. HBx mRNA expression was significantly higher compared with the MPC5 and pEX-neo groups ( $P < 0.01$ ; Table II). HBx expression was determined to be significantly higher in the pEX-HBx group compared with that in the pEX-neo and MPC5 groups, while there was no difference in expression between the pEX-neo and MPC5 groups ( $P > 0.05$ ; Table II). Using western blotting to examine the expression of HBx protein and verify the transfection efficiency, corresponding results were obtained as those for qPCR (Fig. 1A and B). Based on expression increase, the 48-h post-transfection podocytes were used for the follow-up experiments.

**Nephrin expression is downregulated in HBx-transfected podocytes.** As expected, the expression of nephrin protein in the pEX-HBx podocytes was lower than that in the pEX-neo and MPC5 groups ( $P < 0.01$  and  $P < 0.01$ , respectively). Additionally, the western blot results demonstrated that there was no difference in nephrin protein expression between the pEX-neo and MPC5 groups ( $P > 0.05$ ; Fig. 2).

**Overexpression of the HBx gene suppresses podocyte viability.** The viable cell rate of the pEX-HBx podocytes was  $52.2 \pm 2.4\%$ , which was the lowest rate observed compared with that of the MPC5 ( $67.2 \pm 3.0\%$ ) and pEX-neo ( $63.4 \pm 3.4\%$ ) groups ( $P < 0.01$  and  $P < 0.01$ , respectively). No significant difference was identified between the rates of viable cells in the pEX-neo and MPC5 groups ( $P > 0.05$ ; Fig. 3).

**Overexpression of HBx increases podocyte apoptosis.** The rate of cell apoptosis in the pEX-HBx group was significantly higher than that in the MPC5 and pEX-neo groups ( $P < 0.01$  and  $P < 0.01$ , respectively); the ratio of apoptotic cells in each

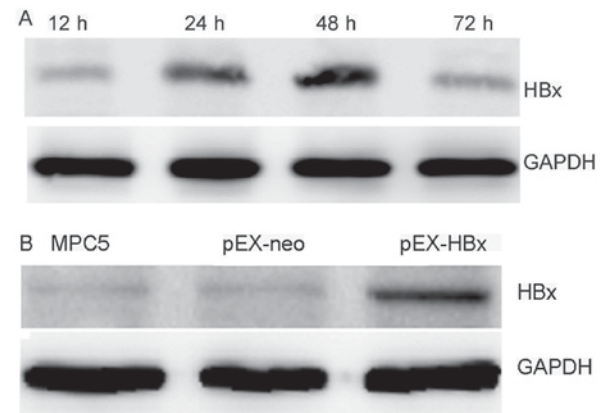


Figure 1. Western blot analysis of HBx protein expression in MPC5 cells following transfection with pEX-HBx. (A) HBx protein expression after transfection for 12-72 h; (B) HBx expression in different groups after transfection for 48 h. HBx, hepatitis B virus X protein.

of the MPC5, pEX-neo and pEX-HBx groups was  $3.46 \pm 0.17$ ,  $4.86 \pm 0.55$  and  $10.82 \pm 0.45$ , respectively. There was no difference between the MPC5 and pEX-neo groups ( $P > 0.05$ ; Fig. 4).

**HBx stimulates STAT3 production in MPC5 cells.** The expression of STAT3 and p-STAT3 was highest in the pEX-HBx group; no difference was observed in the expression levels between the MPC5 and pEX-neo groups ( $P > 0.05$ ; Fig. 5). p-STAT3/STAT3 ratios in the MPC5, pEX-neo and pEX-HBx groups were  $1.160 \pm 0.017$ ,  $0.877 \pm 0.014$  and  $1.411 \pm 0.008$ , respectively (Table III). The proportion of STAT3 phosphorylation in the pEX-HBx group was significantly increased compared with that in the other two groups ( $P < 0.01$  and  $P < 0.01$ , respectively; Fig. 5; Table III).

## Discussion

HBV-GN is generally considered to be caused by immune complex deposition, as well as HBV replication and direct virus infection, accompanied by the renal and immune dysfunction caused by the infection, which is also considered a main pathogenic mechanism underlying HBV-GN (21). To date, little information has been established regarding the potential effects of HBx protein in terms of the damage and dysfunction observed in renal podocytes during chronic HBV infection. HBx is considered the most important determinant in viral pathogenesis (22,23); it is a necessary transcription factor for HBV replication, having an trans-activation effect, which

Table II. Efficiency of *HBx* gene transfection in MPC5 cells.

Group	<i>HBx</i> expression at different time points, h			
	12	24	48	72
MPC5	0.48±0.14	0.51±0.12	0.54±0.12	0.44±0.10
pEX-neo	0.53±0.13	0.65±0.11	0.64±0.20	0.58±0.21
pEX-HBx	0.64±0.11 <sup>a,c</sup>	18.3±0.25 <sup>b,d</sup>	593.42±0.56 <sup>b,d</sup>	88.59±0.33 <sup>b,d</sup>

Values are presented as mean ± standard deviation (n=3). <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs. MPC5 group; <sup>c</sup>P<0.05, <sup>d</sup>P<0.01 vs. pEX-neo group. HBx, hepatitis B virus X protein.

Table III. Effect of HBx on the protein expression of STAT3 and p-STAT3.

Group	STAT3	p-STAT3	p-STAT3/STAT3
MPC5	0.312±0.012	0.362±0.015	1.160±0.017
pEX-neo	0.342±0.018	0.301±0.011	0.877±0.014
pEX-HBx	0.680±0.036 <sup>a,b</sup>	0.960±0.015 <sup>a,b</sup>	1.411±0.008 <sup>a,b</sup>

Values are presented as mean ± standard deviation (n=3). Actual grayscale value of the target protein=the target protein average grayscale value/the GAPDH average grayscale value. <sup>a</sup>P<0.01 vs. MPC5 group; <sup>b</sup>P<0.01 vs. pEX-neo group. HBx, hepatitis B virus X protein; STAT3, signal transducer and activator of transcription 3; p-, phosphorylation.

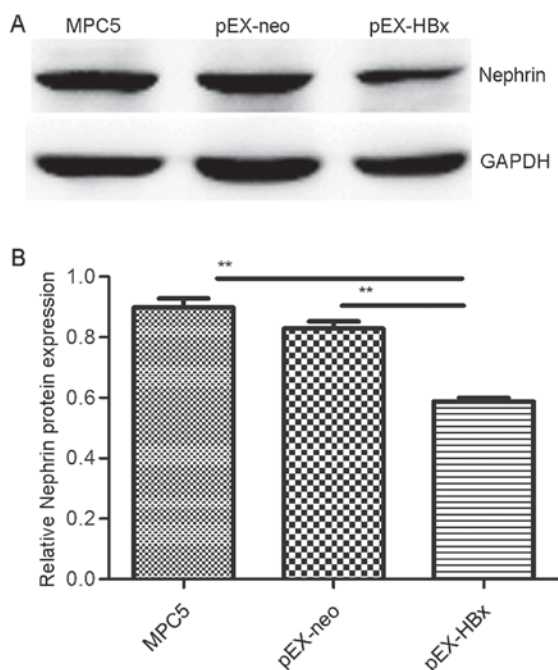


Figure 2. Effects of HBx on the expression of nephrin protein. Values are presented as the mean ± standard deviation (n=3). (A) Western blot gel; (B) graphical representation of the results. <sup>\*\*</sup>P<0.01. HBx, hepatitis B virus X protein.

can promote viral replication and induce apoptosis directly, and subsequently induce the occurrence of an inflammatory reaction and serve an important role in cell transformation and proliferation (24,25). Due to the widely trans-activation properties of the *HBx* gene and its effect on glomerular foot

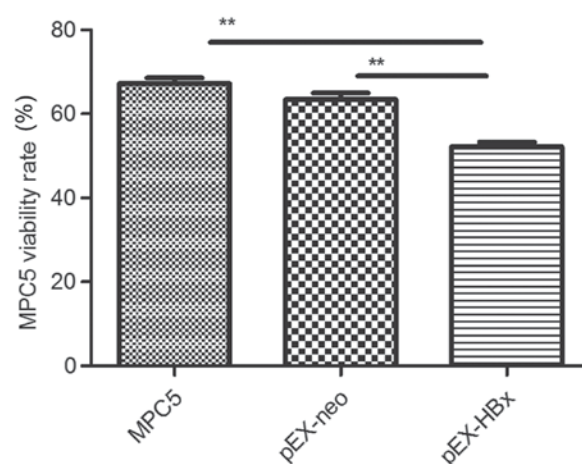


Figure 3. Podocyte viability rate comparison between the groups. Values are presented as the mean ± standard deviation (n=3). <sup>\*\*</sup>P<0.01. HBx, hepatitis B virus X protein.

cells, mesangial cells and renal tubule epithelial cells (26-29), it is considered to have an important role in the pathogenesis of HBV-GN (28,29). A previous study by Zhang *et al* (15) demonstrated that podocyte number was significantly decreased in the glomeruli of children with HBV-GN, while HBx protein was visualized within the glomerulus in 71% children with HBV-GN, where the expression of HBx protein was localized mainly in the cytoplasm of podocytes, with lower expression in the nuclei of the podocytes (19). Therefore, in the present study it was examined whether HBx could affect the apoptosis and proliferation of renal podocytes and change the expression of nephrin.



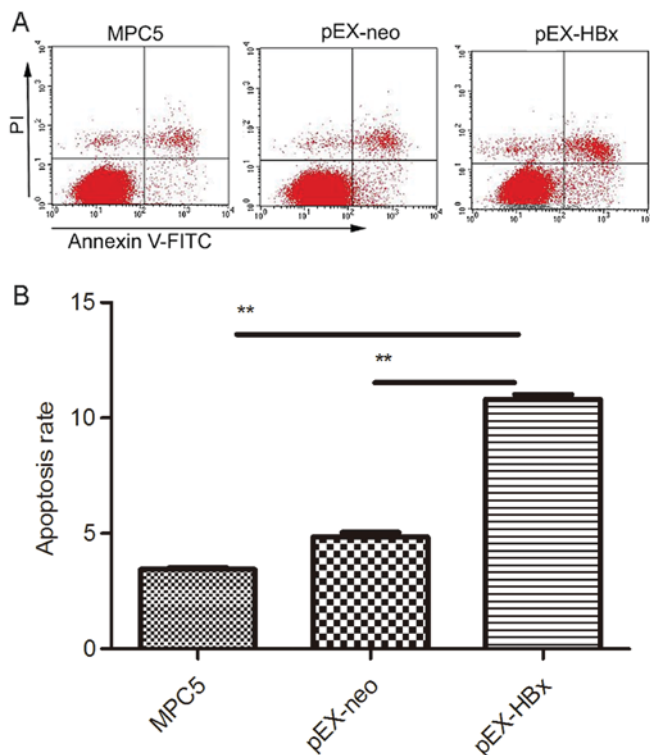


Figure 4. Apoptotic rates of podocytes with or without HBx transfection. Values are presented as the mean  $\pm$  standard deviation (n=3). (A) Flow cytometry results; (B) graphical representation of the results. \*\*P<0.01. HBx, hepatitis B virus X protein. HBx, hepatitis B virus X protein; FITC, fluorescein isothiocyanate; PI, propidium iodide.

In the current study, the highest level of *HBx* mRNA expression in podocytes occurred at 48 h post-*HBx* transfection. Thus, these cells at 48 h were selected to detect the viability of podocytes, and the data indicated that the viability of the *HBx* group was significantly lower than that of the blank control and negative control groups. This indicated that overexpression of *HBx* may suppress podocyte viability, which is consistent with the report of Zhang *et al* (19). Nephron is a main marker of foot cell damage; previous research has confirmed it serves an important role in recovering membrane integrity and in cytoskeletal remodelling (30). Furthermore, nephron downregulation is considered a main mechanism involved in proteinuria (31). We therefore studied the relationship between nephron and *HBx* protein, and observed that the expression of nephron protein in the *HBx* transfection group was decreased to a greater extent than that in the negative and blank control groups, suggesting that the *HBx* gene may induce proteinuria through downregulation of nephron protein.

Proteinuria is a common symptom of glomerular filtration membrane damage, and podocytes are highly differentiated epithelial cells that serve a key role in preventing the urinary leakage of plasma proteins; thus, podocyte injury or loss leads to proteinuria (12). To date, a number of studies have demonstrated that podocyte injury, loss and dysfunction serve an important role in diseases including FSGS and MN, among others (32-34). Therefore, maintenance of the integrity of podocyte structure and function, and protection of foot cells from injury have become potential therapeutic methods for the treatment of proteinuria. Apoptosis is a major method

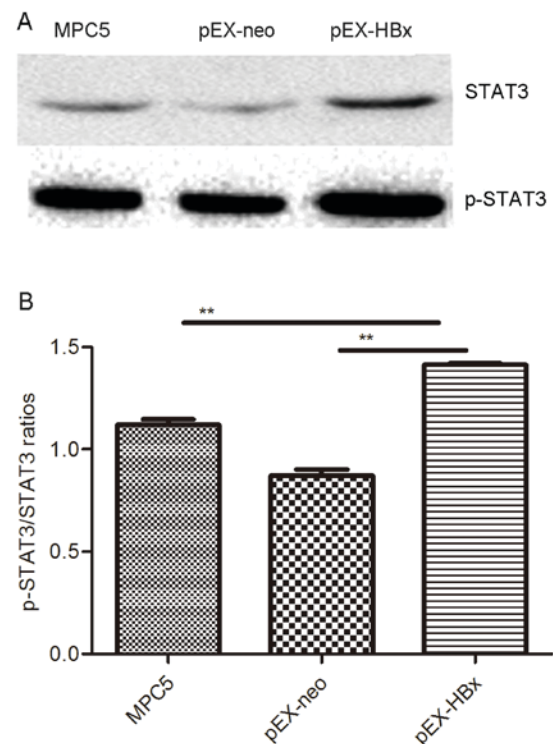


Figure 5. Effects of HBx on the protein levels of STAT3 and p-STAT3. Values are presented as the mean  $\pm$  standard deviation (n=3). (A) Western blot gel; (B) graphical representation of the results. \*\*P<0.01. HBx, hepatitis B virus X protein; STAT3, signal transducer and activator of transcription 3; p-, phosphorylation.

involved in podocyte decline (35,36), and the present study confirmed that *HBx* could increase the apoptosis of podocytes. Recently, studies have investigated the mechanisms underlying *HBx*-induced apoptosis of renal tubular cells (29,37), and He *et al* (28) suggested that *HBx* could reduce podocyte adhesion via downregulation of  $\alpha 3 \beta 1$  integrin.

STATs are transcription factors located in the cytoplasm, where they can become activated by extracellular stimuli. Activated STATs, through regulation of gene expression, are able to regulate a series of biological processes, including cell proliferation, survival, apoptosis and differentiation, among others, and thus serve an important regulatory role in physiological and pathological reactions in cells (38-40). STAT3 is an important member of the STAT family; it is widely expressed in different tissue and cell types, and is responsible for transferring extracellular signals to the nucleus and for inducing the transcriptional expression of target genes, which involves tyrosine phosphorylation of STAT3, for instance at tyrosine 705 near the SH2 domain, to establish the activated form of STAT3 (p-STAT3) (41,42). The proportion of STAT3 phosphorylation in the pEX-*HBx* group was higher than that in the control groups, indicating *HBx* overexpression may activate the STAT3 protein. Although previous literature suggests that activation of STAT3 is associated with inhibition of apoptosis, particularly in cancers (43-45), in the present study, the activation of STAT3 was concomitant with podocyte apoptosis; a finding consistent with a study by He *et al* (29) in renal tubular epithelial cells. Therefore, it may be speculated that the apoptosis of podocytes in HBV-GN is associated with STAT3 activation, although the specific mechanism is

unclear and requires further investigation; in future research, our group will conduct immunoprecipitation to screen protein interactions and provide further information about HBx in inducing HBV-GN via STAT signaling pathways.

In conclusion, these findings supported that HBx was involved in the pathogenic mechanism that HBV directly damages nephridial tissue. Additionally, find STAT3 related signal pathway and use specific inhibitors may be useful as new therapeutics for the treatment of HBV-GN.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XYL and XXC designed the study, analyzed the data and wrote the manuscript. YHSun and MDG conducted the experiments. XXH and YHSuo assisted with the technical performance of experiments and contributed to the writing of the manuscript. All authors read and approved the final manuscript. XYL and XXC contributed equally to the present study as co-first authors. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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