

## Original Article

# Hepatitis B virus X protein maintains hepatic stellate cell activation by regulating peroxisome proliferator-activated receptor $\gamma$

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**Abstract:** Background: Chronic hepatitis B virus (HBV) infection is a major cause of hepatic fibrosis, and the activation of hepatic stellate cells (HSCs) is the main mechanism of fibrosis. However, the mechanism of hepatic fibrosis induced by HBV is not well elucidated. Hepatitis B virus X protein (HBx), one of the HBV-related proteins, induces fibrosis in a paracrine way. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) inhibits the activation of HSCs and even switches the cell phenotype from activated to quiescent. The aim of this study was to determine the interaction of HBx and PPAR $\gamma$  in stellate cell activation. Methods: A stable cell line, LX-2-X, which expressed HBx, was established by infecting LX-2 cells with lentivirus. The Cell Counting Kit-8 (CCK-8) assay was used to detect cell proliferation. The expression of PPAR $\gamma$ , transforming growth factor- $\beta$ 1 (TGF $\beta$ 1),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I was measured by quantitative real-time PCR (qRT-PCR), Western blot or ELISA. For the interaction of HBx and PPAR $\gamma$ , co-immunoprecipitation and luciferase reporter assays were performed. Results: LX-2-X cells showed increased proliferation compared to control cells, and the PPAR $\gamma$  ligand troglitazone (0, 5, 10  $\mu$ mol/L) inhibited LX-2-X cell proliferation in a dose-dependent manner. The expression of TGF- $\beta$ 1,  $\alpha$ -SMA, and collagen I increased, while PPAR $\gamma$  decreased in LX-2-X cells. HBx bound to PPAR $\gamma$  and suppressed the transcriptional activity of PPAR $\gamma$ . Conclusions: HBx can maintain stellate cell activation by down-regulating PPAR $\gamma$  *in vitro*.

**Keywords:** Hepatitis B virus X protein, peroxisome proliferator-activated receptor  $\gamma$ , hepatic stellate cell, fibrosis

## Introduction

Fibrosis, characterized by an excessive deposition of extracellular matrix (ECM), is a wound-healing response to a variety of chronic stimuli [1-3]. Although several other cells are fibrogenic, hepatic stellate cells still have dominated the study of fibrosis [1, 2]. Following a variety of fibrogenic stimuli, hepatic stellate cells (HSCs) are activated, changing from a quiescent vitamin A-storing phenotype to a myofibroblast-like phenotype, and are associated with the expression of smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA) [3]. Two specific aspects of stellate cell activation are critical in the fibrogenic response: increased cell proliferation and increased synthesis of ECM constituents, of which collagen I predominates [3, 4]. Platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) are the most potent stellate cell mitogen [5, 6] and profibrogenic cytokine [7-9], respectively.

HBV infection is a major public health problem worldwide and is strongly associated with the development of hepatitis, hepatic fibrosis and hepatocellular carcinoma (HCC) [10, 11]. HBV is a hepatotropic, partially double-stranded DNA virus. The X gene is the smallest fragment among the four overlapping open reading frames (ORFs) of the HBV genome, coding for the X protein [12, 13]. HBx regulates a variety of viral replication and cellular functions through different signaling pathways, both in the cytoplasm and nucleus [14]. Previous studies have suggested that HBx is associated with the development of hepatic fibrosis [8, 15, 16].

Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a transcription factor of the nuclear receptor superfamily. PPAR $\gamma$  plays a critical role in regulating energy homeostasis, especially lipid and glucose metabolism [17]. PPAR $\gamma$  needs to combine with retinoid X receptor (RXR),

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forming a PPAR/RXR heterodimer, to function. Upon ligand binding, the heterodimer binds to specific DNA regions, peroxisome proliferator response elements (PPREs), to regulate gene transcription [18, 19]. A variety of studies about PPAR $\gamma$  were carried out to uncover the mechanism of hepatic fibrosis because of the anti-fibrotic effect of this protein [20-24]. Ligands of PPAR $\gamma$  inhibit the proliferation and activation of hepatic stellate cells and may induce a phenotypic switch from activated to quiescent [25]. Troglitazone, one antidiabetic drug of the thiazolidinedione group, could bind and activate PPAR $\gamma$ .

HBx inhibits the effect of PPAR $\gamma$  through protein-protein interaction in HCC cells [26], and it can activate hepatic stellate cells through paracrine action [27-29]. Although HBV infects mainly hepatic parenchymal cells, it can also infect stellate cells directly and affect their proliferation and activation [30]. In this study, we examined how HBx affected HSCs via modulating PPAR $\gamma$ , and we also investigated whether ligands of PPAR $\gamma$  could counter the HBx-induced fibrogenesis. Our data may provide novel insight about and suggest potential therapies for hepatic fibrosis.

### Material and methods

#### *Plasmids and cell line*

To express HBx with a Flag tag, the HBx fragment was subcloned into the Nhe I and Sac I site of the pCMV-N-Flag plasmid (Beyotime technology, Nantong, China). The plasmid TK-PPRE $\times$ 3-LUC was purchased from Zhongyuan LTD (Beijing, China). It contains PPRE and luciferase gene, and could express luciferase when binded with PPAR $\gamma$ . All the plasmids used for lentivirus construction were purchased from GenePharma (Shanghai, China). We constructed an LV5-X lentivirus containing target gene (HBVgp3 X protein). A stable LX-2-X cell line was established by infecting LX-2 cells with lentivirus LV5-X, and the control cell line was named LX-2-C. The LX-2 cell line was purchased from the Shanghai Cell Bank (Shanghai, China). All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/L streptomycin, 100 U/ml penicillin at 37°C and 5% CO $_2$ .

#### *Cell proliferation assay*

The cell proliferation was determined by CCK-8 assay. Briefly, 5 $\times$ 10 $^3$  cells were seeded into 96-

well plates, treated with or without troglitazone (Sigma, St. Louis, MO, USA) for various times. After treatment, 10  $\mu$ l CCK-8 solution (Beyotime Technology, Nantong, China) was added to each well, and the cells were incubated for 2 h at 37°C. The optical density (OD) of each well at 450 nm was measured by an ELISA reader.

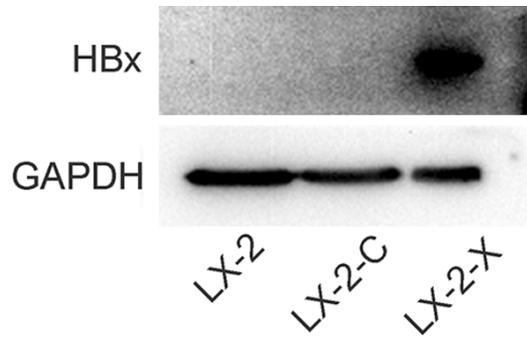
#### *Real-time quantitative RT-PCR*

Total RNA was extracted from cells by Trizol Reagent (Takara, Dalian, China). The reverse transcription for complementary DNA was performed by the PrimeScript RT kit (Takara, Dalian, China). Real-time quantitative PCR was performed by the FastStart Universal SYBR Green Master (ROX) (Roche, USA) on an ABI 7500 (Applied Biosystems, Life Technologies Corporation, CA, USA). The relative mRNA expression was examined as the inverse log of the  $\Delta\Delta$ CT, which was normalized to the reference gene GAPDH. The reaction conditions of quantitative RT-PCR were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60 s at 60°C. At the end of the program a melting curve analysis was made. All primers for quantitative RT-PCR were synthesized by Invitrogen (Shanghai, China). The sequences of primers were as follows: GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' (sense); 5'-TCCACCACCCTGTTGCTGTA-3' (antisense); HBx: 5'-CAGCAATGTCAACGACCGAC-3' (sense) and 5'-GTTGCATGGTGCTGGTGAAC-3' (antisense); PPAR $\gamma$ : 5'-GGGATCAGCTCCGTGGATCT-3' (sense) and 5'-TGCACTTTGGTACTCTTGAAGTT-3' (antisense); TGF $\beta$ 1: 5'-CTAATGGTGAAACCCACAACG-3' (sense) and 5'-TATCGCCAGGAATTGTTGCTG-3' (antisense);  $\alpha$ -SMA: 5'-AAAAGACAGCTACGTGGGTGA-3' (sense) and 5'-GCCATGTTCTATCGGGTACTTC-3' (antisense); collagen I: 5'-GTGCGATGACGTGATCTGTGA-3' (sense) and 5'-CGGTGTTTCTTGGTCGGT-3' (antisense).

#### *Co-immunoprecipitation and Western blot*

To detect the expression of fibrosis-related molecules, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer. Total protein concentration was measured by NanoDrop 2000 protein assay (Thermo Scientific, USA). After boiling for 5 minutes, equal amounts of protein (30  $\mu$ g) were loaded, and lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes (Millipore, Bedford, USA), using a trans-blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in Tris-buffered sa-

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**Figure 1.** LX-2-X cells express HBx protein stably. Analysis of HBx expression by Western blot.

line with Tween-20 (TBST) containing 5% non-fat milk at room temperature for 2 hours and subsequently incubated with primary antibodies against PPAR $\gamma$  (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HBx, TGF $\beta$ 1 (Abcam, Cambridge, MA, USA), and  $\alpha$ -SMA (Epitomics, Burlingame, CA, USA) at 4°C overnight. After rinsing three times for 30 minutes in TBST, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (Beyotime Technology, Nantong, China) at room temperature for 2 hours. GAPDH (Kang-Chen Bio-Tech, Shanghai, China) was used as an internal control. Electrochemiluminescence was performed with a Chemilmager 5500 imaging system (Alpha Innotech Co., San Leandro, CA, USA).

For the co-immunoprecipitation assay of HBx and PPAR $\gamma$ , LX-2 cells were transiently transfected with pCMV-FLAG-HBx plasmid using Lipofectamine 2000 (Invitrogen, Shanghai, China). Cells were lysed with RIPA buffer. Cell lysates were then incubated with anti-Flag antibody overnight at 4°C and incubated with G-coupled Sepharose beads at 4°C under rotary agitation for 4 hours. After centrifugation, the precipitates were dissolved in 1 $\times$  loading buffer and boiled for 5 min. The samples were separated by SDS-PAGE, followed by immunoblot analysis.

### Luciferase reporter assay

PPAR $\gamma$  needs to form a heterodimer, and then binds PPRE to start the gene transcription. To determine whether HBx protein expressed by lentivirus decreased the transcriptional activity of PPAR $\gamma$ , cells were transiently transfected with luciferase reporter plasmid TK-PPRE $\times$ 3-LUC which contains PPRE and luciferase gene using Lipofectamine 2000 (Invitrogen, Shang-

hai, China). The pRL-TK Renilla luciferase reporter plasmid (Promega, WI, US) was an internal control. After 6 hours, the transfection medium was changed to DMEM with 10% FBS, and the cells were incubated with or without troglitazone (10  $\mu$ M/L) for 72 hours. The cells were analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega).

### ELISA

For the analysis of collagen I, the supernatant was collected from the culture medium. The amount of collagen I was determined by ELISA (Takara, Dalian, China) according to the manufacturer's instructions.

### Statistical analysis

Data were analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Each experiment was conducted at least three times. The results were expressed as the mean  $\pm$  SD. For three or more groups, statistical significance was determined with one-way ANOVA, and for two groups with Student's t-test.  $p < 0.05$  was considered statistically significant.

## Results

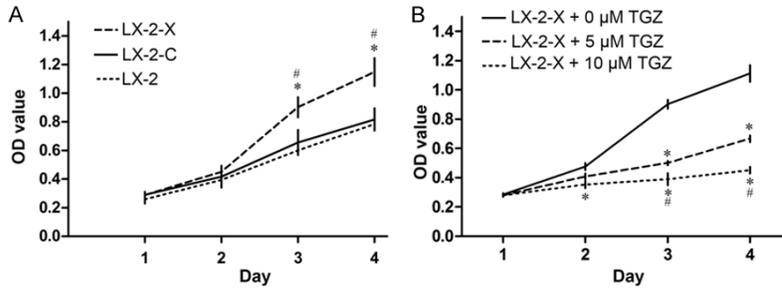
### *HBx promotes the proliferation of HSCs, and troglitazone counters this effect*

The LX-2-X cells expressed HBx protein stably (**Figure 1**). To determine the effect of HBx on the proliferation of LX-2 cells, a CCK-8 assay was performed. LX-2-X cells showed increased proliferation from 72 h compared to LX-2-C cells and LX-2 cells (**Figure 2A**). However, treated with troglitazone of different folds (0, 5, 10  $\mu$ M/L), the proliferation of LX-2-X cells was inhibited in a dose-dependent manner (**Figure 2B**).

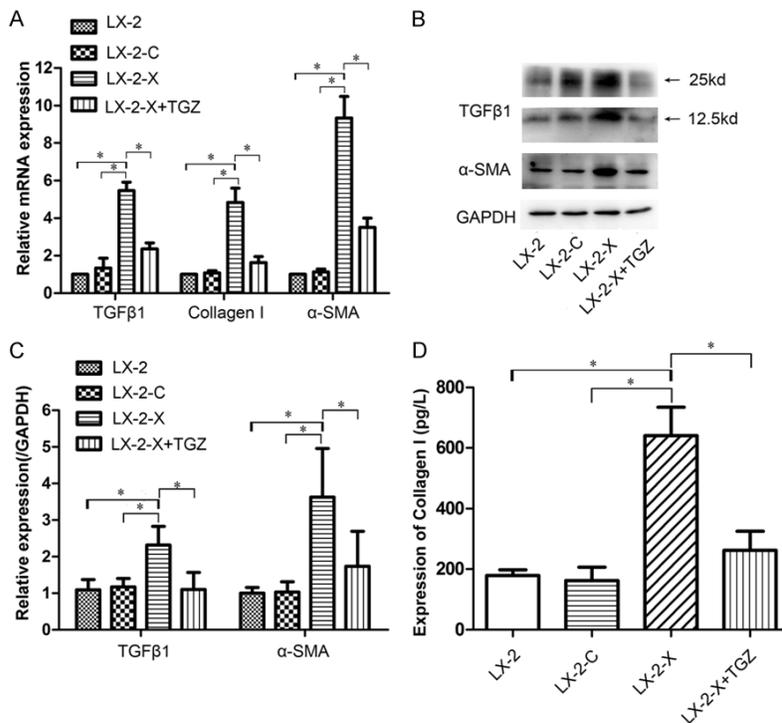
### *HBx-expressing HSCs show increased expression of TGF- $\beta$ 1, $\alpha$ -SMA, and collagen I*

Liver fibrosis is characterized by the deposition of collagen I. TGF- $\beta$ 1 is the most potent fibrogenic cytokine, and  $\alpha$ -SMA is specifically expressed by activated HSCs [1-4, 7-9]. To determine whether HBx could maintain the stellate cell activation, we detected the expression of these fibrotic markers. RT-PCR and western blotting showed that the LX-2-X cells expressed more TGF- $\beta$ 1 and  $\alpha$ -SMA compared to the LX-

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**Figure 2.** HBx promotes the proliferation of LX-2-X HSCs, and troglitazone counters this effect. A: LX-2, LX-2-C and LX-2-X cells were cultured in 96-well plates for the indicated time, and cell proliferation was determined by CCK-8 assay. \*:  $p < 0.05$  (vs. LX-2-C group); #:  $p < 0.05$  (vs. LX-2 group). B: LX-2-X cells were treated with troglitazone (0, 5, 10  $\mu$ M/L) for the indicated time. \*:  $p < 0.05$  (vs. control group); #:  $p < 0.05$  (vs. 5  $\mu$ M/L group).



**Figure 3.** HBx increases the mRNA expression of fibrotic markers. A: The mRNA expression of TGF- $\beta$ 1,  $\alpha$ -SMA and collagen I in LX-2, LX-2-C and LX-2-X cells was detected by qRT-PCR. B: TGF- $\beta$ 1 and  $\alpha$ -SMA were measured by Western blot. C: Grayscale analysis of the Western blot data. D: The concentration of collagen I in the supernatant was detected by ELISA assay. There were no differences between group LX-2 and LX-2-C. \*:  $p < 0.05$ .

2-C cells and LX-2 cells (Figure 3A-C). Moreover, we found increased secretion of collagen I in the supernatant of the LX-2-X cells using ELISA (Figure 3D), consistent with the mRNA expression in the LX-2-X cells (Figure 3A). However, LX-2-X cells treated with troglitazone (10  $\mu$ M/L) showed less expression of TGF- $\beta$ 1,  $\alpha$ -SMA, and collagen I.

*HBx binds to PPAR $\gamma$  in LX-2 cells and suppresses the transcriptional activity of PPAR $\gamma$  in HSCs*

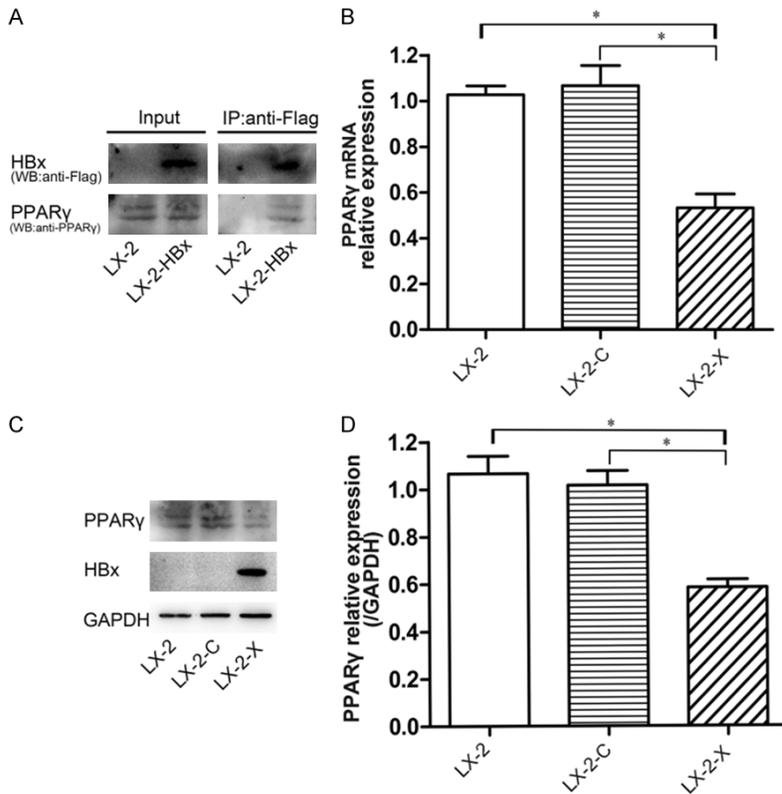
HBx can bind to PPAR $\gamma$ , thereby affecting the nuclear localization of PPAR $\gamma$  [26, 31]. We demonstrated that HBx bound to PPAR $\gamma$  in our LX-2-X stellate cell line (Figure 4A), decreasing PPAR $\gamma$  expression at both the gene and protein levels (Figure 4B-D). Furthermore, to determine whether HBx influenced the transcriptional activity of PPAR $\gamma$ , a luciferase activity reporter assay was performed. Compared to the LX-2-C and LX-2 cells, the luciferase activity was decreased in the LX-2-X cells. Although HBx inhibited the transcriptional activity of PPAR $\gamma$ , troglitazone countered this inhibition (Figure 5).

## Discussion

Activation of stellate cells from vitamin A-storing cells into contractile myofibroblasts is the dominant pathway leading to hepatic fibrosis. HBV infection, along with HCV infection and ethanol consumption, is a major cause of fibrosis [1-4]. The present hypothesis of HBV-related fibrosis is that HBV infects hepatocytes, which release profibrogenic factors to activate the neighboring HSCs [32].

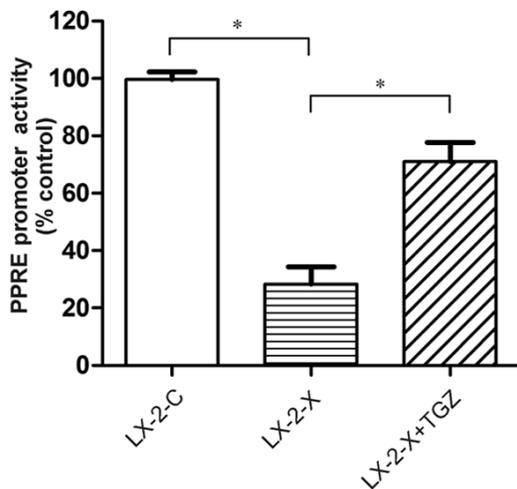
Among the four proteins of HBV, polymerase, surface, core, and X, HBx has been associated with HBV-related pathogenesis [13]. Several studies have explored the efficacy of HBx in the management of hepatic fibrosis [27-29]. What these studies have in common is that they established a "contacting" system of HSCs and hepatic cell lines infected with HBx to investigate the paracrine function

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**Figure 4.** Interaction of HBx and PPAR $\gamma$ . A: The lysate was immunoprecipitated with anti-Flag monoclonal antibody and separated by 12% SDS-PAGE. Western blot was performed with anti-Flag and anti-PPAR $\gamma$  antibodies. Input: input control in CO-IP assay. Before adding IP antibody, cell lysates are obtained for common western blot. B: The expression of PPAR $\gamma$  was determined by qRT-PCR. C: PPAR $\gamma$  was measured by Western blot. D: Grayscale analysis of the Western blot data. There were no differences between group LX-2 and LX-2-C. \*;  $p < 0.05$ .

central to the activation of HSCs. Interestingly, HBV can be found in non-hepatic cells [33, 34], and HBV can infect hepatic stellate cells *in vitro* [30]. HBx expression has been observed in hepatic mesenchymal cells from patients with chronic hepatitis or cirrhosis, which may be important in the pathogenesis of chronic infection [16]. To further investigate this topic, we established a hepatic stellate cell line that expressed HBx stably, allowing us to explore the direct role of HBx in liver fibrosis. The cell line we used in this study was a human hepatic cell line, LX-2, which keeps key features of *in vivo* HSCs [35]. Most fibrosis advances continuously and moderately, and we demonstrated that HBx was involved in the perpetuation of stellate cell activation in LX-2 cells, in spite of the fact that LX-2 cells keep the activated phenotype.



**Figure 5.** HBx suppresses the transcriptional activity of PPAR $\gamma$ . The transcriptional activity of PPAR $\gamma$  was determined by luciferase reporter assay. \*;  $p < 0.05$ .

Initiation and perpetuation are the two major phases of stellate cell activation. Perpetuation results from the effects of stimuli on maintaining the activated phenotype and generating fibrosis. Perpetuation involves autocrine as well as paracrine loops, while initiation mostly results from paracrine stimulation. Proliferation, contractility and fibrogenesis are pivotal and discrete responses of perpetuation [2]. We demonstrated that HBx could promote the growth of HSCs. The PDGF pathway is involved in HBx-stimulated HSC growth. PDGF is the most potent stellate cell mitogen, and the downstream pathways of PDGF signaling have been carefully characterized in stellate cells [5, 6]. Among liver cells, the cytoskeletal protein  $\alpha$ -SMA is a specific feature of activated stellate cells and confers increased contractile potential [2]. In our study,  $\alpha$ -SMA increased under the stimulation of HBx (Figure 3).

of HBx in hepatic fibrosis. The elegant results of these previous studies suggested that HBx is

Fibrogenesis is another pivotal event of liver fibrosis [3, 4]. Increasing ECM deposition per

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cell contributes to fibrosis. Collagen I is the best-studied component of matrix in hepatic scars. The most potent stimulus for production of collagen I and other matrix constituents by stellate cells is TGF- $\beta$ 1, which is derived from both paracrine and autocrine sources [2, 7-9]. Our results show that HBx increased the expression of TGF- $\beta$ 1 and collagen I (**Figure 3**). HBx is thought to upregulate TGF $\beta$ 1, activating the HSCs in a paracrine way [8, 27]. However, in our study, TGF $\beta$ 1 maintained the perpetuation of stellate cell activation more likely in an autocrine way in stellate cells.

PPAR $\gamma$  ligands inhibit PDGF-induced proliferation of hepatic stellate cells [36], and PPAR $\gamma$  agonists block TGF $\beta$ 1-mediated activation of Smad3 and induction of ECM gene expression in adipocytic human HSCs [20]. Since PPAR $\gamma$  interacts with the downstream pathways of stellate cell activation, we proposed that the interaction of HBx and PPAR $\gamma$  might account for the HBx-induced fibrosis. PPAR $\alpha$ /RXR $\alpha$  can inhibit the transcription and replication of HBV [37]. Conversely, the HBx protein can also modulate the PPARs. Choi demonstrated that HBx bound to the DNA-binding domain of PPAR $\gamma$ . This protein-protein interaction interferes with the nuclear localization of PPAR $\gamma$  and its binding to PPREs, but the endogenous PPAR $\gamma$  protein level is unaffected [26]. Kim et al demonstrated that HBx induces lipid accumulation, and they proposed that this phenomenon is induced by upregulating the SREBP1 and PPAR $\gamma$  expression [31]. In this study, we demonstrated that HBx protein bound to PPAR $\gamma$  in LX-2 cell line (**Figure 4A**). Interestingly, the expression of PPAR $\gamma$  in the LX-2-X cells was significantly lower compared to the control groups (**Figure 4**), in contrast to previous studies. Furthermore, the luciferase activity reporter assay showed that HBx inhibited the transcriptional activity of PPAR $\gamma$  (**Figure 5**). We propose that HBx not only interacted with PPAR $\gamma$  to interfere with its function but also decreased its expression in some way. The disruption and the downregulation of PPAR $\gamma$  removed the protection of the quiescent phenotype of HSCs. Treatment with the PPAR $\gamma$  ligand troglitazone made HSCs less activated in the presence of HBx.

Taken together, our data suggest that HBx could maintain the perpetuation of stellate cell activation by regulating PPAR $\gamma$  and that troglitazone could attenuate the HBx-induced cell

growth promotion and fibrogenesis, which might provide a potential therapy for fibrosis. However, because of the *in vitro* nature of our study, these data cannot be generalized to the situation of *in vivo* fibrosis. Further studies should clarify why PPAR $\gamma$  is downregulated by HBx and investigate the possible interactions between other HBV proteins and PPAR $\gamma$ .

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### Disclosure of conflict of interest

None.

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