# Parallel induction of cell proliferation and inhibition of cell differentiation in hepatic progenitor cells by hepatitis B virus X gene

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**Abstract.** Increasing evidence has shown that normal stem cells may contribute to the development and progression of cancer by acting as cancer-initiating cells. The hepatitis B virus X (HBX) protein has been implicated in the hepatitis B virus (HBV)-associated liver carcinogenesis. However, the role of HBX in hepatic progenitor cells (HPCs) is poorly understood. In this study, we aimed to determine the role of HBX in regulating HPC proliferation and differentiation. Using MTT analysis, we showed that HPCs infected with adenovirus expressing HBX (Ad-HBX) grew more rapidly compared to HPCs infected with adenovirus expressing green fluorescent protein (Ad-GFP). To reveal the mechanism for the increased cell number after HBX treatment, we searched for possible alterations in the cell cycle and apoptosis by flow cytometry. We found that HBX treatment resulted in an increase in the S phase cell cycle fraction and a decrease in apoptosis. In addition, we examined the differentiation of HPCs infected with Ad-HBX and found that the HBX expression in HP14.5 cells led to an increased expression of early progenitor markers and a decreased expression of late hepatocyte markers. Furthermore, HBX inhibited glycogen synthesis in HP14.5 cells, indicating that HBX is capable of inhibiting terminal hepatic differentiation. Therefore, our results strongly suggest that HBX plays an important role in regulating HPC proliferation and differentiation. This is the potential mechanism of HBX-mediated liver carcinogenesis.

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## Introduction

Hepatocellular carcinoma (HCC) is among the top 5 most frequent cancers worldwide. Approximately 250,000 new cases of HCC are diagnosed yearly, and the majority of individuals succumb to the disease within a year of diagnosis. The hepatitis B virus (HBV) is one of the major pathogens of HCC. Approximately 25% of people who are chronically infected with HBV develop HCC. The results of numerous studies suggest that the mechanism of HBV-associated HCC is a complex process. An HBV infection may induce a host immune response and a destruction of HBV-infected hepatocytes that induce repeated liver regeneration. At the same time, the integration of the HBV and host cell genome may directly result in a frame-shift mutation, where the activation of a proto-oncogene, and the integration result in the persistent expression of the HBV genome in the host cell.

HBV encodes a partially double-stranded DNA genome that contains 4 open reading frames (ORFs) which encode the viral polymerase/reverse transcriptase, core protein, surface antigen and the non-structural hepatitis B virus X (HBX) protein. HBX, a small 17-kDa soluble protein, is believed to play a key role in HBV biology and in the development of liver cancer. HBX, a multi-function transactivator, despite its inability to bind to DNA directly, may stimulate the HBV genome replication and thus regulate the viral life cycle. The knockdown of HBX by the transfection of small interfering RNA (siRNA) in the HepG2.2.15 cell line previously has been shown to disturb HBV replication *in vitro* (1). Moreover, HBX controls several signal transduction pathways, such as MAPK, JNK and Src and upregulates several genes which mediate oncogenesis, proliferation and immune responses (2). HBX also modulates the levels of the cell cycle regulators, p16, p21, p27, cyclin D1, cyclin A and cyclin B1, and increases the activity of CDK2 in various cell lines and primary hepatocytes (3,4), and accelerates the cell cycle by inducing the exit of quiescent cells from G0 and the transition from the G1 to the S phase and from the G2 to the M phase (5). Additionally, HBX prevents apoptosis by interfering with pathways that are activated by Fas and TGF-β (6,7) or by interacting directly with p53 (8) or caspase-3 (9).

Nonetheless, the carcinogenic role of HBX in HCC remains unclear. In recent years, cancer stem cell (CSC) research has drawn much attention. CSCs have been accepted as key players in tumorigenesis. Due to CSCs and normal stem cells having similar properties, such as differentiation, self-renewal, similar surface markers and sharing tightly regulated self-renewal pathways, including the Notch (10), Wnt (11) and sonic hedgehog (Shh) pathways (12), CSCs are considered to originate from normal stem cells or progenitor cells (13). In liver cancer research, hepatic progenitor cells (HPCs) isolated from 3,5-diethoxycarbonyl-1,4- dihydrocollidine (DDC)-treated p53-null mice liver were able to induce tumors with characteristics of both HCC and cholangiocarcinoma in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (14). This indicated that HPCs may be the initiation cells in liver tumor generation. Although CSCs have differentiation properties, they may not be able to display the lineage of cell development (15) and may result from normal stem cell maturation arrest after the acquisition of multiple mutations.

The positive correlation between HBX and HCC has been confirmed; however, the correlation between HBX and HPCs remains unclear. We hypothesized that HBX may affect the proliferation and differentiation of HPCs, and result in HPC maturation arrest and malignant transformation, which may be the mechanism of HBX-mediated liver carcinogenesis. These findings shed new light on the role of HBX in HPCs and the correlation between chronic hepatitis infection and liver cancer.

## Materials and methods

*Cell culture and chemicals*. L02, HepG2 and HEK-293 lines were obtained from the American Type Culture Collection (Manassas, VA). HPCs (HP14.5) were isolated from the E14.5 mouse embryo fetal liver and infected with a retrovirus packaged SSR no. 69, which employs the overexpression of SV40 large T antigen flanked by loxP sites, to establish reversible stable progenitor lines. We selected the single clone cell expression early marker and late markers and induced the cells to differentiate into hepatic and bile duct cells designated as HP14.5 (16,17). All cell lines were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin at 37˚C in 5% $CO_2$. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

*Recombinant adenoviruses expressing green fluorescent protein (Ad-GFP) and HBX (Ad-HBX)*. GFP and HBX. Recombinant adenoviruses expressing GFP and HBX were provided by Dr T.C. He of the University of Chicago Medical Center.

*Cell proliferation assay*. Liver cells ($1x10^4$) infected with Ad-GFP or Ad-HBX were plated into 96-well plates and incubated for 1, 2, 3 or 4 days. After incubation, 20 $\mu$l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT reagent, 5 mg/ml dissolved in PBS) were added to each well, and the samples were incubated for 4 h. Formazan precipitates were dissolved in DMSO, and the absorbance was measured with a Victor3 1420 Multilabel Counter (Perkin-Elmer, Waltham, MA). All experiments were conducted in 6 replicates and were repeated at least 3 times.

*Cell cycle and apoptosis assays*. For cell cycle and apoptosis analyses, cells were cultured using 6-well plates and were analyzed 72 h after being infected with Ad-GFP or Ad-HBX. Cells were synchronized using a serum-free medium for 24 h and stimulated by replacing with a complete medium. Propidium iodide staining was used to examine the cell cycle with flow cytometry (FCM). Annexin V-PE and 7-amino-actinomycin D (7-AAD) were used to measure the number of apoptotic cells at the early phase. All experiments were repeated at least 3 times.

*Isolation of total RNA*. Subconfluent cells were seeded in 75-$cm^2$ cell culture flasks or 100 mm dishes in a medium supplemented with 0.5% FBS. Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions.

*Reverse transcription and quantitative real-time PCR (qPCR) analysis*. Reverse transcription PCR was carried out as previously described (16). Briefly, 10 $\mu$g of total RNA weres used to generate cDNA templates by reverse transcription with hexamer and Superscript II reverse transcriptase (Invitrogen). The first-strand cDNA products were further diluted 5- to 10-fold and used as PCR templates. The qPCR primers were 18-mer primers, designed by the program Primer3, to amplify the fragment (~120 bp) of the mouse cytokeratin 19 (CK)19, CK18, dual leucine zipper-bearing kinase (DLK), α-fetoprotein (AFP) or albumin (ALB) genes. SYBR-Green-based qPCR analysis was carried out using the ABI 7500. The specificity of each qPCR reaction was verified by the melting curve analysis and further confirmed by resolving the PCR products on 1.5% agarose gels. Triplicate reactions were carried out for each sample. All samples were normalized to the expression levels of GAPDH.

*Immunofluorescence staining*. Immunofluorescence staining was carried out as previously described (18). Briefly, cells were fixed with methanol at -20˚C for 15 min and washed with PBS. The fixed cells were permeabilized with 1% NP-40 and blocked with 10% bovine serum albumin, followed by the incubation with DLK and ALB antibodies (Santa Cruz Biotechnology, Inc.) for 60 min. After washing, cells were incubated with DyLight 594-labeled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 min. The proteins of interest were examined under a fluorescence microscope. Stains without the primary antibody, or with the control IgG, were used as the negative controls.

*Periodic acid-Schiff (PAS) staining*. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and were then washed with $ddH_2O$. The fixed cells were stained with 0.5% periodic acid solution for 5 min at room temperature, and rinsed with $ddH_2O$ for 3 min. Cells were incubated with Schiff's reagent (Sigma-Aldrich) for 15 min at room temperature, washed with tap water for 3 min, and then counterstained with hematoxylin solution for 2 min, followed by a thorough rinsing with tap water. Positive stain (purple red) was recorded using a microscope.

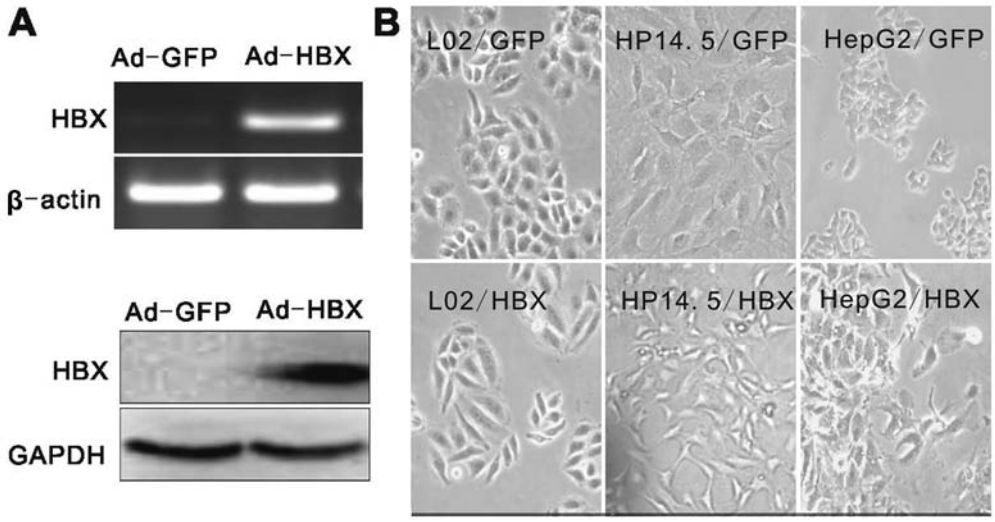


Figure 1. HBX promoted morphological changes in liver cells. (A) The expression of HBX in HP14.5 cells infected with Ad-HBX at the mRNA level and protein level. All samples were normalized to GAPDH in PCR testing and to β-actin in western blot analysis testing. (B) Morphological changes in L02, HP14.5 and HepG2 cells infected with Ad-HBX. All cells appear larger in size and more narrow, particularly the HP14.5 cells.

*Statistical analysis*. We used the Microsoft Excel program to calculate standard deviation (SD) and statistically significant differences between samples using the two-tailed Student's t-test.

## Results

*HBX promotes morphological changes in liver cells*. To examine the potential role of HBX in the proliferation and differentiation of HPCs, we first prepared the adenovirus expressing HBX. We infected HP14.5 HPCs with Ad-HBX and confirmed the expression of HBX by RT-PCR and western blot analysis (Fig. 1A). Interestingly, after being infected with Ad-HBX, the cell morphology was clearly changed compared with the Ad-GFP group, (L02 , HP14.5 or HepG2 cells). The morphological changes were mainly cells that were narrower and longer in size (Fig. 1B).

*HBX induces liver cell growth*. Firstly, we evaluated the changes in proliferation of liver cells infected with Ad-HBX by MTT assay. Cells were divided into a mock group, infected with Ad-GFP, and an HBX group, infected with Ad-HBX, and their growth was monitored for several days. A statistically significant growth induction was observed for all cell lines. This growth induction effect was observed at 24 h and more clearly at 48 h (20%) after the HP14.5 cells were infected with Ad-HBX (Fig. 2A).

To reveal the mechanism for the increased cell number following HBX treatment, we searched for possible alterations in the cell cycle and apoptosis in the liver cells by FCM. In the cell cycle, the HBX treatment resulted in a statistically significant increase in the S cell cycle fraction consistent in all of the studied cell lines at day 2, including the L02, HP14.5 and HepG2 cells (Fig. 2B). The data also showed that the cells treated with HBX grew more rapidly compared to the controls. The proliferation index (PI) is the sum of the S and G2/M phase activities of the cell cycle expressed as a fraction of the total cell population, that is PI = [(S + G2/M)/(G0/G1 + S + G2/M)] x100%. A high PI value corresponds to a rapid cell proliferation. The results showed that the PIs of the cells infected with Ad-HBX were higher than those of the mock cells (Fig. 2C). Thus, the HBX transfection significantly accelerated the proliferation of L02, HP14.5 and HepG2 cells. In addition to the cell cycle alteration, the overall cell growth promotion induced by HBX may be due to increased anti-apoptosis. Cells treated with HBX for 48 h were harvested and stained with Annexin V-PE and 7-AAD. FCM was used to evaluate the apoptotic cells at the early phase. After treatment with HBX, the number of apoptotic cells clearly decreased in the HP14.5 cells (Fig. 2D).

*HBX inhibits hepatic differentiation of HPCs*. We then aimed to determine the role of HBX in hepatic differentiation. We analyzed the hepatic markers that indicate the different stages of hepatic differentiation upon HBX stimulation. Using RT-PCR, we demonstrated that the early markers, DLK and AFP, had increased in the HP14.5 cells infected with HBX, compared with the HP14.5 cells infected with GFP (Fig. 3A). CK19 serves as a bipotential stem cell marker for both hepatic stem cells and biliary epithelial progenitors (19). The CK19 expression decreases in differentiated hepatocytes whereas the expression increases in pancreatic islet and biliary ductal cells. As shown in Fig. 3A, the CK19 expression increased after a 6-day treatment of HBX, while the late hepatocyte markers, CK18 and ALB (19,20), had decreased after HBX treatment (Fig. 3B). Immunofluorescence staining revealed that the expression of ALB was significantly inhibited after a 9-day treatment of HP14.5 cells with HBX, while the expression of the early marker, DLK, was induced upon HBX treatment (Fig. 3C).

Finally, we examined the effect of HBX on the terminal differentiation of HPCs by using the PAS staining method. PAS staining is primarily used to identify glycogen in tissues, although glycoprotein and proteoglycans may also be posi-

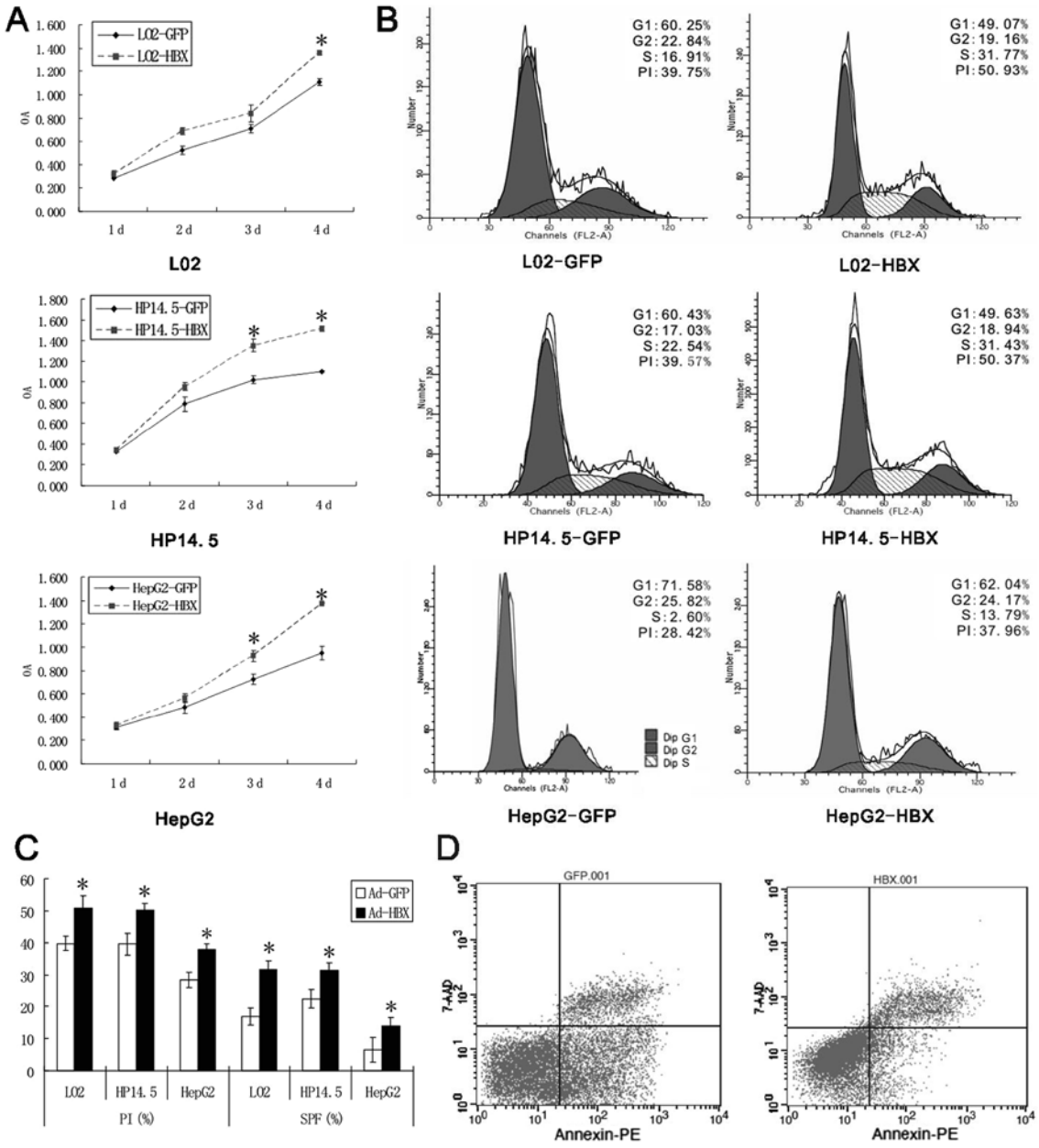


Figure 2. HBX is responsible for the proliferation of liver cells. (A) Changes in proliferation in L02, HP14.5 and HepG2 cells due to the expression of HBX were evaluated using a growth curve. (B) FCM analysis of the cell cycle in L02, HP14.5 and HepG2 cells expressing HBX. Propidium iodide staining was used to examine the cell cycle with FCM. (C) HBX induced L02, HP14.5 and HepG2 cell proliferation as measured by the proliferation index [PI = (S + G2M)/(G0/G1 + S + G2M) x100%] and the S phase fraction [SPF = S/(G0/G1 + S + G2M) x100%]. (D) FCM analysis revealed that HP14.5 cells expressing HBX had lower apoptotic rates than the mock cells. Annexin V-PE and 7-amino-actinomycin D (7-AAD) were used to measure the number of apoptotic cells at the early phase. All results represent the means ± SE. $^{*}P<0.05$.

tively stained. We treated HP14.5 cells with HBX for 10 days. When the cells were fixed and subjected to PAS staining, positive stains were significantly decreased in HBX-treated HP14.5 cells (Fig. 3D). Taken together, our results demonstrate that HBX may effectively inhibit the terminal differentiation of HPCs.

## Discussion

In the present study, we aimed to investigate the role of the HBX protein in regulating hepatic proliferation and differentiation of HPCs. We established reversible stable HPCs derived from the E14.5 mouse fetal liver through the retroviral integration of SV40 large T cells (21) (designated as HP14.5 cells). The HP14.5 cells were shown to express high levels of early liver stem cell markers (e.g., Oct-3/4, DLK and c-kit), but low levels of late liver markers (e.g., ALB and UGT1A). We further infected the HP14.5 cells with Ad-HBX and named them HPCs-HBX. Compared with the control group, the HPCs-HBX group developed faster and had more cell transition from the G0/G1 to the S phase, while apoptosis inhibition of HPCs-HBX was evaluated by FCM analysis. We also examined the differentiation of HPCs and found that HBX-expressing HP14.5 cells had an increased expression of early progenitor markers and a decreased expression of late hepatocyte markers. Furthermore, HBX inhibited glycogen

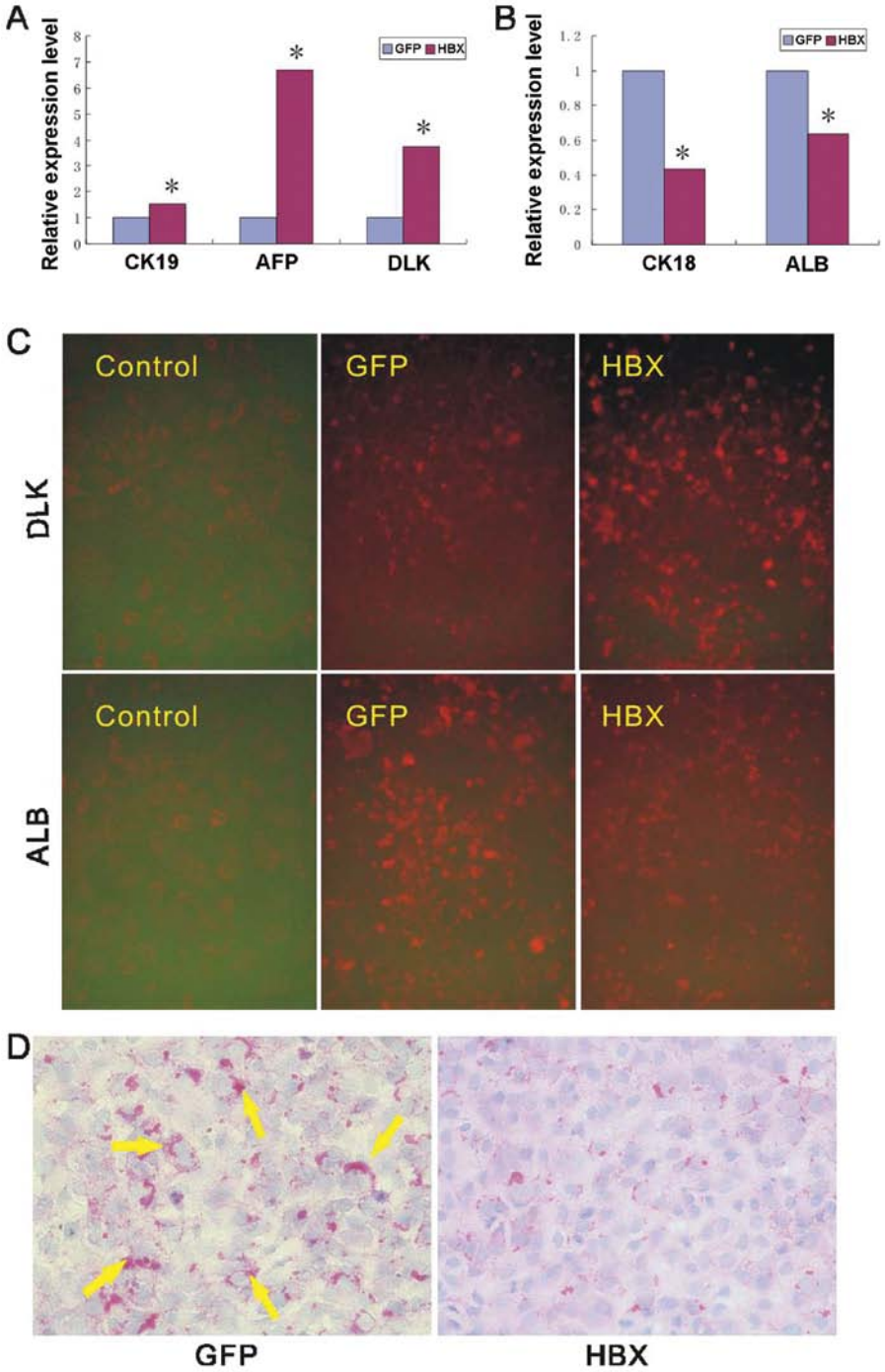


Figure 3. HBX inhibited terminal differentiation of hepatic progenitor cells (HPCs). (A) RNA was isolated from the cells treated with HBX at the indicated time-points and subjected to RT-PCR analysis (see Materials and methods). The expression of the early hepatic markers, AFP, DLK and CK19, was analyzed. All samples were normalized to GAPDH. (B) HBX inhibited the expression of the late hepatic markers, CK18 and ALB. All samples were normalized to GAPDH. (C) The change in the epxression of the hepatic markers, DLK and ALB, in HP14.5 cells treated with HBX was measured by immunofluorescence. Cells were fixed and stained with DLK and ALB antibodies, followed by staining with DyLight 594-labeled secondary antibodies. Fluorescence signals were recorded with a fluorescent microscope. (D) HBX inhibited terminal differentiation of HPCs. HP14.5 cells were treated with HBX for 10 days. Cells were fixed and subjected to PAS staining. Positive stains (purple red) are indicated by arrows. The staining results were confirmed by at least 3 batches of independent experiments and representative results are shown. All results represent the means ± SE. *P<0.05.

synthesis in HP14.5 cells, indicating that HBX is able to inhibit terminal hepatic differentiation. Therefore, our results strongly suggest that the HBX protein may play an important role in regulating hepatic proliferation and differentiation. HBX results in maturation arrest of HPCs that may be the key mechamism of HBX-mediated liver carcinogenesis. The results from the present study shed new light on the role of HBX in HPCs and the correlation between chronic hepatitis infection and liver cancer.

CSCs are defined as 'a small subset of cancer cells within a cancer that constitute a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor' (22). The first convincing evidence for CSCs was

demonstrated in acute myelogenous leukemia (AML) in 1994 by Lapidot *et al* (23) and Bonnet *et al* (24). The first solid tumor stem cells, breast CSCs, were reported by Al-Hajj *et al* in 2003 (25). Since then, CSCs have drawn much attention and have been widely studied and characterized in many different types of human tumor, including brain tumors (26), multiple myeloma (27), colon cancer (28), prostate cancer (29), head and neck cancer (30), melanoma (31) HCC (32), pancreatic cancer (33) and lung cancer (34).

Although CSC research has drawn much attention for improving the understanding of cancer biology and providing new approaches for cancer treatment, it is still at its infancy. The origin of CSCs is largely unknown. In recent years it has been supported that CSCs may be initiated from the different maturation levels of normal cells (35). In HCC (32), CSCs have been found to arise from the dedifferentiation of mature hepatocytes or from the arrested maturation of determined stem cells (36). HBX has been extensively studied in the normal liver or liver cancer cells, but the effect of HBX on the normal liver stem cell or liver CSC is poorly understood. It has been reported that HepG2 cells stably transduced with HBX highly express the Oct-4, Nanog, Klf4, β-catenin and epithelial cell adhesion molecule (EpCAM) *in vitro* and *in vivo*. Moreover, HBX stimulated cell migration, growth in soft agar and spheroid formation. Therefore, HBX contributes to hepatocarcinogenesis, at least in part, by promoting the dedifferentiation of mature hepatocytes and the appearance of liver CSCs (37). In L02 cells, the stable HBX transfection also resulted in a malignant phenotype (38). However, whether HBX is capable of inducing hepatic stem cell maturation arrest remains unknown. In HBX transgenic mice, the elevated number of EpCAM(+) cells with characteristics of HPCs was observed, and all HBX transgenic mice developed liver tumors characterized with histological features of HCC and cholangiocarcinoma after 7 months of DDC feeding. This indicated that HBX may induce a malignant transformation of HPCs that contributes to tumorigenesis (39). In our study, using HPCs derived from the E14.5 mouse fetal liver, we directly observed that HBX is capable of arresting the maturation of HPCs and may cause the transformation of HPCs and induce the appearance of CSC.

The HBX activation of the development of HCC depends partly on its ability to regulate cell proliferation and apoptosis. HBX may modulate the levels of the cell cycle regulators, p16, p21, p27, cyclin D1, cyclin A and cyclin≈B1, and increase the activity of CDK2 in various cell lines and primary hepatocytes, and accelerate the cell cycle by inducing the exit of quiescent cells from G0 and the transition from the G1 to the S phase and from the G2 to the M phase. Few studies have examined the effect of HBX on cell proliferation in HPCs, the liver cancer initiation cells. In our study, we confirm that HBX treatment may result in a statistically significant increase in the S cell cycle fraction not only in L02 and HepG2 cells, but also in HPCs. The high PI value in all 3 cell lines expressing HBX indicated that HBX may accelerate the proliferation of L02, HP14.5 and HepG2 cells. The correlation between HBX and apoptosis is a topic of HBV biology that illustrates its complex and paradoxical effects. The HBX expression prevents Fas-mediated apoptosis in DP-16 cells (a mouse erythroleukemia cell line), mouse embryonic fibroblasts, HepG2 hepatoblastoma cells, and Chang liver cells that constitutively express HBX (6). In another anti-apoptotic mechanism, HBX decreases the caspase activity through its association with survivin, an anti-apoptotic protein that is overexpressed in most human cancers (40). Our results are in accordance with this finding, and we confirm that in HPCs, HBX also has an anti-apoptotic property, although whether the mechanism is similar to that in L02 or HepG2 cells remains unclear.

In conclusion, we demonstrate that the HBX treatment may induce the proliferation of HP14.5 cells. Furthermore, we show that HBX expression in HP14.5 cells leads to an increased expression of early progenitor markers and a decreased expression of late hepatocyte markers. HBX inhibited glycogen synthesis in HP14.5 cells, indicating that HBX is able to inhibit terminal hepatic differentiation. Therefore, our results strongly suggest that HBX plays an important role in regulating HPC proliferation and differentiation and may be involved in the HBX-mediated liver carcinogenesis. Future studies should focus on understanding the molecular basis of HBX that triggers the malignant transformation of HPCs, which may help identify a potential drug target for HCC.

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