HBx drives alpha fetoprotein expression to promote initiation of liver cancer stem cells through activating PI3K/AKT signal pathway

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Introduction

The development of hepatocellular carcinoma (HCC) is closely related to hepatitis B virus infection.5 HCC is the fifth most common malignancy worldwide, accounting for approximately 1 million deaths annually.7 The high mortality ratio of HCC is attributed to the failure of early-stage diagnosis, lack of effective treatment and relapse.3,4 Chronic hepatitis B virus (HBV) infection is considered a prominent risk factor for the development of HCC.5 >170 million people are infected with HBV, and HBV-related liver disease is increasing worldwide.6,7 Although a strong relationship between HBV-induced chronic liver disease and HCC development is widely accepted, the molecular mechanism of HBV-induced hepatocarcinogenesis is still unclear. HBV cannot directly induce hepatocarcinogenesis,8,9 but HBV X protein (HBx) plays a critical role in promoting HCC development.10–12 HBx not only inhibits the transcriptional activity of p5313,14 but also stimulates growth signal pathways, such as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)15 and the Wnt/β-catenin signalling pathways to induce the malignant transformation of liver cells.16 Studies have suggested that pathologic microorganisms were able to induce the natural reprogramming of normal mature cells to generate cancer stem cells,17 suggesting that HBx-induced functional alterations in transcription and signal transduction in infectious cells that significantly contribute to HCC development and progression.
What’s new?
Although the relationship between hepatitis B virus (HBV)-induced chronic liver disease and hepatocellular carcinoma (HCC) development is widely accepted, the molecular mechanism of HBV-induced hepatocarcinogenesis remains unclear. The early HCC biomarker alpha fetoprotein (AFP) has been suggested to promote generation of HCC stem cells. Here, expression of reprogramming factors Oct4, Klf4, Sox2 and c-myc was positively associated with AFP(+)/HBV(+) HCC tissues, with stemness markers CD44, CD133 and EpCAM expression being significantly higher in those tissues. AFP could stimulate expression of reprogramming-related genes in normal liver cells through PI3K/AKT signalling, suggesting a pivotal role in promoting initiation of HCC stem cells.

Alpha fetoprotein (AFP) is an early biomarker of HCC diagnosis, and previous studies have found that AFP can stimulate HCC cell proliferation. In addition, we have found that AFP can suppress apoptosis signal transduction, such as caspase activation, or activate growth signals, such as PI3K/AKT, leading to HCC cell drug resistance and the promotion of proliferation. AFP expression is closely associated with the initiation of cancer stem cells, and HBx can stimulate AFP expression by inhibiting p53 transcriptional activity. AFP also plays a role in activating PI3K/AKT signal in HCC cells. Recent studies have shown that PI3K/AKT signalling can induce the expression of the reprogramming factor Oct4 to stimulate the specification of cancer stem cells. These results suggest that AFP can promote generation of HCC stem cells. Because HCC development is closely related to HBV infection and because HBx plays a pivotal role in promoting hepatocarcinogenesis, we examined the effect of HBx-expressing vectors on the expression of AFP, reprogramming molecules and other stemness markers in HCC cells, and examined the expression of these markers in liver tissues from HBV-related HCC patients. We demonstrated that HBx stimulated AFP expression, predisposing cells to the acquisition of HCC progenitor/stem cell characteristics. We further found that AFP played a critical role in promoting occurrence of HCC.

Material and Methods
Patients and specimens
Archived clinical specimens were originally collected during hepatectomy of 47 patients, including 8 cases of liver trauma patients (normal liver specimens) and 39 HCC cases (28 HBV(+)/AFP(+) cases and 11 HBV(-)/AFP(-) cases) at Hainan Provincial People’s Hospital (Haikou, Hainan, China) and the Affiliated Hospital of the Hainan Medical College (Haikou, Hainan, China) between January 2010 and December 2014. Of the 47 patients, 31 were men and 16 were women, with an average age of 52.3 (range, 32–78) years. All enrolled patients were treated with radical surgery and received no other treatments. Circulating HBsAg and serum AFP levels were measured by enzyme-linked immunosorbent assay (ELISA) to confirm AFP and HBV expression. Clinical data were obtained by retrospective chart review, and follow-up care was available for all patients. A section of liver tissue approximately 2.0 cm × 2.0 cm × 2.0 cm in size was obtained from each patient immediately after surgery. Approximately 1.0 cm × 1.0 cm × 1.0 cm of each sample was fixed in 10% formalin, embedded in paraffin, and routinely stained with haematoxylin and eosin. The 1.0 cm × 1.0 cm × 1.0 cm tissue specimens were stored in liquid nitrogen. All specimens were assessed blindly and independently by two pathologists. In the case of interobserver disagreement, final decisions were achieved by general consensus. All selected patients were diagnosed by histopathological evaluation and computerized tomography (CT). The study protocol was approved by the Ethical Committee of Hainan Provincial People’s Hospital and the Science Investigation Ethical Committee of Hainan Medical College. Written informed consent was obtained from all participants. The methods were performed in accordance with the approved guidelines.

Immunohistochemical analysis
The expression and cellular distribution of phosphorylated AKT(Ser743) (pAKT(Ser743)), Oct4, phosphorylated Oct4(Thr235) (pOct4(Thr235)), Klf4, Sox2, c-myc, AFP, CD44, CD133 and EpCAM proteins in HCC specimens were assessed by immunohistochemical analysis. Five-micrometre-thick paraffin sections were deparaffinized and re-hydrated according to standard protocols, and heat-induced antigen retrieval was performed in sodium citrate buffer (10 mmol/L, pH 6.0). Endogenous peroxidase was inhibited by 0.3% H2O2, and non-specific protein binding was blocked with 10% goat serum. The sections were then incubated with primary antibody against pAKT(Ser743), Oct4, Klf4, Sox2, c-myc, AFP, CD44, CD133 or EpCAM (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. Non-immune immunoglobulin G (IgG) was used as a negative control, and antigenic sites were localized using an SP9000 Polymer Detection System and a 3,3’-diaminobenzidine kit (ZSGB-BIO, Beijing, China). The methods were performed in accordance with the approved guidelines.

Cell culture
The normal human liver L-02 cell and Chang liver (CHL) cell lines were purchased from the Institution of Cell Biology, Shanghai Life Academy of Science (Shanghai, China), and
the non-AFP-producing human HCC HLE cell line was a gift from the Department of Cell Biology, Peking University Health Science Center (Beijing, China) and were grown in Dulbecco’s modified Eagle’s medium (DMEM)(Gibco, Carlsbad, CA) supplemented with 10% foetal calf serum (FCS) (Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

**Generation of an HBx-expression vector and AFP-expression vector construct and transfection**

The full-length HBx gene was inserted into the lentiviral pEB-3xflag-GP-Puro vector, and the HBx gene was identified by PCR, restriction endonuclease digestion and DNA sequencing methods. The HBx-expression vector was termed MCV-HBx. MCV-HBx vectors were infected into L-02 and CHL cells, and puromycin was applied to screen stable cell clones. The stable HBx-expressing cells were termed L-02-X and CHL-X. The AFP-expression vector (pcDNA3.1-afp) was generated as described previously.22 Transient transfections were conducted in non-AFP-producing HLE Cells (1 × 10⁵ cells/well in a 12-well plate for a confluent cell layer) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM reduced serum medium (Invitrogen, Carlsbad, CA).

**RNA interference**

For RNA interference (RNAi) experiments, the AFP-specific siRNA-expressing vectors (AFP-siRNA) directed at the 923–944 region of the AFP gene and a corresponding scrambled sequence as the negative control were used as described previously.21,22 AFP-siRNA vectors were transfected into L-02-X and CHL-X cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

**Protein localization analysis by laser confocal microscopy**

Cells were stained as described previously.21 Briefly, cells were fixed in 4% paraformaldehyde and incubated with mouse anti-human AFP, CD44, CD133 or EpCAM antibody for 12 hr. Fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse immunoglobulin G or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary anti-mouse immunoglobulin G was added and incubated for 2 hr, followed by the addition of 100 µL DAPI (1 µg/mL) for 30 min. Cells were visualized with a Leica TCS-NT SP2 laser confocal microscope (Leica Camera, Wetzlar, Germany).

**Soft agar colony formation assay**

Soft agar formation assays were performed to compare the clonogenic potential of L-02 cells and L-02-X cells, and CHL cells and CHL-X cells in semisolid medium. Briefly, 5,000 cells were mixed with 0.5% soft agar and plated on a layer of 0.8% bottom agar in six-well plates. A total of 2 mL complete medium was added to the top of the agar. Cells were fed twice a week, and the plates were incubated for 14 days at 37°C with 5% CO₂. Colonies were photographed and counted with a Nikon inverted microscope (Nikon Corp., Tokyo, Japan).

**Xenograft tumourigenesis analysis**

Xenograft experiments were performed as previously described.23 Briefly, L-02, L-02-X, CHL or CHL-X cells were implanted into the right leg fat pads (2 × 10⁶ cells) of 8-week-old male nude mice. Tumourigenesis was assessed 21 days after inoculation by sacrificing the mice and dissecting out the tumours. Proteins of interest were analyzed by immunohistochemistry and Western blotting. Animal handling procedures were approved by the Hainan Medical College Institutional Animal Care and Use Committee. The methods were performed in accordance with the approved guidelines.

**Western blotting analysis**

L-02 cells and CHL cells were infected with MCV-HBx vectors for 0, 7, 14, 21 or 28 days. Western blotting assay was performed to detect the expression of AFP, pAKT(Ser473), pOct4(Thr235), Sox2, Klf4 and c-myc at each time point. Western blotting assays were also applied to evaluate the expression of target proteins in liver tissues or cells. Briefly, total proteins were extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The proteins (50 µg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubating with 5% skim milk in Tris-buffered saline and Tweek-20 (TBST) at 37°C for 30 min, the membranes were probed for the following primary antibodies: mouse anti-AFP (1:500), -CD44 (1:400), -CD133 (1:400), -Sox2 (1:500), -c-myc (1:500), -Klf4 (1:500), -Src (1:500), -Ras (1:500) or β-actin (1:1000); rabbit anti-EpCAM (1:500), -pOct4(Thr235) (1:500) or -pAKT(Ser473) (1:500) antibody (all from Santa Cruz Biotechnology, Inc.) overnight at 4°C. After three washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hr at 37°C. The bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher, Rockford, IL) and analyzed with a gel analysis system (VersDoc TM5000MP System; BIO-RAD, Guangzhou, China). β-actin was used as a loading control.

**Statistical analysis**

The results of multiple observations are presented as the mean ± SD of at least three independent experiments. Statistical significance was determined using Student’s t test and One-way ANOVA (SPSS 11.5 software for Windows, SPSS, Inc., Chicago, IL).

**Results**

**HBV(+) /AFP(+) clinical HCC patient tissues express high levels of reprogramming proteins and stemness markers**

To observe the expression of reprogramming proteins, PI3K/ AKT signalling molecules, and stemness markers in HCC
tissues, we performed immunohistochemical analysis of pAKT(Ser473), Oct4, Klf4, Sox2 and c-myc in normal cases (liver trauma) and clinical HCC patient samples. The results indicated that HBV(AFP+) tissues expressed higher levels of pAKT(Ser473), Oct4, Klf4, Sox2 and c-myc than normal liver tissues or HBV(AFP-) tissues (Fig. 1a). Western blotting assays revealed that HBV(AFP+) HCC tissues expressed higher levels of the stemness markers CD44, CD133 and epithelial cell adhesion molecule (EpCAM) compared to normal liver tissues or HBV(AFP-) HCC tissues (Fig. 1b). Further analysis demonstrated that the stemness markers AFP, CD44, CD133 and EpCAM were expressed in a small subset of cells in HBV(AFP+) HCC tissues (Fig. 1c). The results showed that the high expression of reprogramming proteins and stemness markers closely correlated with HBV(AFP+) HCC tissues.

HBx induces the expression of AFP and subsequent expression of reprogramming proteins and stemness markers in normal liver cells

We infected normal human L-02 cell and CHL liver cell lines with a lentiviral pEB-3xflag-GP-Puro vector (MCV-HBx). The results indicated that AFP began to be expressed by Day 7, then progressively increased on days 14, 21 and 28. However, pOct4(Thr235), Sox2, Klf4 and c-myc were expressed on Day 14 and continued to increase on days 21 and 28. pAKT(Ser473) expression significantly increased on Day 21, and continued to increase on Day 28 (Fig. 2a). Laser confocal microscopy showed that MCV-HBx-infected L-02 cells (Fig. 2b) and CHL cells (Fig. 2c) expressed the stem cells markers AFP, CD44, CD133 and EpCAM by 14 days post-infection (red stain). AFP and EpCAM localized to the cytoplasm and nucleus, and CD44 and CD133 localized to the membrane. These results suggest that HBx induces AFP expression, which promotes the expression of reprogramming proteins and stemness markers.

HBx induces normal liver cells to produce cancer stem/progenitor cells

To estimate the stemness and tumourigenic capabilities of L-02-X cells and CHL-X cells (stably expressing HBx cells), we performed soft agar culture and xenograft analysis. Soft agar culture experiments demonstrated that L-02-X cells formed more colonies than L-02 cells (primary cells) (Fig. 3a). We observed a similar outcome for CHL-X cells (Fig. 3b). Xenograft tests indicated that L-02-X and CHL-X-induced tumourigenesis in nude mice, while the control L-02 cells and CHL cells did not induce tumourigenesis (Fig. 3c). We examined the expression of reprogramming proteins and HBx in tumour tissues by immunohistochemical analysis. Tumour tissues expressed higher levels of pOct4(Thr235) and pAKT(Ser473), and a small subset of tumour cells expressed pOct4(Thr235). Western Blotting analysis demonstrated that the tumour...
HBx stimulates oncogenes expression and AFP promotes the expression of reprogramming-related genes

We infected L-02 cells and CHL cells with MCV-HBx vectors and treated the cells with puromycin for 28 days to generate stable clones. We then used the stably expressing HBx cells to observe AFP and pOct4(Thr235) co-expression. Laser confocal microscopy results showed that AFP(red) and pOct4(Thr235)(green) were co-expressed in some L-02-X cells and CHL-X cells (white arrow), but not in primary cells (L-02 and CHL) (Fig. 4a). To determine whether HBx could induce malignant transformation of L-02 cells and CHL cells, we detected the expression of the critical reprogramming molecule pOct4(Thr235), the oncogenes Src and Ras, and the growth signalling molecule pAKT(Ser473). Src and Ras expression was higher in L-02-X cells and CHL-X cells compared to primary cells (Fig. 4b). To determine whether AFP regulated the expression of reprogramming-related genes, we transfected the non-AFP-producing human hepatoma HLE cell line with pcDNA3.1-afp. The results indicated that pcDNA3.1-afp was able to promote the expression of reprogramming genes, including pOct4(Thr 235), Klf4, Sox2 and c-myc and stimulate the expression of pAKT(Ser473). Treatment with the specific PI3K inhibitor Ly294002 suppressed AFP-induced expression of reprogramming-related genes and pAKT(Ser473)(Fig. 4c). These results demonstrate that HBx plays a pivotal role in stimulating oncogene expression and that AFP promoted the expression of reprogramming-related proteins through activating PI3K/AKT signal pathway.

AFP promotes stem cell colony formation and activation of PI3K/AKT to stimulate reprogramming-related genes expression

Previous results indicated that L-02-X cells and CHL-X cells produced AFP. To demonstrate that AFP expression affected colony formation, we transfected the cells with AFP-siRNA vectors and performed soft agar culture assays. The results demonstrated that silenced AFP expression in L-02-X cells and CHL-X cells significantly decreased the number of colonies (Figs. 5a and 5b). AFP-siRNA vectors significantly inhibited the expression of pAKT(Ser473), pOct4(Thr235), Klf4, Sox2 and c-myc in L-02-X cells and CHL-X cells. Treatment with the specific PI3K inhibitor Ly294002, the Ly294002 played the same effect as the effect of AFP-siRNA vectors (Fig. 5c). These results further to demonstrate that AFP plays a critical role in promoting colony formation of L-02-X cells and CHL-X cells and that AFP stimulates the expression of reprogramming-related proteins through activation of the PI3K/AKT signal pathway. The potential role of HBV in
driving AFP expression to promote HCC stem cell generation is illustrated in Figure 6.

**Discussion**

Reprogramming factors such as Oct4, Klf4, Sox2 and c-myc play pivotal roles in inducing stem cell origination.30–33 Cancer is caused by the presence of cancer stem cells (CSCs).34,35 AFP acts as a CSC marker in HCC, and its high expression suggests that AFP plays a role in promoting HCC stem cell generation. In our study, we performed immunohistochemical analysis and Western blotting to examine the expression of reprogramming-related proteins and stemness markers in tissue samples. The results demonstrated that HBV(+)/AFP(+) HCC tissues expressed higher levels of the reprogramming-related proteins Oct4, Klf4, Sox2 and c-myc and the stem cell markers CD44, CD133, EpCAM compared to HBV(−)/AFP(−) HCC tissues and normal liver tissues, suggesting that AFP expression closely correlated with presence of CSCs. We had previously found that HBx drives AFP expression to stimulate the expression of Ras and Src,36 and AFP mediated HBx-induced hepatocarcinogenesis.37 These data indicate that HBx promotes AFP expression and plays a pivotal role in HCC development.

Expression of reprogramming-related proteins and stemness markers are important traits of stem cell origination. CD44, CD133 and EpCAM expression is a hallmark of stem cells.38–40 To explore the effects of HBx and AFP on CSC formation, we transfected HBx-expressing vectors into the normal human liver L-02 cell and CHL cell lines. The results
demonstrated that HBx-expressing vectors induced AFP expression, which promoted the expression of reprogramming-related proteins. HBx also promoted the expression of stemness markers, such as CD44, CD133 and EpCAM, in normal liver cells. These results showed that HBx can induce the generation of stem cells. Previously, studies have shown that cancer develops due to the existence of CSCs.41 Although our results indicated that HBx-induced generation of stem cells, the tumourigenesis properties of these stem cells was still unclear. Therefore, we performed soft agar culture and xenograft experiments to analyze the tumourigenic capacity of these stem cells. The results suggest that expression of HBx conferred stemness on L-02-X cells and CHL-X cells based on their increased colony formation ability and tumourigenic capacity. These results demonstrated that the stem cells were competent to develop liver cancer.

Figure 4. Effects of HBx and AFP on the expression of oncogenes and reprogramming-related genes. (a) Laser confocal microscopy was used to observe the expression of AFP and pOct4(Thr235) in L-02, CHL cells and L-02-X, CHL-X cells. The expression of AFP (red) and pOct4(Thr235) (green) was examined in L-02, CHL, L-02-X and CHL-X cells. A large number of double-positive cells (yellow) were observed in L-02-X cells or CHL-X cells. Cells expressing both markers were detected in 74.6% of L-02-X cells and 67.2% of CHL-X cells. The white arrow indicates co-expression of AFP and pOct4(Thr235) in L-02-X cells or CHL-X cells. The images are representative of three experiments. (b) The expression of Src, Ras, pAKT(Ser473) and pOct(Thr235) in L-02, L-02-X, CHL and CHL-X cells was detected by Western blotting; (c) HLE cells were transfected with pcDNA3.1-afp for 48 hr and treated with the PI3K inhibitor Ly294002 for 48 hr. The expression of pAKT(Ser 473), pOct(Thr235), Klf4, Sox2 and c-myc in HLE cells was examined by Western blotting. The results are representative of three independent experiments.
Previous studies have shown that the reprogramming-related protein Oct4 plays a critical role in promoting stem cells generation, and pOct4(Thr235) is the active form of Oct4. Activation of the PI3K/AKT signalling pathway contributes to the phosphorylation of Oct4(Thr235). Previous studies have demonstrated that the co-expression of AFP and Oct4 in hepatoma stem cells and liver stem cells. In our study, we found that co-expression of AFP and pOct4(Thr235) or HBx in L-02-X cells and CHL-X cells stimulated the expression of oncogenes such as Src and Ras and promoted the expression of pAKT(Ser473). We hypothesize that asymmetric cell divisions occur during stem cells generation. Overexpression of AFP in the non-AFP-producing HLE human hepatoma cells induced the expression of reprogramming-related proteins and pAKT(Ser473). The specific PI3K inhibitor Ly294002 blocked the effects of AFP overexpression. We recently found that AFP activated the PI3K/AKT signal pathway by inhibiting the activity of phosphatase and tensin homologue (PTEN), and PI3K plays critical role in promoting initiation of cancer stem cells. These results demonstrated that HBx can induce the malignant transformation of stem cells, and HBx-driven expression of AFP contributes to the activation of the PI3K/AKT signal pathway to induce the expression of reprogramming-related proteins. AFP plays a role in driving liver stem cells production and inducing stem cells transformation into liver cancer. We recently found that AFP also plays a role in promoting the malignant behaviours.

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of HCC cells. To explore the role of AFP in stem cells transformation into malignant cells, we silenced AFP expression by RNA interference in L-02-X cells and CHL-X cells, and found that inhibition expression of AFP by AFP-siRNA vectors was able to suppress colony formation and inhibited the expression of reprogramming-related proteins and pAKT(Ser473). Ly294002 treatment showed similar effects as AFP-siRNA vectors. These results suggest that AFP plays a critical role in inducing stem cells production and promoting the malignant transformation of liver cells through activation of the PI3K/AKT signal pathway. As shown in Figure 6, we suggest that HBV induces the development of HCC by driving AFP expression to generate stem cells, and AFP acts to stimulate the production of liver CSCs.

Our study is the first to report that HBx can induce normal human liver cells to generate stem cells. HBx drives AFP expression to activate PI3K/AKT signal pathway to stimulate the expression of reprogramming-related proteins and oncogenes, which promotes the malignant transformation of liver cells. AFP may be applied as a novel therapeutic target in the treatment of HCC.

Conflict of Interest
The authors declare that they have no competing interests.

Author Contributions
M.Z., W.L., X.D., B.L., Y.C. and X.Z. performed the experiments; J.G. and M.L. analyzed clinical data and discussed the results; M.L. drafted the article; M.L. designed experiments and revised the results; J.G. and M.L. wrote the article. All authors contributed to article editing and approval.

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