Hepatitis B virus (HBV) infection is one of the most serious and prevalent global health problems, accounting for approximately 2 billion people with past or present infection. Despite the availability of highly effective vaccines against hepatitis B since 1982, more than 350 million chronic HBV carriers, 75% of who reside in the Asia-Pacific region, are still at increased risk of developing lethal hepatic decompensation, cirrhosis, and hepatocellular carcinoma (HCC). Among all proteins encoded by HBV, hepatitis B virus X protein (HBx) is a key regulatory non-structural protein of the virus that is at the intersection of HBV infection, replication, pathogenesis, and carcinogenesis. Our previous studies have revealed that HBx could promote HBV-associated hepatocarcinogenesis through upregulating GalTI transcription, inhibiting Notch1 cleavage, and activating p21-activated kinase 1 (PAK1). In addition to hepatocarcinogenesis facilitation by engendering a migratory phenotype in a CD44-dependent manner, promoting matrix metalloproteinase and COX-2 expression, repressing E-cadherin expression, as well as inducing epithelial–mesenchymal transition (EMT) through signal transducer and activator of transcription5b (Stat5b) activation.

Epithelial–mesenchymal transition is an intricate biological process by which polarized epithelial cells, which normally interact with the basement membrane through its basal surface, undergo multiple biochemical changes that enable them to lose their epithelial characteristics and acquire a highly motile mesenchymal-like phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and highly increased production of ECM components. This dramatic phenotypic switch has now emerged as a recognized mechanism for dispersing cells during embryonic development, forming fibroblasts or mesenchymal cells in chronic inflammation and fibrosis, as well as initiating invasive and metastatic behavior for epithelial cancer progression. Accumulating evidence suggests that hepatocellular EMT plays a pivotal role in the dissemination of malignant hepatocytes which accounts for tumor invasion and metastasis during HCC progression.

Although the pathological relevance and significance of HBx in HBV-associated hepatocarcinogenesis attracted much attention in recent years, the role and molecular mechanism for HBx in hepatoma invasion and metastasis remain poorly understood. In the present investigation, we tested the hypothesis whether HBx could promote hepatoma invasion and metastasis through EMT induction. We found for the first time that HBx expression could stabilize Snail protein by activating the phosphatidylinositol 3-kinase/protein kinase B/glycogen synthase kinase-3β (PI3K/AKT/GSK-3β) signaling pathway, which contributed to facilitating EMT and promoting hepatoma invasion in vitro and in vivo. This study may be useful in the future to define PI3K/AKT/GSK3β/Snail as a crucial molecular mechanism and potential therapeutic target for tumor invasion and metastasis in HBV-associated HCC.

Materials and Methods

Cell lines and patient samples. Huh7 and Chang cells were cultured in DMEM supplemented with 10% FBS in a 5% CO2 incubator at 37°C. Twenty-four pairs of frozen fresh tumor liver tissues and their peripheral non-tumor tissues were collected after surgical resection from 20 HCC patients with chronic HBV infection and four patients without chronic HBV.
infection in the Nantong Cancer Hospital (Nantong University, Jiangsu, China) with informed consent and Institutional Review Board approval.

**Construction of plasmids.** The plasmids containing HBx amplified from the HBV genome were generated as described previously.(8) The GSK3β WT and GSK3β (S9A) CA plasmids were kindly provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX, USA). All plasmid constructs were confirmed by DNA sequencing.

**Plasmid transfection and RNA interference.** Transient and stable transfections with various plasmids were carried out as previously described.(8) The siRNAs against SNAI1 gene Snail siRNA (human) and corresponding control siRNA-A (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for RNA interference as described previously.(31) The gene silencing effect was confirmed by Western blot analysis and RT-PCR at 72-h post-transfection.

**Western blot, quantitative real-time PCR, immunofluorescence staining, and confocal microscopy.** Western blot and quantitative real-time PCR (qRT-PCR) were carried out as described previously.(32,33) Antibodies used here are listed in Table S1. The PCR primer sets used here are shown in Table S2. Immunofluorescence staining and confocal microscopy were carried out as described previously.(9) Fluorescein isothiocyanate-conjugated phalloidin (Sigma, St. Louis, MO, USA) was used for F-actin staining. Antibodies against E-cadherin, N-cadherin, vimentin, and fibronectin were used in immunofluorescence.

**Wound healing, cell migration, and cell invasion assays.** Wound healing assay was carried out as described previously.(34) Cell migration and cell invasion assays were carried

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![Image](image_url)

**Fig. 1.** Hepatitis B virus X protein (HBx) induces epithelial–mesenchymal transition in hepatoma and hepatic cells. (A) Representative images of cell morphology in Huh7 and Chang cells after HBx transfection. (B) Expression of epithelial marker (E-cadherin), mesenchymal markers (N-cadherin, vimentin, and fibronectin), and HBx in Huh7 cells after HBx transfection. (C) mRNA fold change of E-cadherin, N-cadherin, vimentin, and fibronectin in Huh7 cells after HBx transfection. (D) Immunofluorescence staining of E-cadherin, N-cadherin, vimentin, and fibronectin, F-actin staining in Huh7 cells after HBx transfection. Nuclei were stained and visualized with DAPI. Con, control.
out with a Transwell Assay (Corning, Lowell, MA, USA) and Collagen-Based Cell Invasion Assay (Colorimetric; Millipore, Billerica, MA, USA), respectively, according to the manufacturers’ instructions.

**Experimental metastasis model.** Four- to five-week-old male athymic nude \((\text{Foxn}^{nu/nu}, \text{BALB/c background})\) mice were purchased from the Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). Cells were injected into the splenic vein or the tail vein of 6-week-old nude mice at \(1 \times 10^6\) cells per injection. The mice were killed after 6 weeks and the number of metastatic tumors in liver and lung were separately counted. Intrahepatic and pulmonary metastatic tumor tissues were collected for Western blot analysis.

**Statistical analysis.** Experimental data were analyzed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and presented as the mean ± standard deviation \((X \pm SD)\). Statistical significance of the differences in the experimental data was determined by Student’s \(t\)-test. Differences were considered significant at values of \(P < 0.05\).

**Results**

Hepatitis B virus X protein expression promoted hepatocellular EMT. To address the potential contribution of HBx expression on HBV-associated HCC progression, we investigated whether HBx overexpression affects hepatocellular EMT in hepatoma and hepatic cell lines. First, we found that the cell morphological phenotype of Huh7 and Chang cells transfected with HBx plasmid changed from a cobblestone-like monolayer of epithelial-like cells to dispersed spindle-shaped mesenchymal-like cells (Fig. 1A), which suggests that these cells underwent a rearrangement of the cytoskeleton due to HBx overexpression. As rearrangement of the cytoskeleton is often regarded as a *bona fide* hallmark of EMT, the effect of HBx expression on hepatocellular EMT was investigated. The expression of one epithelial marker (E-cadherin) decreased, whereas the expression of three mesenchymal markers (N-cadherin, vimentin, and fibronectin) increased, in HBx-expressing Huh7 cells compared with empty vector-transfected Huh7 cells (Fig. 1B). Furthermore, qRT-PCR analysis showed decreased mRNA levels of epithelial marker E-cadherin and increased mRNA levels of mesenchymal markers N-cadherin, vimentin, and fibronectin in HBx-transfected Huh7 cells compared with empty vector-overexpressed Huh7 cells (Fig. 1C). Immunofluorescence staining also confirmed decreased expression of epithelial marker E-cadherin and increased expression of mesenchymal markers N-cadherin, vimentin, and fibronectin in HBx-transfected Huh7 cells compared with control Huh7 cells (Fig. 1D). F-actin staining showed a dramatic F-actin cytoskeleton reorganization from the typical cortical organization of epithelial-like control Huh7 cells to stress fibers and a dynamic lamellipodia-like organization, characteristic of mesenchymal-like Huh7-HBx cells, and suggestive of substantial reorganization of actin in Huh7-HBx cells (Fig. 1D). All of these results indicated that...
HBx expression could promote EMT in hepatoma and hepatic cells.

Hepatitis B virus X protein expression facilitated EMT through Snail protein stabilization. Induction of EMT is distinguished by the dynamic repression of E-cadherin transcription by zinc-finger proteins (e.g., Snail, Slug) or basic helix-loop-helix family transcriptional factors (e.g., Twist) through interaction with specific E-boxes of the proximal E-cadherin promoter. To further characterize transcriptional factors involved in the hepatocellular EMT promotion by HBx expression, we examined the expression profiles of Snail, Slug, and Twist in Huh7 cells transfected with HBx. An increased protein level of Snail but no significant change in Slug and Twist protein levels were found in HBx-transfected Huh7 cells compared with empty vector-transfected Huh7 cells (Fig. 2A). However, qRT-PCR analysis showed no significant changes in the mRNA levels of all three transcriptional factors, which suggests that HBx expression increased Snail protein level in a transcription-independent manner (Fig. 2B). To investigate the effect of HBx expression on the protein stability of Snail, a protein translational inhibitor (CHX) and two proteosome inhibitors (MG132, lactacystin) were used to evaluate Snail protein stability after HBx transfection. A slow downregulation of Snail protein level was observed in HBx-transfected Huh7 cells compared with control Huh7 cells after CHX treatment, indicating that HBx expression might stabilize Snail protein (Fig. 2C). Compatible with this conception, we also found a slight upregulation of Snail protein level in HBx-transfected Huh7 cells compared with control Huh7 cells after MG132 or lactacystin treatment (Fig. 2D). Taken together, these results strongly suggest that increased Snail expression level by HBx expression could be due to upregulation of Snail protein stability.

To further clarify whether Snail protein stabilization by HBx expression is responsible for HBx-induced EMT, siRNA was used to silence Snail expression in HBx-transfected Huh7 cells. Morphological change from an epithelial-like phenotype to a mesenchymal-like phenotype in HBx-transfected Huh7 cells was dramatically reversed after Snail siRNA cotransfection compared with control siRNA cotransfection, which suggests that HBx-promoted EMT is dependent on Snail protein stabilization (Fig. 3A). Consistent with this notion, HBx-promoted EMT was also blocked by Snail siRNA treatment, as evidenced by increased E-cadherin and decreased N-cadherin, vimentin, and fibronectin protein expression as well as mRNA levels in HBx-transfected Huh7 cells (Fig. 3B,C). All of these data further indicate that HBx-induced EMT is mediated through Snail protein stabilization by HBx expression.

Hepatitis B virus X protein expression stabilized Snail protein by activating PI3K/AKT/GSK-3β signal pathway. The central role of Snail in the regulation of EMT has been underscored by recent studies showing Snail undergoes post-translational modifications to control its stability, subcellular localization, and function through different phosphorylation events. These modifications include phosphorylation at two Ser residues on Snail by GSK3β, which is inhibited by the PI3K/AKT signal pathway and could lead to Snail nuclear export for ubiquitination and degradation. To examine whether Snail protein stabilization by HBx expression was mediated through regulation of the PI3K/AKT/GSK3β signal pathway, we treated HBx-transfected Huh7 cells with two PI3K inhibitors (LY294002 and wortmannin) and two GSK3β plasmids (GSK3β WT plasmid and GSK3β CA plasmid; the latter lacks GSK3β phosphorylation at Serine 9, which enhances its activity). We found that induction of Snail protein by HBx expression, as well as increased pAKT and pGSK3β protein, could be inhibited by LY294002 or wortmannin treatment in HBx-transfected Huh7 cells compared with empty vector-transfected Huh7 cells, which suggested that the stabilization of Snail and induction of EMT by HBx expression was dependent on PI3K/AKT activation (Fig. 4A,B). Similarly, HBx expression induced stability of Snail protein and increased pGSK3β protein could be inhibited when GSK3β activity was enhanced by GSK3β CA cotransfection in HBx-overexpressing Huh7 cells compared with control Huh7 cells (Fig. 4C). Neither treatment
Fig. 4. Hepatitis B virus X protein (HBx) increases Snail expression through activating the phosphatidylinositol 3-kinase/protein kinase B/glycogen synthase kinase-3β (PI3K/AKT/GSK-3β) signal pathway. (A) Expression of pAKT, AKT, pGSK3β, GSK3β, Snail, and HBx in Huh7 cells after HBx transfection with or without LY294002 treatment. (B) Expression of pAKT, AKT, pGSK3β, GSK3β, Snail, and HBx in Huh7 cells after HBx transfection with or without wortmannin treatment. (C) Expression of pAKT, AKT, pGSK3β, GSK3β, Snail, and HBx in Huh7 cells after HBx transfection or cotransfected with GSK3β WT plasmid or GSK3β CA plasmid together. (D) mRNA fold change of Snail in Huh7 cells after HBx transfection with or without LY294002 (LY) or wortmannin (Wort) treatment or cotransfected with GSK3β WT plasmid or GSK3β CA plasmid. Con, control.

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with PI3K/AKT inhibitors nor GSK3β CA cotransfection showed any regulatory effect on Snail mRNA levels in HBx-overexpressing Huh7 cells compared with control Huh7 cells (Fig. 4D). All of these data indicate that HBx expression could stabilize Snail protein through activating the PI3K/AKT/GSK3β signal pathway.

**Hepatitis B virus X protein expression promoted hepatoma cell migration and invasion through Snail protein in vitro.** Snail-induced EMT converts epithelial-like cells into mesenchymal-like cells with migratory properties that contribute to the acquisition of invasive properties in epithelial tumors. To test whether HBx-transfected Huh7 cells acquired higher migratory and invasive capabilities through stabilizing Snail protein, in vitro scratch wound healing assays, Transwell cell migration assays, and collagen-based cell invasion assays were carried out in HBx-overexpressing Huh7 cells after Snail siRNA treatment. The wound healing assay and Transwell cell migration assay indicated that ectopic expression of HBx increased cell mobility, which could be reversed by Snail siRNA cotransfection in HBx-transfected Huh7 cells compared with control Huh7 cells (Fig. 5A,B). Similarly, the collagen-based cell invasion assay also showed that the increased invasiveness by HBx expression could also be reversed by Snail siRNA treatment in the HBx-overexpressing Huh7 cells compared with empty vector-transfected Huh7 cells (Fig. 5C). These results show that HBx expression could promote hepatoma cell migration and invasion through stabilizing Snail protein in vitro.

**Stable HBx expression facilitated hepatoma cell metastasis through increasing Snail protein in nude mice.** To further elucidate the invasive function of HBx in vivo, we examined whether hepatoma cells with high expression of HBx undergo EMT during tumor progression. For this purpose, we generated stable transfection Huh7 cells overexpressing HBx plasmid or empty vector and analyzed Snail expression and morphological changes in these two stable Huh7 cells. Consistent with results stated above in transiently transfected Huh7 cells, increased Snail protein level and phenotypic changes from epithelial-like cells to mesenchymal-like cells were found in HBx-transfected Huh7 cells compared with control Huh7 cells, which indicated that stable HBx expression could also promote EMT in hepatoma cells (Fig. 6A,B). To determine whether EMT played a
role in HCC intrahepatic and distant metastasis induced by HBx transfectants in vivo, an experimental metastasis model was established by injecting HBx stable transfection HuH7 cells into the splenic vein or tail vein in nude mice. Increased intrahepatic and pulmonary metastatic tumor nodules, as well as enhanced Snail, pAKT, and pGSK3β protein levels were observed in metastatic tumors induced by HBx-transfected HuH7 cells compared with tumors induced by control HuH7 cells (Fig. 6C,D). These results suggest that EMT promotion by HBx expression could play a crucial role in HCC invasion and metastasis in vivo.

Activated PI3K/AKT/GSK-3β signaling correlated with increased Snail protein in HCC patient tumor, metastatic tumor, and HBV-associated HCC patient tumor tissues. To further confirm the effect of the PI3K/AKT/GSK3β/Snail pathway on HBV-associated HCC progression, the correlation among pAKT, pGSK3β, and Snail protein levels was investigated in eight pairs of primary HCC tumor tissues and their peripheral non-tumor, four pairs of metastatic and non-metastatic HCC samples, and four pairs of HBV-associated and non-HBV-associated HCC specimens. Results showed that increased pAKT, pGSK3β, and Snail protein levels were observed in all eight HCC tumor tissues compared with the relevant adjacent non-tumor tissues, indicating the contribution of the PI3K/AKT/GSK3β/Snail pathway to clinical HCC progression (Fig. 7A). Similarly, as shown in Figure. 7B, increased expression of pAKT and pGSK3β, accompanied with upregulated expression of Snail, was also detected in all four metastatic HCC tumor specimens compared with four non-metastatic HCC tumor specimens, suggestive of the involvement of the PI3K/AKT/GSK3β/Snail pathway in HCC invasion and metastasis. More importantly, compared with non-HBV-associated HCC samples, increased expression of pAKT, pGSK3β, and HBx, as well as enhanced Snail protein level, was observed in HBV-associated HCC samples (Fig. 7B). These results strongly support that the molecular pathway PI3K/AKT/GSK3β/Snail most likely played a critical role in HCC progression, especially in HBV-associated HCC invasion and metastasis.

Discussion

Increasing evidence indicates that hepatocellular EMT is a crucial event during HCC progression, which causes an increase in malignancy of hepatocytes associated with tumor cell invasion and metastasis.27–30 Our present work compliments and extends growing evidence implicating EMT in hepatocarcinogenesis. More importantly, this study provides several insights regarding the regulation of EMT and metastasis by HBx expression in HBV-associated HCC.

First, our study indicated that Snail-induced EMT is critical for HBx-initiated migration, invasion, and metastasis in HBV-associated HCC. Knockdown of Snail expression dramatically impedes HBx-induced hepatoma cell migration and invasion in vitro. Consistent with previous observations indicating the contribution of Snail overexpression to hepatoma invasion and progression,37–39 our present work revealed that HBx expression could significantly increase Snail expression, induce EMT, and contribute to migration and invasion capacity upregulation in hepatoma cells. However, our experimental model system to evaluate the effect of HBx on Snail-induced EMT has several limitations, as the observed effects of HBx could be due to the overexpression of HBx which could not reflect the pathological roles of HBx in HBV-associated hepatocarcinogenesis and/or the EMT phenotype of HCC. In addition, the findings might only be observed in HuH7 and Chang cells.

Second, our current study also revealed that Snail protein stability was regulated by the PI3K/AKT/GSK3β signal...
pathway in the process of HBx-induced EMT and hepatoma invasion. In addition to being tightly modulated at the transcriptional level, EMT inducer Snail undergoes post-translational modifications that control its nuclear localization or degradation. Snail stability and nuclear translocation can be positively modulated by PAK1, LIV1, or LOXL2, whereas GSK3β phosphorylation promotes Snail cytoplasmic export and ubiquitin-mediated proteasome degradation. As HBx expression has been reported to induce PI3K/AKT/GSK3β signal activation, it is speculated that stabilized Snail protein by HBx expression indicated in our present study might be due to upregulation of the PI3K/AKT/GSK3β pathway. Specific inhibition of the PI3K/AKT signal or increased GSK3β activity in the presence of HBx expression.

Fig. 7. Phosphatidylinositol 3-kinase/protein kinase B/glycogen synthase kinase-3β (PI3K/AKT/GSK-3β) signaling activation correlates with elevated Snail protein in hepatocellular carcinoma (HCC) patient tumor, metastatic tumor, and hepatitis B virus (HBV)-associated HCC patient tumor tissues. (A) Expression of Snail, pAKT, pGSK3β, and hepatitis B virus X protein (HBx) in eight pairs of HCC patient tumor tissues (T) and relevant adjacent non-tumor tissues (N). (B) Expression of Snail, pAKT, pGSK3β, and HBx in four pairs of HCC patient metastatic tumor tissues compared with four pairs of HCC patient non-metastatic tumor tissues (left) and in four pairs of HBV-associated HCC patient tumor tissues compared with four pairs of non-HBV-associated HCC patient tumor tissues (right).

Fig. 8. Schematic model for hepatitis B virus X protein (HBx)-induced epithelial–mesenchymal transition (EMT) mediated by the phosphatidylinositol 3-kinase/protein kinase B/glycogen synthase kinase-3β (PI3K/AKT/GSK3β)/Snail pathway plays a pivotal role in hepatoma invasion and metastasis in hepatitis B virus-associated hepatocellular carcinoma (HBV-HCC).
significantly repressed Snail expression in this work, and reinforces our hypothesis that the PI3K/AKT/GSK3β pathway most likely mediated Snail protein stabilization induced by HBx. These findings are in agreement with the notion that Snail is a labile protein and is subjected to delicate regulation by multiple extracellular signal pathways to control its ubiquitination and degradation. Although Snail has been shown to be inhibited at the transcriptional level by the GSK3β pathway, our results indicated that Snail stabilization by HBx expression also occurred at the post-transcriptional level. Furthermore, we have also found that HBx expression could upregulate and activate pAK1 in hepatoma cells. Whether increasing pAK1 expression is involved in stabilized Snail protein by HBx expression, and therefore participates in HBV-associated HCC progression, merits further examination. The molecular mechanism underlying HBx-mediated PI3K/AKT activation during Snail-induced EMT will be addressed in our future investigations.

Finally, in addition to endowing tumor cells with migratory and invasive properties, Snail-induced EMT could also prevent apoptosis and senescence, confer stem cell properties, and contribute to immunosuppression. Our previous study, which indicated HBx-blunted senescence mediated by Notch1 signal repression, also implies the potential role of HBx-induced EMT in HBV-associated hepatocarcinogenesis. However, the interaction between repressed Notch1 signal and Snail-induced EMT needs further investigation. The question whether HBx-

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Disclosure Statement

The authors have no conflict of interest.
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of antibodies used in Western blot analysis.

Table S2. List of primers used in quantitative RT-PCR analysis.