Hepatitis B Virus X Protein Stabilizes Cyclin D1 and Increases Cyclin D1 Nuclear Accumulation through ERK-Mediated Inactivation of GSK-3β

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Abstract

The Hepatitis B virus (HBx) contributes centrally to the pathogenesis of hepatocellular carcinoma (HCC). It has been suggested that the transcriptional activation of cyclin D1 by HBx is implicated in the development of HCC. However, numerous studies have shown that overexpression of cyclin D1 alone is not sufficient to drive oncogenic transformation. Herein, we investigated whether HBx can stabilize cyclin D1 and induce cyclin D1 protein nuclear accumulation, and thereby accelerate hepatocarcinogenesis. The effects of HBx on cyclin D1 stabilization were assessed in cell-based transfection, Western blot, immunoprecipitation, immunocytofluorescence staining, and flow-cytometric assays. The results demonstrated that ectopic expression of HBx in HCC cells could extend the half-life of cyclin D1 protein from 40–60 minutes to 80–110 minutes. HBx stabilized cyclin D1 primarily in the S phase of the cell cycle, in a manner dependent on the inactivation of GSK-3β, which was mediated by ERK activation. HBx also prompted the nuclear accumulation of cyclin D1, and cotransfection of the constitutively active mutant of GSK-3β along with HBx could reverse the nuclear accumulation and subsequent cell proliferation induced by HBx. Further, a positive correlation between HBx and nuclear cyclin D1 level was established in HCC specimens detected by an immunohistochemical assay. Taken together, our results indicated that HBx could stabilize and increase cyclin D1 nuclear accumulation through ERK-mediated inactivation of GSK-3β. This HBx-induced cyclin D1 upregulation might play an important role in HCC development and progression.

Introduction

Hepatitis B virus (HBV) infection is the main risk factor for hepatocellular carcinoma (HCC) worldwide. Epidemiologic studies have shown that individuals who are chronically HBV carriers have a greater than 100-fold increase in the risk of developing liver cancer (1). The HBV genome is a partially double-stranded, circular DNA containing four overlapping genes: S/preS, C/preC, P, and X. The X gene encodes a 17-kd HBV X protein (HBx), which is a multifunctional transactivator of both viral and cellular genes (2). It is widely accepted that HBx plays crucial roles in the pathogenesis of HBV-induced HCC, as X gene-transgenic mouse developed liver cancer promptly (3, 4). However, a full understanding of the molecular mechanism(s) responsible for HBx-induced HCC has yet to be achieved.

Perturbations in the regulation of the core cell cycle machinery are frequently observed in human cancers. As a key regulator of G1 phase reentry and progression, cyclin D1 is one of the most frequently altered cell cycle regulators in cancers (5). Newly synthesized cyclin D1 associates with CDK4/6, and the cyclin D1/CDK complexes transport to and accumulate in the nucleus during mid-G1 phase. The nuclear cyclin D1/CDK complexes can phosphorylate the retinoblastoma protein (Rb) and its related family members, thereby triggering E2F-dependent transcription of genes required for S-phase entry (6). During S-phase, cyclin D1 level rapidly declines as a consequence of threonine-286 (Thr286) phosphorylation by glycogen synthase kinase-3β (GSK-3β; ref. 7), and this promotes the nuclear export, poly-ubiquitylation, and degradation of cyclin D1 by the 26S proteasome (8, 9). This timely expression and accumulation of cyclin D1 is crucial for maintaining normal cell cycle progression, and disruption of it may result in uncontrolled cell proliferation.

Therefore, it is not a surprise to find frequently overexpressed cyclin D1 in a variety of human malignancies, including HCC (10–14). The overexpression of cyclin D1 can be attributed to many factors, including increased transcription, translation, and protein stability. Although cyclin D1 overexpression is clearly implicated in the affected cancers, simple overexpression of cyclin D1 is not sufficient to drive oncogenic transformation. This stems in part from the fact that in a number of experimental transgenic mouse models of carcinogenesis, cyclin D1 overexpression alone failed to induce tumors (8, 15). Rather, emerging evidence suggests that nuclear retention of cyclin D1 resulting from altered nuclear export and proteolysis is critical for the manifestation of its oncogenicity (8, 15, 16).
Previous studies have suggested a link between HBx and cyclin D1 upregulation in HCC tumors (17–19). Park and colleagues (17) reported that HBx may upregulate cyclin D1 in an immortalized hepatocyte cell line Chang liver cells, as well as in HepG2.2.15 cells, which is an HBV-producing cell line derived from an HCC cell line HepG2. A more detailed biochemical analysis has shown that the upregulation of cyclin D1 by HBx is mediated by the NF-κB2 (p52)/BCL-3 complex in the nucleus, which in turn activates the transcription of the cyclin D1 gene (CCND1). In addition, Ding and colleagues (18) and Khattar and colleagues (19) found that HBx-activated Akt or ERKs could phosphorylate and inactivate the downstream target GSK-3β, leading to stabilization of β-catenin, which subsequently triggered CCND1 gene transcription and hence increased cyclin D1 production. Although these findings suggested that cyclin D1 overexpression in HCC might result from the increased CCND1 gene transcription by HBx, these studies lacked information regarding the role of HBx in the proteolysis and nuclear accumulation of cyclin D1. Given the important role of GSK-3β in cyclin D1 degradation (7), we hypothesized that the inactivation of GSK-3β by HBx not only upregulate cyclin D1 at transcriptional level, but it may also stabilize cyclin D1 protein and prompt cyclin D1 to accumulate in the nucleus.

In this study, we identified a mechanism to stabilize cyclin D1 and prompt its nuclear accumulation through GSK-3β phosphorylation and inactivation by the HBx-activated ERK pathway, which will help us further understand the roles of HBx in the HBV-related HCC.

Materials and Methods

Sample collection
A total of 36 paraffin-embedded tissues of primary human HCCs were obtained from patients who underwent surgical resection in Henan Cancer Hospital between 2012 and 2013. All patients were Han Chinese and had a background of chronic hepatitis B infection and cirrhosis. This study was approved by the Ethics Committee of Peking University Health Science Center, and the informed consent was obtained from each participant.

Recombinant plasmid preparation
The Flag-tagged pFlex-cyclin D1, pFlex-D1-T286A, and HA-tagged pCMV GSX-3β (S9A) plasmids were all kindly provided by professor J. Alan Diehl from Medical University of South Carolina (20, 21). The HA-tagged full-length HBx plasmid (pCMV-HA-HBx) was constructed by inserting a PCR-amplified full-length HBx fragment into the EcoRI/KpnI sites of the pCMV-HA vector, using the following primers: forward 5'-TATGGC-CATCGAGGCAGCCGAAATCGG-3' and reverse 5'-ATCCCCGCGG-CGCGCGTACCCG-3'.

Cell culture, synchronization, and transfection
The human HCC cell line HepG2 and immortalized mouse embryonic fibroblast cell line NIH3T3 were purchased from the American Type Culture Collection, and human HCC cell line SMCC7721 was from Cell Resources Center of Peking Union Medical College (Beijing, P. R. China). HepG2 cells were authenticated by DNA (STR) profiling in November 2013. No authentication for NIH3T3 and SMCC7721 cells was done by the authors. All cells were maintained in DMEM supplemented with 10% FBS (Gibco). Cells were transfected with the indicated plasmids by using Lipofectamine Plus (Invitrogen) or 4D-Nucleofector X Unit (Lonza) according to the manufacturer's instructions. S-phase synchronization was achieved by culturing cells in medium containing 2.5 mmol/L thymidine for 28 hours, after which cells were released in complete media, and harvested for 4 hours after release.

Quantitative real-time reverse transcription PCR
Quantitative real-time reverse transcription (RT) PCR was performed as previously described (22). The primers used for real-time RT-PCR were listed in Supplementary Table S1.

Immunoprecipitation and Western blot assays
For Western blot analysis, proteins were extracted from cultured cells using a radioimmunoprecipitation assay buffer. Protein samples (30 μg each) were loaded onto 15% sodium dodecyl sulfate-polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% dried milk for 2 hours, the membranes were incubated with primary antibody. The protein–antibody complexes were visualized using the Odyssey Imager (LI-COR Biosciences).

Immunoprecipitation (IP) assay was carried out on clarified cell lysates in IP buffer. The clarified supernatant was incubated with the primary antibody overnight at 4°C, and then immunocomplexes were bound to protein-G Sepharose 4B for 1 hour at 4°C. Proteins bound to the protein-G Sepharose 4B were eluted by adding Laemml-SDS sample buffer and boiling for 5 minutes. After centrifugation for 2 minutes, the immunoprecipitated proteins present in the supernatant were analyzed using the basic Western blot protocol described above in this section. The antibodies used in IP and Western blot assays were listed in Supplementary Table S2.

Flow-cytometric analysis
For cell cycle analysis, cells were harvested by trypsinization and fixed by treatment in 70% ethanol at 4°C overnight. The fixed cells were rinsed twice with PBS, and resuspended in a propidium iodine (PI) solution (50 μg/mL) containing 0.5 mg/mL RNase A (Sigma), and incubated at 37°C for 30 minutes in the dark. The fluorescence of the PI-labeled cells was then measured using a FACScalibur system (Nippon Becton Dickinson), and the percentages of cells in G0–G1, S, and G2–M phase were determined using the ModFit program (Nippon Becton Dickinson).

Immunofluorescence cell staining
Cells were plated on glass coverslips and after 24 hours were permeabilized with Methanol/Acetone (1:1), washed with PBS, and incubated in primary anti-cyclinD1 antibody for 2 hours. After washing and application of the secondary FITC-conjugated anti-mouse antibody, slides were mounted using Hoechst 33258 dye and analyzed by fluorescent microscopy (Nikon Eclipse E800).

Immunohistochemistry assay
Immunohistochemical (IHC) assay was performed on 5-μm thick serial sections derived from formaldehyde-fixed, paraffin wax–embedded HCC tissue blocks. The immunostaining of cyclin D1 or HBx protein in these tissues was detected as described previously (21). The antibodies used for IHC analysis were listed in Supplementary Table S2.
Statistical analysis

For statistical analyses, the differences between two groups were analyzed by the two-tailed Student t test. The correlation between HBx and cyclin D1 expression was analyzed by Pearson correlation analysis. \( P < 0.05 \) was considered statistically significant. All analyses were performed using the SPSS suite of computer software (version 11.0).

Results

HBx upregulates cyclin D1 expression through inhibiting its degradation

To investigate the ability of HBx to upregulate cyclin D1 expression, the HepG2 and SMMC7721 cells were transfected with either control or HA-tagged HBx expressing plasmid. As shown in Fig. 1A and B, ectopic expression of HBx could increase cyclin D1 level both at the protein and the mRNA levels. We then tested whether HBx could affect the stability of cyclin D1. We used cycloheximide to block protein synthesis and detected the turnover rate of cyclin D1 protein in the endogenous cyclin D1 protein in both HepG2 and SMMC7721 cells after cycloheximide (50 \( \mu \)g/mL) treatment for the indicated time. The turnover rate of cyclin D1 protein was quantitatively analyzed by Quantity One software (Bio-Rad) and shown on the right.

HBx inhibits cyclin D1 proteasomal degradation during S phase of cell cycle

Cyclin D1 has been shown to be degraded primarily via the 26S proteasome in an ubiquitin-dependent manner (9). Consequently, it is possible that HBx stabilizes cyclin D1 by inhibiting its proteasomal degradation. To test this hypothesis, the proteasome inhibitor MG132 was used to block the degradation of proteins by ubiquitin-proteasome machinery. Western blot analysis revealed that MG132 significantly increased the cyclin D1 level in HepG2 and SMMC7721 control cells (Fig. 2A, lane 2 vs. lane 1), but not in the HBx-expressing cells (Fig. 2A, lane 4 vs. lane 3). The simplest interpretation of this result is that HBx inhibits the proteasomal-based degradation of cyclin D1. To provide additional data in support of this conclusion, the ubiquitination of cyclin D1 in the presence of HBx was monitored in an immunoprecipitation assay. As expected, despite the increase in cyclin D1 level in HBx-transfected cells, the ubiquitination levels of the protein dramatically decreased, demonstrating that HBx inhibits the ubiquitination of cyclin D1 protein (Fig. 2B).

During cell cycle progression, the cyclin D1 level rises early in G1 phase and continues to accumulate until the G1–S phase boundary, and then it declines rapidly when DNA replication initiates (5). To investigate the precise cell cycle phase in which HBx upregulated cyclin D1 level, HepG2 and SMMC7721 cells were transfected with either control or HBx expression plasmid were treated with thymidine to block their progression into G2 phase,
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Figure 2.
HBx inhibits cyclin D1 proteasomal degradation mainly in the S phase of the cell cycle. A, Western blot analysis of cyclin D1 protein in HBx-transfected HepG2 and SMMC7721 cells after MG132 (10 μmol/L) treatment for 4 hours. Lower panel, quantitative analysis of cyclin D1 protein. B, Immunoprecipitation analysis of cyclin D1 ubiquitination in pCMV-HBx-transfected HepG2 and SMMC7721 cells. Anti-cyclin D1 antibodies were used to immunoprecipitate the protein from cell lysates, and anti-ubiquitin antibodies were used to detect the ubiquitination of cyclin D1. Ten percent of lysates were subjected to direct Western blot analysis to confirm expression levels of cyclin D1, HBx, and tubulin (input). C, HepG2 and SMMC7721 cells transfected with pCMV-HBx or control plasmids were cultured in medium containing 2.5 mmol/L thymidine for 28 hours. Then, cells were released in complete media for 4 hours (synchronized in S phase) and harvested after being treated with or without MG132 (10 μmol/L) for 4 hours. Western blot analysis was used to detect the expression of cyclin D1 (i), ii, quantitative analysis of cyclin D1 protein in nonsynchronized cells and synchronized in S-phase cells. iii, quantitative analysis of cyclin D1 protein in S-phase cells with or without MG132 treatment.

therby synchronizing the cell population in S phase (confirmed by FACS analysis). As expected, in comparison with nonsynchronized HepG2 cells (68.33% in G0–G1, 28.72% in S, and 2.95% in G2–M), cyclin D1 levels were dramatically reduced in synchronized cells (9.45% in G0–G1, 88.98% in S, and 1.57% in G2–M) transfected with control plasmid (Fig. 2Ci, lane 2 vs. lane 1). In contrast, such reduction was much less apparent in synchronized cell populations that express ectopic HBx (Fig. 2Ci, lane S vs. lane 4). This result showed that HBx significantly increased the cyclin D1 level in the S phase of the cell cycle (Fig. 2Ci). It was further confirmed by examining the cyclin D1 level in synchronized cell populations expressing ectopic HBx that had been treated with MG132. Treatment of such cells with MG132 failed to produce any significant increase in cyclin D1 levels (Fig. 2Ci, lane 6 vs. lane 5), while similar treatment of synchronized S-phase cells transfected with control plasmid showed a dramatic increase of cyclin D1 level (Fig. 2Ci lane 3 vs. lane 2, and Fig. 2Ciii). Additionally, no decrease of CCND1 mRNA during S phase was observed in either control HepG2 cells or those expressing ectopic HBx (Supplementary Fig. S1). A similar phenomenon was obtained in SMMC7721 cells. These results indicated that the observed decrease in cyclin D1 protein level seen in S-phase cells resulted primarily from the accelerated degradation of the protein and, most likely, HBx increases cyclin D1 level during S phase primarily via the inhibition of proteasomal degradation of cyclin D1.

HBx stabilizes cyclin D1 through blocking GSK-3β-mediated cyclin D1 Thr286 phosphorylation

GSK-3β-mediated cyclin D1–specific phosphorylation at Thr286 is the initiating step for the rapid turnover of cyclin D1 protein.
HBx Prompts Cyclin D1 Nuclear Accumulation

We next investigated whether the decreased Thr286 phosphorylation of cyclin D1 in HBx-expressing cells resulted from the inhibition of GSK-3β kinase activity. To this end, we compared the GSK-3β kinase activity in HepG2 and SMMC7721 cells with or without HBx overexpression. We found that the phosphorylation of the GSK-3β Ser9 residue, which is known to inactivate GSK-3β kinase, was much stronger in both HepG2 and SMMC7721 cells transfected with HBx than that detected in control cells (Fig. 4A and B, lane 2 vs. lane 1). Furthermore, cotransfected with HBx, cyclin D1 and different doses of constitutively active S9A mutant of GSK-3β kinase (GSK-3β-S9A) into HepG2 and SMMC7721 cells resulted in a dose-dependent suppression of exogenous cyclin D1 expression, indicating that constitutively active GSK-3β can reverse HBx-induced cyclin D1 stability (Fig. 4C). These data suggested that HBx stabilized cyclin D1 and increased cyclin D1 expression through inactivation of GSK-3β.

Active ERKs are required for the HBx-mediated inactivation of GSK-3β

It has been reported that HBx can activate either Akt or ERKs, and both Akt and ERKs can phosphorylate the Ser9 residue and inactive GSK-3β (18, 19). To delineate which kinase is responsible for the phosphorylation of GSK-3β and cyclin D1, specific inhibitors of the Akt and ERK pathways were used to block these kinases. As shown in Fig. 4, increasing expression of active phosphorylated ERKs, coupled with inactive phosphorylation of GSK-3β, was found in the presence of HBx in both HepG2 and SMMC7721 cells. Pretreatment of cells with the ERK inhibitor U0126 significantly reduced HBx-mediated increase of endogenous cyclin D1, accompanied with the decrease of the phospho-GSK-3β (Ser9) (Fig. 4A, lane 3 vs. lane 2), whereas the PI3K/Akt inhibitor LY294002 did not (Supplementary Fig. S2). Consistently, Western blot assays showed that the ERK inhibitor U0126 also inhibited upregulated ectopic expression of cyclin D1 by HBx (Fig. 4B, lane 3 vs. lane 2). Taken together, these results suggested that the HBx-activated ERK pathway played a major role in the inactivation of GSK-3β and upregulation of cyclin D1 expression.

HBx-induced cyclin D1 nuclear accumulation prompts HCC cell proliferation

The cyclin D1 T286A mutant, which localizes specifically to the nucleus of the cell, exhibits a cellular transformation capacity, suggesting that disruption of cyclin D1 nuclear export is an oncogenic event (23). To investigate whether the oncogenic potential of HBx is linked with the nuclear localization of cyclin D1, we detected the subcellular distribution of cyclin D1 in HBx-transfected cells by immunocytofluorescent staining. As shown in Fig. 5A, cyclin D1 accumulates predominantly in the nucleus of HepG2 cells and mouse fibroblast NIH3T3 cells transfected with HBx during S phase, whereas in control cells cyclin D1 showed an exclusively cytoplasmic pattern of localization. Cotransfection of the constitutively active GSK-3β-S9A together with HBx was able to reverse the nuclear accumulation of cyclin D1 induced by HBx. Consistently, flow-cytometric analysis confirmed the more rapid progression in HBx-expressing HepG2 cells, whereby the percentage of cells in S-phase entry was greater than control cells (5.13% vs. 3.53%) and the percentage of cells in G0–G1 phase decreased from 67.85% to 40.45% (Fig. 5B). A similar phenomenon was observed in SMMC7721 cells. It is also noteworthy that, consistent with the reversal of HBx-induced cyclin D1 nuclear localization, cotransfection of GSK-3β-S9A was able to reverse the binding of cyclin D1 to cyclin E and Cdk2, which is required for the G1-S transition in cell cycle progression (24). These results suggest that HBx-mediated inactivation of GSK-3β and phosphorylation of cyclin D1 at Thr286 is essential for the nuclear accumulation of cyclin D1 and its interaction with cyclin E and Cdk2, which is required for cell cycle progression. Therefore, inhibition of GSK-3β by HBx may contribute to the nuclear accumulation and cell cycle progression of cyclin D1, thereby promoting HCC cell proliferation.
proliferative activity induced by HBx alone. These results demonstrated that the nuclear localization of cyclin D1 induced by HBx was required for the cell proliferation and might be an oncogenic driver in HCC development.

The nuclear cyclin D1 expression is significantly correlated with HBx in HBV-related HCC

To further examine whether this relationship identified in vitro can also be observed in human tumor tissues, we compared the levels of HBx and nuclear expressions of cyclin D1 in 36 pairs of HBV-related HCC tissues by IHC. The results showed that the positive rates of nuclear cyclin D1 and HBx in HCC tissues were 33.3% (12 of 36) and 38.9% (14 of 36), respectively. A significant correlation was found between the accumulation of cyclin D1 in nucleus and the expression of HBx in these HBV-related HCC samples ($P = 0.0002$; Fig. 6A and B), suggesting that the nuclear localization of cyclin D1 induced by HBx may be a general phenomenon in HBV-related HCC.

**Discussion**

Although the multifunctional characteristics of viral protein HBx have been extensively studied, the pathogenic mechanism of HBx in human liver cancer remains largely unknown (2, 3). In this study, we provide the first evidence that by inactivating GSK-3β via inhibiting the ERK pathways, HBx is able to inhibit cyclin D1 nucleus export and subsequent proteasomal degradation. Importantly, our data suggest that the nuclear accumulation of cyclin D1 induced by HBx is an oncogenic driver in HCC cells. These observations highlighted the essential role of the cross-talk between HBx and cyclin D1 in HBV-related hepatocarcinogenesis.

In normal cells, the proteolysis of cyclin D1 protein is subject to a precise regulation by several mechanisms involving GSK3β-mediated phosphorylation of Thr286 (7), nuclear export by CRM1 (8, 24), and subsequent ubiquitylation by SCF3β-Trk-crystallin ligase targeting the cyclin D1 to the 26S proteasome (9). Deregulations of cyclin D1 proteolysis can lead to aberrant accumulation of cyclin D1 independent of alterations in CCND1 gene expression and translation. Indeed, mutations that directly disrupt on the phosphorylation of Thr286 (25, 26) or inactivate SCF3β-Trk-crystallin ligase have been identified in esophageal cancer and endometrial cancer (9, 27). In addition to these mutations, an alternative splice variant of cyclin D1, cyclin D1b, was identified in human cancer–derived cell lines. The characterization of cyclin D1b revealed that cyclin D1b lacked Thr286 and remained in nucleus through the cell cycle, where its constitutive expression facilitates cellular transformation (21). Additionally, the upstream oncogenic events, such as those targeting the Ras or Wnt pathway, can inhibit GSK-3β activity and thereby decrease cyclin D1 turnover (7, 28). Because no mutations at Thr286 of cyclin D1 in HCC tissues have been reported, it is reasonable to predict that frequent alterations in regulation of GSK-3β activity may play a major role in the nuclear accumulation of cyclin D1. Consistent with this notion, we observed that in HCC cell lines,
HBx can stabilize cyclin D1 and increase cyclin D1 nuclear accumulation through inactivation of GSK-3β by Ser9 phosphorylation. Importantly, more than 30% of primary HBV-related HCC specimens examined in this study showed an abnormal cyclin D1 accumulation in the nucleus, and a positive correlation between HBx and nuclear cyclin D1 protein level was established in these HCC specimens. These data provide the first evidence of HBx involvement in the GSK-3β–mediated cyclin D1 stabilization.

GSK-3β is a serine/threonine kinase that can phosphorylate several proteins that are involved in controlling the structural characteristics of the cells. Aberrant activity of GSK-3β has been implicated in the pathologies of many diseases and disorders (29). GSK-3β can be inactivated via phosphorylation at Ser9 by diverse kinases, including Akt and ERKs. It is known that the insulin signaling causes inactivation of GSK-3β (Ser9) by activated Akt following receptor tyrosine kinase (RTK) and G protein–coupled receptor stimulation of PI3K, while growth factors cause inactivation of GSK-3β (Ser9) by activated Raf/MEK/ERK signaling (30, 31). Recent work has revealed that both Akt and ERKs can be activated by HBx (18, 19); we therefore predicted that the inactivation of GSK-3β might correlate with the activation of Akt or ERKs triggered by HBx. Our analysis using specific inhibitors of the Akt and ERK pathways revealed that the HBx-activated ERK pathway plays an indispensable role in the inactivation of GSK-3β and nuclear accumulation of cyclin D1.

Accumulating data suggest that oncogenic functions of cyclin D1 depend upon its nuclear accumulation during S phase (16, 23). To date, there seem to be at least two major mechanisms of cyclin D1–mediated tumorigenesis attributed to its association with CDKs. The first mechanism is based on the capacity of cyclin D1 to activate CDKs and consequently promote S-phase entry. This hypothesis is supported by the observations that the constitutively active nuclear cyclin D1–T286A/CDK4 complex may perturb critical temporal and spatial timing of Rb-dependent growth control, resulting in S-phase entry and continuous proliferation (8, 32). Recently, experimental evidence linking the nuclear cyclin D1/CDK4 complex with induction of genomic instability (33) provided a novel mechanism wherein cyclin D1 stabilization initiates tumor formation. In mouse models and human cancer-derived cells, the cyclin D1–T286A/CDK4 complex was found to be able to trigger DNA rereplication during S phase, resulting from Cdt1 stabilization, which in turn induced genomic instability characterized by aneuploidy (33). Although the precise mechanism of this regulation remains to be uncovered, this

Figure 5.
The nuclear cyclin D1 prompted HCC cell proliferation. A, immunofluorescence assay was performed to measure the expression and cellular distribution of cyclin D1 in HepG2 and NIH-3T3 cells transfected with pCMV-HBx alone or pCMV-HBx plus pCMV-GSK-3β-S9A. B, flow-cytometric analysis of HepG2 and SMMC7721 cells transfected with plasmids as in A.
observation suggests that the cyclin D1/CDK4 kinase is likely to participate more directly in the neoplastic process. Consistent with these findings, our study demonstrated that the nuclear localization of cyclin D1 induced by HBx was required for the S-phase entry and might be an oncogenic driver in HCC cells. Further study should investigate whether the HBx-induced nuclear cyclin D1/CDK complex could trigger genomic instability and contribute to neoplastic growth.

In conclusion, our results, together with those in previous reports (18), provided a plausible mechanism for how HBx might facilitate cell proliferation and malignant transition (Fig. 6C). On the one hand, HBx activates ERKs, resulting in GSK-3β inactivation and consequently β-catenin stabilization, which in turn activates CCND1 transcription; on the other hand, the HBx-mediated GSK-3β inactivation abolishes its capability to phosphorylate cyclin D1 at Thr286 and blocks the phosphorylation-dependent nuclear export of the protein. As a consequence, cyclin D1 protein accumulates in the nucleus, where it can induce genomic instability and neoplastic growth. The nuclear retention of cyclin D1 may contribute to the malignant transformation of hepatocytes in HBV-related hepatocarcinogenesis and may be a potential target in the design of HCC therapy. However, further studies are needed to verify these results in HBx-expressing HCC cell lines by knocking down the expression of HBx protein.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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