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HBx Inhibits the Growth of CCL13-HBX-Stable Cells via the GSK-3β/β-Catenin Cascade

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Key Words
GSK-3β/β-catenin cascade · Hepatitis B virus X protein · Proliferation

Abstract
Objective: The hepatitis B virus X protein (HBx) plays critical roles in cell survival via modulation of signaling pathways. In our previous studies, we reported that HBx inhibited the growth of CCL13-HBx-stable cells (Chang-HBx cells) in vitro and tumor formation in vivo in CCL13-HBx-cell-injected nude mice; however, this inhibition mechanism is unclear. ***Methods:*** To investigate the role of HBx in Wnt-3/β-catenin signaling pathways, we focused on the key molecules GSK-3β and β-catenin, and analyzed by Western blotting and immunofluorescence staining. ***Results:*** Results indicated that following HBx induction, GSK-3β activity was up-regulated, the expression and accumulation of β-catenin in the nucleus were decreased, and cell proliferation was suppressed. Inhibition of GSK-3β activity by pharmacological inhibitors rescued the expression and accumulation of β-catenin in the nucleus and facilitated cell proliferation and growth following HBx induction. The localization of β-catenin, which is involved in cell proliferation, and mediated by GSK-3β activation was also demonstrated. ***Conclusion:*** Our findings suggest that HBx negatively regulated proliferation of CCL13-HBx-stable cells via the GSK-3β/β-catenin cascade.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. The hepatitis B virus X protein (HBx) is a well-known risk factor and plays critical roles in the development of HCC. To study the roles of HBx in HCC, the expression of the HBx antigen and anti-HBx antibodies in cases of HCC was examined. The results indicated that the sera from 70% of HCC patients and 5% of chronic hepatitis (CH) patients contained antibodies that demonstrated significant binding to the HBx protein. Further, 85% of HCC patients' liver tissues contained a specific HBx protein [1]. Moreover, we have also demonstrated that HBx inhibits the growth of CCL13-HBx-stable liver cells and that it inhibits tumorigenicity in CCL13-HBx-cell-injected nude mice [2]. Currently, it is known that HBx mediates cell growth via apoptosis or proliferation and that it is associated with signal transduction cascades, which include the ras/raf/MAPK, PKB/Akt, and JAK/STST pathways. Although HBx has been regarded as a multifunctional protein that is involved in transcriptional factors and signaling pathways [3], the critical roles of HBx remain unclear. Recently, there are reports regarding an association between the Wnt/β-catenin pathway and the development of HCC. Mutations in β-catenin or CTNNB1 or AXIN-1 or AXIN-2 causing β-catenin activation were observed in 20–90% of HCC cases [4]. Also, overexpression of the inactive form of GSK-3β and β-catenin was higher in HCC tissues than in surrounding tissues [5]. Merle et al. [6] reported that FZD7 was highly

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overexpressed in HCC and occurred in dysplasia. In addition, the results also indicated a correlation between HBx and β-catenin accumulation in HCC tumor tissues [7].

The acceleration of liver cancer, accumulation of β-catenin, and activation of Wnt/β-catenin were observed in an animal model, namely, c-Myc/E2F1 double transgenic mice [8]. Furthermore, the downregulation of GSK-3β activity in HCC cells was reported [9]. Further, Cha et al. demonstrated that HBx is essential for the activation of Wnt-1 signaling in Huh7 cells [10]. These findings raise the possibility of an association between HBx and the Wnt/β-catenin signaling pathway in HCC development.

GSK-3β and β-catenin are the key molecules in the Wnt/β-catenin signaling pathway. In the absence of Wnt, the downstream molecule GSK-3β is activated, β-catenin is degraded by ubiquitination, and the resulting transcriptional inactivation inhibits cell proliferation and alters cell fate [11]. It has been reported that GSK-3β activity is significantly facilitated by Tyr216 phosphorylation and reduced by the phosphorylation of an N-terminal Ser9 [12, 13]. Furthermore, both Cha et al. [10] and Ding et al. [7] suggested that HBx may cause β-catenin stabilization via GSK-3β inactivation. Further, nuclear translocation of β-catenin was observed following the cotransfection of Huh7 cells with HBx and Wnt-1 [10]. The nuclear accumulation of β-catenin provides a proliferative advantage during tumor formation in c-Myc/E2F1 double transgenic mice [8]. However, according to our previous report [2], which is consistent with many other studies, HBx medicates or sensitizes cells to apoptosis [14–23] and causes cell cycle arrest in the G1 phase [24]. Consequently, in this study, we explored the possibility that HBx negatively regulated the proliferation of CCL13-HBx-stable cells via the GSK-3β/β-catenin cascade. In the activated GSK-3β state, we investigated the expression and nuclear accumulation of β-catenin in CCL13-HBx-stable cells following HBx induction. We also used two specific inhibitors TDZD-8 and BIO to study the effects on GSK-3β activity. In this study, we propose that HBx downregulates the expression and nuclear accumulation of β-catenin in a CCL13-HBx-stable cell line via GSK-3β activation.

Materials and Methods

Cell Lines and Cell Culture

A CCL13-HBx-stable cell line was established in our laboratory under the culture conditions described by Wang et al. [2]. Briefly, cells were grown and maintained in DMEM supplement with 10% fetal bovine serum (FBS) and 1.5 μg/ml tetracycline. The culture medium was replaced every alternate day.

Nuclear Fraction Extraction

The nuclear fraction was extracted from the CCL13-HBx-stable cells. The cells were collected and resuspended in a hypotonic buffer (10 mM HEPES, pH7.9; 10 mM KCl; 1.5 mM MgCl2; 0.2 mM PMSF; 20 μg/ml aprotinin; 0.5 mM DTT; and 0.5% NP-40) on ice for 15 min. After centrifuging at 6,000 *g* for 15 min at 4°, the pellet was collected and then washed with basal buffer (hypotonic buffer without 0.5% NP-40). After centrifuging again at 6,000 *g* for 15 min at 4°, the pellet was collected and resuspended in a hypertonic buffer (20 mM HEPES, pH 7.9; 400 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM PMSF; 20 μg/ml aprotinin; 0.5 mM DTT; 0.2 mM EDTA; 10% glycerol) at room temperature for 30 min. After centrifuging at 10,000 *g* for 30 min at 4°, the nuclear fraction contained in the supernatant was collected.

Western Blotting

Proteins extracted from the CCL13-HBx-stable cells were subjected to Western blotting. The membranes were immunodetected using anti-HBx (ab235, Abcam), anti-GSK-3β (#9315, cell signaling), anti-phospho-GSK-3β^{Tyr216} (#612313, BD), anti-β-catenin, anti-proliferating-cell nuclear antigen (PCNA), anti-actin (A5316, Sigma), and anti-histone H1 antibodies (sc-56694, Santa Cruz). An ECL kit (Millipore) was used according to the manufacturer's instructions.

Cell Viability

Cells were seeded in a 6-well culture dish at a concentration of 0.5×10^5 cells per well and were grown in the presence of 1.5 μg/ml tetracycline in DMEM culture medium containing 10% FBS. The culture medium was replaced once in 2 days. Further, the culture medium lacking 1.5 μg/ml tetracycline was replaced every alternate day, and the cells were counted until they attained approximately 60–70% confluence. After the cells were counted, they were stained with trypan blue.

Immunofluorescence Staining

The CCL13-HBx-stable cells were fixed in 3% formaldehyde, blocked with PBS containing 3% FBS, and incubated with anti-HBx antibody (ab235, Abcam) or anti-β-catenin antibody (sc-7963, Santa Cruz). Subsequently, the cells were incubated with a FITC-labeled secondary antibody (Jackson Immunoresearch Laboratories). The nuclei were stained with diamidino-2-phenylindole (DAPI, Molecular Probes), which is a DNA groove-binding dye, and the cells were examined under a Nikon E400 microscope.

Results

Expression of Nuclear β-Catenin Was Downregulated following HBx Induction

Nuclear β-catenin has been reported as the key molecule of the activated Wnt pathway [25]. In this study, we used the CCL13-HBx-stable cell line established in our laboratory in an HBx-inducible expression system that is regulated by a tetracycline-dependent expression system (tet-off system) [2, 26]. To elucidate the effect of HBx on the Wnt pathway, the expression of nuclear β-catenin was

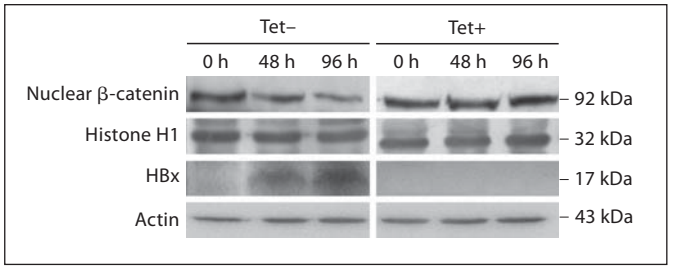

Fig. 1. In the tet-off system, HBx was expressed following tetracycline deprivation. Expression of nuclear β-catenin was downregulated following HBx induction. The nuclear fraction was extracted from cells with time in the absence (Tet–, left panel) and presence (Tet+, right panel) of tetracycline. The expression of nuclear β-catenin was downregulated following HBx induction at 48 and 96 h (Tet–, left panel), but its expression was unaltered in the presence of tetracycline (Tet+, right panel). Histone H1 and actin were used as the internal controls.

measured using Western blotting (fig. 1). The data revealed that the expression of nuclear β-catenin was downregulated following HBx induction (fig. 1, Tet–) but was not altered in the presence of tetracycline (fig. 1, Tet+). To conclusively illustrate the effect of HBx on β-catenin, we used immunofluorescence staining to detect the localization of β-catenin in a time-dependent manner (fig. 2). The data showed that the cells were intensely stained at the nucleus by anti-β-catenin in the absence of HBx induction and in the presence of tetracycline at 0 and 96 h (fig. 2, Tet+, middle panel) or at 0 h under conditions of tetracycline deprivation (fig. 2, Tet–, upper panel). Contrastingly, the membrane and prenucleus was stained following HBx induction at 96 h under conditions of tetracycline deprivation (fig. 2, Tet–, upper panel). The two major functions that β-catenin is involved are cell adhesion and the Wnt pathway. Localization-dependent functions of β-catenin have been reported. In the cell membrane, β-catenin acts as a structural component of cadherin-mediated cell adhesion; it also plays the role of a transcriptional coactivator involved in the Wnt pathway and is usually found in the cytoplasm and nucleus [27]. Therefore, the results suggested that the accumulations of nuclear β-catenin were decreased following the HBx induction of CCL13-HBx-stable cells.

HBx Negatively Regulated the Proliferation of CCL13-HBx-Stable Cells via the GSK-3β/β-Catenin Cascade

β-Catenin is the target of GSK-3β and is degraded more rapidly by GSK-3β expression [28]. To demonstrate the effects of GSK-3β on β-catenin in the presence of

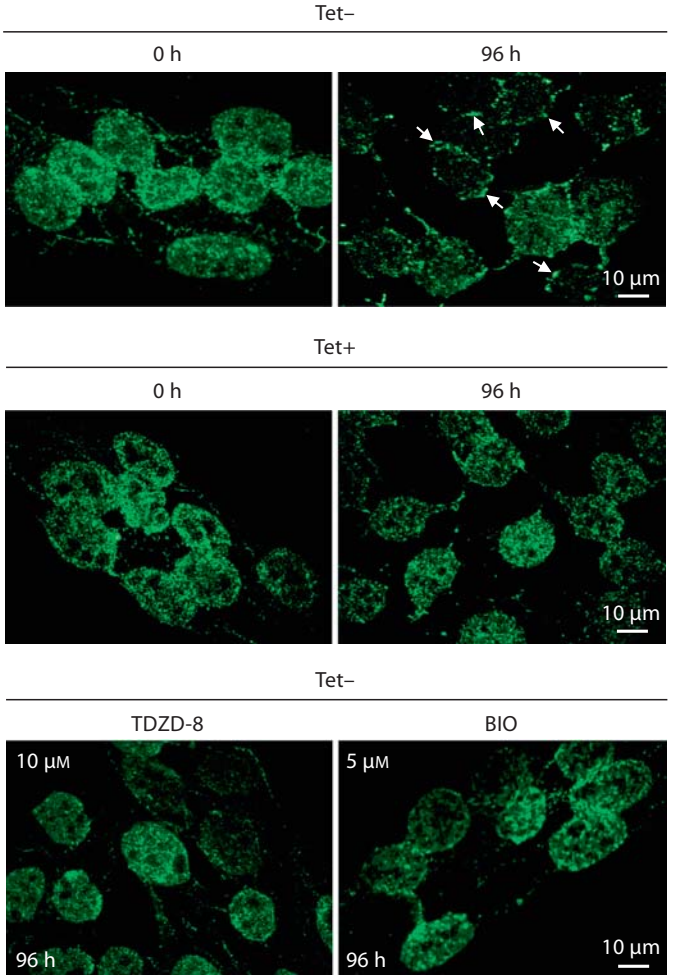

Fig. 2. Subcellular localization of β-catenin was observed in CCL13-HBx-stable cells and affected by GSK-3β inhibitors. The β-catenin signal was intensely stained in the nucleus in the absence of HBx induction at 0 and 96 h, in the presence of tetracycline (Tet+, middle panel) or 0 h after tetracycline deprivation (Tet–, upper panel). The distribution of β-catenin localized in the prenucleus was observed in HBx induction at 96 h (Tet–, upper panel, arrows indicated). The distribution of β-catenin localized in the nucleus was observed in the presence of inhibitors following HBx induction at 96 h (Tet–, lower panel); a similar pattern was observed under natural culture conditions at 0 h without inhibitors (Tet–, 0 h). Bar = 10 μm.

HBx in CCL13-HBx-stable cells, we used two specific GSK-3β inhibitors to analyze the expressions of β-catenin in the total lysate and nuclear extract by using Western blotting (fig. 3, 4). TDZD-8 and BIO are both well-established inhibitors of GSK-3β [29, 30]. To demonstrate the inhibitory effects of these two inhibitors on GSK-3β activity in CCL13-HBx-stable cells, we detected the expres-

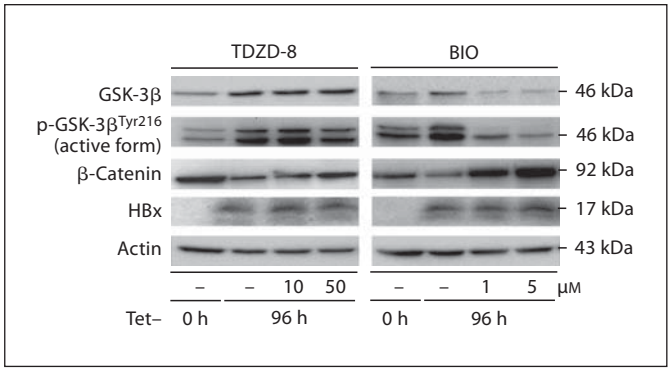

Fig. 3. Effects of treatment with different concentrations of the GSK-3β inhibitors TDZD-8 and BIO following HBx induction (Tet–) were detected by Western blotting. Including the active form of GSK-3β (phospho-GSK-3β^{Tyr216}), the total GSK-3β was up-regulated, while β-catenin was downregulated following HBx induction without inhibitors. In the presence of the inhibitor BIO, the active form of GSK-3β (phospho-GSK-3β^{Tyr216}) was downregulated in a dose-dependent manner; however, there was only a slight decrease in its levels in the presence of 50 μM TDZD-8. The total GSK-3β was downregulated in the presence of 1 or 5 μM BIO; however, its levels were not altered in the presence of TDZD-8. The expressions of β-catenin were downregulated following HBx induction in the absence of GSK-3β inhibitors and up-regulated in the presence of the inhibitors TDZD-8 and BIO in a dose-dependent manner. Actin was used as the internal control.

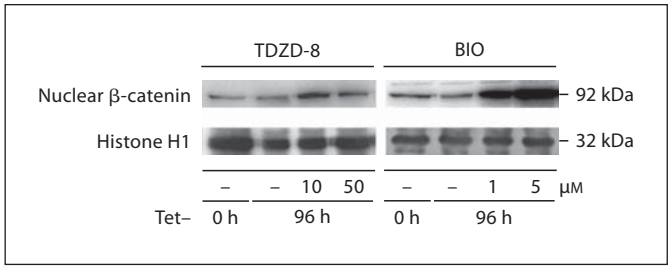

Fig. 4. Expression of nuclear β-catenin was up-regulated by the inhibition of GSK-3β activity following HBx induction. The nuclear fractions were extracted from cell culture having different concentrations of the GSK-3β inhibitors TDZD-8 and BIO after HBx induction (Tet–), and Western blotting was then performed. The expression of nuclear β-catenin was up-regulated in the presence of the inhibitors TDZD-8 and BIO. Histone H1 was used as the internal control.

sion of phospho-GSK-3β^{Tyr216}, an active form of GSK-3β [12, 13], in a dose-dependent manner (fig. 3). In absence of inhibitors, the expressions of phospho-GSK-3β^{Tyr216} and total GSK-3β were up-regulated following HBx induction. In the presence of BIO, the expression of phospho-GSK-3β^{Tyr216} was decreased in a dose-dependent manner but only slightly decreased in the presence of 50 μM TDZD-8. The expression of total GSK-3β was decreased in the presence of 1 and 5 μM BIO but not altered in the presence of TDZD-8 (fig. 3, upper panel). Yoshino et al. [31] have demonstrated that the inhibitor TDZD-8 affected the phosphorylation of GSK-3β but had no effect on the expression of total GSK-3β. Moreover, the results also showed that the expressed β-catenin, which is the target of GSK-3β, was degraded more rapidly following the expression of activated GSK-3β [28]. Further, while β-catenin expression was downregulated following HBx induction in the absence of GSK-3β inhibitors, it was up-regulated in the presence of GSK-3β inhibitors (fig. 3, middle panel).

The nuclear accumulation of β-catenin is one of the best indications of activation of the Wnt pathway [25]. In addition, activated GSK-3β causes the phosphorylation, subsequent degradation, and decreasing nuclear accumulation of β-catenin [32]. In this study, the data showed that following HBx induction, the expression of nuclear β-catenin was downregulated (fig. 1, Tet–), while that of phospho-GSK-3β^{Tyr216}, an active form of GSK-3β, was up-regulated (fig. 3). The inhibitory effect of the inhibitors on phospho-GSK-3β^{Tyr216} and GSK-3β is observed (fig. 3). Further, the expression of nuclear β-catenin was up-regulated in the presence of inhibitors (fig. 4).

To conclusively demonstrate the effect of inhibitors on β-catenin, we used immunofluorescence staining to detect the localization of β-catenin in the CCL13-HBx cells (fig. 2, Tet–, lower panel). The results showed that following HBx induction, the cells were intensely stained in the prenucleus in the absence of inhibitors; however, following HBx induction, β-catenin in the nucleus was stained in the presence of inhibitors at 96 h; a similar pattern of staining was observed at 0 h under natural culture conditions without inhibitors (fig. 2, Tet–, 0 h, upper panel). Accordingly, the data suggested that the GSK-3β inhibitors TDZD-8 and BIO suppressed GSK-3β activation and rescued the expression and nuclear accumulation of β-catenin in the CCL13-HBx-stable cell line. To determine whether GSK-3β activation is effective in the HBx-mediated repression of cell proliferation, we detected the expression of PCNA by Western blotting. PCNA is considered a marker of cell proliferation during the G1 phase and reaches maximal levels during the S phase [33]. Based on our data, at 96 h, GSK-3β downregulated the expression of PCNA following HBx induction, whereas PCNA expression was up-regulated in the presence of 50 μM TDZD-8 and 5 μM BIO (fig. 5). GSK-3β suppressed cell

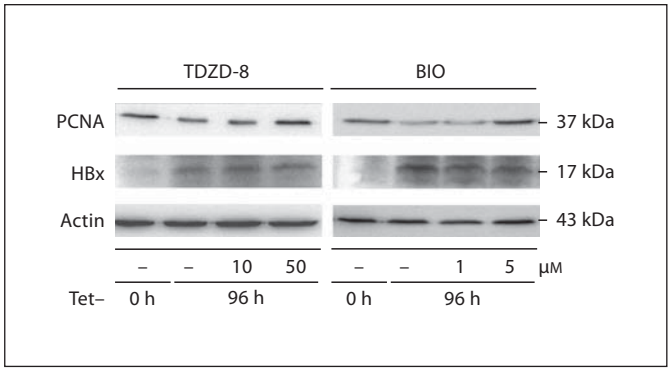

Fig. 5. Using Western blotting, PCNA expression was detected in the CCL13-HBx-stable cell line in the presence of the GSK-3β inhibitors. PCNA expression was downregulated in absence of the inhibitors TDZD-8 and BIO at 96 h, but was up-regulated in the presence of 50 μM TDZD-8 and 5 μM BIO following HBx induction. Actin was used as the internal control.

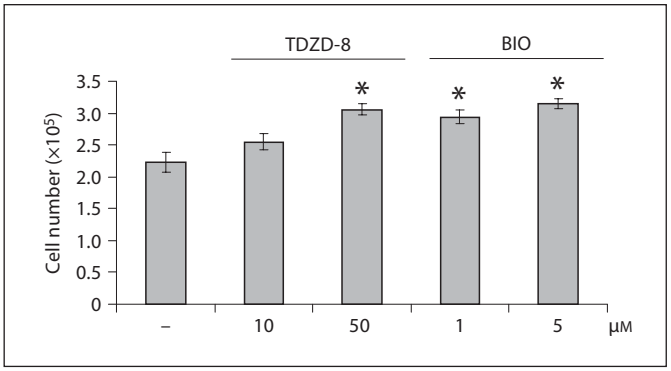

Fig. 6. Cell growth was detected in the presence of GSK-3β inhibitors. As compared to in the absence of the inhibitors, the cell number was significantly increased in the presence of 50 μM of inhibitor TDZD-8, and 1 and 5 μM of inhibitor BIO. The asterisk indicates a statistically significant difference between the two groups (* $p < 0.05$). The cells were counted as the mean of triplicate.

growth following HBx induction in the absence of the inhibitors TDZD and BIO, whereas it promoted cell growth in the presence of 50 μM TDZD-8 and in the presence of 1 or 5 μM BIO (fig. 6). The data suggested that the inhibition of GSK-3β activity by pharmacological inhibitors promoted the proliferation of CCL13-HBx-stable cells after HBx induction.

Discussion

The aim of this study was to investigate the possibility that HBx negatively regulates the proliferation of CCL13-HBx-stable cells via the GSK-3β/β-catenin cascade. The data indicated that following HBx induction, activated GSK-3β suppressed the expression and nuclear accumulation of β-catenin in CCL13-HBx-stable cells. However, the role of HBx in cell proliferation or apoptosis remains debatable and conflicting. HBx is involved in multiple signaling pathways, and it is possible that the reason for conflicting results may be because studies have investigated different signaling cascades in various cell types or experimental systems [16, 24]. Many studies have demonstrated that HBx mediates or sensitizes cells to apoptosis [14–23]; however, there are also reports that HBx protects cells from or suppresses apoptosis [34–36]. Recently, Jung et al. [37] reported that the contradictory effects of HBx on β-catenin ubiquitination depend on the status of p53. They also suggested that the β-catenin stabilization was mediated by the classical $SCF^{\beta\text{-}TrCP}$-ubiquitin ligase complex in a p53-independent manner or the Siah-$1^{SIP\text{-}Skp1\text{-}Ebi}$ complex in a p53-dependent manner following HBx induction. The $SCF^{\beta\text{-}TrCP}$-ubiquitin ligase complex is involved in Wnt signaling; phosphorylated β-catenin is recognized by this complex and finally degraded in proteasomes in the absence of Wnt signaling [38]. The report by Jung et al. [37] also provides a good basis for speculation regarding the dual effects of HBx on the proliferation or apoptosis in various types of hepatic cell lines.

Following HBx induction, we observed the inhibition of growth of CCL13-HBx-stable cells and tumorigenicity in CCL13-HBx-cell-injected nude mice in a previous study [2]. In addition, studies have demonstrated that the expression of GSK-3β is up-regulated, that of β-catenin is downregulated upon HBx induction, and growth inhibition following HBx induction in CCL13-HBx-stable cells [26]. The results of this study showed that following HBx induction, the expression and nuclear accumulation of β-catenin were suppressed by activated GSK-3β. Therefore, we suggest that HBx negatively regulates the proliferation of CCL13-HBx-stable cells via the GSK-3β/β-catenin cascade.

GSK-3 is a multifunctional serine/theronine kinase and a key regulator of the Wnt signaling pathway, the cellular response to the receptor of tyrosine kinase and G-protein-coupled receptors. Further, it is involved in cell cycle regulation and proliferation. GSK-3 dysregulation has been reported to be involved in the development of human diseases such as Alzheimer's disease, diabetes, and cancer [12]. GSK-3α and GSK-3β are the two iso-

forms of GSK-3 [39]. Currently, the role of GSK-3β in the tumorigenesis of colorectal cancer, pancreatic cancer, and hepatocellular carcinoma has also been reported [40–42]. Beurel et al. [40] suggested that GSK-3β triggered the apoptosis of hepatoma cells by PI3K inhibition and that it could be a possible mechanism that protected cancer cells from programmed cell death during chemotherapy. In addition, a report also demonstrated that GSK-3β overexpression increased apoptosis in cultured cortical neurons [43]. However, Shakoori et al. [42] reported that the inhibition of GSK-3β expression and activity attenuated cell proliferation and survival and induced apoptosis in colon cancer cells. GSK-3β has been demonstrated to positively regulate the proliferation and survival of ovarian cancer cells [43]. Reports regarding the inhibition of TNF-α-induced NF-κB activation and apoptosis in hepatocytes mediated by GSK-3β have been published [45]. Consequently, the dual role of GSK-3β in cell proliferation and apoptosis has been reported. Therefore, it is possible that GSK-3β is involved in various signaling pathways and that it may act as a tumor suppressor in the Wnt signaling pathway while also playing a role in promoting the survival and proliferation of cancer cells in the presence of activated NF-κB [44–47].

In summary, the results of this study showed that following HBx induction, GSK-3β negatively regulated the expression and accumulation of nuclear β-catenin and suppressed the proliferation of CCL-13-HBx-stable cells. We also observed that the inhibition of GSK-3β activity by pharmacological inhibitors increased the expression and accumulation of nuclear β-catenin and promoted the proliferation of CCL13-HBx-stable cells after HBx induction. Therefore, we propose that HBx negatively regulates the proliferation of CCL13-HBx-stable cells via the GSK-3β/β-catenin cascade.

Acknowledgments

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