Notch1 promotes hepatitis B virus X protein-induced hepatocarcinogenesis via Wnt/β-catenin pathway

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Abstract. Hepatitis B virus X protein (HBx) is implicated in the pathogenesis of hepatocellular carcinoma (HCC) via a network of signaling pathways. Notch pathway is a major member of the network. Notch signaling may generate opposing effect in different steps of carcinogenesis, depending on the tumor cell type and the status of other signaling pathways, such as Wnt signaling pathway. Our previous studies have shown that activated Notch1 signaling is required for HBx to promote proliferation and survival of human hepatic cell line L02. However, the exact mechanisms remain vague. Here, we used L02/HBx cell lines as a cell model to study the relationship between Notch and Wnt/β-catenin pathways in promoting proliferation. We observed that activated Notch1 and Wnt/β-catenin signaling pathways and L02 cell malignant transformation were induced by HBx. Inhibition of the Notch1 pathway decreased the activation of Wnt/β-catenin pathway and cell proliferation, while inhibition of the Wnt/β-catenin pathway impaired cell proliferation, but did not significantly affect Notch1 signaling pathway in L02/HBx cells. Furthermore, inhibition of the Wnt/β-catenin pathway overcame the inhibition effect of knockdown Notch1 on proliferation and survival in L02/HBx cells. Additionally, the activity of Wnt/β-catenin signaling appears to be consistent with Fzd10 expression. Therefore, we demonstrate that Wnt signaling is downstream of the Notch pathway in regulating proliferation of L02/HBx cells, and which may be related to Fzd10 instead of Fzd7. These data suggest a new model of HBx-related HCC via cooperation between Wnt and Notch pathways.

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Abbreviations: HCC, hepatocellular carcinoma; HBx, hepatitis B virus X protein; Hes, hairy and enhancer of split family; qRT-PCR, quantitative real-time RT-PCR; Fzd, Frizzled family of Wnt receptors

Key words: Notch1, hepatitis B virus X protein, hepatocarcinogenesis, Wnt/β-catenin pathway

Introduction

As one of the most common malignancies in the world, hepatocellular carcinoma (HCC) has a very high morbidity and mortality (1). Risk factors of HCC have been well established, and chronic hepatitis B virus (HBV) infection is a major cause in China. The smallest open reading frame of the HBV genome, HBX, which encodes the hepatitis B virus X protein (HBx), has been implicated in hepatocarcinogenesis and considered to be oncogenic (2). HBx interacts with many signal pathways in the initiation of hepatocarcinogenesis, including AKT/PKB, ERK1/2, SAPK, NF-κB signal transduction pathway (3). Recent studies have indicated the role of Notch pathway in HBx-related HCC (4-8). According to the conventional model, Notch itself is a cell-surface receptor that transduces short-range signals by interacting with transmembrane ligands such as Delta (termed Delta-like in humans) and Serrate (termed Jagged in humans) on neighboring cells. Proteolytic cleavage within the transmembrane subunit of the Notch receptor results in translocation of the intracellular domain of Notch (ICN) to the nucleus where it interacts with the transcriptional repressor CSL, also known as CBF-1 or RBP-Jκ. Binding of ICN displaces co-repressor complexes, thereby activates transcription by promoters with CSL binding elements and then modulates the expression of many genes, such as Hes1 (9-11). Notch signaling may generate opposing effect in different steps of carcinogenesis, depending on the tumor cell type and the status of other signaling pathways, such as Wnt signaling pathway (12).

The effect of Notch in HCC is highly controversial. Some studies have described a direct role of Notch in promoting HCC. Giovannini *et al* (13) reported aberrant nuclear expression of Notch1 and Notch3 in HCC tissues compared with the surrounding cirrhotic tissues. Furthermore, silencing of Notch3 in HCC cells enhanced sensitivity to doxorubicin-induced cell death via a p53-dependent mechanism. In keeping with the view, Villanueva *et al* (14) reported that Notch signaling was activated in human HCC samples and promoted formation

of liver tumors in mice. Other groups, however, have pointed out the different role of Notch signaling in HCC. Qi *et al* (15) demonstrated that Notch1 induced apoptosis of HCC cells by altering the balance between p53 and Bcl-2. Consistent with the observation, upregulation of p53 induced by Notch1 sensitized HCC cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (16). These discoveries were further confirmed in a mouse model of HCC generated by genetic inactivation of the retino blastoma pathway, activation of the Notch signaling reduced HCC cell proliferation and tumor growth (17). In a word, contradictory data exist on the effect of Notch signaling in HCC, and its role in HBx-related HCC is even less documented. Our previous studies have shown that activated Notch1 signaling is required for HBx to promote proliferation and survival of L02 (4,7). However, the exact mechanisms remain elusive.

To address this question, we used L02/HBx cell lines as a cell model to study the relationship between Notch and Wnt/β-catenin pathways in promoting proliferation. These data suggested that Wnt pathway might be involved in Notch1-mediated effects.

The Wnt signaling pathway has long been recognized for its role in regulating embryonic development, and has recently been linked to cancer in adults (18). Canonical Wnt signaling is the most well-known pathway, which is centered around β-catenin. At the cell membrane, Wnt binds to Frizzled receptors and co-receptors such as low density lipoprotein receptor-related proteins (LRP). This results in stabilization of β-catenin through disheveled-mediated inhibition of the destruction complex. Soluble β-catenin then translocates to the nuclei and displaces groucho-related repressor from T cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factor. TCF/LEF is then able to regulate expression of target genes, such as cyclin D1 (19).

There is now emerging evidence of cross-talk between Notch and Wnt pathways in cancer. Camps *et al* (20) identified LNX2, a gene that was not associated with cancer before, as mediating cross-talk between WNT and NOTCH signaling cascades in colorectal cancer. Li *et al* (21) found evidence of cross-talk in non-small cell lung cancer. However, little is known about cross-talk between Notch and Wnt signaling pathways in HCC, and in HBx-related HCC cross-talk and the potential role of Wnt signaling are even less documented.

In this study, we address the question of how Notch signaling can modulate HBx-related HCC and discover novel observations that close cooperation between Notch and Wnt pathways is important in HBx-related HCC.

Materials and methods

Cell culture. The human non-tumor hepatic cell line L02 was a generous gift from Dr Xinyuan Liu (Shanghai Institutes for Biological Sciences, Shanghai, China). This cell line was originated histologically from normal human liver tissue immortalized by stable transfection with the hTERT gene, which has been used previously (22). L02/HBx and L02/pcDNA3.1 cell lines, which derived from L02 cells by transfection with HBx expression plasmid or its empty plasmid (pcDNA3.1(+)/V5-HisB), respectively, were successfully established previously (23). All cell lines were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in humidified incubator at 37˚C in a 5% CO_2 atmosphere, L02/pcDNA3.1 and L02/HBx cell lines were supplemented with 250 μg/ml G418 (Invitrogen, Carlsbad, CA, USA).

RNA isolation, quantitative real-time PCR (qRT-PCR). Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) and cDNA was synthesized from 100 ng RNA using PrimeScript RT reagent kit (Takara, Japan). Real-time quantitative PCR using SYBRP remix (Takara) was performed as described previously (8). Amplifications were performed in a Step One Real-Time PCR system (Applied Biosystems, USA) following the manufacturer's instructions. The expression of RNA was determined from the threshold cycle (Ct) and the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. All samples were assayed in triplicate. The primer sequences used to amplify specific target genes are listed in Table I.

Western blot analysis. Cells were lysed as described (8). Protein (40 μg) from each sample was examined by SDS-12% PAGE and then electrotransferred to nitrocellulose membranes using a semidry transfer apparatus (Bio-Rad). Nitrocellulose membranes were subsequently blocked with 5% BSA in TBST for 2 h at room temperature, and incubated with each primary antibody overnight. Rabbit anti-Notch1, anti-Fzd7, anti-Fzd10, anti-β-catenin, anti-cyclin D1 were purchased from Proteintech Group (Proteintech Group, Chicago, IL, USA), and rabbit anti-Hes1, anti-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were washed and incubated with horseradish peroxidase-labeled secondary antibody (1:4,000; Santa Cruz Biotechnology), visualization was performed by an enhanced chemiluminescence kit (Pierce) and exposure to X-ray film (Kodak, Rochester, NY, USA). Immunoblotting with anti-actin antibody was used as an internal control to confirme quivalent protein loading. Each experiment was repeated three times.

Transfection and RNA interference. L02/HBx cells were seeded in 6-well plates. The next day the cells (30-50% confluence) were treated with Notch1 siRNAs or Fzd10 siRNAs, and control siRNA (which does not match any known mammalian GenBank sequences). The siRNA sequences are listed in Tables II and III. All siRNAs were purchased from RiboBio (Guangzhou, China). Cells were transiently transfected with Notch1 siRNAs or Fzd10 siRNAs using Lipofectamine™ 2000 (Invitrogen). Media were replaced 6 h after transfection. Cells were allowed to grow for 48 h and harvested to choose the sequence of maximum inhibition effect on Notch1 and Fzd10, based on which recombinant plasmids pH1-MCS-CMV-GFP-Hygro-Notch1 shRNA and -Fzd10 shRNA were constructed for further stable transfection. After 48 h, transfected cells were selected for 3 weeks with 100 μg/ml hygromycin B (Sigma, St. Louis, MO, USA) to obtain stable Notch1-knockdown cell lines (Notch1-shRNA), Fzd10-knockdown cell lines (Fzd10-shRNA) and negative control cells (NC). Individual colonies were picked and then the stable cells were expanded with 50 μg/ml hygromycin B.

Table I. Primer sequences for quantitative RT-PCR analysis.

Gene	Sequences	PCR product (bp)	GenBank accession no.
Notch1			
Sense	5'-CCGCAGTTGTGCTCCTGAA-3'	109	NM_017617
Antisense	5'-ACCTTGGCGGTCTCGTAGCT-3'		
Hes1			
Sense	5'-GCTAAGGTGTTTGGAGGCT-3'	122	NM_005524
Antisense	5'-CCGCTGTTGCTGGTGTA-3'		
Fzd7			
Sense	5'-TTCTCGGACGATGGCTACC-3'	132	NM_003507
Antisense	5'-GAACCAAGTGAGAGACAGAATGACC-3'		
Fzd10			
Sense	5'-CCCGATTATGGAGCAGTTCA-3'	117	NM_007197
Antisense	5'-TCGTCCGAGCCGTTGTT-3'		
cyclin D1			
Sense	5'-GGCTGAAGTCACCTCTTGGTTACAG-3'	177	NM_053056
Antisense	5'-TAGCGTATCGTAGGAGTGGGACAG-3'		
Actin			
Sense	5'-GTTGCGTTACACCCTTTCTTG-3'	157	NM_001101
Antisense	5'-GACTGCTGTCACCTTCACCGT-3'		

Table II. Three siRNA sequences against Notch1 sequence (NM_017617).

Target sequences		siRNA sequences
siRNA1		
GGTGTCTTCCAGATCCTGA	Sense	5'-GGUGUCUUCCAGAUCCUGA dTdT-3'
	Antisense	3'-dTdT CCACAGAAGGUCUAGGACU-5'
siRNA2		
TGGCGGGAAGTGTGAAGCG	Sense	5'-UGGCGGGAAGUGUGAAGCG dTdT-3'
	Antisense	3'-dTdT ACCGCCCUUCACACUUCGC-5'
siRNA3		
GGACCAACTGTGACATCAA	Sense	5'-GGACCAACUGUGACAUCAA dTdT-3'
	Antisense	3'-dTdT CCUGGUUGACACUGUAGUU-5'

Table III. Three siRNA sequences against Fzd10 sequence (NM_007197).

Target sequences		siRNA sequences
siRNA1		
CGATTATGGAGCAGTTCAA	Sense	5'-CGAUUAUGGAGCAGUUCAA dTdT-3'
	Antisense	3'-dTdT GCUAAUACCUCGUCAAGUU-5'
siRNA2		
GCTACAACATGACTCGTAT	Sense	5'-GCUACAACAUGACUCGUAU dTdT-3'
	Antisense	3'-dTdT CGAUGUUGUACUGAGCAUA-5'
siRNA3		
CCATCCAGTTGCACGAGTT	Sense	5'-CCAUCCAGUUGCACGAGUU dTdT-3'
	Antisense	3'-dTdT GGUAGGUCAACGUGCUCAA-5'

Construction of Fzd10-expression vector and transfection. Fzd10 gene was obtained from cDNA library via PCR, and then the Fzd10 gene was cloned into *Xho*I and *Kpn*I sites of pCMV-MCS-3FLAG-SV40-Puromycin (pCV061) expression vector using T4 DNA ligase at 16˚C overnight. The resulting construct was confirmed by DNA sequencing. cDNA library and pCV061 were purchased from GeneChem (Shanghai, China). The primerse quences used to amplify Fzd10 gene: sense 5'-TCCGCTCGAGATGCAGCGCCCGGGCCCCC GCCTGT-3', antisense 5'-ATGGGGTACCGACACGCAGG TGGGCGACTGGGCAG-3'. Ninety percent-confluent Notch1-shRNA cells were transfected with pCV061-Fzd10 plasmids or pCV061 control vector using Lipofectamine 2000, respectively (Invitrogen). After 6 h of transfection, cells were washed and allowed to recover in DMEM medium supplemented with 10% fetal calf serum. After 48 h, transfected cells were selected for 3 weeks with 2 μg/ml puromycin (Sigma) to obtain stable Fzd10-overexpressed cell lines (pCV061-Fzd10) and control cells (pCV061). Individual colonies were picked and then the stable cells were expanded with 1 μg/ml puromycin.

Cell proliferation and viability assay. Cells were seeded in a 96-well plate with 10^4 cells/well and Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) was used to test cell proliferation at the indicated times (24, 48, 72 and 96 h). The absorbance value at 450 nm was measured using a spectrophotometer after incubation with WST-8 reagent (10 μl) for 2 h; 630 nm was the reference wavelength. All experiments were repeated eight times.

Analysis of colony formation. For clonogenicity analysis, 1000 viable cells were placed in 6-well plates and maintained in complete medium for 2 weeks. Colonies were fixed with methanol and stained with methylene blue.

Xenograft tumor model. Twelve male BALB/c-nu/nu mice (BW, approximately 19 g; age, 4 weeks) were purchased from the animal centre of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Huazhong University of Science and Technology. The mice were randomized into two groups (n=6 in each group), and housed in the Animal Institute of Tongji Medical College in laminar flow cabinets. Cells ($2x10^6$) (NC and Fzd10-shRNA) in 0.2 ml each were injected subcutaneously into the posterior neck of each nude mouse. The length (L) and width (W) of the tumors were measured externally with a vernier caliper every 3 days. Tumor volume was calculated with the formula: $V = (L \times W^2)/2$. After 20 days, tumor-bearing mice and controls were sacrificed, and the tumors were excised and measured.

Histology and immunohistochemistry. For histological analysis, tissues were fixed in 4% paraformaldehyde at 4˚C, embedded in paraffin, cut into 5-μM sections and transferred to silicon-coated slides. Tissue sections were then stained with hematoxylin and eosin. For immunohistochemical analysis, staining for β-catenin was carried out, using rabbit polyclonal antibodies against β-catenin (Proteintech Group) at a dilution of 1:100. Visualization was performed using the 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), followed by counterstaining with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Immunofluorescence analysis. Cells seeded on 24-well plates were fixed in 4% paraformaldehyde for 30 min at 37˚C. Then cells were permeabilized with 0.5% Triton-X 100 for 20 min, and blocked with 5% BSA for 30 min. Cells were incubated with anti-β-catenin (1:100, Proteintech Group) at 4˚C overnight. Primary antibody was visualized with Cy3-conjugated secondary antibodies (1:100, Boster, China) for 1 h in the dark. Nuclei were stained with DAPI (Boster) for 5 min. Images were collected using an LSM410 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Statistics. Each experiment was repeated at least three times. The data are presented as mean ± SEM. SPSS version 17.0 software (SPSS for Windows, Inc., Chicago, IL, USA) was used for all statistical analyses. Statistical analysis of data was performed using standard One-way ANOVA or One-way ANOVA for repeated measures, followed by Bonferroni post-hoc test. A two-tailed Student's paired t-test was also used to compare the difference in values between 2 groups. A P-value of <0.05 is considered as statistically significant.

Results

HBx activates Notch1 and Wnt/β-catenin signaling pathways in L02 cells, and increase Fzd10 expression. We first used qRT-PCR and Western blot analysis in L02, L02/pcDNA3.1 and L02/HBx cells. As shown in Fig. 1, the mRNA and protein expression levels of Notch1, Hes1 in L02/HBx cells were elevated relative to controls (Fig. 1A; $^{**}P<0.01$). In addition, we analyzed the mRNA and protein expression levels of Fzd7, Fzd10 and cyclin D1, along with the protein expression of β-catenin. We found that their expression levels were increased in L02/HBx cells as compared with controls except Fzd7 (Fig. 1B; $^{**}P<0.01$). Moreover, β-catenin translocated from the membrane and cytoplasm to the nuclei (Fig. 1C). Taken together, these results suggest that HBx may activate Notch1 and Wnt/β-catenin signaling pathways upregulating Fzd10 expression.

Inhibition of Notch1 signaling pathway decreases the activity of Wnt/β-catenin signaling pathway, and reduces the Fzd10 expression in L02/HBx cells. In order to identify the effective siRNA target gene, the mRNA expression of Notch1 in L02/HBx cells was determined by qRT-PCR 48 h after transfection with siRNAs. Comparing to the non-transfected cells, transfection of Notch1-siRNA1, 2 or 3 (siRNA1, 2, 3) into L02/HBx cells resulted in downregulating of Notch1 mRNA expression up to 64.2, 72.9 and 22.4%, respectively (Fig. 2; $^{**}P<0.01$). So the target sequence of Notch1-siRNA2 was chosen for construction of Notch1-shRNA.

We successfully constructed the pH1-MCS-CMV-GFP-Hygro-Notch1 shRNA (Notch1-shRNA) and the pH1-MCS-CMV-GFP-Hygro negative control vector (NC). Comparing to the non-transfected cells, transfection of Notch1-shRNA into L02/HBx cells resulted in dramatically downregulating of Notch1 mRNA and protein expression, but

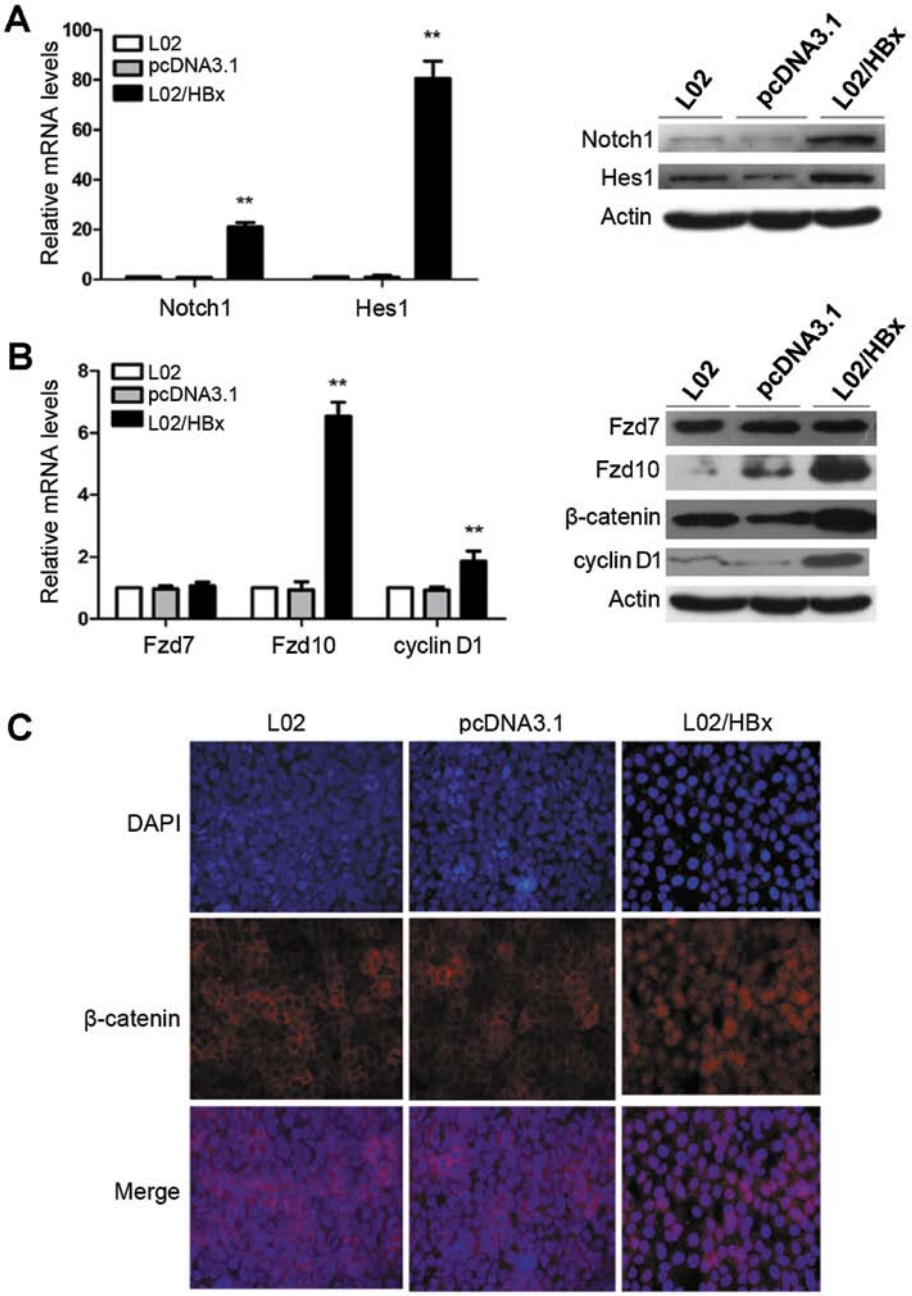

Figure 1. Notch signaling pathway components and Wnt signaling pathway components are upregulated in HBx-pcDNA3.1 transfected cells. (A) Notch signaling pathway components are upregulated in L02/HBx cells. The mRNA and protein expression levels of Notch1 and Hes1 were assessed by qRT-PCR and western blot analysis, respectively. (B) Wnt signaling pathway components are upregulated in L02/HBx cells. The mRNA and protein expression levels of Fzd7, Fzd10, cyclin D1 and β-catenin were assessed by qRT-PCR and western blotting, respectively. (C) The location of β-catenin was analysed by immunofluorescence images at x400 magnification. Actin was used as a loading control for both quantitative RT-PCR and western blot analysis. Data are shown as the mean ± SEM from three independent experiments. Statistically significant differences are indicated as: $^{**}P<0.01$ vs. L02 cells.

there was no significant change in NC-transfected group. Hes1, a target gene of Notch signaling, was downregulated after transfected with Notch1-shRNA (Fig. 3A; $^{**}P<0.01$). These results indicated that Notch1 signaling pathway in L02/HBx cells had been partially blocked by Notch1-shRNA. Then, we tested the mRNA and protein expression levels of Fzd7, Fzd10 and cyclin D1, along with the protein expression of β-catenin. We found that their expression levels except Fzd7 were decreased in L02/HBx cells as compared with controls (Fig. 3B; $^{**}P<0.01$). These results suggested that downregulation of Notch1 expression by shRNA decreases the activation of Wnt/β-catenin pathway in L02/HBx cells.

Inhibition of Wnt/β-catenin signaling pathway via Fzd10 shRNA does not affect the activity of Notch1 signaling pathway in L02/HBx cells. In order to identify the effective siRNA target gene, the mRNA expression of Fzd10 in L02/HBx cells was determined by qRT-PCR 48 h after transfection

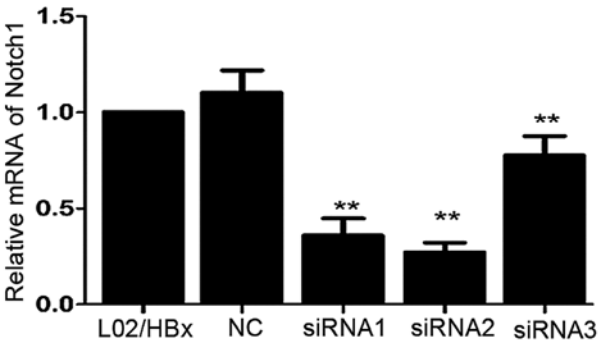

Figure 2. Identification of effective siRNA targeting Notch1 gene. The Notch1 mRNA was measured by qRT-PCR in L02/HBx cells 48 h after transient transfection with Notch1-siRNA1, -siRNA2, -siRNA3 or negative control-siRNA (NC), respectively. Data are shown as the mean ± SEM from at least three independent experiments. Statistically significant differences are indicated as: **P<0.01 vs. L02 cells.

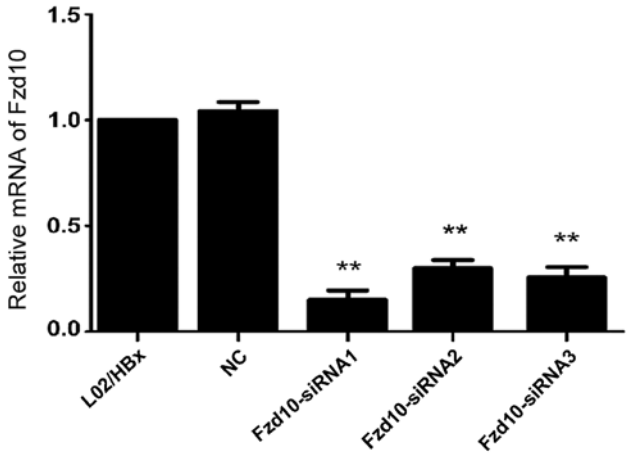

Figure 4. Identification of effective siRNA targeting Fzd10 gene. The Fzd10 mRNA was measured by qRT-PCR in L02/HBx cells 48 h after transient transfection with Fzd10-siRNA1, -siRNA2, -siRNA3 or negative control-siRNA (NC), respectively. Data are shown as the mean ± SEM from at least three independent experiments. Statistically significant differences are indicated as: **P<0.01 vs. L02 cells.

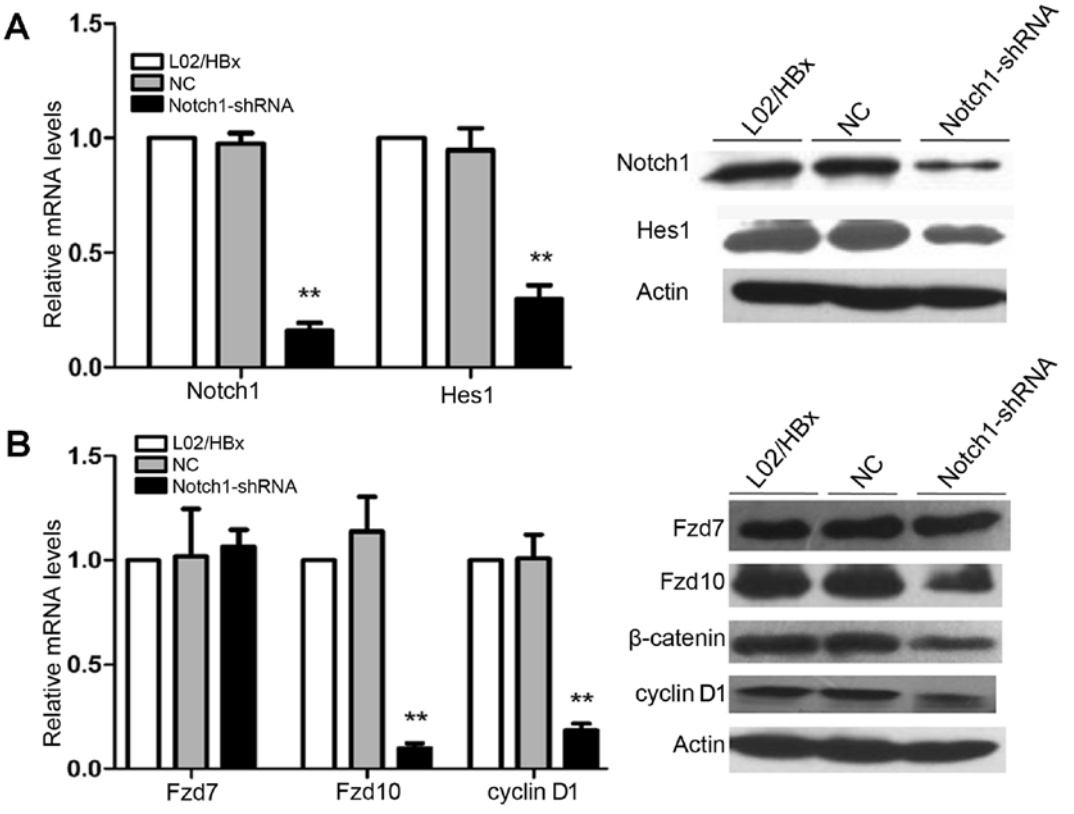

Figure 3. Downregulation of Notch1 expression by shRNA decreases the activation of Wnt/β-catenin pathway in L02/HBx cells. (A) The components of Notch1 signaling pathway are downregulated in L02/HBx-Notch1 shRNA cells. The mRNA and protein expression levels of Notch1 and Hes1 were assessed by qRT-PCR and western blot analysis, respectively. (B) The components of Wnt signaling pathway are downregulated in L02/HBx-Notch1 shRNA cells. The mRNA and protein expression levels of Fzd7, Fzd10, cyclin D1 and β-catenin were assessed by qRT-PCR and western blotting, respectively. Actin was used as a loading control for both quantitative RT-PCR and western blot analysis. Data are shown as the mean ± SEM from at least three independent experiments. Statistically significant differences are indicated as: **P<0.01 vs. L02/HBx cells.

with siRNAs. Comparing to the non-transfected cells, transfection of Fzd10-siRNA1, 2 or 3 into L02/HBx cells resulted in downregulating of Fzd10 mRNA expression up to 85.0, 70.0 and 74.3%, respectively (Fig. 4; **P<0.01). So the target sequences of Fzd10-siRNA1 were chosen for construction of Fzd10-shRNA.

We successfully constructed the pH1-MCS-CMV-GFP-Hygro-Fzd10 shRNA (Fzd10-shRNA). Comparing to

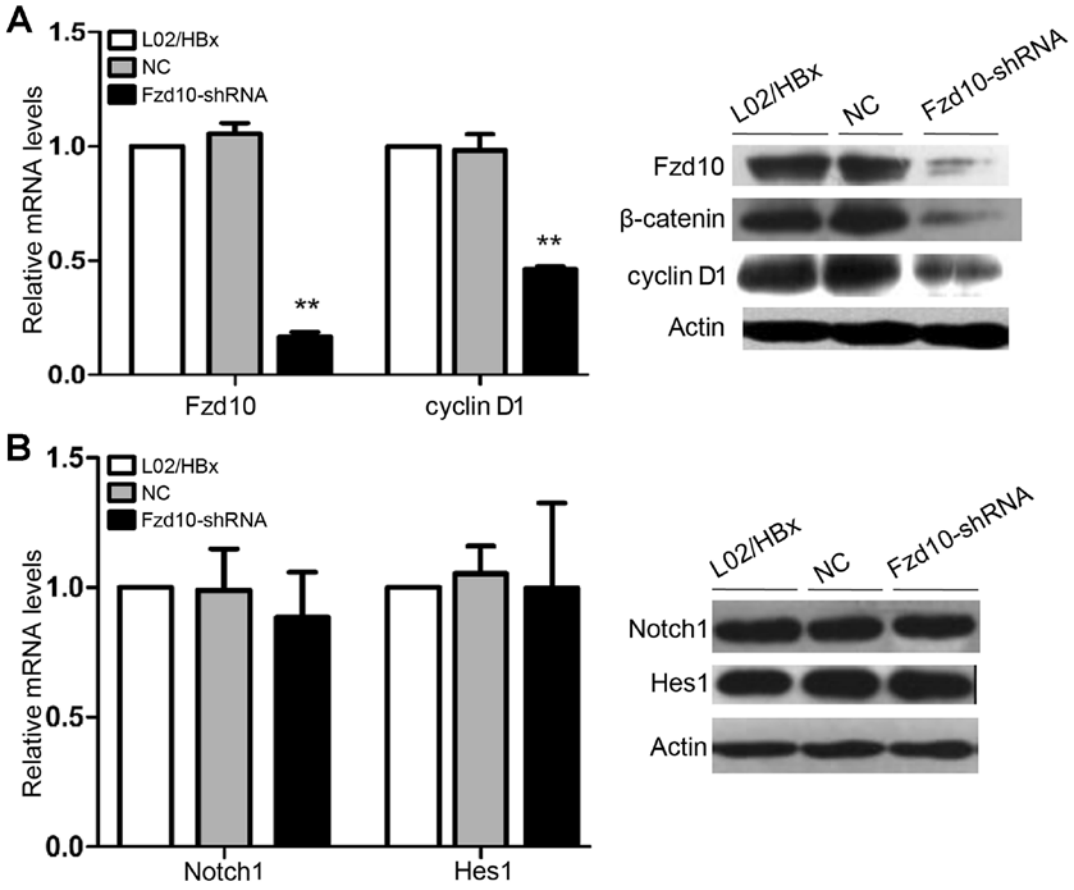

Figure 5. Downregulation of Fzd10 expression by shRNA does not significantly affect the Notch signaling pathway in L02/HBx cells. (A) The components of Wnt signaling pathway are downregulated in L02/HBx-Fzd10 shRNA cells. The mRNA and protein expression levels of Fzd10, cyclin D1 and β-catenin were assessed by qRT-PCR and western blotting, respectively. (B) The components of Notch signaling pathway are downregulated in L02/HBx-Fzd10 shRNA cells. The mRNA and protein expression levels of Notch1 and Hes1 were assessed by qRT-PCR and western blot analysis, respectively. Actin was used as a loading control for both quantitative RT-PCR and western blot analysis. Data are shown as the mean ± SEM from at least three independent experiments. Statistically significant differences are indicated as: **P<0.01 vs. L02/HBx cells.

the non-transfected cells, transfection of Fzd10-shRNA into L02/HBx cells resulted in dramatic downregulation of Fzd10 mRNA and protein expressions, but there was no significant change in NC-transfected group. β-catenin, a dominant effector and cyclin D1, a target gene of Wnt/β-catenin signaling, were downregulated after transfected with Fzd10-shRNA (Fig. 5A; **P<0.01). These results indicated that Wnt/β-catenin pathway in L02/HBx cells had been partially blocked by Fzd10-shRNA. Then, we tested the mRNA and protein expression levels of Notch1 and Hes1. We found that their expression levels were not changed in L02/HBx cells as compared with controls (Fig. 5B). These results indicated that downregulation of Fzd10 expression by shRNA does not significantly affect the Notch signaling pathway in L02/HBx cells.

Activated Wnt/β-catenin pathway is required for L02/HBx cell proliferation in vivo and in vitro. L02/HBx cells lacking Notch1 have impaired ability of proliferation and survival (7). We asked whether activation of Wnt signaling can overcome this defect. To address this question, we constructed pCV061-Fzd10. After transfection of pCV061-Fzd10, L02/HBx-Notch1 shRNA cells that previously expressed low levels of Fzd10, cyclin D1 and β-catenin, showed significantly increased levels of mRNA and protein compared to non-transfected cells (Fig. 6A; **P<0.01). There was no remarkable change in the pCV061 empty vector-transfected group, suggesting that the transfection was successful and Wnt signaling was activated with Fzd10-expression vector. Moreover, CCK-8 assay (Fig. 6B; *P<0.05, **P<0.01) and colony formation assay (Fig. 6C) showed that activation of Wnt signaling restored proliferation that was inhibited in L02/HBx-Notch1 shRNA cells. These data suggest that Wnt signaling is downstream of Notch in regulation of cell proliferation.

To confirm the above observations, we used an alternative experimental system where activated Notch1 signaling exists in L02/HBx cells, and is required for HBx to promote proliferation and survival of L02 cells (4,7). To verify that the Notch effect on cell proliferation was mediated via Wnt pathway, L02/HBx cells were transfected with either NC or Fzd10-shRNA and then carried out CCK-8 (Fig. 7A; *P<0.05, **P<0.01) and colony formation assays (Fig. 7B). Notch1 may induce a significant increase in the proliferation of L02 (7).

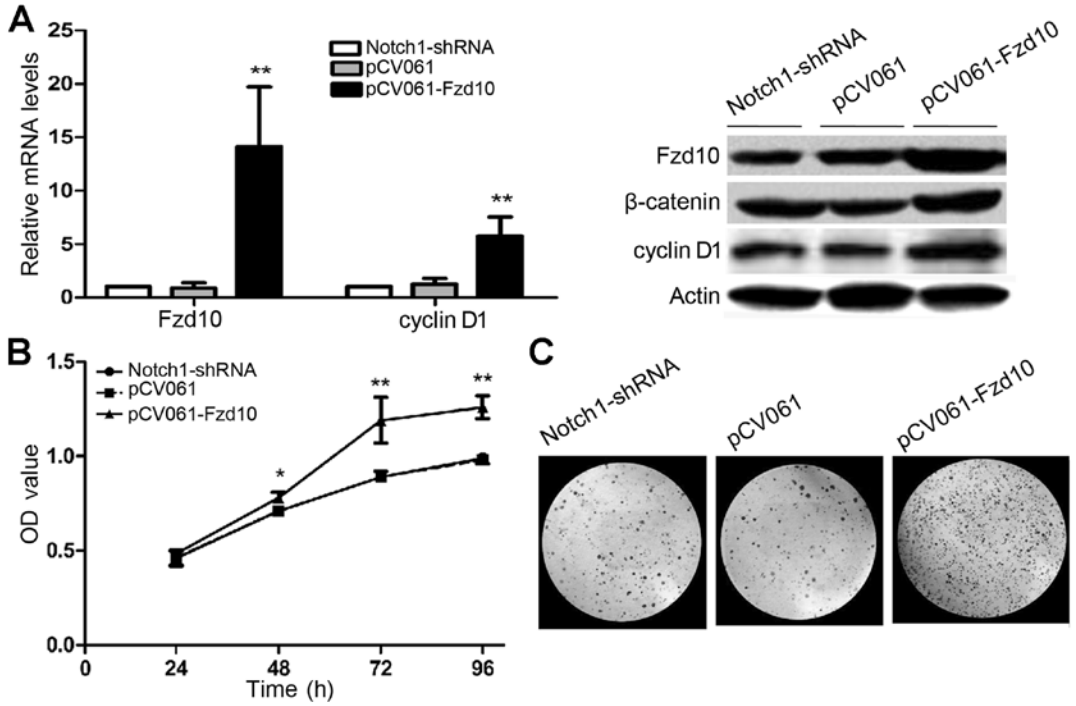

Figure 6. Upregulation of Wnt signaling restored the proportion of proliferation generated from Notch1-shRNA cells to the level of L02/HBx cells. (A) Upregulation of Wnt signaling pathway by pCV061-Fzd10. The mRNA and protein expression levels of Fzd10 and cyclin D1, along with the protein expression of β-catenin were assessed by qRT-PCR and western blot analysis, respectively. (B) CCK-8 assay and (C) colony formation assay for Notch1-shRNA and stably transfected with pCV061-Fzd10 and pCV061 empty vector.

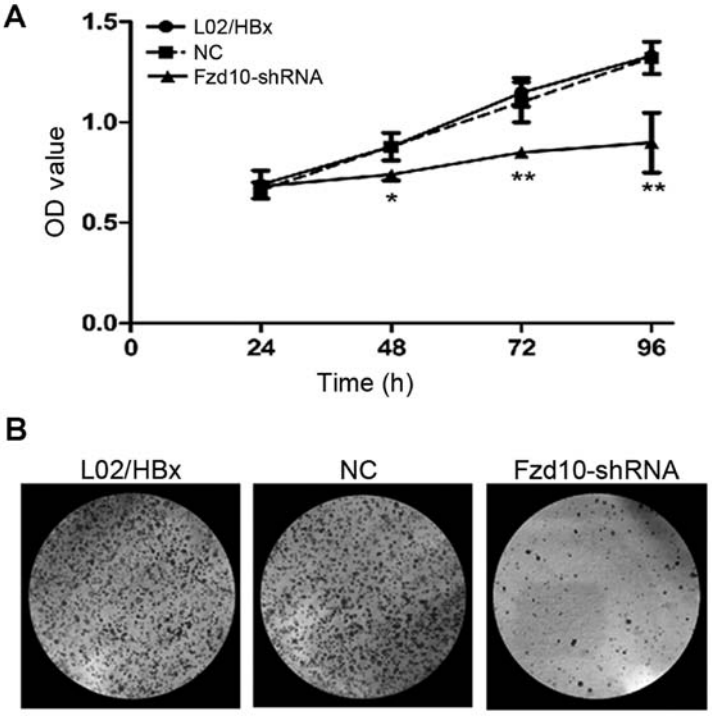

Figure 7. Fzd10-shRNA suppresses L02/HBx cell proliferation *in vitro*. (A) CCK-8 assay and (B) colony formation assay for L02/HBx stably transfected with control or Notch1 shRNA2. Data are shown as the mean ± SEM from at least three independent experiments. Statistically significant differences are indicated as: $^{*}P<0.05$, $^{**}P<0.01$ vs. L02/HBx cells.

This effect was absent when L02/HBx cells were transfected with Fzd10-shRNA. Fzd10-shRNA abrogated the effect of Notch1 on proliferation. These data were consistent with the results of experiments with L02/HBx-Notch1 shRNA cells and

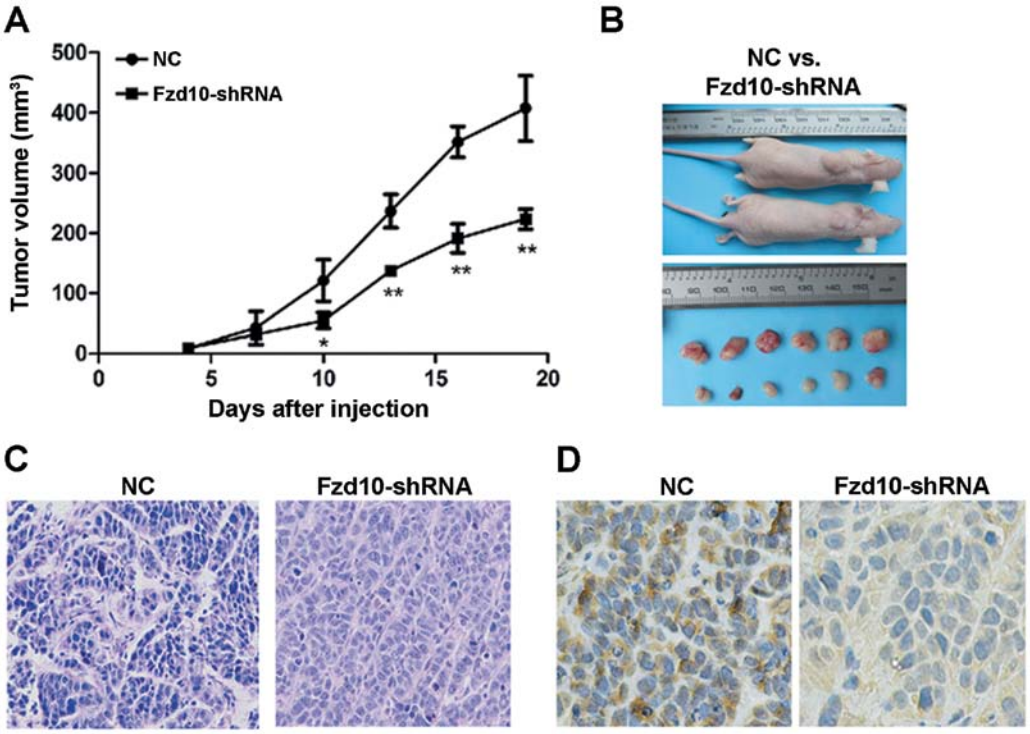

Figure 8. Fzd10-shRNA suppresses tumor growth in nude mice. (A) The growth curve of the tumors transplanted with L02/HBx cells pretreated with Fzd10-shRNA or control-shRNA in nude mice. Data represent means ± SEM of six samples. *P<0.05, **P<0.01. (B) Photographs of representative mice and dissected tumors from nude mice. (C) Tumor tissues from nude mice inoculated with NC or Fzd10-shRNA cells were stained with hematoxylin and eosin (H&E). Original magnification, x200. (D) Immunohistochemistry of β-catenin expression in tumor tissues from nude mice. Original magnification, x400.

indicate that Wnt pathway is downstream of Notch signaling in proliferation of L02/HBx cells, and Wnt pathway is required for Notch1 to promote proliferation of L02/HBx cells.

Coincidentally, the tumorigenicity was remarkably suppressed in nude mice injected with L02/HBx cells pretreated with Fzd10-shRNA *in vivo* (Fig. 8A and B; *P<0.05, **P<0.01). Pathological examination of the primary tumors revealed malignant phenotypes in both groups by hematoxylin and eosin (H&E) staining (Fig. 8C). The effective inhibition of Wnt signaling pathway was confirmed by immunoreactivity analysis with β-catenin in mouse tumor models *in vivo* (Fig. 8D).

Discussion

Notch signaling was previously reported to be implicated in the development of HCC. However, the available data were controversial. Some groups found aberrant nuclear expression of Notch1 and Notch3 in human liver cancer tissue compared to the surrounding cirrhotic tissue, and Notch signaling promoted formation of liver tumors in mice. Utilizing experiments with 'loss of function' based on knockdown of Notch3 in HCC cells showed enhanced sensitivity to doxorubicin-induced cell death (13,14). On the contrary, other studies demonstrated that the Notch1 signaling results in significant growth inhibition of HCC cells both *in vitro* and *in vivo* (15-17). So contradictory data exists in respect to the effect of Notch signaling on HCC, and scarce data exist on its effect on HBx-related HCC. Our research may be helpful to resolve some of those contradictions and clarify the role of Notch signaling in HBx-related HCC.

We have reported that activated Notch1 signaling is required for HBx to promote malignant transformation of L02 cells (4,7). In this study, we showed that Notch1 may exert its effect on HBx-related HCC primarily via activation the Wnt pathway. Activation of Wnt signaling restored the proportion of proliferation inhibited by L02/HBx-Notch1 shRNA, and inhibition of Wnt signaling impaired the effect of Notch1 on proliferation and tumor formation of L02/HBx cells in BALB/c nude mice.

Our data provide a potential new model for explaining the machanism of HBx-related HCC. It appears that Notch signaling may affect Wnt signaling in L02/HBx via regulating expression level of Fzd10 instead of Fzd7. Several lines of evidence have demonstrated that Notch modulates Wnt/β-catenin pathway by regulating Fzd10 expression in HBx-expressing L02 cells: HBx upregulated Notch signaling pathway and Wnt/β-catenin pathway, along with Fzd10 rather than Fzd7; inhibition of Notch pathways resulted in the decreased expression of Fzd10 and the activation of Wnt pathway; Fzd10-shRNA significantly reduced the activation of Wnt pathway, but did not apparently affect the Notch signaling pathway.

Human FZD10 has been found to be expressed in adult normal placenta, skeletal muscle, brain, heart, lung, pancreas, spleen, prostate and fetal kidney, lung and brain (24). Our data are in agreement with observations from some groups that FZD10 expression was higher in some cancer

tissues (colon, lung squamous cell carcinoma) (25-27). Moreover, the effectiveness of anti-FZD10 antibody therapy was reported in synovial sarcomas since FZD10 increases cell growth in synovial sarcoma (28,29). However, it was previously demonstrated that FZD7 activated Wnt/β-catenin signaling in several cancer types including hepatocellular carcinoma (24,30), and the Fzd7 steady-state mRNA levels were upregulated in hepatitis B, C and non-viral-induced HCC cell lines and mouse models (30-33), and inhibition of Fzd7 with small interfering peptides displayed antitumor properties in hepatocellular carcinoma (34). Given that our data are inconsistent with these reports, we speculate Fzd10 is related to the initial progression of HBx-related HCC, and Fzd7 is linked to the development of HCC.

Our results demonstrate a critical role of the canonical Wnt pathway for malignant transformation of human hepatic cells induced by HBx. Downregulation of Wnt pathway in L02/HBx cells inhibited proliferation and tumor formation of L02/HBx cells in BALB/c nude mice. Our results are consistent with the recent observations that HBx competitively binds adenomatous polyposis coli (APC) to displace β-catenin from its degradation complex, which results in the activation of Wnt signaling (35); ectopic expression of HBx along with Wnt-1 activated Wnt/β-catenin signaling in hepatoma cells, which was achieved by suppressing glycogen synthase kinase3 activity via the activation of Src kinase, suggesting that HBx could contribute to hepatocarcinogenesis via the activation of Wnt/β-catenin signaling (36).

Our data suggest that the Wnt pathway can be downstream of Notch in malignant transformation of human hepatic cells induced by HBx. This model may also explain the discrepancy in reports of Notch effects on HCC. On one hand, activation of the Notch pathway by itself is not sufficient for HCC and requires the Wnt pathway. However, activation of the Wnt pathway may overcome the absence of Notch signaling. This cooperation between Notch and Wnt signaling may provide a novel field of vision for research on the pathogenesis of HBx-related HCC.

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