

Full-length article

Involvement of hepatitis B X-interacting protein (HBXIP) in proliferation regulation of cells¹

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Abstract

Aim: To investigate the effect of Hepatitis B X-interacting protein (HBXIP) on cell proliferation. **Methods:** A rabbit antibody against HBXIP was generated. The RNA interference (RNAi) fragment of the HBXIP gene was constructed in the pSilencer-3.0-H1 vector termed pSilencer-hbxip. Plasmids of the pcDNA3-hbxip encoding HBXIP gene and pSilencer-hbxip were transfected into human breast carcinoma MCF-7 cells, hepatoma H7402 cells, and the normal human hepatic cell line L-O2, respectively. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and 5-bromo-2-deoxyuridine incorporation assay were applied to detect cell proliferation. MCF-7 cells and L-O2 cells in the cell cycle were examined by flow cytometry. The proteins involved in cell proliferation and cell cycle were investigated by Western blot. **Results:** Overexpression of HBXIP resulted in the promotion of proliferation of MCF-7, H7402, and L-O2 cells. Flow cytometry showed that the overexpression of HBXIP promoted the cell proliferation of MCF-7 and L-O2 cells, and led to an increased cell proliferative index in MCF-7 cells (from 46.25% to 58.28%) and L-O2 cells (from 29.62% to 35.54%). Western blot showed that expression levels of c-Myc, Bcl-2, and proliferating cell nuclear antigen were upregulated in MCF-7, H7402, or L-O2 cells, whereas that of p27 was downregulated. However, the RNAi of HBXIP brought opposite results. **Conclusion:** One of the functions of HBXIP is its involvement in cell proliferation.

Introduction

The hepatitis B X-interacting protein (HBXIP), encoding a 9.6 kDa protein, was originally identified by its interaction with the C-terminus of the hepatitis B virus (HBV) X protein (HBX) and located at human chromosome 1p13.3^[1]. It contained a putative leucine zipper motif and 2 consensus phosphorylation sites at threonines 12 and 36 for protein kinase C and casein kinase II. Investigation for the role of HBXIP in the HBV replication in hepatoma cells revealed that HBXIP reduced the replication of the wild-type HBV following transfection with a HBX-minus virus^[1]. The transactivation effect of HBX on an activating protein-1 (AP-1) binding site as well as on the HBV enhancers was abolished by co-expression with the HBXIP. HBXIP could form complex with survivin, an anti-apoptotic protein that is overexpressed in

most human cancers^[2]. The complex of HBXIP and survivin binded pro-caspase-9 to prevent its recruitment to Apaf I, and thereby selectively suppressing apoptosis initiated via the mitochondrial/cytochrome c pathway. HBXIP was required for bipolar spindle formation and was a regulator of centrosome dynamics and cytokinesis in cells^[3]. Recently, an interaction factor of HBXIP was identified, which was a human ATP-dependent RNA/DNA helicase hSuv3p. The nucleotide triphosphate (NTP)-dependent DNA/RNA DExH box helicase was predominantly localized in the mitochondria. It was found that the HBXIP-binding domain was important for mitochondrial import and the stability of the Suv3 protein^[4]. Moreover, we found that HBXIP inhibited apoptosis induced by HBX in hepatoma cells^[5]. We previously demonstrated that 8 types of the HBXIP gene were homologous (data not shown), such as (i) human fetal tissues of skeletal muscle,

cardiac muscle and uterus muscle; (ii) human adult uterus muscle; (iii) mouse tissues of skeletal muscle, cardiac muscle and uterus muscle; and (iv) SM3 cells (a derivation of rabbit vascular smooth muscle), suggesting that HBXIP was a conserved protein in evolution.

Although some binding proteins of HBXIP have been identified in the last few years, the basic biological functions of HBXIP are still unclear. In the present study, we are interested in the basic biological functions of HBXIP and address whether HBXIP is able to influence cell proliferation by transfection with HBXIP gene in cells, such as breast cancer MCF-7 cells and hepatoma H7402 cells, and in normal liver L-O2 cells.

Materials and methods

Cancer cells and cell culture MCF-7 cells, H7402 cells^[6], and L-O2 cells^[6] were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mmol/L glutamine and 100 U/mL penicillin, and 100 µg/mL streptomycin in humidified 5% CO₂ at 37 °C.

Construction of the HBXIP gene in vectors Total RNA was extracted from H7402 cells using Trizol (Invitrogen, Carlsbad, CA, USA) reagent following the manufacturer's recommendations. Prior to the first cDNA strand synthesis, total RNA was digested with RNase-free DNase I (TaKaRa, Tokyo, Japan) at 37 °C for 20 min and inactivated at 60 °C for 10 min. With 2 µg of total RNA as the template and oligo (dT) as the primers, the first cDNA strand was synthesized in a 20 µL reaction system with M-MLV reverse transcriptase (TaKaRa). One µL of cDNA template was used in a 50 µL reaction volume with *rTaq* DNA polymerase (TaKaRa) and oligonucleotide primers as follows: 5'-GACGAATTCATGGAGGCGACCTTGGAGCA-3' (forward) and 5'-GATCTC-GAGTCAAGAGGCCATTTGTGCA-3' (reverse). The resultant cDNA fragments were ligated into pET30a vector (termed pET30a-hbxip) and pGEX-4T-1 encoding glutathione S-transferase (GST) vector (termed pGEX-4T-hbxip), respectively. The pET30a-hbxip plasmids and pGEX-4T-hbxip plasmids were induced by 0.4 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) in *Escherichia coli* BL21 (DE3) at 37 °C for 4 h. The purified fusion protein of GST-HBXIP (35.6 kDa) expressed by pGEX-4T-hbxip plasmids was used to identify the specificity of rabbit anti-HBXIP antibody as the antigen.

Generation of antibody of rabbit anti-HBXIP pET30a-hbxip was induced by 0.4 mmol/L IPTG in *Escherichia coli* BL21 (DE3) at 37 °C for 4 h, and the HBXIP band was collected from the gel after SDS-PAGE electrophoresis, which was used as the immunogen. Equal amounts of antigen

solution and adjuvant were mixed thoroughly. HBXIP antigen (0.1 mg) was administered by an intradermal injection of rabbit antibody. First immunizations were done with complete Freund's adjuvant (Sigma, St Louis, MO, USA) and subsequent immunizations were done employing incomplete Freund's adjuvant (Sigma) weekly for 5 times. The serum was harvested by bleeding at the sixth week. The rabbit antibody against HBXIP was purified from the immune sera by affinity purification.

Construction of RNA interference (RNAi) targeting the HBXIP gene in the vector According to a report^[2], a synthesized 63 mer oligonucleotide containing a specific sequence for a targeting region of the HBXIP open reading frame was inserted into the pSilencer-3.0-H1 of the RNAi vector. The sequence of oligonucleotide 1 was as follows: 5'-GATCCGC-AGCTAAGCTAACCTCTGTTCAAGAGACAGAGGTTAG-CTTAGCTGCTTTTTTGGAAA-3'; the sequence of oligonucleotide 2 was as follows: 5'-AGCTTTTCCAAAAAG-CAGCTAAGCTAACCTCTGTCTCTTGAACAGAGGTTA-GCTTAGCTGCG-3'. The 2 annealed complementary oligonucleotides were inserted into the *Bam*H I/*Hind* III site of the pSilencer 3.0 H1 vector. After PCR and enzyme digestion, identification of the RNAi fragment of HBXIP inserted in the vector was performed by sequencing.

Transfection One day before transfection, MCF-7 cells, H7402 cells, and L-O2 cells were collected, and seeded into 6-well plates at 1×10⁵ cells per well (*n*=3, each group). MCF-7, H7402, or L-O2 cells were transfected with 2 µg plasmids such as pcDNA3 empty vector, pcDNA3-hbxip encoding HBXIP^[5], and pSilencer-hbxip respectively, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The transfection mixture was removed after 6 h. Transfection efficiency in the cells was monitored by co-transfection of 0.2 µg pEGFP-C2 plasmid, which expresses green fluorescence protein (GFP). After 24 h transfection, 5-bromo-2-deoxyuridine (BrdU) incorporation assay was performed, and after 48 h transfection, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry analysis, and Western blot analysis were followed to detect cell proliferation, cell cycle, and expression of proteins associated with proliferation.

MTT assay Cell proliferation was measured by MTT assay^[7]. Briefly, an amount of 200 µL cell suspensions (5×10³ cells/mL) was added to each well of 96-well plates and incubated at 37 °C for 48 h. After 48 h transfection (as described earlier), an amount of 20 µL MTT (5 mg/mL, Genview, Houston, TX, USA) was added and incubated at 37 °C for 4 h. After removing the supernatant, 200 µL dimethyl sulfoxide was added to resolve formazan crystals, and the value of the

optical density was detected at 570 nm. The results are based on the cleavage of the tetrazolium salt by viable cells that were proportional to the number of living cells in the wells.

BrdU labeling and immunofluorescent staining The detailed procedures were followed accordingly^[8]. In brief, the cells were seeded into 6-well plates and were grown overnight prior to transfection. All groups ($n=3$ in every group) were incubated with fresh medium containing 10 $\mu\text{mol/L}$ BrdU (Sigma) for 4 h prior to immunofluorescence staining with mouse anti-BrdU antibody. The cells were fixed for 15 min with 4% paraformaldehyde in phosphate buffered saline (PBS). After 1 h incubation with PBS containing 2 mol/L HCl to denature DNA, cover slips were washed 3 times with 0.5% bovine serum albumin (BSA) and 0.5% Tween 20 in PBS, and incubated overnight (4 °C) with a mouse anti-BrdU antibody (NeoMarkers, Fremont, CA, USA) at 1:300 dilution. Reactions were developed using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) at 1:100 dilution for BrdU staining. The BrdU labeling index was assessed by point counting through a Nikon TE200 inverted microscope (Nikon, Tokyo, Japan) using a 40 \times objective lens. A total of 700–800 nuclei were counted in 6–8 representative fields. The labeling index was expressed as the number of positively-labeled nuclei/total number of nuclei. Propidium iodide (PI) (Sigma) staining for nuclei in 50 $\mu\text{g/mL}$ was used as the control to all cells in each group.

Flow cytometry analysis After 48 h transfection as earlier described, the cells (1×10^6) were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in 0.6 mL PBS (pH 7.4), and fixed by the addition of 1.4 mL 100% ethanol at 4 °C overnight. The fixed cells were rinsed twice with PBS, and resuspended in propidium iodide (PI) solution, including 50 $\mu\text{g/mL}$ PI and 50 $\mu\text{g/mL}$ RNaseA (Sigma) in PBS without calcium and magnesium, and incubated at 37 °C for 30 min in the dark. Stained cells were passed through a nylon-mesh sieve to remove cell clumps and analyzed by a FACScan flow cytometer and Cell Quest analysis software (Becton Dickinson, San Jose, CA, USA). Flow cytometry analysis was repeated 3 times.

Western blot analysis For the identification of the generated rabbit anti-HBXIP antibody, cell lysates from MCF-7 and purified GST-HBXIP from BL21 (DE3) cells were examined by Western blot analysis. After washing twice in cold PBS, the cells were lysed with ice-cold lysis buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 0.1% SDS, 1.0% Nonidet P-40, 0.5% Na-deoxycholate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and protein inhibitor cocktails). Lysates were centrifuged at 12 000 $\times g$ for 20 min, and the superna-

tants were used as total cell lysates. The protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). A quantity of 30 μg total protein per lane was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5.0% milk powder in 0.05% Tween-PBS, incubated with the specific antibodies such as rabbit anti-HBXIP (1:1000 dilution) or anti-GST antibody (Tiangen, Beijing, China, 1:800 dilution), at room temperature for 2 h, followed by a peroxidase-conjugated secondary antibody diluted in 0.3% BSA/Tween-PBS at room temperature for 1 h. Detection of the target proteins on the membranes was performed using the enhanced chemiluminescence (ECL) system Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK). All experiments were repeated at least 3 times. After 48 h transfection, Western blot analysis was performed as above, the primary antibodies were mouse anti-p27 (NeoMarkers, Fremont, CA, USA, 1:500 dilution), mouse anti-c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500 dilution), and mouse anti-Bcl-2 (NeoMarkers, Fremont, CA, USA, 1:500 dilution), rabbit anti-proliferating cell nuclear antigen (PCNA) (NeoMarkers, Fremont, CA, USA, 1:1000 dilution), mouse anti-survivin (Chemicon, Temecula, CA, USA, 1:1000 dilution) and mouse anti- β -actin (Sigma, 1:20000 dilution).

Statistical analysis All data were expressed as mean \pm SD. Statistical analysis was performed by the Student's *t*-test. $P < 0.05$ was indicated to be statistical significant.

Results

Identification of antibody of rabbit anti-HBXIP To examine HBXIP, we generated rabbit anti-HBXIP antibody recognizing HBXIP. Western blot analysis showed that this anti-HBXIP antibody specifically recognized HBXIP in MCF-7 cells, and the GST-HBXIP fusion protein expressed in *Escherichia coli* BL21 (DE3) and GST in the fusion protein GST-HBXIP from BL21 (DE3) cells could also be detectable using antibody of anti-GST (Figure 1).

Promotion of cell proliferation by HBXIP overexpression Transfection efficiency revealed that approximately 70%–80% of cells showed green fluorescence (Figure 2). To evaluate whether HBXIP expression correlated with fundamental cellular processes, we investigated the cell proliferation by MTT assay, BrdU incorporation assay, flow cytometry analysis, and Western blot analysis after transfection. MTT assay showed that transfection with pcDNA3-hbxip plasmid promoted cell proliferation ($P < 0.05$ vs control), whereas transfection with the pSilencer-hbxip plasmid decreased cell

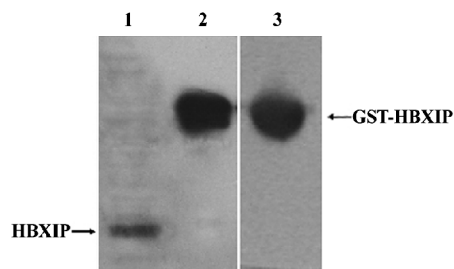


Figure 1. Identification of rabbit anti-HBXIP antibody by Western blot analysis in MCF-7 cells (lane 1) and *Escherichia coli* BL21 (DE3) (lane 2). GST in GST-HBXIP fusion protein from BL21 (DE3) cells was detectable using antibody of anti-GST (lane 3).

proliferation by MTT assay in MCF-7 cells ($P < 0.05$ vs control, data not shown). To further confirm the results, we repeated the same experiments in hepatoma H7402 cells and L-O2 cells. BrdU incorporation analysis showed that the induction of DNA synthesis by HBXIP was assessed in MCF-7, H7402, and L-O2 cells. The results showed that the percentage of cells in the S phase increased significantly in MCF-7, H7402, and L-O2 cells transfected with pcDNA3-hbxip compared with the cells transfected with pcDNA3 ($P < 0.05$), and was reduced significantly in cells transfected with pSilencer-hbxip plasmids (Figure 3). No statistically significant difference was observed between the control cells and cells transfected

with pcDNA3 empty vector.

HBXIP overexpression significantly accelerated the cell proliferation compared with untransfected and pcDNA3 transfected cells. Cell proliferation was quantified by PI. PI is the sum of the S and G₂/M phase activities of the cell cycle expressed as a fraction of the total cell population, that is, $PI = [(S + G_2/M) / (G_0/G_1 + S + G_2/M)] \times 100\%$. Flow cytometry analysis showed that overexpression of HBXIP, transfected with pcDNA3-hbxip plasmids, led to an increased cell PI from 46.25% to 58.28% in MCF-7 cells. However, the downregulation of HBXIP, transfected with pSilencer-hbxip plasmid, resulted in a decreased cell PI (from 46.25% to 31.67%) and increased percentage of cells in the G₁ phase (from 53.75% to 68.33%, $P < 0.01$; Figure 4A). Flow cytometry analysis was also repeated in L-O2 cells (Figure 4B), and the PI of the L-O2 cells transfected with pcDNA3-hbxip plasmids increased significantly compared with the pcDNA3 control (from 29.62% to 35.54%, $P < 0.01$). The PI in pSilencer-hbxip transient transfection cells was only 22.72%, so HBXIP also accelerated the cell proliferation of L-O2 cells. Flow cytometry was repeated 3 times.

Involvement of proteins in the promotion of cell proliferation To investigate the mechanism, we examined some proteins related to cell proliferation and cell-cycle regulation, such as c-Myc, Bcl-2, PCNA, and p27. Western blot analysis showed that transient transfection with pcDNA3-hbxip

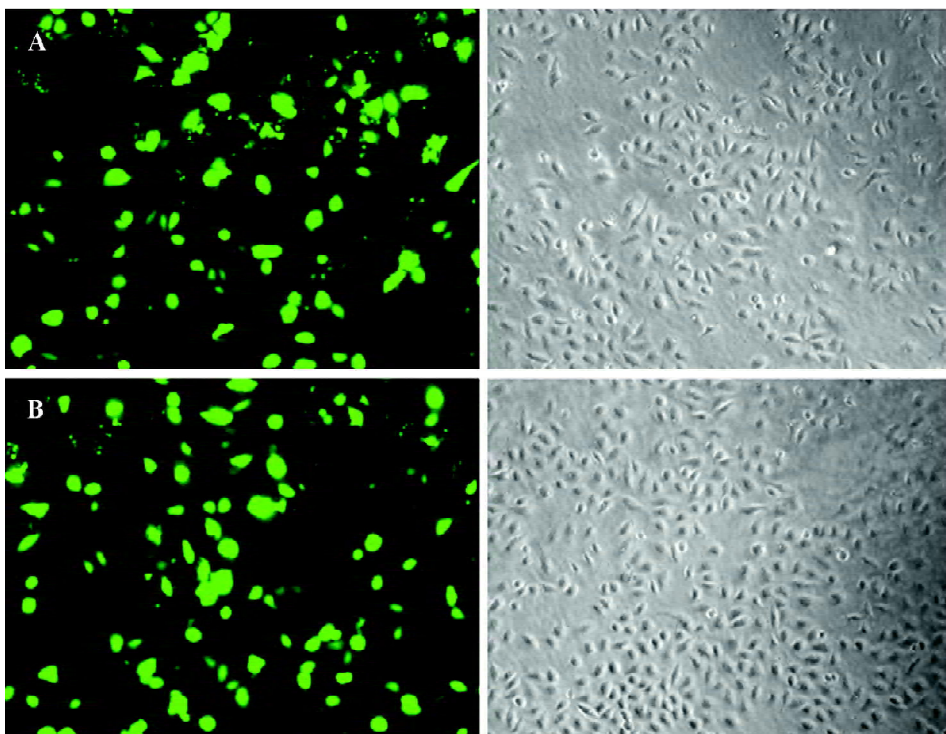


Figure 2. Detection of transfection efficiency of the pcDNA3-hbxip plasmid. Co-transfection transiently was performed in H7402 cells, such as (A) pcDNA3 plasmid and pEGFP-C2 plasmid, and (B) pcDNA3-hbxip plasmid and pEGFP-C2 plasmid. $\times 100$.

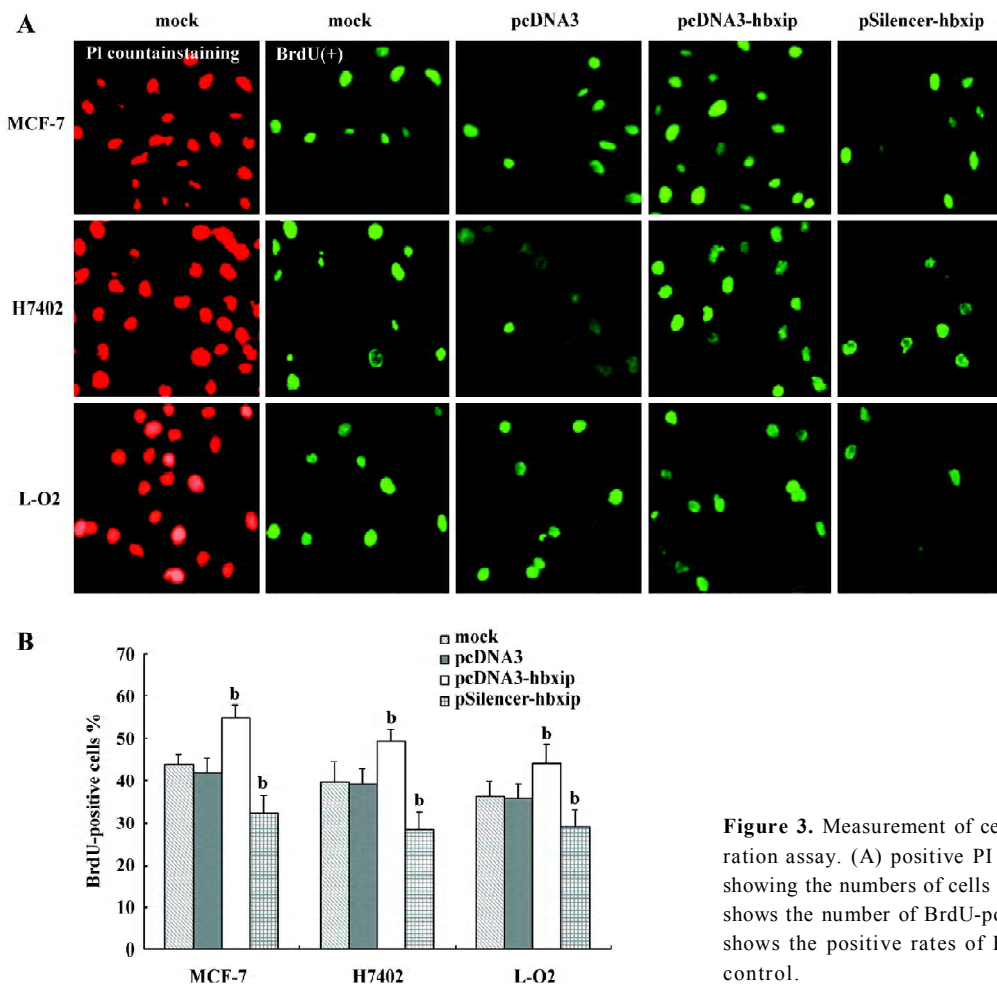


Figure 3. Measurement of cell proliferation by BrdU incorporation assay. (A) positive PI staining is in red in the nucleus, showing the numbers of cells as the control. Green fluorescence shows the number of BrdU-positive cells. $\times 100$. (B) histogram shows the positive rates of BrdU-positive cells. ^b $P < 0.05$ vs control.

plasmid upregulated expression of c-Myc, Bcl-2, and PCNA, but downregulated p27 in MCF-7, H7402, and L-O2 cells. However, RNAi targeting HBXIP mRNA with the pSilencer-hbxip plasmid reduced the expression levels of c-Myc, Bcl-2, PCNA, whereas the p27 expression level was upregulated with the downexpression of HBXIP in these cells. Since HBXIP was a cofactor for survivin, we also detected the expression of survivin in the same condition. The expression level of survivin was not affected by the overexpression or depression of HBXIP, which was consistent with the previous report^[2]. We further confirmed this finding by applying Glyco Band-Scan software (PROZYME, San Leandro, CA, USA; Figure 5).

Discussion

The functions of HBXIP were investigated in breast cancer MCF-7 cells, hepatoma H7402 cells, and the normal

hepatic cell line, L-O2. We overexpressed HBXIP in the above cells by transfection with the pcDNA3-hbxip plasmid and followed with an investigation of cell proliferation by MTT, BrdU incorporation assay, and flow cytometry analysis. The findings showed that the overexpression of HBXIP was able to promote cell proliferation. The depression of cell proliferation was found by RNAi targeting HBXIP mRNA in the cells. As we know, cell proliferation is functionally linked to the expression of genes associated with growth control. The maintenance of normal cell function and tissue homeostasis is depend on the precise regulation of multiple signaling pathways that control cellular decisions to either proliferate, differentiate, arrest cell growth, or apoptosis. Previous studies indicated that HBXIP was a necessary cofactor of survivin in the process of suppression of apoptosis in cancer cells, and increased HBXIP was found in both cancerous and non-malignant liver tissue of humans with chronic HBV infection^[2], so we supposed that the function of HBXIP might relate to

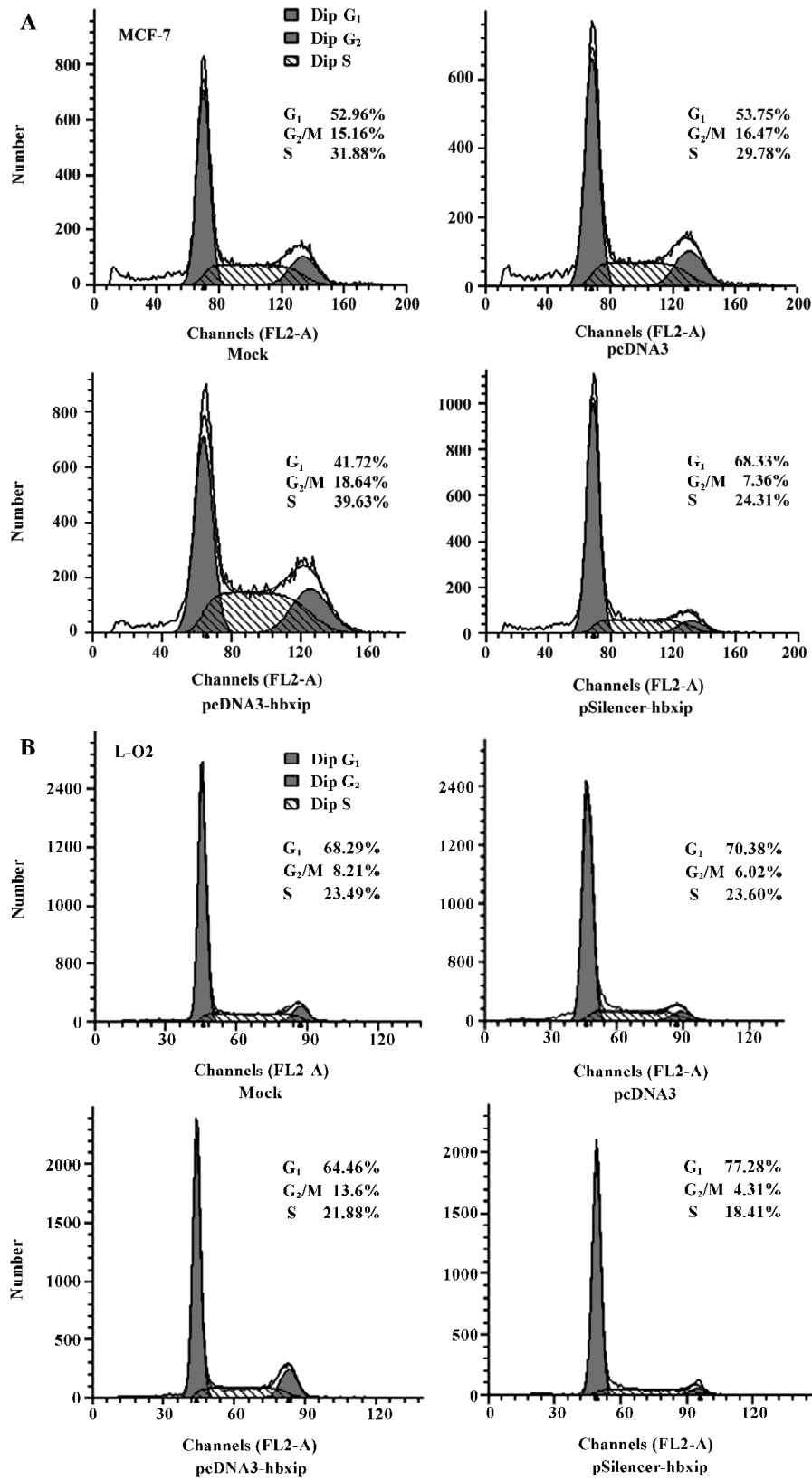


Figure 4. Examination of cell cycle by flow cytometry analysis in (A) MCF-7 cells and (B) L-O2 cells.

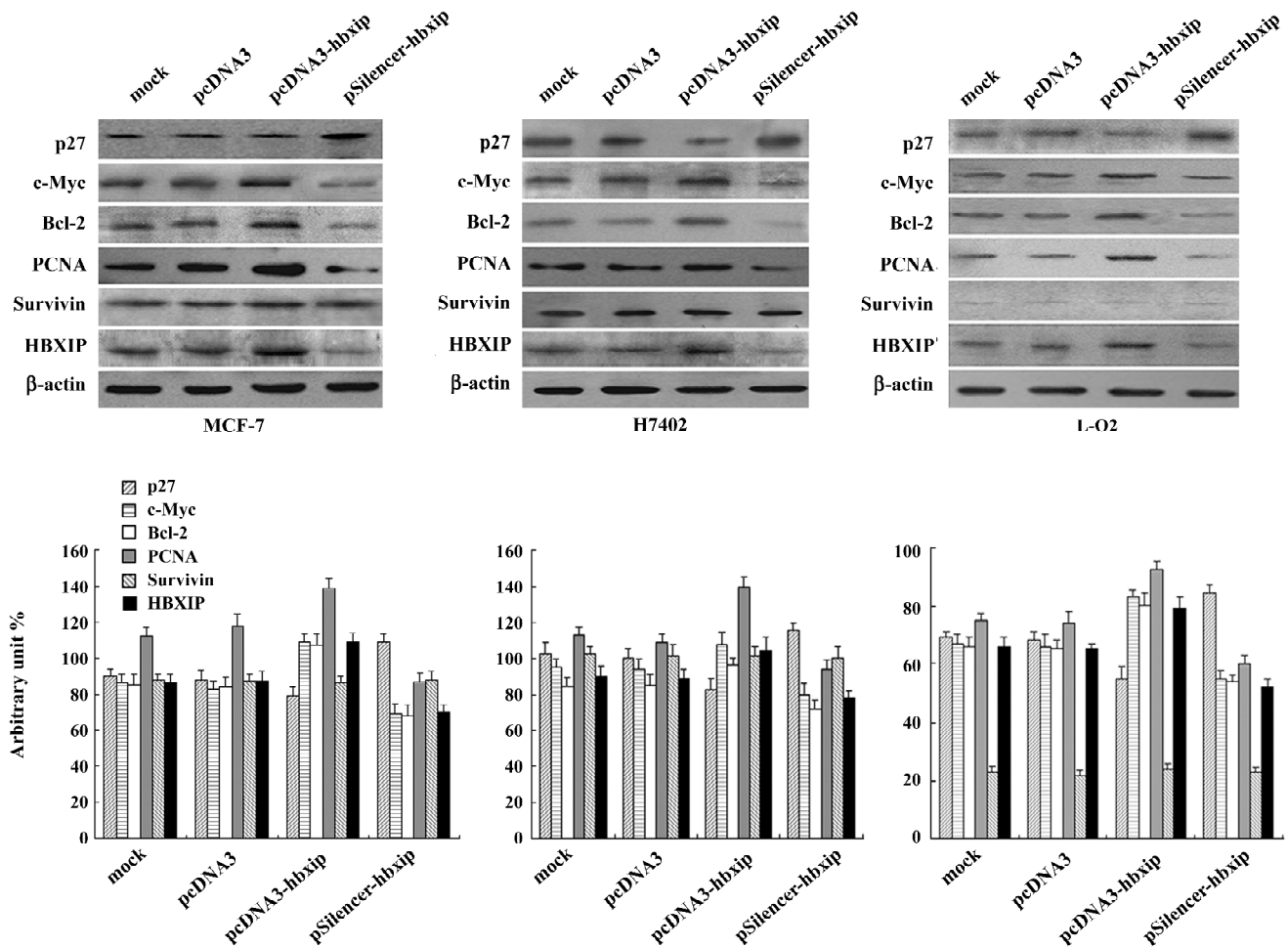


Figure 5. Examination of expression levels of p27, c-Myc, Bcl-2, PCNA, and survivin by Western blot analysis in MCF-7 cells, H7402 cells, and L-O2 cells.

the cell proliferation.

Marusawa *et al* reported that HBXIP was a link in bridging HBX and survivin^[2]. HBX plays an important role in the development of liver disease and exhibits effects on gene transcription, cell proliferation, survival, and apoptosis^[9,10]. It stimulates cell-cycle progression, shortening the emergence of cells from quiescence (G₀) and entry into the S phase by stimulating the Ras-signaling pathway, and accelerating transition through checkpoint controls at the G₀/G₁ and G₂/M phases^[11]. Survivin is a member of IAP (inhibitor of apoptosis protein) family and has been implicated in anti-apoptosis, cell division, and cell-cycle control^[12-15]. Under normal physiological conditions, survivin is involved in coordinating chromosomes and mitosis^[16].

In the present study, our data indicated that overexpression of HBXIP resulted in more MCF-7 cells going into the S phase

of the cell cycle. p27 is a member of the Cip/Kip family of cyclin-dependent kinase inhibitor (CDKI) that regulate cell-cycle progression, thus inhibiting various cycle-CDK complexes. Physiologically, p27 is believed to primarily regulate the progression of cells from late G₁ into the S phase by interacting with the cyclin E-CDK2 complex^[17,18]. We found that transient transfection with the pcDNA3-hbxip plasmid upregulated the expression of c-Myc, Bcl-2, and PCNA, but downregulated the expression of p27 in these cell lines. c-Myc is a nuclear phosphoprotein that functions as a transcription factor stimulating both cell-cycle progression and apoptosis. It plays a critical role in normal cell-cycle progression, especially during transition from G₁ to the S phase^[19]. c-Myc is also an early response gene, which responds directly to mitogenic signals to push cells in the G₁ phase of the cell cycle^[20]. The proto-oncoprotein Bcl-2 is a powerful

antagonist of the mitochondrial pathway of apoptosis initiated by a variety of extra- and intra-cellular stresses. As the Bcl-2 family members reside upstream of irreversible cellular damage and focus much of their efforts on the level of mitochondria, they play a pivotal role in deciding whether a cell will live or die^[21]. PCNA is a highly conserved 36 kDa acidic nuclear protein that is expressed during cell replication and DNA repair, and is correlated with cell proliferation^[22]. The same results, that is, upregulation of c-Myc, Bcl-2, and PCNA, were also observed in H7402 cells and L-O2 cells by overexpression of HBXIP.

Protein-protein interactions are crucial for all biological processes. Using systematic, automated, large-scale Y2H matrix interaction mating, Ulrich Stelzl *et al*^[23] screened other interacting proteins of HBXIP, such as eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), glyceraldehyde-3-phosphate dehydrogenase (GAPD), G protein-coupled receptor kinase interactor 1 (GIT1), KIAA1377, and rap1-interacting factor 1 (RIF1). These proteins in cellular functions are different. Therefore, HBXIP may also contribute more besides its effects on apoptosis and cell proliferation. Additionally, the expression of HBXIP could be regulated by some factors, such as garlic^[24], gonadotropins, and forskolin^[25].

Taken together, our findings indicate that one of the functions of HBXIP is its involvement in proliferation regulation in cancer cell lines and the normal liver cell line, which is related to cell-cycle transition through checkpoint controls at the G₀/G₁ or G₂/M phases and the downregulation of p27. In addition, the upregulation of c-Myc by HBXIP may also play an important role in cell-cycle transition.

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