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PES1 is overexpressed in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of common cancer with high mortality around the world, especially in China. PES1 was found to be overexpressed and played important roles in several kinds of human cancer, but its roles in HCC development remain unclear. We firstly reported here that PES1 was overexpressed in HCC tissues. Repression of endogenous PES1 suppressed cell proliferation and resulted in cell arrest in G1 phase in two HCC cell lines (HepG2 and HuH-7). Ablation of endogenous PES1 inhibited expressions of cell proliferation- and angiogenesis-related genes. Meanwhile, ablation of endogenous PES1 decreased alpha-fetoprotein (AFP) expression of HepG2 and HuH-7 cells. Re-expression of PES1 in these two kinds of PES1-knockdown cells rescued these effects. In addition, hepatitis B virus x (HBx) protein could upregulate PES1 expression of HCC cells in vitro. These results show that PES1 may have multiple functions in the development of HCC. PES1 may be a potential target for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of most common cancer with the second most common cause of cancer deaths around the world [1]. At the time of finally confirmed diagnosis, most HCC cases are locally advanced with or without distant metastasis, so they are difficultly treated and show poor prognosis. Targeted therapy has already been a new weapon against cancer, and targeted therapy plus chemotherapy or radiotherapy has been recently applied for the treatment of HCC. However, majority of these explorations didn't show exhilarating outcomes [2,3]. So breakthroughs in underlining molecular mechanisms of HCC progression will be very meaningful for HCC therapy.

Pescadillo is a gene originally identified in the investigation of embryonic development of zebra fish [4]. Pescadillo is highly conserved in sequence and functions among different species. Its homologues were identified and named in human (PES1), yeast (YPH1, Nop7p), and mouse (Pes1) [4-6]. Previous researches showed that pescadillo was necessary in biogenesis of ribosome and played an essential role in cell proliferation and cell cycle [7-14]. It has been shown that pescadillo could immortalize or transform some kinds of mammalian cells [15,16]. Our previous

researches and other investigators' researches showed that PES1 was overexpressed in many kinds of human cancer such as malignant astrocytomas and glioblastomas [6], breast cancer [16-18], prostatic cancer [19,20], head and neck squamous cell cancer [21], colon cancer [22], ovarian cancer [23], neuroblastoma [24] and gastric cancer [25]. These results suggest that PES1 may play an important role in tumor progression. But, so far, the functions of PES1 in human HCC progression are totally unknown.

Pes1 was expressed in embryonic liver but not in livers of adult mice. However, Pes1 expression in liver obviously increased at 16 hours after posthepatectomy in adult mice and was prior to expression of proliferating cell nuclear antigen, a well-known marker of cell proliferation [9]. As aberrant cell proliferation is one of the major properties of cancer cells, we wonder if PES1 play a role in the development of HCC. In this paper, we investigated the expression of PES1 in HCC tissues and effects of PES1 on cell proliferation and its related gene expressions. Meanwhile, the relationships between PES1 expression and alpha-fetoprotein (AFP) or hepatitis B virus x (HBx) protein expression in HCC cells were also observed in vitro.

Materials and Methods

Reagents and Plasmids

PES1 antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Antibodies against cyclin D1, HIF-1 α , p21 WAF1 and VEGF were from Bioworld Biotechnology (Louis Park, MN, USA). Pescadillo shRNA plasmid (h) (Santa Cruz Biotechnology; Santa Cruz, CA, USA) was packaged into pLVX-shRNA2 (Clontech; Mountain View, CA, USA). Expression vector for shRNA-resistant PES1 has been described previously [25]. HBx expression vector was a kind gift from Dr. Xu Lin (Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Research Center for Molecular Medicine, Fujian Medical University, Fuzhou, Fujian, People's Republic of China). HCC tissue array (Lot LV631) was from US Biomax (Rochville, MD, USA).

IHC staining

IHC staining was performed as described previously [23,25] using anti-PES1 as primary antibodies.

Cell culture and transfection

HCC cell lines HuH-7 and HepG2 were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China). HepG2 cells were cultured in MEM (GIBCO) with 10% fetal bovine serum (FBS) in a humidified atmosphere with 5% CO₂ at 37 °C. HuH-7 and HEK293T cells were cultured in DMEM (Invitrogen) containing 10% FBS (Hyclone). Lipofectamine 2000 reagent was used for the transfections according to the manufacturer's protocol (Invitrogen). 1 µg/ml puromycin was used to select stable cell lines after the lentiviruses were added to culture medium. Pooled clones or individual clones were screened by immunoblotting.

Inhibition of PES1 by RNA interference

As described before [25], to stably knock down endogenous PES1 expression, lentiviruses were prepared by co-transfected a Pescadillo shRNA plasmid and the pPACK Packaging Plasmid Mix (Clontech) into HEK293T cells. Lentiviruses were then added to the medium of target cells with 8 µg/ml polybrene.

Western blot analysis

Hepatocellular cancer cells were collected and lysed on ice in RIPA buffer. After detection of protein concentration, protein samples were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Blotted membranes were blocked overnight at 4 °C in TBST containing 5% non-fat milk. Then primary antibodies were used to probe blots for 1 h at room temperature. After washing with TBST, membranes were incubated with appropriate secondary antibody, followed by chemiluminescent detection.

Anchorage-dependent growth assay

Anchorage-dependent growth was analyzed as described [25]. Cells were plated on 24-well plates (2×10² cells per well) and cultured for 2 weeks. The colonies were stained with 0.5% crystal violet for 30 min after fixation with methanol for 30 min at room temperature.

Cell growth assay

Cell growth was analyzed by crystal violet assay as described previously [25]. Briefly, hepatocellular cancer cells were fixed by 1% glutaraldehyde for 15 min and stained with 0.5% crystal violet for 15 min at room temperature. The dye was eluted by Sorenson's solution for 30 min at room temperature. OD values of eluant aliquots were measured with microplate reader at 590 nm.

Cell cycle analysis

The cell cycle was analyzed by FACS [25]. Briefly, cells were harvested and washed twice with PBS and fixed in 75% ethanol (in PBS) at 4 °C overnight. Then cells were re-suspended in PBS after twice washing. Cells were incubated at 37 °C for 30 min and re-suspended in PBS containing 50 mg/ml propidium iodide. The cell cycle distribution was calculated using Cell Quest and Mod-fit software by analysis of flow cytometer.

Quantitative PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). The following primers were designed for quantitative PCR as described previously [25,26]: for PES1, forward 5'-TCATCAGGAGTTTGGTGGGGAAGT-3' and reverse 5'-TGGGTGATGCGGGAGTCT

GTGA-3'; HIF-1a, forward 5'-ATCCATGTGACCATGAG-

GAAATG-3' and reverse 5'-TCG-GCTAGTTAGGTA-CACTTC-3'; VEGF, forward 5'-CGCAGCTACTGCCATC-CAAT-3' and reverse 5'-GTGAGGTTTGTATCCGCATAATCT-3'; Cyclin D1, forward 5'-CCCACGATTT-CATTGAAC-3' and reverse 5'-AGGGCGGATTGGAAATGAAC-3'; p21 WAF1, forward 5'-TGGTGGCAGTAGAGGCTATG-3' and reverse 5'-AGTCCAGGCCAGTATGTTAC-3'; AFP, forward 5'-ACCCTGGTGTGGCCAGTGC-3' and reverse 5'-GCAGCGCTACACCCTGAGCT-3'; b-actin, forward 5'-TGGCACCCAGCACAAATGAA-3' and reverse 5'-CTAAGT-CATAGT CCGCCTAGAAGCA-3'. The thermocycling conditions were as follows: 42 °C for 5 min, 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 31 s. The relative target gene mRNA levels were normalized to b-actin. The experiment was repeated in triplicate.

Measurement of AFP in culture medium

AFP was measured by ELISA using ACCESS immuno-assay system (Beckman Coulter, USA). HepG2 and HuH-7 Cells were cultured in 24-well plates for 48 hours with or without transfection. Media were collected and cell number/well was determined. The amount of AFP secreted into culture media was detected with ELISA using immuno-assay. The experiment was repeated three times.

Statistical analysis

Differences between variables were assessed by a 2-tailed Student's *t*-test. Statistical calculations were performed using SPSS17.0. *P*-values of less than 0.05 were considered statistically significant.

Results

PES1 expression was upregulated in HCC tissues

In order to investigate PES1 expression in HCC, HCC tissue arrays were used to detect PES1 expression by IHC. As showed in Figure 1, PES1 was strongly stained in hepatocellular nucleus. About 66.7% of the detected cancer tissues showed PES1 positive, but all of the non-cancerous tissues exhibited negative staining. These results indicated that PES1 was overexpressed in HCC tissues (*p*<0.01).

Inhibition of endogenous PES1 expression suppressed cell growth and proliferation of HCC cells

Our previous results showed that PES1 was expressed at different levels in several kinds of HCC cell lines in our lab [27] (some data not shown) and PES1 expressions in HuH-7 and HepG2 cells were relatively higher. So these two kinds of cell lines were used for further experiments. In order to investigate the functions of PES1 in HCC progression, HuH-7 and HepG2 cell lines with stably ablated endogenous PES1 were established by transduction of PES1 shRNA. As showed in Figure 2A, PES1 shRNA significantly inhibited endogenous PES1 expression in these two cell lines. Compared to control cells, HuH-7 cells and HepG2 cells with stably ablated PES1 grew significantly slower (Figure 2B). PES1-ablated HuH-7 cells and HepG2 cells showed obviously less colonies in anchorage-dependent growth assay compared to control cells (Figure 2C). Meanwhile, ablation of endogenous PES1 in HuH-7 and HepG2 cells resulted in G1 phase arrest analyzed by FACS (Figure 2D&2E). Re-expression of PES1 in these kinds of cell lines was showed to rescue the effects caused by PES1 ablation. These results indicate that PES1 is very important to cell proliferation and growth of HCC cells.

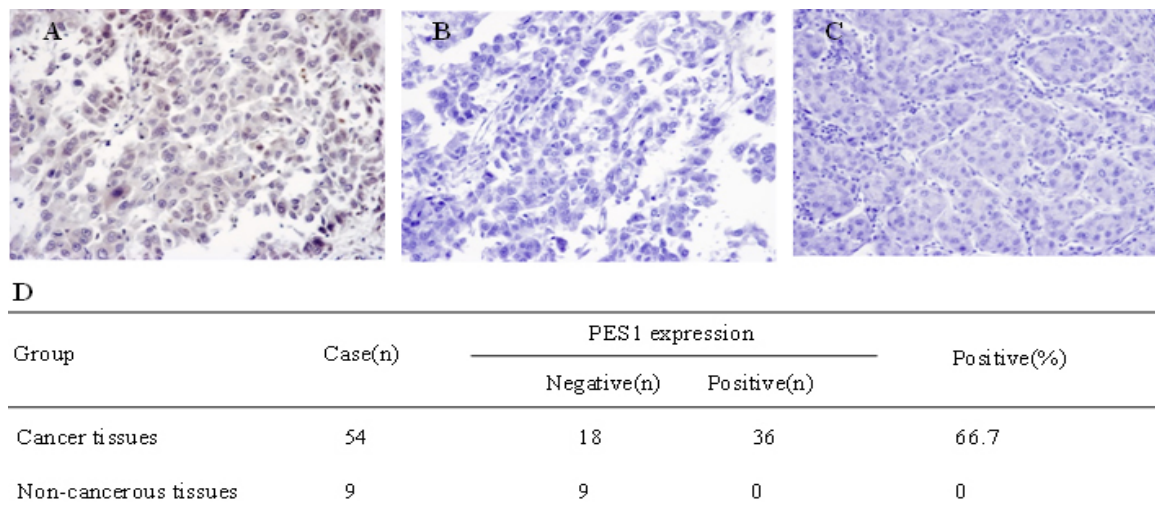


Figure 1: Expression of PES1 in HCC tissues. Immunohistochemical analysis of PES1 expression in human HCC tissues. The figures show the strongly nuclei staining of PES1 in HCC tissues (A). Negative staining of PES1 in non-cancerous tissues was shown in (B). Normal mouse IgG was used as a negative control in HCC tissues and shown in (C). Original magnification, $\times 40$. Scale bar: 100 μm . (D) Summary of PES1 expression in human HCC tissues and non-cancerous tissues

Ablation of endogenous PES1 altered expressions of genes related to cell proliferation and angiogenesis

As ablation of endogenous PES1 expression of HCC cells changed the cell proliferation and resulted in cell arrest, some genes related were detected in these two stably PES1-ablated cell lines. As showed in Figure 3A and Figure 3B, compared to control cells, mRNA and protein levels of Cyclin D1 in stably PES1-ablated HuH-7 and HepG2 cells were dramatically decreased, but p21WAF1 significantly increased at both mRNA and protein levels. It is well known that angiogenesis is closely related with the development of HCC, so HIF-1 α and VEGF expression were also detected in these two kinds of cell lines. The mRNA and protein levels of HIF-1 α and VEGF dramatically decreased in both kinds of stably PES1-ablated cell lines. Re-expression of PES1 in these two kinds of cell lines rescued these effects.

Inhibition of endogenous PES1 suppressed AFP expression of HCC cells in vitro

AFP is a widely used biomarker in diagnosis of HCC, so it is interesting to know if PES1 could affect the expression of AFP. As showed in Figure 4A, AFP mRNA levels were significantly decreased in both PES1-ablated HuH-7 and HepG2 cell lines analyzed by Q-PCR. Further detection of secreted AFP in the culture media showed that there were significant decreases of secreted AFP from both two kinds of stably PES1-ablated HCC cells compared to control cells (Figure 4B). Re-expression of PES1 could rescue these effects in PES1-ablated HepG2 and HuH-7 cells.

HBx protein increased PES1 expression of HCC cells in vitro

Chronic hepatitis B virus (HBV) infection is one of the most associated factors in hepatocarcinogenesis, HBx protein plays a crucial a role in the pathogenesis of HCC[28]. To investigate if there was a relationship between HBx protein and PES1 expression, HBx expression vector was transfected into HuH-7 and HepG2 cells in vitro. As showed in Figure 5A and Figure 5B, HBx protein increased PES1 expression at both mRNA and protein levels in both HuH7 and HepG2 cells. These results suggest PES1 may also play a role in the development of HBV-related HCC.

Discussion

We firstly showed here that PES1 expression was expressed in HCC tissues but not in non-cancerous tissues. In vitro, ablation of endogenous PES1 expression in HCC cells inhibited the growth of HCC cells, changed some genes related to cell proliferation and angiogenesis and resulted in cell cycle arrest in G1 phase. Meanwhile, ablation of endogenous PES1 repressed AFP expression in HCC cells. HBx protein could increase PES1 expression in vitro.

It is well known that Cyclin D1 and p21WAF1 play important roles in G1/M and G2/M transition [29,30]. Our results showed that ablation of PES1 differentially regulated p21WAF1 and Cyclin D1 expression and resulted in G1 phase arrest in HCC cells which was in concern with other researchers' results in other kinds of cancer cells [22,25]. However, repression of PES1 could result in G2 phase arrest in ovarian cancer cells and gastric cancer cells [23,25]. So it may be very interesting to investigate the precise mechanisms of cell cycle regulated by PES1.

Our previous results and other investigators' results showed that PES1 as a transcriptional factor could be recruited to the promoters of Cyclin D1 and heme oxygenase-1 genes and directly regulated the expressions of these genes [16,31]. Here we showed that PES1 changed the expression of genes related to cell cycle and angiogenesis such as Cyclin D1, p21WAF1, HIF-1 α and VEGF. As VEGF is one of the downstream target genes of HIF-1 α , it is very interesting to further investigate the precise mechanisms of the expression of this gene regulated by PES1. These results indicate that PES1 may have multiple functions and play important roles in the development of HCC.

Determination of serum AFP level is the gold standard in HCC diagnosis and has been widely used to complement HCC surveillance [33]. Our results showed that repression of PES1 expression in HCC cells resulted in an obvious decrease of AFP expression in vitro. These results suggested that alteration of PES1 expression may be an earlier event than change of AFP expression in the development of HCC. It has been clearly showed that HBx protein promoted the pathogenesis of HCC by interacting with several signaling pathway, such as p53, Wnt, and

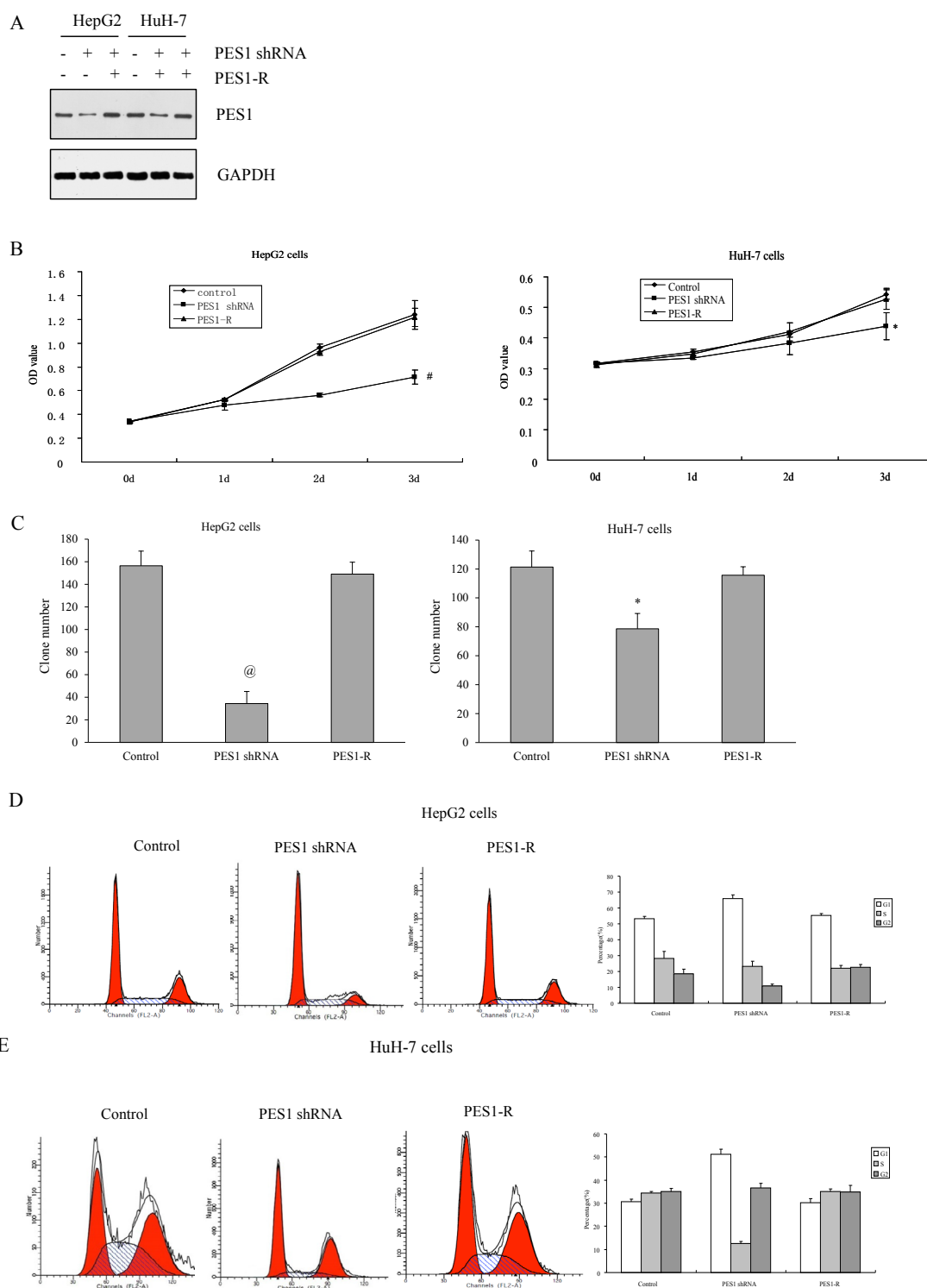


Figure 2: Effects of PES1 on the growth and proliferation of HCC cells. (A) Western blotting analysis of lysates from HepG2 and HuH-7 cells that have undergone the lentivirus-mediated stable ablation of PES1 or PES1 shRNA plus transiently transfected shRNA-resistant PES1. (B) Growth curves of HepG2 and HuH-7 cells stably transfected with PES1 shRNA or PES1 shRNA plus transiently transfected shRNA-resistant PES1. Cells were analyzed by the MTT assay. * $P < 0.01$ vs. shRNA control on day 4. # $P = 0.001$ vs. shRNA control on day 4. Values represent means \pm SD from three independent experiments. (C) Silencing of PES1 decreased colony formation in HepG2 and HuH-7 cells. * $P < 0.01$ vs. shRNA control. @ $P < 0.001$ vs. shRNA control. (D and E) Cell cycle was analyzed by FACS in HepG2 and HuH-7 cells with ablation or re-expression of PES1. Quantification of the cell cycle distribution was derived from three independent experiments. Values represent the means \pm SD

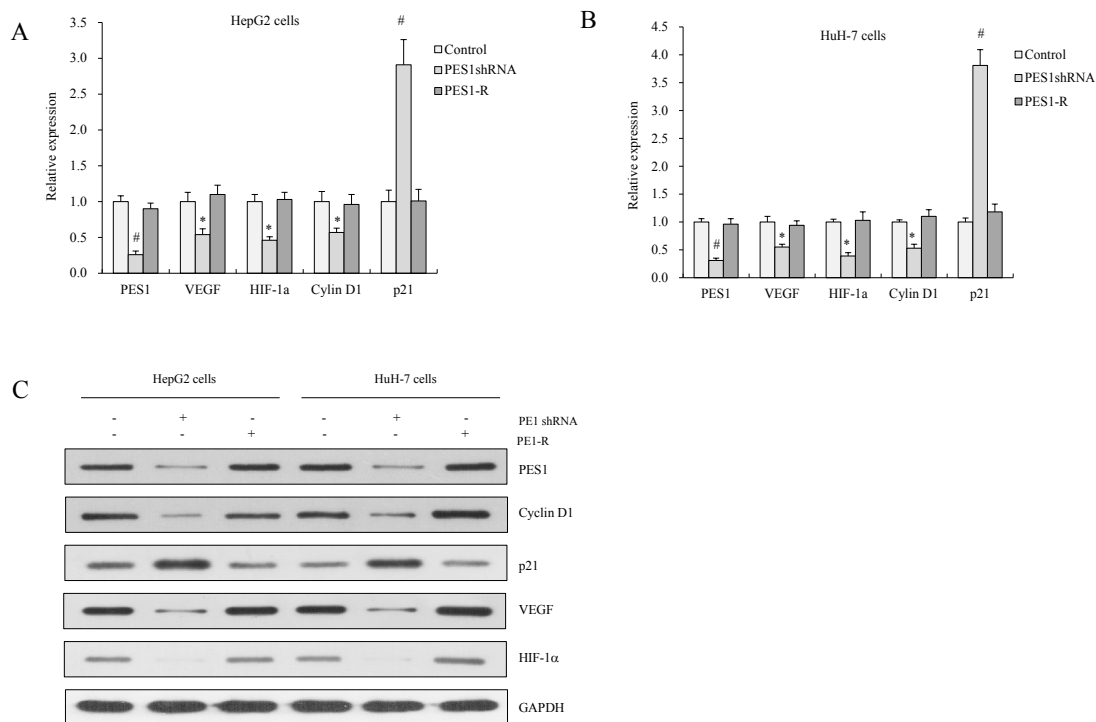


Figure 3: PES1 regulates downstream target genes expressions. (A and B) Real-time PCR analysis of target genes in HepG2 and HuH-7 cells stably transduced with shRNA control, PES1 shRNA or PES1 shRNA plus transiently transfected shRNA-resistant PES1. * $P < 0.01$ vs. shRNA control. # $P < 0.001$ vs. shRNA control. Values represent means \pm SD from three independent experiments. (C) Immunoblot analysis of the protein expressions of target genes in HepG2 and HuH-7 cells stably transduced with shRNA control, PES1 shRNA or PES1 shRNA plus transiently transfected shRNA-resistant PES1

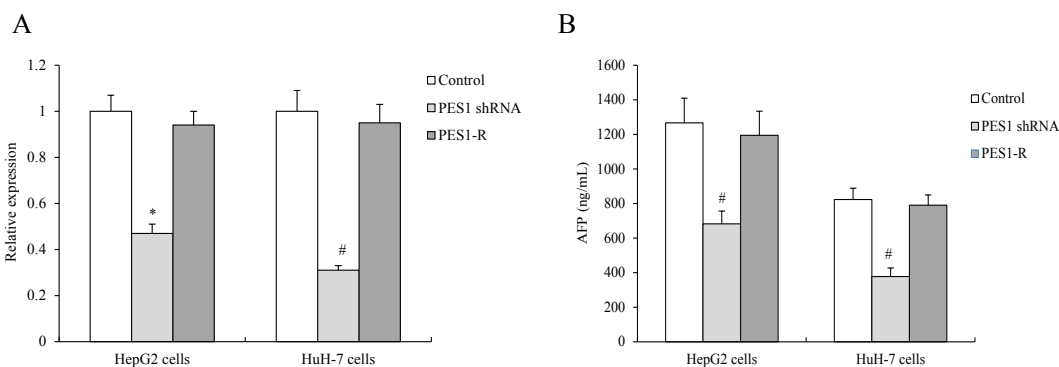


Figure 4: Repression endogenous PES1 expression decreased AFP expression in different kinds of HCC cells. (A) Real-time PCR analysis of AFP mRNA and (B) ELISA analysis of AFP secretion in HepG2 and HuH-7 cells stably transduced with shRNA control, PES1 shRNA or PES1 shRNA plus transiently transfected shRNA-resistant PES1. * $P < 0.01$ vs. shRNA control. # $P < 0.001$ vs. shRNA control. Values represent means \pm SD from three independent experiments

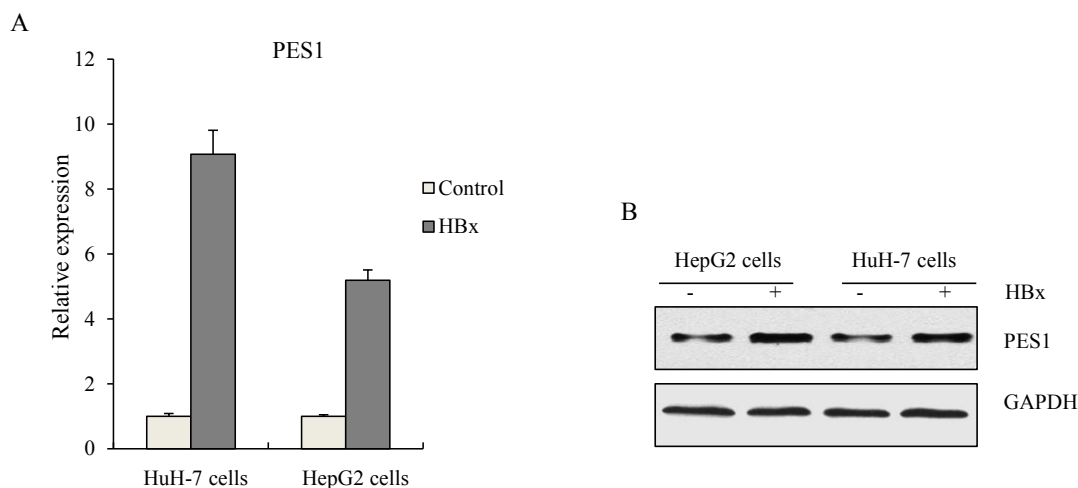


Figure 5: HBx protein increases PES1 expression in HCC cells. Real-time PCR (A) and western blotting (B) assay of PES1 expression in different HCC cells transfected with HBx expression vector or empty vector. Values represent means \pm SD from three independent experiments

nuclear factor-kB pathway [28]. Our results showed that HBx protein increased PES1 expression in vitro, suggesting PES1 may at least partly contribute to the carcinogenesis of HBV-reduced HCC. It has been showed that HBx protein could induce AFP expression and critically promoted malignant transformation of liver cells through activation of PI3K/mTOR signaling [34]. So it would be very interesting to investigate the precise mechanisms on how HBx protein regulates the expression of AFP and how PES1 participates to the signaling pathway interacted with HBx protein in HBV-related HCC.

Even though PES1 expression was showed higher expression in HCC tissues than non-cancerous tissues in this paper, small sample study was used. It is necessary to use larger sample to assay PES1 expression in HCC tissues and further its relationship with the outcome of HCC in future.

In summary, PES1 was overexpressed in HCC tissues. In vitro, repression of PES1 expression inhibited cell proliferation of HCC cells and altered some genes related cell cycle and angiogenesis. Suppression of endogenous PES1 of HCC cells significantly decreased AFP expression in vitro. Meanwhile, HBx protein could up-regulate PES1 expression in HCC cells in vitro. PES1 may play important roles in development of non-HBV- and HBV-related HCC.

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