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In vitro transfection of the hepatitis B virus PreS2 gene into the human hepatocarcinoma cell line HepG2 induces upregulation of human telomerase reverse transcriptase

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Abstract

The preS2 domain is the minimal functional unit of transcription activators that is encoded by the Hepatitis B virus (HBV) surface (S) gene. It is present in more than one-third of the HBV-integrates in HBV induced hepatocarcinoma (HCC). To further understand the functional role of PreS2 in hepatocytes, a PreS2 expression plasmid, pcS2, was constructed and stably transfected into HepG2 cells. We conducted growth curve and colony-forming assays to study the impact of PreS2 expression on cell proliferation. Cells transfected with PreS2 proliferated more rapidly and formed colonies in soft agar. PreS2 expressing cells also induced upregulation of human telomerase reverse transcriptase (hTERT) and telomerase activation by RT-PCR and the modified TRAP assay. Blocking expression of hTERT with antisense oligonuleotide reversed the growth rate in cells stably transfected with PreS2. Our data suggest that PreS2 may increase the malignant transformation of human HCC cell line HepG2 by upregulating hTERT and inducing telomerase activation.

Keywords: Hepatitis B virus; PreS2; Human telomerase reverse transcriptase; Hepatocellular carcinoma

Hepatitis B virus (HBV) is regarded as a major etiological factor in the development of human hepatocellularcarcinoma (HCC) [1,2]. Almost all HBV-associated HCCs harbor chromosomally integrated HBV DNA [3]. Integrated HBV DNA can encode two types of transcriptional activators, HBx protein and PreS2 activators [large surface proteins (LHBs) and truncated middle surface proteins ($MHBs^t$)] [4–7]. Clinical studies show that more than one-third of HBV-integrates in HBV related HCC might encode functional $MHBs^t$ transactivators [8,9]. This demonstrates the biological significance of PreS2 activators.

The sequence encoding the PreS2 activator is localized in the HBV surface gene. This gene consists of a single open reading frame which is divided into three coding regions, preS1, preS2, and S, that all start with an in-frame ATG codon. A large (LHBs; preS1+preS2+S), middle (MHBs; preS2+S), and small (SHBs; S) envelope glycoprotein can be synthesized through alternate translational initiation at each of the three ATG codons. Previous reports have shown that the preS2 region is sufficient to generate transcriptional activator function [10,11. However, the effect of the minimum functional PreS2 region on malignant transformation of hepatocytes remains unknown.

Telomerase is a multisubunit complex that forms the ends of eukaryotic chromosomes using complementary RNA sequence as a template [12,13]. Telomerase is generally inactive in normal somatic cells but is expressed in most human cancers and immortal cell lines [14–16].

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Recent reports suggest that telomerase activation may be an important step in the development of most malignant tumors [17,18], including human HCC. Studies demonstrate that, in contrast to normal human liver, over 80% of human HCC and 100% of HBV related HCC contain a high level of telomerase activity [19]. These findings provide new insight into the role of HBV in liver carcinogenesis.

In this study, we stable transfected the preS2 gene into the HCC cell line, HepG2, to study the effects of PreS2 on malignant transformation of HCC. Cells transfected with PreS2 proliferated more rapidly and were able to form a larger number of colonies in soft agar. This enhancement in proliferation was accompanied by an upregulation in hTERT expression and increased the telomerase activity. Blocking hTERT expression with antisense oligonuleotide reversed the growth rate in cells that were stably transfected with PreS2.

Materials and methods

Cell lines and cell culture. The HCC cell line, HepG2, was purchased from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences. It was maintained in MEM medium supplemented with 10% fetal bovine serum. All cell lines were cultivated in a 37 °C incubator with 5% CO_2.

Plasmid construction. The preS2 gene was amplified using sense (5′-CTAAGCTTCAGTCATCCTCAGG-3′) and antisense (5′-CTCTAG ACCTAAGCGTAGTCTGGTACGTCGTAAGGGTATGTGTTCTCC ATGTTCGGTG-3′) primers from the plasmid, as a gift from Dr. Akira Nishizono (Oita Medical University, Japan) pUC19/3HBV-wt(adr), which contained three copies of HBV (adr subtype, GenBank Number: gi59404). The primers were so designed that the endogenous M protein translation initiation codon could be used to initiate translation of our PreS2 expression construct. To allow the expressed protein to be detected, the antisense primer was fused with the HA sequence (underlined). The amplified 239 bp fragment was subcloned into the *Hin*dIII/*Xba*I site of pcDNA3 (Invitrogen, USA) to construct the PreS2 expression plasmid-pcS2.

Stable expression of the preS2 gene. A total of 2×10^5 HepG2 cells were seeded into each well of a 24-well plate. When the cells had reached 70–80% confluence one day later, they were transfected with the appropriate plasmids using lipofectam6ine™2000 (Invitrogen, USA) according to the manufacturer's protocol. After 48 h, stably transfected cells were screened with 380 μg/ml of G418 (Sigma) for two weeks. Untransfected HepG2 cells and cell clones that were stably transfected with pcDNA3 were established as controls. All transfected cell clones were maintained in the presence of G418 (380 μg/ml) throughout the experiments. These cells were used to assess the effects of PreS2 on HCC cells as described below.

RNA extraction and reverse transcription PCR. Total RNA was extracted by TRIZOL (TaKaRa Co.) from 5×10^5 cells and quantified using a spectrophotometer (Eppendorf Co. German). Three micrograms of total RNA was reverse transcribed into cDNA using Murine Moloney Leukemia virus (M-MLV) reverse transcriptase (Promega, USA). PCR was carried out according to the manufacturer's instructions. The following sequence-specific primers were used for PCR amplification: (1) the housekeeping gene β-actin (sense: 5′-GGCATCGTGATGGACTCCG-3′, and antisense: 5′-GCTGGAAGGTGGACAGCGA-3′), (2) PreS2 gene primer pairs (see above), and (3) the hTERT gene (sense: 5′-CGGAAG AGTGTCTGGAGCAA-3′, and antisense: 5′-GGATGAAGCGGAG TCTGGA-3′). The PCR products were electrophoretically separated on a 2% agarose gel and visualized by ethidium bromide staining.

Western blot analysis. Cells were lysed in gel-loading buffer containing 50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, and 10% glycerol. Fifty micrograms of protein was resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and electrically blotted onto a nylon filter (Millipore). The filters were blocked with phosphate-buffered saline (PBS) containing 15% nonfat milk and incubated with monoclonal antibody directed against HA (Covance). After washing with PBS containing 0.5% Tween 20, bound primary antibody was detected with anti-mouse IgG (Zhongshan, Beijing, China). After washing, the anti-HA antibody bound proteins were visualized using enhanced chemiluminescence.

Cell growth analysis. The cell growth curve and colony forming assays were adopted to measure cellular proliferation and colony formation. Stably transfected cells (5×10^3) were plated into each well of a 96-well plate. Each group had three wells for every time point. The Cell Counting Kit-8 (Dojindo Molecular Technologies, Shanghai, China) was used to measure cellular proliferation at different time points as previously described [20]. In brief, 10 μl of a CCK-8 solution was added to the cell culture and incubated for 15 min, and the optical density (OD) value of the wells was read using an enzyme-labeled minireader (Bio-Rad, Japan) at A450.

For the soft agar colony formation assay, approximately 500 cells were suspended in medium containing 0.35% low melt agarose, seeded into a six-well plate that was overlaid with 0.5% low melt agarose, and allowed to grow for two weeks at 37° C in 5% CO_2. The colonies containing more than 50 cells were counted under a microscope. Three to five wells were analyzed for each experiment.

Telomerase activity assay. To measure telomerase activity, the telomeric repeat amplification protocol (TRAP) assay was performed as previously described [21]. Samples were suspended in phosphate-buffered saline (PBS) and after centrifugation, the pellets were homogenized in 20 μl ice-cold lysis buffer (10 mmol/L Tris/HCl (pH 7.5), 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L b-mercaptoethanol, 0.5% CHAPS (Sigma), and 10% glycerol). After incubating on ice for 30 min, the lysate was centrifuged at 15,000*g* for 30 min at 4° C, and the supernatant was frozen and stored at −80° C. The protein concentration in the extract was measured using the Bradford assay (Beyotime Biotechnology), and 5 μg of protein was used for the TRAP assay. Assay tubes were prepared using 0.2 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′). Each extract was assayed in 50 μl of a reaction mixture containing 20 mmol/L Tris/HCl (pH 7.9), 10 mmol/L $MgCl_2$, 50 mmol/L KCl, 1 mmol/L DTT, 50 μmol/L dNTPs, and 1 μg of T4g32 protein (New England Biolabs). After telomerase mediated extension of the TS primer for 30 min at 23 °C, 0.2 μg CX primer (5′-CCCTTACCCTTACCCTTACCCTTAA-3′) and 2.5 U of Taq DNA polymerase (Takara Biotechnology) were added. The reaction mixture was heated at 90 °C for 3 min and subjected to 30 PCR cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s. The PCR products were electrophoresed on a 12% polyacrylamide gel and visualized by silver-staining.

Antisense oligonucleotide blocking assay. Antisense oligodeoxynucleotides directed against hTERT mRNA (as-hTERT: 5′-GCCACG TGG GAAGCG-3′) were synthesized (Bioasia Biotechnology, Shanghai, China) and used in experiments to specifically block expression of hTERT. A non-complementary sequence (random: 5′-TTGCCGAGCGGGGTA-3′) was used as a control. For the antisense blocking assay, 5×10^3 cells/well were seeded in a 96-well plate. After 24 h, 10 μmol/L as-hTERT or non-complementary oligodeoxyonucleotides were added to the PreS2 stably transfected HepG2 cells. Cell numbers were counted in triplicate every day for four days.

Results

Identification of preS2-transfected cells

The preS2 expression plasmid, pcS2, which contained the second translational initiation of the HBV (adr type) surface gene was constructed (data not shown) and stably transfected into HepG2 cells. HepG2 cells that were stably transfected with pcDNA3 served as a control. RT-PCR

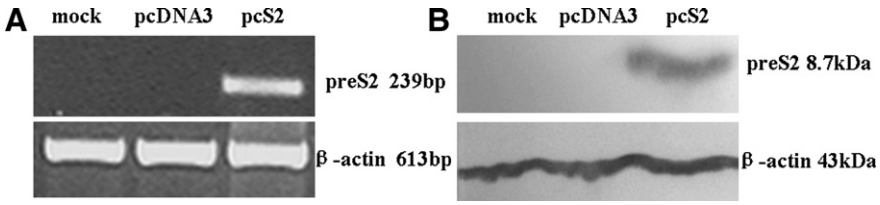

Fig. 1. Expression of PreS2 in HepG2 cells. (A) RT-PCR was performed on total RNA to determine preS2 expression (top) and β-actin mRNA levels (bottom) in HepG2 cells transfected with the indicated plasmids. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. (B) Western blot was performed to determine PreS2 (top) and β-actin expression (bottom) in HepG2 cells. Bound proteins were visualized using enhanced chemiluminescence.

illustrated that preS2 mRNA was expressed in cells transfected with the preS2-expression construct (Fig. 1A), but not in untransfected HepG2 cells or cells transfected with pcDNA3 vectors. The RT-PCR results were confirmed at the protein level using Western-blot analysis (Fig. 1B).

PreS2 expression increases cell proliferation in vitro

To investigate the effects of PreS2 gene expression on the growth of HepG2 in vitro, properties of the HepG2 cells carrying the preS2 gene were examined, and compared to empty vector transfected and untransfected HepG2 cells. Growth curves indicated that cells expressing the preS2 gene showed a more rapid cell proliferation rate than cells transfected with the empty vector and untransfected cells (Fig. 2A).

To confirm the effects of PreS2 on cell growth, the colony forming assay was used. The colony forming ability of HepG2 cells carrying the PreS2 was 19–21% while that of untransfected HepG2 cells was approximately 15% ($p < 0.05$). No differences were observed in the colony forming ability of untransfected and empty transfected cells (Fig. 2B). These results indicated that preS2 gene expression enhanced the growth rate and malignant tendency of cells.

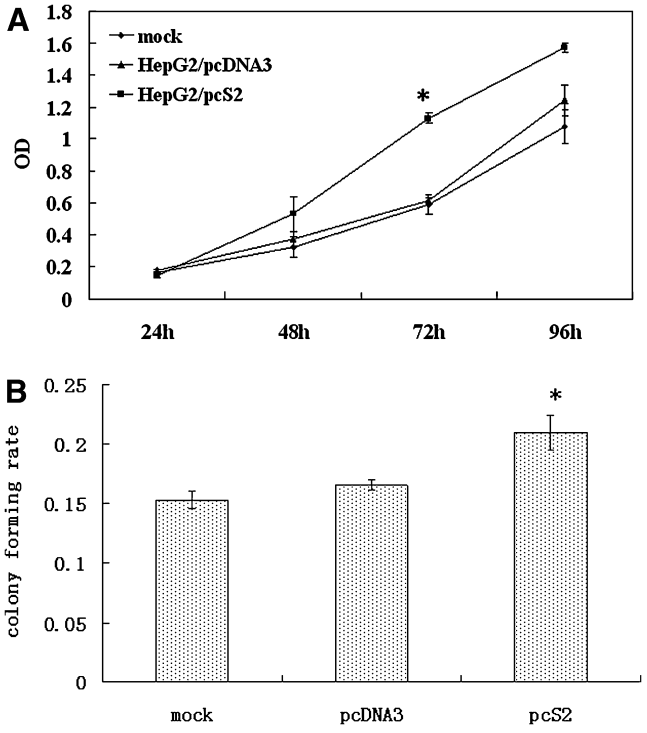

Fig. 2. HBV PreS2 accelerates the growth of HepG2 cells. The data show the means of four independent experiments. *, indicates significant differences in relation to HepG2 cells, $p < 0.05$. (A) Growth curve of HepG2 cells transfected with the indicated plasmids. Stably transfected cells (5×10^3) plated into each well of a 96-well plate and the cell number was counted every day for four days using the CCK-8 assay. (B) Soft agar colony formation of HepG2 cells transfected with the indicated plasmids. Cells (5×10^2) were seeded on low melt agarose medium. Cell colony number was counted after two weeks. Three to five wells were analyzed for each experiment.

PreS2 expression upregulates hTERT mRNA and telomerase activity

A number of studies have shown that in addition to HBV infection, telomerase activation plays an important role in HCC. To further investigate the mechanism of the increase in cell growth rate induced by the PreS2 gene, we first measured telomerase activity in HepG2 cells. The TRAP assay was used to detect telomerase activity in preS2-transfected cells. As shown in Fig. 3A and B, cells expressing preS2 exhibited high levels of telomerase activity. No differences were observed in telomerase activity between HepG2 and pcDNA3 transfected HepG2 cells ($p > 0.05$).

We next determined whether this increase in telomerase activity correlated with hTERT transcription. RT-PCR revealed that hTERT mRNA expression of PreS2 stably transfected HepG2 cells was significantly higher than that of untransfected HepG2 cells. hTERT mRNA expression was found to be approximately fivefold higher than that of control cells. No differences were observed in hTERT mRNA expression between HepG2 and pcDNA3 transfected HepG2 cells ($p > 0.05$) (Fig. 3C and D). These results suggest that PreS2 increases the telomerase activity by upregulating hTERT transcription.

Blocking hTERT expression decreases PreS2 stable transfected cell proliferation in vitro

Our studies showed that the increased growth rate of cells expressing PreS2 was accompanied by upregulated telomerase activity. To investigate the correlation between

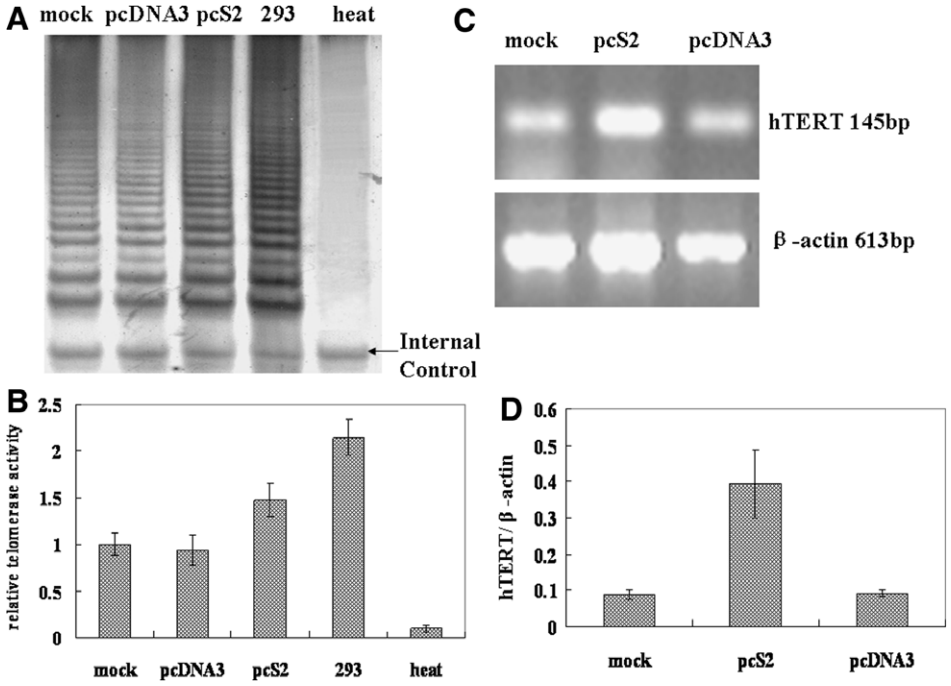

Fig. 3. Telomerase activity and hTERT mRNA expression were activated by PreS2 expression in HepG2 cells. (A) TRAP assay products were electrophoresed on a 12% polyacrylamide gel and visualized by silver-staining. 293 cell line were used as a telomerase-positive control. 293 cells were heat treated prior to measuring telomerase-specific activity. (B) Telomerase activity was quantified by applying Glyco BandScan software. The telomerase activity is expressed as the value relative to that of mock cells that was normalized to 1.0. Data are means ± SD of at least three separate experiments. (C) RT-PCR was performed on total RNA to determine hTERT expression (top) and β-actin mRNA levels (bottom) in HepG2 cells transfected with the indicated plasmids. (D) RT-PCR products were quantified on digitized images of DNA gels using the ImageMaster TotalLab v 1.00. The pixel density of each individual PCR reaction was normalized to the average pixel density for actin-β. The gene/actin-β density ratios were averaged for independent experiments. Each bar corresponds to means ± SD for at least three independent experiments. Results were analyzed using the Student's *t*-test.

increased hTERT transcription and the rapid cell proliferation enhanced by preS2, we used antisense oligonucleotide against hTERT (as-hTERT). RT-PCR revealed that as-hTERT suppressed hTERT expression but had no effect on β-actin expression (Fig. 4A). The TRAP assay showed that as-hTERT inhibited telomerase activity while non-complementation had no effect on HepG2 expression in preS2 cells (Fig. 4B). The effect of as-hTERT on the growth of preS2 transfected cells was also examined. The increase in cell proliferation induced by preS2 was reversed by as-hTERT (Fig. 4C). However, as-hTERT treated cells showed a slightly higher growth rate than untransfected and pcDNA3 transfected HepG2 cells. These results suggest that preS2 promotes HCC malignant transformation at least in part through enhancing the expression of hTERT.

Discussion

In this study, we used preS2 stable transfected HepG2 cells to study the transactivation effects of the PreS2 protein on HCC cells. Results showed that cells transfected with PreS2 proliferated rapidly and had increased colony-forming ability. In previous studies, we found that antisense RNA targeted to PreS2 specifically suppressed PreS2 expression and significantly inhibited tumor growth in mice injected with HCC HepG2.2.15 cells [22]. These results indicate the important role of PreS2 in the development of HBV related HCC.

It is well known that some virus-encoded proteins contribute to human cell transformation and carcinogenesis by modulating cellular signaling pathways that regulate cell-cycle progression, proliferation, differentiation, and death. Recent evidence suggests that human tumor-associated viruses can also induce cancer formation through transcriptional activation of the hTERT gene and subsequent activation of telomerase. Previous reports show the tumor promoter-like function of preS2 activators. PKC-dependent activation of the c-Raf-1/MAP2-kinase signaling cascade can induce PreS2 activators that go on to activate transcription factors like AP-1 and NF-κB [23–25]. An increased incidence of liver tumor is found in MHBs^t transgenic mice that are older than 15 months [25]. To identify the impact of the minimal function PreS2 region on the malignant transformation of HCC, we first analyzed the effects of PreS2 on telomerase activation. Our results demonstrated that the HepG2 cells carrying the PreS2 gene showed increased telomerase activity and hTERT expression.

To further investigate the impact of PreS2 expression and telomerase activation on the increase in cell growth, we used antisense against hTERT (as-hTERT). The high rate of cell proliferation induced by PreS2 expression was

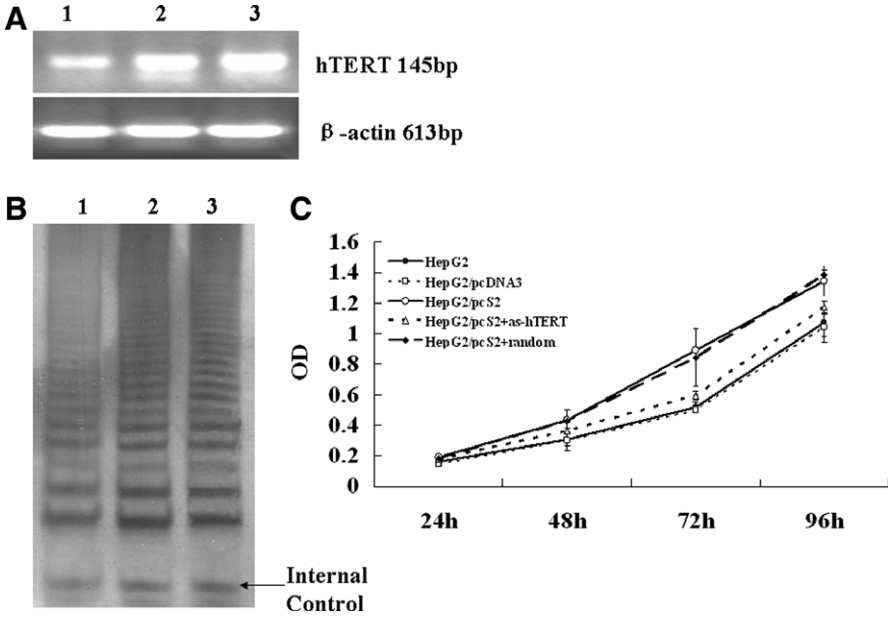

Fig. 4. Blocking hTERT expression decreased the proliferation of cells stably transfected with PreS2 in vitro. Data represent the means of four independent experiments. (A) PreS2 transfected HepG2 cells were cultured with 10 μmol/L as-hTERT or non-complementary oligdeoxyonucleotides for 48 h. RT-PCR was performed on total RNA to determine hTERT expression (top) and β-actin mRNA levels (bottom). Lane 1, cells with as-hTERT; lane 2, cells with non-complement; and lane3, HepG2/pcS2 cells. (B) PreS2 transfected HepG2 cells were cultured with 10 μmol/L as-hTERT or non-complementary oligdeoxyonucleotides for 48 h. The TRAP assay was used to measure telomerase activity. Lane 1, cells with as-hTERT; lane 2, cells with non-complement; and lane 3, HepG2/pcS2 cells. (C) Growth curve of HepG2 cells transfected with the indicated plasmids. After 24 h, 10 μmol/L as-hTERT or non-complementary oligodeoxyonucleotides were added to preS2 transfected HepG2 cells. Cell numbers were counted in triplicate every day for four days using the CCK-8 assay. HepG2/pcS2+as-hTERT vs. HepG2/pcS2 cell $p < 0.05$, HepG2/pcS2+random vs. HepG2/pcS2 $p > 0.05$, HepG2/pcS2 vs. HepG2 $p < 0.05$, HepG2 vs. HepG2/pcDNA3 $p > 0.05$.

greatly reversed by as-hTERT treatment. These results indicated that preS2 promotes tumor growth at least in part through inducing hTERT transcription. However, cell proliferation after as-hTERT treatment of preS2 transfected cells was still slightly higher than in untransfected and pcDNA3 transfected cells, indicating that preS2 promotes tumor growth using mechanisms other than hTERT. This conclusion is consistent with the way in which some viruses contribute to carcinogenesis by modulating cell signaling pathways that regulate cell-cycle progression, proliferation, differentiation, and death.

hTERT expression is primarily regulated at the transcriptional level and the core promoter of hTERT encompasses numerous transcription factor binding sites [26–28]. All these factors, which regulate the hTERT promoter region individually or together, comprise a complex regulation system. The human papillomavirus type (HBV)16 E6 protein activates telomerase and hTERT through c-Myc and GC-rich Sp1 binding sites [29] The latency associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus (KSHV) appears to target and affect the Sp1 protein bound to the hTERT promoter [30]. Our study indicated that the HBV virus transactivator, PreS2, upregulates hTERT mRNA expression and telomerase activity. Whether or not the PreS2 protein can transactivate the hTERT promoter and which *cis* element is responsible for activation, however, requires further investigation.

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