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HBV core promoter mutations promote cellular proliferation through E2F1-mediated upregulation of S-phase kinase associated protein 2 transcription

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Abstract

Background & Aims—Hepatitis B virus (HBV) core promoter (CP) mutations have been associated with an increased risk of hepatocellular carcinoma (HCC) in clinical studies. We previously reported that a combination of CP mutations seen in HCC patients, expressed in HBx gene, increased SKP2 (S-phase kinase-associated protein 2) expression thereby promoting cellular proliferation. Here, we investigate the possible mechanisms by which CP mutations up-regulate SKP2.

Methods—We used immunoblotting and ATPlite assay to validate the effect of CP mutations in full length HBV genome on cell cycle regulator levels and cell proliferation. Activation of SKP2 mRNA was assessed by quantitative real time PCR in primary human hepatocytes and HCC cell lines. Effect of CP mutations on SKP2 promoter activity was determined by luciferase assay. Target regulation of E2F1 on SKP2 was analyzed by siRNAs.

Results—CP mutations in full length HBV genome up-regulated SKP2 expression thereby down-regulating cell cycle inhibitors and accelerating cellular proliferation. CP mutations enhanced SKP2 promoter activity but had no effect on SKP2 protein stability. Mapping of the SKP2 promoter identified a region necessary for activation by CP mutations that contain an E2F1 response element. Knocking down E2F1 reduced the effects of CP mutations on SKP2 and cellular proliferation. Effect of CP mutations on E2F1 might be mediated through hyperphosphorylation of RB.

Conclusions—HBV CP mutations enhance SKP2 transcription by activating the E2F1 transcription factor and in turn down-regulate cell cycle inhibitors thus providing a potential mechanism for an association between CP mutations and HCC.

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Keywords

hepatocellular carcinoma; HBx gene; cell cycle regulators

1. Introduction

Several viral factors including hepatitis B virus (HBV) genotype C, persistently high HBV DNA level and HBV core promoter (CP) mutations have been shown to be independently associated with HBV-related hepatocellular carcinoma (HCC) [1]. In a study of 2762 persons with chronic HBV infection followed for 13 years, the common double mutation A1762T/G1764A (TA) in the HBV CP region was associated with a hazard ratio of 1.73 for HCC development when compared to wild type CP [1]. Longitudinal studies indicate that TA is selected early, many years before HCC diagnosis, while other CP mutations, notably T1753V and T1768A appear later, near the time of HCC diagnosis [2, 3]. These other CP mutations are almost always accompanied by TA double mutations in HCC patients.

How CP mutations induce HCC has not been established. CP region overlaps with HBV X gene (HBx). HBx is a multifunctional protein that can regulate HBV replication, cellular transcription, signal transduction pathway, proteasome activity, cell progression, and apoptosis [4–6]. The TA double mutation in the CP region results in amino acid changes in HBx protein (K130M/V131I) and may increase the risk of HCC by modulating the biological functions of HBx protein.

Down-regulation of cell cycle inhibitors has been suggested to contribute to HCC development. The S-phase kinase associated protein 2 (SKP2) in the SCF^{SKP2} E3 ligase complex targets many key cell cycle regulatory proteins for proteasomal degradation. Studies have shown that SKP2 is required for many steps in cell cycle progression, including G1/S transition, S phase progression, and S/G2 transition [7, 8]. Given its central role in promoting S phase entry, SKP2 may act as an oncogene. Overexpression of SKP2 has been detected in many human tumors including HCC [9]. We and others have shown that SKP2 increases cell proliferation by enhancing cell cycle promoters such as CDK2 and Cyclin E and decreasing cell cycle inhibitors such as p21, p27, and p57 [9, 10]. SKP2 is also a common downstream effector of multiple mitogenic cascades including Ha-Ras, AKT, JAK/STAT, and Wnt/beta-catenin, which had been implicated in human hepatocarcinogenesis. High level of SKP2 had been shown to correlate with poor prognosis in HCC patients [9]. Recent studies showed that aberrant activation of SKP2 cooperates with other oncogenes in hepatocarcinogenesis and may decrease efficacy or confer resistance to therapy with mammalian target of rapamycin (mTOR) inhibitors [11]. Studies with animal models showed that pharmacologically targeting SKP2 in combination with other anti-cancer drugs may improve response to HCC treatment [8, 11, 12].

In an earlier study, we showed that a combination of CP mutations typically seen at the time of HCC diagnosis (TA plus T1753V and T1768A) up-regulates SKP2 which in turn down-regulates cell cycle inhibitor p21, leading to accelerated cell cycle progression, anchorage-independent cell growth, and increased cellular proliferation. Knocking down SKP2 abrogated the effect on p21 and cellular functions [10]. However, those studies were conducted in the context of HBx gene alone and the mechanisms leading to increase SKP2 expression were not determined. In the current study, we determined whether the effect of CP mutations on SKP2, p21 and other cell cycle regulators could be reproduced in the context of the full length HBV genome. We also investigated the possible pathways by which CP variant increases SKP2 expression. Comparable results were observed in cells expressing HBx alone or full length HBV genome. We then demonstrated that CP variant

increased SKP2 mRNA and protein levels via activation of its promoter function. Finally, we showed that CP variant increased SKP2 transcription through the transcription factor E2F1.

2. Materials and Methods

2.1 Plasmid construction

HBx expressing plasmid was constructed by amplifying the open reading frame and the upstream enhancer/promoter region (nt1060–1838) of the X gene from 1.5-mer HBV genome. A FLAG epitope was introduced at the C-terminus of HBx and this amplified fragment was inserted into KpnI and XbaI sites of pGL3-Basic vector. Variants with HBx K130M/V131I (abbreviated as HBx TA) and HBx with I127N/K130M/V131I/F132Y (abbreviated as HBx Combo) were generated by site-directed mutagenesis as previously described [10]. Tandem dimers of full length HBV genome wild type (abbreviated HBV WT) and variant with K130M/V131I (abbreviated as HBV TA) were provided by Dr. Tong [13]. A combination variant with I127N/K130M/V131I/F132Y corresponding to the changes of T1753A/A1762T/G1764A/T1768A in the CP region (abbreviated as HBV Combo) was generated from the HBV TA variant by overlap extension PCR using Expand high-fidelity PCR system (Roche, Pleasanton, CA). All mutations were confirmed by sequencing.

2.2 Cloning of SKP2 promoter and luciferase reporter assay

A 3.98-kb fragment of human SKP2 promoter was cloned from human genomic DNA (Lonza, Walkersville, MD) (bases –3980 to +1) into the XhoI site of the pGL3 firefly luciferase vector (Promega, Madison, WI). Truncated SKP2 promoter fragments p(–3980/+1), p(–316/+1), p(–197/+1), and p(–108/+1) were generated by PCR using p(–3980/+1) as template (Primer sets used are shown in Supplementary Table 1). A series of SKP2 promoter variants were generated using Quickchange kit (Stratagene) and p(–316/+1) as a template (sequences of primers in Supplementary Table 1). All constructs were verified by DNA sequence analysis. For dual-luciferase assays, the cells were transiently cotransfected with the firefly luciferase reporter constructs along with the pRL-tk plasmid (Promega) containing the Renilla luciferase gene for internal normalization.

2.3 Cells, transfection, and Western blotting

Human hepatoma cell lines and primary human hepatocytes (PHH) were used in this study. All transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transduction of cultured PHH with lentivirus based HBx WT and HBx variants and generation of HBx expressing stable cell lines were performed as described previously [10]. For Western blotting, whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and immunoblotted with the following primary antibodies: p21 (BD Biosciences, San Jose, CA), p27 (BD Biosciences), anti-Flag (Stratagene, La Jolla, CA), anti-SKP2 (Cell Signaling, Danvers, MA), anti- β -actin (Sigma-Aldrich, St. Louis, MO), anti-Cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA), anti-E2F1 (Cell Signaling), anti-Rb (Cell Signaling), anti-phospho-Rb (Sigma), and anti-HBV core (Dako, Carpinteria, CA). After washing, the membranes were incubated with donkey anti-mouse IgG-horseradish peroxidase or goat anti-rabbit (Santa Cruz Biotechnology). The bound antibody was visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Relative protein levels were quantified by densitometry analysis using NIH Image J (version 1.41).

2.4 Quantitative RNA analysis

Primary human hepatocytes (PHH) were infected with lentivirus preparations containing HBx WT and variants as described previously [10]. Huh7 cells were transfected with HBx WT and variants. On the day of harvest, total cellular RNA in PHH and Huh7 cells, respectively, was isolated using TRIzol reagent (Invitrogen) and was reverse transcribed by using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on an IQ5 Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green kit Supermix (Bio-Rad) according to the manufacturer's instructions. Relative levels of mRNA were standardized to beta-actin. Primer sequences used in this study are listed in Supplementary Table 1. Each reaction was repeated at least three times.

2.5 Cycloheximide chase analysis

Huh7 cells were seeded onto 60mm dishes and transfected with HBx WT, TA, and Combo variants. 48 hours after transfection, cells were treated with cycloheximide (CHX) 60 µg/ml to stop *de novo* protein synthesis. Cell lysates were harvested at 0, 60, 120, 180, and 240 min following CHX treatment and then subjected to immunoblotting.

2.6 RNA interference

Cells were plated at 5×10^5 cells/ml in a 6-well plate. 24 hrs after plating, the cells were transfected with E2F1 siRNA (Dharmacon, Chicago, IL). Scrambled siRNA oligonucleotides were used as control for E2F1 RNA interference experiments (sequences shown in Supplementary Table 1). Transfection of siRNA was carried out according to the LipofectamineTM RNAiMAX transfection reagents guidelines (Invitrogen).

2.7 ATPlite cell proliferation assay

Cells transfected with HBV/HBx WT and CP variants were split and seeded into 96-well plates with 3,000 cells per well in quadruplicate for ATPlite cell proliferation assay at various time points (Beckman Coulter).

2.8 HBsAg and HBeAg detection

HBsAg secreted into the culture supernatant was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Abazyme, Needham, MA). Briefly, 20ul of culture supernatant was diluted with phosphate-buffer saline to 100ul for the detection of HBsAg. The Optical Density (O.D.) was read at a wavelength of 450nm. HBeAg in the supernatants was quantified by the ETI-EBK plus enzyme immunoassay (Diasorin, Stillwater, MD) according to the manufacturer's instructions. Briefly, 100ul of 1:5 diluted culture supernatant, calibrator and controls were added to their respective microwells. The absorbent value was read at a wavelength of 450nm. The linear range of absorbance in this assay is from 0.12 to 2.50. Samples with absorbance that exceeded the upper limit were retested after further dilution to bring the reading to the range of calibration curve. Samples with absorbance below detection were retested undiluted. Results were evaluated based on sample absorbance to cutoff ratio. The relative titers of HBsAg and HBeAg for each HBV variant were calculated by comparing to that for wild type HBV.

2.9 Statistical Analysis

Values were expressed as mean $\pm$ standard deviation (SD). Comparison between experimental and control groups was performed using unpaired Student's t-test. In all cases, $p < 0.05$ was considered significant.

3. Results

3.1 CP mutations in the context of full length HBV genome dysregulates cell cycle regulators and cellular proliferation

We have previously shown that HBx variants with a combination of CP mutations seen in HCC patients increased SKP2 (S-phase kinase-associated protein 2) expression to promote cellular proliferation and transformation [10]. To address whether CP mutations affect cell cycle related proteins when they are expressed in the context of full length HBV genome, we first confirmed that HBV genomic constructs encoding the WT, TA, and Combo HBx variants maintain expression of HBsAg, HBeAg and hepatitis B core protein. All of the HBV constructs produce HBsAg, HBeAg and HBV core protein. Compared to HBV WT, HBV TA and Combo variants decreased HBeAg secretion (Supplementary Figure 1). We next determined expression of p21 and p27, members of the CIP/KIP family of cell cycle inhibitors. Similar to our findings with HBx alone, expression of the Combo and TA mutations in full length HBV genome expressing Huh7 cells decreased the levels of both p21 and p27 compared to HBV WT, with the Combo variant having a greater effect than the TA variant. We also found that HBV Combo and TA variants increased SKP2 (Fig. 1A and B).

To further verify whether the effect of CP mutations on cell proliferation in HBx-expressing cells can be replicated when these mutations are expressed in the context of full length HBV genome, we measured cellular proliferation by ATPlite proliferation assay. The HBV Combo variant accelerated cell growth in Huh7 cells ($P<0.05$ for Combo vs WT or TA, Fig. 1C), similar to that in cells transfected with HBx CP variant (Fig. 1D). Similar effects of CP mutations on cell cycle regulators and cellular proliferation were also observed in HepG2 cells (data not shown). Taken together, these results demonstrate that CP mutations expressed in the context of full length HBV genome decrease levels of cell cycle inhibitors, increase SKP2 protein, and enhance cell proliferation. Therefore, subsequent experiments were performed using HBx alone to reveal how HBx CP variants upregulate SKP2 protein expression.

3.2 HBx CP variants increase SKP2 transcription

The observed increase in SKP2 protein expression could be due to increased transcription of SKP2 mRNA or to decreased degradation of SKP2 protein. We first examined the effect of HBx CP variants on endogenous SKP2 mRNA levels in both primary human hepatocytes (PHH) and Huh7 cells by quantitative real-time RT-PCR. The HBx Combo variant but not HBx TA variant, increased SKP2 mRNA levels in PHH (Fig. 2A; $P<0.05$ for Combo vs WT or TA). Both HBx Combo and TA variants increased SKP2 mRNA levels in Huh7 cells compared to HBx WT (Fig. 2A, $P<0.01$ and $P<0.05$ for Combo variant vs WT or TA, respectively, $P<0.05$ for TA vs WT). A time course study indicated that increase in SKP2 mRNA induced by HBx Combo variant preceded increase in SKP2 protein levels in Huh7 cells (Fig. 2B).

We then focused on the effect of CP mutations on SKP2 protein stability by performing cycloheximide (CHX) chase experiments. As shown in Figure 2C, SKP2 protein stability was similar in cells expressing HBx WT or CP variants, as well as vector control. These data indicate that HBx CP variants enhance SKP2 protein expression by increasing SKP2 transcription and not by modulating SKP2 protein degradation.

3.3 HBx CP variants enhance SKP2 promoter activity

Having shown that HBx CP variants upregulate SKP2 levels by promoting its transcription, we next identified the regions of the SKP2 promoter required for transcriptional activation

by HBx. Luciferase reporter plasmids containing various lengths of the SKP2 promoter (p(-3980/+1), p(-316/+1), p(-197/+1), and p(-108/+1)) were transiently transfected into Huh7 cells to determine their promoter activity. Results indicated that constructs p(-3980/+1), p(-316/+1) and p(-197/+1) exhibited strong luciferase activity while construct (-108/+1) displayed weak promoter activity when compared to the pGL3-Basic vector lacking upstream promoter sequences (Fig. 3A). We then identified the region in the SKP2 promoter responsible for HBx CP variant modulation of SKP2 transcription by cotransfection of HBx variants with our SKP2 promoter reporter constructs. As shown in Figure 3B, HBx TA and Combo variants increased SKP2 promoter activity of the (-3980/+1), (-316/+1), and (-197/+1) reporter constructs to a similar extent while no enhancement was observed for the -108/+1 construct. Thus, the region between -197 to -108 of the SKP2 promoter is necessary for HBx CP variant enhancement of SKP2 transcription. These data indicate that CP variant enhances SKP2 promoter activity which in turn increases transcription of SKP2 mRNA and translation of SKP2 protein

To further investigate the mechanisms by which HBx regulates SKP2 transcription, we searched the -300 to +1 sequence of the SKP2 promoter for transcription factor binding sites using the TRANSFAC[®] 7.0 database (BIOBASE Co., Beverly, MA). This search identified three putative E2F recognition sites (abbreviated as ERS1, ERS2, and ERS3, respectively) suggesting that E2F might play a role in CP variant regulation of SKP2 transcription (Fig. 3C). Because the region -197 to -108 is necessary for the enhancement of SKP2 promoter activity by CP variant, we hypothesized that ERS2 is a critical element for HBx CP variant activation of the SKP2 promoter. We created a series of SKP2 promoter constructs encoding mutations in each of the three E2F recognition sites (abbreviated as mERS1, mERS2, mERS3, and mERS1+mERS3, respectively). As shown in figure 3D, mutation of ERS1, ERS3 or both ERS1 and ERS3 had no effect on the ability of HBx CP variants to enhance SKP2 transcription, while mutation of ERS2 abolished the transactivation of SKP2 promoter by HBx CP mutant variant. These data indicate the essential role of the ERS2 element in mediating activation of SKP2 promoter by HBx CP variants.

3.4 HBx CP variant up regulates SKP2 promoter activity through E2F1

Because SKP2 is a target of the E2F1 transcription factor(14) and E2F1 plays a role in driving cell cycle progression, we asked whether E2F1 activity is required for enhancement of SKP2 expression by HBx CP variants. First, we evaluated E2F1 expression in HepG2 cells expressing HBx WT and CP variants. HBx CP variants increased E2F1 protein expression, with the Combo variant having a more pronounced effect (Figure 4A). We also examined the effect of HBx expression on retinoblastoma protein (RB), a repressor of E2F1. Both HBx TA and Combo variants increased the levels of phospho-Rb but had no effect on the total amount of RB when compared to HBx WT (Fig. 4A). Next, we knocked down E2F1 by RNA interference and determined the effect on SKP2 level and cell proliferation in Huh7 cells expressing HBx WT and CP variants or the vector control. E2F1 silencing blocked the induction of E2F1 by HBx CP variant (Figure 4B) and abrogated the increase in SKP2 levels. Next, HepG2 cells stably expressing HBx WT or CP variants were transfected with E2F1 siRNA (E2F1si) or a scrambled siRNA (SC), and cellular proliferation was measured. E2F1 silencing blocked the enhancement of cell proliferation by HBx CP variant (Figure 4C). These results indicate that HBx CP variants regulate SKP2 transcription through E2F1.

4. Discussion

In this study, we confirmed that HBV CP mutations that are often seen in patients with HCC, in the context of full length HBV genome had the same effect as they did in HBx

alone in increasing SKP2 expression and cellular proliferation and in decreasing cell cycle inhibitors p21 and p27. Studies have shown that SKP2 contributes to hepatocarcinogenesis and tumor aggressiveness [9, 10, 12, 15], and HBV CP mutations have been reported to be associated with an increased risk of HCC (1–3).

In this study, we investigated how HBV CP mutations enhanced SKP2 expression. We found that this was achieved through increased SKP2 transcription and not increased stability of SKP2 protein. We then identified a region in the SKP2 promoter that was necessary for interaction with CP mutations. This region contained an E2F1 recognition site. Silencing of E2F1 confirmed that enhancement of SKP2 promoter activity by HBV CP mutations is dependent on E2F1.

E2F1 has been shown to play an important role in cell cycle progression and cellular growth via its association with RB. RB repression of E2F transcription factors acts as a master switch for cell cycle progression, holding cells in G1 until they are ready to progress into S phase. At the G1/S checkpoint, RB phosphorylation leads to E2F release with subsequent progression into S phase. Thus, deregulation of RB/E2F activity favors aberrant DNA replication and cellular growth [16]. Given our finding that HBx CP variant increases phospho-Rb levels, we propose that HBV CP mutations activate E2F1 by increasing Rb phosphorylation, which in turn promotes SKP2 transcription. Increased SKP2 accelerates degradation of cell cycle inhibitors inducing cell cycle progression (10). Some studies showed that E2F1 may interact with other regulatory proteins besides RB [17]. Further studies are needed to determine the pathway by which HBV CP mutations interact with E2F1.

Deregulation of E2F1 has been implicated in the development of HCC [18, 19]. In addition to the effects of E2F1 on cell cycle inhibitors, overexpression of E2F1 in both transgenic mice and human liver activates the Akt signaling pathway to suppress apoptosis [19–21]. E2F1 also interacts with the mTOR cascade to enhance carcinogenesis via accelerating cellular proliferation [22]. Besides regulating cell growth and apoptosis, E2F1 combined with c-Myc may modulate specific metabolic functions in the hepatocyte that contribute to hepatocarcinogenesis [23]. CP mutations have also been reported to be associated with more severe hepatic inflammation and may contribute to HCC development through increased inflammation and accelerated progression to cirrhosis. It is likely that CP variants represent a subgroup within the HBV quasispecies. The ratio of CP variants to wild type might evolve during the progression from pre-malignant to malignant liver disease. It would be interesting to further study the effect of changes in proportion of CP variants due to immune pressure selection on cellular proliferation.

In conclusion, the present study revealed that HBV CP mutations frequently present in patients with HCC stimulate transcription of SKP2 that in turn decrease expression of cell cycle inhibitors such as p21 thereby promoting cellular proliferation. We showed that HBV CP mutation enhancement of SKP2 expression is E2F1 dependent and may be mediated through increase RB phosphorylation. Elucidating the pathway by which HBV CP mutations interact with E2F1 may advance our understanding of the oncogenic role of HBV CP mutations and provide potential drug targets for HCC treatment. These findings need to be confirmed in humans or in animal models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

CP	Core promoter
HCC	hepatocellular carcinoma
HBx	hepatitis B virus X-protein
TA	mutant, A1762T/G1764A mutant
Combo mutant	T1753A/A1762T/G1764A/T1768A mutant
WT	wild type
SKP	S-phase kinase-associated protein
PHH	primary human hepatocytes
ERS	E2F recognition site
RB	retinoblastoma protein

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Fig. 1.

Effect of HBx CP mutations on cell cycle regulators and cellular proliferation in Huh7 cells. (A) Levels of p21, p27, Cyclin E and SKP2 in cells expressing full length HBV genome. (B) Levels of p21, p27, and SKP2 in cells expressing HBx alone. (C) Full length HBV genome with CP mutations accelerated cell proliferation. Asterisk: Combo vs WT, $P < 0.01$; Dot: Combo vs TA or V, $P < 0.05$. (D) HBx with CP mutations increased cellular growth. Asterisk: Combo vs WT, $P < 0.01$, Dot: Combo vs TA or V, $P < 0.05$, dagger: TA vs WT, $P < 0.05$. V: control vector

Fig. 2.

HBx CP variant increased SKP2 mRNA levels but had no effect on SKP2 protein degradation. (A) Effect of CP variants on SKP2 mRNA level in PHH (left panel, Asterisk: Combo vs WT or TA, $P < 0.05$) or Huh7 cells (right panel, Asterisk: Combo vs WT, $P < 0.01$, Dot: Combo vs TA, $P < 0.05$, dagger: TA vs WT, $P < 0.05$), V: vector control. (B) Combo variant increased SKP2 mRNA followed later by increase in SKP2 protein expression. (C) Effect of CP variant on SKP2 protein degradation. The protein levels at different time points were quantified by densitometric analysis and calculated as percentage of the initial protein level (lower panel).

agtcagccgtagcgccgcccgccttccgcctccccgc -343
ctaccccgtgggcccgcaccagtcccgcctcccgcgggg -307
ggttgtgggtatctcgaagGCGGGTAAgctgcaag-271
E2F (ERS1)
ggaaggaagtaagggcgagcccgtcggactacgaag -235
cgggacggaaactacaattcccagcaggccttggggc -199
ctcagtgcggccgcgaagcagagcgggctgtagagc -163
cTTGCGCGCGcagtggggatggaacgttgctaggct -127
E2F (ERS2)
tagcgggtctggctgctggggggcccgcagcagcacgc -91
tcggagccgcccgCGCGCCAAAgcggggaatctgggag-55
E2F (ERS3)
gcgagcagctctgcagttaatgcacgtattttaaac -19
tcccgggcctgcggacgctatg +3

Fig. 3.

Effect of HBx WT and CP variants on SKP2 promoter activity. (A) Identification of human SKP2 promoter. (B) CP variant enhanced SKP2 transcription. (C) Alignment of human SKP2 promoter sequences (~300-bp) upstream of translation start sites. Bold with arrows: putative transcription factor binding sites. (D) Mutation analysis of SKP2 promoter. Upper panel: positions of wild type and mutant ERS within plasmid p(-316/+1). Middle panel: specific mutation of each ERS site. Bottom panel: effect of ERS mutations on E2F1 transactivation of SKP2 promoter. Asterisk: Combo vs WT, $P < 0.01$, Dot: Combo vs TA, $P < 0.05$, dagger: TA vs WT, $P < 0.05$, V: vector control.

Fig. 4. HBx CP variant regulates SKP2 expression through E2F1. (A) Effect of HBx WT and CP variants on protein levels of E2F1 and RB (total and phosphorylated form) in HepG2 cells. (B) Knocking down E2F1 eliminated effects of CP variant on SKP2 promoter activity and protein level. Asterisk: Combo vs WT, $P < 0.01$, Dot: Combo vs TA, $P < 0.05$, dagger: TA vs WT, $P < 0.05$. (C) Knocking down E2F1 eliminated effects of CP variant on cellular proliferation.