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Cyclin D2 plays a regulatory role in HBV replication

Chun-li Song [a,1], Ji-hua Ren [a,1], Long-kuan Ran [a,1], Yong-guo Li [b], Xiao-song Li [a], Xiang Chen [a], Wan-yu Li [a], Ai-long Huang [a], Juan Chen [a,*]

[a] The Second Affiliated Hospital and the Key Laboratory of Molecular Biology of Infectious Diseases designated by the Chinese Ministry of Education, Chongqing Medical University, Chongqing, China
[b] Department of Forensic Medicine, Chongqing Medical University, Chongqing, China

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ABSTRACT

Hepatitis B virus (HBV) infection is the leading cause of liver diseases. However, the molecular mechanisms of HBV infection and carcinogenesis have not been fully elucidated. In this study, we found that cyclin D2 was upregualted in HBV-expressing cells and liver tissues of HBV-transgenic mice. Gene silencing of cyclin D2 inhibited HBV DNA replicative intermediates, 3.5 kb mRNA, core protein level, as well as the secretions of HBsAg and HBeAg. On the contrary, overexpression of cyclin D2 promoted HBV replication. Furthermore, cyclin D2 regulated HBV replication by enhancing the activity of HBV core and Sp1 promoters by targeting transcription factor CREB2. Silencing of CREB2 abolished enhancement of HBV replication induced by cyclin D2. Together, our study has uncovered a positive role of cyclin D2 in HBV replication. It is conceivable that therapeutic application of cyclin D2 inhibitor in HBV infection therapy.

Introduction

HBV is the leading cause of acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), affecting more than 240 million people worldwide (Ott et al., 2012). It is particularly common in Asia, Africa, Southern Europe and Latin America (Lin et al., 2005). However, the molecular mechanism of HBV replication remain elusive. Currently, there are two major strategies including immunomodulatory therapy and direct antiviral therapy applied for HBV treatment. However, longer term treatment of HBV patients with nucleoside/nucleotide analogs often results in drug resistance and with interferon brings about notorious side effects (Jiao et al., 2012; Micco et al., 2013). Therefore, there is an urgent need to understand HBV replication at the molecular level and to probe novel molecular targets for HBV therapy.

HBV carries a small (ca. 3.2 kb), relaxed circular (RC), partially double-stranded DNA genome (Summers and Mason, 1982). Upon viral entry into hepatocyte, the viral DNA is first converted into a covalently closed circular DNA, the cccDNA. The cccDNA then serves as the viral transcriptional template for the synthesis of pg RNA and subgenomic RNAs that translate into the viral surface protein, core protein, the viral polymerase, and x protein respectively (Neuveut et al., 2010).

Cyclin D2 (CCND2) is a member of the D-type cyclin family, which is the main integral mediators associating the extracellular signaling environment with cell-cycle progression (Zhang et al., 2013). Cyclin D2 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6 and shortens the G1 phase (Kato and Sherr, 1993). Cyclin D2 mainly participates in cell cycle and cancer progression (Song et al., 2008). Wang et al. demonstrated that hypermethylation intensity of cyclin D2 promoter was increased in HBV-related HCC (Wang et al., 2012). Additionally, other cell-cycle regulators p53 and p21 protein has been reported to be associated with HBV-related HCC (Choi et al., 2001). These findings suggested the association between cell-cycle regulators and HBV-related HCC. So far, studies focused on the direct relation between cyclin D2 and HBV infection are very limited.

In this study, we analyzed the differentially expressed genes in HBV replication cell model HepG2.2.15 (Bird and Sells, 1987) and HepG2 cells using gene microarray analysis. The results showed that cyclin D2 was significantly upregulated in the presence of the replicating HBV genome. Knockdown of cyclin D2 inhibited HBV replication, whereas overexpression of cyclin D2 promoted HBV replication. Furthermore, we discovered that cyclin D2 could regulate activities of HBV core and Sp1 promoters possibly by targeting transcription factor CREB2. Our findings implicated a role

* Corresponding author. Tel.: +86 2368818112.
E-mail address: yixin_xinyuan@163.com (J. Chen).
[1] These authors contribute equally to this work.

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of cyclin D2 in HBV replication and have identified a host factor in HBV life cycle.

Results

Upregulation of cyclin D2 in HBV-expressing cells

The gene expression profile was compared in HepG2.2.15 which is a HBV stably transfected cell line constitutively producing HBV and HepG2 cells by using gene expression array. Array-based transcriptional mapping indicated that transcription of the HBV genome altered several genes expression involved in cell cycle regulation. Among them, the expression of cyclin D2 (CCND2) was upregulated more than 14 folds in HepG2.2.15 compared with HepG2 (Zhang et al., 2010). To determine whether cyclin D2 was specially upregulated by HBV, the mRNA level of cyclin D2 was first examined in HepG2.2.15, HepG2 cells transiently transfected with HBV expressing plasmid pCH9/3091 (containing 1. 1-unit length HBV genome driven by CMV promoter) or pGEM-HBV1.3 (containing 1.3-unit length HBV genome driven by endogenous viral promoter) and control cells. The real-time PCR confirmed that cyclin D2 was upregulated nearly 16 folds in HepG2.2.15 and nearly 2 folds in HepG2 cells transfected with HBV expression plasmid compared with HepG2 cells (Fig. 1A). In addition, several other cyclin members including cyclin A2, cyclin B1, cyclin B2, cyclin D1, cyclin C and cyclin E1 were also examined in HepG2.2.15, HepG2, HepG2 cells transfected with pGEM-HBV1.3 or control vector. The results found that HepG2.2.15 cells showed higher level of cyclin C and cyclin B1, however, no differentially expressed cyclins investigated were found in HepG2 cells transfected with pGEM-HBV1.3 (Supplementary Fig. 1A,B). Consistently, western blotting analysis revealed that HBV replication indeed upregulated the protein level of cyclin D2 (Fig. 1B). To substantiate the association between HBV and cyclin D2, the mRNA and protein level of cyclin D2 were compared in liver tissues of HBV-transgenic mice and control mice. All the HBV-transgenic mice showed elevated mRNA and protein level of cyclin D2 in liver tissue (Fig. 1C,D). Taken together, these data revealed that transcription of HBV genome upregulated the expression of cyclin D2, suggesting cyclin D2 might play a role in the HBV replication process.

Gene silencing of cyclin D2 inhibited HBV replication

To investigate the possible regulatory effect of cyclin D2 on HBV replication, HepG2.2.15 was transfected with shRNA targeting cyclin D2 (shcyclin D2-1 or shcyclin D2-2) or scramble control shRNA (shCont). Western blotting analysis showed that cyclin D2 was markedly downregulated in HepG2.2.15 cells after transfected with the two cyclin D2 shRNAs (Fig. 2A). Gene silencing of cyclin D2 resulted in a 50% decrease in HBV DNA replicative intermediates in HepG2.2.15 cells as measured by real time PCR (Fig. 2B). Southern blot also confirmed that significant downregulation of HBV DNA replicative intermediates in cyclin D2-silencing cells (Fig. 2C). Moreover, real-time PCR revealed that cyclin D2-silencing cells showed more than 50% reduction of 3.5 kb mRNA compared with control cells (Fig. 2D). The expression of HBV core protein was also significantly inhibited in HepG2.2.15 cells expressing cyclin D2 shRNAs compared with shCont (Fig. 2E). Knockdown of cyclin D2 also inhibited the secretion of both HBsAg and HBeAg (Fig. 2F). On the other hand, the effect of cyclin D2 silencing on HBV replication was further verified in Huh-7 cells transiently transfected with pGEM-HBV1.3. Consistently, silencing of cyclin D2 inhibited HBV DNA replicative intermediates as evidenced by real-time PCR (Fig. 3A) and Southern blot (Fig. 3B). Cyclin D2 suppression also inhibited HBV 3.5 Kb mRNA (Fig. 3C) as well as secretion of both HBsAg and HBeAg (Fig. 3D). Taken together, these data suggest that knockdown of cyclin D2 may exert the suppressive effect on HBV replication.

Cyclin D2 enhanced HBV replication

To further elucidate the role of cyclin D2 in HBV replication, plasmid expressing cyclin D2 or pcDNA3.1 was transfected into HepG2.2.15 cells respectively. The transfection efficiency was first confirmed by using western blotting analysis (Fig. 4A). Real-time PCR showed that overexpression of cyclin D2 increased the copies of viral DNA replication by nearly 1.6-fold (Fig. 4B). Southern blot confirmed that upregulation of HBV DNA replicative intermediates induced by cyclin D2 overexpression (Fig. 4C). Importantly, HepG.2.2.15 cells overexpressing cyclin D2 showed 2-fold higher expression of 3.5 kb mRNA (Fig. 4D). Furthermore, overexpression of cyclin D2 also increased the expression of HBV core protein (Fig. 4E) as well as HBsAg and HBeAg secreted in supernatant (Fig. 4F). These results demonstrated that cyclin D2 enhanced HBV replication and expression of viral proteins.

Cyclin D2 regulated activities of HBV promoters by targeting transcription factor CREB2

To uncover how cyclin D2 affected HBV replication, we sought to identify whether cyclin D2 plays a role in the activity of the HBV core, X, Sp1 and Sp2 promoters. Luciferase reporter assays showed that overexpression of cyclin D2 enhanced the activity of core and Sp1 promoter, but did not affect Xp and Sp2 activities (Fig. 5A). In contrast, cyclin D2 knockdown inhibited the activity of core and Sp1 promoters (Fig. 5B). These results suggested that promotive action of cyclin D2 on HBV replication was possibly due to activation of HBV core and Sp1 promoters. To further examined the mechanism of inhibition of cyclin D2 on HBV promoters, a panel of transcription factors including PPAR-alpha,CREB1, CREB2, AP-1, COUP-TF1, HNF4, NFκB, p53, p56 and TR2, were screened by real-time PCR. The result found that cyclin D2 overexpression increased CREB2 mRNA level (Fig. 6A). Consistently, western blotting analysis confirmed that overexpression of cyclin D2 promoted CREB2 protein level while cyclin D2 knockdown inhibited CREB2 expression (Fig. 6B). Importantly, the enhancement of HBV replicative intermediates and 3.5 Kb mRNA induced by cyclin D2 was partially abolished when CREB2 was suppressed (Fig. 6C, D). Taken together, these results suggested that cyclin D2 is a positive regulator of CREB2 and it may upregulate activity of HBV promoters via CREB2.

Discussion

Host immune system is to clear exogenous pathogens through immune response and cytokines, while pathogens are to devise ways to escape host attack and immune surveillance, such as down-regulating host genes. Zhang et al. demonstrated that host factor HS3ST3B1 effectively inhibited HBV replication and viral protein expression (Zhang et al., 2010). Cyclin D2 is known to be involved in immune responses. Cyclin D2 is first induced in T and B lymphocytes and controls the proliferation of lymphocytes by regulating the cell cycle (Morito et al., 2011). These reminded us that cyclin D2 may play an important role in the immune system between host cells and virus. Recently, a study reported that the expression of cyclin D2 was reduced in HIV patient $CD4^+$ cells (Sieg et al., 2001). Another study found that the mRNA level of cyclin D2 decreased in the HRSV-infected primary airway epithelial cells (Wu et al., 2011). TaeJoon Park et al. found that cyclin D2 polymorphisms associated with clearance of HBV infection

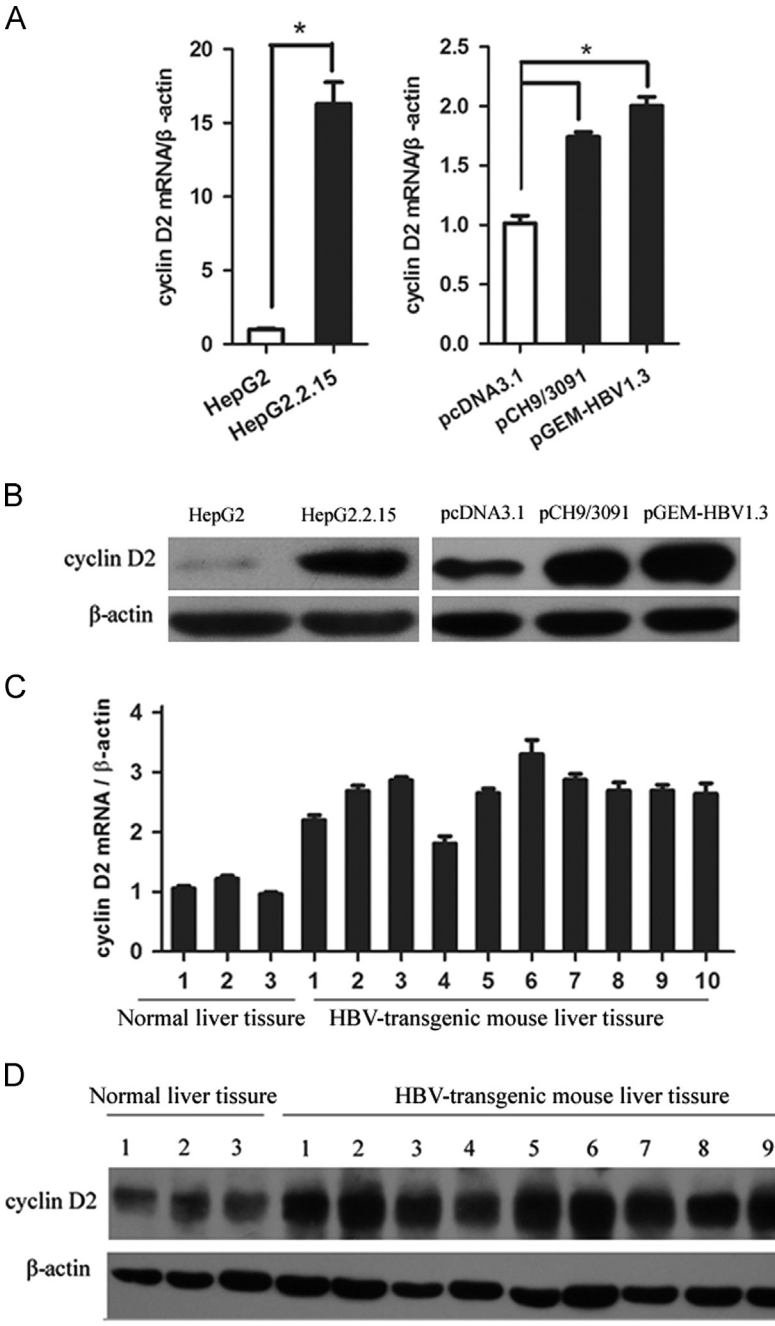

Fig. 1. Expression of cyclin D2 in HBV-expressing cells and liver tissue from HBV-transgenic mice. (A) The mRNA level of cyclin D2 in HepG2.2.15, HepG2 and HepG2 cells transiently transfected with plasmid pCH9/3091 or pGEM-HBV1.3. β-actin mRNA was used as an internal control. $*p < 0.01$. (B) Western blotting analysis of cyclin D2 protein level in HepG2.2.15, HepG2 and HepG2 cells transiently transfected with plasmid pCH9/3091 or pGEM-HBV1.3. β-actin was used as a loading control. (C,D) The mRNA and protein level of cyclin D2 in liver tissues of HBV-transgenic mice (C57BL/6J) and control mice was analyzed by western blot analysis. β-actin was used as a loading control.

(Park et al., 2010). This finding suggested that cyclin D2 may be associated with life cycle of HBV replication. In this study, we found cyclin D2 was closely related to the HBV replication. Gene microarray analysis found the markedly upregulation of cyclin D2 in cell model of HBV infection. This prompted our investigation into the role of cyclin D2 in HBV replication. Gain-of-function and lose-of-function studies found that cyclin D2 could regulate HBV DNA replicate intermediates, 3.5 kb mRNA, core protein and HBsAg and HBeAg secretion.

The molecular mechanism of cyclin D2-meidated regulation of HBV replication was further investigated. The apparent regulation of HBV replication by cyclin D2 was correlated with regulation of HBV core and Sp1 promoter activities. HBV transcription is under the control of four promoter including Core, Sp1, Sp2 and X promoter. Core promoter is responsible for the transcription of 3.5 kb mRNA. Xp controls the transcription of the mRNA of the X protein, a promiscuous viral regulator (Murakami, 2001). Sp1 launches transcription of the mRNA encoding the large surface protein, which is essential for virion formation and infectivity (Seeger and Mason, 2000). Sp2 guides the synthesis of the preS2/S transcript encoding the middle and small surface proteins. Consistent with its regulation of core promoter, cyclin D2 regulated the expression level of core protein and 3.5 kb mRNA transcribed from core promoter. Similarly, cyclin D2 regulated the HBsAg

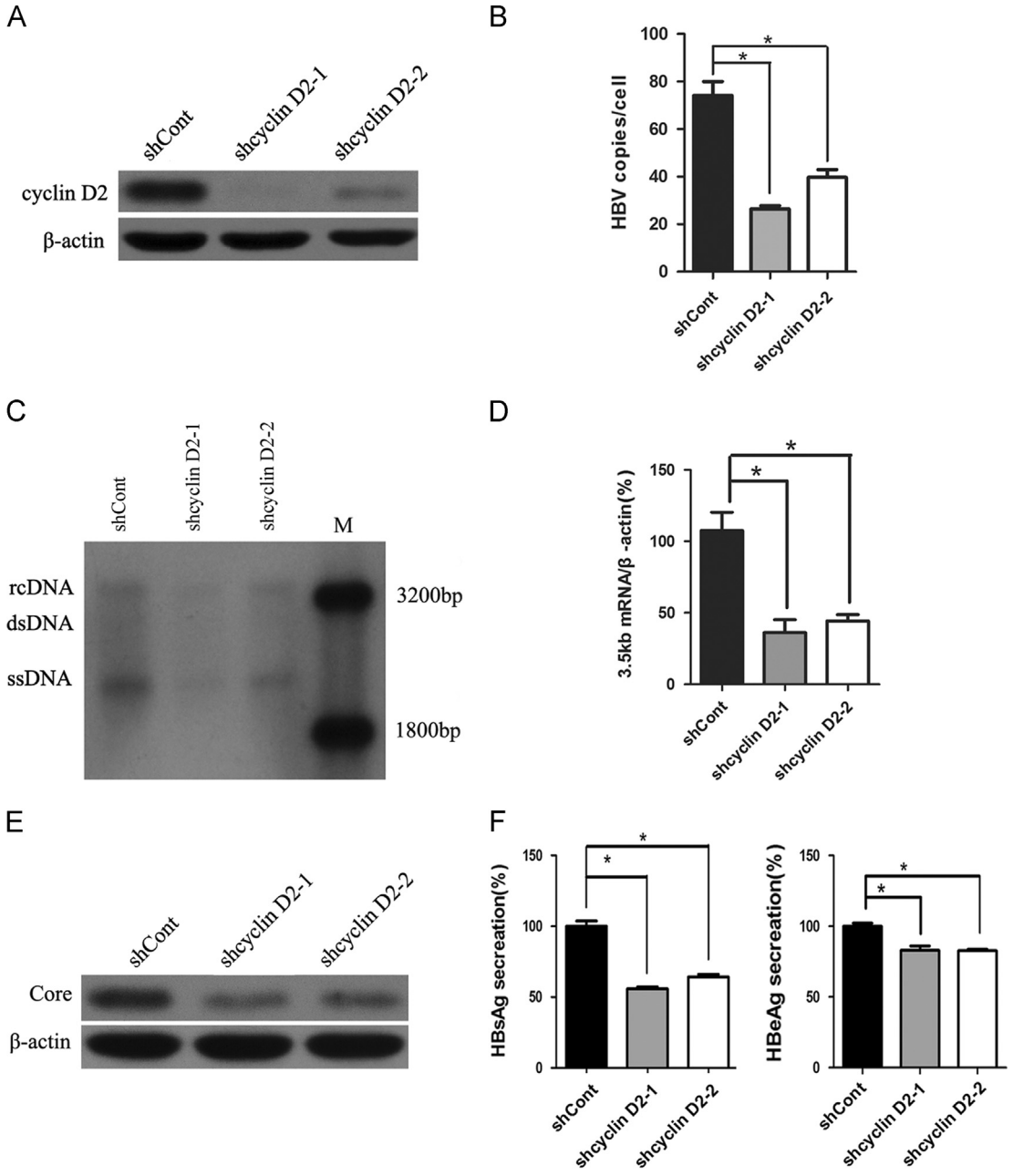

Fig. 2. Gene silencing of cyclin D2 inhibited HBV replication in HepG2.2.15 cells. (A) The knockdown efficiency of shcyclin D2-1 and shcyclin D2-2 in HepG2.2.15 cells was analyzed by using western blotting analysis. HepG2.2.15 cells was transfected with plasmid expressing scramble (shCont) or cyclin D2-targeting shRNAs (shcyclin D2-1 or shcyclin D2-2). Cells were harvested 5 days after transfection. (B,C) Effects of cyclin D2 knockdown on HBV DNA replicative intermediates in HepG2.2.15 cells. HBV replicative intermediates were extracted from nucleocapsid 5 days after transfection. B, Real-time PCR of HBV DNA. Results are expressed as number of HBV DNA copies per cell. $^{*}p < 0.001$. C, Southern blot analysis. M, ladder; RC, intracellular HBV relax circle; DS, double strand DNA; SS, single strand DNA. (D) 3.5 kb mRNA level was analyzed by using selective primers and β-actin primers (equal loading of each RNA samples). $^{*}p < 0.01$. (E) Cyclin D2 knockdown down-regulated HBV core protein expression. (F) The expression of HBsAg and HBeAg in supernatant were detected by using ELISA assay.The O.D. values were expressed as percentage relative to the shCont. $^{*}p < 0.01$.

expression associated with Sp1 promoter. These two promoters of HBV may act as molecular switches, determining the gene activity. The "switches" further influence the transcription and translation of HBV gene, resulting in the overall effect on HBV replication. However, results are still preliminary, and detailed molecular mechanism of regulation of HBV replication mediated by cyclin D2 needs to be determined.

Cyclin D2 is well-known to be involved in the regulation of cell cycle progression. Importantly, it is also an important signal molecule in several signaling pathways. Trans-activator protein (Tax) of Human T-cell leukemia virus type I (HTLV-I) induced activation of cyclin D2 through NFκB (Iwanaga et al., 2008). Cyclin D2 is an important downstream target of the PI3-K/PKB signaling pathway (Fernandez et al., 2004). Myc-cyclin D2-p27 signaling pathway plays an important role in cell cycle progression of quiescent cells (Bouchard et al., 1999). MiR-206 inhibits proliferation of breast cancer cell through up-regulation of cyclin D2 (Zhou et al., 2013). Efficient transcription HBV genes require a number of host transcription factors. Various transcription factors are reported to bind to HBV core promoter region including specificity protein 1(Sp1), Peroxisome proliferator-activated receptor alpha (PPAR-alpha), hepatocyte nuclear factor 4 (HNF4),

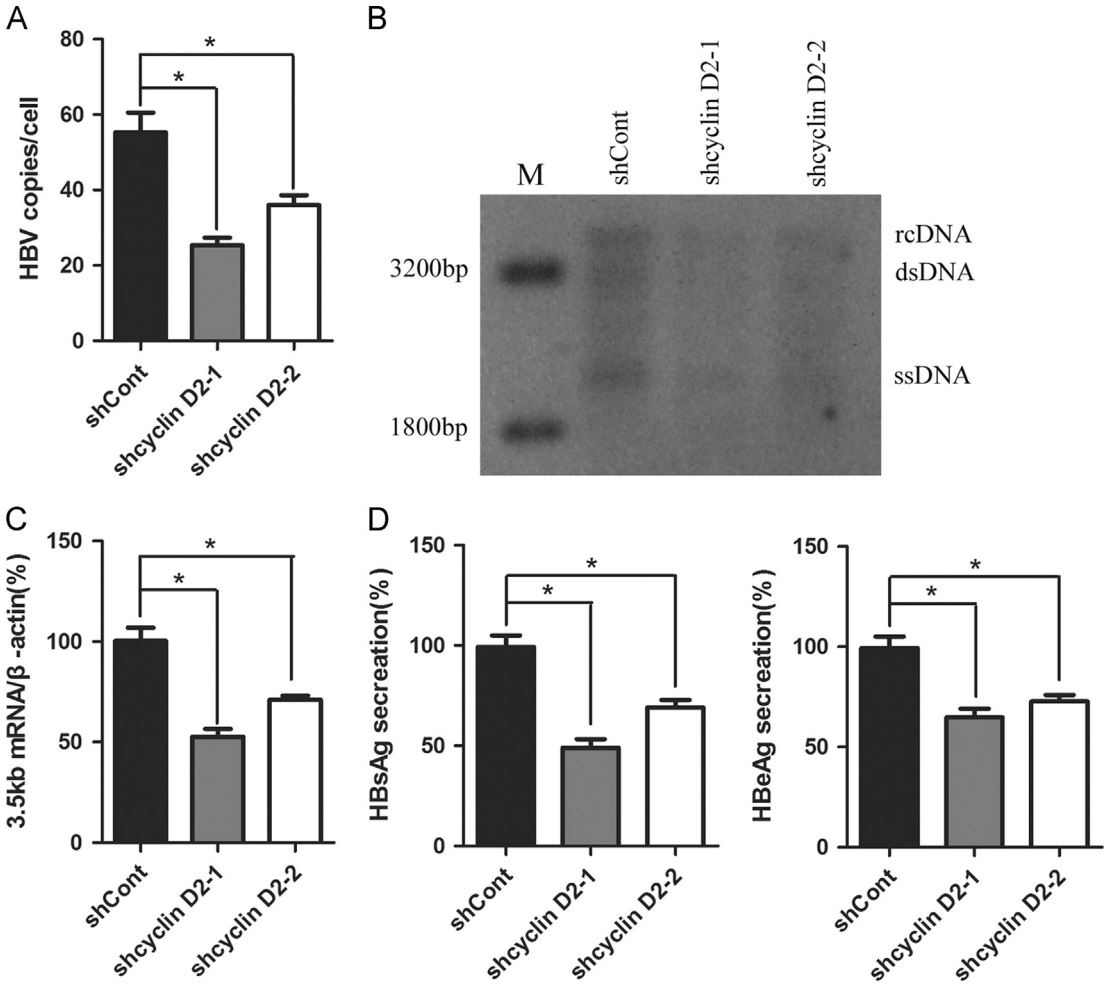

Fig. 3. Cyclin D2 knockdown inhibited HBV replication in Huh-7 cells transiently expressing HBV. (A,B) Effect of cyclin D2 suppression on HBV DNA replicative intermediates in Huh-7 cells transfected with pGEM-HBV1.3. Huh-7 cells transfected with cyclin D2 shRNAs 5 days after transfection with pGEM-HBV1.3. The HBV replicative intermediates were then analyzed by real-time PCR (A) and southern blot (B). *$p < 0.01$. (C) Cylin D2 inhibited HBV 3.5 Kb mRNA by real-time PCR. (D) HBsAg and HBeAg secreted in the supernatant were analyzed by using ELISA assay. The O.D. values were expressed as percentage relative to the shCont. *$p < 0.01$.

C-AMP-response element binding protein (CREB), nuclear factor-kappa B (NFκB), et al. HBV Sp1 promoter contains binding sites of HNF1, HNF3a and HNF3β, Octamer transcription factor 1(Oct1), et al. Based on the finding that cyclin D2 could regulate the core and Sp1 promoter activities, we hypothesized that cyclin D2 alone or combined with other signaling molecules may be involved in the regulation of certain HBV-related transcription factors. To test this hypothesis, we screened various transcription factors related to HBV transcription, and found that CREB2 was possibly involved in the regulation of HBV promoter by cyclin D2. CREB transcription factors are key regulators of homeostatic functions in the liver (Servillo et al., 2002), and CRE binding is increased in hepatic inflammation (Servillo et al., 2002). In terms of HBV, Tacke et al. found that CREB inducer protein kinase A (PKA) resulted in HBV-S mRNA induction and enhanced small envelope protein expression (Tacke et al., 2005). Kim et al. showed that both replication and gene expression of HBV require a functional CREB and HBV-CRE (Kim et al., 2008). In this study, we found that cyclin D2 could regulate CREB2 mRNA and protein levels. Gene silencing of CREB2 abolished partially enhancement of HBV replication induced by cyclin D2. Although the mechanism by which cyclin D2 promoted HBV replication remains to be further investigated, this study suggests that activation of CREB2 is probably involved.

Briefly, we identified a novel host gene, cyclin D2, facilitates efficiently HBV replication by enhancing its core and Sp1 promoters' activities. Our study provided valuable information to understand the interaction between host factors and HBV. Further study should be devoted to investigate the detailed mechanism of cyclin D2-mediated HBV regulation and explore if cyclin D2 could be an anti-HBV target.

Materials and methods

Plasmids and antibodies

HBV replication plasmid pCH9/3091 was a gift from Prof. Lin Lan (The Third Military Medical University, China). pCH9/3091 plasmid containing HBV1.1 was driven by CMV promoter. pGEM-HBV1.3 was a gift from Dr. Protzer (University of Heidelberg, Heidelberg, Germany). pcDNA3.1-cyclin D2 was purchased from addgene (addgene, USA), plasmid plentilox3.7 was from Invitrogen Life Technologies (Invitrogen, USA), cyclin D2 antibody was from Cell Signaling Technology (CST, USA), CREB2 antibody was from Santa Cruz Technology (SC-200) and HBV core antibody was from Dako (Dako, Denmark).

Cell culture and transfection

HepG2 was purchased from ATCC (American Type Culture Collection, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (gibco,

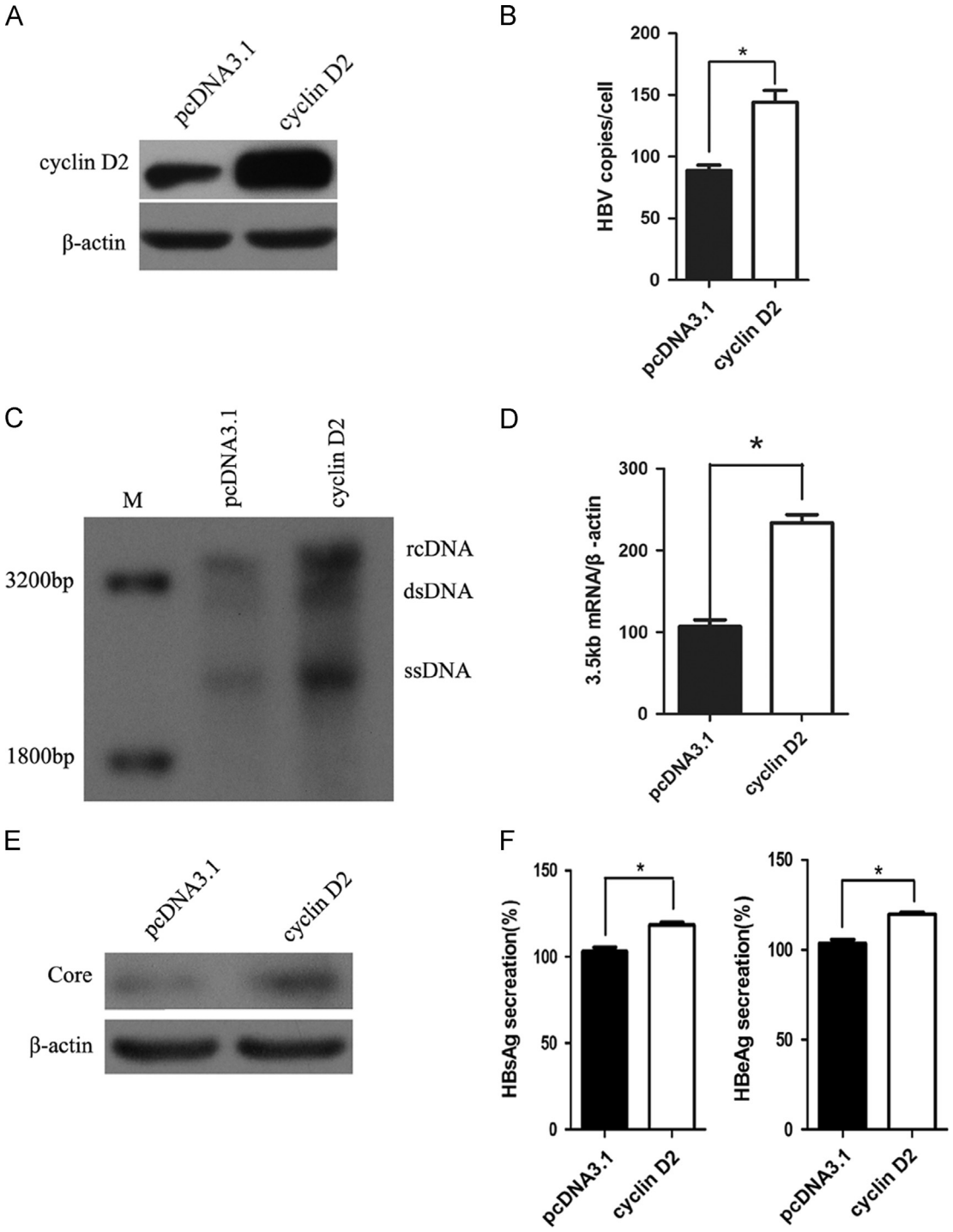

Fig. 4. Overexpression of cyclin D2 enhanced HBV replication. (A)Western blotting analysis was used to confirm the transfection efficiency of cyclin D2 in HepG2.2.15 cells. HepG2.2.15 cells were transfected with plasmid expressing cyclin D2 or corresponding empty vector (pcDNA3.1), respectively. Cells were harvested 5 days after plasmid transfection. (B,C) The level of HBV DNA replicative intermediates was analyzed in cells ectopically expressing cyclin D2 by using real-time PCR (B) and Southern blot analysis (C). (D) HBV 3.5 kb mRNA level was analyzed by real-time PCR using 3.5 kb mRNA selective primers. β-actin mRNA was used as an internal control. $^{*}p < 0.01$. (E) HBV core protein expression was analyzed by western blot analysis. (F) The expression of HBsAg and HBeAg in the culture supernatant were quantified by using ELISA assay. $^{*}p < 0.01$.

USA). HepG2.2.15 was purchased from Shanghai Second Military Medical University and was maintained in MEM (gibco, USA) containing 10% fetal bovine serum and 500 μg/ml G418. The two cell lines were authenticated by short tandem repeat profiling analysis and maintained in a humidified incubator at 37 °C with 5% CO_2. Transfection was carried out using Lipofectamine 2000TM (Invitrogen, USA).

Knockdown of cyclin D2 with short hairpin RNA (shRNA) interfering

shRNA targeting cyclin D2 was generated by inserting short hairpin RNA (shRNA) sequence into lentivirus plasmid vector plentilox3.7. Oligonucleotide sequence of shcyclin D2-1: positive-sense strand, 5′-TCGTCGATGATCGCAACTGGAATTCAAGAGATTC-CAGTTGCGATCATCGACGTTTTTTC-3′; Oligonucleotide sequence of

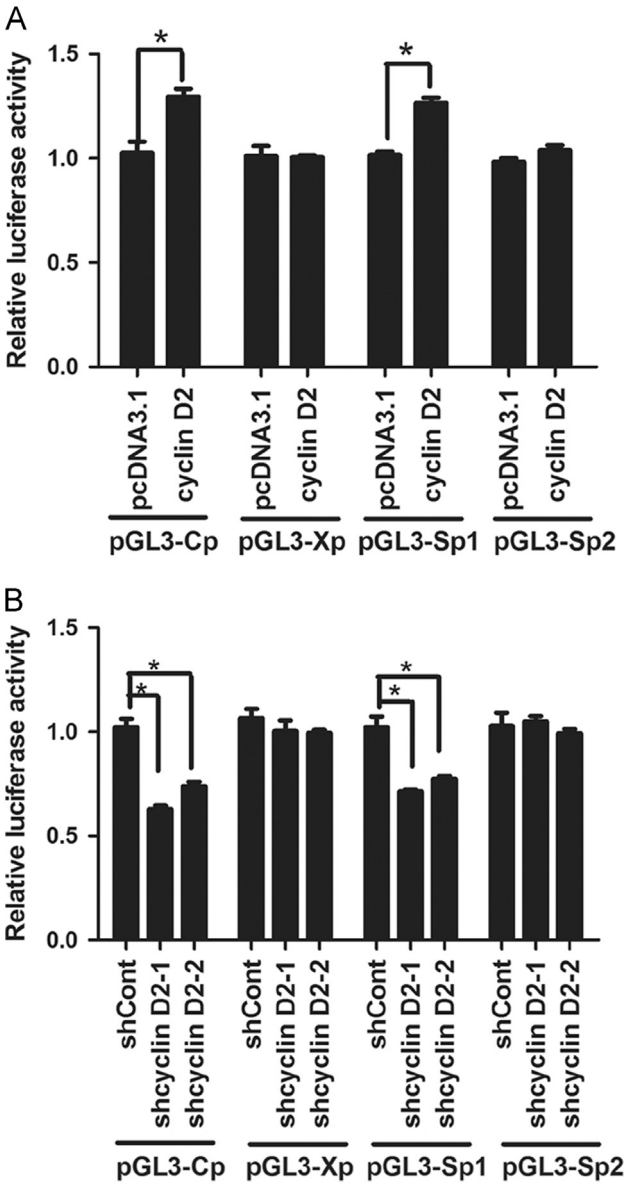

Fig. 5. Cyclin D2 regulated activities of HBV promoters. (A) Different luciferase reporter vectors were co-transfected with pcDNA3.1-cyclin D2 into HepG2 cells. The plasmid RL-TK was co-transfected to normalize the transfection efficiency. Luciferase activity was measured 72 h after transfection. For each group, three independent experiments were conducted. $*p < 0.001$. (B) Different luciferase reporter vectors were co-transfected with shcyclin D2-1 or shcyclin D2-2 into HepG2 cells. The plasmid RL-TK was co-transfected to normalize the transfection efficiency. Luciferase activity was measured 72 h after transfection. $*p < 0.001$.

shcyclin D2-2: positive-sense strand, 5′-TGAAGGACATCCAACCCTA-CATTTCAAGAGAATGTAGGGTTGGATGTCCTTCTTTTTTC-3'; Sequence of control shRNA (shCont) is 5′-GCAACAAGATGAAGAGCACCAA-3′.

Western blotting analysis

Total protein was extracted using RIPA buffer containing protease inhibitors (Roche, Germany). Concentration of proteins was determined by the protein assay Reagent (Bio-Rad, USA). Protein lysates with 30 μg of protein were separated by SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare, UK), and immunoblotted with antibodies as indicated (Anti-cyclin D2 1:800; Anti-core protein 1:1000; Anti-β-actin 1:3000). Blots were developed with ECL Western blotting reagents (Millipore, USA). β-actin (Santa Cruz, USA) was used as loading reference for data analysis.

Real-time PCR

Absolute quantification of HBV replicative intermediates were carried out using Fast Start Universal SYBR Green Master (Roche, Germany). Relative quantification of cyclin D2 and the 3.5Kb mRNA were performed using the SYBR Green Master and the reactions were carried out using an IQTM 5 Multicolor Real-Time PCR Detection system (Bio-Rad, USA). The cDNA was synthesized from 1 μg of total RNA using iScript™ cDNA Synthesis Kit according to manufacturer's protocol (Bio-Rad, USA). β-actin mRNA was used as endogenous control. The level of expression in different treatments was quantified using an optimized comparative Ct ($^{\Delta\Delta}$Ct) value method. Cyclin D2 specific primers: forward primer, 5′-CACCGACTTTAAGTTTGCCA-3′, reverse primer, 5′-TTGGTGATCTTAGCCAGCAG-3′. 3.5 kb mRNA specific primers: forward primer, 5′-GCCTTAGAGTCTCCTGAGCA-3′, reverseprimer, 5′-GAGGGAGTTCTTCTTCTAGG-3′. The primers for quantifying β-actin are 5′-CTCTTCCAGCCTTCCTTCCT-3′ and 5′-AGCACTGTGTTGGCG-TACAG-3′. HBV specific primers: forward primer, 5′-CCTAGTAGTCAGT-TATGTCAAC-3′,reverse primer, 5′-TCTATAAGCTGGAGGAGTGCGA-3′. All the samples were cycled once 95 °C for 2 min, then 34 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. Values represent the mean ± SD of three independent experiments.

Enzyme linked immunosorbent assay (ELISA)

Five days after transfection, the expression of HBsAg and HBeAg in cell culture medium were detected using two different commercial ELISA kits (KHB, Shanghai, China). All assays were performed according to the manufacturer's instructions.

Luciferase reporter assay

pGL3-Cp, pGL3-Xp, pGL3-Sp1, pGL3-Sp2 were constructed through replace SV40 promoter with 4 of HBV inner promoters into vector pGL3-basic. These four different plasmids were transfected with cyclin D2 or pcDNA3.1 vector control, respectively. Three days after transfection; cells were harvested and assayed by the Dual Luciferase Report Assay System (Promega, USA) after transfection three days, according to the manufacturer's instructions. pRL-TK was cotransfected with reporter plasmid to normalize transfection efficiency. Luciferase activity was determined by a GloMax microplate luminometer (Promega, USA).

Detection of HBV DNA replicative intermediates by Southern blot

HBV DNA replicative intermediates in HBV core particles isolated from transfected HepG2.2.15 cells were analyzed using Southern blot. The normalized viral DNA replicative intermediates were electrophoresed onto a 0.9% agarose gel. Then DNA was transferred onto a positive nylon membrane (Roche, Germany) in 20 × SSC. After fixing at 120 °C for 30 min, the membrane was prehybridized for 1 h at 42 °C in DIG Easy hyb Granules, and then hybridized with full-length HBV DNA probes labeled

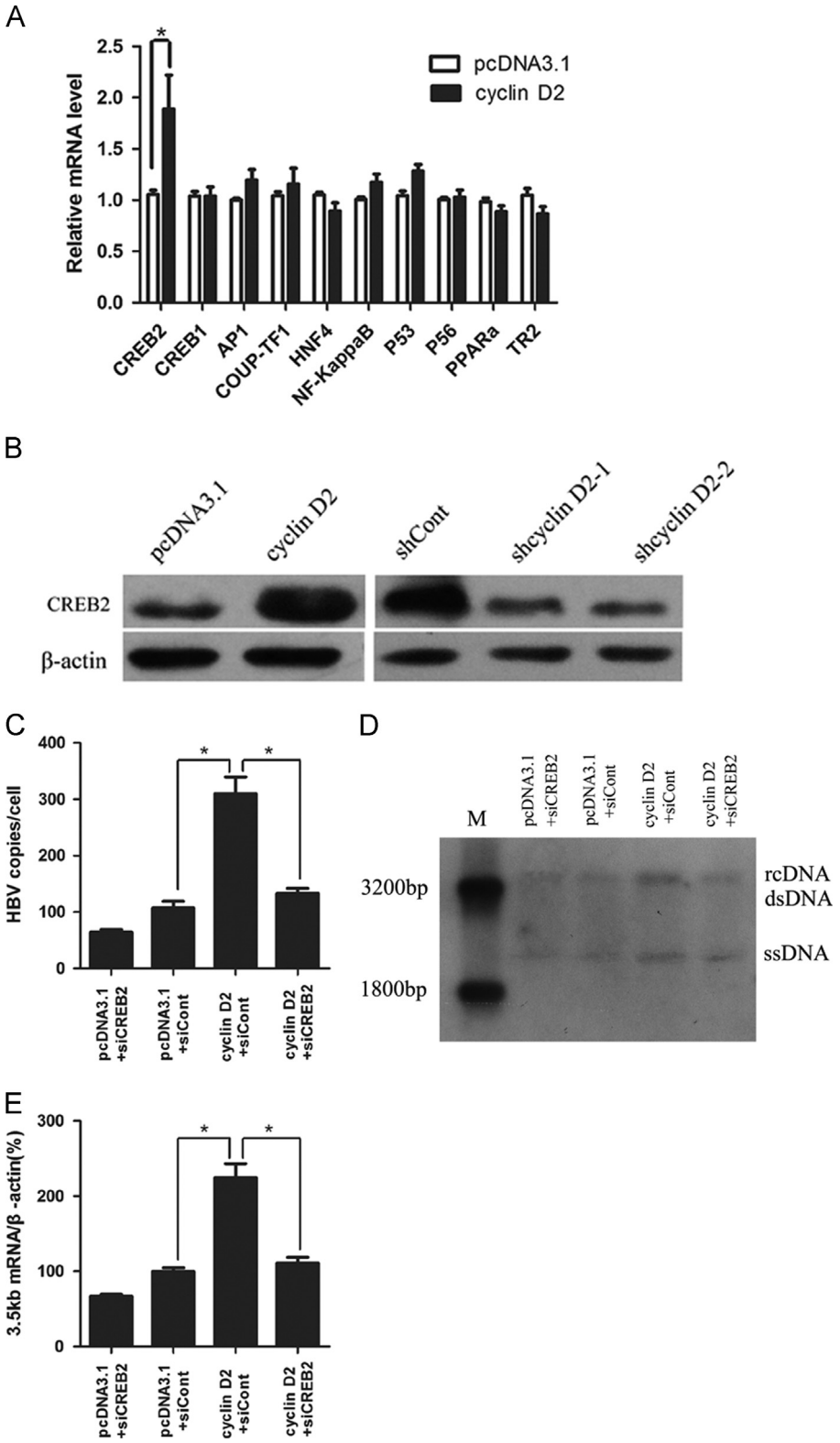

Fig. 6. Cyclin D2 regulated transcription factor CREB2 expression. (A) A panel of HBV-related transcription factors were screened in HepG2.2.15 cells overexpressing cyclin D2 by using real-time PCR. *$p < 0.01$. (B) The effect of cyclin D2 on CREB2 expression was analyzed by western blotting analysis. Overexpression of cyclin D2 promoted CREB2 protein level (Left panel) while cyclin D knockdown inhibited CREB2 expression (Right panel). (C,D) The effect of CREB2 silencing on HBV DNA replicative intermediates in HepG2.2.15 cells overexpressing cyclin D2. HepG2.2.15 co-transfected with indicated plasmids and siRNA were harvested for HBV replicative intermediates analysis by real-time PCR (C) and Southern blot (D). (E) HBV 3.5 kb mRNA level in HepG2.2.15 cells transfected with indicated plasmids and siRNA was analyzed by real-time PCR. *$p < 0.01$.

with DIG by DIG High prime DNA labeling and detection starter kit (Roche, Germany) under the same condition of prehybridization overnight. The signal was detected by exposing on an X-ray film.

Statistical analysis

Values were expressed as means ± SD. Differences were evaluated by two-tailed Student's *t* tests or one-way ANOVA.

All statistical analysis was carried out by the statistical program, SPSS version 16.0 (SPSS, Inc, Chicago, IL). P value <0.05 was considered statistically significant.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.05.027.

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