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Tumor and Stem Cell Biology

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E2F8 Contributes to Human Hepatocellular Carcinoma via Regulating Cell Proliferation

Qing Deng[1,2], Qun Wang[1,2], Wei-Ying Zong[2], Da-Li Zheng[1,2], Yi-Xin Wen[2], Ke-Sheng Wang[2], Xiao-Mei Teng[1,2], Xin Zhang[2], Jian Huang[2], and Ze-Guang Han[1,2]

Abstract

The E2F family member of transcription factors includes the atypical member E2F8, which has been little studied in cancer. We report that E2F8 is strongly upregulated in human hepatocellular carcinoma (HCC), where it was evidenced to contribute to oncogenesis and progression. Ectopic overexpression of E2F8 promoted cell proliferation, colony formation, and tumorigenicity, whereas E2F8 knockdown inhibited these phenotypes, as documented in Huh-7, Focus, Hep3B, and YY-8103 HCC cell lines. Mechanistic analyses indicated that E2F8 could bind to regulatory elements of cyclin D1, regulating its transcription and promoting accumulation of S-phase cells. Together, our findings suggest that E2F8 contributes to the oncogenic potential of HCC and may constitute a potential therapeutic target in this disease. *Cancer Res; 70(2); 782–91. ©2010 AACR.*

Introduction

As known, the members of E2F family function as transcription factors that control the expression of genes involved in cell cycle progression, apoptosis, DNA repair, and differentiation (1, 2). To date, eight members, E2F1 to E2F8, of this family have been recognized. In general, E2F1, E2F2, and E2F3 are considered as transcriptional activators, whereas E2F4, E2F5, E2F6, and E2F7 play an inhibitory role in transcriptional expression of downstream target genes (3–5). However, the function of a novel member, E2F8, is still obscure. Unlike known E2F1 to E2F6, E2F8 just has two distinct E2F-related DNA-binding domains (DBD) but lacks DP-dimerization, retinoblastoma-binding, and transcriptional activation domains included in E2F1 to E2F5 members (6–8). E2F8 was initially considered as a cell cycle–regulated transrepressor that was activated by E2Fs at G_1–early S phase of the cell cycle (6), which was capable of blocking cellular proliferation (8). However, recent evidence suggests that the synergistic function of E2F8 with E2F7 is essential for cell survival and embryonic development of mouse, where the deficiency of both *E2F7* and *E2F8* leads to an increase of E2F1 and p53 that can result in massive apoptosis and dilation of blood vessels, culminating in embryonic lethality (9). This suggests that both E2F7 and E2F8 can serve as a unique repressive arm to balance the E2F transcriptional network that is critical for embryonic development and control of the E2F1-p53 apoptotic axis (10). Moreover, E2F8 was induced in those cells treated with DNA-damaging agents, suggesting that it has an important role in dictating the outcome of the DNA damage response (11).

In addition to physiologic activities, increasingly convincing lines of evidence revealed that some E2F members were deregulated in various human cancers, where they can function to activate or repress transcription, accelerate or impede cell cycle progression, and enhance or inhibit cell death, in a cellular context-dependent manner (1, 2, 12, 13). With regard to hepatocellular carcinoma (HCC), the fifth most common cancer worldwide and the third most common cause of cancer mortality, both E2F1 and E2F3 were found to be upregulated in animal and human HCC specimens (14, 15). E2F1 overexpression was considered as a critical antiapoptotic factor in both human and rodent liver cancer through its ability to counteract c-Myc–driven apoptosis (16–18). Except for E2F1, the contribution of other E2F members to hepatocarcinogenesis was rarely investigated in depth. However, the present work, for the first time, indicated that E2F8 was significantly upregulated in human HCC specimens and regulated the transcriptional expression of cyclin D1, a well-documented oncogene that contributes to HCC development.

Materials and Methods

Tissue specimens and cell lines. All specimens were harvested from patients who suffered from HCC with informed consent. Fifteen liver tumor–derived cell lines and the fetal liver–derived cell line L02 were used in this study. The project and protocol for any investigation involving humans and animals have been approved by the ethics committee of the Chinese National Human Genome Center at Shanghai.

Authors' Affiliations: [1]National Human Genome Center, Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine; [2]Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China

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Q. Deng and Q. Wang contributed equally to this work.

Corresponding Author: Ze-Guang Han, Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China. Phone: 86-21-50801325; Fax: 86-21-50800402; E-mail: hanzg@chgc.sh.cn.

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Semiquantitative and real-time reverse transcription-PCR. Semiquantitative PCR and real-time quantitative reverse transcription-PCR (RT-PCR) were performed with primers listed in Supplementary Table S1. β-Actin served as a loading control.

Immunohistochemical staining. Tissue array containing 100 pairs of HCC samples (Shanghai OUTDO Biotech Co. Ltd.) was used in this study. The slides were incubated with mouse anti-E2F8 monoclonal antibody (3E9-2F5, 1:50 dilution; Abnova) at 37°C for 2 h, where normal mouse IgG1κ was used as negative control, followed by incubation with a horseradish peroxidase–conjugated anti-mouse secondary antibody (Dako Japan Ltd.) at 37°C for 1 h. The signals were detected using diaminobenzidine substrate kit (Vector Laboratories). Counterstaining was performed with hematoxylin.

Construction of recombinant plasmids and adenoviral vectors. E2F8 open reading frame (ORF) was inserted into the pcDNA3.1 (Invitrogen). The c-Myc–tagged *E2F8* was then subcloned into pTRE2 (Clontech). Plasmid pShuttle-CMV-*E2F8-IRES-hrGFP* was constructed, and then E2F8 ORF was subcloned into AdEasy XL Adenoviral Vector System (Stratagene).

Cell transfection. Cell transfection was performed by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA interference. Small interference RNAs (siRNA) were chemically synthesized (GenePharma Co. Ltd.). Synthesized DNA nucleotide fragment encoding short hairpin RNA (shRNA) for knockdown of endogenous E2F8 was inserted into pSUPER (OligoEngine) and adenoviral vector containing H1 element (Ad-H1-sh796), respectively. The same vectors (pSUPER-shNC and Ad-shNC) with irrelevant nucleotides not targeting any annotated human genes were used as negative controls. The sequences of these synthesized oligonucleotides for RNA interference (RNAi) were listed in Supplementary Table S2.

Cell cycle analysis and cell synchronization. Flow cytometry was used to analyze cell cycle. For DNA content analysis, cells were fixed in 70% ethanol, rehydrated in PBS, and treated for 30 min with RNase A (10 mg/mL) and for 5 min with propidium iodide (10 μg/mL). Two chemical drugs, thymidine and hydroxyurea (Sigma-Aldrich), were used to cause cell cycle G_1-S–phase arrest. After 24 h of plating cells, the cells were treated with 2 mmol/L thymidine for 17 to 18 h, released for 9 h, and treated again. After 18 h, the cells were released and collected at different time points. The cells were treated with 2 mmol/L hydroxyurea for 16 to 18 h, washed with PBS thrice, released, and harvested at different time points.

Bromodeoxyuridine incorporation assay. To evaluate the cell population in S phase of the cell cycle, cells were incubated with bromodeoxyuridine (BrdUrd; Sigma-Aldrich) for 2 h and then harvested and fixed in 75% ice-cold ethanol. The cells were denatured in 2 mol/L HCl for 20 min at room temperature, neutralized with 0.1 mol/L sodium borate ($Na_2B_4O_7$; pH 8.5), washed in PBS, and then incubated with FITC-conjugated mouse anti-BrdUrd monoclonal antibody (BD Pharmingen), where FITC-conjugated mouse IgG1κ monoclonal isotype was used as negative control. Subsequently, the cells were incubated with propidium iodide in the presence of RNase for 30 min at 37°C. The samples were analyzed by flow cytometry.

Western blot analysis. Western blot analysis was performed according to the manufacturer's recommended protocol (Santa Cruz Biotechnology). Briefly, cell extracts were prepared in lysis buffer [25 mmol/L Tris (pH 6.8), 1% SDS, 5 mmol/L EDTA, protease inhibitor cocktail (Sigma)]. The blot is incubated with blocking solution (5% nonfat milk and 0.1% Tween 20 in PBS) for 2 h at room temperature. E2F8 (3E9-2F5) and cyclin D1 (Santa Cruz Biotechnology) antibodies were used in this study.

Cell proliferation and colony formation. Cell viability was measured using the Cell Counting Kit-8 (Dojindo Laboratories) according to the instructions of the manufacturer. HCC cells transfected by vector containing *E2F8* were cultured on 10-cm plate for colony formation, and then G418 (Life Technologies, Inc.) was added to the medium with serum at a final concentration of 0.6 to 1 mg/mL.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assay was performed as previously described. Briefly, cells were cross-linked by the addition of formaldehyde to 1% final concentration, and the reaction was stopped by addition of glycine. Sonicated chromatin was incubated at 4°C overnight with the antibodies against E2F1, E2F8, and Flag tag, respectively. Immunocomplexes were then incubated with protein A/G agarose beads (Santa Cruz Biotechnology). After extensive washes, immunocomplexes were eluted from the beads, cross-linked, and harvested by phenol/chloroform extraction and ethanol precipitation. The bound DNA fragments were analyzed by PCR using HotStart Taq enzyme (Qiagen) with primers against E2F-binding element of human *cyclin D1* promoter (−995/−676; forward, 5′-GAGGGGACTAATATTTCCAGCAA-3′; reverse, 5′-TAAAGGGATTTCAGCTTAGCA-3′; ref. 19).

Electrophoretic mobility shift assay. Both E2F8 and E2F1 proteins were obtained by the TNT Quick Coupled Transcription/Translation Systems (Promega), and protein concentration was determined by bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Electrophoretic mobility shift assay (EMSA) was performed using EMSA kit (Molecular Probes, Invitrogen), where the purified PCR product of E2F-binding element located on *cyclin D1* promoter was used as probe. Binding reactions were carried out in 5× binding buffer [750 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L EDTA, 50 mmol/L Tris (pH 7.4)].

Luciferase reporter assay. To evaluate the effect of E2F8 on transcriptional activity of cyclin D1, 4×10^4 PLC/PRF/5 cells were seeded in 24-well tissue culture plates and then transfected with a combination of reporter plasmid and expression vector containing E2F8. Dual-Luciferase Reporter Assay System (Promega) was used according to the instructions of the manufacturer.

Tumor xenograft in animals. Male BALB/c nude mice or nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice (5–6 wk old) were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences. Kinetics of tumor formation was estimated by

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measuring tumor size and volume at every 3-d interval. Tumor size was measured with calipers, and tumor volume was determined using the following formula: volume = 0.5 × width^2 × length.

***Gene therapy* in vivo.** Male BALB/c nude mice were used for a subcutaneous tumor model. Once tumor xenografts reached ~200 mm^3, the injection of Ad-shNC and Ad-sh796 (5×10^9 plaque-forming units) for E2F8 knockdown was administered intratumorally every other day for four times, respectively.

Statistical analysis. Mann-Whitney *U* test was used for comparison of tumor weight and volume. Categorical data were evaluated with the χ^2 test or Fisher's exact test. *P* values of <0.05 were considered to be significant.

Results

E2F8 was frequently upregulated in HCC. In the present work, we first investigated the relative expression level of all known members of E2F family, E2F1 to E2F8, in 24 pairs of human HCC specimens through real-time RT-PCR. The data showed that mRNA levels of E2F1, E2F2, E2F7, and E2F8 were significantly upregulated in HCC specimens as compared with that of corresponding noncancerous livers (data not shown). The expression level of E2F8, a novel and atypical member of E2F family, was further evaluated in additional 48 paired human HCC specimens by quantitative and semiquantitative RT-PCR (Fig. 1*A*; Supplementary Fig. S1*A*), confirming the observation that E2F8 was obviously upregulated in HCC specimens. Moreover, tissue array containing 100 pairs of HCC samples was also examined by immunohistochemical staining with a specific antibody against E2F8. The resulting data showed that E2F8 was significantly overexpressed in 68 of 100 HCC specimens as compared with that of corresponding noncancerous livers, where the intensity and proportion of E2F8-positive cells in HCC specimens were generally scored to the scale of 3+ (Fig. 1*B*; Supplementary Fig. S1*B*). Taken together, upregulation of E2F8 was observed in 112 of 172 (65%) HCC specimens examined. Furthermore, we evaluated the expression pattern of E2F8 in available HCC cell lines and adult and fetal human livers by RT-PCR. The result showed that E2F8 was highly expressed in fetal livers and the majority of these HCC cell lines but very low in healthy adult livers and PLC/PRF/5 cells (Fig. 1*C*; Supplementary Fig. S1*C*). Expectedly, E2F8 was located on nucleus in HepG2 and Huh-7 cells, as indicated by immunofluorescence assay (Fig. 1*D*).

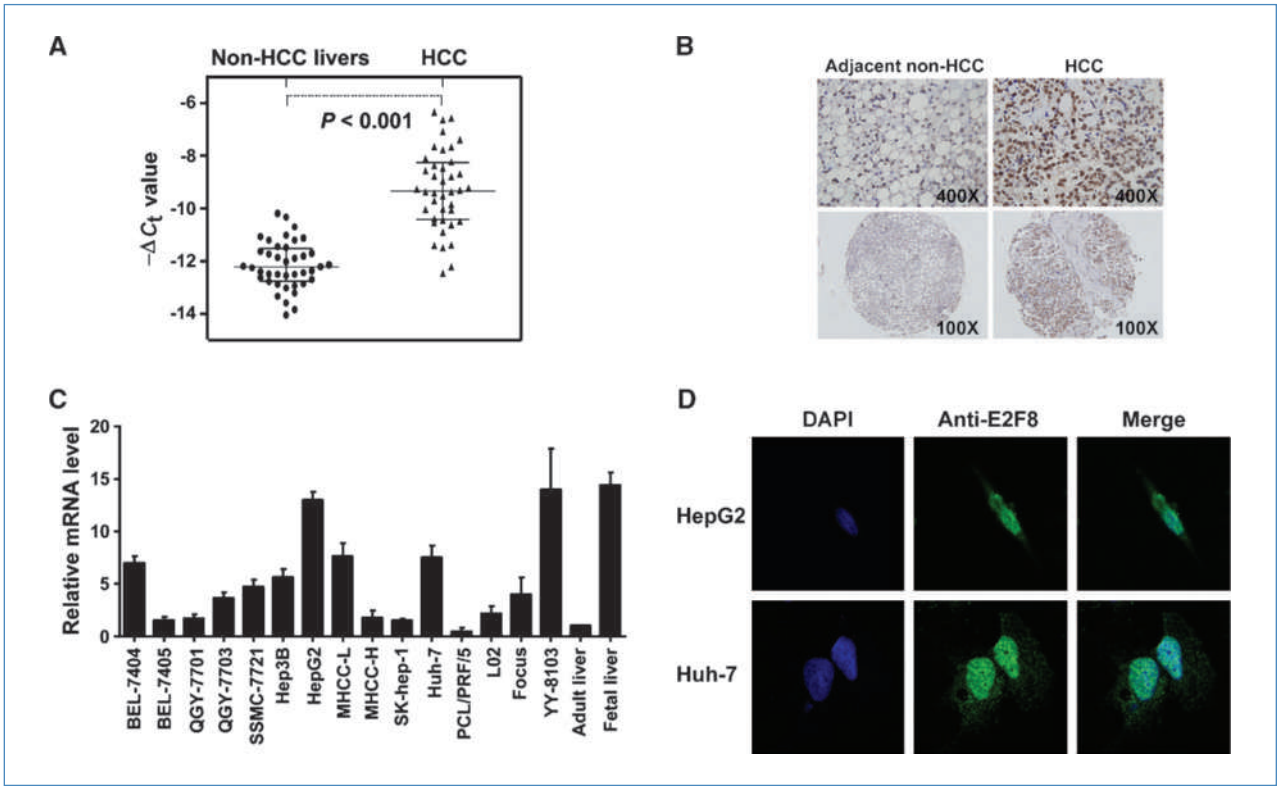

Figure 1. Expression pattern of E2F8 in HCC specimens and cell lines. *A,* the transcript level of E2F8 was measured in 40 HCC specimens and corresponding adjacent noncancerous livers by quantitative RT-PCR, where β-actin was used as internal reference. Each scatter plot displays the expression level of a given HCC specimen or adjacent liver, where the lines represent the median with interquartile range of $-\Delta C_t$ value; *P* value was calculated by Student's *t* test. *B,* representative immunohistochemical staining on HCC samples with E2F8 antibody. *C,* relative mRNA level of E2F8 was evaluated in HCC cell lines and fetal and adult human livers by quantitative RT-PCR, where β-actin was used as internal control. *D,* immunofluorescence assay indicated that E2F8 protein was located within nuclei in HepG2 and Huh-7 cells. The nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*).

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E2F8 overexpression promotes the cellular proliferation and tumorigenicity. To evaluate the effect of E2F8 on HCC cells, recombinant pcDNA3.1-*E2F8* was transiently transfected into PLC/PRF/5 cells. The result showed that cellular growth and colony formation were significantly promoted by the enforced E2F8 overexpression as compared with that of those transfected with empty vector ($P < 0.01$; Fig. 2*A* and *B*). To exclude the artificially experimental errors, we constructed a recombinant vector pcDNA3.1-*E2F8*(–) with a reversal *E2F8* sequence as an alternative control. Likewise, as compared with this control, ectopic E2F8 exhibited a significantly enhanced effect on cellular growth and colony formation of PLC/PRF/5 cells (Supplementary Fig. S2*A* and *B*). To further confirm the promotion of E2F8 to cellular proliferation and colony formation, we used a doxycycline-induced Tet-on expression system to assess the effect of E2F8 on PLC/PRF/5 cells. We first evaluated the expression level of E2F8 in PLC/PRF/5 cells in response to different doxycycline dosage, where the expression levels of endogenous E2F8 in Huh-7 and HepG2 cells were used as references (Supplementary Fig. S2*C*), and found that the near or up to endogenous E2F8 level of Huh-7 and HepG2 cells promoted the cell proliferation (Fig. 2*C*). Moreover, to evaluate the effect of E2F8 on tumorigenicity, recombinant adenovirus carrying *E2F8* was introduced into PLC/PRF/5 cells. As expected, PLC/PRF/5 cells (8×10^6) infected with Ad-*E2F8* (multiplicity of infection, 40) could result in tumorigenicity *in vivo* in six of nine NOD-SCID mice tested, whereas those infected with empty Ad-*GFP* used as controls had no visible tumors in the same mice under observation within 2 months ($P < 0.05$; Fig. 2*D*). The immunoblotting assay validated the ectopic Flag-tagged E2F8 expression in these tumors (Supplementary Fig. S2*D*). These collective data suggested that E2F8 overexpression plays an important role in promoting cell growth, colony formation, and tumorigenicity of HCC cells.

E2F8 knockdown inhibits cellular proliferation and colony formation. To further investigate the effect of E2F8 on cellular proliferation and colony formation, we used chemically synthesized siRNAs and shRNA derived from recombinant pSUPER to knock down endogenous E2F8 in some HCC cell lines. After evaluating the efficiency of RNAi, si-796 was considered to be appropriate for E2F8 knockdown. To exclude the off-target effect of RNAi, besides the si-NC without homology with human genes, the synthesized si-mut796 with ribonucleotide transversion was used as an additional control. Expectedly, si-796, not si-mut796 and si-NC, significantly knocked down the endogenous E2F8 and inhibited the cell growth of YY-8103 and Focus cells (Fig. 3*A* and *B*). To evaluate the effect of E2F8 on colony formation, recombinant pSUPER producing shRNA-796 was constructed and transfected to HCC cell lines YY-8103, Focus, Huh-7, and Hep3B. The resulting data showed that shRNA-796 significantly inhibited the colony formation of these cell lines as compared with the control sh-NC ($P < 0.001$; Fig. 3*C*). Besides, another siRNA, si-1770, also was used, although it has lower knockdown efficiency than si-796. Like si-796, si-1770 inhibited cell growth and colony formation (Supplementary Fig. S3*A*–*D*). These collective data implied that endogenous E2F8 could

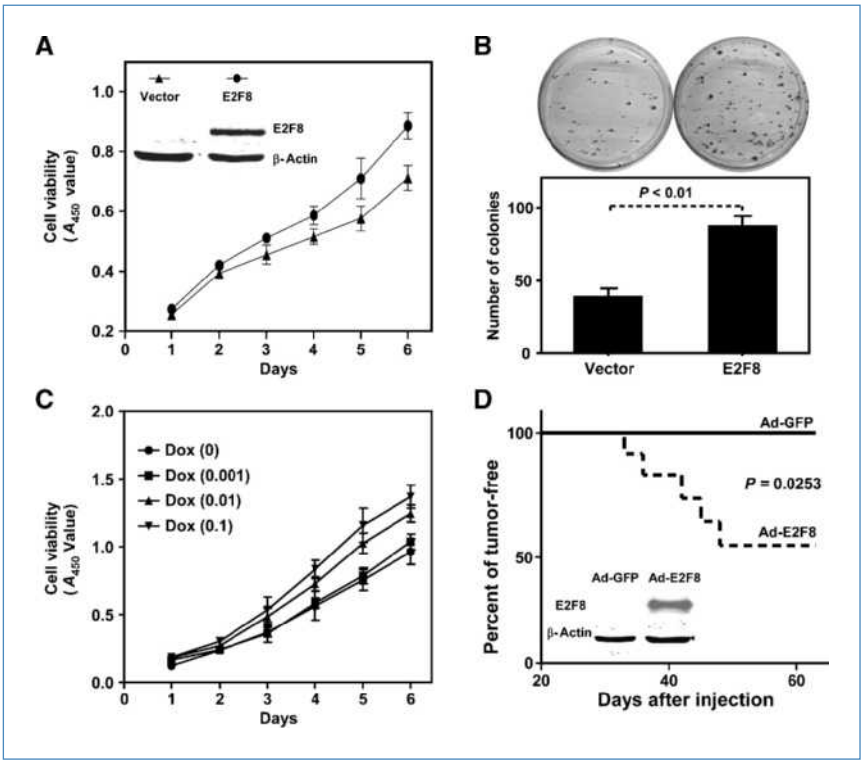

Figure 2. E2F8 overexpression promotes the cellular proliferation, colony formation, and tumorigenicity. *A,* ectopic E2F8 promoted the proliferation of PLC/PRF/5 cells, where E2F8 expression was evaluated by immunoblotting assay. *B,* overexpressed E2F8 enhanced the colony formation of PLC/PRF/5 cells, as shown by representative dishes of colony formation of those transfected with pcDNA3.1-*E2F8* (*right*) and pcDNA3.1 used as control (*left*). The histograms (*bottom*) represent the numbers of colonies. *Columns,* mean ($n = 3$); *bars,* SD. *C,* doxycycline-induced E2F8 expression promoted the cellular proliferation. *D,* Ad-*E2F8*–infected PLC/PRF/5 cells were injected s.c. into a flank of NOD-SCID mice ($n = 9$), where those cells infected with Ad-*GFP* were used as controls. The tumor-free survival curve of mice was statistically analyzed by the Kaplan-Meier method and log-rank test.

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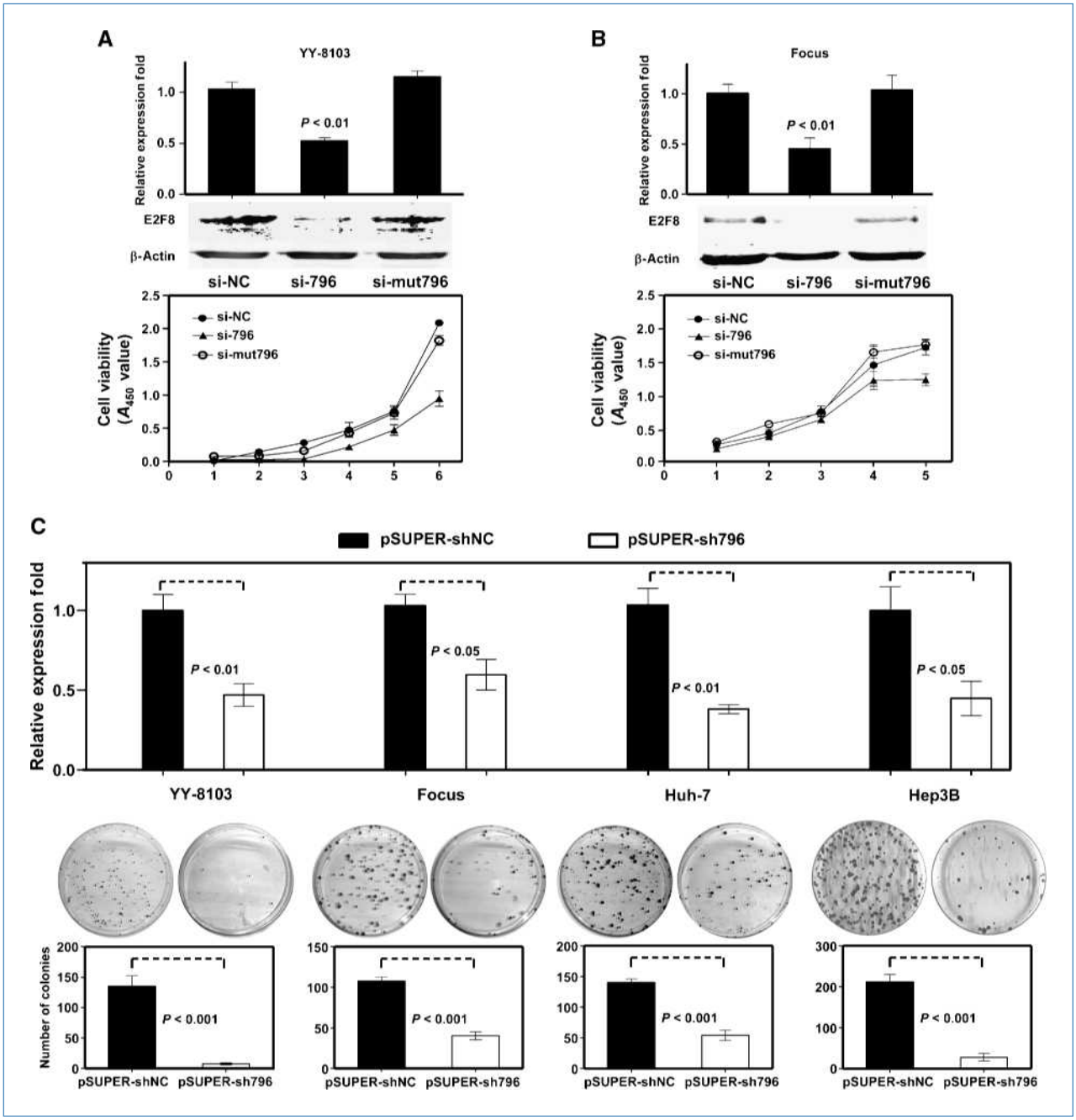

Figure 3. E2F8 knockdown suppresses the growth and colony formation of HCC cells. *A* and *B,* synthesized si-796 knocked down the endogenous E2F8 in YY-8103 (*A*) and Focus cells (*B*), as indicated by quantitative RT-PCR and immunoblotting assay (*top*), and inhibited their cellular proliferation (*bottom*), where si-NC and si-mut796 were used as controls. *Points,* average value of triplicate wells; *bars,* SD. *C,* RNAi efficacy of pSUPER-sh796 was evaluated in YY-8103, Focus, Huh-7, and Hep3B cells by real-time RT-PCR (*top*), where pSUPER-shNC was used as control. Relative mRNA level of E2F8 was normalized based on that of the control. The dishes of colony formation were stained, and the numbers of colonies were counted from three independent experiments. *Bottom, columns,* average numbers statistically analyzed by Student's *t* test; *bars,* SD.

be essential for maintaining cellular proliferation and colony formation of HCC cells.

E2F8 knockdown suppresses the tumorigenicity and reduces tumor burden. To address the effect of E2F8 knockdown on tumorigenicity *in vivo*, recombinant adenoviral vector producing shRNA-796 (Ad-sh796) was used to infect Huh-7 cells (Supplementary Fig. S4*A*) and then those cells were inoculated s.c. into a flank of mice, where those infected with adenoviral vector carrying sh-NC used as control were inoculated s.c. into the opposite flank of the same mice.

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Expectedly, Ad-sh796 can significantly suppress the tumorigenicity *in vivo*, where tumor size and weight were obviously reduced as compared with that of the controls (Fig. 4*A* and *B*). The quantitative PCR data showed that endogenous E2F8 was indeed significantly decreased in these tumors as compared with that of the controls ($P < 0.05$; Supplementary Fig. S4*B*). Furthermore, to evaluate the antitumor activity of Ad-sh796, a subcutaneous liver tumor model was first established by the implantation of Huh-7 cells in nude mice. When the tumors reached 200 mm^3, an intratumoral injection of Ad-sh796 was performed. The resulting data showed that Ad-sh796 significantly reduced tumor volume as compared with that of Ad-shNC as controls ($P < 0.05$; Fig. 4*C* and *D*). This implied that E2F8 could be considered as a potential therapeutic target for HCC.

E2F8 influences G_1-S transition of cell cycle progression. As known, the E2F family exerts an important role in cell cycle control (4, 13, 20). To evaluate the function of E2F8 on cell cycle progression, *de novo* DNA synthesis was determined by pursuing the transfected cells with BrdUrd incorporation. Flow cytometry analysis showed that E2F8-transfected PLC/PRF/5 cells exhibited significant increases of BrdUrd-incorporating cells; in contrast, si-796 reduced BrdUrd-incorporating cell population of Huh-7 and Focus cells (Fig. 5*A*). Moreover, immunofluorescence assay with BrdUrd antibody also supported the observation (Supplementary Fig. S5*A*), implying that E2F8 could be involved in G_1-S transition of cell cycle progression. To confirm the hypothesis, we synchronized those HCC cells, by the addition of thymidine or hydroxyurea, and then observed the cell cycle progression after release. Herein, the enforced E2F8 pushed PLC/PRF/5 cells to enter into S phase in advance; conversely, si-796 targeting endogenous E2F8 led to significant G_1 arrest or delayed the entry of S phase in Huh-7 and Focus cells (Fig. 5*B*; Supplementary Fig. S5*B*) as compared with si-NC used as control. These data supported the notion that E2F8 could contribute to cell proliferation of HCC via promoting the entry of S phase in cell cycle.

E2F8 mediates cyclin D1 transcription in a dominant-negative manner. To explore the molecular mechanisms by which E2F8 overexpression contributes to the promotion of G_1 to S transition, we analyzed some known important factors, including cyclin D1, cyclin E, E2F1, p16, p53, p21, and NF-κB, by real-time RT-PCR and/or immunoblotting assay (Supplementary Fig. S6*A* and *B*). Herein, as E2F8 overexpression, only cyclin D1 was significantly upregulated in PLC/PRF/5 cells; in contrast, as E2F8 knockdown by si-796, cyclin D1 was obviously decreased in YY-8103 and Huh-7 cells (Fig. 6*A*, *left*). This suggested that *cyclin D1*, a well-documented oncogene and key factor responsible for the entry of S phase (21–23), could be crucial to the promotion of G_1 to S transition that was triggered by E2F8 overexpression.

To address the mechanisms responsible for the enhanced cyclin D1 transcription, we analyzed the upstream

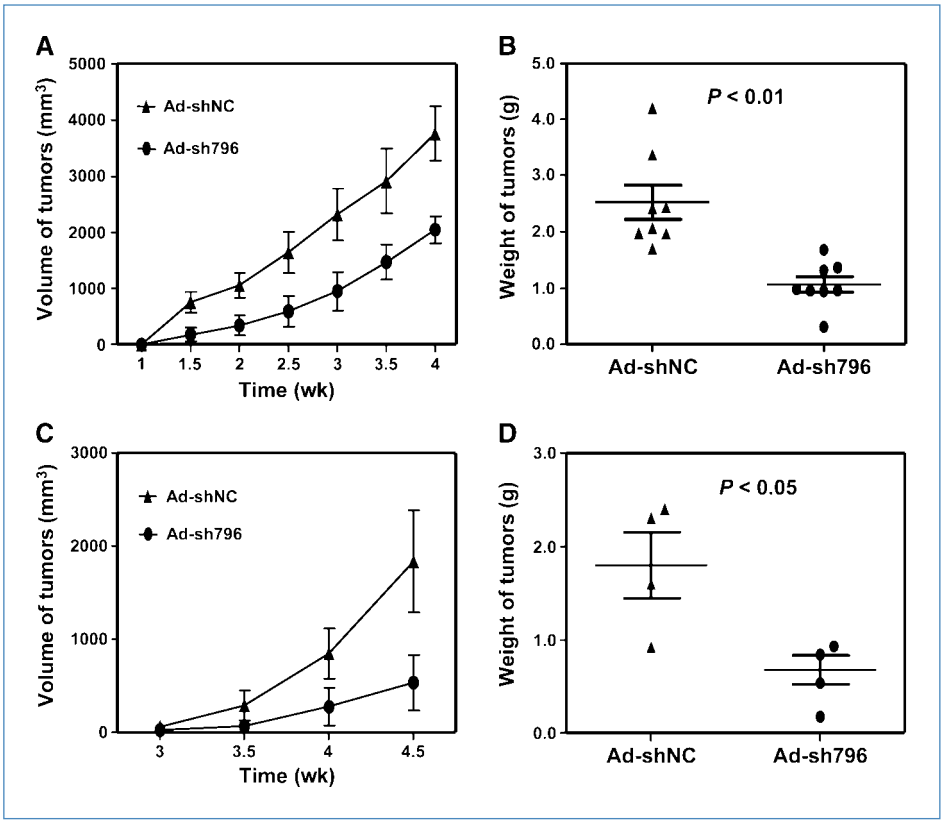

Figure 4. Adenovirus-mediated RNA inference against E2F8 curbs tumorigenicity and reduces tumor burden *in vivo*. *A* and *B,* Ad-sh796 significantly suppressed the tumorigenicity of Huh-7 cells in a flank of nude mice ($n = 8$), where those cells infected with Ad-shNC used as controls were injected s.c. into the opposite flank of the same mice. Tumor size was estimated by serial calipation (*A*) and tumors were weighted (*B*). The results showed the statistically significant difference ($P < 0.01$, Mann-Whitney test; $n = 8$). *C* and *D,* to assess the possibility that E2F8 serves as a therapeutic target, the intratumoral injection of Ad-sh796 was performed on xenograft tumors from Huh-7 cells. Tumor size and weight was significantly reduced after Ad-sh796 administration ($P < 0.05$, Mann-Whitney test; $n = 4$), where Ad-shNC was used as a control.

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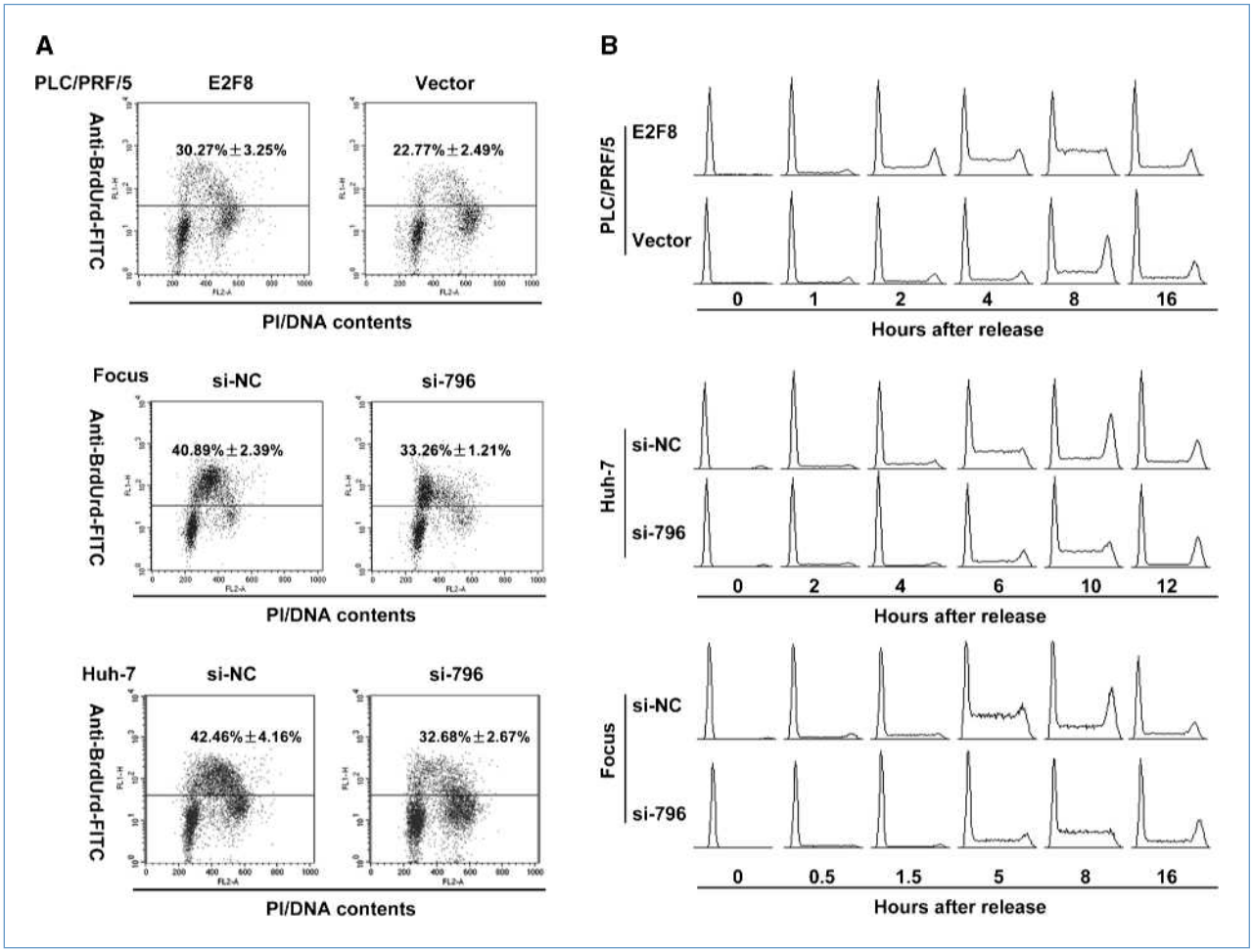

Figure 5. E2F8 regulates cell cycle progression of HCC cells. *A,* the scattergrams showed the percentage of BrdUrd-incorporated population in HCC cells, as shown by fluorescence-activated cell sorting analysis with BrdUrd antibody, when E2F8 overexpression in PLC/PRF/5 cells or knockdown in both Focus and Huh-7 cells. Empty vector and si-NC were used as controls, respectively. *B,* the delineation showed the cell cycle progression at indicated time points after these HCC cells were released from the synchronous status, as indicated by flow cytometry analysis. Ectopic E2F8 significantly promoted the transition of G_1 to S phase of PLC/PRF/5 cells (*top*) as compared with that of empty vector used as controls. E2F8 knockdown by si-796 resulted in the significant delay of G_1-S transition in Huh-7 and Focus cells (*bottom*), where si-NC was used as a control.

regulatory elements of *cyclin D1*. A consensus E2F-binding site was found to be located on *cyclin D1* promoter (19, 24, 25), implying that E2F8 could bind to the motif to regulate cyclin D1 transcription. To confirm the hypothesis, EMSA assay was executed and indicated that E2F8 protein could directly bind the E2F-binding site on *cyclin D1* promoter *in vitro* (Fig. 6*B, right*). To further confirm the *in vivo* binding of endogenous E2F8 to *cyclin D1* promoter, ChIP assay was performed with E2F8 antibody. The ChIP-PCR showed that the *cyclin D1* promoter was immunoprecipitated by endogenous E2F8 in Huh-7 cells (Fig. 6*B, left*), and the immunoprecipitation was weakened as endogenous E2F8 was knocked down by Ad-sh796.

However, as known, E2F8 has only two E2F-related DBDs and lacks known transcriptional activation domains (6–8). Thus, we proposed that E2F8 overexpression could upregulate cyclin D1 transcription in a dominant-negative manner, where E2F8 could competitively bind *cyclin D1* promoter with other E2F family members, which could attenuate or block the effect of some transrepressors on its transcription. To confirm this, a recombinant vector pcDNA3.1 containing *E2F8 DBDs* alone was first constructed and transfected into PLC/PRF/5 cells. Expectedly, ectopic E2F8 DBDs alone can significantly promote the cyclin D1 expression (Fig. 6*A, middle*). Meanwhile, luciferase reporter system under the control of *cyclin D1* promoter was constructed and then transfected along with vectors encoding whole E2F8 and E2F8 DBDs into PLC/PRF/5 cells. Herein, like whole E2F8, E2F8 DBDs activated the luciferase activity (Fig. 6*A, right*). As known, both E2F1 and E2F4, the E2F family members, were also associated with cyclin D1 transcription (24, 26), and subsequently, we examined cyclin D1 expression as E2F1 or E2F4 knockdown in PLC/PRF/5 cells by siRNAs. The resulting data showed that E2F1 knockdown, not E2F4, promoted cyclin D1 expression (Fig. 6*C*; Supplementary Fig. S7). Accordingly, we assumed

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that E2F8 overexpression might displace E2F1 on *cyclin D1* promoter and then promote the transcription of this gene. To confirm the hypothesis, we first used ChIP and EMSA assays to define whether E2F1 can bind to *cyclin D1* promoter. The result indicated that, like E2F8, E2F1 could bind the same regulatory element of *cyclin D1* (Fig. 6*B*). Subsequently, we assessed the competitive binding of both E2F8 and E2F1 to the consensus E2F-binding site *in vivo* and *in vitro* via ChIP and EMSA assays. The resulting data revealed that E2F8 overexpression attenuated the E2F1 binding to the regulatory element, as shown by ChIP assay (Fig. 6*B, middle*), possibly through displacing E2F1, as indicated by EMSA assay (Fig. 6*B, right*). These collective data support the postulate that E2F8 mediates cyclin D1 expression in a dominant-negative manner through abolishing the transrepressive effect of E2F1 on transcription of cyclin D1 (Fig. 6*D*).

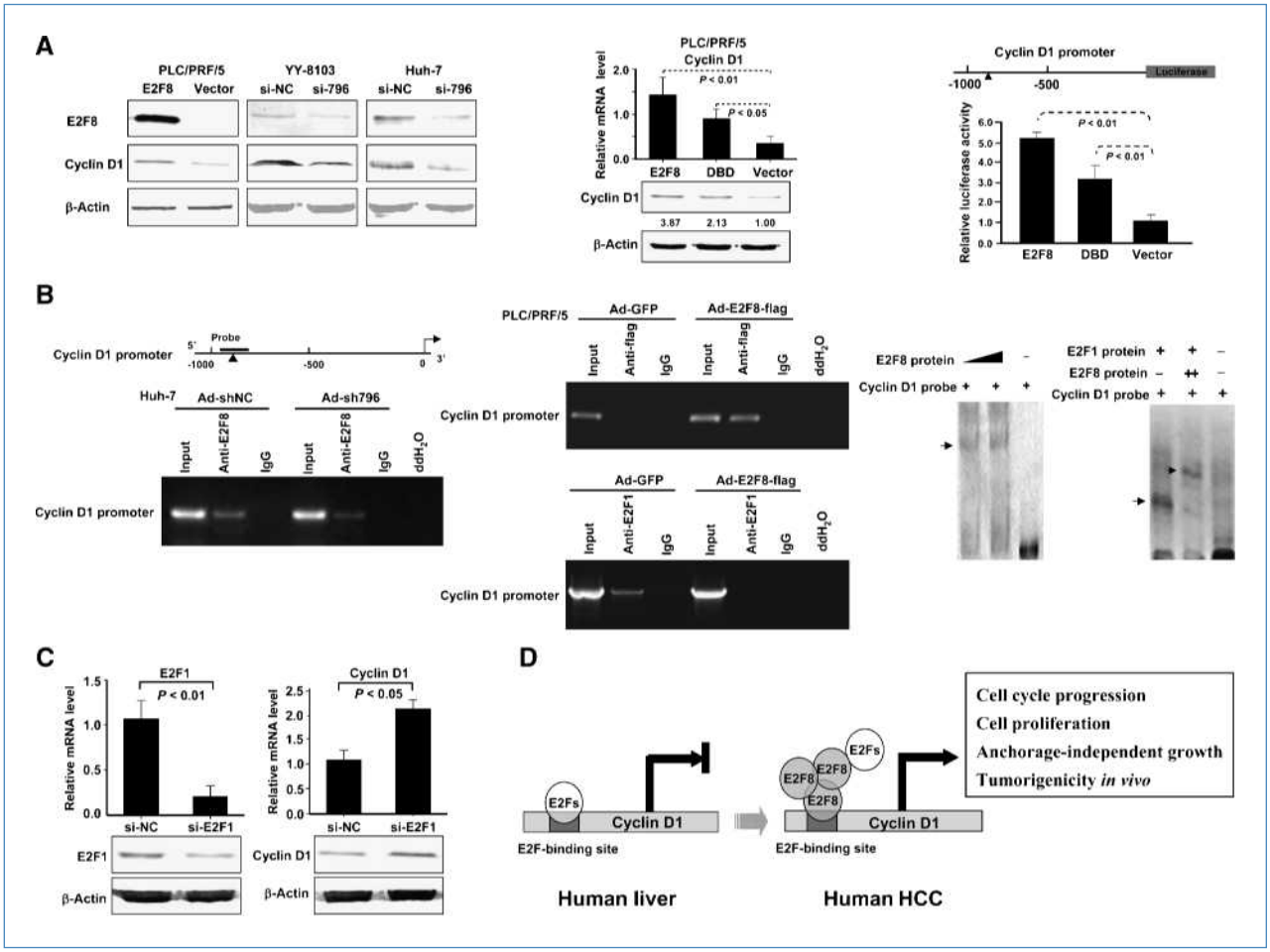

Figure 6. E2F8 mediates cyclin D1 transcription in a dominant-negative manner. *A, left,* the expression level of cyclin D1 was examined by immunoblotting assay, as E2F8 overexpression in PLC/PRF/5 or knockdown in YY-8103 and Huh-7 cells. The mRNA and protein levels of cyclin D1 were also measured by real-time PCR and immunoblotting assay, when pcDNA3.1-*E2F8-DBD* containing two DBDs alone was transfected into PLC/PRF/5 cells (*middle*), where pcDNA3.1-*E2F8* and empty vector were used as positive and negative controls, respectively. The relative protein level of cyclin D1 was normalized as the ratio to the β-actin used as a loading control. A luciferase reporter system under control of cyclin D1 promoter, as shown by a sketch map, was used to evaluate the effect of whole E2F8 or E2F8 DBDs on cyclin D1 transcription in PLC/PRF/5 cells. *Right,* the relative luciferase activity was calculated as the ratio to that of empty vector used as control, where standard derivation and statistical significance were included. *B,* EMSA and ChIP assays showed the binding of both E2F8 and E2F1 to the consensus E2F-binding site of *cyclin D1* promoter, as indicated by a triangle. ChIP-PCR assay showed that the consensus E2F-binding motif was immunoprecipitated by antibodies against E2F8, Flag, and E2F1 in Huh-7 and PLC/PRF/5 cells, respectively (*left* and *middle*), where irrelevant IgG was used as control for antibodies, and double-distilled water (ddH_2O) was used as a negative control. Herein, the amplified regulatory element was used as probe for EMSA assay. *Right,* the shifted bands indicated that both E2F8 and E2F1 can bind the probe, as shown by arrows. *C,* E2F1 knockdown upregulated the cyclin D1 expression in PLC/PRF/5 cells. The cyclin D1 expression was assessed by real-time RT-PCR and immunoblotting assay, where si-NC was used as a control and standard derivation was included. *D,* the schematic diagram of possible mechanism by which E2F8 overexpression upregulates cyclin D1 in HCC cells. In normal adult human liver, the E2F-binding site of cyclin D1 could be occupied by some E2Fs as transrepressors such as E2F1. However, in HCC cells, the overexpressed E2F8 could abolish the transrepression effect of some E2Fs on cyclin D1 through competitive binding to the E2F-binding site. The cyclin D1 overexpression can confer some hallmarks of tumor cells by enhancing cell cycle progression, anchorage-independent growth, and tumorigenicity *in vivo*.

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Discussion

The deregulation of E2F family members has been found in various human malignancies. The expression levels of E2F1, E2F2, and E2F8 were elevated in the ovarian cancer cells (27). Besides, E2F1 was overexpressed in breast cancers (28), pancreatic ductal carcinoma (29), and gastric cancer (30). E2F5 was also overexpressed in breast cancer (31). In HCC, only both E2F1 and E2F3 were reported to be upregulated in cancerous tissues (14, 15). Significantly, this study is the first report that E2F8 was upregulated in the majority of HCC specimens examined. To address the mechanism relevant to upregulation of E2F8, we analyzed chromosomal location and genomic structure of this gene. E2F8 is located on human chromosome 11p15.1 that has not been reported to be relevant to genomic amplification in HCC thus far. Moreover, we also reanalyzed our published array comparative genomic hybridization data (32). The data did not support that the genomic amplification occurred in this chromosomal region in HCC samples. Thus, it is reasonable that upregulation of E2F8 seems not to be ascribed to genetic events involving the genomic locus.

The functions of E2F8 on tumorigenesis and cell cycle progression are still unclear. In the present work, our data revealed that the enforced E2F8 inhibited the growth of QBI-HEK 293A cells (data not shown) but promoted proliferation, colony formation, and tumorigenicity of PLC/PRF/5 cells. This implied that, like other E2F family members, E2F8 could function as an activator or repressor on cell cycle progression in a cellular context–dependent manner (1, 2, 12, 13, 33). In addition, E2F8 could be essential for maintaining the hallmark of human HCC cells. Herein, E2F8 knockdown inhibited the cellular proliferation and tumorigenicity of Huh-7, Focus, Hep3B, and YY-8103 cells. These data indicated that the upregulation of E2F8 could contribute to oncogenesis and progression of HCC. In HCC cells, ectopic E2F8 promoted the transition of G_1 to S phase of cell cycle progression, whereas E2F8 knockdown delayed the transition, which was not consistent with data in human TIG3 fibroblasts (6). As known, E2F family members could orchestrate the expression of genes required for cell cycle progression. Here, we found that E2F8 functions as a transactivator of cyclin D1 in HCC cells. Cyclin D1 is a well-documented important regulator that promotes the G_1- to S-phase transition of cell cycle progression and functions as an oncogene involved in many cancers, including HCC (21–23). This study proposed a possible mechanism that E2F8 overexpression enhances the upregulation of oncogenic cyclin D1 in HCC cells by a dominant-negative manner, where E2F8 can displace partially or completely some E2F transrepressors, such as E2F1, on the E2F-binding site of *cyclin D1* promoter (Fig. 6*D*). Subsequently, cyclin D1 overexpression can promote cell cycle progression and confer some hallmarks of tumor cells with anchorage-independent growth and tumorigenicity *in vivo*. Although this study is the first to uncover the upregulation of E2F8 as an enhancer for cell cycle progression and hepatocarcinogenesis via promoting the cyclin D1 expression, the complexity of E2F8 contribution to HCC is worthy of further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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