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HepaCAM Induces G1 Phase Arrest and Promotes c-Myc Degradation in Human Renal Cell Carcinoma

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ABSTRACT

Hepatocyte cell adhesion molecule (hepaCAM) encodes a generally inactive phosphorylated glycoprotein which mediates cancer cell proliferation, migration, and differentiation. We have reported that hepaCAM is down-regulated in renal cell carcinoma (RCC) and takes responsibility of cell growth inhibition. However, the precise mechanisms of hepaCAM inhibits cell growth is still unknown. In this study, we demonstrated that re-expression of hepaCAM can cause an accumulation in G0/G1 phase in 786-0 cells. This reaction was accompanied by a substantial reduction of c-Myc expression through using an ectopic hepaCAM expression system. Furthermore, we found a comparable decrease in proliferation and G0/G1 accumulation of 786-0 and RC-2 cells after treatment with a small molecule c-Myc inhibitor, 10058-F4. This indicated that the down regulation of c-Myc was an essential process in controlling growth inhibitory actions of hepaCAM. Nevertheless, re-expression of hepaCAM results in apparent reduction of c-Myc protein with no corresponding reduction of c-Myc mRNA. This suggests that this reaction might take place at a post-transcriptional level rather than transcriptional one. Consistent with these findings, hepaCAM decreased c-Myc stability by increasing the proportion of c-Myc phosphorylation on T58 which can be abrogated by a proteasomal inhibitor (MG132). Thus, our research implies that the decrease in c-Myc protein expression, resulting from ectopic expression of hepaCAM, may contribute to the inhibition of proliferation in these cells. J. Cell. Biochem. 112: 2910–2919, 2011.

KEY WORDS: HEPACAM; RENAL CELL CARCINOMA; c-Myc; CELL CYCLECELL CYCLE

HepaCAM is a newly found N-linked glycoprotein. It is originally found in hepatocellular carcinoma by using the technique of suppression subtractive hybridization and gene amplification [Moh et al., 2005]. It displays a typical structure of immunoglobulin (Ig)-like adhesion molecules including two extracellular Ig-like domains, a transmembrane segment, and a cytoplasmic tail [Chung et al., 2005]. The expression level of hepaCAM mRNA is low in many tumor tissues and cell lines [Chung et al., 2005; Shuzhe et al., 2010; Xun et al., 2010]. The exon 2 region of hepaCAM is hypermethylated in bladder cancer tissues and cell lines. Re-expression of the gene occurs in T24 and BIU87 cells after 5-aza-2′-deoxycytidine (5-Aza-Cdr) treatment [Cuicui et al., 2010]. Besides, hepaCAM is also involved in modulating cell adhesion, growth, migration, and invasion [Lee et al., 2009; Moh et al., 2009; He et al., 2010].

RCC is one of the most lethal urologic malignancies. It accounts for 2–3% of cancers worldwide [Jemal et al., 2009]. Thirty percent of RCC patients presented with metastasis at their initial diagnosis. Approximately 40% of patients undergoing nephrectomy for clinically localized RCC will develop to metastasis [Flanigan et al., 2004]. Conventional therapies like chemotherapy, radiotherapy, and immunotherapy are successful only in small number of patients. Therefore, understanding the genetic basis of RCC will be helpful in finding more efficient therapies for this disease.

Overexpression of c-Myc, as determined by immunohistochemistry, is related to the development of RCC [Lovisolo et al., 2006; Gumz et al., 2007]. Moreover, MYC pathway is activated in clear cell renal cell carcinoma, and c-Myc shows a prominent role in the highest scoring network [Tang et al., 2009]. Thus, c-Myc plays an important role in RCC. Treatment which can reduce its expression is of great importance. The regulation of c-Myc is complex. At the transcriptional level, HIF-α (hypoxia-inducible factor-α), a critical regulator for tumor growth, angiogenesis, invasion, and metastasis, modulates the proliferation of RCC through enhancing or

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attenuating c-Myc promoter binding, transcriptional effects on both activated and repressed target genes [John et al., 2007; Zhang et al., 2007]. At the protein level, the c-Myc protein has a relatively short half-life and its stability is regulated by a complex series of phosphorylation and dephosphorylation followed by proteasome mediated degradation [Vervoorts et al., 2006]. Besides, c-Myc needs to dimerize with Max, a helix–loop–helix leucine zipper protein, to bind the specific DNA sequences (called E-box with a central CACGTG motif) in the promoters of its target genes [Amati et al., 1993]. 10058-F4, a small-molecule c-Myc inhibitor, prevented the binding of c-Myc/Max dimers to its DNA targets and inhibited the proliferation of hepatocellular carcinoma and leukemia [Huang et al., 2006; Lin et al., 2007]. However, the functional role of the c-Myc protein and the mechanism, whereby its expression is regulated in RCC, are largely unexplored.

c-Myc is involved in many cell adhesion molecules (CAMS)-mediated tumor cell proliferation. For example, quantity change of E-cadherin is associated with increased cell proliferation and higher microvessel density in c-Myc transgenic mouse models [Calvisi et al., 2004], N-cadherin overexpression inhibits the expression of c-Myc in osteoblasts [Haÿ et al., 2009]. c-Myc has been found to be regulated by epithelial cell adhesion molecule (EpCAM) in both normal and cancer cells [Lu et al., 2010]. In our previous study, we found that hepaCAM was absent or low in RCC tissues and cell lines and its re-expression inhibits the proliferation of RCC [Xun et al., 2010], however, the mechanism that how hepaCAM inhibits RCC proliferation has not been investigated. Whether c-Myc is involved in hepaCAM-induced growth inhibition in RCC is still unknown.

In this study, the role of hepaCAM and c-Myc in RCC proliferation was investigated and the relationship between them was established.

MATERIALS AND METHODS

CELLS AND CULTURE

Human renal cell carcinoma RC-2 and 786-0 cell lines were purchased from American Type Cultrue Collection (ATCC). Cells were grown in RPMI 1640 (Gibco, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C.

HUMAN NORMAL RENAL TISSUE SPECIMEN

The normal renal tissues were obtained from the Department of Urinary Surgery, First Hospital of Chongqing Medical University. Informed consent was obtained from all patients with approval of the Research Ethics Committee of our institution. The tissues were snapping frozen and stored in liquid nitrogen before experiment.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted with Trizol reagent (TaKaRa, Japan). One microgram of total RNA was reverse transcribed with reverse transcriptase and cDNA was subjected to PCR for 35 cycles of amplification using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Japan). PCR primers for hepaCAM were as follows: The forward primer was 5′-AGC GGG AAA TCG TGC GTG-3′, and the reverse primer was 5′-CTT CTG GTT TCA GGC GGT C-3′, 461 bp; c-Myc 5′-CCT ACC CTC TCA ACG ACA GC-3′, 5′-CTC TGA CCT TTT GCA AGG AG-3′, 248 bp; β-actin: 5′-CAC CAC ACC TTC TAC AAT GAG C-3′, 5′-GTG ATC TCC TTC TGC ATC CTG T-3′, 700 bp. After the PCR amplification, 9 μl of the samples with 1 μl of a tracking dye were run on a 1.5% agarose gel containing 1 μg/ml ethidium bromide.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QRT-PCR)

Total RNA was extracted as described. Total RNA aliquots (0.5 μg) were used for reaction. qRT-PCR was preformed with SYBR® PrimeScript® RT-PCR Kit II (TaKaRa, Japan). Transcript quantification was done in triplicate for every sample and reported relative to β-actin. The expression level of HepaCAM as ΔCt = Ct value of gene of interest (HepaCAM) − Ct value of β-actin.

PLASMIDS AND TRANSFECTION

PEGFPN2-hepaCAM recombinant and control pEGFPN2 plasmids were kindly provided by professor Shali shen (National University of Singapore). PEGFPN2-hepaCAM encoded the full length cDNA of hepaCAM. Transfection was carried out by using the reagent of Lipofectamine 2000 Reagent (Invitrogen, Carlsbad) according to the manufacturer's instructions. To generate stable clones, 786-0 cells transfected with pEGFPN2-hepaCAM were subjected to G418 selection at a concentration of 600 μg/ml. Independent single clones were obtained after 2–3 weeks of post-transfectional drug selection. Three clones were used at minimum, each was done in triplicates. Both total RNA and protein were extracted from the clones and RT-PCR and Western blotting analyses were used to screen for positive clones. The pEGFPN2 vector alone was transfected to 786-0 cells as described above and those stable clones served as controls.

CELL GROWTH ASSAYS

Cells were plated in six-well plates at a density of 10^4 cells/well. At indicated time, cells were washed with PBS, harvested by scraping in PBS, and counted by using a Coulter Particle Counter Z1 (Hialeah, FL).

CYTOTOXICITY ASSAYS

Cells were plated in quadruplicate at a density of 10^5 cells/well in 96-well plates and treated with indicated concentrations of 10058-F4 and cultured under standard conditions (37°C in a humidified atmosphere containing 5% CO_2). At indicated time cell viability was determined by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, Santa clara, CA) assay. A volume of 20 μl/well MTT (5 mg/ml in phosphate buffered saline) was added into each well. After 4 h of incubation at 37°C, cells were lysed and formazan crystals were dissolved in 96-well plates, and then added to 100 μl/well dimethyl sulfoxide (DMSO). Optical density (OD) was read at 570 nm on an ELX-800 spectrometer reader (Bio-Tek instruments Inc, Winooski, Vermont)). Cells treated with equivalent amounts of DMSO were used as controls.

CELL CYCLE ANALYSIS

1×10^6 786-0 cells transfected with pEGFPN2 vector and pEGFPN2/hepaCAM plasmids and treated with 100 μM 10058-F4 were

cultured in six-well plates for 24 h. Cells were harvested in cold phosphate buffer solution (PBS), fixed in 70% ethanol, and stored at 4°C for subsequent cell cycle analysis. Fixed cells were washed with PBS once and suspended in 1 ml propidium iodide-staining reagents (20 mg/L ribonuclease and 50 mg/L propidium iodide). Samples were incubated in the dark for 30 min before cell cycle analysis and the distribution of cells in the various phases of the cell cycle was measured with a flow cytometer (Becton Dickinson, Mountain View). The percentage of cells in the G1, S, and G2/M phases was then calculated by the CellQuest software on the flow cytometer.

WESTERN BLOT ANALYSIS

Preparation of cell lysates, protein fractionation, and transfer were as described [Moh et al., 2008]. After transferring to polyvinylidene difluoride membranes (pore size, 0.45 Am; Millipore, Billerica, MA), the membranes were blocked with 5% skim milk and then incubated with a primary antibody followed by a secondary antibody. Both antibodies were diluted in Odyssey blocking buffer containing 0.1% Tween-20. Proteins were visualized by using an Odyssey scanner instrument.

c-Myc STABILITY

Three groups of 786-0 cells (transected with pEGFPN2-hepaCAM and pEGFPN2 and blank control), plated in six-well plates at a density of 5×10^6 cells/well, incubated with 10 μg/ml cyclohexiimide (CHX) for the indicated times period. After cells extraction done, c-Myc protein was detected as described above.

ANTIBODIES AND REAGENTS

The mouse monoclonal antibody to GFP, p21, cyclin D1, c-Myc, and the rabbit polyclonal antibodies to P-c-Myc were from Santa Cruz Biotechnology (Santa Cruz, CA). The inhibitor of c-Myc: 10058-F4, the inhibitor of glycogen synthase kinase-3β (GSK-3β): 6-bromoindirubin3-oxime (BIO) and CHX were from Sigma-Aldrich (Sigma).

STATISTICAL ANALYSIS

Statistical analysis of the results was carried out with SPSS13.0 software. All data were expressed as the mean ± SD. Statistical significance was evaluated by using the one-way ANOVA followed by the Bonferroni's *t*-test for multiple comparisons; $P < 0.05$ was considered as statistically significant.

RESULTS

REINTRODUCTION OF FULL-LENGTH HEPACAM INTO 786-0 CELLS INDUCES THE EXPRESSION OF HEPACAM

It has been previously identified that the expression of hepaCAM is very low in renal cell carcinoma tissues and its re-expression can inhibit 786-0 cell proliferation. In this study, we try to study the mechanism in several RCC cell lines. Firstly, the expression level of hepaCAM mRNA in RCC 786-0, RC-2 cell lines and normal renal tissues was detected by RT-PCR and qRT-PCR (Fig. 1A, B). HepaCAM mRNA expression was decreased in 786-0 and RC-2 cells compared with normal renal tissues and was lower in 786-0 cells than in RC-2 cells. Then, 786-0 cells were transfected with vector alone (GFP) and GFP containing the full-length cDNA of hepaCAM (hepaCAM). About 3 weeks after selection with complete RPMI 1640 containing 600 μg/ml G418, independent single clones were obtained and the number of positive clones was lower for hepaCAM than for the control. To confirm that 786-0 transfected cells can express exogenous hepaCAM, RT-PCR and western blotting experiments were performed. HepaCAM mRNA was detected in 786-0 cells transfected with exogenous full-length hepaCAM, whereas hardly any hepaCAM mRNA expression was detectable in the cells with the empty vector transfected (Fig. 1C). QRT-PCR showed that hepaCAM mRNA was significantly increased in 786-0 cells transfected with hepaCAM than in 786-0 cells transfected with GFP and 786-0 cells untreated (Fig. 1D). The anti-GFP antibody is used indirectly to detect hepaCAM protein indirectly to further confirm the expression of hepaCAM protein (Fig. 1E).

HEPACAM-MEDIATED GROWTH INHIBITION IS ACCOMPANIED BY DOWN-REGULATION OF c-Myc

Cell growth assays were used to monitor the hepaCAM-mediated growth inhibition in RCC 786-0 cells. It showed that the proliferation of cells stably expressed hepaCAM was markedly inhibited compared with control (Fig. 2A). The time course of growth inhibition was about to start at the third day. Furthermore, cell cycle distribution was evaluated by flow cytometry which showed that hepaCAM arrested 786-0 cell growth in the G1 phase of the cell cycle (Fig. 2B, C, D, E). To determine the relationship between hepaCAM and c-Myc, we detected the c-Myc mRNA level in 786-0 cells and found that the c-Myc mRNA was unaffected by hepaCAM (Fig. 2F). These data suggested that hepaCAM regulates c-Myc expression at a post-transcriptional level. To determine whether the regulation of c-Myc expression by hepaCAM occurred at protein levels, western blotting was performed. As showed in (Fig. 2G), the overexpressing of hepaCAM can down-regulate c-Myc expression and at the protein expression as well.

A SMALL MOLECULE c-Myc INHIBITOR, 10058-F4, INHIBITS CELL PROLIFERATION AND ARRESTS THE CELL CYCLE AT G0/G1 PHASE

c-Myc mainly needs to dimerize with Max to transactivate its target genes to modulate cell proliferation, apoptosis, and cell differentiation [Amati et al., 1993]. 10058-F4, a small molecule c-Myc inhibitor, inhibits proliferation in human hepatocellular carcinoma cells and human acute myeloid leukemia through inhibiting c-Myc/Max dimerization [Huang et al., 2006; Lin et al., 2007]. To investigate the role of c-Myc protein level reduction in inhibition of hepaCAM-mediated growth in RCC, 10058-F4 was used to detect if reduction of c-Myc/Max dimerization was sufficient to inhibit proliferation and induce G1 accumulation. MTT is used to detect the activity of 10058-F4 in RC-2 cells and 786-0 cells. The results showed that both cell lines proliferation were inhibited and 786-0 cells were more sensitive. Moreover, RC-2 cells and 786-0 cells treated with 100 μM 10058-F4 for 24 h can obviously inhibit cells proliferation and 786-0 cells exhibited marked morphological changes (Fig. 3A, B). Then, 1×10^6 786-0 cells were exposed to 100 μM 10058-F4 for 24 h and its cell cycle was detected. DMSO was used as control. (Fig. 3C, D, E) showed that the significant increase in G0/G1 accompanied by a decrease in S in 10058-F4 treated cells was

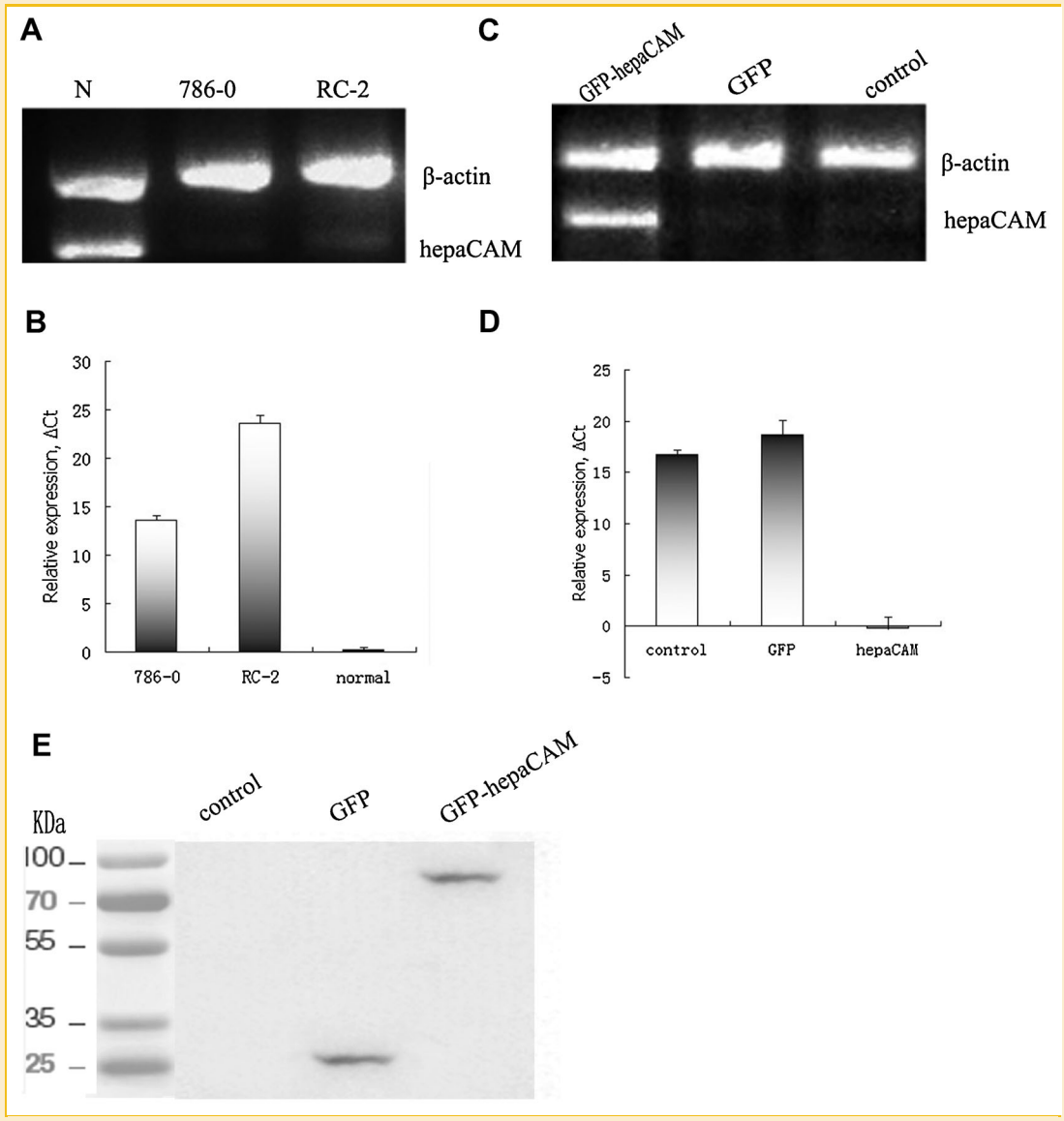

Fig. 1. Reintroduction of full-length hepaCAM into 786-0 cells induces the expression of hepaCAM. A, B: RT-PCR and QRT-PCR were used to detect the expression of hepaCAM in cell lines and normal renal tissues. The expression level of HepaCAM as ΔCt = Ct value of gene of interest (HepaCAM) – Ct value of β-action. C, D: 786-0 cells transfected with pEGFPN2 (GFP) vector and pEGFPN2/hepaCAM plasmids (hepaCAM) were selected by G418 for 3 weeks. Then, the expression of hepaCAM was examined by RT-PCR and QRT-PCR. E: Protein expression of pEGFPN2 vector and GFP-fused full-length hepaCAM in 786-0 cells was detected by Western blotting using anti-GFP antibody. N/normal: Normal renal tissues group; control: 786-0; GFP: 786-0/GFP; hepaCAM: 786-0/GFP-hepaCAM. All blots and RT-PCR data are representative of three independent experiments performed in triplicate.

found. These results indicated that 10058-F4 can inhibit proliferation in RCC and it is sufficient to inhibit RCC proliferation when the activity of c-Myc was suppressed.

EFFECT OF HEPACAM ON c-Myc STABILITY IN RCC

Our initial studies suggested that hepaCAM-mediated growth inhibition was accompanied by down-regulation of c-Myc protein. c-Myc is a short-life molecule, but it is overexpressed in renal cell carcinoma [Lovisolo et al., 2006]. To clarify that whether hepaCAM can reduce c-Myc protein stability, 786-0 cells transfected with hepaCAM were treated with CHX to inhibit de novo protein synthesis and cells were harvested at the indicated times. The results showed that the total amount of c-Myc protein was decreased and the c-Myc expression decreased more quickly in 786-0 cells stabely expressed hepaCAM (Fig. 4A). In hepaCAM-transfected cells, the half-life of c-Myc was less than 30 min, by contrast, that of c-Myc in nontransfected cells and GFP-transfected cells was longer than 45 min (Fig. 4B). Ubiquitin-mediated proteolysis by the 28S proteasome has been shown to be responsible for the relatively short life of the c-Myc protein [Salghetti et al., 1999]. To determine whether the degradation process was associated with loss of c-Myc, cells were treated with proteasomal inhibitor (MG132) for 24 h. It showed that the proteasomal inhibitor prevented the hepaCAM-induced loss of c-Myc (Fig. 4C, D).

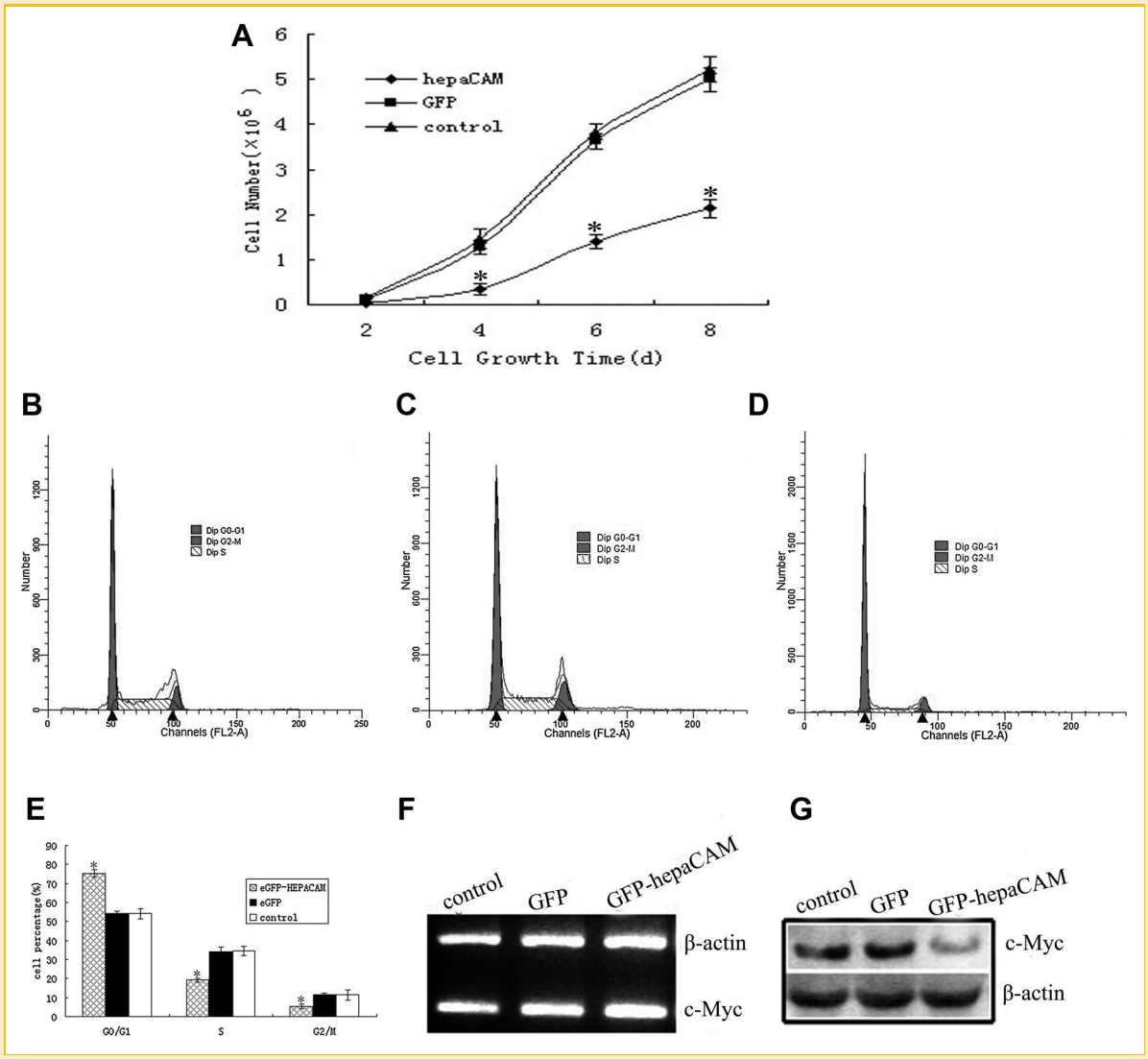

Fig. 2. HepaCAM-mediated growth inhibition is accompanied by down-regulation of c-Myc. A: Proliferation measured by serial cell counts in 786-0 cells transfected with GFP and hepaCAM. Cells were plated in six-well plates at a dose of 1×10^5/well in duplicates and counted as indicated. B, C, D: The effect of hepaCAM on cell cycle distribution was analyzed by flow cytometry. B: Blank control, (C) cells transfected with GFP, (D) cells transfected with hepaCAM. E: Cell percentage in each phase according to the diploid analysis using a flow cytometer. Data are reported as mean ± SD ($n = 3$). $^*P < 0.05$ versus control. F: 786-0 cells were transfected with GFP and hepaCAM. c-Myc mRNA was measured by RT-PCR and β-actin was used as control. G: 786-0 cells were transfected with GFP and hepaCAM. Cells were harvested for protein analysis, extracts run on an SDS-PAGE gel and proteins (c-Myc and β-actin) detected by western blotting. Control: 786-0; GFP: 786-0/GFP; hepaCAM: 786-0/GFP-hepaCAM. Triplicate experiments were performed in a parallel manner and representative results are shown.

HEPACAM INCREASED THE PHOSPHORYLATION OF c-Myc IN RCC

The steady state level of c-Myc ubiquitination depends on an enzymatic ubiquitin conjugation and deconjugation. Phosphorylation of T58 in c-Myc serves as a signal to induce degradation through binding of SCF^{Fbw7} (E3 ubiquitin ligase complexes) [Welcker et al., 2004]. To examine the mechanism by which hepaCAM induced c-Myc degradation, the cellular levels of phosphorylation of c-Myc at T58 were measured in 786-0 cells. After treatment, the cells were lysed and 50 μg protein was analyzed by western blotting using a specific antibody to P-T58-Myc. The results indicated that hepaCAM increased phosphorylation of c-Myc levels in 786-0 cells (Fig. 5A, B). GSK-3β is the only known kinase that phosphorylates c-Myc at T58 [Pulverer et al., 1994]. The role of GSK-3β in hepaCAM-induced c-Myc degradation was examined by using GSK-3β inhibitor (BIO). We tested the level of c-Myc and P-T58-c-Myc protein after treated with BIO for 6 h in 786-0 cells stably expressed hepaCAM. (Fig. 5C, D) showed that the GSK-3β inhibitor (BIO) did not prevent hepaCAM-induced c-Myc degradation nor decrease the level of P-T58-Myc. This suggested that the absence of GSK-3β can not prevent hepaCAM-induced c-Myc degradation and other kinases which phosphorylate c-Myc at T58 may be involved in this pathway.

EFFECT OF HEPACAM ON THE EXPRESSION OF P21 AND CYCLIN D1

P21 and cyclin D1 are both cell cycle regulators, which are important for cell proliferation. They are all the target genes of

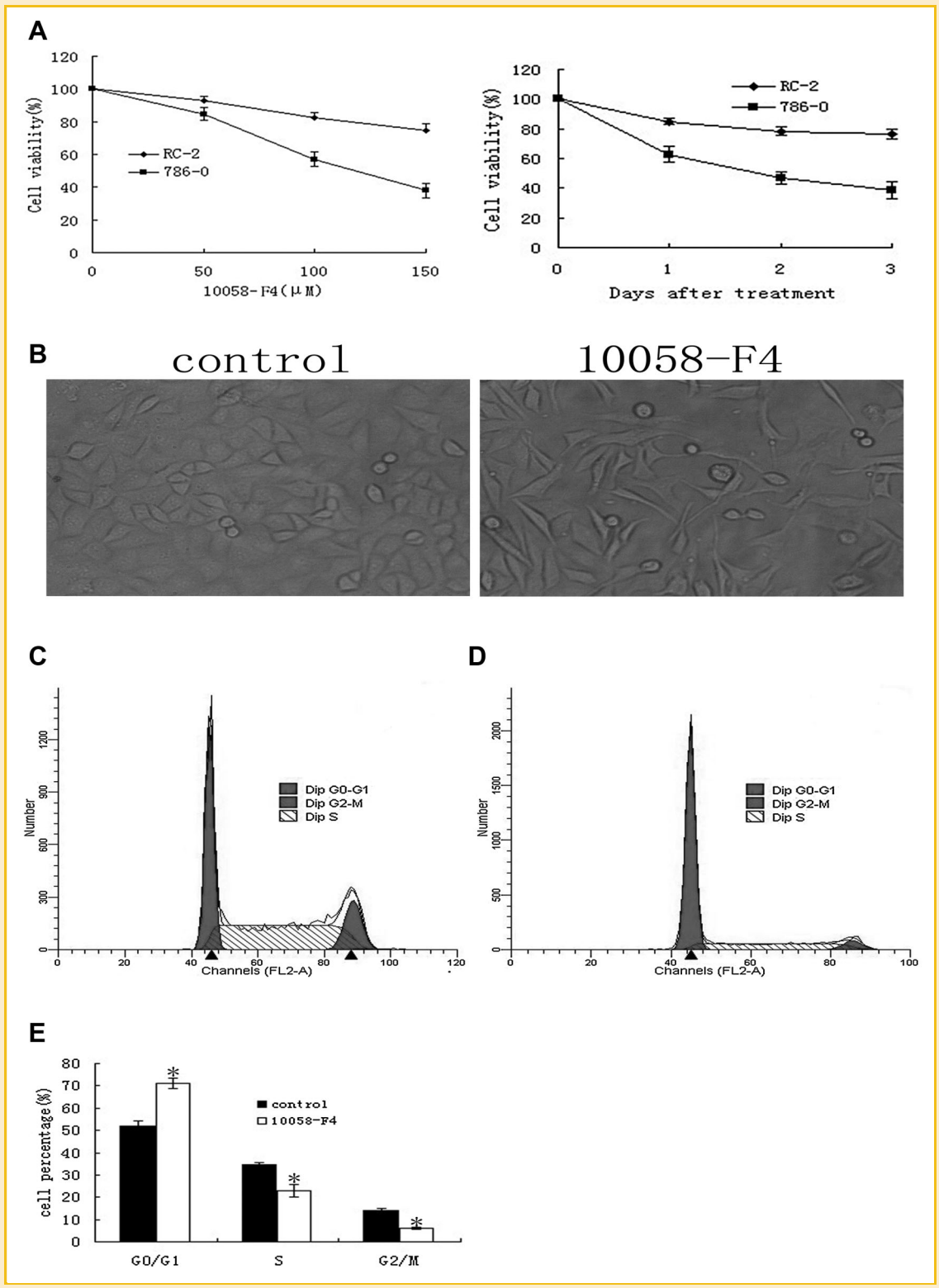

Fig. 3. A small molecule c-Myc inhibitor, 10058-F4, inhibits cells proliferation and arrests the cell cycle at G0/G1 phase. A: Growth inhibition of 786-0 and RC-2 cells by 10058-F4 was assessed by MTT assays after exposure to 100 μM of 10058-F4 for up to 3 days and to indicated concentrations of 10058-F4 for 24 h. Data represent the mean ± SD of experiments in quadruplicates. B: Morphological changes of 786-0 cells after being treated with 100 μM 10058-F4 for 24 h. C, D: The cell cycle distribution in 786-0 cells treated with 100 μM 10058-F4 for 24 h was analyzed by flow cytometry. C: Control group; (D) Cells treated with 10058-F4. E: Cell percentage in each phase according to the diploid analysis using a flow cytometer. Data are reported as mean ± SD ($n = 3$). $^{*}P < 0.05$ versus control.

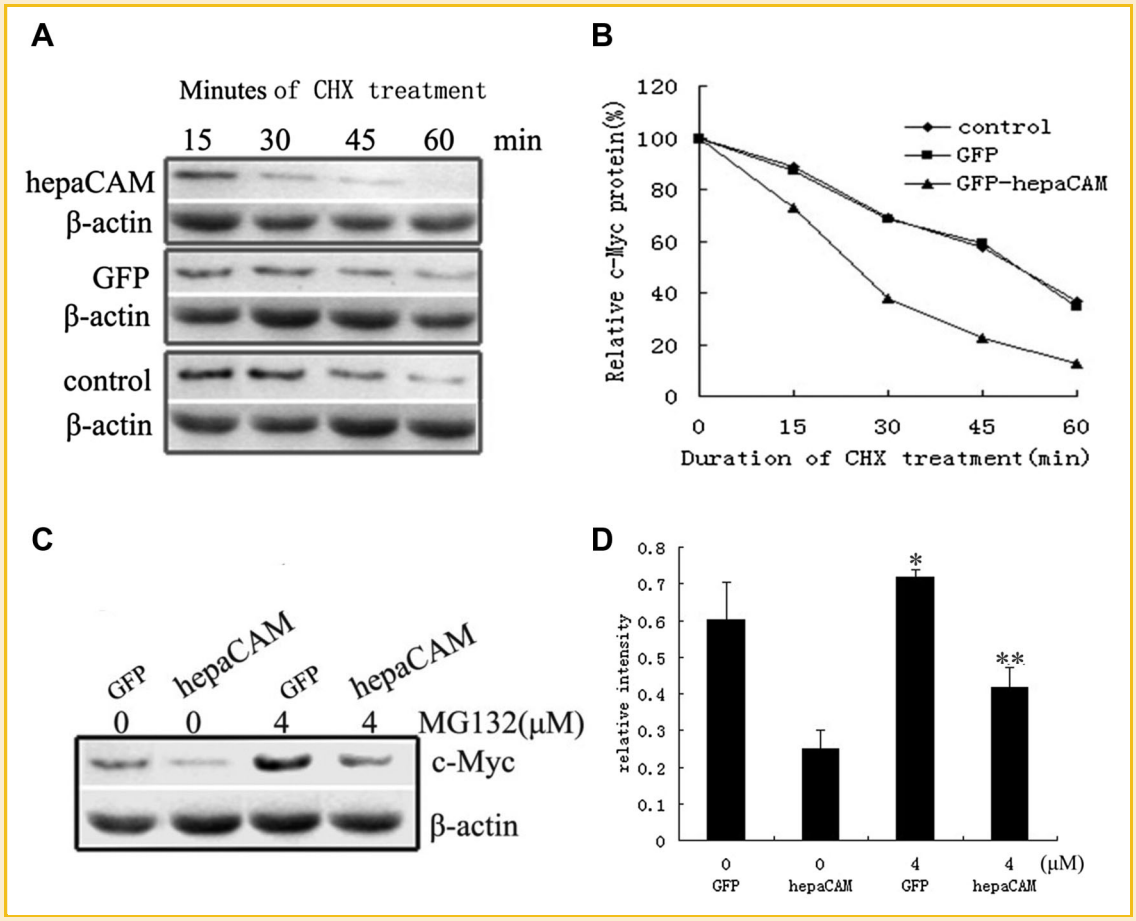

Fig. 4. Effect of hepaCAM on c-Myc stability in RCC. A: 786-0 cells were transfected with GFP and hepaCAM. Prior to harvesting, cells were treated with cycloheximide (CHX) at the concentration of 10 μg/ml for 0–60 min. Cells were harvested for protein analysis at the indicated times, extracts run on an SDS-PAGE gel and c-Myc and β-actin protein levels were detected by western blotting. B: Cells were treated as described above and the relative c-Myc protein was calculated from western blotting after normalization to β-actin levels. C, D: 786-0 cells transfected with GFP and hepaCAM were treated with 4 μM MG132 for 2 h. After treatment, cells were lysed and 50 μg of whole cell lysate was analyzed by western blotting, the relative intensities of target bands were scanned by Quantity one software, $^{*}P < 0.05$, compared with control. Control: 786-0; GFP: 786-0/GFP; hepaCAM: 786-0/GFP-hepaCAM.

c-Myc. To further investigate the role of c-Myc in hepaCAM-mediated cell proliferation, the protein levels of p21 and cyclin D1, transfected with GFP and hepaCAM, in 786-0 cells were detected by western blotting. The results showed that the expression of p21 protein was increased and the expression of cyclin D1 was decreased comparing with control groups (Fig. 6A, B).

DISCUSSION

HepaCAM, a member of immunoglobulin superfamily, acts as a cell adhesion molecule in cell adhesion and a key molecule in tumor process [Moh et al., 2009]. The expression level of its mRNA is absent or low in many tumors and cell lines and re-expression of it can inhibit multiple tumor cell proliferation [Lee et al., 2009; He et al., 2010]. Re-expression of hepaCAM can also inhibit RCC cell proliferation. However, the mechanism of hepaCAM regulating RCC proliferation is still unexplored. In this study, we showed that the expression of c-Myc was decreased in hepaCAM-mediated growth inhibition. Furthermore, we found that c-Myc stability was down-regulated when hepaCAM re-expressed in renal cell carcinoma cell line 786-0 and the phosphorylation of T58 in c-Myc was increased. Moreover, a small molecule c-Myc inhibitor, 10058-F4, inhibited cell proliferation and arrested the cell cycle at G0/G1 phase in RCC.

High-expressed hepaCAM is involved in mediating growth inhibition in human breast cancer cells via G2/M phase arrest and a p53/p21-dependent pathway [Moh et al., 2008], and in human glioblastoma with no significant alteration in the cell cycle distribution [Lee et al., 2009]. However, our data here showed that the high expression of hepaCAM caused a G1 accumulation. Such a phenomenon has also been observed in other tumor suppressors such as PTEN, which arrested prostatic cancer cell cycle at G1 phase [Van Duijn et al., 2010], arrested melanoma at G2 phase [Jacob et al., 2009] and had no effect on the cell cycle of leukemia [Seminario et al., 2003]. These data suggested that hepaCAM may inhibit the proliferation of different cancer through different approaches.

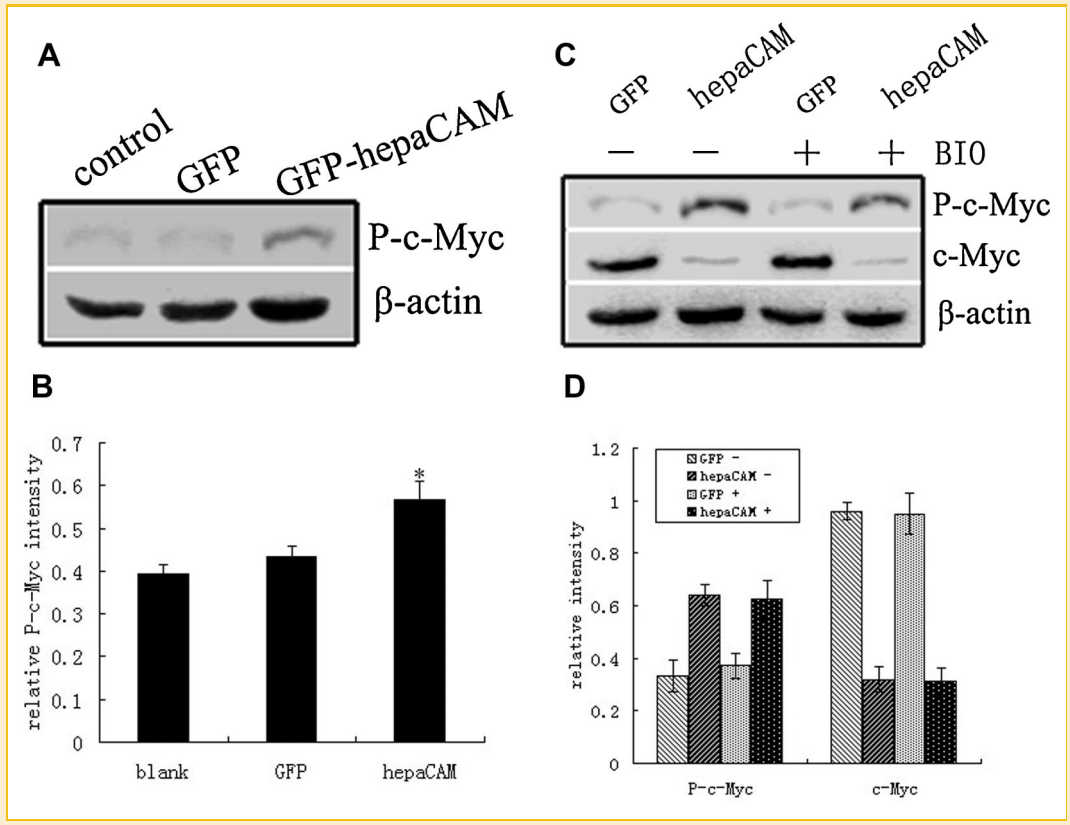

Fig. 5. HepaCAM increased the phosphorylation of c-Myc in RCC. A: Phosphorylated c-Myc (T58) was detected by western blotting in 786-0 cells transfected with GFP or hepaCAM. C: 786-0 cells transfected with GFP and hepaCAM were treated with vehicle (−) or GSK-3β inhibitor BIO (+) 2 μM for 6 h, extracts run on an SDS-PAGE gel, and then c-Myc and phosphorylated c-Myc (T58) protein were detected by western blotting. B, D: Results showed a representative result, and data form three independent experiments are shown; the relative intensities of target bands were scanned by Quantity one software, $^{*}P < 0.05$, compared with control. Control: 786-0; GFP: 786-0/GFP; hepaCAM: 786-0/GFP-hepaCAM.

Our research showed in the first time that reducing c-Myc/Max dimerization with a c-Myc inhibitor 10058-F4, which has been proved being able to inhibit the proliferation of human hepatocellular carcinoma cells and human acute myeloid leukemia [Huang et al., 2006; Lin et al., 2007], is sufficient to cause both the growth inhibition and G1 accumulation in RCC cell lines. Previous reports using c-Myc-specific siRNA to reduce c-Myc expression in renal cell carcinoma also showed growth inhibition [Zhang et al., 2007].

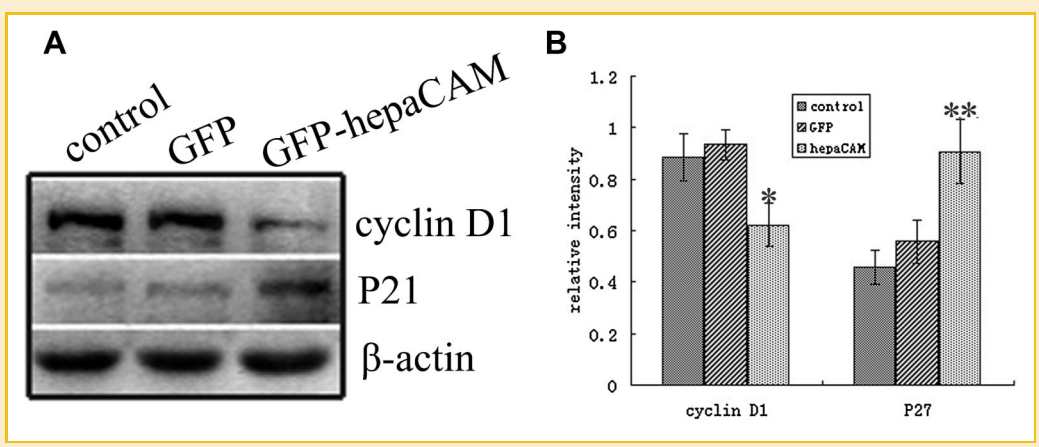

Fig. 6. HepaCAM regulates the expression of p21 and cyclin D1. A: 786-0 cells transfected with GFP and hepaCAM were lysed and 50 μg of whole cell lysate was analyzed by western blotting. B: Results showed a representative result, and data form three independent experiments are shown; the relative intensities of target bands were scanned by Quantity one software, $^{**}P < 0.01$, $^{*}P < 0.05$, compared with control. Control: 786-0; GFP: 786-0/GFP; hepaCAM: 786-0/GFP-hepaCAM.

Expression analysis revealed that the mRNA and protein levels of c-Myc were increased in renal cell carcinoma [Kinouchi et al., 1989], and MYC pathway was activated in RCC and c-Myc played a vital role in it [Tang et al., 2009]. And Anke Geick et al. reported that transgenic mouse expressing c-Myc can frequently develop renal cell carcinoma [Geick et al., 2001]. We find that hepaCAM-mediated growth inhibition is accompanied by down-regulation of c-Myc protein, and moreover, cells reducing c-Myc by 10058-F4 can cause a G1 accumulation which was similar to cells transfected with hepaCAM. This suggests that hepaCAM is a key point in the development of RCC and the regulation of c-Myc.

The c-Myc protein level but not mRNA level was down-regulated in RCC cells stably expressed hepaCAM which suggested that the regulation of c-Myc was done at the level of protein expression. We found that the half-life of c-Myc protein was reduced obviously in RCC cells which was transfected with hepaCAM. Phosphorylation of c-Myc at T58 serves as a signal for proteasome-mediated degradation [Welcker et al., 2004]. T58 is phosphorylated by GSK-3β, which requires a prior phosphorylation at S62. In order to phosphorylate T58, S62 is then dephosphorylated and the protein ubiquitinated and degraded [Pulverer et al., 1994]. We found that GSK-3β did not appear to be involved in the degradation of c-Myc induced by hepaCAM for knocking down GSK-3β by its inhibitor BIO, did not decrease P-T58-Myc levels nor increase c-Myc levels. This paradoxical result indicates that GSK-3β is not the only enzyme to phosphorylate c-Myc at T58 in renal cell carcinoma 786-0 cells.

We also detected the expression of p21 and cyclin D1. Results showed that the protein level of p21 was increased and that of cyclin D1 was decreased in renal cell carcinoma stably expressed hepaCAM. p21and cyclin D1 are both cell cycle regulator, involved in mediating cell proliferation and G1 phase of cell cycle [Caldon et al., 2006].We found that the expression of p21 and cyclin D1 was recovered when c-Myc inhibitor 10058-F4 was used (data not shown). Thus, p21 and cyclin D1, served as targets of c-Myc, had been suggested as contributors to hepaCAM mediated growth inhibition in renal cell carcinoma.

The necessity for tightly regulating the expression of c-Myc in normal cells has resulted in the evolution of a complex series of regulatory mechanisms. The expression of c-Myc is often regulated by Wnt signaling through β-catenin/TCF/LEF [He et al., 1998]. Many adhesion molecules mediated cell proliferation through Wnt/β-catenin pathway such as N-cadherin, which mediated cell proliferation by inhibiting the expression of Wnt3a ligand, c-Myc served as a target of Wnt pathway involved in it [Haÿ et al., 2009]. However, in our study, the c-Myc mRNA is not obviously changed by hepaCAM, and we find that reduction of β-catenin expression is insufficient to inhibit proliferation (data not show). This suggests that there are additional mechanisms regulating c-Myc. c-Myc is also involved in some other pathway such as MAPK and ERK [Inamura et al., 2005; Kaizu et al., 2008]. Whether these pathways are redundant with down-regulation of c-Myc or cooperate in producing the down regulation in renal cell carcinoma stably expressed hepaCAM remains to be undetermined. The down-regulation of c-Myc protein level may also occur at the translational level. The 5′-UTR region of c-Myc mRNA contains a complex secondary structure that limits the efficiency of cap-dependent translation, which is weakly translated when the activity of the PI3K/AKT/mTORC1 pathway is suboptimal [Gingras et al., 2001]. HepaCAM may influence c-Myc expression at translational level directly or indirectly, which needs to be determined.

In summary, we have demonstrated that the growth suppression ability of ectopic hepaCAM, 10058-F4 and the role of hepaCAM in maintaining the stability of c-Myc protein in renal cell carcinoma cell lines. Down-regulation of c-Myc by hepaCAM significantly contributes to the inhibition of G1-S transition and maintains the 786-0 cells in the G0/G1 phase. The antiproliferaion effect of hepaCAM gene on renal cell carcinoma is partially via degradation of c-Myc protein. Further highlights may reside in the relevance of hepaCAM and c-Myc in renal cell carcinoma development, which will allow us to have a better understanding of the function of hepaCAM gene function in the development of renal cell carcinoma and to have an appropriate choice of therapeutic method.

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