Prostaglandin E_2 promotes the cell growth and invasive ability of hepatocellular carcinoma cells by upregulating c-Myc expression via EP4 receptor and the PKA signaling pathway

SHUKAI XIA, JUAN MA, XIAOMING BAI, HAI ZHANG, SHANYU CHENG, MIN ZHANG, LI ZHANG, MINGZHAN DU, YIPIN WANG, HAI LI, RONG RONG, FENG SHI, QINYI YANG and JING LENG

Cancer Center, Department of Pathology, Nanjing Medical University, Nanjing, Jiangsu 210029, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) represents a major health problem worldwide. Prostaglandin E_2 (PGE_2), the predominant product of cyclooxygenase-2, has been implicated in hepatocarcinogenesis. However, the underlying molecular mechanisms remain to be further elucidated. *c-myc*, a cellular proto-oncogene, is activated or overexpressed in many types of human cancer, including HCC. The present study was designed to investigate the internal relationship and molecular mechanisms between PGE_2 and c-Myc in HCC, and to define its role in HCC cell growth and invasion. Our results showed that PGE_2 significantly upregulated c-Myc expression at both the mRNA and protein levels, and knockdown of c-Myc blocked PGE_2-induced HCC cell growth and invasive ability in human HCC Huh-7 cells. The effect of PGE_2 on c-Myc expression was mainly through the EP4 receptor, and EP4 receptor-mediated c-Myc protein upregulation largely depended on *de novo* biosynthesis of c-Myc mRNA and its protein. EP4 receptor signaling activated G_S/AC and increased the intracellular cAMP level in Huh-7 cells. The adenylate cyclase (AC) activator forskolin mimicked the effects of the EP4 receptor agonist on c-Myc expression, while the AC inhibitor SQ22536 reduced EP4 receptor-mediated c-Myc upregulation. These data confirm the involvement of the G_S/AC/cAMP pathway in EP4 receptor-mediated c-Myc upregulation. Moreover, the phosphorylation levels of CREB protein were markedly elevated by EP4 receptor signaling, and by using specific inhibitor and siRNA interference, we demonstrated that PKA/CREB was also involved in the EP4 receptor-mediated c-Myc upregulation. In summary, the present study revealed that PGE_2 significantly upregulates c-Myc expression at both mRNA and protein levels through the EP4R/G_S/AC/cAMP/PKA/CREB signaling pathway, thus promoting cell growth and invasion in HCC cells. Targeting of the PGE_2/EP4R/c-Myc pathway may be a new therapeutic strategy to prevent and cure human HCC.

Correspondence to: Professor Jing Leng, Cancer Center, Department of Pathology, Nanjing Medical University, 140 Hanzhong Road, Nanjing, Jiangsu 210029, P.R. China
E-mail: lengjing@njmu.edu.cn

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Introduction

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is one of the most prevalent malignancies and the third leading cause of cancer-related mortality worldwide with ~700,000 deaths each year (1). The incidence of HCC is dramatically increasing, and to date there are no effective chemoprevention or systemic treatments available. Therefore, it is of utmost importance to elucidate the molecular mechanisms of HCC. An increasing number of studies have indicated that chronic inflammation plays a crucial role in the occurrence and development of HCC (2-4), yet the precise mechanisms are still unclear.

Prostaglandin E_2 (PGE_2), a bioactive lipid which is produced predominantly from arachidonic acid by cyclooxygenases (COXs) and prostaglandin E synthases (PGES), is generally considered to be a potent pro-inflammatory mediator (5). Furthermore, substantial evidence has shown that PGE_2 is also associated with several serious human diseases, including malignant tumors (6,7). PGE_2 may promote cancer cell growth, adhesion, invasion, metastasis and angiogenesis (8), and thus participate in the tumorigenesis and progression of numerous human cancers, such as HCC (9-13), breast (14,15), gastrointestinal (16) and prostate cancer (17). PGE_2 exerts diverse biological effects through four cognate E prostanoid receptors (EP receptor) from EP1 to EP4 (5,18), among which, the EP4 receptor is believed to be closely associated with cancer cell proliferation and invasion in many types of human cancer (19-23).

Moreover, the activation or overexpression of proto-oncogenes is involved in HCC (24). The *c-myc* proto-oncogene is the human cellular homologue of avian myelocytomatosis viral oncogene (*v-myc*), encoding a transcription factor c-Myc protein that upregulates the expression of many target genes and thus promotes cell proliferation and tumorigenesis. In addition, *c-myc* expression itself is regulated at multiple levels

including transcription, post-transcription and post-translation (25,26). In HCC, the activation or overexpression of the *c-myc* proto-oncogene has been well documented (27,28). In addition, research supports a central role for c-Myc in human hepatocarcinogenesis (29). Thus, it is vital to explore the detailed molecular mechanisms of *c-myc* activation in HCC.

Given our previous results showing that PGE_2 could notably enhance the cell growth and invasive ability of HCC cells (10-13), and the involvement of *c-myc* activation in hepatocarcinogenesis, the present study was designed to evaluate our hypothesis that PGE_2 may promote the cell growth and invasion of HCC cells through upregulation of c-Myc protein expression. Data from the present study revealed that PGE_2 significantly upregulated the expression of c-Myc at the mRNA and protein levels both via the EP4 receptor and the coupled G_S/AC/cAMP/PKA/CREB signaling pathway, thus promoting cell growth and invasiveness in HCC cells.

Materials and methods

Materials. The human HCC cell line Huh-7 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and Lipofectamine 2000 were from Life Technologies (Grand Island, NY, USA). PGE_2, PGE_1 alcohol, GW627368X and the cyclic AMP EIA kit were from Cayman Chemical Co. (Ann Arbor, MI, USA). SQ22536, forskolin, H89, actinomycin D (Act D) and cycloheximide (CHX) were from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). High Pure RNA isolation kit was from Roche (Mannheim, Germany). PrimeScript RT reagent kit was from Takara (Dalian, China). Anti-c-Myc (no. 5605), anti-CREB (no. 9104), anti-phosphorylated CREB (no. 9198, Ser133) antibodies were from Cell Signaling Technology (Danvers, MA, USA); anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Wuhan, China), and anti-β-tubulin antibody (no. 21335) was from SAB (Signalway Antibody; College Park, MD, USA). The protein assay dye reagent was from Bio-Rad (Hercules, CA, USA). ECL Prime Western Blotting Detection reagent was from GE Healthcare (Piscataway, NJ, USA). The Transwell unit was from Corning (Cambridge, MA, USA). Matrigel was from BD Biosciences (San Jose, CA, USA).

Cell line and culture. Human HCC Huh-7 cells were cultured in DMEM with 10% fetal calf serum (FCS) at 37˚C in a humidified 5% CO_2 incubator. The experiments were performed when cells reached 80% confluency and were conducted in serum-free medium.

Cell proliferation assay. Cell proliferation was determined using the CCK-8 kit from Dojindo Laboratories. This kit contains the WST-8 reagent, a tetrazolium salt that can be reduced by mitochondrial dehydrogenases in viable cells to produce an orange colored formazan dye. Briefly, 600 μl of cell suspension ($1x10^5$ cells) was plated in each well of 24-well plates. After a 24-h culture to allow reattachment, the cells were then incubated with different treatments at the indicated concentrations and time periods. Cell proliferation reagent, WST-8 (60 μl), was subsequently added to each well. The incubation was continued from 30 min to 4 h at 37˚C and absorbance at 450 nm was measured using an automatic ELISA plate reader.

Cell invasion assays. Cell invasion assays were performed in Matrigel-coated 24-well Transwell units. Cells ($5x10^4$) were added to the upper Transwell chamber and media with 10% FCS were added to the lower Transwell chamber. The serum-free media plus pharmacological agents were added in the upper Transwell chamber. After 24 h of incubation at 37˚C, the cells were fixed with ethanol and then stained by 0.1% crystal violet for 30 min at room temperature. After washing the wells with PBS, the cells on the upper surface of the filter were removed with a cotton swab. The invaded cells on the lower surface of the membrane were solubilized with 10% acetic acid for 10 min and quantified by measuring the absorbance at 550 nm.

Western blotting. Cells were treated with different pharmacological agents at 37˚C for various times, as indicated in the experiments. The cells were collected into lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, sodium orthovanadate, sodium fluoride and aprotinin) and placed on ice for 30 min. Cell lysates were sonicated on ice for at least 30 sec and then cleared by centrifugation at 15,000 x g for 30 min at 4˚C. Protein concentrations of lysates were measured using Bio-Rad protein assay dye reagent. Equal amounts of proteins (40 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk-TBST buffer for 1 h at room temperature and incubated with the corresponding primary antibodies overnight at 4˚C with gentle shaking. Then, the membranes were washed by TBST and incubated for 2 h with the peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies at room temperature. The signals were detected using enhanced chemiluminescent reagent (ECL) and analyzed using Image Lab 4.0 analysis software from Bio-Rad.

RNA isolation and real-time PCR. Total RNA from the cultured cells was isolated using the High Pure RNA isolation kit according to the manufacturer's instructions. Reverse transcription was carried out with the PrimeScript RT reagent kit according to the standard protocol. For quantification of mRNA expression, real-time PCR was performed using the following primer pairs: c-Myc, 5'-AGGCTATTCTGCCCATTT-3' (forward) and 5'-TCGTAGTCGAGGTCATAGTTC-3' (reverse); EP4, 5'-CATCTTACTCATTGCCACCT-3' (forward) and 5'-TACTGAGCACTGTCTTTCTC-3' (reverse); GAPDH, 5'-TTCCAGGAGCGAGATCCCT-3' (forward), and 5'-CACCCATGACGAACATGGG-3' (reverse). Real-time PCR analysis was performed on Roche LightCycler Nano instrument using FastStart Essential DNA Green Master Mix from Roche, and GAPDH was used as the endogenous control. PCR conditions were pre-incubation at 95˚C for 10 min (1 cycle) followed by 40 cycles of 95˚C for 20 sec, 60˚C for 20 sec and 72˚C for 20 sec. All treatments and conditions were performed in triplicate to calculate the statistical significance.

siRNA interference. The siRNA targeting human EP4 receptor (siRNA ID: s11455) was purchased from Life Technologies.

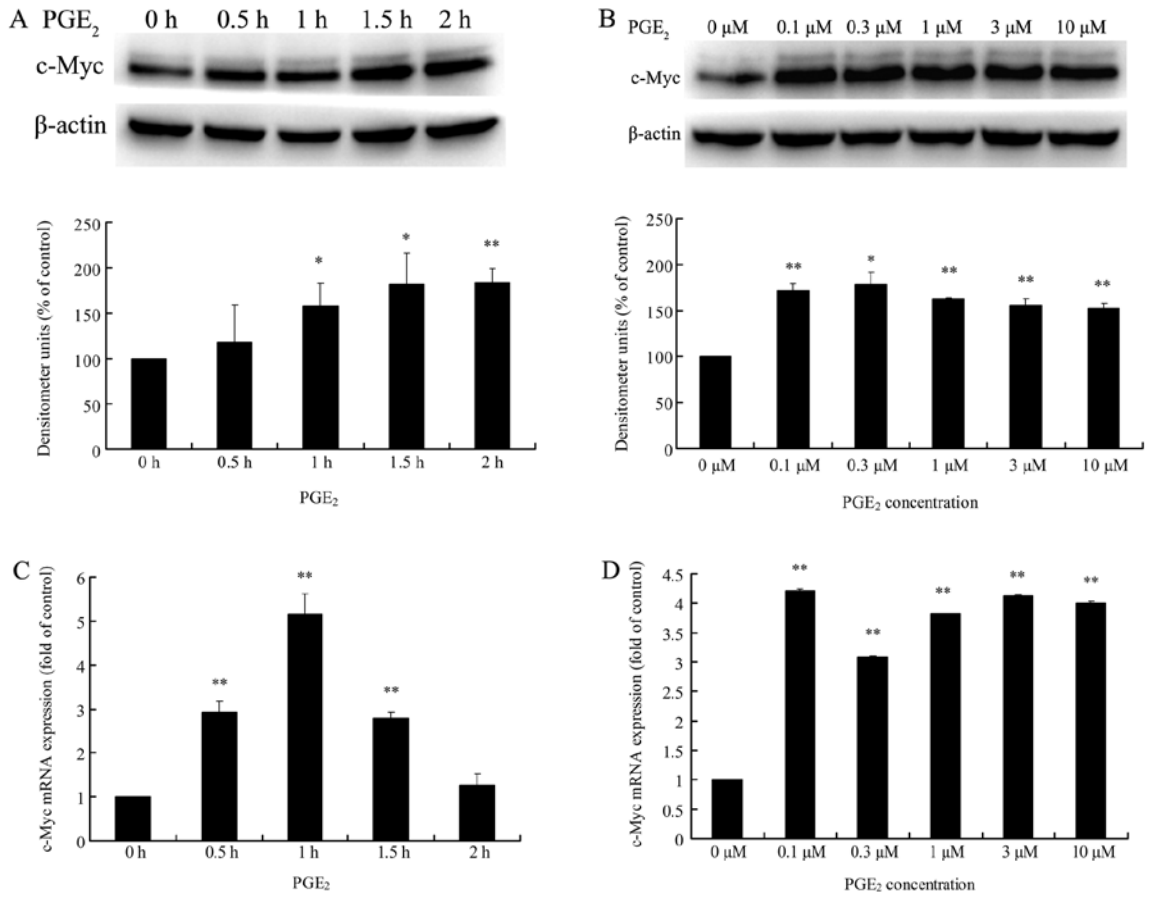

Figure 1. PGE_2 upregulates c-Myc expression at the mRNA and protein levels in Huh-7 cells. (A) Time course of the effects of PGE_2 on c-Myc protein expression. Huh-7 cells were treated with 3 μM PGE_2 for the indicated times, and protein expression of c-Myc was determined by western blotting. (B) Dose effect of PGE_2 on c-Myc protein expression. Huh-7 cells were incubated with PGE_2 at the indicated doses for 2 h, and protein expression of c-Myc was determined by western blotting. Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. (C) Time course of the effects of PGE_2 on c-Myc mRNA expression. Huh-7 cells were treated with 3 μM PGE_2 for the indicated times, and mRNA expression of c-Myc was examined by real-time PCR. (D) Dose effect of PGE_2 on c-Myc mRNA expression. Huh-7 cells were incubated with PGE_2 at the indicated doses for 1 h, and mRNA expression of c-Myc was examined by real-time PCR. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control. PGE_2, prostaglandin E_2.

The siRNA reagents specific to c-Myc (no. 6341) and CREB (no. 6588) were from Cell Signaling Technology. Huh-7 cells ($2x10^5$) were plated in 6-well plates for 24 h, resulting in a 30-50% confluent cell monolayer. The cells were then transfected with the targeting siRNA or the negative control siRNA (N.C. siRNA) from GenePharma (Shanghai, China) using Lipofectamine 2000. After transfection, depletion of target protein was confirmed by western blotting or real-time PCR analysis, and the cells were subsequently used for further experiments.

cAMP assay. Intracellular cAMP levels were measured using an enzyme immunoassay kit. Briefly, Huh-7 cells were cultured in 35-mm dishes until they reached 80% confluency and were then treated with PGE_1 alcohol or vehicle at 37˚C for different times. The cells were harvested in 0.1 M HCl solution, and incubated for 20 min at room temperature. After centrifugation at 1,000 x g for 10 min, the supernatant (50 μl) was analyzed for cAMP content according to the manufacturer's instructions.

Statistical analysis. Data are presented as means ± SD. P-values were calculated using the Student's t-test for unpaired samples with MS Excel software. The results were considered significantly different at P<0.05.

Results

PGE_2 upregulates c-Myc mRNA and protein expression in HCC cells. To determine the direct effect of PGE_2 on c-Myc expression in HCC cells, Huh-7 cells were treated with PGE_2 at various doses or for different times, and then the levels of mRNA and protein expression of c-Myc were analyzed by real-time PCR and western blotting, respectively. Results from the western blotting experiments showed that 3 μM PGE_2 treatment significantly increased c-Myc protein expression from 0.5 to 2 h, and at 2 h it reached a maximum value which was 183% fold of the value at 0 h (Fig. 1A). Moreover, the expression levels of c-Myc protein were all upregulated by 2 h PGE_2 treatment with a dose from 0.1 to 10 μM (Fig. 1B). On the other hand, the mRNA level of c-Myc was obviously elevated by 3 μM PGE_2 from 0.5 h, and it peaked at 1 h which was 5.16-fold of that at 0 h and then declined to the basal level at 2 h (Fig. 1C). In addition, treatment of cells with 0.1 to 10 μM PGE_2 for 1 h markedly increased the mRNA level of

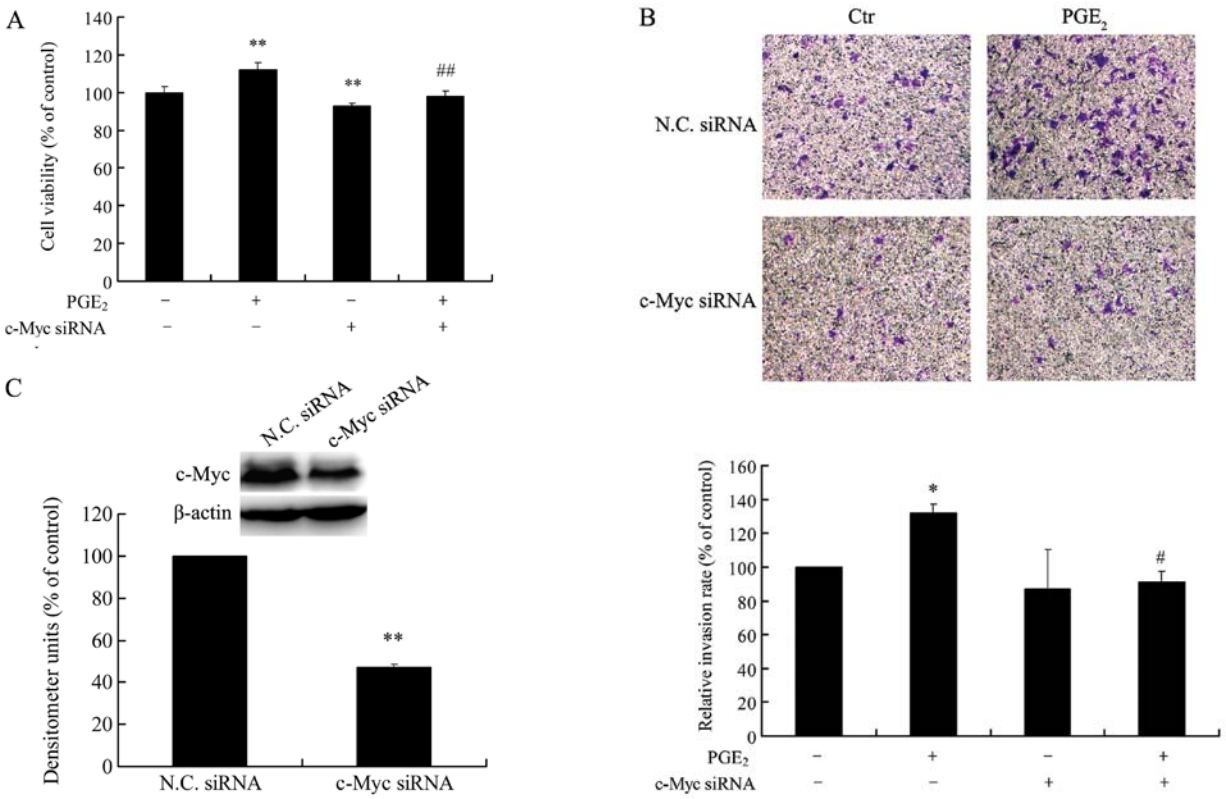

Figure 2. Knockdown of c-Myc blocks PGE_2-induced HCC cell growth and invasion. (A) Knockdown of c-Myc blocked PGE_2-induced HCC cell growth. Huh-7 cells were transfected with c-Myc siRNA or N.C. siRNA (siRNA concentration 80 nM) for 24 h and then stimulated with PGE_2 (10 μM) in serum-free medium for 24 h, and cell proliferation was measured using the CCK-8 kit. (B) Knockdown of c-Myc blocked PGE_2-induced HCC cell invasion. Huh-7 cells were transfected with c-Myc siRNA or N.C. siRNA (siRNA concentration 80 nM), and 24 h post-transfection cell suspensions were prepared. Serum-free medium (100 μl) containing $5x10^4$ cells were seeded in the upper chamber in the presence or absence of 10 μM PGE_2, and regular medium containing 10% FCS was added to the lower chamber. The cell invasion assay was conducted after 24 h. Representative images of invaded cells from the different groups are shown in the upper panel. Quantitative analysis of invaded cells is shown in the lower panel. (C) RNAi efficiency of c-Myc siRNA in Huh-7 cells. After transfection of Huh-7 cells with c-Myc siRNA or N.C. siRNA (siRNA concentration 80 nM) for 24 h, the protein expression of c-Myc was determined by western blotting. Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. Results are presented as the means ± SD of three independent experiments. $^*P<0.05$, $^{**}P<0.01$ compared with the control; $^{\#}P<0.05$; $^{\#\#}P<0.01$ compared with PGE_2 treatment. PGE_2, prostaglandin E_2; N.C., negative control.

c-Myc in the Huh-7 cells (Fig. 1D). These data indicate that PGE_2 upregulates c-Myc expression at the mRNA and protein levels in the HCC cells.

Knockdown of c-Myc blocks PGE_2-induced HCC cell growth and invasion. Our previous results demonstrated that PGE_2 promotes cell growth and invasion in HCC cells (10-13). Now, we revealed that PGE_2 directly upregulated c-Myc protein expression in the HCC cells, a key transcription factor with cancer-promoting effects. Thus, the role of c-Myc upregulation in PGE_2-induced HCC cell growth and invasion need to be further investigated. As shown in Fig. 2A, c-Myc siRNA significantly lowered the basal proliferation and blocked PGE_2-induced proliferation in the HCC Huh-7 cells. In addition, c-Myc siRNA showed a similar inhibitory effect on PGE_2-induced HCC cell invasion (Fig. 2B). Depletion of c-Myc protein by siRNA transfection was confirmed by western blot analysis (Fig. 2C). These results showed that the upregulation of c-Myc protein plays an important role in PGE_2-induced HCC cell growth and invasion.

EP4 receptor is involved in PGE_2-induced c-Myc expression in HCC cells. EP4 receptor is closely associated with cancer cell growth and invasion in various human cancers (19-23). Thus, we investigated whether the EP4 receptor is also involved in PGE_2-induced c-Myc expression in HCC cells. As shown in Fig. 3A and B, treatment of Huh-7 cells with PGE_1 alcohol, the EP4 receptor selective agonist, significantly increased the expression level of c-Myc protein in a time- and dose-dependent manner. In addition, the mRNA expression level of c-Myc was also upregulated by PGE_1 alcohol in Huh-7 cells in a dose-dependent manner (Fig. 3C and D). These results suggest a key role of the EP4 receptor in the regulation of c-Myc expression in HCC cells.

To further confirm the involvement of the EP4 receptor in PGE_2-induced c-Myc expression, we examined the effects of an EP4 receptor selective antagonist or EP4 receptor siRNA on PGE_2-induced c-Myc expression in HCC cells. As shown in Fig. 4A and B, pretreatment of Huh-7 cells with GW627368X, the EP4 receptor selective antagonist, markedly reduced PGE_2- or PGE_1 alcohol-induced c-Myc expression at the protein and mRNA levels. In addition, EP4 receptor siRNA also blocked the upregulation of c-Myc protein expression by PGE_2 or PGE_1 alcohol in the Huh-7 cells (Fig. 4C). The interference efficacy of EP4 receptor siRNA was verified by real-time PCR analysis, showing that EP4 siRNA significantly lowered the mRNA

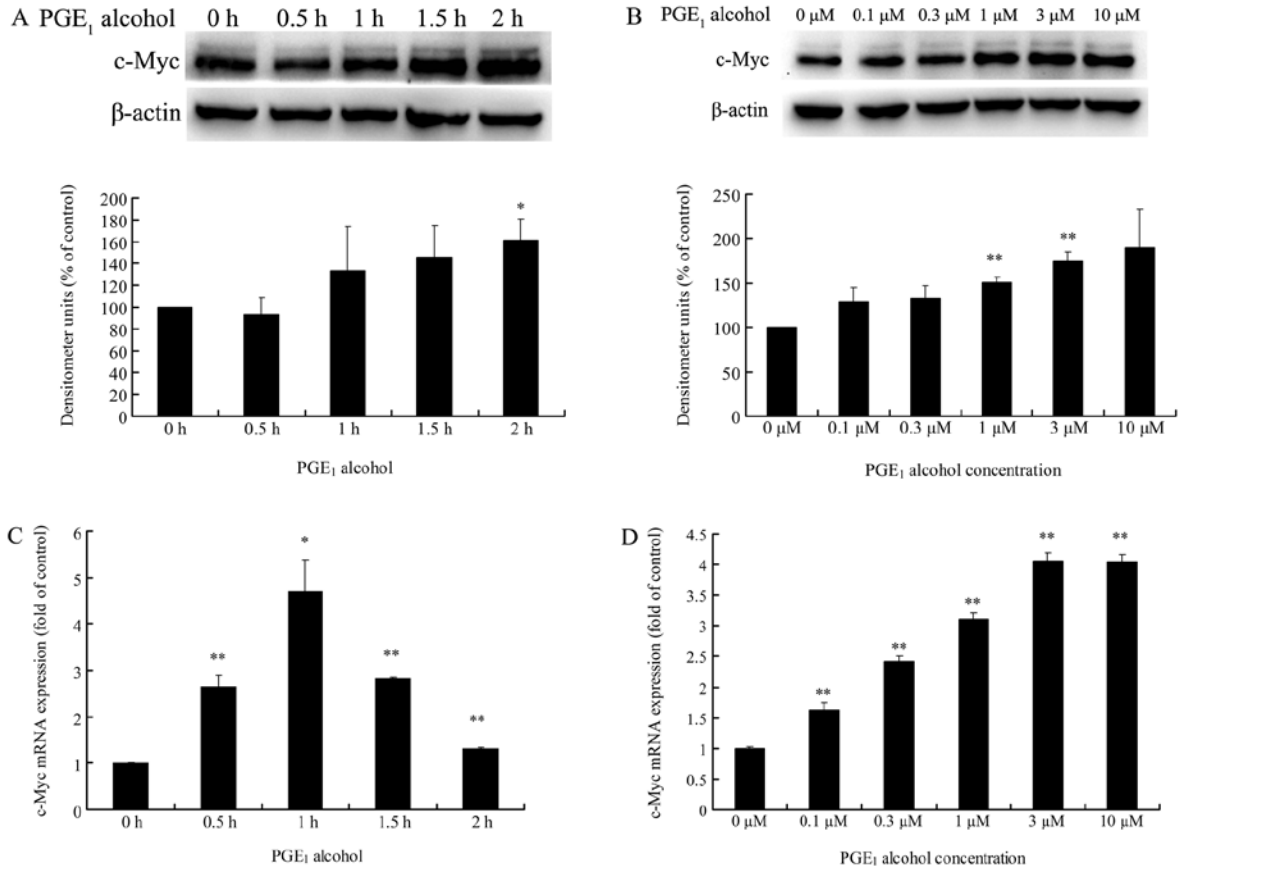

Figure 3. The EP4 receptor selective agonist PGE_1 alcohol mimics the upregulatory effect of PGE_2 on c-Myc expression in Huh-7 cells. (A and B) Time effect and dose effect of PGE_1 alcohol on c-Myc protein expression. Huh-7 cells were treated with PGE_1 alcohol (3 μM) for the indicated times (A) or PGE_1 alcohol at the indicated doses for 2 h (B), and protein expression of c-Myc was determined by western blotting. Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. (C and D) Time effect and dose effect of PGE_1 alcohol on c-Myc mRNA expression. Huh-7 cells were treated with PGE_1 alcohol (3 μM) for the indicated times (C) or PGE_1 alcohol at the indicated doses for 1 h (D), and mRNA expression of c-Myc was examined by real-time PCR. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control. PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2.

expression of the EP4 receptor in the Huh-7 cells (Fig. 4D). These observations indicate that the EP4 receptor is involved in PGE_2-induced c-Myc expression in HCC cells.

EP4 receptor-mediated c-Myc protein upregulation depends on de novo biosynthesis of c-Myc mRNA and its protein in HCC cells. As a key protein in cells, c-Myc expression is tightly regulated at multiple levels, including transcription, post-transcription and post-translation (25,26). To elucidate the mechanisms of EP4 receptor-mediated c-Myc protein upregulation, Act D and CHX, inhibitors of *de novo* RNA synthesis and *de novo* protein synthesis, respectively, were used. As shown in Fig. 5A, pretreatment with Act D or CHX significantly reduced EP4 receptor-mediated c-Myc protein upregulation in the Huh-7 cells, suggesting a crucial role of *de novo* biosynthesis of c-Myc mRNA and its protein in EP4 receptor-mediated c-Myc protein upregulation. The pharmacological effects of Act D or CHX on the mRNA level of c-Myc were further examined by real-time PCR experiment, and the results showed that Act D markedly lowered the basal and EP4 receptor-mediated c-Myc mRNA expression, while CHX, the inhibitor of *de novo* protein synthesis, had no such effect (Fig. 5B). These findings indicate that EP4 receptor-mediated c-Myc protein upregulation greatly depends on the *de novo* biosynthesis of c-Myc mRNA and its protein in HCC cells.

The G_S/AC/cAMP signaling pathway is involved in EP4 receptor-mediated c-Myc upregulation in HCC cells. The EP4 receptor, a G protein coupled receptor, is usually coupled with G_S protein to activate adenylate cyclase (AC) and elevate intracellular cAMP levels (5,18). To confirm the downstream signaling pathway of the EP4 receptor in HCC cells, we measured the direct effect of the EP4 receptor agonist on the intracellular cAMP level in Huh-7 cells. As shown in Fig. 6, stimulation of cells with PGE_1 alcohol notably increased the intracellular cAMP level in a time-dependent manner, and at around 30-45 min the cAMP level reached a maximum value which was ~15-fold of the control. By contrast, the vehicle DMSO had no effect on the cAMP level. These data demonstrated that the EP4 receptor is coupled to the G_S/AC/cAMP signaling pathway in HCC cells.

To determine the role of the G_S/AC/cAMP signaling pathway in EP4 receptor-mediated c-Myc upregulation, Huh-7 cells were treated with a specific activator or inhibitor of AC, and then their effects on c-Myc expression in HCC cells were examined. As shown in Fig. 7A and B, treatment of Huh-7 cells with forskolin, the specific activator of AC, resulted in

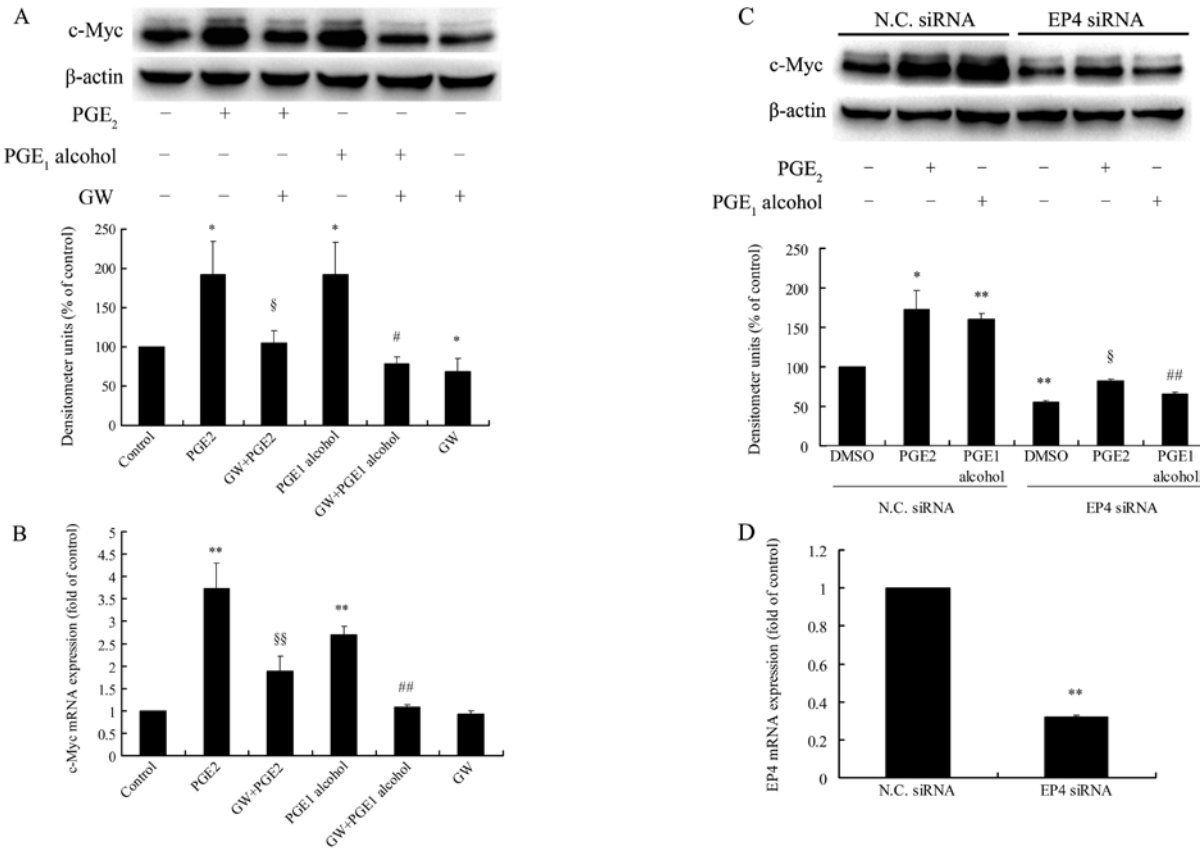

Figure 4. EP4 receptor antagonism or siRNA interference attenuates PGE_2-induced c-Myc expression in Huh-7 cells. (A and B) Effect of the EP4 receptor selective antagonist GW627368X on PGE_2-induced c-Myc expression. Huh-7 cells were pretreated for 1 h with GW627368X (4 μM) followed by stimulation with PGE_2 (3 μM) or PGE_1 alcohol (3 μM), and protein expression of c-Myc was determined by western blotting after 2 h (A) and mRNA expression of c-Myc was examined by real-time PCR after 1 h (B). (C) Effect of EP4 receptor siRNA on PGE_2-induced c-Myc protein expression. Huh-7 cells were transfected with EP4 siRNA or N.C. siRNA for 72 h and then stimulated with PGE_2 (3 μM) or PGE_1 alcohol (3 μM) in serum-free medium for 2 h, and protein expression of c-Myc was determined by western blotting. Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. (D) RNAi efficiency of EP4 siRNA in Huh-7 cells. After transfection of Huh-7 cells with EP4 siRNA or N.C. siRNA for 72 h, the mRNA expression of the EP4 receptor was examined by real-time PCR. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control; §P<0.05, §§P<0.01 compared with the PGE_2 treatment; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the PGE_1 alcohol treatment. GW, GW627368X. PGE_2, prostaglandin E_2; PGE_1, prostaglandin E_1; N.C., negative control.

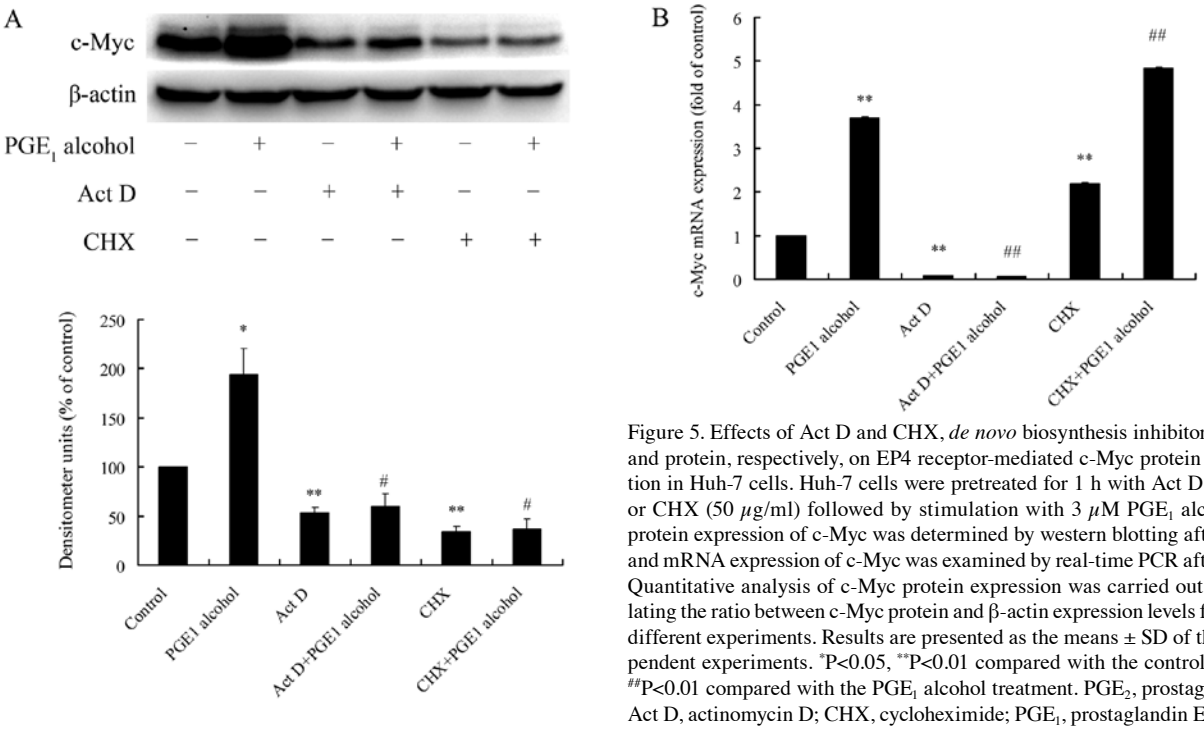

Figure 5. Effects of Act D and CHX, *de novo* biosynthesis inhibitors of RNA and protein, respectively, on EP4 receptor-mediated c-Myc protein upregulation in Huh-7 cells. Huh-7 cells were pretreated for 1 h with Act D (5 μg/ml) or CHX (50 μg/ml) followed by stimulation with 3 μM PGE_1 alcohol, and protein expression of c-Myc was determined by western blotting after 2 h (A) and mRNA expression of c-Myc was examined by real-time PCR after 1 h (B). Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the PGE_1 alcohol treatment. PGE_2, prostaglandin E_2. Act D, actinomycin D; CHX, cycloheximide; PGE_1, prostaglandin E_1.

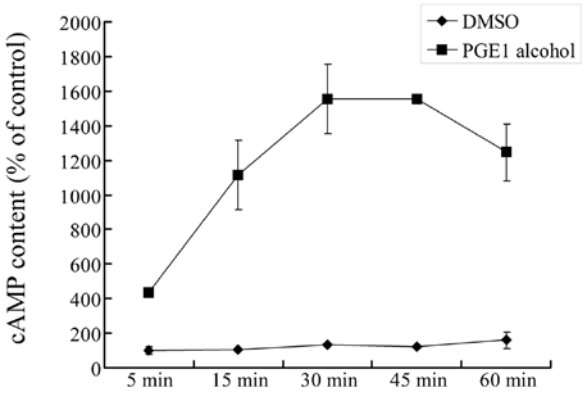

Figure 6. EP4 receptor agonist PGE_1 alcohol elevates the intracellular cAMP level in Huh-7 cells. Huh-7 cells were incubated with PGE_1 alcohol (3 μM) or the vehicle DMSO for the indicated times, and the intracellular cAMP level was measured by EIA assay. Results are presented as the means ± SD of three independent experiments. PGE_1, prostaglandin E_1.

a dose-dependent increase in c-Myc expression at the protein and mRNA levels. Pretreatment of cells with SQ22536, the specific inhibitor of AC, markedly reduced EP4 receptor-mediated c-Myc upregulation at the protein level and partly at the mRNA level (Fig. 7C and D). These results revealed that the G_S/AC/cAMP signaling pathway is involved in EP4 receptor-mediated c-Myc upregulation in HCC cells.

Involvement of the PKA/CREB pathway in EP4 receptor-mediated c-Myc upregulation in HCC cells. An elevated intracellular cAMP level leads to protein kinase A (PKA) activation, while activated PKA could transfer into the cell nucleus and phosphorylate transcription factor CREB protein, thus regulating gene expression (30). Firstly, we investigated the involvement of PKA activation in EP4 receptor-mediated c-Myc upregulation in HCC cells by using the PKA-specific inhibitor H89. As shown in Fig. 8A and B, pretreatment of Huh-7 cells with H89 significantly reduced EP4 receptor-mediated c-Myc upregulation at the protein and mRNA levels, suggesting an important role of PKA in EP4 receptor-mediated c-Myc upregulation. Secondly, the role of CREB in EP4 receptor-mediated c-Myc upregulation was also examined. Stimulation of Huh-7 cells with the EP4 receptor agonist PGE_1 alcohol led to a significant increase in the phosphorylation of CREB at Ser133 (Fig. 9A), a crucial event for transcriptional activation by CREB. Meanwhile, the CREB siRNA markedly

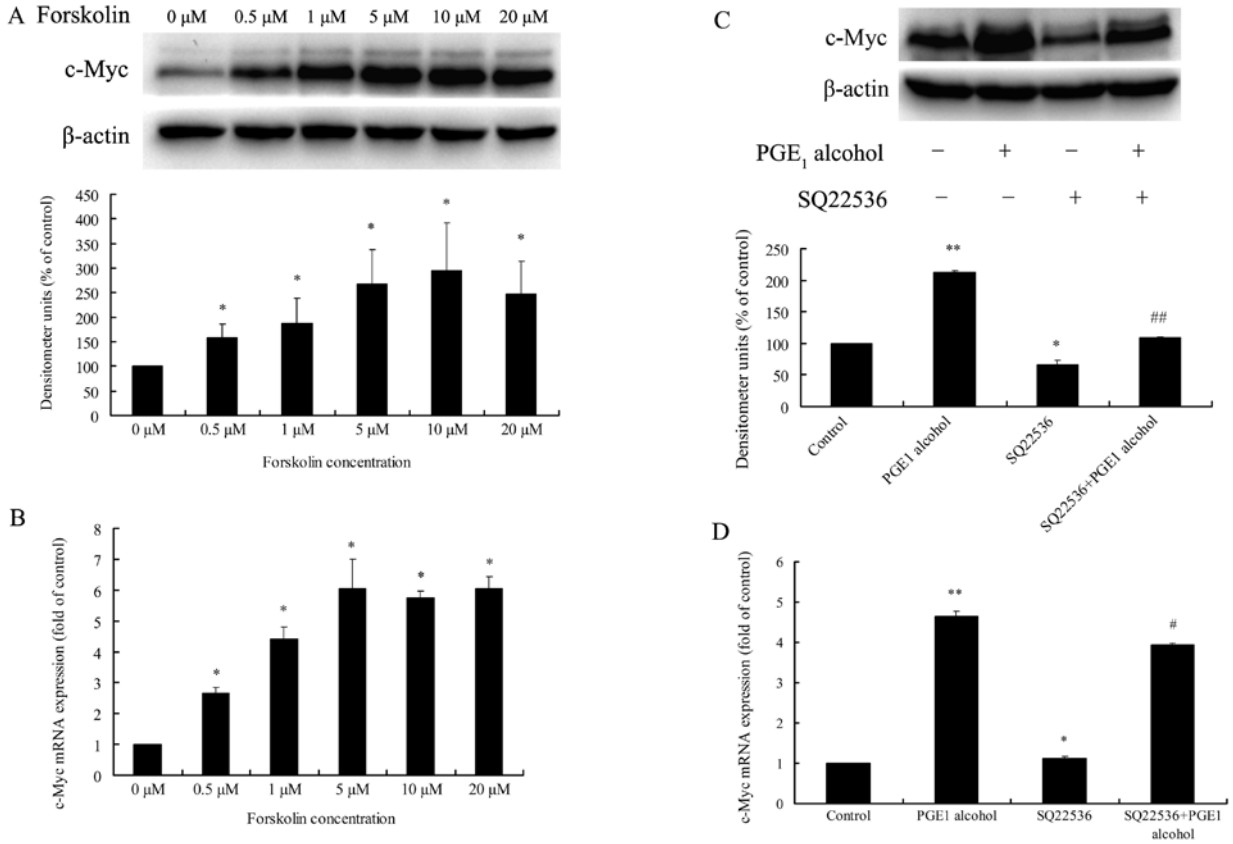

Figure 7. Involvement of the G_S/AC/cAMP signaling pathway in EP4 receptor-mediated c-Myc upregulation in Huh-7 cells. (A and B) The specific AC activator forskolin induced c-Myc expression at the mRNA and protein levels. Huh-7 cells were treated with forskolin at the indicated doses, and protein expression of c-Myc was determined after 2 h by western blotting (A) and mRNA expression of c-Myc was examined after 1 h by real-time PCR (B). (C and D) The specific AC inhibitor SQ22536 reduced EP4 receptor-mediated c-Myc upregulation. Huh-7 cells were pretreated for 1 h with SQ22536 (200 μM) followed by stimulation with 3 μM PGE_1 alcohol, and protein expression of c-Myc was determined after 2 h (C) and mRNA expression of c-Myc was examined after 1 h (D). Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the PGE_1 alcohol treatment. AC, adenylate cyclase; PGE_1, prostaglandin E_1.

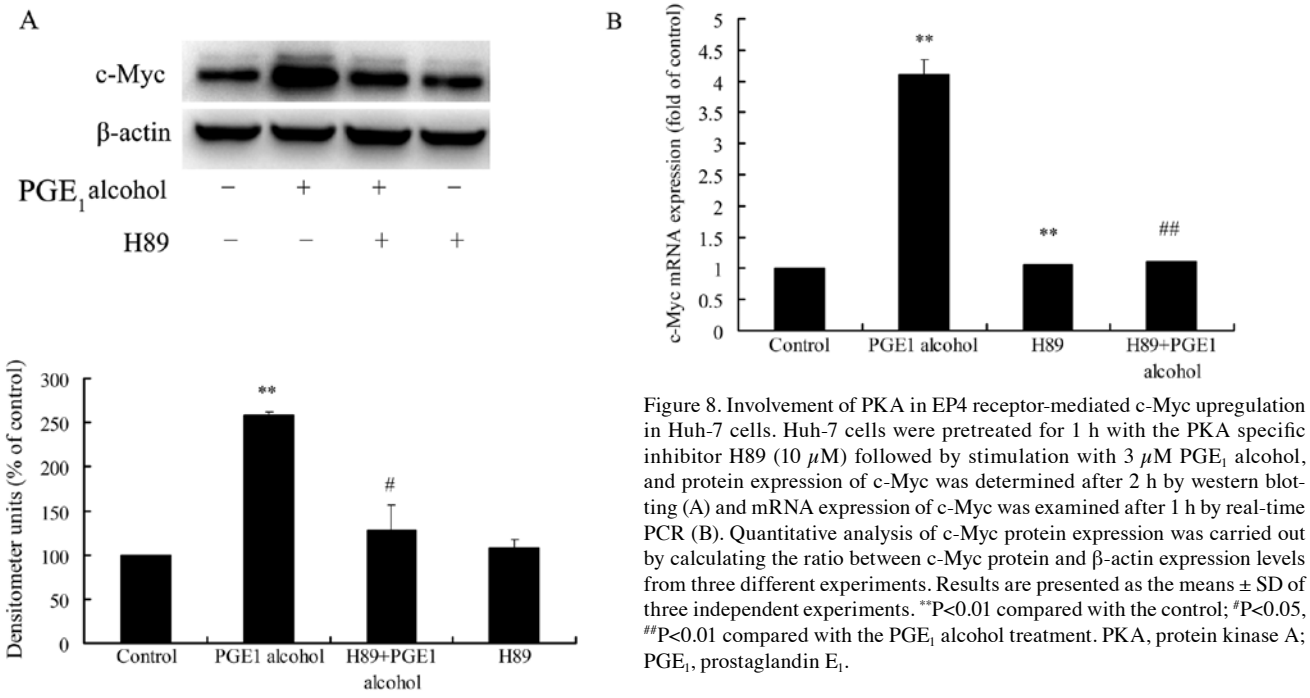

Figure 8. Involvement of PKA in EP4 receptor-mediated c-Myc upregulation in Huh-7 cells. Huh-7 cells were pretreated for 1 h with the PKA specific inhibitor H89 (10 μM) followed by stimulation with 3 μM PGE_1 alcohol, and protein expression of c-Myc was determined after 2 h by western blotting (A) and mRNA expression of c-Myc was examined after 1 h by real-time PCR (B). Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. Results are presented as the means ± SD of three independent experiments. **P<0.01 compared with the control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 compared with the PGE_1 alcohol treatment. PKA, protein kinase A; PGE_1, prostaglandin E_1.

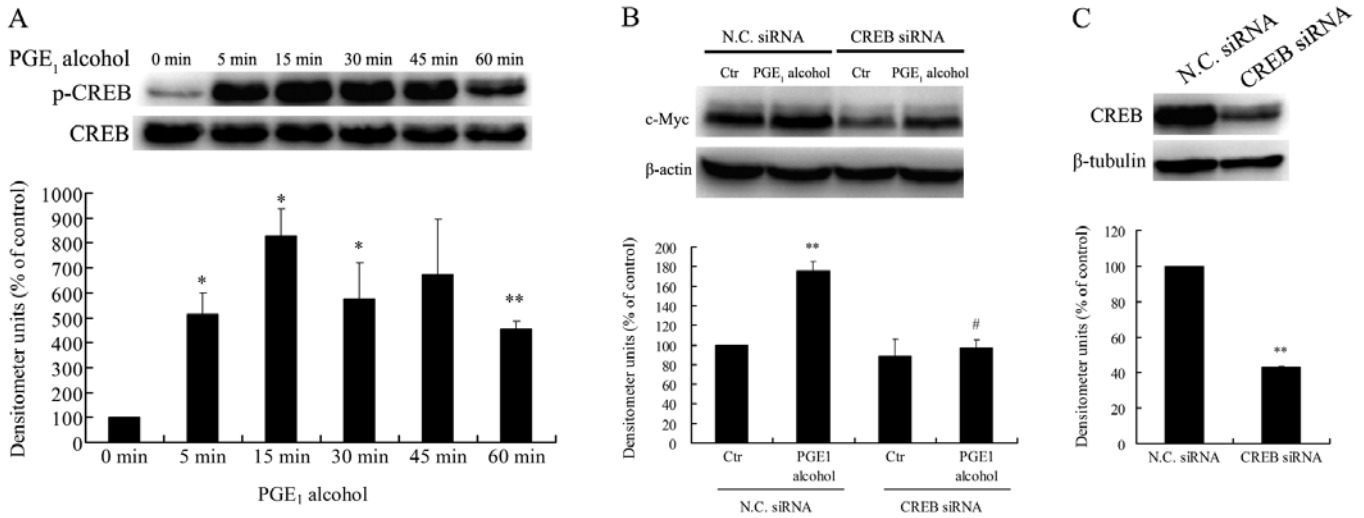

Figure 9. Involvement of CREB in EP4 receptor-mediated c-Myc upregulation in Huh-7 cells. (A) CREB phosphorylation at Ser133 induced by PGE_1 alcohol. Huh-7 cells were treated with PGE_1 alcohol (3 μM) for indicated times, and the phosphorylated CREB and total CREB protein expression were determined by western blotting. Quantitative analysis of CREB phosphorylation was carried out by calculating the ratio between the CREB protein and phosphorylation levels from three different experiments. (B) CREB siRNA reduced EP4 receptor-mediated c-Myc protein upregulation. Huh-7 cells were transfected with CREB siRNA or N.C. siRNA (siRNA concentration 80 nM) for 48 h and then stimulated with PGE_1 alcohol (3 μM) in serum-free medium for 2 h, and protein expression of c-Myc was determined by western blotting. Quantitative analysis of c-Myc protein expression was carried out by calculating the ratio between c-Myc protein and β-actin expression levels from three different experiments. (C) RNAi efficiency of CREB siRNA in Huh-7 cells. After transfection of Huh-7 cells with CREB siRNA or N.C. siRNA (siRNA concentration 80 nM) for 48 h, the protein expression of CREB was determined by western blotting. Quantitative analysis of CREB protein expression was carried out by calculating the ratio between CREB protein and β-tubulin expression levels from three different experiments. Results are presented as the means ± SD of three independent experiments. *P<0.05, **P<0.01 compared with the control; $^{\#}$P<0.05 compared with the PGE_1 alcohol treatment. PGE_1, prostaglandin E_1; N.C., negative control.

reduced EP4 receptor-mediated c-Myc protein upregulation in Huh-7 cells (Fig. 9B). The downregulation of CREB protein by siRNA transfection in the Huh-7 cells was confirmed by western blotting (Fig. 9C). Based on these findings, the PKA/CREB pathway is also involved in EP4 receptor-mediated c-Myc upregulation in HCC cells.

Discussion

Hepatocellular carcinoma (HCC) represents a major health problem worldwide. To date, our understanding of the molecular mechanisms of this disease remains rudimentary. A large body of studies support a pivotal role of chronic inflammation

in the pathogenesis of HCC (2-4). Yet, the underlying mechanisms are not well understood.

PGE_2, an inflammatory mediator and the predominant product of COX-2, has been shown to be involved in various human cancers, including HCC. Our previous results revealed that PGE_2 significantly enhanced the cell growth, migration and invasion in HCC cells (10-13). Although several signaling pathways have been identified such as transactivation of EGFR receptor (31), phosphorylation of FAK kinase (12,13), the detailed mechanisms of PGE_2 in HCC remain to be further studied.

c-Myc protein encoded by the proto-oncogene *c-myc* functions as a transcription factor. After dimerizing with its partner protein Max, c-Myc binds to E box sequence elements (5'-CACGTG-3') to activate the transcription of many target genes, and thus promotes cell proliferation and tumorigenesis (26). In addition, substantial studies confirmed that *c-myc* is involved in human hepatocarcinogenesis. Approximately 30% of human HCC samples exhibit gene amplification of *c-myc* (27,28), and the major etiological factors of HCC including hepatitis C or B virus infection, and aflatoxin could induce overexpression of c-Myc protein (32-34). However, the exact mechanisms of *c-myc* activation in HCC are largely unknown.

Since PGE_2 and c-Myc are both involved in HCC, and have the potential for causing tumorigenesis, the internal relationship between PGE_2 and c-Myc in HCC is of particular interest to us. In the present study, we found that PGE_2 directly upregulated c-Myc expression at the mRNA and protein levels, and knockdown of c-Myc protein greatly suppressed PGE_2-induced HCC cell growth and invasion in Huh-7 cells. These findings firmly confirm that c-Myc is a critical regulator in PGE_2-induced HCC cell growth and invasion, suggesting a probable molecular mechanism for inflammation-induced hepatocarcinogenesis.

PGE_2 exerts biological effects through four types of EP receptors on the cell surface membrane, designated as EP1, EP2, EP3 and EP4 (5,18). Studies indicate that the EP4 receptor plays a crucial role in PGE_2-mediated tumorigenesis in many types of cancer. For example, PGE_2 promotes renal cancer cell invasion via the EP4 receptor and small GTPase Rap signaling (19). In colon cancer cells, PGE_2 induces S100p expression to enhance cancer cell growth and migration via the EP4 receptor signaling (20). In lung cancer cells, PGE_2 promotes cell migration via EP4-β Arrestin1-c-Src signalsome (21). Likewise, we found that PGE_2 induced c-Myc expression to promote cell growth and invasion in HCC cells mainly through the EP4 receptor.

Usually, c-Myc expression is tightly controlled by a number of mechanisms at the transcriptional, post-transcriptional and post-translational levels. Many transcription factors such as TCF, FBP, CNBP, NF-κB and AP-1 can bind to the promoter of the human *c-myc* gene, and upregulate its expression at the transcriptional level (25,41). In addition, the c-Myc protein itself can be post-translational modified at multiple sites by phosphorylation or ubiquitination. Two key phosphorylation sites of Ser62 and Thr58 located within the N terminus are important for the regulation of c-Myc protein stabilization, yet have opposite function. Ser62 phosphorylation stabilizes c-Myc protein, while T58 phosphorylation leads to its protein degradation (35). In human esophageal squamous cell carcinoma cells, PGE_2 was shown to upregulate c-Myc protein expression by stimulating Ser62 phosphorylation and then stabilizing its protein at the post-translational level (36). However, the present study revealed that in HCC cells the EP4 receptor-mediated c-Myc protein upregulation largely depended on *de novo* biosynthesis of c-Myc mRNA and its protein.

As a G protein coupled receptor, the EP4 receptor is believed to be coupled with G_S protein to activate AC and elevate intracellular cAMP levels (5,18). Our results confirmed that the EP4 receptor activated the G_S/AC/cAMP signaling pathway in HCC cells. In addition, we demonstrated that this canonical pathway is involved in EP4 receptor-mediated c-Myc upregulation in HCC cells. This finding is similar to a study in human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs), which showed that PGE_2 upregulated the expression of c-Myc and VEGF via EP2/cAMP signaling and thus promoted cell proliferation of hUCB-MSCs (37).

In cells, an elevated cAMP level subsequently activates three main targets including protein kinase A (PKA), the exchange protein activated by cAMP (Epac) and the cyclic-nucleotide-gated ion channels (CNGCs) (30). Among them, PKA has been shown to regulate many aspects of cell functions, such as metabolism, signal transduction and gene expression (38). The effect of PKA on gene transcription is mainly achieved by direct phosphorylation of the transcription factor CREB at the site of Ser133 following stimulation of gene transcription by activated CREB (39,40). By using a specific inhibitor and siRNA, we demonstrated that the signaling pathway of PKA/CREB is also involved in the EP4 receptor-mediated c-Myc upregulation in HCC cells.

CREB, one member of the bZIP (basic domain/leucine zipper) transcription factor family, can activate numerous target genes including c-fos and JunD through cAMP response elements (CREs). Full CRE is an 8 bp palindrome (5'-TGACGTCA-3'), and the half motif (CGTCA) is also active for CREB binding and cAMP responsiveness. It is reported that of 105 genes with functional CREs identified in the literature, approximately half contain a full palindrome, with the other half containing a single CGTCA motif (40). In the present study, we found that CREB is involved in the upregulation of c-Myc expression in HCC cells. Yet, there are no data or study which shows that the promoter of the human *c-myc* gene contains a or more full CRE (41). Based on sequence analysis, we found that two half CGTCA motifs locate in the upstream of transcription start site (TSS) of the human *c-myc* gene, at -3,005 and -3,759 bp, respectively. Therefore, we speculated that CREB upregulates the expression of *c-myc* by following possible mechanisms: by binding to these two half CGTCA motifs; indirect action, by stimulation of the expression of other transcription factors such as AP-1 and then activation of c-Myc expression or other unknown mechanisms. Thus, the exact mechanisms involved in c-Myc upregulation by CREB need to be further investigated.

In summary, the present study revealed that PGE_2 directly upregulated c-Myc expression via the EP4R/G_S/AC/cAMP/PKA/CREB signaling pathway to promote cell growth and invasion in human HCC cells. This finding provides a further insight into the mechanisms by which PGE_2 enhances HCC

cell growth and invasion. Targeting of the PGE_2/EP4R/c-Myc pathway may be a new therapeutic strategy to prevent and cure human HCC.

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Nakagawa H and Maeda S: Inflammation- and stress-related signaling pathways in hepatocarcinogenesis. World J Gastroenterol 18: 4071-4081, 2012.
3. Ramakrishna G, Rastogi A, Trehanpati N, Sen B, Khosla R and Sarin SK: From cirrhosis to hepatocellular carcinoma: new molecular insights on inflammation and cellular senescence. Liver Cancer 2: 367-383, 2013.
4. Berasain C, Castillo J, Perugorria MJ, Latasa MU, Prieto J and Avila MA: Inflammation and liver cancer: new molecular links. Ann NY Acad Sci 1155: 206-221, 2009.
5. Hata AN and Breyer RM: Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 103: 147-166, 2004.
6. Wang D and Dubois RN: Prostaglandins and cancer. Gut 55: 115-122, 2006.
7. Legler DF, Bruckner M, Uetz-von Allmen E and Krause P: Prostaglandin E_2 at new glance: novel insights in functional diversity offer therapeutic chances. Int J Biochem Cell Biol 42: 198-201, 2010.
8. Rizzo MT: Cyclooxygenase-2 in oncogenesis. Clin Chim Acta 412: 671-687, 2011.
9. Mayoral R, Fernández-Martínez A, Boscá L and Martín-Sanz P: Prostaglandin E_2 promotes migration and adhesion in hepatocellular carcinoma cells. Carcinogenesis 26: 753-761, 2005.
10. Leng J, Han C, Demetris AJ, Michalopoulos GK and Wu T: Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis. Hepatology 38: 756-768, 2003.
11. Ma J, Chen M, Xia SK, *et al*: Prostaglandin E_2 promotes liver cancer cell growth by the upregulation of FUSE-binding protein 1 expression. Int J Oncol 42: 1093-1104, 2013.
12. Bai XM, Zhang W, Liu NB, *et al*: Focal adhesion kinase: Important to prostaglandin E_2-mediated adhesion, migration and invasion in hepatocellular carcinoma cells. Oncol Rep 21: 129-136, 2009.
13. Bai X, Wang J, Zhang L, *et al*: Prostaglandin E_2 receptor EP1-mediated phosphorylation of focal adhesion kinase enhances cell adhesion and migration in hepatocellular carcinoma cells. Int J Oncol 42: 1833-1841, 2013.
14. Reader J, Holt D and Fulton A: Prostaglandin E_2 EP receptors as therapeutic targets in breast cancer. Cancer Metastasis Rev 30: 449-463, 2011.
15. Timoshenko AV, Xu G, Chakrabarti S, Lala PK and Chakraborty C: Role of prostaglandin E_2 receptors in migration of murine and human breast cancer cells. Exp Cell Res 289: 265-274, 2003.
16. Wu WK, Sung JJ, Lee CW, Yu J and Cho CH: Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. Cancer Lett 295: 7-16, 2010.
17. Jain S, Chakraborty G, Raja R, Kale S and Kundu GC: Prostaglandin E_2 regulates tumor angiogenesis in prostate cancer. Cancer Res 68: 7750-7759, 2008.
18. Sugimoto Y and Narumiya S: Prostaglandin E receptors. J Biol Chem 282: 11613-11617, 2007.
19. Wu J, Zhang Y, Frilot N, Kim JI, Kim WJ and Daaka Y: Prostaglandin E_2 regulates renal cell carcinoma invasion through the EP4 receptor-Rap GTPase signal transduction pathway. J Biol Chem 286: 33954-33962, 2011.
20. Chandramouli A, Mercado-Pimentel ME, Hutchinson A, *et al*: The induction of S100p expression by the Prostaglandin E_2 (PGE_2)/EP4 receptor signaling pathway in colon cancer cells. Cancer Biol Ther 10: 1056-1066, 2010.
21. Kim JI, Lakshmikanthan V, Frilot N and Daaka Y: Prostaglandin E_2 promotes lung cancer cell migration via EP4-βArrestin1-c-Src signalsome. Mol Cancer Res 8: 569-577, 2010.
22. Oshima H, Popivanova BK, Oguma K, Kong D, Ishikawa TO and Oshima M: Activation of epidermal growth factor receptor signaling by the prostaglandin E_2 receptor EP4 pathway during gastric tumorigenesis. Cancer Sci 102: 713-719, 2011.
23. Robertson FM, Simeone AM, Mazumdar A, *et al*: Molecular and pharmacological blockade of the EP4 receptor selectively inhibits both proliferation and invasion of human inflammatory breast cancer cells. J Exp Ther Oncol 7: 299-312, 2008.
24. Zender L, Villanueva A, Tovar V, Sia D, Chiang DY and Llovet JM: Cancer gene discovery in hepatocellular carcinoma. J Hepatol 52: 921-929, 2010.
25. Levens D: You don't muck with MYC. Genes Cancer 1: 547-554, 2010.
26. Dang CV: *MYC* on the path to cancer. Cell 149: 22-35, 2012.
27. Kawate S, Fukusato T, Ohwada S, Watanuki A and Morishita Y: Amplification of c-*myc* in hepatocellular carcinoma: correlation with clinicopathologic features, proliferative activity and p53 overexpression. Oncology 57: 157-163, 1999.
28. Chan KL, Guan XY and Ng IO: High-throughput tissue microarray analysis of c-*myc* activation in chronic liver diseases and hepatocellular carcinoma. Hum Pathol 35: 1324-1331, 2004.
29. Kaposi-Novak P, Libbrecht L, Woo HG, *et al*: Central role of c-Myc during malignant conversion in human hepatocarcinogenesis. Cancer Res 69: 2775-2782, 2009.
30. Fimia GM and Sassone-Corsi P: Cyclic AMP signalling. J Cell Sci 114: 1971-1972, 2001.
31. Han C, Michalopoulos GK and Wu T: Prostaglandin E_2 receptor EP_1 transactivates EGFR/MET receptor tyrosine kinases and enhances invasiveness in human hepatocellular carcinoma cells. J Cell Physiol 207: 261-270, 2006.
32. Higgs MR, Lerat H and Pawlotsky JM: Hepatitis C virus-induced activation of β-catenin promotes c-Myc expression and a cascade of pro-carcinogenetic events. Oncogene 32: 4683-4693, 2013.
33. Iizuka N, Tsunedomi R, Tamesa T, *et al*: Involvement of c-myc-regulated genes in hepatocellular carcinoma related to genotype-C hepatitis B virus. J Cancer Res Clin Oncol 132: 473-481, 2006.
34. Tashiro F, Morimura S, Hayashi K, *et al*: Expression of the c-Ha-ras and c-myc genes in aflatoxin B1-induced hepatocellular carcinomas. Biochem Biophys Res Commun 138: 858-864, 1986.
35. Junttila MR and Westermarck J: Mechanisms of MYC stabilization in human malignancies. Cell Cycle 7: 592-596, 2008.
36. Yu L, Wu WK, Li ZJ, Li HT, Wu YC and Cho CH: Prostaglandin E_2 promotes cell proliferation *via* protein kinase C/extracellular signal regulated kinase pathway-dependent induction of c-Myc expression in human esophageal squamous cell carcinoma cells. Int J Cancer 125: 2540-2546, 2009.
37. Jang MW, Yun SP, Park JH, Ryu JM, Lee JH and Han HJ: Cooperation of Epac1/Rap1/Akt and PKA in prostaglandin E_2-induced proliferation of human umbilical cord blood derived mesenchymal stem cells: involvement of c-Myc and VEGF expression. J Cell Physiol 227: 3756-3767, 2012.
38. Cheng X, Ji Z, Tsalkova T and Mei F: Epac and PKA: a tale of two intracellular cAMP receptors. Acta Biochim Biophys Sin 40: 651-662, 2008.
39. Sands WA and Palmer TM: Regulating gene transcription in response to cyclic AMP elevation. Cell Signal 20: 460-466, 2008.
40. Mayr B and Montminy M: Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2: 599-609, 2001.
41. Wierstra I and Alves J: The *c-myc* promoter: still MysterY and challenge. Adv Cancer Res 99: 113-333, 2008.