Prostaglandin E₂ promotes the cell growth and invasive ability of hepatocellular carcinoma cells by upregulating c-Myc expression via EP4 receptor and the PKA signaling pathway

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Abstract. Hepatocellular carcinoma (HCC) represents a major health problem worldwide. Prostaglandin E₂ (PGE₂), 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 PGE₂ and c-Myc in HCC, and to define its role in HCC cell growth and invasion. Our results showed that PGE₂ significantly upregulated c-Myc expression at both the mRNA and protein levels, and knockdown of c-Myc blocked PGE₂-induced HCC cell growth and invasive ability in human HCC Huh-7 cells. The effect of PGE₂ 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 Gₛ/AC and increased the intracellular cAMP level in Huh-7 cells. The adeny late 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 Gₛ/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 PGE₂ significantly upregulates c-Myc expression at both mRNA and protein levels through the EP4R/Gₛ/AC/cAMP/PKA/CREB signaling pathway, thus promoting cell growth and invasion in HCC cells. Targeting of the PGE₂/EP4R/c-Myc pathway may be a new therapeutic strategy to prevent and cure human HCC.

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 E₂ (PGE₂), 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 PGE₂ is also associated with several serious human diseases, including malignant tumors (6,7). PGE₂ 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). PGE₂ 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 PGE2 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 PGE2 may promote the cell growth and invasion of HCC cells through upregulation of c-Myc protein expression. Data from the present study revealed that PGE2 significantly upregulated the expression of c-Myc at the mRNA and protein levels both via the EP4 receptor and the coupled Gz/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). PGE1, PGE2, alcohol, GW627368X and the cyclic AMP EIA kit were from Cayman Chemical Co. (Ann Arbor, MI, USA). SQ22556, 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 (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 (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China). Anti-β-actin mouse monoclonal antibody (BM0627) was from Boster (Dalian, China).

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'-AGGCTATTCTGTGCCCCAT TT-3' (forward) and 5'-TCGATAGTGTAGTCATGCTTT-3' (reverse); EP4, 5'-CATCTTACTGTTGATGTTT-3' (forward) and 5'-TACTGAGCTGTCTTCTTC-3' (reverse); GAPDH, 5'-TTCAGAGCGAGATCCCC-3' (forward), and 5'-CACC CATGACGAACATGGG-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.
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.

**Results**

**PGE₂ upregulates c-Myc mRNA and protein expression in HCC cells.** To determine the direct effect of PGE₂ on c-Myc expression in HCC cells, Huh-7 cells were treated with PGE₂ 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 PGE₂ 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 PGE₂ treatment with a dose from 0.1 to 10 µM PGE₂ for the indicated times, and mRNA expression of c-Myc was examined by real-time PCR. Huh-7 cells were incubated with PGE₂ 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. PGE₂, prostaglandin E₂.

**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 PGE₂ 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.

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**Figure 1.** PGE₂ upregulates c-Myc expression at the mRNA and protein levels in Huh-7 cells. (A) Time course of the effects of PGE₂ on c-Myc protein expression. Huh-7 cells were treated with 3 µM PGE₂ for the indicated times, and protein expression of c-Myc was determined by western blotting. (B) Dose effect of PGE₂ on c-Myc protein expression. Huh-7 cells were incubated with PGE₂ 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 PGE₂ on c-Myc mRNA expression. Huh-7 cells were treated with 3 µM PGE₂ for the indicated times, and mRNA expression of c-Myc was examined by real-time PCR. (D) Dose effect of PGE₂ on c-Myc mRNA expression. Huh-7 cells were incubated with PGE₂ 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. PGE₂, prostaglandin E₂.
c-Myc in the Huh-7 cells (Fig. 1D). These data indicate that PGE₂ upregulates c-Myc expression at the mRNA and protein levels in the HCC cells.

**Knockdown of c-Myc blocks PGE₂-induced HCC cell growth and invasion.** Our previous results demonstrated that PGE₂ promotes cell growth and invasion in HCC cells (10-13). Now, we revealed that PGE₂ 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 PGE₂-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 PGE₂-induced proliferation in the HCC Huh-7 cells. In addition, c-Myc siRNA showed a similar inhibitory effect on PGE₂-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 PGE₂-induced HCC cell growth and invasion.

**EP4 receptor is involved in PGE₂-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 PGE₂-induced c-Myc expression in HCC cells. As shown in Fig. 3A and B, treatment of Huh-7 cells with PGE₁ 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 PGE₁ 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 PGE₂-induced c-Myc expression, we examined the effects of an EP4 receptor selective antagonist or EP4 receptor siRNA on PGE₂-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 PGE₂- or PGE₁ 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 PGE₂ or PGE₁ 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 expression of c-Myc.
expression of the EP4 receptor in the Huh-7 cells (Fig. 4D). These observations indicate that the EP4 receptor is involved in PGE2-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 Gs/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 Gi 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 PGE1 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 Gs/AC/cAMP signaling pathway in HCC cells.

To determine the role of the Gs/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 PGE2-induced c-Myc expression in Huh-7 cells. (A and B) Effect of the EP4 receptor selective antagonist GW627368X on PGE2-induced c-Myc expression. Huh-7 cells were pretreated for 1 h with GW627368X (4 µM) followed by stimulation with PGE2 (3 µM) or PGE1 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 PGE2-induced c-Myc protein expression. Huh-7 cells were transfected with EP4 siRNA or N.C. siRNA for 72 h and then stimulated with PGE2 (3 µM) or PGE1 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 PGE2 treatment; #P<0.05, ##P<0.01 compared with the PGE1 alcohol treatment. GW, GW627368X. PGE2, prostaglandin E2; PGE1, prostaglandin E1; 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 PGE2, 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 PGE2 treatment; ††P<0.05, ‡‡P<0.01 compared with the PGE1 alcohol treatment. Act D, actinomycin D; CHX, cycloheximide; PGE2, prostaglandin E2; PGE1, prostaglandin E1; N.C., negative control.
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 Gs/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 PGE1 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...
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.

PGE₂, 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 PGE₂ 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 PGE₂ 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 PGE₂ and c-Myc are both involved in HCC, and have the potential for causing tumorigenesis, the internal relationship between PGE₂ and c-Myc in HCC is of particular interest to us. In the present study, we found that PGE₂ directly upregulated c-Myc expression at the mRNA and protein levels, and knockdown of c-Myc protein greatly suppressed PGE₂-induced HCC cell growth and invasion in HuH-7 cells. These findings firmly confirm that c-Myc is a critical regulator in PGE₂-induced HCC cell growth and invasion, suggesting a probable molecular mechanism for inflammation-induced hepatocarcinogenesis.

PGE₂ 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 PGE₂-mediated tumorigenesis in many types of cancer. For example, PGE₂ promotes renal cancer cell invasion via the EP4 receptor and small GTPase Rap signaling (19). In colon cancer cells, PGE₂ induces S100p expression to enhance cancer cell growth and migration via the EP4 receptor signaling (20). In lung cancer cells, PGE₂ promotes cell migration via EP4-β Arrestin1-c-Src signaling (21). Likewise, we found that PGE₂ 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, PGE₂ 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 Gβγ protein to activate AC and elevate intracellular cAMP levels (5,18). Our results confirmed that the EP4 receptor activated the Gβγ/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 PGE₂ 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 palindromic (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 palindromic, 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 PGE₂ directly upregulated c-Myc expression via the EP4/Gβγ/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 PGE₂ enhances HCC
cell growth and invasion. Targeting of the PGE2/EP4/c-Myc pathway may be a new therapeutic strategy to prevent and cure human HCC.

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