

Prostaglandin E2 promotes human cholangiocarcinoma cell proliferation, migration and invasion through the upregulation of β -catenin expression via EP3-4 receptor

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Abstract. Prostaglandin E2 (PGE2) is involved in cholangiocarcinoma cell proliferation, migration and invasion through E prostanoid receptors, including EP1, EP2 and EP4. However, the functions and the mechanisms of those splice variants of EP3 receptors in promoting liver cancer cell growth and invasion remain to be elucidated. In our previous studies, four isoforms of EP3 receptors, EP3-4, EP3-5, EP3-6 and EP3-7 receptors, were detected in CCLP1 and HuCCT1 cells. However, the functions of these receptors in these cells have yet to be determined. It was reported that β -catenin is closely correlated with malignancy, including cholangiocarcinoma. The present study was designed to examine the effects of 4-7 isoforms of EP3 in promoting cholangiocarcinoma progression and the mechanisms by which PGE2 increases β -catenin protein via EP3 receptors. The results showed that PGE2 promotes cholangiocarcinoma progression via the upregulation of β -catenin protein, and the EP3-4 receptor pathway is mainly responsible for this regulation. These findings reveal that PGE2 upregulated the cholangiocarcinoma cell β -catenin protein through the EP3-4R/Src/EGFR/PI3K/AKT/GSK-3 β pathway. The present study identified the functions of EP3 and the mechanisms by which PGE2 regulates β -catenin expression and promoted cholangiocarcinoma cell growth and invasion.

Introduction

Cholangiocarcinoma is a common primary hepatobiliary malignancy that often arises from longstanding bile duct inflammation, injury, and reparative biliary epithelial cell proliferation (1,2). Prostaglandin E2 (PGE2), a key product of cyclooxygenase-2, participates in the development of

inflammatory reactions and in oncogenesis (3,4). PGE2 was able to promote tumor cell proliferation, migration, invasion and anti-apoptosis. In previous studies, PGE2 significantly promoted proliferation and invasion by upregulating the expression of survivin (5), focal adhesion kinase (4), Snail (6,7), YB-1 (8), c-Myc (9) and β 1-integrin (10) in hepatocellular carcinoma cells, and by upregulating the expression of MMP2 (11) and the FUSE-binding protein 1 (12) in cholangiocarcinoma cells. It was also reported that, PGE2 promoted cell proliferation and invasion in breast (13), colon (14), ovarian (15) cancer and several other types of tumors.

The activity of PGE2 is mediated by four E prostanoid receptors (EPs), known as EP1R, EP2R, EP3R and EP4R. These constitute the G protein-coupled receptors (GPCRs). Although previous studies have examined the relationship between tumor growth, invasion and EP1R, EP2R, EP4R but few studies have focused on the role EP3R. The EP3 receptor is more complicated (12,16). EP3R mRNA has several splice variants that generate multiple isoforms. Five isoforms of the human EP3 receptors: EP3-4, EP3-5, EP3-6, EP3-7 and EP3-8 have been identified. Evidence of different signal transduction pathways and the regulation of gene expression among different EP receptors has also been demonstrated in a number of studies (17). Thus, the specific target of PGE2 in regulating cancer cell proliferation via EP receptors have yet to be clarified.

It was reported that β -catenin is highly correlated with cancer progression and poor prognosis. For example, the expression level of β -catenin has been shown to correlate with progression in colorectal (18), cervical (19) and prostate cancer (20). The activation of β -catenin was also involved in various stages of hepatic carcinoma (21-24) and cholangiocarcinoma (25,26). β -catenin leads to the activation of target genes such as c-Myc (27), Snail (28) and cyclin D1 (29). Although β -catenin has been regarded as a useful biomarker of cancer progression, the detailed mechanisms for its tumor-promoting function remain to be elucidated.

In a previous study, EP3-4R, EP3-5R, EP3-6R and EP3-7R were observed in human CCLP1 and HuCCT1 cholangiocarcinoma cells (12). Overexpression of the stable transfection of EP3-4, EP3-5, EP3-6 and EP3-7 receptors to CCLP1, HuCCT1 and HEK 293T cells showed that the EP3-4 receptor is the most significant receptor in promoting the proliferation, migration

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and invasion of the cholangiocarcinoma cells. The aim of the present study was to assess the effects of the EP3R isoforms 4-7 on promoting cholangiocarcinoma cell growth and invasion and the signaling pathway whereby PGE2 upregulates β -catenin protein. Our results revealed that PGE2 upregulated β -catenin protein via the EP3-4 receptor and involved the Src EGFR, AKT and GSK-3 β pathway. Upregulated β -catenin eventually caused increased c-Myc and Snail expression.

Materials and methods

Antibodies and reagents. Human CCLP1 and HuCCT1 cholangiocarcinoma cell lines were obtained from the Department of Transplantation Pathology, University of Pittsburgh Medical Center (UPMC; Pittsburgh, PA, USA). HEK 293T cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, and LipofectamineTM 2000 were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). PGE2, EP3 receptor selective antagonist L-798106, EP3 receptor agonist sulprostone, anti-EP3 antibody were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Src inhibitor PP2 and G418 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phosphorylated EGFR (Tyr1173) and anti-GAPDH antibodies were purchased from SAB Signalway Antibody, (Nanjing, China). Anti-phosphorylated AKT (Ser473), anti-phosphorylated GSK-3 β (Ser9), anti-phosphorylated β -catenin (Ser33/37Thr41), anti-EGFR, anti-AKT, anti-GSK-3 β , anti- β -catenin, anti-Snail, anti-c-Myc antibodies, PI3K/AKT inhibitor LY294002, and EGFR inhibitor AG1478 were purchased from Cell Signaling Technology (Danvers, MA, USA). The protein assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Electrochemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). The Transwell unit was purchased from Costar Corning (Cambridge, MA, USA), while Matrigel was purchased from BD Biosciences, (Discovery Labware, Bedford, MA, USA). The cell proliferation assay reagent CCK-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Primer sequences were determined using the PrimeScriptTM RT reagent kit (Takara, Dalian, China). FastStart Essential DNA Green Master Mix was purchased from Roche Life Science (Indianapolis, IN, USA). pcDNA3.1, pcDNA-EP3-4, pcDNA-EP3-5, pcDNA-EP3-6 and pcDNA-EP3-7 plasmids were purchased from Jinsite Science and Technology Co. (Nanjing, China).

Cell culture. The cells were maintained at 37°C in a humidified 5% CO₂ incubator. CCLP1 and HEK 293T cells were cultured in DMEM and HuCCT1 cells in RPMI-1640 medium containing 10% fetal bovine serum (FBS).

Overexpression of EP3 receptor. The CCLP1, HuCCT1 and HEK 293T cells were exposed to a mixture of Lipofectamine 2000 and pcDNA-EP3/pcDNA3.1 control vector for 4 h. Following removal of the transfection mixtures, fresh medium with 10% fetal bovine serum was added. On the second day, the medium was changed, and the cells were incubated with medium containing G418 sulfate for 14 days. Subsequent cultures of selected cells were routinely grown in the presence

of selective pressure. Western blotting and quantitative PCR (qPCR) analyses were performed in the selected cells permanently transfected with EP3 receptors or control plasmids. The selected cells with the successful increase in EP3 proteins and mRNA expression were used for subsequent experiments.

Cell proliferation assay. Cell proliferation was determined using the CCK-8 kit. 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, 100 μ l of cell suspension was plated in each well of 96-well plates. After a 24-h culture to allow reattachment, the cells were incubated with sulprostone at the indicated concentrations (0, 0.1, 1, 5 and 10 μ M) and time periods. The cell proliferation reagent, WST-8 (10 μ l), 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. These experiments were repeated in six individual wells.

Cell migration assay. Cells in 100 μ l serum-free medium in the presence or absence of sulprostone were seeded in the upper chamber. Regular medium containing 10% FBS was added in the lower chamber as the chemoattractant. After incubation at 37°C for 12 h, the cells were fixed with ethanol and stained with 0.1% crystal violet for 30 min. After washing the cells with PBS, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The invading cells on the lower surface were solubilized with 300 μ l 10% acetic acid. The absorbance was measured at 570 nm. These experiments were repeated three times, and three wells were used for each treatment.

Cell invasion assay. The Matrigel was diluted with serum-free medium at the indicated concentrations and then 40 μ l of diluted Matrigel was added to the upper chamber, and incubated at 37°C for 4 h to solidify the gel. Cells in 100 μ l serum-free medium in the presence or absence of sulprostone were seeded in the upper chamber. Regular medium containing 10% FBS was added in the lower chamber as the chemoattractant. After 24 h of incubation at 37°C, the cells were fixed with ethanol and stained with 0.1% crystal violet for 30 min. After washing the cells with PBS, the cells on the upper surface of the filter were mechanically removed with a cotton swab. The invading cells on the lower surface were solubilized with 300 μ l 10% acetic acid. The absorbance was measured at 570 nm. These experiments were repeated three times, and three wells were used for each treatment.

Western blotting. At the end of each treatment, the cells were washed twice with ice-cold PBS and then sonicated on ice in a 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). Cell lysates were centrifuged at 12,000 \times g for 10 min at 4°C, and the supernatants were collected for western blotting. Protein concentration was measured using a protein assay. After boiling for 5 min in the loading buffer with 10% 2-mercaptoethanol, the samples containing 30 μ g protein were separated by SDS-PAGE and transferred onto nitrocellulose

membranes. The 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 agitation. The membranes were washed in TBST and incubated for 2 h with secondary antibodies at room temperature. The signals were detected using ECL reagent and analyzed using Image Lab 4.0 analysis software from Bio-Rad.

RNA isolation and qPCR. Total RNA from the cultured cells was isolated using the TRIzol reagent according to the manufacturer's instructions. Reverse transcription was carried out with PrimeScript™ RT reagent kit according to the standard protocol. The primer sequences used were: EP3-4R, 5'-TGC ATCCAGCTCCACCTCCT-3' (forward) and 5'-GCAAAT TCAGGGAAGCAGGAATTGC-3' (reverse); EP3-5R, 5'-TGT TCGCCTGGCTTCACTGAACC-3' (forward) and 5'-AGA GCAGCTGGAGACAGCATTTGC-3' (reverse); EP3-6R, 5'-TGTTCGCCTGGCTTCACTGAACC-3' (forward) and 5'-TGTGATCCTGGCAGAAAGGCAGG-3' (reverse); EP3-7R, 5'-TGTGATCCTGGCAGAAAGGCAGG-3' (forward) and 5'-TTTGGGAGGTGGGTGTTTCTGTGA' (reverse); c-Myc, 5'-AGGCTATTCTGCCCATTT-3' (forward) and 5'-TCGTAGTCGAGGTCATAGTTC-3' (reverse); Snail, 5'-AAGGACCACCGCATCTCTAC-3' (forward) and 5'-CC AGCTCCTTGAAGCAGAAGA-3' (reverse); β -catenin, 5'-GC CAAGTGGGTGGTATAGAG-3' (forward) and 5'-TGGGAT GGTGGGTGTAAG-3' (reverse); GAPDH, 5'-TTCCAGGAG CGAGATCCCT-3' (forward) and 5'-CACCCATGACGAA CATGGG-3' (reverse). qPCR analysis was performed on a Roche LightCycler Nano instrument using FastStart Essential DNA Green Master Mix, and GAPDH was used as the endogenous control. The qPCR conditions were: pre-incubation at 95°C for 10 min (1 cycle) followed by denaturation of 40 cycles at 95°C for 20 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. Treatments and conditions were performed in triplicate to calculate the statistical significance.

Statistical analysis. Data are presented as the means \pm SD. Statistical analysis was performed with the Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Isoforms of EP3 receptors are overexpressed in cholangiocarcinoma and HEK 293T cell. The CCLP1, HuCCT1 and HEK 293T cells were transfected with EP3-4R, EP3-5R, EP3-6R, EP3-7R or the pcDNA control plasmids and then selected by G418. The qPCR analysis results showed that mRNAs of EP3-4R, EP3-5R, EP3-6R and EP3-7R were increased significantly in pcDNA-EP3R-transfected cells respectively (Fig. 1A). Western blot analysis results showed that EP3 receptors were overexpressed in the pcDNA-EP3R transfected cells (Fig. 1B). The selected cells with increased EP3 proteins and mRNAs were used for subsequent experiments.

EP3 receptor isoforms play a key role in the proliferation of cholangiocarcinoma cells. The EP3R or control plasmids pcDNA3.1 transfected CCLP1 cells were examined for their response to treatment with the EP3 agonist, sulprostone. After treatment with different concentrations of sulprostone

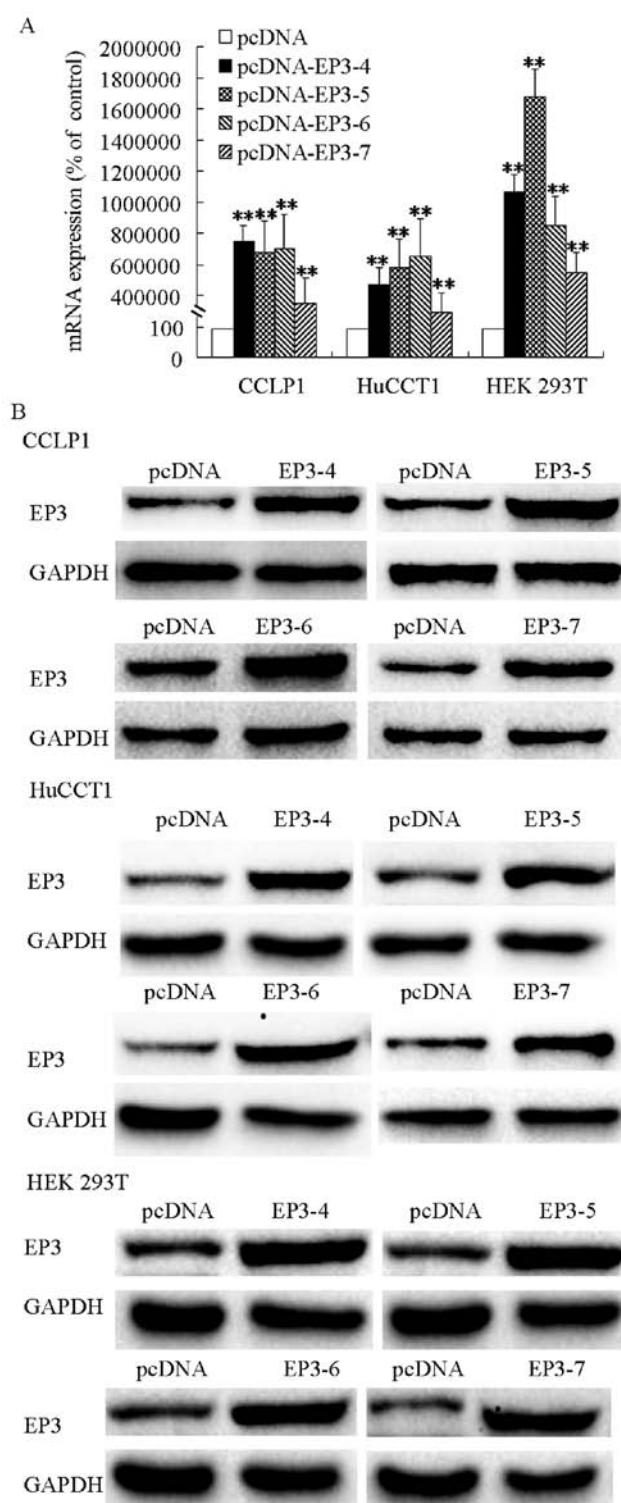


Figure 1. EP3 receptor isoforms mRNAs and proteins were overexpressed in pcDNA-EP3-transfected cells. (A) EP3 receptor isoforms mRNAs were overexpressed in CCLP1, HuCCT1 and HEK 293T cells. Cells were transfected with the EP3-4, EP3-5, EP3-6, EP3-7 or the pcDNA control plasmids. CCLP1 cells transfected with EP3R were selected by 500 μ g/ml G418. HuCCT1 cells were selected using 1,000 μ g/ml G418 and HEK 293T cells by 200 μ g/ml G418. The expression of the EP3R isoforms mRNAs were confirmed by qPCR analysis. Levels of GAPDH served as the control. (B) EP3 receptor isoforms proteins were overexpressed in CCLP1, HuCCT1 and HEK 293T cells. The cells were transfected with the EP3-4, EP3-5, EP3-6, EP3-7 or the pcDNA control plasmids. The expression of the EP3R isoforms proteins were determined by western blotting with anti-EP3R antibody, and GAPDH as the loading control with anti-GAPDH antibody. $^{**}P < 0.01$, compared with the control. Data are presented as the mean \pm SD of three independent experiments.

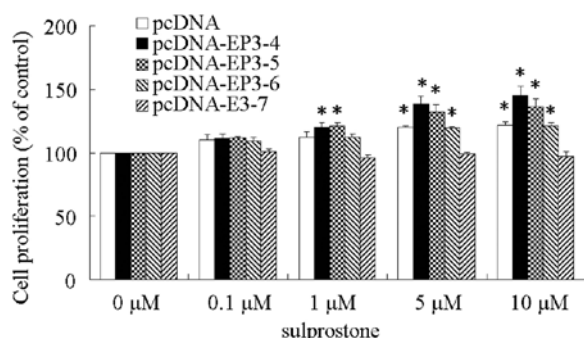


Figure 2. Effects of EP3 receptor isoforms on sulprostone induced CCLP1 cell proliferation. The EP3R- and pcDNA3.1-transfected CCLP1 cells (1×10^4) were seeded in 96-well plates in 100- μ l serum-supplemented medium for 24 h. The cells were treated with the indicated concentrations with EP3R agonist sulprostone for 24 h, and cell proliferation was assessed by using the cell proliferation reagent WST-8. * $P < 0.05$ compared with the control. The data were obtained from six individual experiments and are presented as the mean \pm SD as a percentage of the respective control.

for 24 h, the cell proliferation rate was detected by WST-8 analysis. The results showed that the proliferation of CCLP1 cells treated with sulprostone was increased compared with the control in pcDNA-, pcDNA-EP3-4-, pcDNA-EP3-5- and pcDNA-EP3-6-transfected cells. Of these, the effect of pcDNA-EP3-4-transfected cells was the most significant. pcDNA-EP3-7-transfected cells did not exert any effect on cell proliferation (Fig. 2). Similar results were observed in the HuCCT1 and HEK 293T cells (data not shown).

EP3 receptor isoforms mediate the migration of cholangiocarcinoma cells. The EP3Rs or control plasmids pcDNA3.1-transfected CCLP1 cells were examined to determine their response to treatment with sulprostone. Following treatment with 10 μ M sulprostone for 12 h, the cell migration rate was detected by Transwell analysis. The results showed that the migration of CCLP1 cells treated with sulprostone was increased compared with the control in pcDNA-,

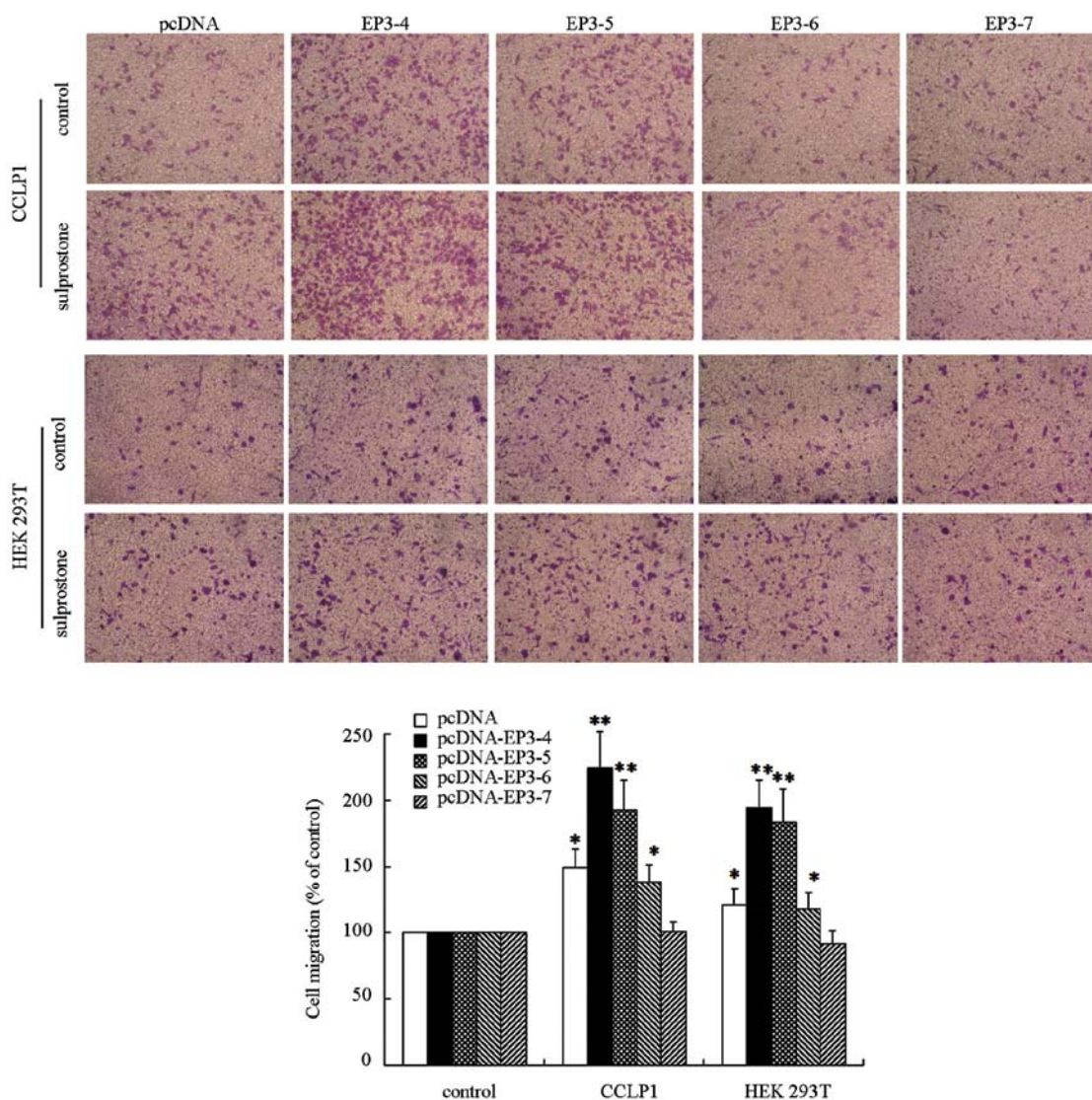


Figure 3. Effects of EP3 isoforms on sulprostone induced CCLP1 and HEK 293T cells migration. CCLP1 (5×10^4) and HEK 293T (1×10^5) cells transfected with pcDNA or EP3 receptor isoforms in 100 μ l serum-free medium were seeded in the upper chamber in the presence or absence of 10 μ M sulprostone, and the regular medium containing 10% FBS was added to the lower chamber. After 12 h, the cell migration assay was conducted. These experiments were repeated three times, and three wells were used for each treatment. Representative images of invaded cells from the independent groups are shown in the upper panels. * $P < 0.05$, ** $P < 0.01$ comparison with control.

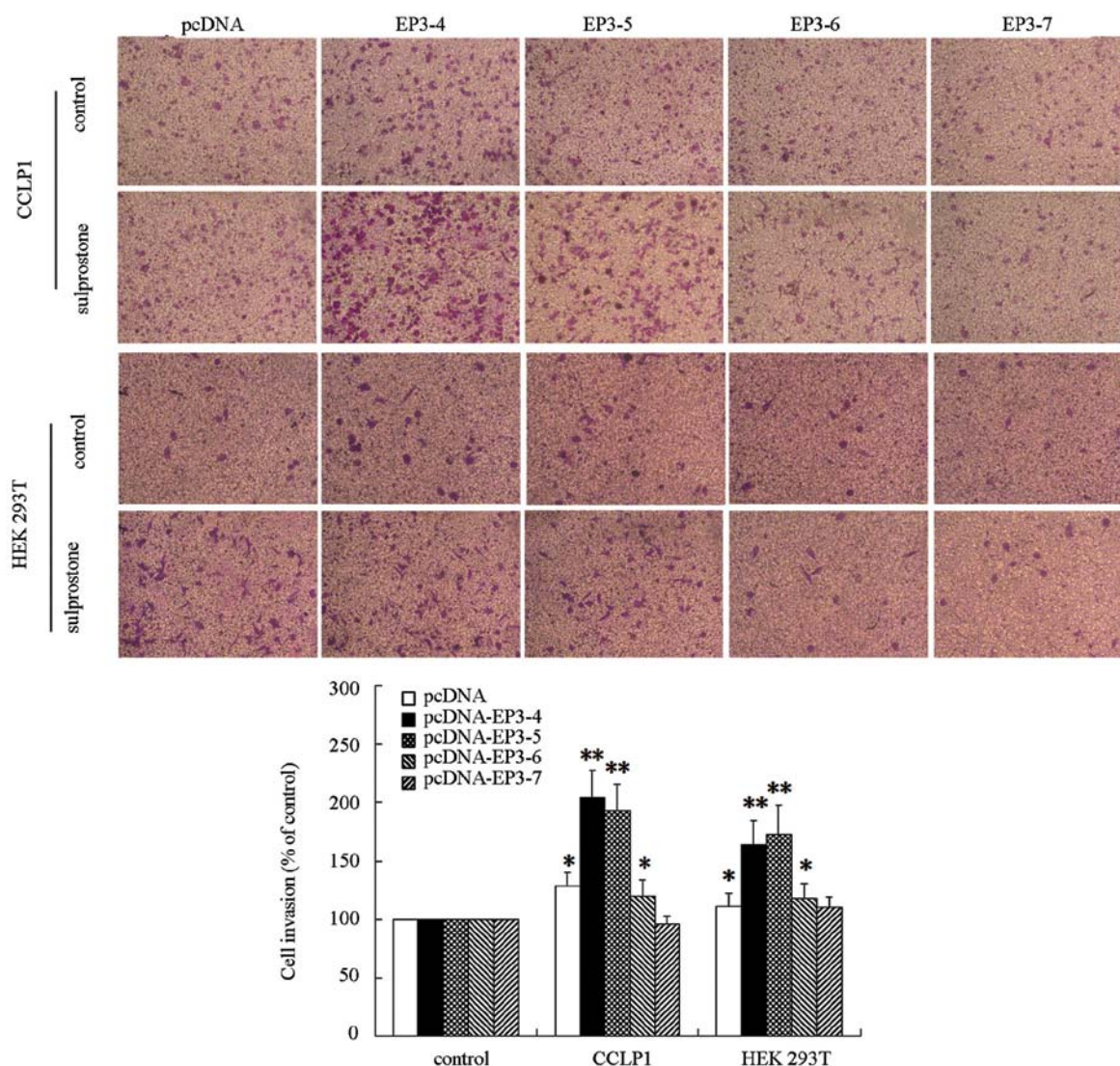


Figure 4. Effects of EP3 isoforms on sulprostone induced CCLP1 and HEK 293T cell invasion. The cell invasion assay was performed in Matrigel (1:6 for CCLP1 and 1:9 for HEK 293T)-coated Transwell chamber. CCLP1 (5×10^4) and HEK 293T (1×10^5) cells transfected with pcDNA or EP3 receptor isoforms in 100 μ l serum-free medium were seeded in the upper chamber in the presence or absence of 10 μ M sulprostone, and the regular medium containing 10% FBS was added to the lower chamber. After 24 h, a cell invasion assay was conducted. These experiments were repeated three times, and three wells were used for each treatment. Representative images of invaded cells from independent groups are shown in the upper panels. * $P < 0.05$, ** $P < 0.01$ comparison with the control.

pcDNA-EP3-4-, pcDNA-EP3-5- and pcDNA-EP3-6-transfected cells. Of these, the effect in pcDNA-EP3-4-transfected cells was the most significant. The pcDNA-EP3-7-transfected cells did not significantly affect the cell migration rate in the sulprostone treatment group compared with the control (Fig. 3). Similar results were observed in HuCCT1 (data not shown) and HEK 293T cells (Fig. 3).

EP3 receptor isoforms are responsible for invasion in cholangiocarcinoma cells. The EP3R or control plasmid pcDNA3.1-transfected CCLP1 cells were examined to determine their response to treatment with sulprostone. Following treatment with 10 μ M sulprostone for 24 h, the cell invasion rate was detected by Transwell analysis. The results showed that the invasion of CCLP1 cells treated with sulprostone was increased compared with the control in pcDNA-, pcDNA-EP3-4-, pcDNA-EP3-5- and pcDNA-EP3-6-transfected cells. Of these, the effect in pcDNA-EP3-4-transfected cells was

the most significant. The pcDNA-EP3-7-transfected cells did not significantly affect the cell invasion rate in the sulprostone treatment group compared with the control (Fig. 4). Similar results were observed in HuCCT1 (data not shown) and HEK 293T cells (Fig. 4).

EP3-4 receptor pathway is involved in upregulation of PGE2-induced β -catenin. PGE2 enhanced the malignancy of cancer cells (8). Additionally, β -catenin has been reported to correlated with malignancy of cancer (25). We hypothesized that PGE2 promoted cholangiocarcinoma progression through the upregulation of β -catenin protein level. To evaluate this hypothesis, we treated pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells with PGE2 with the indicated concentrations for 24 h. The results showed that treatment of pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells with PGE2 greatly increased the level of β -catenin protein and the effect on pcDNA-EP3-4-transfected CCLP1 cells was more significant

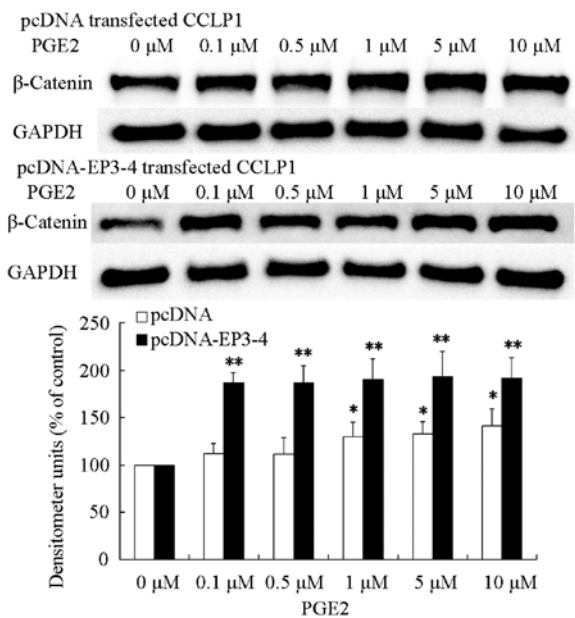


Figure 5. PGE2 upregulates β -catenin protein in CCLP1 cells. The pcDNA and pcDNA-EP3-4-transfected CCLP1 cells which cultured in serum-free medium for 24 h were treated with PGE2 at indicated concentration for 24 h. β -catenin protein was determined by western blotting with anti- β -catenin antibody, and GAPDH as the loading control with anti-GAPDH antibody. Quantitative analysis of β -catenin expression level was carried out by calculating the percentage of β -catenin protein and GAPDH levels from three different experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control. Data are presented as the mean \pm SD of three independent experiments.

than that in pcDNA-transfected CCLP1 cells (Fig. 5). These findings indicated that PGE2 was able to upregulate the β -catenin protein level in CCLP1 cells.

To investigate the role of EP3R isoforms in the upregulation of β -catenin, the protein was determined in pcDNA-, pcDNA-EP3-4-, pcDNA-EP3-5-, pcDNA-EP3-6- and pcDNA-EP3-7-transfected CCLP1 cells. As shown in Fig. 6A, β -catenin was significantly increased in pcDNA-EP3-4-transfected CCLP1 cells. Previous findings suggested that sulprostone promotes cholangiocarcinoma cell progression via the EP3-4 receptor, and PGE2 upregulated the β -catenin protein level. Thus, we hypothesized that PGE2 was able to upregulate β -catenin protein via the EP3-4 receptor. To evaluate this hypothesis, the pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells were treated with the indicated concentrations of sulprostone for 24 h. The results showed that treatment of pcDNA- or pcDNA-EP3-4-transfected CCLP1 cells with sulprostone greatly increased the level of β -catenin protein in a concentration-dependent manner (Fig. 6B). Pretreatment of pcDNA-EP3-4-transfected CCLP1 cells with EP3 selective antagonist L-798106 inhibited the upregulation of β -catenin induced by PGE2 (Fig. 6C). These results suggested that the EP3-4 receptor plays an important role in PGE2-induced β -catenin upregulation.

PGE2-associated β -catenin induces the upregulation of downstream c-Myc and Snail expression in cholangiocarcinoma cells. β -catenin was accumulated in the nucleus induced by PGE2 in cholangiocarcinoma cells (26). The accumulation of β -catenin in the nucleus induced transcription

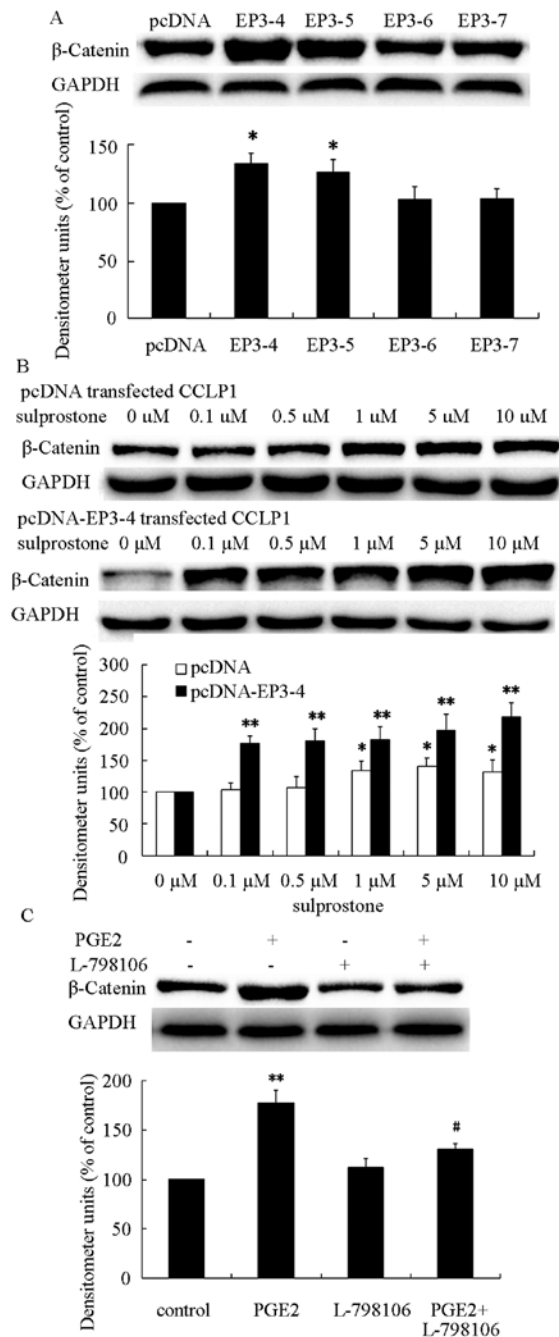


Figure 6. EP3-4 receptor is involved in PGE2 induced β -catenin expression. (A) β -catenin protein level in EP3Rs transfected CCLP1 cells. The pcDNA-, pcDNA-EP3-4-, pcDNA-EP3-5-, pcDNA-EP3-6- and pcDNA-EP3-7-transfected CCLP1 cells were cultured. β -catenin expression level was determined by western blotting with anti- β -catenin antibody. GAPDH as the loading control was determined with anti-GAPDH antibody. (B) Effects of sulprostone on β -catenin upregulation in CCLP1 cells. The pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at the indicated concentration for 24 h. β -catenin expression level was determined by western blotting with anti- β -catenin antibody. GAPDH as the loading control was determined with anti-GAPDH antibody. (C) Effects of EP3 receptor selective antagonist L-798106 on β -catenin expression induced by PGE2. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were pretreated with 10 μ M L-798106 for 1 h, and then treated with 10 μ M PGE2 for 24 h. β -catenin expression level was determined by western blotting with anti- β -catenin antibody, and GAPDH as the loading control with anti-GAPDH antibody. Quantitative analysis of the β -catenin expression level was carried out by calculating the percentage of β -catenin protein and GAPDH expression levels from three different experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control; # $P < 0.05$ compared with the PGE2 treatment. Data are presented as the mean \pm SD of three independent experiments.

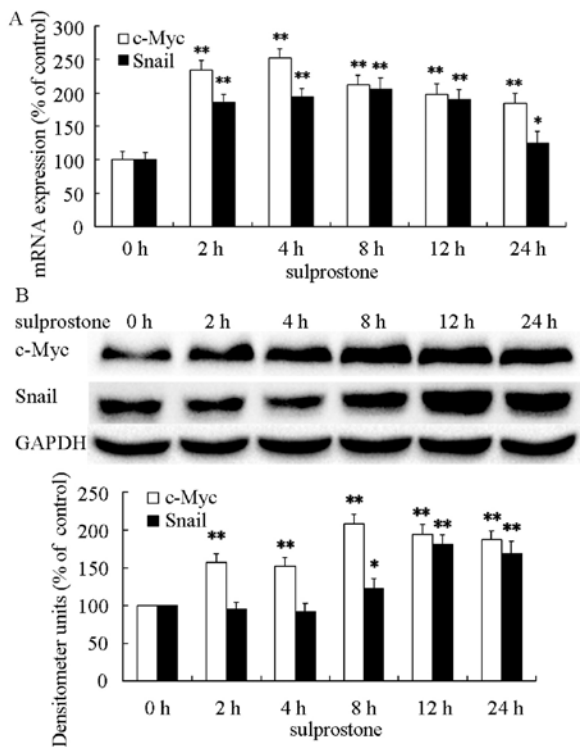


Figure 7. Sulprostone induces c-Myc and Snail expression. (A) Sulprostone upregulates c-Myc and Snail mRNAs in pcDNA-EP3-4-transfected CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with 5 μ M sulprostone for the indicated time periods. The c-Myc and Snail mRNAs were determined by qPCR. GAPDH as the loading control was determined by qPCR. (B) Sulprostone upregulates c-Myc and Snail proteins in pcDNA-EP3-4-transfected CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with 5 μ M sulprostone for the indicated time periods. The c-Myc and Snail proteins were determined by western blotting with anti-c-Myc and anti-Snail antibodies, and GAPDH as the loading control with anti-GAPDH antibody. Quantitative analysis of c-Myc and Snail expression levels were carried out by calculating the percentage of c-Myc, Snail proteins and GAPDH levels from three different experiments. * P <0.05, ** P <0.01 compared with the control. Data are presented as the mean \pm SD of three independent experiments.

of important downstream target genes, such as *c-Myc* and *Snail* (27,28). The protein and mRNA levels were determined after pcDNA-EP3-4 transfected CCLP1 cells treated with 5 μ M sulprostone for the indicated time periods. The results showed that treatment of pcDNA-EP3-4-transfected CCLP1 cells with sulprostone increased the levels of c-Myc and Snail mRNAs and the levels of c-Myc and Snail proteins (Fig. 7).

The mechanism of the upregulation of β -catenin protein induced by PGE2. There are two main ways associated with the increase of β -catenin protein: upregulation of mRNA and downregulation of ubiquitin-dependent degradation. The degradation of β -catenin depends on β -catenin phosphorylation. Previous findings suggest that, PGE2 and sulprostone increased the level of β -catenin protein in a concentration-dependent manner. To confirm the β -catenin protein upregulation, the pcDNA-EP3-4-transfected CCLP1 cells were treated with 5 μ M sulprostone for the indicated time periods. The levels of β -catenin and phosphorylated β -catenin proteins were analyzed by western blotting and β -catenin mRNAs by qPCR. The results showed that treatment of

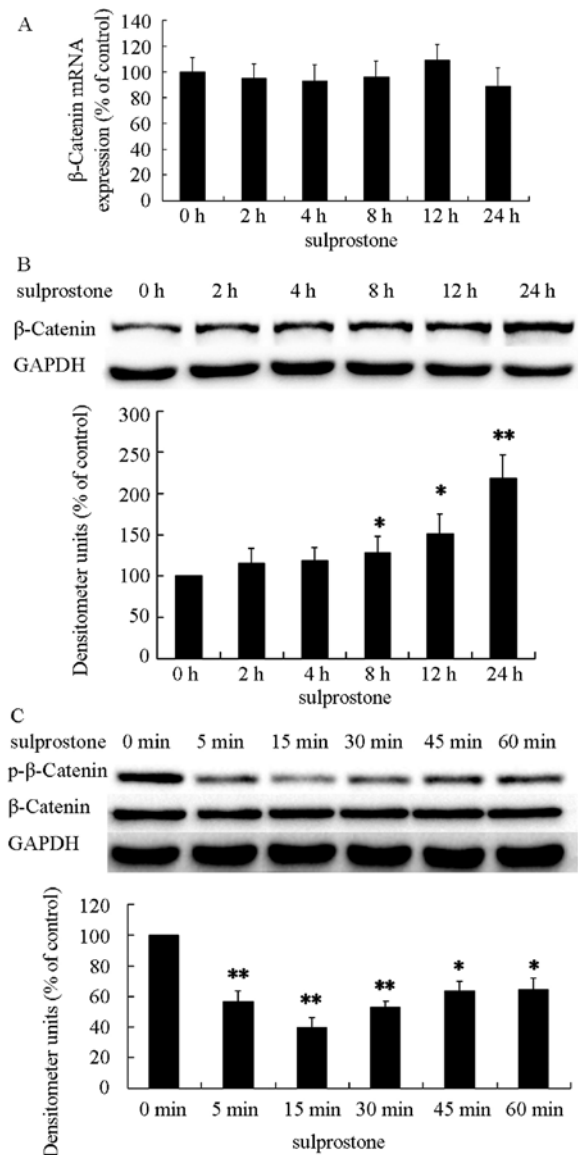


Figure 8. β -catenin protein upregulation induced by sulprostone in CCLP1 cells. (A) Effects of sulprostone on β -catenin mRNA level in pcDNA-EP3-4-transfected CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at 5 μ M for the indicated time points. The β -catenin mRNA was determined by qPCR. GAPDH as the loading control was determined by qPCR. (B) Sulprostone upregulates β -catenin protein in pcDNA-EP3-4-transfected CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at 5 μ M for the indicated time points. β -catenin expression level was determined by western blotting with anti- β -catenin antibody, and GAPDH as the loading control with anti-GAPDH antibody. (C) Sulprostone downregulates the phosphorylation of β -catenin in pcDNA-EP3-4-transfected CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at 5 μ M for the indicated time points. The phosphorylated- β -catenin protein level was determined by western blotting with anti-p- β -catenin antibody, and total β -catenin as the loading control with anti- β -catenin antibody. Quantitative analysis of protein and mRNA level was carried out from three different experiments. * P <0.05, ** P <0.01 compared with the control. Data are presented as the mean \pm SD of three independent experiments.

pcDNA-EP3-4-transfected CCLP1 cells with sulprostone increased the level of β -catenin protein and decreased the level of phosphorylated β -catenin protein in a time-dependent manner (Fig. 8B and C). However, the change of β -catenin

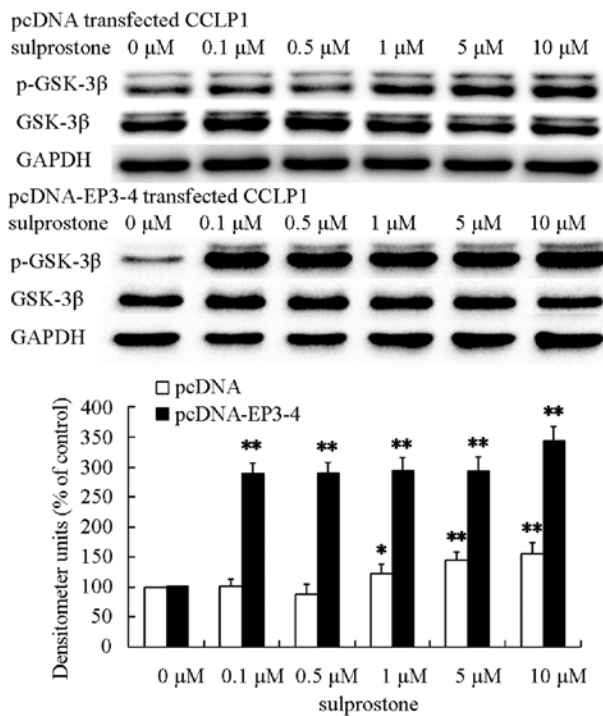


Figure 9. Sulprostone induces the phosphorylation of GSK-3 β in CCLP1 cells. The pcDNA and pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at the indicated concentration for 30 min. Phosphorylated-GSK-3 β and GSK-3 β expression was determined by western blotting with anti-p-GSK-3 β and anti-GSK-3 β antibodies. GAPDH was determined with anti-GAPDH antibody. Quantitative analysis of the p-GSK-3 β protein level was carried out by calculating the percentage of p-GSK-3 β and GSK-3 β protein levels from three different experiments. *P<0.05, **P<0.01 compared with the control. Data are presented as the mean \pm SD of three independent experiments.

mRNA was not observed in the experiment (Fig. 8A). These results indicated that the degradation of β -catenin is the main reason for the upregulation of β -catenin induced by PGE2.

PI3K/AKT and GSK-3 β are involved in the upregulation of β -catenin induced by PGE2. GSK-3 β associates in a complex with β -catenin, then β -catenin is phosphorylated and targeted for degradation (30). The phosphorylation of GSK-3 β on serine 9 inhibits its kinase activity. Therefore, the phosphorylation of GSK-3 β results in the loss of phosphorylation of β -catenin and the accumulation of β -catenin protein. Previous findings suggested that loss phosphorylation of β -catenin was induced by PGE2. Thus, we postulated that PGE2 could upregulate β -catenin protein via GSK-3 β . To evaluate this hypothesis, the pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells were treated at the indicated concentrations of sulprostone for 30 min. The levels of GSK-3 β and phosphorylated-GSK-3 β proteins were determined by western blotting. The results showed that treatment of pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells with sulprostone increased the levels of phosphorylated GSK-3 β proteins (Fig. 9).

It has been reported that AKT is the kinase responsible for the phosphorylation of GSK-3 β (31,32). We postulated that PGE2 upregulated β -catenin protein and inactivated GSK-3 β via AKT. To evaluate this hypothesis, the pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells were treated

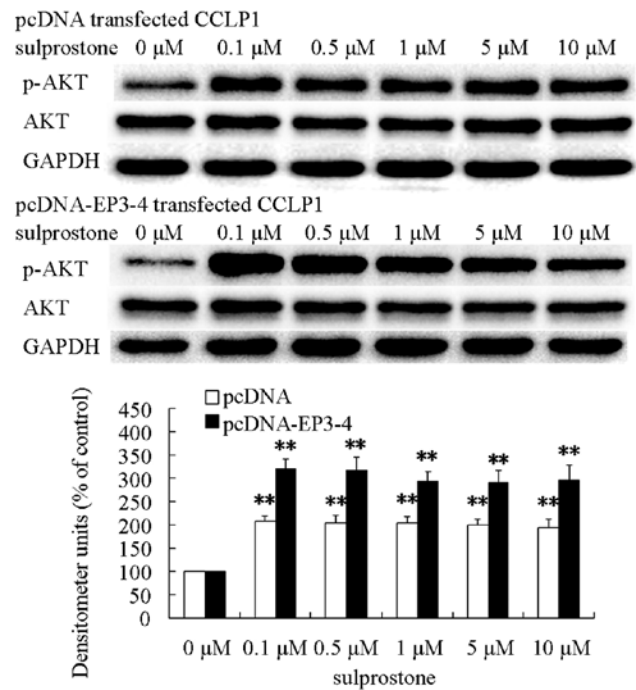


Figure 10. Sulprostone induces the phosphorylation of AKT in CCLP1 cells. The pcDNA and pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at the indicated concentrations for 30 min. Phosphorylated-AKT and AKT expression was determined by western blotting with anti-p-AKT and anti-AKT antibodies. GAPDH was determined with anti-GAPDH antibody. Quantitative analysis of the p-AKT level was carried out by calculating the percentage of p-AKT and AKT protein levels from three different experiments. **P<0.01 compared with the control. Data are presented as the mean \pm SD of three independent experiments.

at the indicated concentrations of sulprostone for 30 min. The levels of AKT and phosphorylated AKT proteins were analyzed by western blotting. The results showed that treatment of pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells with sulprostone increased the levels of phosphorylated-AKT proteins (Fig. 10).

The pcDNA-EP3-4-transfected CCLP1 cells were pretreated with the PI3K/AKT inhibitor LY-294002 or GSK-3 β inhibitor LiCl, and then treated with sulprostone. The β -catenin expression level was determined by western blotting. It was also shown that pretreatment of pcDNA-EP3-4-transfected CCLP1 cells with LY-294002 inhibited the upregulation of β -catenin protein and with LiCl promoted the upregulation of β -catenin protein induced by sulprostone (Fig. 11).

These results indicated that the upregulation of β -catenin induced by PGE2 is mediated through the activation of PI3K/AKT and GSK-3 β .

EGFR and Src are involved in AKT activation. EGFR and Src have been reported to be downstream of the EP receptors, and are important for progression of cancer (7,8,33). Therefore, we hypothesized that upregulation of β -catenin induced by sulprostone was mediated by EGFR and Src. To evaluate this hypothesis, the pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells were treated with the indicated concentrations of sulprostone for 45 min. The levels of EGFR and phosphorylated-EGFR proteins were analyzed by western blotting.

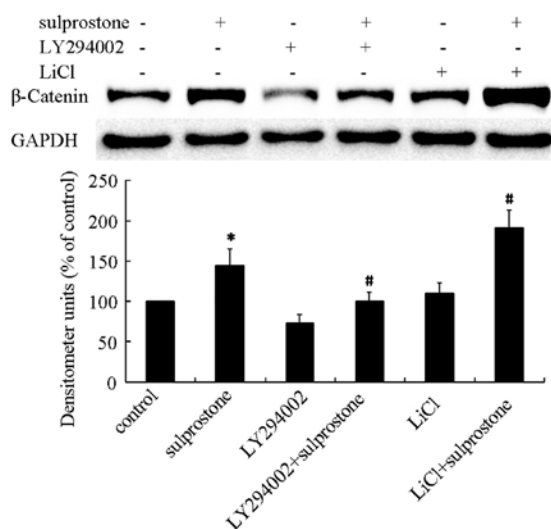


Figure 11. Effects of PI3K/AKT antagonist LY294002 and GSK-3 β antagonist LiCl on β -catenin upregulation induced by sulprostone. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were pretreated with 10 μ M LY294002 or 40 mM LiCl for 1 h, and then treated with 5 μ M sulprostone for 24 h. The β -catenin expression level was determined by western blotting with anti- β -catenin antibody, and GAPDH as the loading control with anti-GAPDH antibody. Quantitative analysis of the β -catenin expression level was carried out by calculating the percentage of β -catenin protein and GAPDH levels from three different experiments. * P <0.05, compared with the control. # P <0.05 compared with the sulprostone treatment. Data are presented as the mean \pm SD of three independent experiments.

The results showed that treatment of pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells with sulprostone increased the levels of phosphorylated-EGFR proteins (Fig. 12). The pcDNA-EP3-4-transfected CCLP1 cells were pretreated with Src inhibitor PP2 or EGFR inhibitor AG1478, and then treated with sulprostone. The phosphorylation of AKT was determined by western blotting after 30 min of treatment with sulprostone and β -catenin was determined after 24 h of treatment with sulprostone. Pretreatment of pcDNA-EP3-4-transfected CCLP1 cells with PP2 or AG1478 inhibited the phosphorylation of AKT and the upregulation of β -catenin protein (Fig. 13). These results indicated that PGE2 upregulates β -catenin expression is mediated through the activation of Src and EGFR.

Discussion

Prostaglandin E2 (PGE2) appears to participate in the development of inflammatory reactions and in oncogenesis (3,4). Our previous results revealed that PGE2 significantly enhanced the cell proliferation, migration and invasion in cholangiocarcinoma cells, and several signaling pathways were identified such as the upregulation of MMP-2 expression through the CREB pathway (11), upregulation of FBP1 expression through the cAMP/PKA pathway (12) and stimulation of Erk through the phosphorylation of EGFR (34). PGE2 significantly promoted hepatocellular carcinoma cell proliferation and invasion by upregulating the expression of Snail (6,7), YB-1 (8), c-Myc (9), β 1-integrin (10) and other factors. Additionally, PGE2 promoted cell proliferation and invasion in other types of cancer (13-15).

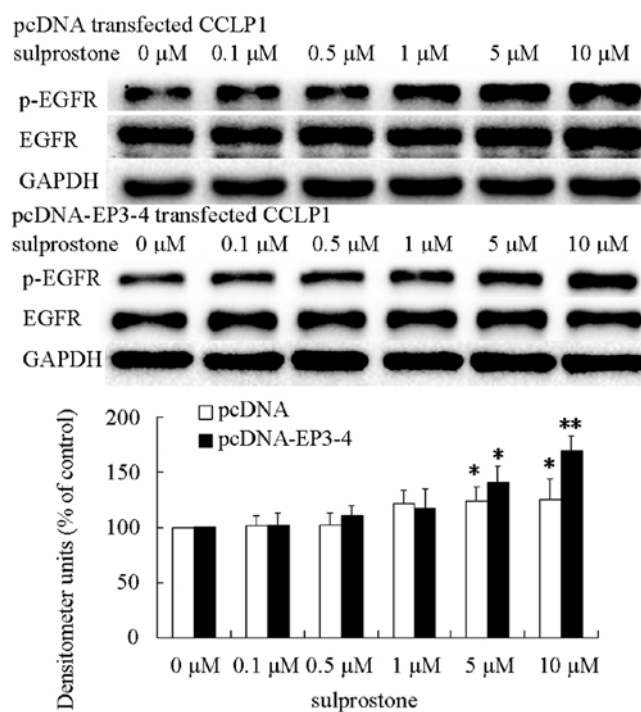


Figure 12. Sulprostone induces the phosphorylation of EGFR in CCLP1 cells. The pcDNA- and pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were treated with sulprostone at the indicated concentrations for 45 min. Phosphorylated-EGFR and EGFR expression was determined by western blotting with anti-p-EGFR and anti-EGFR antibodies. GAPDH was determined with anti-GAPDH antibody. Quantitative analysis of the p-EGFR protein level was carried out by calculating the percentage of p-EGFR and EGFR protein expression levels from three different experiments. * P <0.05, ** P <0.01 compared with the control. Data are presented as the mean \pm SD of three independent experiments.

The action of PGE2 is mediated by four G protein-coupled receptors (GPCRs): EP1, EP2, EP3 and EP4 (35), which activate multiple signal transduction pathways leading to downstream responses. The EP1 receptor mainly couples to G α_q protein and upregulates the level of intracellular Ca²⁺. The EP2 and EP4 receptors couple to G α_s protein, activate adenylate cyclase (AC) and increase the production of cyclic AMP (cAMP). However, the EP3 receptor which couples to G protein is more complicated (12,16). Among EP receptors, EP3 is unique in that alternative mRNA splicing leads to multiple isoforms including EP3 subtype isoforms 4, 5, 6, 7, and 8 (also known as EP3-4, -5, -6, -7 and -8). In a previous study (12), EP3-4, -5, -6 and -7 receptors were observed in CCLP1 and HuCCT1 cells. Those results indicated that, EP3-4 and EP3-5 receptors significantly promoted cholangiocarcinoma cell proliferation, migration and invasion, whereas no significant increase in EP3-6 and EP3-7 receptors was discernable. EP3 isoforms may mediate different intracellular responses to PGE2, contributing to the different roles played by the progression of cancer. EP3 isoform receptors that promote cancer progression may be coupled with G α_s and stimulate the AC-cAMP-PKA pathway (12). EP3 isoform receptors that exhibit no significant increase in cancer progression may be coupled with G α_i and downregulate the AC-cAMP-PKA pathway (36). All the EP3 isoforms share a common N-terminal sequence, which includes hormone binding and membrane spanning regions. However, each isoform has a unique amino acid composition in the C-terminal

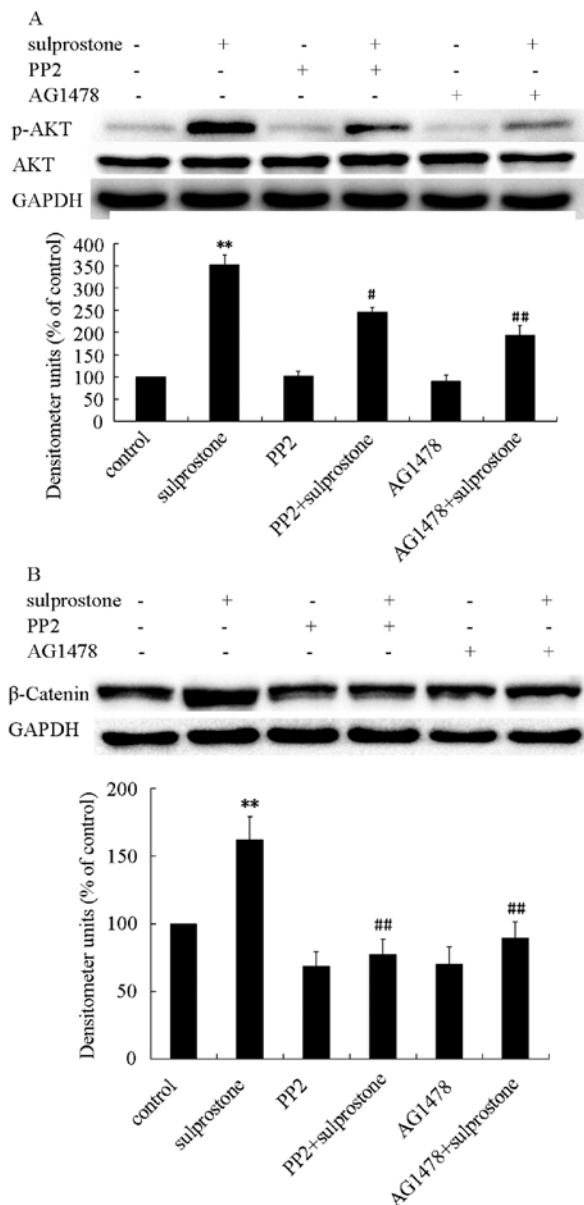


Figure 13. Effects of Src inhibitor PP2 and EGFR inhibitor AG1478 on AKT activation and β -catenin upregulation induced by sulprostone in CCLP1 cells. (A) The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were pretreated with 10 μ M PP2 or 5 μ M AG1478 for 1 h, and then treated with 0.1 μ M sulprostone for 30 min. The p-AKT and AKT protein level was determined by western blotting with anti-p-AKT and anti-AKT antibodies. GAPDH was determined with anti-GAPDH antibody. (B) Effects of PP2 and AG1478 on β -catenin upregulation induced by sulprostone in CCLP1 cells. The pcDNA-EP3-4-transfected CCLP1 cells cultured in serum-free medium for 24 h were pretreated with 10 μ M PP2 or 5 μ M AG1478 for 1 h, and then treated with 5 μ M sulprostone for 24 h. The β -catenin protein level was determined by western blotting with anti- β -catenin antibody. GAPDH as the loading control was determined with anti-GAPDH antibody. Quantitative analysis of the p-AKT protein level was carried out by calculating the percentage of p-AKT and AKT protein levels from three different experiments. Quantitative analysis of the β -catenin expression level was carried out by calculating the percentage of β -catenin and GAPDH protein levels from three different experiments. ** $P < 0.01$ compared with the control. # $P < 0.05$, ## $P < 0.01$ compared with the sulprostone treatment. Data are presented as the mean \pm SD of three independent experiments.

region which regulates the intracellular location and plays a key role in G-protein coupling. These unique sequences in the C-terminal domains may allow each EP3 isoform to localize to

different areas within the cell and trigger different intracellular signaling pathways in response to PGE2. Evidence of different signal transduction pathways and the regulation of gene expression among different EP3 receptor isoforms has also been previously demonstrated (12,17). There are EP1, EP2, EP4 and four EP3 isoforms receptors involved in cholangiocarcinoma progression, and some receptors play a positive role while others do not. The reason that some EP3 isoforms couple with G α_i and do not promote cholangiocarcinoma cell growth and invasion may due to the fact that they are negative regulatory receptors that downregulate the function of other EP receptors.

β -catenin is critically responsible for epithelial cell-cell adhesion (37), and plays a key role in several developmental and pathologic processes including early embryonic development (38), fibrosis (39-42), cystic disease (43), renal failure (44) and cancer. For example, the expression level of β -catenin has been shown to correlate with progression in colorectal (18), cervical (19), prostate (20), renal cell carcinoma (45,46), hepatocellular carcinoma (47,48), cholangiocarcinoma (26) and several other types of tumors. PGE2 and β -catenin are involved in cholangiocarcinoma progression, and our studies confirm that PGE2 upregulates the β -catenin protein through the EP3-4 receptor. There are two main ways leading to the increase of β -catenin protein, upregulation of mRNA and downregulation of ubiquitin-dependent protein degradation. The ubiquitin-dependent degradation is caused by phosphorylation of β -catenin at Ser33, Ser37, and Thr41 or coupled with Ebi which transfers the ubiquitin of ubiquitin-conjugating enzymes UcbH5 to β -catenin. Our results show that the downregulation of ubiquitin-dependent degradation induced by β -catenin phosphorylation is the main reason for the upregulation of β -catenin induced by PGE2 instead of an increasing in mRNA expression. The complex including Axin, APC, Amer1, CK1 α Glycogen synthase kinase 3 β (GSK-3 β) and β -catenin was separated when β -catenin phosphorylated at Ser33, Ser37 and Thr41. As a scaffold protein, Axin, a plurality of the protein interaction site, can combine APC Amer1, CK1 α GSK-3 β and β -catenin together. The main functions of APC and Amer1 enhanced the affinity of β -catenin and the complex (49). Based on the phosphorylation of β -catenin at Ser45 induced by CK1 α , GSK-3 β can phosphorylate β -catenin at Thr41, Ser37, and Ser33, which is discerned by β -TrCP and lead to β -catenin degradation. When β -catenin phosphorylation was downregulated by PGE2 at Ser33, Ser37 and Thr41, the ubiquitin-dependent degradation was inhibited, and β -catenin accumulated in the cytoplasm forming a complex with the transcription factor, lymphoid enhancing factor-1/T-cell factor, which is subsequently transported into the cell nucleus (50). This transcription complex activates the expression of downstream target genes, including c-Jun, c-Fos, c-Myc (27), Snail (28), cyclin D1 (29) and survivin, resulting in abnormal cell proliferation and cell carcinogenesis. Our results show that the expression of c-Myc and Snail which is associated with hepatic carcinoma progression observed in previous studies (6,9) also induced an increase of the EP3 receptor agonist.

The phosphorylation of GSK-3 β at Ser 9 inhibits its kinase activity and results in the accumulation of β -catenin protein (30). Our results indicate that the phosphorylation of GSK-3 β and AKT is responsible for the upregulation of β -catenin induced by PGE2. Phosphatidylinositol 3-kinase

(PI3K) is composed of the p85 regulatory subunit and the p110 catalytic subunit. When PI3K is activated, the p85 subunit is recruited and the p110 subunit is activated. Activated PI3K leads to the consequent activation of PDK and AKT. AKT is the important kinase responsible for the phosphorylation of GSK-3 β (31,32).

EP3-4 receptor activated by PGE2 may couple to G α s protein and activate the AC/PKA pathway (12). Based on this pathway, treatment of CCLP1 cells with the AC inhibitor SQ22536 and the PKA inhibitor H89 inhibit the effects of EP3 receptor agonist-induced β -catenin expression. However, those results have not been observed in our experiments (data not shown). This observation suggests that the G α s/AC/PKA pathway may not be responsible for the EP3-4 receptor-mediated β -catenin protein upregulation.

EP receptors may also modulate activation of the epidermal growth factor receptor (EGFR) (33,34,51). In our previous studies, the EP1, EP2 and EP4 receptors activated the EGFR by forming the complex with EGFR and Src (6-8). The EGFR is a transmembrane tyrosine kinase that belongs to the HER/ErbB protein family. EGFR controls a variety of biological responses such as cell proliferation and migration. These effects are mediated via activation of the downstream molecules, including the PI3K/AKT pathway (52). It was reported that EGFR mediates Grb2 associated binder protein 1 (Gab1) activation in cultured rat hepatocytes. Depletion of Gab1, using siRNA, decreased AKT activation (53). In breast and lung cancer cell lines, PGE2 was associated with activated AKT, through increases in the EGFR-Gab1 complexes (54,55). It was also reported that the EP receptor was able to bind to EGFR directly (33). Our results indicate that PGE2 stimulated EGFR leading to the upregulation of β -catenin. Thus that the ligand combines its specific receptor is not the only manner in which to conduct signaling. The combination and interreaction between two receptors may also mediate the function of the ligand, such as the binding and interaction of EPR and EGFR.

To the best of our knowledge, this is the first study detailing the function of EP3 isoforms in human cholangiocarcinoma cells. The results also show that PGE2 upregulated the β -catenin protein through the EP3-4R/Src/EGFR/AKT/GSK-3 β pathway. Our findings reveal the role of EP3 isoforms in promoting cholangiocarcinoma cell growth and invasion and a new theory that interaction between two receptors can conduct cell signaling without the stimulation of a specific ligand.

Acknowledgements

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