

Prostaglandin E₂ receptor EP4 is involved in the cell growth and invasion of prostate cancer via the cAMP-PKA/PI3K-Akt signaling pathway

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Abstract. Prostate cancer (PCa) is one of the most prevalent diagnosed malignancies globally. Previous studies have demonstrated that prostaglandin E₂ (PGE₂) is closely associated with the tumorigenesis and progression of PCa. However, the underlying molecular mechanisms remain unclear and require further investigation. Matrix metalloproteinases (MMPs), receptor activator of nuclear factor- κ B ligand (RANKL) and runt-related transcription factor 2 (RUNX2), which are involved in cell growth and bone metastasis, are frequently activated or overexpressed in various types of cancer, including PCa. The present study was designed to investigate the associations between PGE₂ and the PGE₂ receptor EP4, and MMPs, RANKL and RUNX2 in PCa, and to define their roles in PCa cell proliferation and invasion in addition to understanding the molecular mechanisms. The results of western blotting and reverse transcription-quantitative polymerase chain reaction demonstrated that the protein and the mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells were significantly upregulated by treatment with PGE₂, respectively, and knockdown of these proteins blocked PGE₂-induced cell proliferation and invasion in PC-3 cells, as determined by Cell Counting Kit-8 and Matrigel invasion assays, respectively. The effect of PGE₂ on the protein and mRNA expression levels was primarily regulated via the EP4 receptor. EP4 receptor signaling activates the cyclic (c)AMP-protein kinase A (PKA) signaling pathway, and forskolin, an activator of adenylate cyclase (AC), exhibited similar effects to an EP4 receptor agonist on the protein expression, while SQ22536, an inhibitor of AC, inhibited the protein expression. These results confirmed that the AC/cAMP pathway may be involved in

EP4 receptor-mediated upregulation of protein expression. By using a specific inhibitor of PKA, it was also demonstrated that cAMP/PKA was also involved in the EP4 receptor-mediated upregulation of protein expression. In addition to the signaling pathway involving PKA, the EP4 receptor also exerts activities through activation of Akt kinase. The results in the present study confirmed the hypothesis that EP4 receptor-mediated protein expression in PCa cells that were pretreated with a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) was significantly inhibited. In conclusion, the results of the present study indicate that PGE₂ significantly upregulated the mRNA and protein expression levels of the MMP-2, MMP-9, RANKL and RUNX2, and the EP4 receptor was involved in the cell proliferation and invasion of PCa via the cAMP-PKA/PI3K-Akt signaling pathway. These results may provide novel insight into potential therapeutic strategies for the prevention and treatment of PCa.

Introduction

Prostate cancer (PCa) is one of the most prevalent diagnosed malignancies and is the sixth leading cause of cancer-associated death in males worldwide (1,2). Globally, it is anticipated that the incidence of PCa may increase to 1.7 million novel cases with 499,000 mortalities by 2030 (3). Furthermore, the 5-year survival rate of patients with PCa is only 29% (4). Despite developments in the treatment of PCa, the annual morbidity rate has increased by 14% since 1990 (5). Although numerous and extensive studies have been performed to investigate PCa, the pathogenesis of the tumorigenesis and progression of PCa has yet to be completely elucidated. Therefore, further investigation of the molecular mechanisms of PCa is required.

In recent decades, an increasing number of studies have demonstrated that inflammation is associated with the tumorigenesis and progression of cancer (6-8), particularly prostaglandins (PGs). PGs are lipid mediators that are derived from arachidonic acid via the cyclooxygenase (COX) pathway, which is an important molecular target in cancer therapy (9-11). In prostate tissue, PGE₂ is the most abundant proinflammatory mediator and excessive levels of PGE₂ have been reported in PCa (12). PGE₂ exerts various effects, including promoting cancer cell growth, proliferation, invasion and metastasis (13), upregulating antiapoptotic proteins and regulating the immune system (14,15). PGE₂, which binds to four cognate E prostanoid

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receptors (EP1, EP2, EP3 and EP4) (16,17), stimulates PCa cell proliferation and modulates various kinase pathways, including those regulated by phosphatidylinositol 3-kinase (PI3K)/Akt and protein kinase A (PKA) (18-22). Of these receptors, EP4 is the most common prostanoid receptor and is closely associated with inflammatory diseases and cancer (23-26). The authors' previous studies demonstrated that EP4 overexpression was associated with the progression of PCa to a castration-resistant form, and the use of an EP4 antagonist, ONO-AE3-208, *in vivo* inhibited the progression of PCa to a castration-resistant form via regulation of androgen receptor activation, which indicated that the expression of the EP4 receptor was positively associated with the metastatic malignant phenotype of PCa (27-30). Although evidence indicates that non-steroidal anti-inflammatory drugs that inhibit COX-2 are effective, they are limited by their well-known side effects, which include cardiovascular complications (31). Therefore, as a downstream factor of PGE₂ and COX-2, evidence has demonstrated that EP4 may be closely associated with PCa and may be a potential novel target for PCa treatment.

Furthermore, metastasis is a complex multistep process that is regulated by various mechanisms. It is considered that increases in migration and the potential for invasion are the most crucial steps in tumor metastasis (32). The activation or overexpression of matrix metalloproteinases (MMPs), receptor activator of nuclear factor- κ B ligand (RANKL) and runt-related transcription factor 2 (RUNX2) is involved in cell growth and bone metastasis via cyclic (c)AMP-PKA and PI3K-Akt signaling pathways (21,33-35). MMPs, which are calcium-dependent zinc-containing endopeptidases (36), have essential functions in tissue remodeling during numerous physiological or pathological processes, which include morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis and metastasis (37). Specifically, MMP-2 and MMP-9 are considered to be implicated in metastasis. Studies have demonstrated that the abnormal expression of MMPs enhances the invasion and metastasis of tumor cells via cAMP-PKA and PI3K-Akt signaling pathways (21,34). In humans, the RANKL protein is the product of the TNFSF11 gene (38,39). Certain reports have indicated that RANKL expression allows favorable microenvironmental conditions for cancer cell migration, and RANKL is also reported to be an important signal regulator in cancer-induced bone loss (40,41). RUNX2 is a transcription factor that is associated with osteoblast differentiation (42). The expression level of RUNX2 was reported to be significantly higher in metastatic PCa and was positively associated with EP4 receptor expression in PCa (19). Therefore, the detailed molecular mechanisms of MMP, RANKL and RUNX2 activation in PCa require further investigation.

Based on the literature and our previous results, the present study was designed to investigate the hypothesis that PGE₂ may upregulate the protein and mRNA expression levels of MMPs, RANKL and RUNX2 by binding to the EP4 receptor and activating cAMP-PKA and PI3K-Akt signaling pathways, thus promoting the cell proliferation and invasion of PCa cells.

Materials and methods

Materials and reagents. The PC-3 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA).

Roswell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PGE₂ and PGE₁ alcohol were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). The EP4 receptor antagonist ONO-AE3-208 was donated by the Department of Urology, Kyoto University (Kyoto, Japan). Lipofectamine 2000 was purchased from Thermo Fisher Scientific, Inc., SQ22536, forskolin, H89 and LY294002 were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The High Pure RNA isolation kit was from Roche Applied Science (Mannheim, Germany) and the PrimeScript RT reagent kit was from Takara Biotechnology Co., Ltd. (Dalian, China). Radioimmunoprecipitation assay (RIPA) lysis buffer was from Beijing Solarbio Science & Technology Co., Ltd., (Beijing, China). The Transwell unit (BD-BioCoat Matrigel Invasion Chambers) were from BD Biosciences (San Jose, CA, USA). TRIzol reagent was from Invitrogen (Thermo Fisher Scientific, Inc.).

Cell treatments. To determine the effect on MMP-2, MMP-9, RANKL and RUNX2 mRNA and protein expression varying doses and concentrations of the EP4 receptor selective agonist PGE₁ were used on cells. PC-3 cells were treated with PGE₁ alcohol (10 μ M) for 0, 0.5, 1, 1.5 and 2 h at 37°C or PGE₁ alcohol at 0, 0.1, 0.3, 1, 3 and 10 μ M for 2 h at 37°C. Then the effect of PGE₁ or PGE₂ and ONO-AE3-208 on protein expression in cells was investigated. PC-3 cells were treated with PGE₁ alcohol (10 μ M) for 0, 0.5, 1, 1.5 and 2 h at 37°C or PGE₁ alcohol at 0, 0.1, 0.3, 1, 3 and 10 μ M for 2 h. PC-3 cells were pretreated for 1 h with the EP4 receptor selective antagonist ONO-AE3-208 (10 μ M) and then stimulated with PGE₂ (10 μ M) or PGE₁ alcohol (10 μ M), and protein expression of MMP-2, MMP-9, RANKL and RUNX2 were determined by western blotting after 2 h. The effect of EP4 siRNA on protein expression was analysed. PC-3 cells were transfected with EP4 siRNA or negative control siRNA for 72 h at 37°C and then stimulated with PGE₂ (10 μ M) or PGE₁ alcohol (10 μ M) in serum-free medium for 2 h, and protein expression of MMP-2, MMP-9, RANKL and RUNX2 were determined by western blotting. To test the effect of adenylyl cyclase activator and inhibitors, PC-3 cells were treated with forskolin (10 μ M), and protein expression of MMP-2, MMP-9, RANKL and RUNX2 WERE determined after 2 h by western blotting. PC-3 cells were pretreated for 1 h with SQ22536 (200 μ M) and then stimulated with PGE₁ alcohol (10 μ M), and protein expression of MMP-2, MMP-9, RANKL and RUNX2 were determined after 2 h by western blotting. The effect of a PKA inhibitor was also investigated on the protein expression of MMP-2, MMP-9 RANKL and RUNX2. PC-3 cells were pretreated for 1 h with the PKA specific inhibitor H89 (10 μ M) and then stimulated with PGE₁ alcohol (10 μ M), and protein expression of MMP-2, MMP-9, RANKL and RUNX2 were determined after 2 h by western blotting.

Cell lines and culture. PC-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified air atmosphere containing 5% CO₂. The cells were digested by trypsin once every 3 days. The

experiments were performed when cells reached 80% confluency and were conducted in serum-free medium.

Small interfering (si)RNA interference. The siRNA targeting human EP4 receptor (siRNA ID: s11455) was purchased from Thermo Fisher Scientific, Inc., siRNA specific to MMP-2, MMP-9, RUNX2 and RANKL were from Cell Signaling Technology, Inc. (Danvers, MA, USA). The siRNA specific to genes were as follows: EP4 siRNA sense, 5'-TTCAGTTCCTTCCTCATCCTCGCC-3' and antisense, 5'-CTGTCCTCCGCA GGAGGATGTATA-3'; MMP-2 siRNA sense, 5'-GUGGCC AACUACAACUUCUTT-3' and antisense, 5'-AGAAGUUGU AGUUGGCCACTT-3'; MMP-9 siRNA sense, 5'-CUAUGG UCCUCGCCUGAATT-3' and antisense, 5'-UUCAGGGCG AGGACCAUAGAG-3'; RUNX2 siRNA sense, 5'-CAAGGA CAGAGUCAGAUUAUU-3' and antisense, 5'-UAAUCUGAC UCUGUCCUUGUU-3'; RANKL siRNA sense, 5'-UCCCAU CGGGUCCCAUAAAdTdT-3' and antisense, 5'-AUUCUG UAGAAGCAGCGCCdTdT-3'; and NC-siRNA sense, 5'-UGG UUUACAUGUCCAAUUAUU-3' and antisense, 5'-UAUUGG AACAUUGAAACCAUU-3'. PC-3 cells (2×10^5) were plated in 6-well plates for 24 h at 37°C, resulting in a 30-50% confluent cell monolayer. The cells were subsequently transfected with the 80 μ M targeting siRNA or negative control (NC) siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China) using Lipofectamine 2000 for 24 h. Following transfection with EP4 or NC siRNA for 72 h, depletion of target protein was confirmed by western blotting or reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and the cells were subsequently used for further experiments.

Cell proliferation assay. A CCK-8 assay was performed to assess the effect of MMP-2, MMP-9, RANKL and RUNX2 siRNA transfection on cell proliferation. PC-3 cells transfected with 80 nM NC or target siRNA (5×10^3) were seeded in 96-well plates. Subsequently, every 24 for 72 h, a batch of cells were stained with 10 μ l CCK-8 reagent at 37°C for 2 h. The reaction was quantified with an automatic plate reader at 450 nm. Each experiment was triplicated and performed three times independently.

Cell invasion assay. RMPI-1640 medium without FBS (100 μ l) containing 3×10^4 cells were seeded in the upper chamber in the presence or absence of 10 μ M PGE₂, and 750 μ l RMPI-1640 culture medium with 1% FBS was added to the lower chamber. The cell invasion assay was conducted following 24 h. The cell invasion activity of PC-3 cells was assessed using BD BioCoat Matrigel Invasion Chambers. The cells were washed with PBS and resuspended in RMPI-1640 medium without FBS at a density of 3×10^4 cells/ml. Cell suspension (500 μ l) was placed onto the upper chamber coated with Matrigel and 750 μ l RMPI-1640 culture medium with 1% FBS was added to the lower chamber of the Transwell. After 24 h incubation at 37°C in a 5% CO₂ incubator, the cells on the upper surface of the filters were removed by wiping with a cotton swab. The filters were fixed in 70% ethanol at 37°C and stained with hematoxylin for 30 min. The stained cells were counted under a light microscope in six randomly selected fields at magnification, $\times 200$. At least three chambers from three different experiments were analyzed.

Western blot analysis. Prior to western blotting, 5×10^6 PC-3 cells were incubated with PGE₂ at 0, 0.1, 0.3, 1, 3 and 10 μ M for 2 h at 37°C to investigate protein expression of MMP-2, MMP-9, RANKL and RUNX2 to varying concentrations of PGE₂. PC-3 cells were also treated with 10 μ M PGE₂ for 0, 0.5, 1, 1.5 and 2 h at 37°C to investigate the effect of different durations of incubation. Total cell lysates were extracted from the cells using RIPA lysis buffer followed by repetitive pipetting and lysis on the ice for 30 min. The contents were collected into centrifuge tubes and centrifuged at $1.6 \times 10^4 \times g$ for 30 min at 4°C. Loading buffer was subsequently added to the supernatant and the samples were treated at 100°C for 5 min to denature the proteins. Protein concentrations of lysates were measured using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples (10 μ l) were loaded into each well for 12% SDS-PAGE and proteins were transferred to a polyvinylidene difluoride membrane. The membranes were soaked with 8% non-fat milk for 1 h at room temperature and incubated with the corresponding primary antibodies overnight at 4°C with gentle agitation. Then, the membranes were washed with 0.1% PBS-Tween-20 (PBST) three times (10 min each time). For western blotting, the primary antibodies were MMP-2 (cat. no. 40994), MMP-9 (cat. no. 13667), RANKL (cat. no. 5312), RUNX2 (cat. no. 12556) and GAPDH (cat. no. 5174), all at 1:10,000, the membranes were washed with PBST three times. Subsequently, membranes were incubated with secondary antibodies [the peroxidase conjugated secondary anti-rabbit IgG (cat. no. 8885) or anti-mouse IgG (cat. no. 8887) antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA), all at 1:5,000] for 2 h at room temperature. The signals were detected using enhanced chemiluminescent western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) and analyzed using Image Lab 4.0 analysis software (Bio-Rad Laboratories, Inc.). GAPDH was used as an internal control.

RT-qPCR. PC-3 cells were incubated at 37°C with PGE₂ at 0, 0.1, 0.3, 1, 3 and 10 μ M for 1 h and mRNA expression of MMP-2, MMP-9, RANKL and RUNX2 was examined. Total RNA was extracted from the human PCa tissues or cells using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from total RNA (1 μ g) using a First-Strand cDNA Synthesis kit (GE Healthcare, Chicago, IL, USA) at 65°C for 5 min. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and monitored using a GeneAmp 5700 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) in triplicate. PCR conditions were: Pre-incubation at 95°C for 10 min (1 cycle) followed by 40 cycles of 95°C for 15 sec (30 cycles), 60°C for 30 sec (30 cycles), and 72°C for 30 sec (30 cycles). The mRNA expression was quantified using the 2^{- $\Delta\Delta$ C_q} method (43), and expressed as the relative quantity of target mRNA normalized to the GAPDH, respectively. Relative expression was calculated using the comparative cross threshold (C_q) method. The following primer pairs were employed in the present study: MMP-2, 5'-AGACATACA TCTTTGCTGGAGACA-3' (forward) and 5'-CTTGAAGAA GTAGCTGTGACCG-3' (reverse); MMP-9, 5'-TTTGAGTCC GGTGGACGATG-3' (forward) and 5'-TTGTGCGGCGATA GGAAGG-3' (reverse); RANKL, 5'-AATAGAATATCA

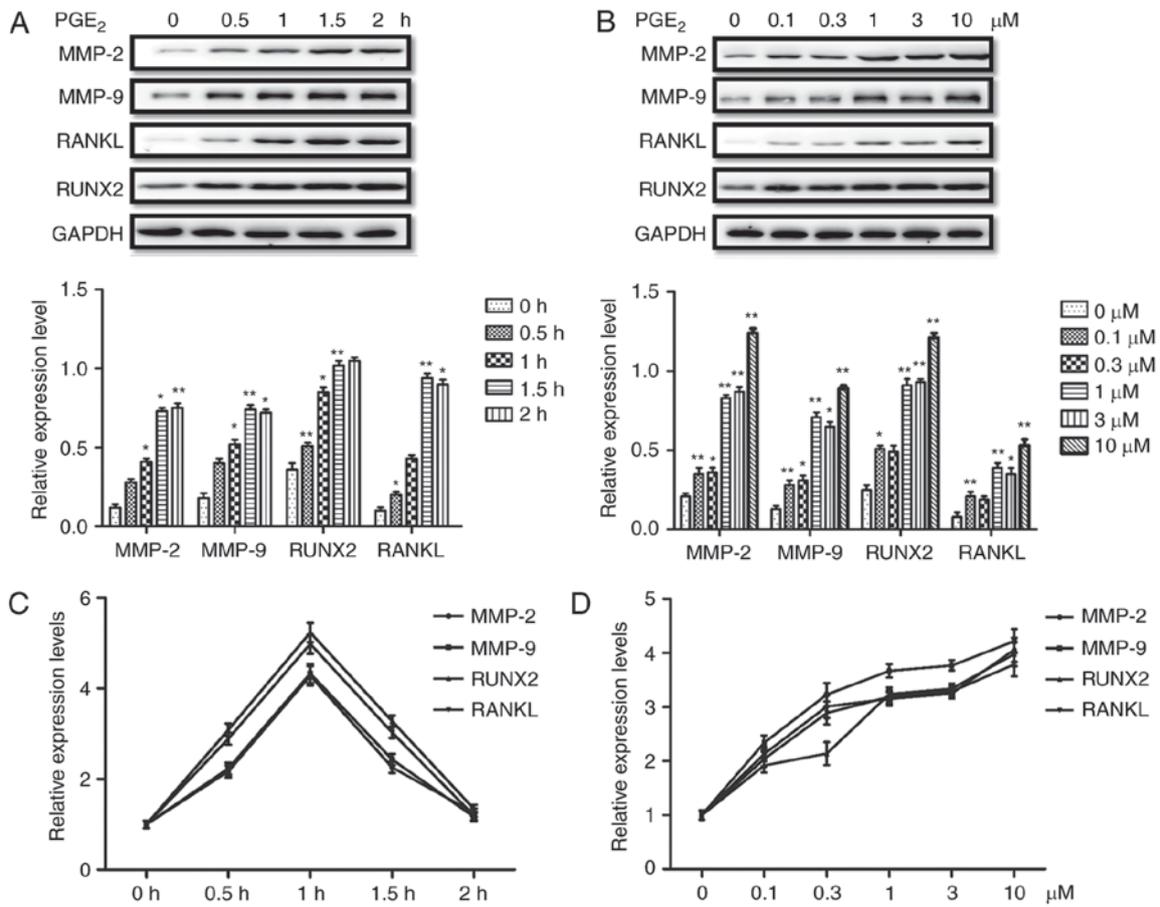


Figure 1. PGE₂ upregulates the protein and mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 prostate cancer cells. (A) Time course of the effects of PGE₂ on the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (B) Effect of various PGE₂ concentrations on the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (C) Time course of the effects of PGE₂ on the mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (D) Effect of various PGE₂ concentrations on the mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. 0 h or 0 μM group. PGE₂, prostaglandin E₂; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RUNX2, runt-related transcription factor 2.

GAAGATGGCACTC-3' (forward) and 5'-TAAGGAGGG GTTGGAGACCTCG-3' (reverse); RUNX2, 5'-GTTTGT TCTCTGACCGCC-3' (forward) and 5'-CCAGTTCTGAAG CACCTGA-3' (reverse); EP4, 5'-CATCTTACTCATTGCCAC CT-3' (forward) and 5'-TACTGAGCACTGTCTTTCTC-3' (reverse); and GAPDH, 5'-TTCCAGGAGCGAGATCCCT-3' (forward) and 5'-CACCCATGACGAACATGGG-3' (reverse). The qPCR reactions were performed in triplicate and included no-template controls.

Statistical analysis. GraphPad Prism 5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze experimental data. Data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) test with post hoc tests performed using Student-Newman-Keuls test. ANOVA or paired t-tests were performed to analyze the intergroup differences. P<0.05 was considered to indicate a statistically significant difference.

Results

PGE₂ induces the protein and mRNA expression of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. PC-3 cells were

treated with various doses of PGE₂ for different durations in order to determine the direct effect of PGE₂ on the expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. Subsequently, the protein and mRNA expression levels of MMP-2, MMP-9, RUNX2 and RANKL were analyzed by western blotting and RT-qPCR, respectively. The results of western blotting demonstrated that the protein expression levels were increased with increasing durations of PGE₂ treatment (0-2 h; Fig. 1A). In addition, the protein expression of all four proteins was upregulated following treatment with 0.1-10 μM PGE₂, compared with the 0 μM group (Fig. 1B). Furthermore, the mRNA expression levels of MMP-2, MMP-9, RUNX2 and RANKL were increased by PGE₂ treatment, with expression peaking at a 1 h duration of treatment with PGE₂ (Fig. 1C). Furthermore, the mRNA levels were increased following treatment with 0.1-10 μM PGE₂ in the PC-3 cells, compared with the 0 μM group (Fig. 1D). These results indicate that PGE₂ upregulated the expression of MMP-2, MMP-9, RUNX2 and RANKL at the protein and mRNA level in PC-3 cells.

Knockdown of MMP-2, MMP-9, RUNX2 and RANKL inhibits the proliferation and invasion of PCa cells. Previous studies have demonstrated that PGE₂ accelerates the proliferation

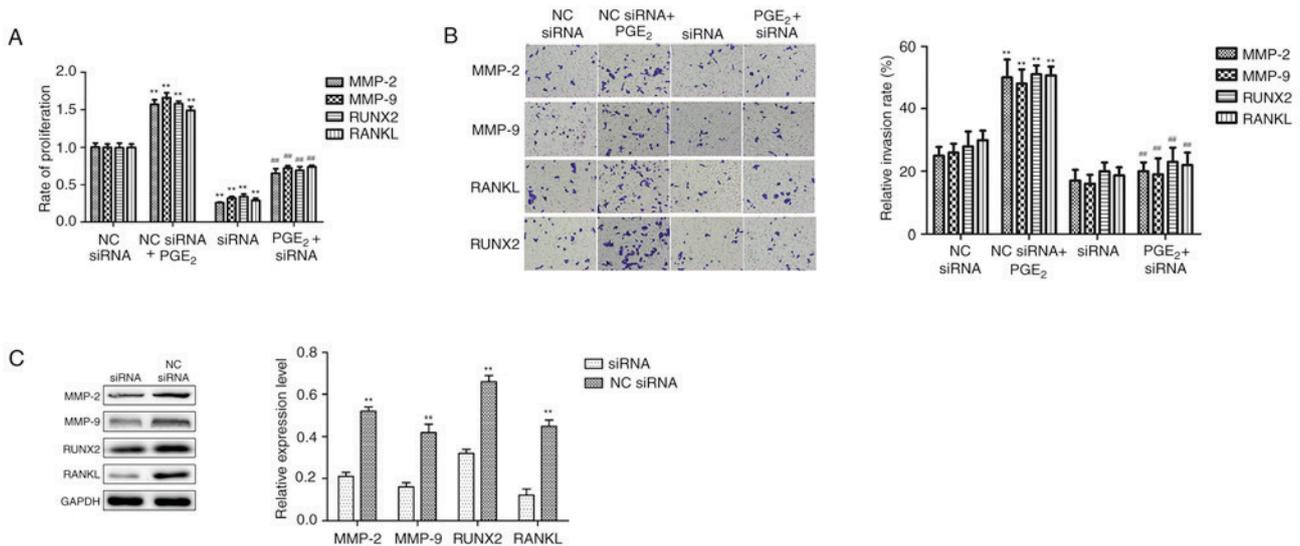


Figure 2. Knockdown of MMP-2, MMP-9, RANKL and RUNX2 using siRNA reduces PGE₂-induced PC-3 cell proliferation and invasion. (A) Knockdown of MMP-2, MMP-9, RANKL and RUNX2 using siRNA reduced PGE₂-induced increases in PCa cell proliferation. (B) Knockdown of MMP-2, MMP-9, RANKL and RUNX2 using siRNA blocked PGE₂-induced increases in PCa cell invasion. The cell invasion assay was conducted after 24 h and magnification was x200. (C) The efficiency of MMP-2, MMP-9, RANKL and RUNX2 siRNA transfection in PC-3 cells was confirmed by western blotting. Results are presented as the mean \pm standard deviation of three independent experiments. **P<0.01 vs. NC siRNA group; #P<0.01 vs. NC siRNA + PGE₂ group. MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor- κ B ligand; RUNX2, runt-related transcription factor 2; siRNA, small interfering RNA; PGE₂, prostaglandin E₂; PCa, prostate cancer; N.C., negative control.

and invasion of PCa cells (44,45), however, the underlying mechanism remains unclear. The results of the present study demonstrated that PGE₂ directly upregulated the protein and mRNA expression levels of MMP-2, MMP-9, RUNX2 and RANKL, which are key transcription factors associated with cell proliferation and the bone metastasis of PCa (33-42). Therefore, further investigation of the role of MMP-2, MMP-9, RUNX2 and RANKL upregulation in PGE₂-induced PC-3 cell proliferation and invasion is required. As demonstrated in Fig. 2A, transfection of PC-3 cells with MMP-2, MMP-9, RUNX2 and RANKL siRNAs significantly blocked PGE₂-induced proliferation. Furthermore, the results in Fig. 2B demonstrate that MMP-2, MMP-9, RUNX2 and RANKL siRNAs inhibited PGE₂-induced invasion in PC-3 cells. Western blot analysis confirmed that the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL were reduced by siRNA transfection in PC-3 cells (Fig. 2C). These results indicate that MMP-2, MMP-9, RUNX2 and RANKL may have an important role in the proliferation and invasion of PCa cells.

The EP4 receptor is involved in the PGE₂-induced protein expression of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. Overexpression of the EP4 receptor has been reported in various types of cancer, and increased EP4 receptor signaling has been previously associated with the migration, invasion and bone metastasis of various tumor types (19,24,46). The authors' previous studies demonstrated that an EP4 receptor antagonist significantly inhibited the proliferation, invasion and metastatic abilities in PCa cells (27,28). Therefore, the present study investigated whether the EP4 receptor may participate in the PGE₂-induced protein expression of MMP-2, MMP-9, RUNX2 and RANKL in PC-3 cells. In PC-3 cells that were treated with PGE₁ alcohol, an EP4 receptor selective

agonist, the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL were increased with increases in treatment concentration and duration (Fig. 3A and B). Furthermore, the mRNA expression levels were also upregulated by PGE₁ alcohol at different concentrations and durations of treatment in PC-3 cells (Fig. 3C and D). These results indicated that PGE₁ alcohol upregulated the protein and mRNA expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells, which was similar to the effects observed following treatment of PC-3 cells with PGE₂. Therefore, the EP4 receptor may have an important role in the regulation of MMP-2, MMP-9, RUNX2 and RANKL expression levels in PC-3 cells.

In order to further confirm the effects of the EP4 receptor on PGE₂-induced protein and mRNA expression, the PC-3 cells were pretreated with an EP4 receptor selective antagonist or EP4 receptor siRNA. As demonstrated in Fig. 4A, pretreatment of PC-3 cells with ONO-AE3-208, the EP4 receptor selective antagonist, markedly reduced PGE₂- and PGE₁ alcohol-induced protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL. Furthermore, transfection of siRNA targeting the EP4 receptor also significantly blocked PGE₂- and PGE₁ alcohol-induced upregulation of the protein expression levels in PC-3 cells (Fig. 4B). RT-qPCR was performed to determine the efficiency of transfection with EP4 receptor siRNA, and the results demonstrated that EP4 siRNA significantly lowered the protein expression of the EP4 receptor in PC-3 cells, compared with cells transfected with NC siRNA (Fig. 4C). These results indicate that the EP4 receptor may be involved in the regulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells.

The cAMP-PKA signaling pathway is involved in the upregulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. As a G-protein-coupled

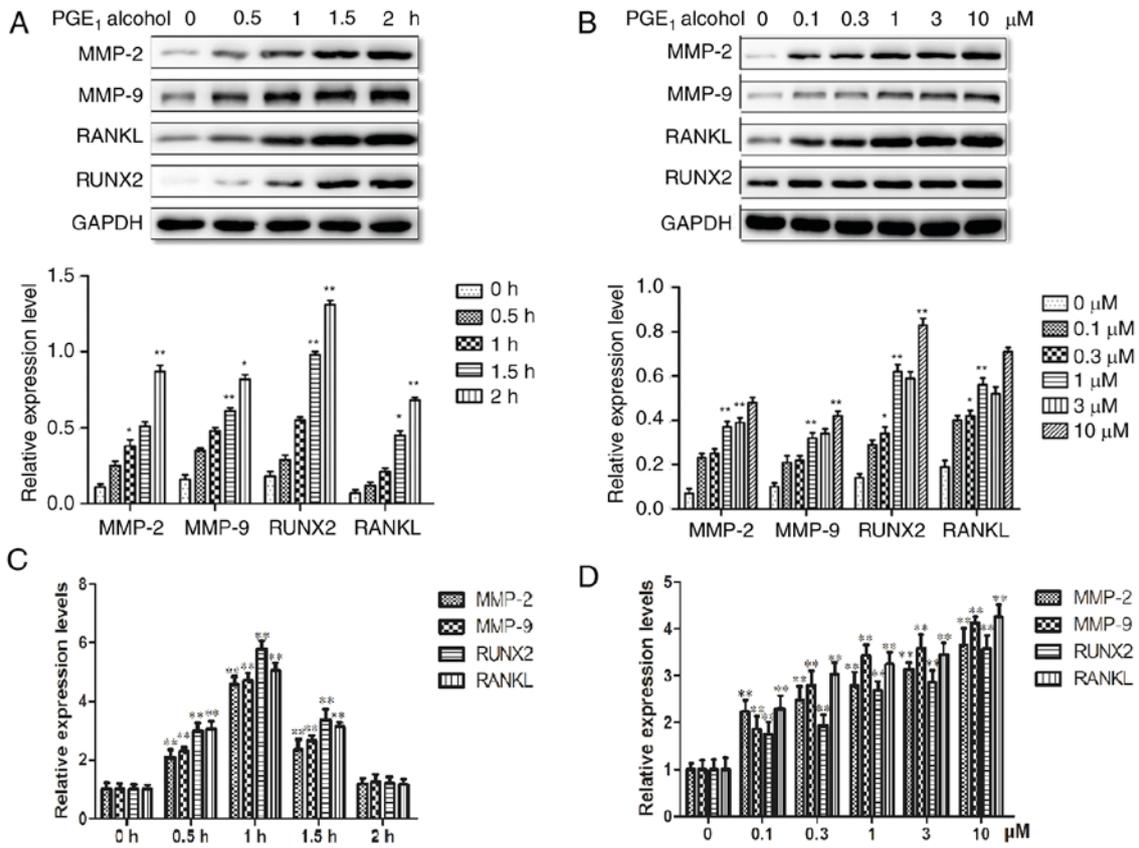


Figure 3. The EP4 receptor selective agonist, PGE₁ alcohol, mimics the upregulatory effect of PGE₂ on the expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 prostate cancer cells. (A) Time course of the effects of PGE₁ alcohol on the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (B) Effect of various concentrations of PGE₁ alcohol on the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (C) Time course of the effects of PGE₁ alcohol on the mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (D) Effect of various concentrations of PGE₁ alcohol on the mRNA expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. 0 h or 0 μM group. PGE, prostaglandin E; EP4, PGE₂ receptor EP4; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RUNX2, runt-related transcription factor 2.

receptor, the EP4 receptor normally couples with G_sα protein to activate adenylate cyclase (AC) and elevate intracellular cAMP levels, which has been associated with the occurrence and development of tumors (16,17). To confirm the role of the cAMP-PKA downstream signaling pathway in the EP4 receptor-mediated upregulation of MMP-2, MMP-9, RUNX2 and RANKL expression, an activator (forskolin) and inhibitor (SQ22536) of AC were employed to treat the PC-3 cells. Following treatment, the effects on the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PC-3 cells were determined by western blotting. The results demonstrated that the protein expression levels in PC-3 cells pretreated with forskolin were significantly upregulated (Fig. 5A), while levels were markedly reduced when pretreated with SQ22536 (Fig. 5B), compared with the respective control groups. In addition, increased cAMP levels lead PKA activation, a key signaling protein, which can regulate gene expression (47). To investigate the involvement of PKA activation in EP4 receptor-mediated upregulation of protein expression in PCa cells, PC-3 cells were pretreated with the PKA specific inhibitor, H89. As demonstrated in Fig. 5C, the PGE₁ alcohol-induced upregulation of protein expression levels in PC-3 cells pretreated with H89 was significantly reduced. These results indicated that PKA may have an

important role in EP4 receptor-mediated protein upregulation in PC-3 cells. Therefore, the cAMP-PKA signaling pathway may be involved in the upregulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells.

The PI3K-Akt signaling pathway is involved in the upregulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. As discussed in the previous paragraph, the results of the current study demonstrated that the cAMP-PKA signaling pathway may be involved in the upregulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells. Furthermore, it has been previously reported that the EP4 receptor activates the Akt signaling pathway to regulate cell proliferation and gene expression (45,46). To determine the involvement of the Akt signaling pathway in the upregulation of the protein expression levels of MMP-2, MMP-9, RUNX2 and RANKL in PCa cells, PC-3 cells were pretreated with a specific inhibitor PI3K, LY294002, and the effects on the protein expression levels in PC-3 cells were determined by western blotting. As demonstrated in Fig. 6, treatment of PC-3 cells with LY294002, the specific inhibitor of PI3K, resulted in a dose-dependent decrease in protein expression levels in PGE₁ alcohol-treated

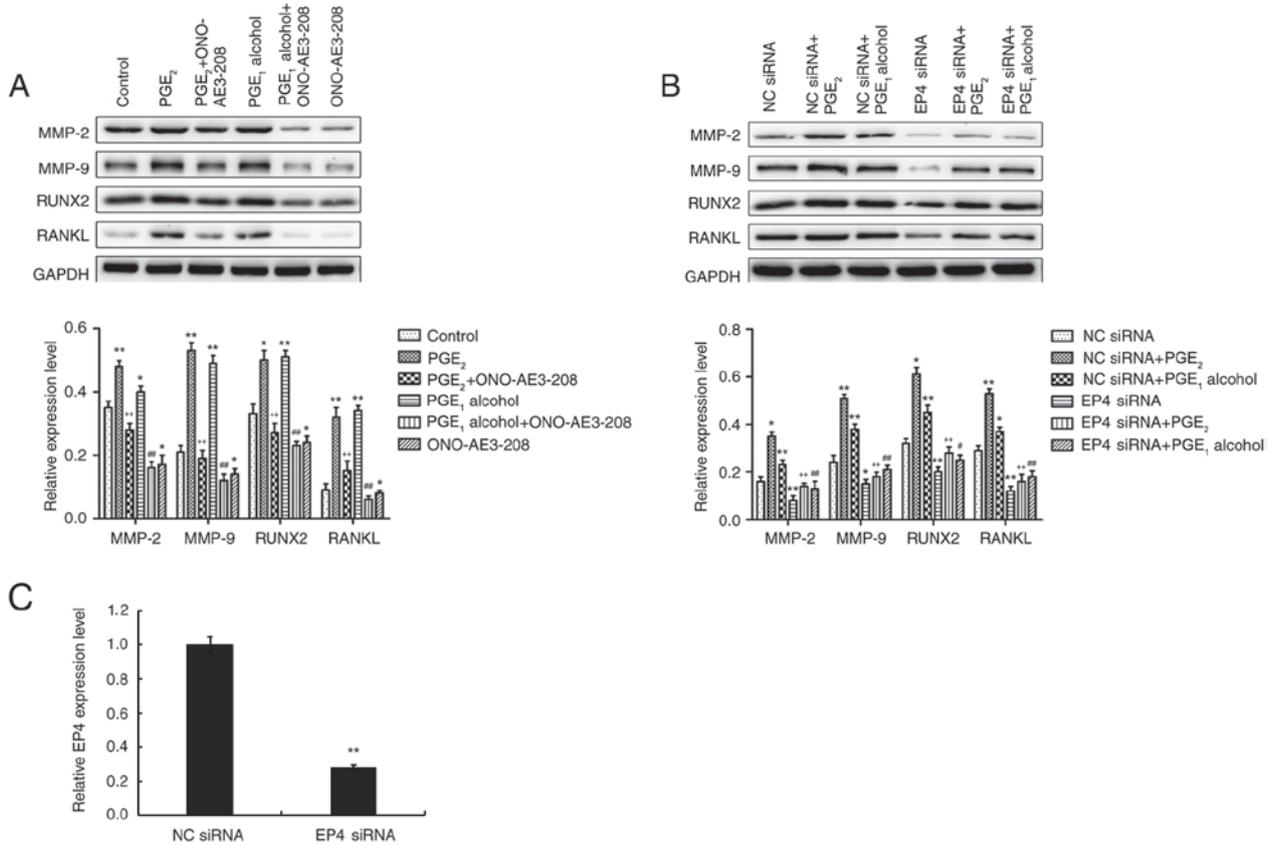


Figure 4. The EP4 receptor antagonist, ONO-AE3-208, or siRNA interference attenuates PGE₂-induced MMP-2, MMP-9, RANKL and RUNX2 expression in PC-3 prostate cancer cells. (A) Effect of the EP4 receptor selective antagonist, ONO-AE3-208, on PGE₂- and PGE₁ alcohol-induced increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (B) Effect of EP4 receptor siRNA on PGE₂- and PGE₁ alcohol-induced increases in the protein expression of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (C) RNAi efficiency of EP4 siRNA in PC-3 cells. Following transfection of PC-3 cells with EP4 or NC siRNA for 72 h, the transfection efficiency was confirmed using reverse transcription-quantitative polymerase chain reaction to detect mRNA levels of EP4. Results are presented as the mean \pm standard deviation of three independent experiments. For parts A and B: * P <0.05 and ** P <0.01 vs. control group; ** P <0.01 vs. PGE₂ group; # P <0.05 and ## P <0.01 vs. PGE₁ alcohol group. For part C: ** P <0.05 vs. NC siRNA group. PGE, prostaglandin E; EP4, PGE₂ receptor EP4; siRNA, small interfering RNA; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor- κ B ligand; RUNX2, runt-related transcription factor 2; NC, negative control.

cells. Based on these results, the PI3K-Akt signaling pathway may be involved in the upregulation of MMP-2, MMP-9, RUNX2 and RANKL protein expression in PCa cells.

Based on the results presented in the current study, we hypothesize that EP4 receptor expression increases the secretion of PGE₂ and the protein and mRNA expression of MMP-2, MMP-9, RANKL and RUNX2 in PCa cells. PGE₂, in turn, acting through the EP4 receptor, activates cAMP-PKA and PI3K-Akt pathways, which is required for the effects of PGE₂ on the cell migration and invasion of PCa cells. A summary of this potential process is presented in Fig. 7. Therefore, targeting of the downstream signaling components regulated by PGE₂ may be a potential therapeutic approach for overcoming severe side effects and health risks associated with the use of COX-2 inhibitors in patients with PCa.

Discussion

Malignant tumors are a serious threat to human health. Clinically, PCa is one of the most common types of malignant tumor, which have become a major health problem globally. However, at present, the detailed molecular mechanisms underlying PCa remain unclear. Previous studies have demonstrated

that PGE₂ is associated with proliferation, invasion and metastasis in PCa cells. Although certain signaling pathways have been identified, including transactivation of epidermal growth factor receptor (EGFR) (47) and phosphorylation of focal adhesion kinase (48), the detailed molecular mechanisms of PGE₂ in PCa require further investigation.

Numerous studies have demonstrated that PGE₂ is closely associated with various human diseases, including malignant tumors (42,49,50). PGE₂ has been reported to significantly enhance cancer cell proliferation, adhesion, invasion, metastasis and angiogenesis, and has therefore been implicated in the tumorigenesis and progression of various cancer types, including prostate (51), breast (52) and liver cancer (53). At the cellular level, PGE₂, as a bioactive lipid, exerts various functions and activates the signal transduction pathway by binding to specific receptors on the cell surface membrane, which occurs in a paracrine or autocrine manner. It has been confirmed that there are four types of PGE₂ receptor (EP1, EP2, EP3 and EP4), which are encoded by different genes. Various studies have reported that the EP4 receptor has an important role in various PGE₂-induced cancers, and the EP4 receptor is usually coupled with G α protein to activate AC and elevate intracellular cAMP levels (54,55).

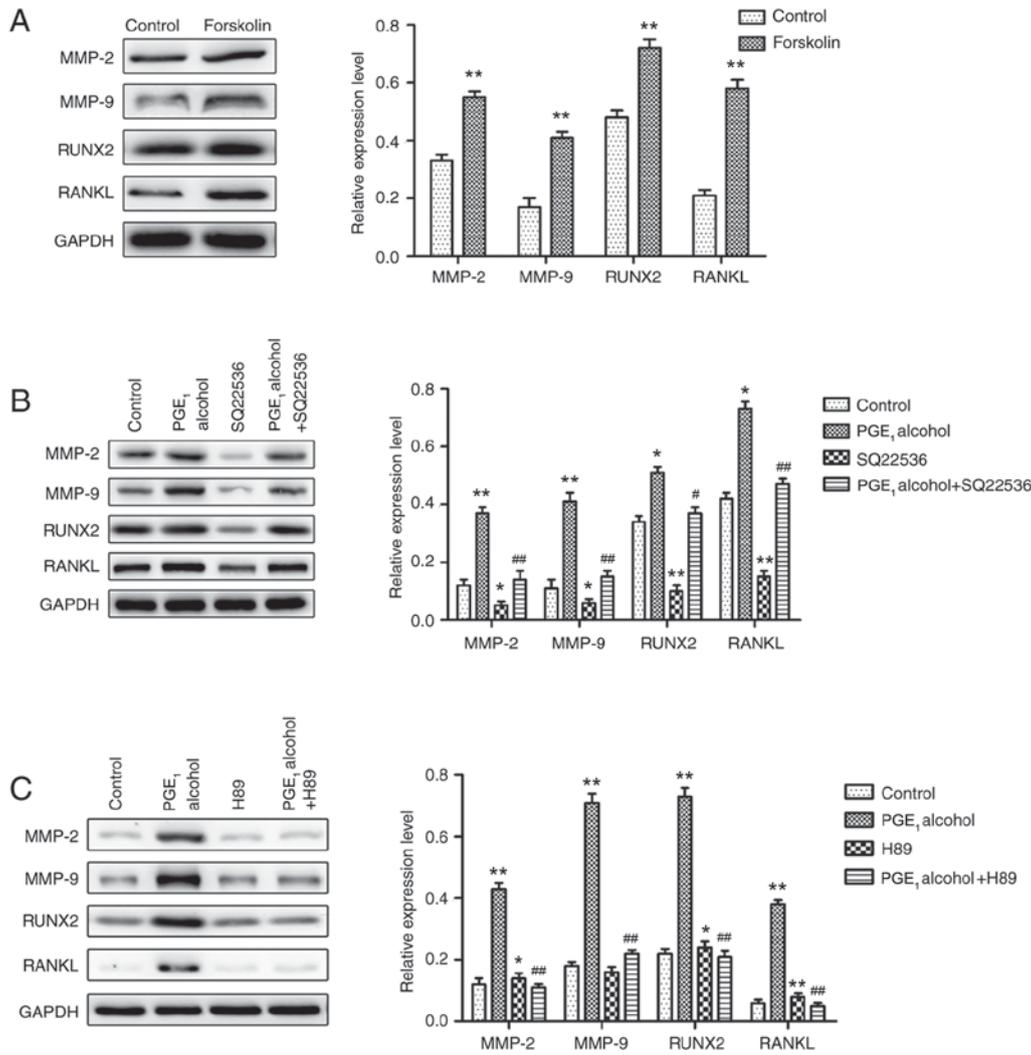


Figure 5. Involvement of the cAMP-PKA signaling pathway in EP4 receptor-mediated increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 prostate cancer cells. (A) The specific AC activator, forskolin, induced increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (B) The specific AC inhibitor, SQ22536, reduced EP4 receptor-mediated increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. (C) The specific PKA inhibitor, H89, reduced EP4 receptor-mediated increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control group; #P<0.05 and ##P<0.01 vs. PGE₁ alcohol group. cAMP, cyclic AMP; PKA, protein kinase A; PGE₁, prostaglandin E1; EP4, PGE₂ receptor EP4; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RUNX2, runt-related transcription factor 2; AC, adenylate cyclase.

Our previous studies demonstrated that the EP4 receptor and its antagonist significantly dominate cell invasion, migration and bone metastasis in PCa, which provided a research direction for the investigation of the underlying mechanisms. The aim of the current study was to investigate the PGE₂-EP4-PKA/PI3K-Akt signaling pathways and the key factors associated with the abnormal proliferation and metastasis of osteoblasts. Numerous studies have reported that MMPs, RANKL and RUNX2 may be involved in bone metastasis which is mediated via the cAMP-PKA and PI3K-Akt signaling pathways, and have been reported to be activated or overexpressed in various cancer types, including PCa (56-59). MMPs exhibit key roles in tissue remodeling, which is observed in various physiological and pathological processes, including morphogenesis, angiogenesis, tissue repair, cirrhosis, arthritis and metastasis (33). MMP-2 and MMP-9 are considered to be associated with metastasis.

RANKL is a protein that is encoded by the TNFSF11 gene in humans. Certain reports have indicated RANKL expression allows optimal microenvironmental conditions to be established to influence cancer cell migration, and RANKL is reported to be an important signal regulator in cancer-induced bone loss (37,38). RUNX2 is a key transcription factor that is associated with osteoblast differentiation. The expression level of RUNX2 was reported to be significantly higher in metastatic PCa and was positively correlated with EP4 receptor expression in PCa (39). Therefore, it is important to investigate the detailed molecular mechanisms of MMPs, RANKL and RUNX2 activation in PCa. In the present study, the results demonstrated that PGE₂ induced the protein and mRNA expression of MMP-2, MMP-9, RANKL and RUNX2, which was associated with increased proliferation and invasion in PCa cells and occurred primarily through the EP4 receptor.

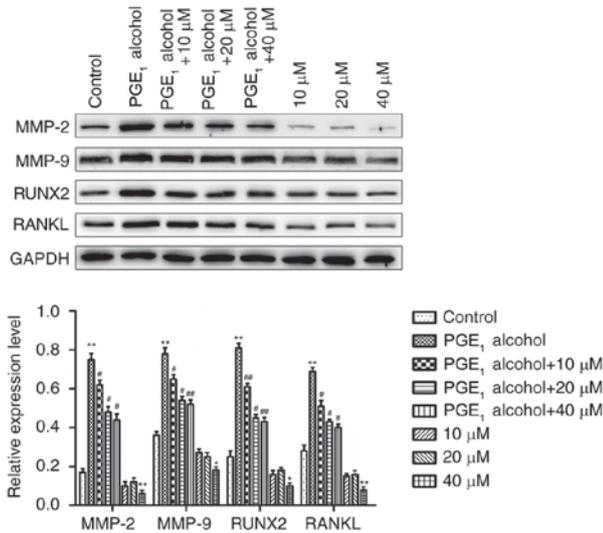


Figure 6. Involvement of the PI3K-Akt signaling pathway in EP4 receptor-mediated increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 prostate cancer cells. The specific PI3K inhibitor, LY294002, reduced the EP4 receptor-mediated increases in the protein expression levels of MMP-2, MMP-9, RANKL and RUNX2 in PC-3 cells. Results are presented as the mean \pm standard deviation of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group; # $P < 0.05$ and ## $P < 0.01$ vs. PGE₁ alcohol group. PI3K, phosphatidylinositol 3-kinase; PGE₁, prostaglandin E₁; EP4, PGE₂ receptor EP4; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor- κ B ligand; RUNX2, runt-related transcription factor 2; 10 μ M, 10 μ M LY294002; 20 μ M, 20 μ M LY294002; 40 μ M, 40 μ M LY294002.

Within the signaling pathway, the EP4 receptor normally couples with G_s α protein to activate AC and elevate intracellular cAMP levels in cells. Subsequently, the elevated cAMP levels lead to the activation of three major targets, including PKA, exchange protein directly activated by cAMP and cyclic nucleotide-gated ion channels (60). Of these targets, PKA has been reported to regulate various cellular processes, including metabolism, signal transduction and gene expression (61). The present study demonstrated that the cAMP-PKA signaling pathway may also be implicated in the EP4 receptor-mediated upregulation of MMP-2, MMP-9, RANKL and RUNX2 protein expression in PCa cells, as determined using an agonist and inhibitor of AC, and a specific inhibitor of PKA.

The PI3K-Akt pathway is associated with migration and survival of cells. In addition to PKA signaling pathway, the EP4 receptor also exerts effects through activation of Akt. Akt, also termed protein kinase B, has been demonstrated to be involved in the regulation of cell survival, proliferation and protein synthesis, and associated with tumor development (55,62). It has also been reported that the EP4 receptor may also function by coupling with non-G-proteins, such as activation of EGFR on the membrane and the intracellular Akt pathway. For example, in intrahepatic cholangiocarcinoma, the EP1 receptor led to the activation of Akt by binding to the Src kinase and EGFR. In a cutaneous tag, the EP2 receptor can activate the Akt pathway through β -arrestin 1-Src to signal transduction. Akt is inhibited by the specific inhibition of PI3K, for example, by using LY294002. Therefore, we hypothesized that the PI3K-Akt pathway may be involved in the EP4 receptor-mediated

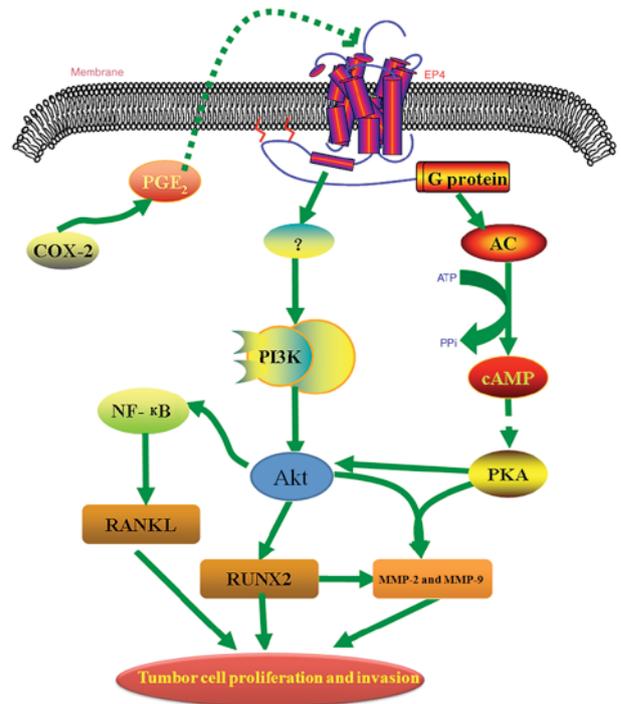


Figure 7. A proposed model indicating that PGE₂ may promote the proliferation and invasion of prostate cancer cells by upregulating the expression of MMP-2, MMP-9, RANKL and RUNX2 via the EP4 receptor and the cAMP-PKA/PI3K-Akt signaling pathways. PGE₂, prostaglandin E₂; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor- κ B ligand; RUNX2, runt-related transcription factor 2; EP4, PGE₂ receptor EP4; cAMP, cyclic AMP; PKA, protein kinase A; PI3K, phosphatidylinositol 3-kinase; COX-2, cyclooxygenase; AC, adenylyl cyclase; NF- κ B, nuclear factor- κ B; PPI, inorganic pyrophosphate.

upregulation of protein expression in PCa cells. The results in present study confirmed the hypothesis that the pretreatment of PCa cells with the specific inhibitor of PI3K significantly inhibited the EP4 receptor-mediated upregulation of protein expression.

The results of the current study confirmed that the EP4 receptor activated the cAMP-PKA/PI3K-Akt signaling pathway in PCa cells. Furthermore, it was demonstrated that this pathway may be involved in the EP4 receptor-mediated upregulation of MMP-2, MMP-9, RANKL and RUNX2 protein expression in PCa cells. In summary, the present study demonstrated that PGE₂ directly upregulated the expression of MMP-2, MMP-9, RANKL and RUNX2, potentially via the EP4 receptor and cAMP-PKA/PI3K-Akt signaling pathways, and thus promoted the proliferation and invasion of PC-3 cells. Therefore, targeting of the EP4 receptor may have potential as a novel therapeutic strategy to prevent and treat PCa; targeting of the downstream signaling components regulated by PGE₂ may be an improved therapeutic approach for overcoming severe side effects and health risks associated with the use of COX-2 inhibitors in patients with PCa.

Acknowledgements

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