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PGE_2 Regulates Pancreatic Stellate Cell Activity Via The EP4 Receptor

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Abstract

Objectives—Pancreatic stellate cells are source of dense fibrotic stroma, a constant pathological feature of chronic pancreatitis (CP) and pancreatic adenocarcinoma (PDAC). We observed correlation between levels of cyclooxygenase-2 (COX-2) and its product prostaglandin E2 (PGE_2) and the extent of pancreatic fibrosis. Aim of this study was to delineate the effects of PGE_2 on immortalized human pancreatic stellate cells (HPSC) and to identify the receptor involved.

Methods—IHC, RT-PCR and Q-RT-PCR were used to assess COX-2, extracellular matrix (ECM) and matrix metalloproteinases (MMP) gene expression. Eicosanoid profile was determined

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by LC/MS/MS. HPSC proliferation was assessed by MTS assay; migration by Boyden chamber assay and invasion using an invasion chamber. Transient silencing was obtained by siRNA.

Results—HPSC express COX-2 and synthesize PGE_2. PGE_2 stimulated HPSC proliferation, migration and invasion; stimulated expression of both ECM and MMP genes. HPSC expressed all four EP receptors. Only blocking the EP4 receptor resulted in abrogation of PGE_2 mediated HPSC activation. Specificity of EP4 for the effects of PGE_2 on stellate cells was confirmed using specific antagonists.

Conclusion—Our data indicate that PGE_2 regulates PSC profibrotic activities via EP4 receptor thus suggesting EP4 receptor as useful therapeutic target for pancreatic cancer to reduce desmoplasia.

Keywords

PGE_2; Pancreatic Cancer; Pancreatic Stellate cells; EP4 receptor

Introduction

Pancreatic fibrosis occurs as the result of a complex process of stellate cell activation, expression of matrix molecules followed by their synthesis, secretion, deposition, maturation and remodeling leading ultimately to a dense fibrous connective tissue (1,2). Such a dense fibrotic stroma is the best known hallmark of chronic pancreatitis and pancreatic cancer. This process which occurs after injury, a reparative or reactive process may be protective. However, when the dense stroma becomes extensive it can interfere with normal pancreatic function (3). Recently it has been suggested that this fibrotic stroma may also act as a physical barrier to drug delivery in pancreatic cancer therapy (4). Therefore, this fibrotic response is generally considered pathological and efforts to reduce the extent of this phenomenon are constantly being sought. However, the specific mechanisms involved in the initiation, remodeling, and resolution of pancreatic fibrosis are poorly understood (3). Fibrotic initiation is generally associated with inflammation and several inflammatory molecules are able to stimulate extracellular matrix production from the pancreatic stellate cells and several have been considered potential therapeutic targets (5). However, which regulators are most critical and might be the best therapeutic targets remains unclear.

Pancreatic inflammation is associated with a high level of COX-2 activity (6,7) which increases the production of prostaglandins including PGE_2 within the inflamed pancreas. PGE_2 has both pro-inflammatory and cell-protective activities (8). COX-2 and PGE_2 are correlated with worse prognosis in many cancers including lung, gastric and pancreatic (9,10). Prostaglandin endoperoxide synthase, commonly referred to as cyclooxygenase (COX), catalyzes the reduction of arachidonic acid (AA), to form prostaglandin E2 by microsomal PGE_2 synthases (11). Currently, there are three known COX isoforms COX-1, COX-2 and COX-3 (a splice variant of COX-1). COX-1 is a ubiquitously and constitutively expressed isoform that is postulated to have “housekeeping” functions with basal production of prostaglandins under homeostatic conditions. In contrast, COX-2 is encoded by an early-response gene and can be rapidly induced by growth factors, cytokines, inflammatory mediators and tumor promoters (12). By immunohistochemistry (IHC), COX-2 was found to be highly expressed in chronic pancreatitis (6), pancreatic adenocarcinoma (7, 13-15) and pancreatic intraepithelial neoplasia (PanIN) (16). The expression of COX-2 in chronic pancreatitis is primarily localized in the cytoplasm of pancreatic acinar cells, islet cells, and ductal cells (6), while in pancreatic adenocarcinoma it is localized primarily to cancer cells (7). Transgenic over-expression of COX-2 in the pancreas led to the development of pancreatic fibrosis and even cellular transformation (17). However, despite the strong

correlation between COX-2 and pancreatic fibrosis, the mechanisms involved in COX-2 mediated effects on the stroma are unclear.

Stellate cells are resident cells of the pancreas, located at the basolateral aspect of acinar cells under control conditions (2). In the normal pancreas, stellate cells are quiescent, identifiable by the presence of vitamin-A containing lipid droplets in the cytoplasm and positive immunostaining for cytoskeletal proteins such as desmin and glial acidic fibrillary protein (18). In health, PSC play a role in extracellular matrix turnover via their ability to both synthesize as well as degrade matrix molecules. During inflammatory injury, PSCs become activated and assume a myofibroblast-like phenotype characterized by the loss of vitamin A droplets, the production of α-SMA and extracellular matrix proteins such as collagen I and III, fibronectin and laminin (3). Factors known to be up-regulated during pancreatic injury such as TGF-β, plateletderived growth factor (PDGF) as well as proinflammatory cytokines, stimulate PSC proliferation and production of extracellular matrix proteins (19). Notably, activated PSC also produce increased amounts of matrix metalloproteinase-2 (MMP2) (20), known to degrade collagen IV, an essential component of the basement membrane, thereby facilitating the deposition of fibrillar collagenla as observed in pancreatic fibrosis. Despite this information, the major mechanisms regulating stellate cells and their development of pancreatic desmoplasia are not fully understood.

In the current study, we sought to elucidate the role of PGE_2 on pancreatic fibrosis in order to determine whether PGE_2 or its receptor might be a useful target for novel therapies designed to interfere with inflammation and reduce fibrosis. Hence, we have investigated the effects of PGE_2 on pancreatic stellate cells. Most of these studies utilized immortalized primary human pancreatic stellate cells (HPSC) which were previously characterized (21). However, key experiments were repeated with primary stellate cells which confirmed the observations. Our studies support the suggestion that HPSCs cells express COX-2 and secrete PGE_2(22). More importantly, we found that PGE_2 is a powerful regulator of stellate cells that increases their proliferation, migration and the production of extra cellular matrix (ECM) molecules. We further identified EP4 as the key prostaglandin receptor using both siRNA and specific antagonists. Our data indicate for the first time that EP4 is of key importance in PSC regulation. Because EP4 is a therapeutically tractable target this may facilitate the development of new approaches to improve therapies for chronic pancreatitis and pancreatic cancer.

Materials and Methods

Materials

Culture media and fetal bovine serum (FBS) were obtained from Life Technologies, Inc. (Rockville, MD). PGE_2, EP1 antagonist (cat # SC18220) and EP2 antagonist (cat # AH 6809) were purchased from Cayman Chemicals (Ann Arbor, MI). The EP4 antagonist (ONOAE3208) was obtained from ONO pharmaceuticals (Osaka, Japan).

Cell Culture

Immortalized human pancreatic stellate cells (HPSC) were isolated using the outgrowth method from pancreatic adenocarcinoma samples from patients undergoing surgical resection and were immortalized (21). HPSCs (HPSC developed from two different patients - lines 1 and 2) were maintained at 37°C in a humidified atmosphere of 5% CO_2 and were grown in 10% DMEM containing 1% antibiotic.

Immunohistochemical Staining for COX-2

Unstained 4micron tissue sections from human patients were deparaffinized with xylene and rehydrated with ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol and non specific binding sites were blocked with protein blocking solution (5% normal horse and 1% normal goat serum). Primary antibody against COX-2 (1:800 dilution; cat # HPA001335, Sigma Chemical) was added and the samples were incubated overnight at 4°C. The secondary antibody was added and incubated for 1 hour at room temperature. Finally, slides were developed with 3,3 diaminobenzidine substrate (DAB) and counterstained with hematoxylin. Then the slides were dehydrated with ethanol, fixed with xylene and mounted. Immunohistochemistry was analyzed using an inverted light microscope (Olympus, Center valley, PA). Images were digitally captured using a charge coupled device camera (Hamamatsu, Bridgewater, NJ) and smartcapture software (Digital Scientific, Cambridge, UK).

LS/MS/MS

Liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed to determine the profile of eicosanoids present in HPSC cellular extracts or HSPC conditioned medium. Media bathing the HPSC cells were collected after 15-20 hours of serum starving and used for the study.

Transient transfection of small interfering RNA

HPSC was plated on 100-mm dishes and transiently transfected with siControl, siEP2 and siEP4 (siRNA IDs # SI03650325, SI02757587, SI00019208, Qiagen, Valencia, CA) at a final concentration of 10 nmol/L with Hiperfect transfection reagent (Qiagen, Valencia, CA), serum starved overnight and treated with and without PGE2 (100nM) and RNA was prepared after 24h for RT-PCR and Q-RT-PCR. Relative expression was calculated after normalizing against β-Actin.

Invasion and Migration assays

For studies of cell invasiveness, BIOCOAT Matrigel invasion chambers (BD Biosciences, Chicago, IL) were used. Briefly, 2×10^4 HPSC cells with and without siRNA transfection were re-suspended in 100ul of serum-free medium were added to the upper chamber and different concentrations of PGE_2 (1–1000 nM) in 0.5% serum containing DMEM were added into the lower chamber. The cells were allowed to invade the Matrigel for 22 h at 37°C in a 5% CO2 atmosphere. DMEM containing 0.5% serum was used as control. The non-invading cells on the upper surface of the membrane were removed with a cotton swab and the invading cells on the lower surface of the membrane were fixed and stained with a Diff-Quick stain kit (BD Biosciences), washed twice with water and air-dried. Invading cells in three adjacent microscope fields for each membrane were imaged at 20× magnification. To assess cellular migratory potential, the protocol described above was used, except that migration chambers devoid of matrigel was used (BD Biosciences, Chicago, IL). Experiments were performed at least in triplicate, and the results were shown as mean ± SD of three independent experiments.

RT-PCR and Quantitative RT-PCR

Total RNA was isolated from HPSC with and without siRNA transfection. DNAse was used to remove contaminating genomic DNA and RNA was purified. Quality of the RNA was confirmed by running on a denaturing gel, and we have observed clear 28S and 18S rRNA bands. RT-PCR and Q-RT-PCR were conducted and the amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide. Primers designed for β-actin was used as a loading control for the PCR reactions. A non-reverse transcribed control was used

to assure that no genomic DNA was amplified. Primers were designed for RT-PCR (Sigma, St.Louis, MO) and Q-RT-PCR (TaqMan Probes, Applied Biosystems, Foster City, CA) Table 1.

Statistical analysis

All experiments were conducted in triplicates. Data presented are means of the three or more independent experiments +/− standard error mean (SEM). Statistical analysis was done using GraphPad Prism (GraphPad Software). Comparisons were made using two-tailed Student's *t* test and significant difference was defined as $P < 0.05$.

Results

COX2 levels are elevated within several cellular compartments of injured pancreas

Immunohistochemical analysis revealed high levels of COX-2 in tissue sections of human pancreatic tumors compared to normal pancreas tissues (Fig. 1A), as previously described (14,23). Additional micrographs of tissue sections are shown in Supplementary Figure 1A&B. Although COX-2 levels in PDAC cells were high, significant levels were also observed in the stroma. To more closely address the expression of COX-2 in pancreatic stroma, in isolated HPSC, we examined COX-2 expression by RT-PCR. We found that HPSC (lines 1 and 2) expressed COX-2 mRNA (Fig. 1B). Full-length gels are shown in Supplementary Figure 1C. To determine whether HPSC COX-2 was functional, we measured the level of PGE_2 produced by HPSC in culture (Fig. 1C). We observed an intracellular concentration of 20 ng/million cells of PGE_2 in stellate cell lysates, which was the highest among the eicosanoids examined. We also examined levels of secreted eicosanoids and again PGE_2 levels were found to be the highest (230 ng/million cells) (Fig. 1D). Similar results were obtained with both the lines of HPSC developed (line 2 data not shown). These data confirm that PGE_2 within pancreatic tumor is derived from both tumor cells and PSCs, as has previously been reported (24). Although, PGE_2 is present in the pancreatic tumor microenvironment, its influence on stellate cell behavior has not been well investigated.

PGE2 stimulates stellate cell proliferation, migration, invasion and stromal gene expression

To determine the effects of PGE_2 on stellate cells, we treated HPSC (line 1) with exogenous PGE_2 *in vitro*. HPSCs re-suspended in 0.5% serum containing media (2×10^4) were transferred to migration and invasion chambers and treated with and without PGE_2 (0-1000nM). Exogenous PGE_2 induced a dose-dependent increase in HPSC cell migration (Fig 2A) and invasion (Fig 2B). Significant effects were consistently noted at 50 nM and maximal effects occurred around 100nM of PGE_2. Higher concentrations of PGE_2 tended to generate reduced effects suggesting high dose inhibition. Similar results were obtained with both the lines of HPSC developed (line 2 data not shown).

In order to see if stimulation of HPSC with PGE_2 influenced stromal gene expression, RNA was extracted from HPSC treated with or without PGE_2 (100nM) and mRNA levels of several key molecules were analyzed. PGE_2 stimulated expression of several structural genes involved in extra cellular matrix formation including collagen1a1, heparan sulfate proteoglycan 2 (HSPG2), fibronectin and elastin as well as vimentin, an intermediate filament cytoskeletal protein found in mesenchymal cells (Fig 2C). Full-length gels are shown in Supplementary Figure 2A-E. Exogenous PGE_2 also stimulated genes involved in matrix turnover and cellular invasion. These molecules included the increased expression of matrix metalloproteinases (MMPs) MMP-2, MMP-3, MMP-9, and tissue inhibitors of

metalloproteinases-1 (TIMP-1) mRNA, whereas the mRNA levels of MMP-11 and TIMP-2 remained unchanged (Fig 2D). ull-length gels are shown in Supplementary Figure 3A-D.

The EP4 receptor is required for the effects of PGE2 on stellate cells

To determine the receptor(s) which might be responsible for the effects of PGE_2 on stellate cells, we first identified which receptors were expressed by these cells. RNA was prepared from HPSC cells and RT-PCR was conducted with primers specific for each of the four types of EP receptors. We observed that mRNA for all four receptors, EP1-4 was expressed in the HPSC cells (Fig 3A). Based on what is known about the effects of PGE_2 on pancreatic cancer cells, we decided to examine the importance of the most likely candidates for these effects, EP2 and EP4 receptors, using siRNA mediated silencing. The siRNA silencing was highly effective at reducing the appropriate mRNAs as indicated by RT-PCR (Fig 3B, C). Full-length gels are shown in Supplementary Figure 4A&B. No effects of these specific siRNAs were noted on the non-targeted EP receptors (data not shown).

Next, we investigated the effects of silencing EP2 and EP4 receptors on PGE_2 mediated biological effect on stellate cells. We observed that transfection with a control siRNA had no effect to reduce PGE_2 stimulated HPSC migration or invasion (Fig 4A, B). Likewise, silencing of EP2 did not show a reduction in these parameters. However, silencing of EP4 dramatically reduced the effects of exogenous PGE_2 on both migration and invasion. We also examined the effects of silencing EP4 receptors on the ability of exogenous PGE_2 to induce expression of Collagen 1A1, MMP-2 and MMP-9 (Fig 4C). Transfection with EP4 targeting siRNA significantly reduced EP4 expression as well as PGE_2 stimulated expression of Collagen 1A1 and the matrix metalloproteinases, MMP 2 & 9. Full-length gels are shown in Supplementary Figure 5A-C. The same results were confirmed by performing quantitative RT-PCR also (Fig 4D). Exogenous addition of PGE_2 significantly stimulated the expression of MMP-2 (0.4 fold), MMP-9 (0.75 fold) and Collagen 1A1 (0.4 fold), while silencing EP4 receptor resulted in significant decrease in the expression of MMP-2 (1 fold), MMP-9 (1 fold) and Collagen 1A1 (0.5 fold). EP4 silencing was also confirmed to be significantly reduced (1 fold).

The role of EP4 as the critical receptor for stellate cell regulation by PGE_2, was confirmed pharmacologically using receptor specific small molecule inhibitors. In these Boyden chamber migration assays, 10μM concentrations of selective EP antagonists (25) were added to the lower chambers 1 hour prior to PGE_2 treatment in serum free media. Treatment of HPSCs with antagonists for either EP1 or EP2 receptors did not affect cell migration as quantified by DAPI staining. In contrast, HPSC pre-treated with the specific antagonist against the EP4 receptor (ONO-AE3-208) significantly reduced cell migration ($P<0.05$) (Fig 5A). Likewise, when HPSCs were treated independently with specific antagonists against either EP1 or EP2 receptors, neither affected matrigel invasion. However, again the EP4 antagonist significantly reduced HPSC invasion through matrigel ($P<0.05$) (Fig 5B).

Discussion

PSCs have recently become the focus of much attention due to their importance in PDAC. The fibrotic stroma that they elaborate serves as the specific niche in which the cancer cells develop. Furthermore, this stroma supports the survival of the cancer cells (21,26) and may provide a physical barrier to some therapies (4). However, stellate cells also play a critical role in chronic pancreatitis (1,27). In both diseases, PSCs produce an abundant desmoplastic response that ends up replacing normal parenchyma and contributing to pancreatic insufficiency. This stroma can also impinge on nerves to cause pain and can block the common bile duct leading to jaundice. Many factors regulate PSC activity including cytokines and several growth factors such as TGFβ, PDGF, and CTGF (28). However, the

regulation of PSCs by prostaglandins has not been extensively evaluated say for one study using normal rat stellate cells in combination with human tumor cells (24). The use of PANC-1 conditioned medium (CM) stimulated rat stellate cells to produce COX-2 that was prevented by U0126, an extracellular signal-regulated kinase (ERK) 1/2 inhibitor. Furthermore, NS398, a selective COX-2 inhibitor, reduced the growth of PSCs by PANC-1 CM. Several studies have indicated that COX-2 expression is high in both chronic pancreatitis and PDAC therefore suggesting a potential role in fibrosis (6,29). COX-2 levels are also known to positively correlate with the aggressiveness of PDAC (30) and that blocking COX-2 significantly reduces pancreatic tumor size and metastatic potential (31) in animal models. No studies to date, however, have examined the downstream role of PGE_2 or the EP receptors in HPSCs. Our data are the first to suggest that targeting EP4 receptors may be a useful way to reduce stellate cell activity associated with pancreatic inflammation and fibrosis.

Previous studies have shown that inhibiting the COX-2 pathway suppresses the development of many cancers including pancreatic cancer (32). Also, COX-2 gene expression and its primary metabolite prostaglandin E2 (PGE_2) were highly expressed in 90% of pancreatic tumors (7). The majority of studies have focused on the tumor cells. Our studies by contrast, highlight the importance of COX-2 and PGE_2 in the profound desmoplastic reaction associated with PDAC. As a pharmacologic intervention, celecoxib is a non-steroidal anti-inflammatory drug (NSAID) and a highly selective COX-2 inhibitor. Traditional NSAIDs can inhibit both COX-1 and COX-2, to varying degrees. Selective inhibition of COX-2 by celecoxib was found to effectively suppress the growth of pancreatic cancer cells *in vitro* (17) and to inhibit PDAC tumor growth and angiogenesis in orthotopic implantation tumor models (33). More recently it was shown that Celecoxib could slow or prevent the progression of early PanIn lesions in mouse models to metastatic disease (32). These data strongly support inhibition of this pathway as a therapeutic approach for PDAC.

Unfortunately, Celecoxib has been found to be a poor therapeutic. We now know that COX-2 inhibition impacts negatively on cardiac and renal, which causes to serious side effects of prolonged inhibition of this pathway (34). In pancreatic cancer clinical trials, celecoxib in combination with standard chemotherapeutic drugs was found to be modestly effective in some studies but in other studies the effectiveness was limited by toxicity (35-39). From all of the available data it appears that inhibition of COX-2/PGE2 pathway is effective, but celecoxib does not appear to be the best therapeutic to inhibit this pathway. In the current study, we found that the effects of PGE_2 on PSC were specifically mediated by the EP4 receptor. These data contrast with those reported for the PDAC cells where the EP2 receptor is thought to be most important (40). Stromal formation in lung cancer has also been reported to involve EP4 receptors (41). Targeting EP4 receptors has recently been found to decrease foci formation, metastasis and tumor incidence in colon cancer (42). To date several toxicity studies have been conducted and no toxicity has been reported for EP4 antagonist. Barring any toxicity issues associated with selective downstream targeting of EP4, this may be an important new approach to control pancreatic fibrosis.

Although the importance of stroma in PDAC is well known, the molecular mechanisms that regulate stromal activity and the downstream signaling remain poorly understood. Our study provides the first evidence that PGE_2 induces major behavioral changes in PSCs. These changes include contribution to the proliferation, migration and activity of PSCs in the formation of the collagen I matrix. These effects are mediated by the EP4 receptor. Furthermore, that fibrotic activity of the stellate cells is tightly regulated by the EP4 receptor as a potential target for the prevention of pancreatic fibrosis or as adjuvant treatment administered along with treatments for PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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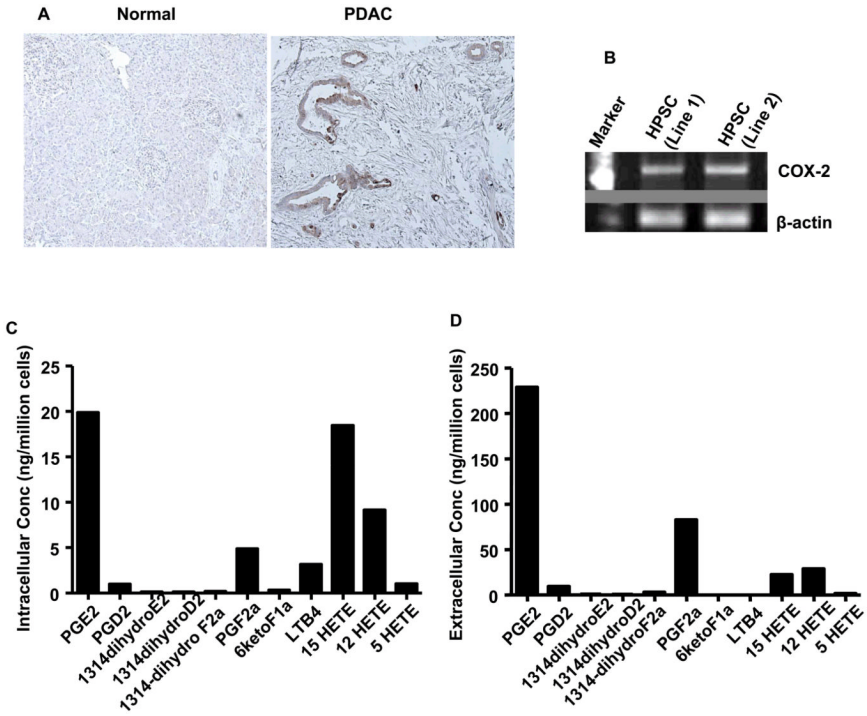

Fig.1. COX-2 expression and eicosanoid levels in PDAC and the stroma

(A) Immunohistochemistry showing the levels of COX-2 in normal and PDAC tissue sections from human samples. (B) RT-PCR showing the expression of COX-2 in HPSC. Liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed to determine the profile of eicosanoids present in HPSC cellular extracts (C) or HSPC conditioned medium (D).

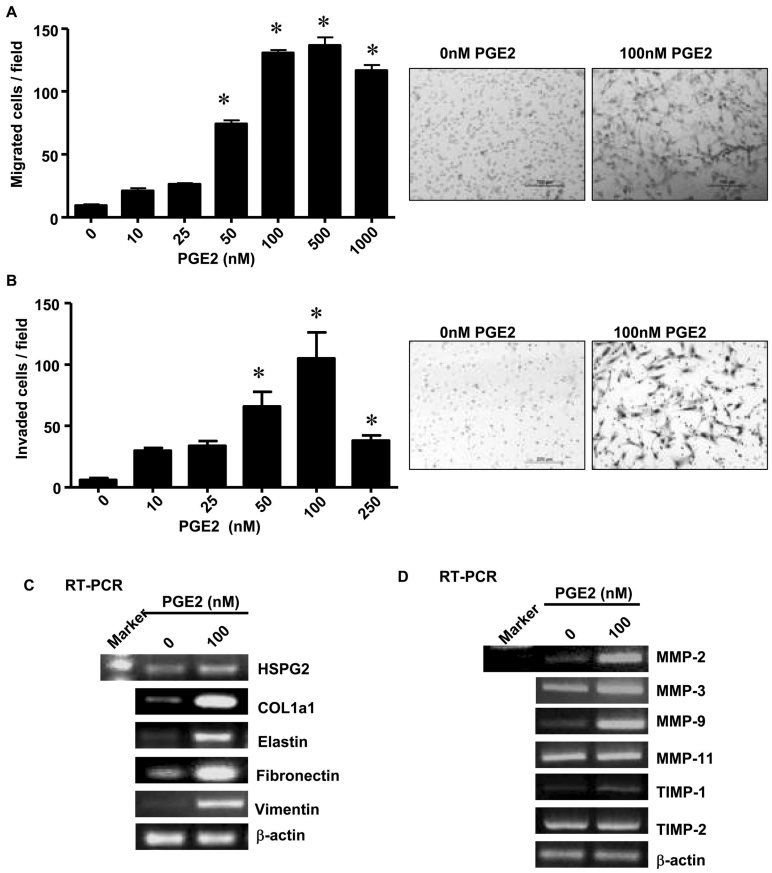

Fig.2. PGE_2 stimulates migration, invasion and gene expression

Cell migration and invasion were assessed by counting the number of stained cells that penetrated the migration membrane after 24h following PGE_2 (100nM) stimulation. (A) membrane alone (right, stained cells; left migration numbers $p<0.05$ versus control) (B) matrigel coated membrane (right, stained cells; left migration numbers $p<0.05$ versus control) (C) RT-PCR showing the expression of HSPG2, COL1A1, elastin, fibronectin, vimentin and (D) the expression of MMP-2, 3, 9, 11 and TIMP-1, 2 before and after stimulation with PGE_2 (100nM). β-actin served as control.

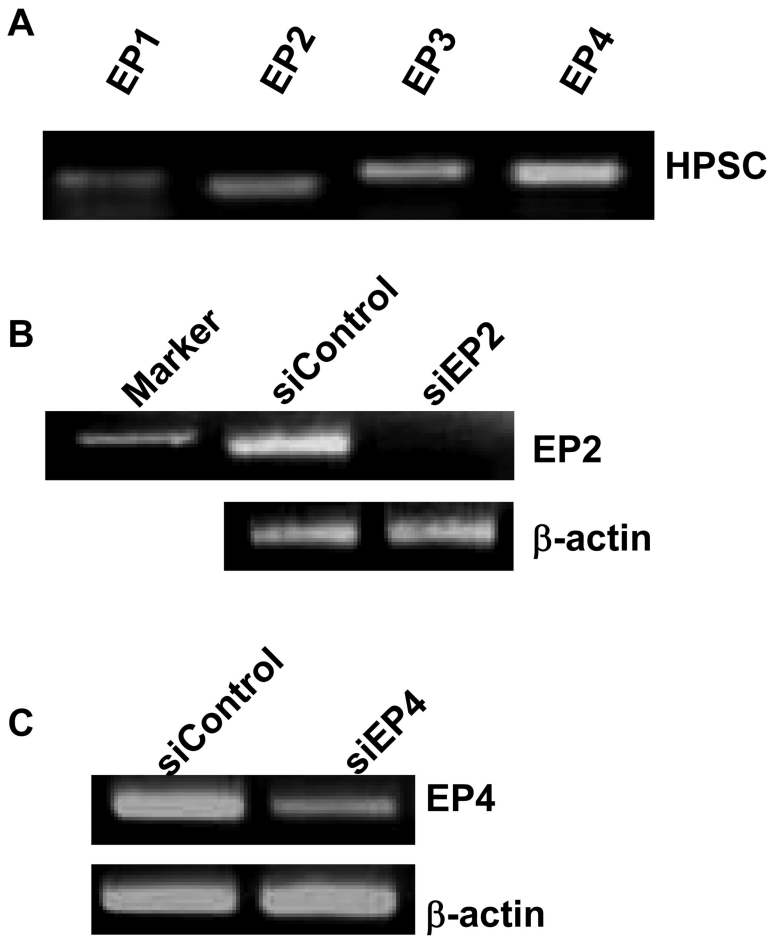

Fig.3. EP receptor differential expression by HPSC and silencing by siRNA

(A) EP1, 2, 3, and 4 receptor mRNA are differentially expressed by HPSC as determined by RT-PCR (B) silencing of mRNA expression was achieved by transient transfection with human siControl and siEP2 (B) or siEP4 (C) β-actin served as control.

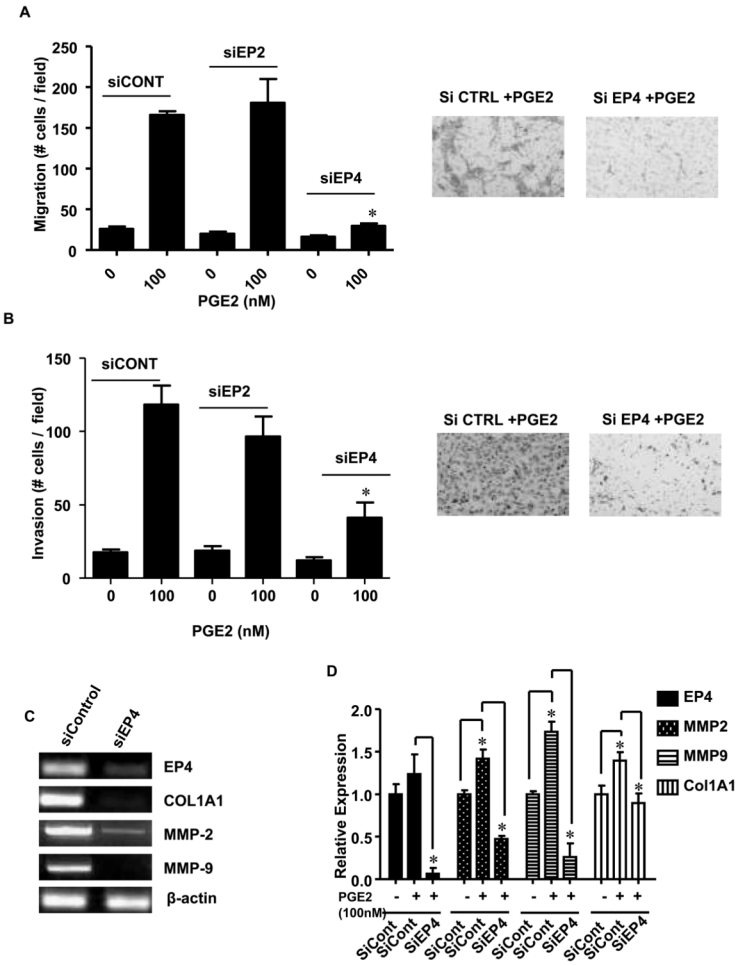

Fig.4. Effects of EP2 and EP4 silencing on PGE_2 mediated HPSC functions

HPSC were transiently transfected with siControl, siEP2 or siEP4. After 48 hours, cell migration and invasion were assessed by counting the number of stained cells that penetrated the migration membrane after 24h following PGE_2 (100nM) stimulation. (A) membrane alone (right, stained cells; left migration numbers $p<0.05$ versus control) (B) matrigel coated membrane (right, stained cells; left migration numbers $p<0.05$ versus control) (C) RT-PCR shows the effects of PGE_2 mediated stimulation of COL1A1, MMP-2 and 9 after the silencing of EP4 receptor. β-actin served as control. (D) Q-RT-PCR shows the effects of PGE_2 mediated stimulation of COL1A1, MMP-2 and 9 and decrease after the silencing of EP4 receptor. β-actin served as control.

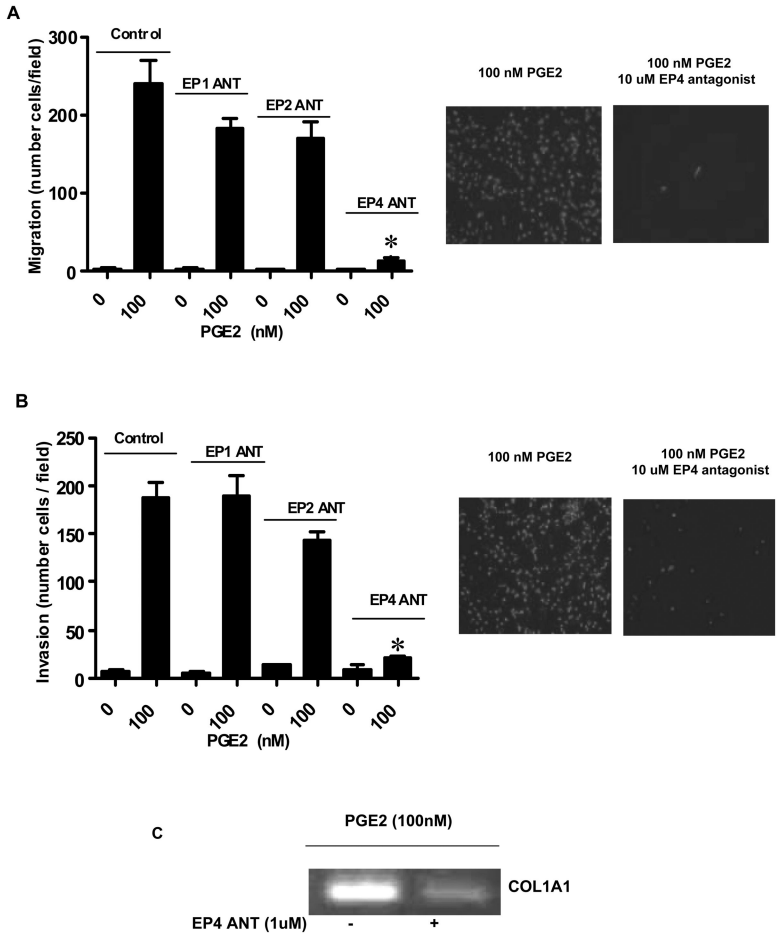

Fig.5. Effects of specific EP receptor antagonists on PGE_2 mediated HPSC functions

HPSC were plated and allowed to settle overnight. Serum containing media was re-plated with serum free media for 24 hours before addition of EP1, EP2 or EP4 antagonists at a dose of 10μM. 1 hour after the antagonist addition, cell migration and invasion were assessed by counting the number of DAPI stained cells that penetrated the migration membrane after 24 h following PGE_2 (100nM) stimulation. (A) membrane alone (right, stained cells; left migration numbers $p<0.05$ versus control) (B) matrigel coated membrane (right, stained cells; left migration numbers $p<0.05$ versus control). Addition of EP4 antagonist showed a significant reduction of PGE_2 mediated HPSC migration and invasion. (C) RT-PCR showing the reduction in collagen 1A1 expression on treatment with EP4 antagonist (10μM) as compared to increase on treatment with PGE_2 (100nM) alone.

Table 1

Primer Sequences

RT-PCR Primers:	
EP1: (5′ atc gct tcg gcc tcc acc ttc ttt 3′)	(3′ gcc agc gcc acc aac acc a 5′)
EP2: (5′ctc gct gcc gct gct gga cta tgg3′)	(3′gca ggc gag cac cga gac aat gag 5′)
EP3: (5′ ggc gct ggc gat gaa caa cga g 3′)	(3′ ggc gct gga gat gaa caa cga g 5′)
EP4: (5′ ccg ccc cca ggt agc cag gag 3′)	(3′tgc ggg agg aca gcg ttc agg t 5′)
MMP-1: (5′ att cta ctg ata tcg ggg ctt tga3′)	(3′ tgt cct tgg ggt atc cgt gtg tag5′)
MMP-2: (5′ ctt ctt gtc gcg gtc gta gtc ctc3′)	(3′ tgg cga tgg ata ccc cct tga5′)
MMP-7: (5′aaa ctc ccc gcg tca tag aaa taa t3′)	(3′ tga gtt gca gca tac agg aag tt 5′)
MMP-9: (5′ gcg ctg ggc tta gat cat tcc tca 3′)	(3′ gca gcg cgg gcc att gtc 5′)
MMP-11: (5′ctg gcg ggc gct ggg aga aga c3′)	(3′cag ggc tgg cca tat agg tgt tga5′)
TIMP-1: (5′ cgt cat cag ggg cca agt tcg tg 3′)	(3′ gag gca ggc agg caa ggt gac 5′)
TIMP-2: (5′ ctg gcg ggc gct ggg aga agac3′)	(3′cag ggc tgg cca tat agg tgt tga3′)
Fibronectin: (5′ccg cca cgt gcc agg att acc3′)	(5′agg ggc tcg ctc ttc tga tta ttc 3′)
Collagen1A1: (5′tgt cca ccg agg ctc cca gaa c3′)	(5′ ccc agg ctc cgg tgt gac tcg tg 3′)
Vimentin: (5′ ggt ccg tgt cct cgt cct cct ac 3′)	(3′ cgc ggg ctt tgt cgt tgg tta 5′)
Elastin: (5′gga ccc ctg act cac gac ctc 3′)	(5′ act tgg ccg ctc ccc tct tgt ttc 3′)
HSPG2: (5′ ccg cca ggg cag gtc a 3′)	(3′ ggt ggg cag cgg tag gaa gga gta 5′)
COX-2: (5′ ggt ctg gtg cct ggt ctg atg atg 3′)	(5′ gtc ctt tca agg aga atg gtg c 3′)
β-actin: (5′ atg ata tcg ccg cgc tcg tcg tc 3′)	(5′ cgc tcg gcc gtg gtg gtg aa 3′)
Q-RT-PCR Primers:	
EP4:	Hs00168761
MMP2:	Hs00234422
MMP9:	Hs00234579
COL1A1:	Hs00164004
β-actin:	4352935E