Prostaglandin E_2 Inhibits Human Lung Fibroblast Chemotaxis through Disparate Actions on Different E-Prostanoid Receptors

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The migration of fibroblasts is believed to play a key role in both normal wound repair and abnormal tissue remodeling. Prostaglandin E $(PGE)_2$, a mediator that can inhibit many fibroblast functions including chemotaxis, was reported to be mediated by the E-prostanoid (EP) receptor EP2. PGE_2, however, can act on four receptors. This study was designed to determine if EP receptors, in addition to EP2, can modulate fibroblast chemotaxis. Using human fetal lung fibroblasts, the expression of all four EP receptors was demonstrated by Western blotting. EP2-selective and EP4-selective agonists inhibited both chemotaxis toward fibronectin in the blindwell assay and migration in a wound-closure assay. In contrast, EP1-selective and EP3-selective agonists stimulated cell migration in both assay systems. These results were confirmed using EP-selective antagonists. The role of both EP2 and EP4 receptors in mediating the PGE_2 inhibition of chemotaxis was also confirmed by small interfering RNA suppression. Furthermore, the role of EP receptors was confirmed by blocking the expected signaling pathways. Taken together, these results demonstrate that PGE_2 can act on multiple EP receptors in human lung fibroblasts, to exert disparate effects. Alterations in EP receptor expression may have the potential to alter PGE_2 action. Targeting specific EP receptors may offer therapeutic opportunities in conditions characterized by abnormal tissue repair and remodeling.

Keywords: human lung fibroblast; cell migration; EP receptors

Cell migration plays a fundamental role in many biological processes, including embryonic development, tissue repair and regeneration, tissue remodeling, immune surveillance, tumor-cell invasion, and metastasis (1, 2). The accumulation of fibroblasts plays a critical role in normal tissue repair and in the development of fibrosis (3, 4). Consequently, an abnormal migration of fibroblasts likely contributes to the pathogenesis of many conditions. The increased migration of fibroblasts derived from lungs of patients with idiopathic pulmonary fibrosis (IPF), compared with normal control subjects, was suggested to contribute to fibrosis (5, 6). Conversely, the reduced migration of fibroblasts cultured from the lungs of patients with chronic obstructive pulmonary disease (COPD) was suggested to contribute to the pathogenesis of emphysema (7, 8). Understanding the mechanisms that regulate lung fibroblast migration will help delineate disease pathogenesis, and may identify molecular targets to treat diseases associated with lung remodeling.

The arachidonic acid cyclooxygenase metabolite prostaglandin E_2 (PGE_2) is a biologically important lipid involved in processes as diverse as pain, fever, and inflammation. It is a potent inhibitor of fibroblast functions, including chemotaxis (9, 10), proliferation (11), matrix production (12, 13), and remodeling (14). PGE_2 is a major product of lung fibroblasts (15) and alveolar macrophages (16). It is present in the lung at concentrations that are near the midrange of its concentration dependence, so that either increases or decreases in PGE_2 may exert a physiologic effect on lung fibroblasts (17, 18). A role for PGE_2 was suggested in a variety of pulmonary diseases, including COPD (7, 19), IPF (17), asthma (18, 20), cystic fibrosis (21), and lung cancer (22).

PGE_2 exerts its actions by acting on a group of four G protein–coupled receptors (designated E-prostanoid [EP] 1, EP2, EP3, and EP4) (23). EP1 regulates Ca^{2+} channel gating via an unidentified G protein. The EP2 and EP4 receptors couple to stimulatory G protein (G_s) and mediate increases in cyclic adenosine monophosphate (cAMP) concentrations. In addition, the EP4 receptor can signal through phosphoinositol-3-kinase (PI3K). The major signaling pathway for the EP3 receptor involves the inhibition of adenylyl cyclase via inhibitory G protein (G_i). However, the EP3 receptor can undergo multiple differential splicing, and can signal via a number of pathways.

PGE_2 was reported to inhibit the migration of normal fibroblasts through actions on the EP2 receptor that signals via the cAMP-dependent protein kinase A (PKA) pathway, and through an increase in phosphatase and tensin homologue on chromosome 10 (PTEN) activity (10). However, whether PGE_2 can also modulate fibroblast migration through other EP receptors is unclear. This study explored the role of the EP1, EP2, EP3, and EP4 receptors in modulating human lung fibroblast migration, using the blindwell chemotaxis assay and a wound-closure assay.

MATERIALS AND METHODS

Materials

Table 1 summarizes the pharmacologic compounds used in this study, and describes their specificity (9, 24–34). PGE_2 was purchased from Sigma (St. Louis, MO) and was dissolved in 100% ethanol as a stock solution of 10^{-3} M, and further diluted in medium to the designated concentrations. The EP1 agonist ONO-DI-004, EP2 agonist ONO-AE1-259, EP3 agonist ONO-AE-248, EP4 agonist ONO-AE1-329, EP1 antagonist ONO-8713, EP3 antagonist ONO-AE3-240, and EP4 antagonist ONO-AE3-208 were gifts from the ONO Pharmaceutical Co. (Lawrenceville, PA). The EP2 antagonist AH6809 was purchased from Cayman Chemical (Ann Arbor, MI). The phosphatidylinositol-3–kinase (PI3K) inhibitor wortmannin and the inhibitory G protein (G_i) inhibitor

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TABLE 1. COMPOUNDS USED AND TARGET SELECTIVITY

Name	Selectivity	Activity	References
ONO-DI-004	EP1	Agonist	(24, 25)
ONO-AE1-259	EP2	Agonist	(25, 26)
ONO-AE-248	EP3	Agonist	(24, 25)
ONO-AE1-329	EP4	Agonist	(25, 26)
ONO-8713	EP1	Antagonist	(27)
AH6809	EP2	Antagonist	(27)
ONO-AE3-240	EP3	Antagonist	(28)
ONO-AE3-208	EP4	Antagonist	(29, 30)
KT-5720	PKA	Inhibitor	(9, 31)
SQ-22536	Adenylyl cyclase	Inhibitor	(31)
Y-27632	Rho kinase	Inhibitor	(32)
Wortmannin	PI3K	Inhibitor	(33)
Pertussis toxin	G_i	Inhibitor	(34)

Definition of abbreviations: EP, E-prostanoid; PKA, protein kinase A; PI3K, phosphatidylinositol-3–kinase.

pertussis toxin (PT) were from Sigma. The PKA inhibitor KT-5720, the adenylyl cyclase inhibitor SQ-22536, and the Rho kinase inhibitor Y-27632 were from Calbiochem (San Diego, CA). Anti-EP1, anti-EP2, anti-EP3, and anti-EP4 antibodies were purchased from Cayman Chemical (Cayman, Ann Arbor, MI). Anti–β-actin antibody was from Sigma. The Rho Activation Assay Kit (catalogue number 17-294) was purchased from Millipore-Upstate Cell Signaling Solutions (Lake Placid, NY).

Cell Culture

Human fetal lung fibroblast (HFL-1) cells were purchased from the American Type Culture Collection (Rockville, MD). Adult human bronchial fibroblasts were obtained from an endobronchial biopsy (35). Cells were cultured in 100-mm tissue culture dishes (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 50 U/ml penicillin G sodium, 50 mg/ml streptomycin sulfate, and 0.25 mg/ml Fungizone, and maintained at 37°C in a humidified 5% CO_2 incubator. Fibroblasts were routinely passaged every 4 or 5 days, and cells were used between passages 13–20 in all experiments. Confluent fibroblasts were detached from culture dishes by treatment with 0.05% trypsin in 0.53 mM EDTA, harvested into DMEM with 10% FCS to inactivate the trypsin, and resuspended in serum-free DMEM (all from Invitrogen, Carlsbad, CA).

Chemotaxis Assay

HFL-1 cell chemotaxis was assessed by the Boyden blindwell chamber technique (11), using a 48-well chamber (Nucleopore, Cabin John, MD). HFL-1 cells (1×10^6 cells/ml in DMEM without serum) were loaded into the upper well of the chamber with the desired concentration of PGE_2, EP agonist, or other additives. The chemoattractant, human fibronectin (5 μg/ml; Invitrogen), was placed in the bottom chamber in DMEM without serum. The two wells were separated by an 8-μm pore filter (NeuroProbe, Gaithersburg, MD). The chamber was incubated at 37°C in a humidified 5% CO_2 incubator for 6 hours, and adherent cells on the upper surface of the membrane were removed by scraping. Migrated cells attaching to the bottom of the membrane were fixed and stained with the Protocol Hema 3 Manual Staining System (Biochemical Science, Swedesboro, NJ). Membranes were then mounted on a glass microscope slide, and the number of migrated cells was counted in five high-power fields with a light microscope. The sum of migrated cells in the five separate fields was used as the measure of chemotaxis for that well. In each experiment, three separate wells were evaluated for each condition. In general, statistical comparisons were performed within each experiment. Each experiment was repeated on separate occasions at least three times, each including triplicate wells per condition.

Wound-Closure Assay

Before plating the cells, the large end of a sterile pipette tip was placed in the central area of a 12-well tissue culture dish to prevent access of cells. Afterward, 1.0 ml of DMEM supplemented with 10% FCS containing 1×10^5 cells/ml was gently placed in the well. Cells were then cultured at 37°C, and the medium was changed every 2 days until confluent (generally for 4 days). The medium was then changed again, and 1 day later, the wound was introduced into the central area of the confluent monolayer by gently removing the pipette tip. Cultures were then incubated in DMEM supplemented with 1% FCS containing PGE_2 or EP receptor agonists at 37°C for various times, after which the cell layers were fixed and stained with the Protocol Hema 3 Manual Staining System (Biochemical Science). Photomicrographs were taken with a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan), equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan). Low-power photomicrographs were taken with a Sony H2 digital camera (Sony, Tokyo, Japan) without magnification. Quantification of wound closure was performed by superimposing a rectangular grid on the digitized images and manually counting boxes that were free of cell nuclei.

Western Blotting

Cell layers were washed twice with ice-cold PBS and homogenized in cell lysis buffer (35 mM Tris-HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 mg/ml aprotinin, and 1 mg/ml leupeptin). The lysates were briefly sonicated and then centrifuged ($13{,}000 \times g$) for 10 minutes to remove debris. The protein concentration in the supernates was determined using the Bio-Rad Protein Assay (Bio-Rad, Minneapolis, MN). Protein samples were separated by 10% SDS-PAGE and transferred to an Immuno-Blot PVDF Membrane (Bio-Rad) with a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were blocked with 5% skim milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature, and incubated with primary antibodies at 4°C overnight. Horseradish peroxidase (HRP)–conjugated IgG secondary antibodies and the ECL Western blot detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for visualization.

Rho Activation Assay

Guanosine triphosphate (GTP)-bound active Rho was assessed by pull-down assays, following the manufacturer's instructions (catalogue number 17-294; Millipore-Upstate). Briefly, HFL-1 cells were cultured to ~ 70–80% confluence, the medium was changed to DMEM without serum, and after an overnight incubation, cell layers were stimulated with EP1 and EP3 agonist (10^{-6} M) for 10 minutes or sphingosine 1–phosphate (S1P; Sigma) (10^{-6} M) for 5 minutes. Precipitated GTP-bound Rho was subjected to 10% SDS-PAGE and immunoblotted, using anti-Rho (A, B, and C) monoclonal antibodies. After washing and applying anti-mouse HRP–conjugated antibody, enhanced chemiluminescence (ECL) reagent was used for visualization, as already described.

RNA Interference

RNA interference was performed using small interfering RNA (siRNA) specific for EP2 and EP4 receptors (SMART Pool; Dharmacon, Lafayette, CO). Transfection reagent–siRNA complexes were prepared by using Lipofectamine 2000 and Opti-MEM (Invitrogen), according to the manufacturer's instructions. HFL-1 cells were plated into 100-mm dishes in DMEM with 10% FCS, and cultured for 24 hours. Cells were 50% confluent at the time of siRNA transfection. Cells were incubated with the siRNA (200 nM) or nontargeting RNA as the control in DMEM without FCS and antibiotics for 6 hours at 37°C. The medium was then changed to DMEM 10% FCS, and cells were cultured for an additional 42 hours at 37°C. The siRNA-treated cells were then used to assess EP2 and EP4 receptor expression by immunoblotting, or to assess the role of PGE_2 in HFL-1 chemotaxis.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) for multiple replica experiments conducted on separate occasions. In general, each experiment was performed in triplicate for each condition. Data were analyzed for significance using one-way ANOVA, followed by the Dunnett test to adjust for multiple comparisons with controls (Stat Mate III software package; ATMS Digitals Medical Station, Tokyo, Japan). Paired data were evaluated by Student *t* test. $P < 0.05$ was considered significant.

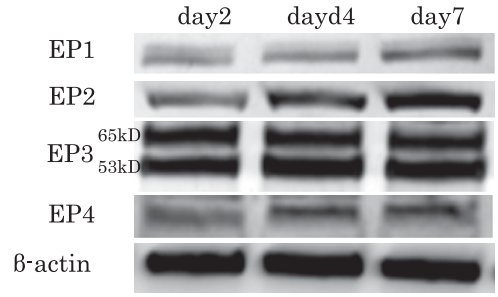

Figure 1. Expression of E-prostanoid (EP) receptors in human fetal lung fibroblast (HFL-1) cells. Cells were seeded in 100-mm tissue culture plates at a cell density of 1×10^5/ml in DMEM with 10% FCS, at 10 ml/dish, on Day 0 and fed again every 2 days. Medium was changed to DMEM without serum and incubated overnight before extracting protein. Cell lysates from HFL-1 cells harvested on Days 2, 4, and 7, and aliquots containing 20 μg total protein, were subjected to immunoblotting for the EP1, EP2, EP3, and EP4 receptors. Aliquots containing 1:10 of the amount loaded for EP receptor staining (2 μg protein) were stained for β-actin and used as a loading control. Molecular weights for two forms of EP3 are indicated.

RESULTS

Expression of EP Receptor in HFL-1 Cells

To examine the receptors through which PGE_2 mediates its effects on HFL-1 chemotaxis, we first assessed the expression of all four EP receptors on HFL-1 cells by Western blotting. All four EP receptors were expressed in HFL-1 cells at all culture time points evaluated. The expression of all four EP receptors increased with increasing time in culture after plating (Figure 1). The expression of receptors was not dramatically affected by cell density, as determined by plating cells at different densities and harvesting after 3 days, or by removing serum for the final 24 hours of culture (shown in Figure E1 in the online supplement).

Cell Density Dependence

Empiric observations suggest that the chemotactic response of HFL-1 cells varies as a function of cell density. This observation was further evaluated by plating cells at low density, followed by sequential harvests as the cells replicated and the cultures became denser. Chemotaxis was greatest at the earliest time point, and decreased as cells became more confluent (Figure 2A). Cells were seeded at a density of 1×10^5/ml, 10 ml/dish, on Day 0, and were cultured in DMEM supplemented with 10% FCS. Two days later, the number of HFL-1 cells that migrated in response to fibronectin was 463 ± 76 per five high-power fields (high migratory capacity cells). By Day 7, the number of HFL-1 cells that migrated to fibronectin was only 65 ± 12 per five high-power fields ($P < 0.002$). PGE_2 inhibited chemotaxis at all time points, although the absolute magnitude of the effect decreased as the baseline chemotaxis decreased. Although a slight tendency was evident for PGE_2 to inhibit less at high density (55% versus 65%), this finding was not significant. The effects of cell density on chemotactic activity were confirmed by plating cells at different densities and then harvesting after 3 days. Chemotactic activity increased as plating density and the final cell number decreased (Figure E2).

Because the four EP receptors appeared to increase with time in culture, the effect of each receptor on chemotaxis was assessed in low-density (high migration) and high-density (low migration) cells. As shown in Figure 2B, for low-density, high-migration cells (the number of migrated cells for the experiment

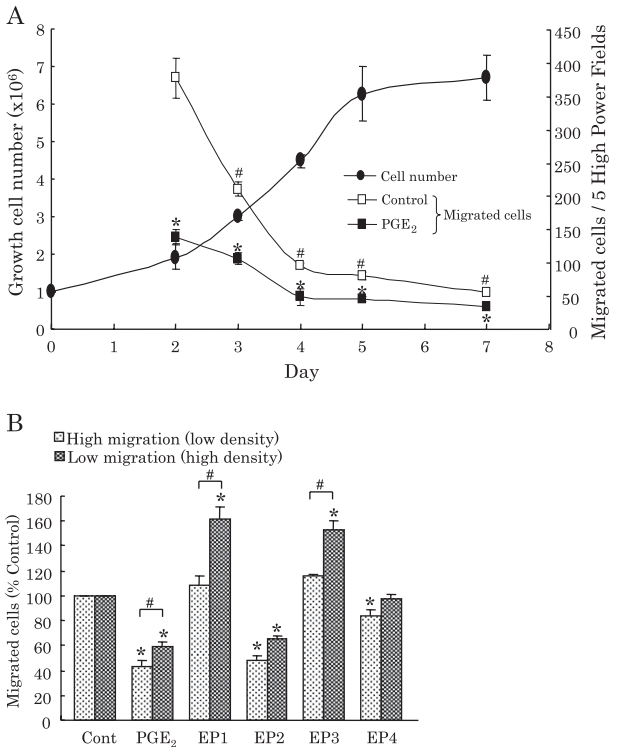

Figure 2. Cell density and fibroblast chemotaxis. (*A*) Relationship between fibroblast chemotaxis and cell density. Cells were seeded in 100-mm tissue culture plates at a cell density of 1×10^5/ml, at 10 ml/dish, on Day 0 in DMEM containing 10% FCS, and medium was changed every 2 days. The cell number (*left vertical axis*) was counted with a Coulter counter (Beckman Coulter, Brea, CA), and fibroblast chemotaxis (*right vertical axis*) was evaluated with or without prostaglandin E_2 (PGE_2) (10^{-7} M) on Days 2, 3, 4, 5, and 7. Chemotaxis data consist of means ± SEM. $^*P < 0.05$ compared with control at respective time points. Data are means ± SEM from three separate experiments, each performed in triplicate. (*B*) Effects of PGE_2 (10^{-6} M) and EP receptor agonists (10^{-6} M) on low-density (Day 2), high-migration and high-density (Day 4), low migration cells. *Vertical axis*, migrated cells, expressed as percent of control (*Cont*). *Horizontal axis*, treatments. $^*P < 0.05$, compared with medium control. $^{\#}P < 0.05$ for comparisons between high migratory capacity cells and low migratory capacity cells. All data represent means of at least three separate experiments, each performed in triplicate.

shown in Figure 2B was 374 ± 42 per five high-power fields), PGE_2, EP2 agonist, and EP4 agonist significantly inhibited chemotaxis. In contrast, EP1 and EP3 agonists stimulated cell migration, but the effect was modest. In high-density, low-migration (the number of migrated cells for the experiment shown in Figure 2B was 84 ± 12 per five high-power fields) cells, PGE_2 and the EP2 agonist inhibited migration, although the inhibitory effects of both were decreased compared with low-density, high-migration cells. The EP4 agonist, which inhibited chemotaxis significantly in low-density, high-migration cells, had no effect on high-density, low-migration cells. The EP1 and EP3 agonists significantly stimulated cell migration in high-density, low-migration cells. In contrast, no significant stimulation occurred in the low-density, high-migration cells.

Effects of PGE_2 and EP Receptor Agonists on the Chemotactic Response of HFL-1 Cells to Fibronectin and Concentration Dependence

As expected, PGE_2 inhibited chemotaxis in a concentration-dependent manner (Figure 3A). The EP receptor agonists

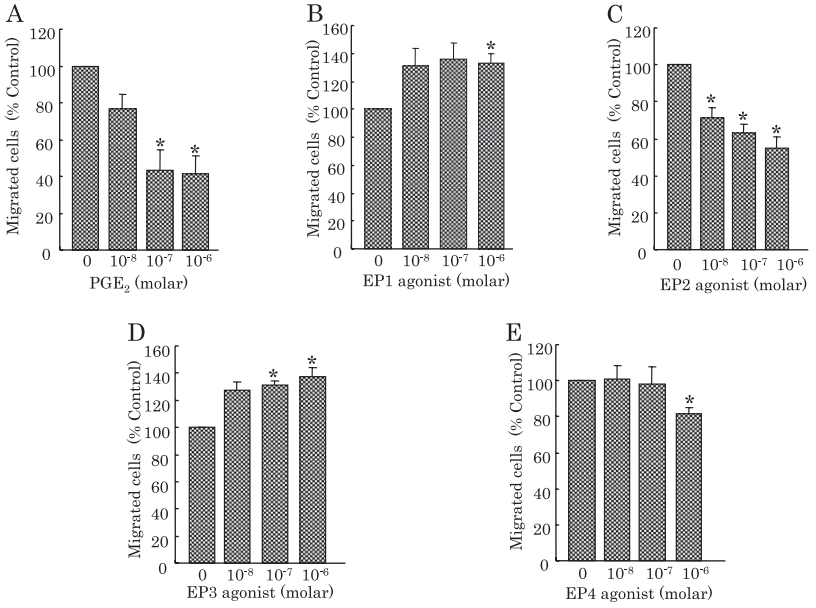

Figure 3. Concentration dependence of PGE_2 and EP receptor agonists on HFL-1 fibroblast chemotaxis toward human fibronectin. Cells were seeded in 100-mm tissue culture plates at a cell density of 1×10^5/ml in DMEM with 10% FCS, at 10 ml/dish, on Day 0. Cells were harvested on Day 2 (low-density) to examine effects of PGE_2 and EP2 and EP4 agonists, and on Day 4 (high-density) to examine effects of EP1 and EP3 agonists. (*A*) Inhibition of chemotaxis by PGE_2. (*B*) Stimulation of chemotaxis by EP1 agonist. (*C*) Inhibition of chemotaxis by EP2 agonist. (*D*) Stimulation of chemotaxis by EP3 agonist. (*E*) Inhibition of chemotaxis by EP4 agonist. Data shown are means ± SEM. $*P < 0.05$ compared with medium-only control. All data represent means of at least three separate experiments, each performed in triplicate.

exerted varied effects on HFL-1 chemotaxis. The EP1 agonist (Figure 3B) and EP3 agonist (Figure 3D) both stimulated HFL-1 chemotaxis. An effect was evident at 10^{-8} M, and the concentration-dependence relationship was relatively flat. In contrast, the EP2 agonist (Figure 3C) and EP4 agonist (Figure 3E) inhibited HFL-1 cell migration toward human fibronectin. The EP2 agonist demonstrated a clear concentration dependence, with a 50% effect at $\sim 10^{-8}$ M, and a maximal effect at the highest concentration tested (10^{-6} M), which resulted in 40% inhibition. The EP4 agonist inhibited chemotaxis but appeared to require higher concentrations than the EP2 agonist, and inhibition at the maximal concentration tested (10^{-6} M) was $\sim 20\%$. When added together, the combination of the EP2 and EP4 agonists resulted in significantly more inhibition than either agonist alone, although the difference was very small (Figure E3). The EP-selective agonists exerted similar effects on adult human bronchial fibroblast chemotaxis toward fibronectin. The EP2 agonist significantly inhibited chemotaxis, and the EP3 agonist significantly stimulated chemotaxis. EP4 agonist–treated cells migrated numerically less than controls, and EP1 agonist–treated cells migrated numerically more than controls, but the differences were not statistically significant (Figure E4).

Effects of PGE_2 and EP Receptor Agonists on HFL-1 Migration in *In Vitro* Wound Repair

To confirm the effects of PGE_2 and the EP receptor agonists on chemotaxis, the effects on cell migration in the *in vitro* wound-closure assay were evaluated. After a "wound" in a cell monolayer was made, progressive cell migration from the edge of the wound was readily observed after 48 hours and 72 hours (Figure 4A). PGE_2 inhibited this migration at both 48 hours and 72 hours (Figure 4A). The effect of the EP receptor agonists paralleled those observed in the blindwell chemotaxis assay. Both the EP1 agonist and the EP3 agonist stimulated HFL-1 cell migration into the wound, whereas PGE_2 and the EP2 agonist inhibited HFL-1 cell migration into the wound (Figures 4 B–4D). The EP4 agonist had a minimal effect on wound closure.

Effect of EP Receptor Antagonists on HFL-1 Chemotaxis

To confirm the effects of specific EP receptors in modulating HFL-1 cell chemotaxis, the effects of EP receptor–specific antagonists were also assessed. The action of each EP-selective agonist was blocked by the corresponding EP-selective antagonist, although the EP2 antagonist only partly blocked the effects of the EP2 agonist (Figure 5A). The antagonists were also assessed for their effects on PGE_2. Neither the EP1-selective nor EP3-selective antagonist modified the inhibitory effect of PGE_2, either when added alone or when added together. In contrast, the EP2-selective and EP4-selective antagonists reduced the inhibitory effect of PGE_2 and, when added together, appeared to be more effective (Figure 5B).

To confirm further that the inhibitory effect of PGE_2 was mediated by both the EP2 and EP4 receptors, we used siRNAs targeting the EP2 and EP4 receptors. Both the EP2 and EP4 siRNA selectively reduced the expression of the corresponding receptor, as assessed by Western blotting. Importantly, the EP2 siRNA did not affect EP4 expression, and the EP4 siRNA did not affect EP2 expression (Figure 6A). When added alone, neither the EP2 nor EP4 siRNA significantly affected chemotaxis (Figure 6B). As expected, PGE_2 inhibited chemotaxis significantly, and this was completely blocked by the EP2 siRNA, and partly blocked by the EP4 siRNA (Figure 6B).

EP Receptor Signaling Pathways Mediating HFL-1 Chemotaxis

The role of specific EP receptors in modulating HFL-1 chemotaxis was further confirmed by assessing the signaling pathways through which the EP agonists modulated chemotaxis. Specifically, the PKA inhibitor KT-5720, the G_i protein inhibitor PT, the Rho kinase inhibitor Y-27632, and the PI3K inhibitor wortmannin were assessed. The pathway inhibitors alone did not affect chemotaxis (Table 2). PGE_2 significantly inhibited HFL-1 migration, and this was blocked by adenylyl cyclase inhibition, by PKA inhibition, and partly by PI3K inhibition, whereas PT numerically potentiated the PGE_2 inhibition of chemotaxis. The EP1 agonist stimulated HFL-1 chemotaxis, and this was blocked by the inhibition of Rho kinase. Interestingly, PKA inhibition numerically increased chemotaxis in the presence of the EP1 agonist. The EP_2 agonist significantly inhibited HFL-1 migration, and this was blocked by PKA inhibition and partly by PI3K inhibition. The EP3 agonist stimulated cell migration, and this was blocked by the inhibition of G_i and Rho kinase. Similar to the EP1 agonist, PKA inhibition in the presence of the EP3 agonist resulted in a numeric stimulation of

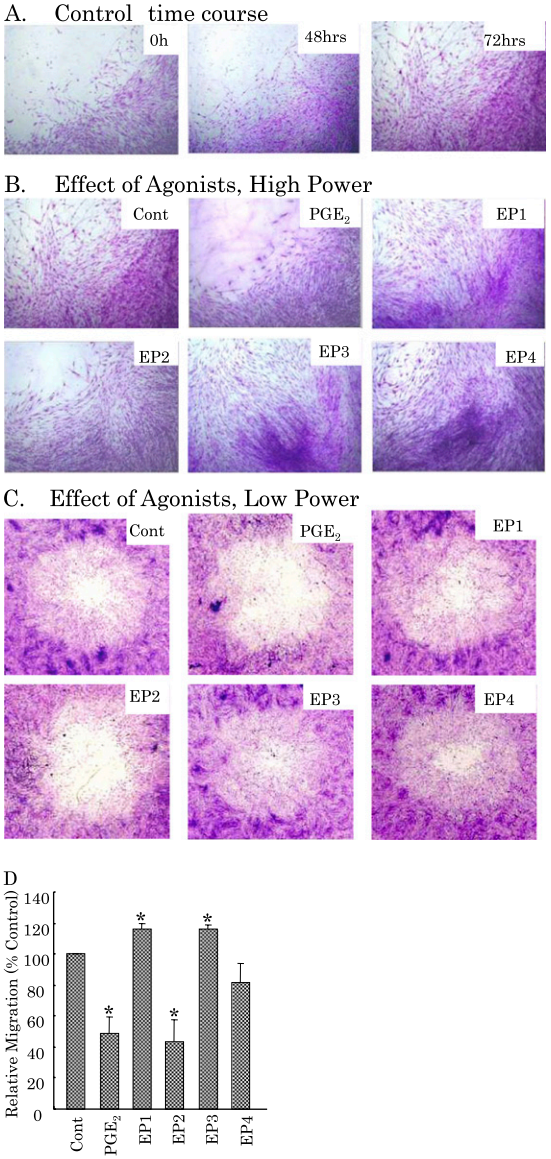

Figure 4. Effects of PGE_2 and EP receptor agonists on fibroblast wound-closure. The wound-closure assay was performed as described in MATERIALS AND METHODS. (*A*) Control cell migration, time course. Images were obtained immediately after removal of the pipette tip (0 hour), as well as 48 hours and 72 hours after removal. (*B*) High-power magnification, 40× original. (*C*) Low-power magnification. Effects of PGE_2 and EP receptor agonists (10^{-6} M) on migration after 72 hours. (*D*) Quantification of low-power photo. *Vertical axis*, cell migration compared with control (%). *Horizontal axis*, treatment group control = 1% FCS-DMEM alone. Data shown are means ± SEM for five separate experiments, each performed in triplicate. *$P < 0.05$ compared with control.

chemotaxis that was not statistically significant. The EP4 agonist inhibited HFL-1 chemotaxis, and this was blocked by PKA inhibition, and in contrast to the effect on EP2-agonist inhibition, the EP4-agonist inhibition was blocked completely by PI3K inhibition.

Because Rho kinase inhibition (Y-27632) significantly blocked the EP1 or EP3 agonist stimulation of chemotaxis, the Rho activation assay was performed after stimulation with the EP1 and EP3 agonists. EP1 and EP3 agonists induced Rho activation, although less robustly compared with the activation by S1P, a known activator of Rho (Figure 7).

DISCUSSION

Here, we demonstrate that PGE_2 modulates human lung fibroblast chemotaxis toward fibronectin in the blindwell chamber assay, and migration in a wound-closure assay, through multiple EP receptors that can either stimulate or inhibit chemotaxis. Two EP receptors, EP2 and EP4, inhibited chemotaxis in the blindwell assay, although only the EP2 agonist inhibited migration in the wound-closure assay. In contrast, both the EP1 and EP3 receptors stimulated chemotaxis and migration in both assays. The activity of these individual receptors was demonstrated by the use of selective agonists and antagonists, and was confirmed, where possible, by demonstrating the effects of expected signaling pathway inhibitors and by the use of siRNA suppression. These effects suggest that PGE_2 can regulate human lung fibroblast migration in complex ways. They also suggest that altered PGE_2 signaling, which could result from differences in receptor expression or function, may lead to alterations in fibroblast recruitment. Such mechanisms could contribute to the pathogenesis of disorders characterized by altered tissue structure, including both fibrotic disorders and conditions characterized by a loss of tissue integrity, such as emphysema of the lung.

PGE_2 was previously reported by several groups, including our own, to inhibit normal fibroblast chemotaxis. Kohyama and colleagues demonstrated that PKA mediated this inhibition (9). White and colleagues further showed that the EP2 receptor agonist butaprost mediated the inhibition of chemotaxis through an EP2 receptor–mediated increase in PTEN activity (10). Our study extends these earlier findings in several important ways. First, the inhibitory effect of PGE_2 was mediated not only by the EP2 receptor but also by the EP4 receptor. The EP2-specific agonist achieved only about half as much inhibition as PGE_2. The EP4 agonist alone had an even more modest effect. Although potential interactions among EP receptors were not assessed in detail, when added together at high concentration, the EP2 and EP4 agonists together inhibited more than either alone, although the additional inhibition was very small. Nevertheless, this suggests the possibility that PGE_2 inhibits chemotaxis through several pathways that may interact. Interactions between receptors in regulating chemotaxis at lower concentrations would be required to evaluate potential EP2–EP4 interactions. In addition, this study demonstrates that the action of PGE_2 on EP1 and EP3 receptors can stimulate chemotaxis, which to the best of our knowledge is a novel finding. The PGE_2 regulation of chemotaxis through multiple receptors is consistent with previous results. White and colleagues (10), for example, noting that the EP2-selective agonist induced PTEN activity to a much greater degree than PGE_2, suggested that the PGE_2 regulation of chemotaxis may be the net result of simultaneous signaling through other EP receptors.

Our study also suggests an explanation for at least some of the varied results obtained in earlier studies. Specifically, the chemotactic response of fibroblasts is dependent on cell density. Low-density cells migrated to a much greater degree than did high-density fibroblasts. This was evident when cells were plated at a single density and then harvested after various times, and when cells were plated at different densities and then harvested at the same time. However, these experiments did not exclude the possibility that time in culture could also affect chemotactic responsiveness. Our study did not explore the mechanistic basis for the density dependence. There are several nonexclusive possibilities. For example, high-density cells may manifest alterations in cell cytoskeleton components. Alternately, not all cells within a culture migrate, and changes could occur with density in a population of cells. Western blots were performed

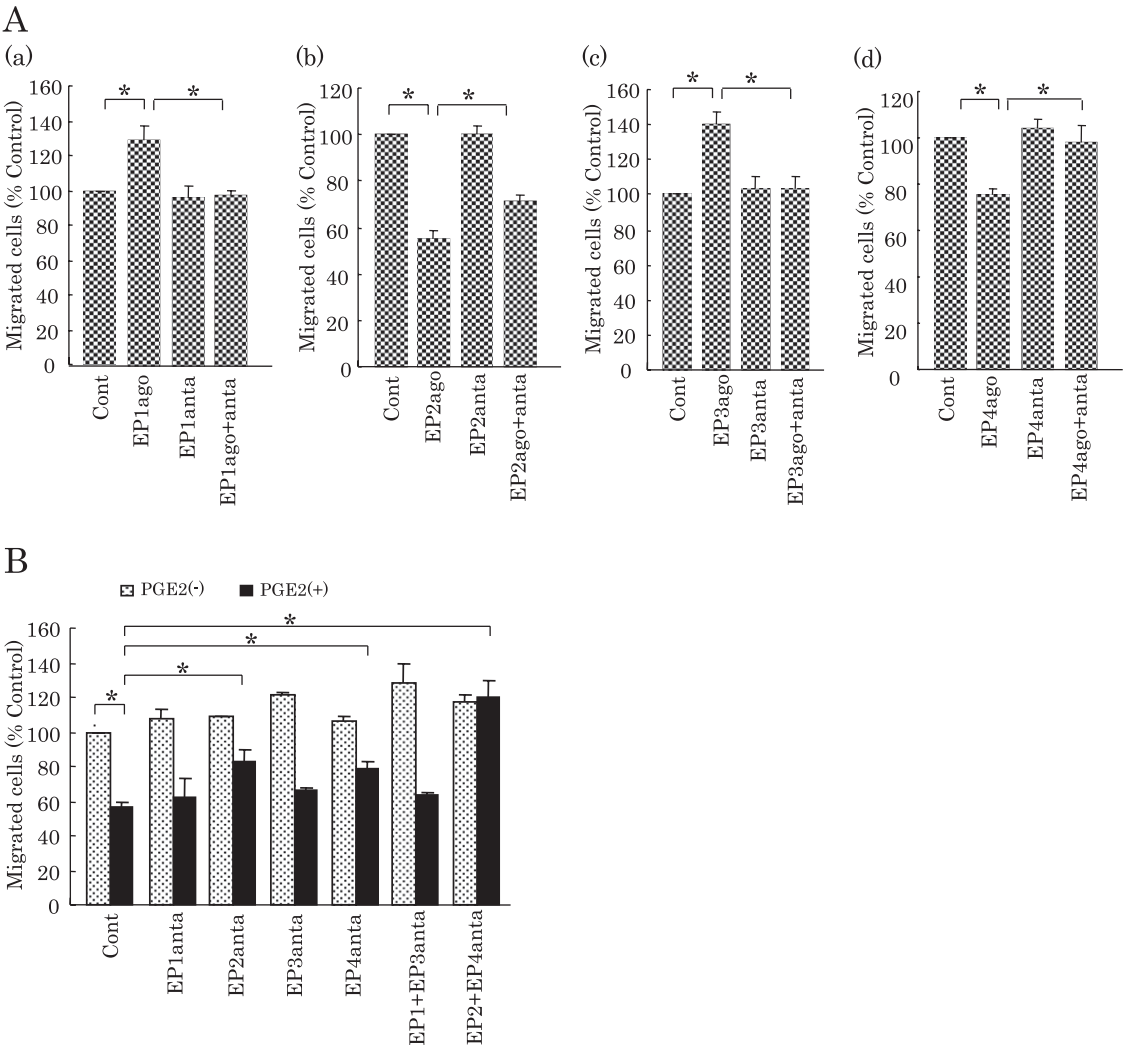

Figure 5. Effects of EP receptor antagonists (10^{-6} M) on HFL-1 chemotaxis. Cells were seeded in 100-mm tissue culture plates at a cell density of 1×10^5/ml in DMEM with 10% FCS, at 10 ml/dish, on Day 0 and harvested on Day 3. (*A*) Blockade by EP-selective agonists. HFL-1 cells were treated with or without EP-selective agonists (10^{-6} M), with corresponding antagonist followed by chemotaxis assay. (*a*) EP1. (*b*) EP2. (*c*) EP3. (*d*) EP4. (*B*) Effects of antagonists on PGE_2-mediated inhibition of chemotaxis. Data shown are means ± SEM for three separate experiments, each performed in triplicate. *$P < 0.05$.

here to demonstrate receptor expression. These experiments suggested an increase in receptor expression with density. However, these experiments were not quantified, and whether changes in EP receptor expression play a role in cell density-modulated cell migration remains unevaluated. Although the mechanisms for the reduction in overall chemotactic response with increasing cell density are unknown, this phenomenon creates technical constraints for evaluating the effects of modulators of chemotaxis. Inhibitors are most easily evaluated in low-density cells, when chemotaxis is brisk and there is a large signal to inhibit. In contrast, stimulators are most easily evaluated when cells are denser and the chemotactic signal is less robust and less likely to obscure further stimulation.

This study also used two assays of fibroblast movement, i.e., chemotaxis in the blindwell assay, and migration to a "wound." In general, the results were similar, although the EP4 agonist inhibited chemotaxis in the blindwell assay and had no effect on wound migration. The assays are not strictly equivalent. The blindwell assay requires cell attachment and occurs during a 6-hour period. In contrast, the wound-closure assay involves cells that are already attached to a matrix and are in close contact with each other. Whether the differences observed for the EP4 agonist represent a biological difference in assay systems remains undefined. Effects in the "wound-closure" assay, which develops over several days, could reflect other responses, such as cell proliferation.

PGE_2 can modulate the migration of other cell types as well as fibroblasts (35, 36, 37, 38, 39, 40). The PGE_2 inhibition of neutrophil (36, 37), lymphocyte (38, 39), and eosinophil (18, 40) chemotaxis was reported. In contrast, PGE_2 was reported to stimulate airway epithelial cell migration (41). The roles played by individual EP receptors in the regulation of chemotaxis in these cells are generally undetermined, although the stimulation of migration of normal human bronchial epithelial cells was suggested to be mediated via the EP1 and EP4 receptors (41). Our study was designed to address the question of which EP receptors could modulate the chemotactic response to PGE_2 in lung fibroblasts. To accomplish this, we first demonstrated the expression of all four EP receptors in HFL-1 cells. We next evaluated the functional effects of EP-selective agonists. Although many traditional agonists for the EP receptors are notoriously nonselective, the agonists in the present study are relatively selective. Nevertheless, confirming the specificity of action was essential, and was undertaken in several ways. First, we confirmed the effect of EP agonists by evaluating the inhibitory effect of EP receptor antagonists. Our results showed that each EP receptor–specific agonist was antagonized by the corresponding EP receptor antagonist. For EP2, the antagonist only partly inhibited the effect of the EP2 agonist. For all other receptors, the antagonist completely blocked the effects of corresponding agonists. Only one EP2 antagonist was available to us. However, we confirmed the result obtained using the

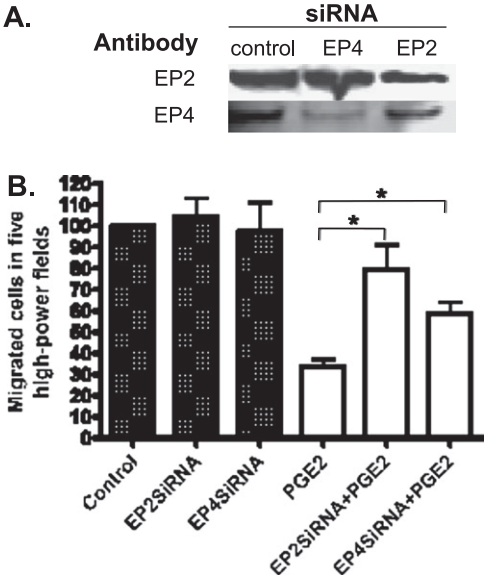

Figure 6. Effects of EP2 and EP4 suppression by small interfering RNA (siRNA) on PGE_2 inhibition of HFL-1 chemotaxis. (*A*) Western blot demonstrates suppression of EP receptor expression. Cells were treated with siRNA for EP2 or EP4, cell layers were extracted, and Western blot analysis was performed for EP2 and EP4, as described in MATERIALS AND METHODS. (*B*) Cells were incubated with siRNA for EP2 or EP4 or with control transfection reagent, as described in MATERIALS AND METHODS. Cells were then trypsinized, and chemotaxis toward fibronectin was assessed in the absence (*hatched bars*) and presence (*open bars*) of PGE_2. *Vertical axis*, chemotaxis expressed as percent of migration to fibronectin control. *Horizontal axis*, conditions. Data are means ± SEM for four separate experiments performed on separate occasions. *$P \leq 0.05$.

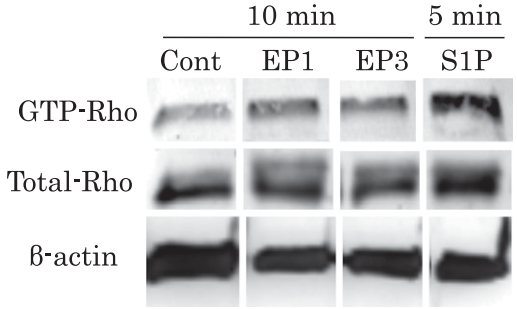

Figure 7. Effects of EP1 and EP3 agonist on Rho activation in HFL-1 cells. Cells were seeded in 100-mm tissue culture plates at a cell density of 1×10^5/ml in DMEM with 10% FCS, at 10 ml/dish, on Day 0. After 3 days (~ 70–80% confluence), medium was changed to DMEM without serum and cultured overnight. Cells were then stimulated with EP1 or and EP3 agonist (10^{-6} M) for 10 minutes before harvesting cell lysates. Sphingosine-1–phosphate (S1P) was used as a positive control. Rho activation was assessed by immunoblotting for guanosine triphosphate (GTP)-bound Rho and total Rho. β-actin was used as a loading control.

ONO EP2 agonist with butaprost (data not shown), another EP2 agonist, and obtained similar results. The mechanism for the partial effect remains undetermined. However, the results obtained with siRNA suppression confirm a role for both EP2 and EP4 in the PGE_2-mediated inhibition of chemotaxis. The siRNA suppression of EP1 and EP3 was not undertaken, because pilot experiments failed to demonstrate a suppression of expression of these receptors by Western blotting (data not shown).

To confirm the roles of different EP receptors further, we took advantage of the predicted signaling pathways activated by EP receptors. The least well-defined signaling mechanism among EP receptors is that of the EP1 receptor. This receptor signals via an unidentified G protein (23), leading to increased cellular Ca^{2+} that is accompanied by modest increases in the generation of inositol 1,4,5-trisphosphate (42, 43). Our results showed that the EP1-selective agonist stimulated HFL-1 cell chemotaxis, and this was accompanied by an activation of Rho and was blocked by the inhibition of Rho kinase. Activation of $G\alpha_{13}$ induces cytoskeletal changes by way of Rho, and participates in the regulation of cell movement in response to specific ligands (44). Therefore, in our system, EP1 receptor–stimulated HFL-1 cell migration may occur via $G\alpha_{13}$-activated Rho and subsequent cytoskeletal changes. The other EP receptors signal through well-defined pathways. The use of inhibitors active in these signaling pathways confirmed the action of PGE_2 signaling through these receptors, which may interact in mediating the effects of nonselective agonists such as PGE_2 (Figure 8).

The present study contains several implications for lung disease. PGE_2 levels were found to be increased in COPD (19, 45, 46), cystic fibrosis (21), and lung cancer (22), and to be reduced in idiopathic pulmonary fibrosis (17). A role for excessive PGE_2 in causing lung disease, particularly leading to deficient repair, was suggested. Conversely, a role for deficient PGE_2 leading to excessive fibroblast activity was suggested as a mechanism of fibrosis. Our results suggest that PGE_2 can modulate at least one fibroblast function, chemotaxis, by action on all four EP receptors, and that these can exert disparate actions. In our studies, PGE_2 was always inhibitory. A different action might ensue if pathologic or physiologic conditions altered the receptor expression or activity. In addition, other ligands such as isoprostanes can signal through EP receptors,

TABLE 2. EFFECTS OF SIGNALING PATHWAY INHIBITORS ON PGE_2 AND EP RECEPTOR–MODULATED HFL-1 CHEMOTAXIS

	Control	PGE_2	EP1	EP2	EP3	EP4
(−)	100 ± 0	43 ± 6.5*	133 ± 6.6*	48 ± 3.5*	140 ± 6.6*	79 ± 1.9*
SQ22536	102 ± 5	68 ± 8†	124 ± 16	109 ± 5†	127 ± 13	101 ± 3†
KT-5720	108 ± 13.8	93 ± 12.1†	168 ± 17.7	81 ± 11.3†	143 ± 3.8	103 ± 6.6†
Pertussis toxin	98 ± 4.8	23 ± 3.9	121 ± 4.5	44 ± 1.6	93 ± 12.0†	84 ± 2.3
Y-27632	91 ± 3.3	27 ± 7.8	78 ± 10.5†	25 ± 7.3†	88 ± 11.8†	78 ± 11.4
Wortmannin	103 ± 8.4	74 ± 7.5†	126 ± 6.5	76 ± 5.4†	120 ± 5.9	109 ± 2.3†

Definition of abbreviations: HFL-1, human fetal lung fibroblast; (−), medium only control; PGE_2, prostaglandin E_2.

HFL-1 chemotaxis was assessed for each inhibitor in the presence and absence of PGE_2 or EP receptor agonists. Effect of the adenylate cyclase inhibitor SQ-22536 (10^{-5} M), the PKA inhibitor KT-5720 (10^{-7} M), the G_i protein inhibitor pertussis toxin (PT) (100 ng/ml), the Rho kinase inhibitor Y-27632 (10^{-6} M) and the PI3K inhibitor wortmanin (10^{-7} M). The number of migrated cells expressed as percent of medium only control.

Data are means ± SEM for three separate experiments, each performed in triplicate.

* $P < 0.05$ compared with medium control (without PGE_2 or EP receptors agonists and inhibitor).

† $P < 0.05$ compared with PGE_2 or EP receptors agonists-treated and inhibitor-untreated group.

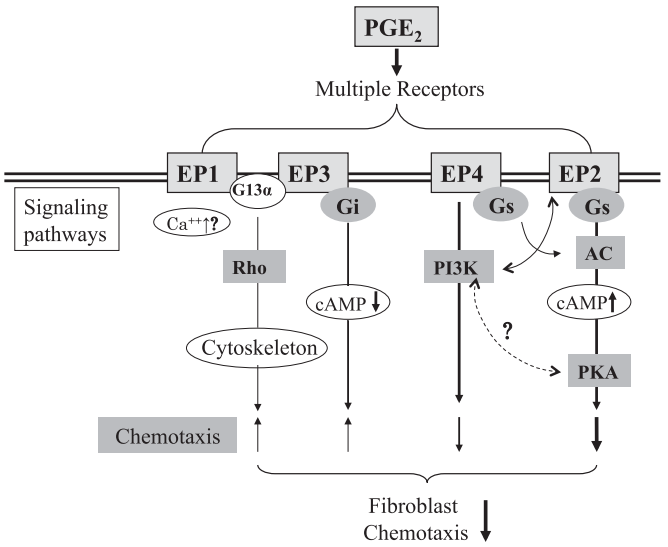

Figure 8. The EP receptor intracellular signaling pathway and its effects on HFL-1 cell migration. PGE_2 binds EP1, EP2, EP3, and EP4 receptors, and can either stimulate or inhibit cell migration. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PI3K, phosphatidylinositol-3–kinase; *solid line*, established signal pathway; *dotted line*, possible signaling pathway; ↓, inhibit; ↑, stimulate.

and could therefore modulate fibroblast chemotaxis (47). Alterations in EP receptor expression or activity, which may result from the presence of inflammatory mediators, toxins, or aging, could contribute to disease pathogenesis. In addition, PGE_2 action could be approached therapeutically by a selective targeting of EP receptors.

In conclusion, this study demonstrates that PGE_2 modulates fibroblast migration through multiple EP receptors that can either stimulate or inhibit migration. PGE_2 signaling through specific EP receptors could be a therapeutic target to augment impaired healing, or to block the development of excessive fibrotic lung disease.

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