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Prostaglandin E₂ Inhibits Fibroblast Migration by E-Prostanoid 2 Receptor–Mediated Increase in PTEN Activity

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Abstract

An increased migratory phenotype exists in lung fibroblasts derived from patients with fibroproliferative lung disease. Prostaglandin E₂ (PGE₂) suppresses fibroblast migration, but the receptor(s) and mechanism(s) mediating this action are unknown. Our data confirm that treatment of human lung fibroblasts with PGE₂ inhibits migration. Similar effects of butaprost, an E-prostanoid (EP) 2 receptor–specific ligand, implicate the EP2 receptor in migration-inhibitory signaling. Further, migration in fibroblasts deficient for the EP2 receptor cannot be inhibited by PGE₂ or butaprost, confirming the central role of EP2 in mediating these effects. Our previous data suggested that phosphatase and tensin homolog on chromosome ten (PTEN), a phosphatase that opposes the actions of phosphatidylinositol-3-kinase (PI3K), may be important in regulating lung fibroblast motility. We now report that both PGE₂ and butaprost increase PTEN phosphatase activity, without a concomitant increase in PTEN protein levels. This contributes to EP2-mediated migration inhibition, because migration in PTEN-null fibroblasts is similarly unaffected by EP2 receptor signaling. Increased PTEN activity in response to EP2 stimulation is associated with decreased tyrosine phosphorylation on PTEN, a mechanism known to regulate enzyme activity. Collectively, these data describe the novel mechanistic finding that PGE₂, via the EP2 receptor, decreases tyrosine phosphorylation on PTEN, resulting in increased PTEN enzyme activity and inhibition of fibroblast migration.

Keywords

fibroblast; eicosanoid; phosphatase; cell migration

Fibroblast migration into wound spaces is an integral part of the wound healing response (1). However, in idiopathic pulmonary fibrosis (IPF) and other fibroproliferative disorders of the lung, there is an excessive influx and accumulation of fibroblasts resulting in progressive tissue distortion and disruption of normal architecture (2). Current therapeutic options for patients with IPF are ineffective at halting the relentless scar formation (3), underscoring the need to identify molecular mechanisms involved in their pathogenesis. We and others have previously shown an increased migratory capacity of fibroblasts derived from lungs of patients with IPF as compared with normal control subjects (4,5), which provides one possible explanation for the profusion of fibroblasts observed in lung biopsies from these patients (3). Therefore,

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dissecting mechanisms that regulate lung fibroblast migration may be beneficial in identifying potential molecular targets to be exploited in the treatment of these diseases.

The arachidonic acid cyclooxygenase metabolite prostaglandin E₂ (PGE₂) is a biologically important lipid involved in processes as diverse as pain, fever, parturition, inflammation, and cancer. At the cellular level, it modulates a number of functions, sometimes in opposing or conflicting fashion. For instance, PGE₂ promotes migration of malignant epithelial cells via activation of the phosphatidylinositol-3-kinase (PI3K) pathway (6,7). However, PGE₂ inhibits migration—via an as yet unidentified mechanism—of normal fibroblasts (8–10). Importantly, lung fibroblasts from patients with IPF have a diminished capacity to both produce PGE₂ (11–14) and to respond to exogenously applied PGE₂ (15). Thus, PGE₂ plays a central role in the regulation of lung fibroblast migration, and aberrant PGE₂ signaling might contribute to the pathogenesis of fibrotic disorders of the lung and other organs. PGE₂ signaling occurs via four identified G-protein–coupled receptors, termed E-prostanoid receptors 1–4 (EP1–4) (16). EP receptors transmit signals by increased cAMP (EP2 and EP4), by mobilization of intracellular calcium (EP1), or by decreased cAMP (EP3). By virtue of these distinct signaling pathways, pleiotropic (or even antagonistic) responses to PGE₂ may be observed in different cells depending on the profile of EP receptors expressed.

Phosphatase and tensin homolog on chromosome ten (PTEN) is a dual-specificity lipid and protein phosphatase that is deleted or mutated in a number of malignancies (17), and thus has been identified as a tumor suppressor. PTEN antagonizes the activity of PI3K by dephosphorylating the D3 position of the inositol ring of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) (18). PIP₃ is a lipid mediator that accumulates at the plasma membrane after PI3K stimulation and allows for recruitment and phosphorylation of proteins containing pleckstrin homology domains, such as protein kinase B/Akt (19), that are important in cellular migration (20,21). As is the case with malignancy, loss of PTEN activity has also been implicated in the pathogenesis of nonmalignant diseases, such as rheumatoid arthritis (22), bronchial asthma (23), and IPF (4), in which inflammation and/or fibrosis disrupts normal tissue architecture. The intracellular regulation of PTEN activity is incompletely described, but is dependent in part on tyrosine phosphorylation (24), C-terminal serine and threonine phosphorylation (25), membrane localization via the C2 domain (26), and ubiquitination and proteasome degradation (27). The multiple means by which PTEN activity is regulated is typical for a critically important determinant of cell function, and presents a range of possibilities by which stimuli might modulate its activity. Delineating the pathways both up- and downstream of PTEN activation may, consequently, allow for the development of more specific therapeutic agents to be used in diseases where PTEN activity is dysregulated.

Because both PGE₂ synthesis (11–14) and PTEN activity (4) are decreased in fibroblasts from the lungs of patients with IPF, we hypothesized that the activities of these two molecules might be related. The results of the present study demonstrate that PGE₂ treatment of normal lung fibroblasts triggers an intracellular pathway through the EP2 receptor leading to increased activity of PTEN and diminished fibroblast migration.

MATERIALS AND METHODS

Cell Culture and Reagents

Normal human fetal lung fibroblasts (IMR-90) were from the Coriell Institute for Medical Research (Camden, NJ). Murine embryonic fibroblasts on a C57Bl/6 background were from ATCC (Rockville, MD). Murine embryonic fibroblasts with a homozygous deletion of the *pten* gene (*pten*-null) have been described previously (28). Primary lung fibroblasts from EP2-null mice or control adult C57Bl/6 mice were generated in a standard fashion (4). Cells were maintained in DMEM with 10% fetal calf serum (FCS), penicillin, streptomycin, glutamine,

fungizone, and HEPES and were used between the 5th and 9th passages. Basic fibroblast growth factor (bFGF) was from R&D Systems (Minneapolis, MN). PGE₂ and the EP2-selective agonist butaprost (supplied as the free acid) were from Cayman Chemical (Ann Arbor, MI). PGE₂ was dissolved in ethanol and butaprost was dissolved in Me₂SO₄; both stock solutions were kept at -80°C until used in assays. Unless otherwise specified, all other reagents were purchased from Sigma (St. Louis, MO).

Measurement of Intracellular cAMP Production

To assess intracellular production of cAMP, IMR-90 cells were plated until subconfluent, serum starved for 24 h, and then treated with varying doses of PGE₂ or butaprost for 15 min. Media was then aspirated and cells were washed, and intracellular cAMP levels were determined by enzyme-linked immunosorbent assay kit according to the manufacturer (Assay Designs, Ann Arbor, MI).

Antibodies

Antibodies to the EP2 receptor were obtained from Cayman Chemical. Antibodies to the EP1, EP3, and EP4 receptors were obtained from Alpha Diagnostic International (San Antonio, TX). Antibodies to total and phosphorylated PTEN, total Akt and S⁴⁷³ phospho-Akt were from Cell Signaling Technologies (Beverly, MA).

Migration Assays

Transwell migration assays were performed and quantitated as previously described (4). Briefly, 8.0 µm pore size polycarbonate transwell inserts (Nalge Nunc, Naperville, IL) were coated on the inner layer with Matrigel (BD Biosciences, Palo Alto, CA) at 1 mg/ml overnight at 4°C. Individual wells of a 24-well plate were then filled with 300 µl serum-free media and transwells were placed within the wells ensuring no air bubbles were trapped beneath. Fibroblasts were placed within the inner well of the transwell and allowed to migrate for 18 h at 37°C with 5% CO₂. At the termination of the experiment, the inner well was wiped with a cotton swab to remove nonmigrating cells and the membranes were fixed, stained, and assessed by light microscopy. Five high-power fields (hpf) per membrane were counted, and migration was expressed as the mean (± SEM) number of cells/hpf. To ensure reproducibility, each condition was tested in three separate wells. For each experiment, cell viability was assessed by trypan blue exclusion before use in assays. In all cases, cell viability exceeded 90%.

PTEN Immunoprecipitation

Serum-starved fibroblasts were treated for the indicated times with the designated stimulus, washed twice, and lysed in lysis buffer containing protease inhibitors. Total protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Immunoprecipitation was performed on 15 µg total protein using Protein G-PLUS (Santa Cruz Biotechnology, Santa Cruz, CA) and 1 µg anti-PTEN antibody (clone A2B1; Santa Cruz).

PTEN Phosphatase Assay

PTEN immunoprecipitates were assessed for phosphatase activity as previously described (4). Briefly, equal volumes of phosphatase reaction buffer containing 200 µM D-*myo*-phosphatidylinositol-3,4,5-trisphosphate (PIP3; Echelon Biosciences, Salt Lake City, UT) was added to immunoprecipitates and the reaction was allowed to proceed at 37°C for 30 min. Immunoprecipitates were centrifuged and supernatants were added to a 96-well plate in triplicate. BIOMOL Green reagent (BIOMOL Research Laboratories, Plymouth Meeting, PA) was added and plates were incubated for 20 min at room temperature. Absorbance of each well was assessed using a colorimetric plate reader at 630 nm wavelength. In each situation, PTEN

activity is quantified as activity observed relative to the control situation. Results were reported as relative PTEN phosphatase activity $\pm$ SEM compared with control conditions.

Western Blot Analysis

Western blot analysis under reducing conditions was performed on equal concentrations of protein from whole-cell lysates and immunoprecipitated proteins as previously described (4).

Statistical Analyses

Statistical analyses were performed using GraphPad InStat 3.05 (San Diego, CA). Differences between groups were evaluated using Student's *t* test. For multiple comparisons, one-way ANOVA with Bonferroni's post-test analysis was used. Data were considered significant if $P < 0.05$. Results were plotted using GraphPad Prism 3.02 (San Diego, CA). Densitometry of visualized bands on Western blot was performed using Image J software (version 1.31; NIH). For migration assays, results are expressed as mean number of migrated cells per hpf $\pm$ SEM. For phosphatase assays, results are expressed as relative activity $\pm$ SEM compared with control conditions.

RESULTS

PGE₂ Inhibits Fibroblast Migration via the EP2 Receptor

PGE₂ inhibits fibronectin- and bovine bronchial epithelial cell conditioned medium-induced HFL1 lung fibroblast migration in a time- and dose-dependent manner (8). Using a second human lung fibroblast line (IMR-90), we observed that basic fibroblast growth factor (bFGF) stimulated migration in a standard transwell assay (Figure 1A). This occurred in a dose-dependent manner (not shown) with a concentration of 50 ng/ml consistently resulting in a 3- to 6-fold increase in number of migrated cells above baseline. Therefore, this concentration was used for further experiments. To test the effect of PGE₂ on fibroblast migration, preliminary dose-response experiments were undertaken. We observed that inhibition of FGF-induced migration occurred with all concentrations of PGE₂, ranging from 100–1,000 nM. Consistent with previous work (8), PGE₂ had no significant effect on baseline migration in our system (data not shown). We observed that maximal inhibition occurred with PGE₂ at a minimum concentration of 500 nM (not shown). Thus, this concentration was used for the remainder of experiments. When PGE₂ (500 nM) was added to cells at the beginning of the assay, bFGF-induced migration over 18 h was significantly inhibited by ~60% ($P < 0.001$) (Figure 1A), which is in agreement with the findings of Kohyama and coworkers (8). To assess the prostaglandin receptor(s) that might mediate this effect, we performed Western blot analysis of whole-cell lysates from IMR-90 cells for the four known PGE₂ receptors EP1–4. We observed, similar to previously published work (29), that EP2 was the predominant EP receptor on IMR-90 cells, whereas EP4 was undetectable and EP1 and EP3 were expressed only minimally (Figure 1B). To determine if EP2 transmitted migration-inhibitory signals from PGE₂, we assessed the ability of butaprost, a selective EP2 agonist (30), to inhibit bFGF-induced fibroblast migration. As expected, butaprost significantly inhibited IMR-90 fibroblast migration in response to bFGF ($P < 0.001$) (Figure 1A). To demonstrate that this effect was specific for the EP2 receptor, we assessed the ability of butaprost or PGE₂ to inhibit bFGF-induced migration in murine lung fibroblasts lacking the EP2 receptor, as compared with wild-type fibroblasts. Consistent with our hypothesis, bFGF-induced migration in EP2-null fibroblasts could not be inhibited by either butaprost or PGE₂, whereas wild-type murine lung fibroblasts exhibited a response similar to that of the IMR-90 fibroblasts (Figure 1C). Together, these data strongly suggest that PGE₂ signals primarily through the EP2 receptor to inhibit fibroblast migration.

Signaling through the EP2 Receptor Increases cAMP Production and PTEN Activity

PTEN plays an integral role in regulating cellular migration (31,32), and we have identified a potentially key role for decreased PTEN activity in the increased migratory phenotype of IPF fibroblasts (4). Because PGE₂ inhibits fibroblast chemotaxis independent of the stimulus, we sought to address the effect of PGE₂ on PTEN activity in the absence of chemoattractant stimuli. To determine whether PTEN activity was affected by PGE₂, we treated IMR-90 cells with 500 nM PGE₂ for varying amounts of time followed by lysis and immunoprecipitation of PTEN. Immunoprecipitated PTEN was then used in an *in vitro* phosphatase assay as described previously (4). We observed a time-dependent increase in relative PTEN activity after treatment with PGE₂ that was significant 6 h after initiation of treatment and maximal at 18 h after treatment (Figure 2A). To confirm that this occurred through the EP2 receptor, we first examined the effects of butaprost on IMR-90 cells. As expected, butaprost treatment increased PTEN activity as well (Figure 2B), although with greater rapidity (3 h versus 6 h for PGE₂) and magnitude (maximal induction $345 \pm 12.3\%$ of control vs. $141 \pm 7\%$ of control for PGE₂). By Western blot, we observed no corresponding time-dependent increase in total PTEN levels after PGE₂ treatment (Figure 2A) or butaprost treatment (Figure 2B), suggesting that increased PTEN levels did not account for increased PTEN activity. To confirm that butaprost increased PTEN activity via the EP2 receptor, we performed similar experiments using primary lung fibroblasts derived from EP2-null mice or control (C57Bl/6) mice. We found, as expected, that EP2-null cells displayed no significant change in PTEN activity after butaprost treatment; in contrast, C57Bl/6 control fibroblasts treated with butaprost displayed a time-dependent increase in PTEN activity following a similar pattern to IMR-90 cells (Figure 2C).

We hypothesized that the disparity in the magnitude of increased PTEN activity following PGE₂ or butaprost treatment was related directly to intracellular cAMP levels downstream of EP2. To address this hypothesis, IMR-90 cells were treated with either PGE₂ or butaprost for 15 min, cells were lysed, and lysates were assayed for intracellular cAMP levels. As shown in Figure 2D, cAMP production in IMR-90 cells increased in a dose-dependent manner after treatment with PGE₂ or butaprost. Consistent with our observations that butaprost increased PTEN activity roughly three times as much as PGE₂, we observed that IMR-90 cAMP levels were ~ 3 times higher after treatment with butaprost than with PGE₂, suggesting that PTEN activity in this system directly corresponds to cAMP levels. Similar experiments demonstrated increased cAMP levels in murine lung fibroblasts after butaprost and PGE₂ challenge, whereas EP2-null cells showed no increase in cAMP levels after butaprost or PGE₂ challenge (data not shown).

PTEN function depends not only on its catalytic activity, but also on its ability to localize to cell membranes in whole-cell systems (25). Because of this, it was important to verify that PGE₂ and butaprost could increase PTEN activity in intact cells. Because PTEN inhibits migration at least partly through its ability to decrease phosphorylation and activation of protein kinase B/Akt on S⁴⁷³ (20,21,33), whole-cell lysates from butaprost-treated IMR-90 cells were separated by electrophoresis and immunoblotted for S⁴⁷³ phospho-Akt. We observed that butaprost caused a time-dependent decrease in Akt phosphorylation (Figure 2E), consistent with upregulated PTEN activity in intact cells. The blot was stripped and probed for total Akt levels to ensure equal protein loading (Figure 2E). Similar results were obtained for treatment with PGE₂ (data not shown).

The correlation between EP2 receptor signaling and increased PTEN activity indicated that increased PTEN activity might account, at least in part, for inhibition of fibroblast migration in our system. To address whether PTEN activity downstream of EP2 is necessary to inhibit fibroblast migration, we assessed the effect of PGE₂ and butaprost on migration in murine fibroblasts with a homozygous deletion of the *pten* gene (*pten*-null) or on control fibroblasts.

These *pten*-null cells display a cAMP production response after PGE₂ and butaprost that is similar to IMR-90 cells (data not shown). As compared with control fibroblasts, *pten*-null cells demonstrated significantly increased baseline migration, which could be increased further by addition of bFGF, but which could not be attenuated by the addition of PGE₂ or butaprost (Figure 3). These data support the concept that EP2-mediated inhibition of fibroblast migration requires the activity of PTEN.

EP2 Receptor Stimulation Results in Decreased Tyrosine Phosphorylation of PTEN

PTEN activity can be negatively regulated by tyrosine, serine, and threonine phosphorylation (24,25). To elucidate whether changes in amino acid phosphorylation of PTEN accounted for the observed increase in PTEN activity, we first assessed the phosphorylation of serine-380 (S³⁸⁰), threonine-382 (T³⁸²), and threonine-383 (T³⁸³) residues located in the C-terminal portion of PTEN that have been implicated in controlling PTEN activity (27,34). Whole-cell lysates from control- and butaprost-treated IMR-90 fibroblasts were separated by electrophoresis and immunoblotted for S³⁸⁰ phospho-PTEN, or S³⁸⁰T³⁸²T³⁸³ phospho-PTEN (Figure 4A). We observed no appreciable difference in the phosphorylation of these residues in response to butaprost treatment. To assess for phosphorylation of tyrosine residues, we next immunoprecipitated PTEN from control- and butaprost-treated IMR-90 cell lysates, separated proteins by electrophoresis, and immunoblotted for phosphotyrosine (24). We found a significant decrease in the phosphorylation of tyrosine residues over time (Figure 4B). Both blots were reprobed for total PTEN to ensure equal protein loading. The finding of decreased phosphorylation of tyrosine on PTEN corresponds to the time course observed for increased PTEN activity, and suggests that PTEN tyrosine dephosphorylation accounts for the increased PTEN activity seen in this model.

Collectively, our data suggest that EP2 stimulation in IMR-90 cells results in decreased PTEN tyrosine phosphorylation, leading to enhanced PTEN activity and inhibition of fibroblast migration.

DISCUSSION

In the current study, we demonstrate for the first time that the biologically important eicosanoid PGE₂ increases PTEN activity, and that this accounts, at least in part, for its ability to inhibit migration of human lung fibroblasts. In our system, we used basic FGF to induce fibroblast chemotaxis. However, studies evaluating the induction of PTEN activity by PGE₂ and butaprost were performed in the absence of FGF, demonstrating that EP2-mediated increases in PTEN activity occur independent of the migratory stimulus. The increase in PTEN activity occurs downstream of the EP2 receptor, and is associated with decreased tyrosine phosphorylation of PTEN. We and others have previously shown an increased migratory phenotype of fibroblasts derived from patients with fibroproliferative lung disorders (4,5), and our previous data suggest that this may be due to decreased PTEN expression and activity in these fibrotic-lung fibroblasts (4). Previous studies have shown PGE₂ levels to be decreased in bronchoalveolar lavage fluid (35) and in cultured fibroblasts from patients with IPF (11–14). Therefore, we hypothesized that the activities of PGE₂ and PTEN in fibroblasts might be interrelated, as was recently shown for PGE₂ and hepatocyte growth factor (13). The data described herein support this hypothesis and provide a plausible mechanism whereby PGE₂ affects PTEN activity to inhibit fibroblast migration.

Our data demonstrate that butaprost, a specific EP2 agonist, results in greater cAMP increases and greater PTEN activity than PGE₂ in our cell-free phosphatase activity assay. However, both compounds appear to have similar inhibitory effects on cellular migration. Because cellular migration is a result of highly orchestrated steps and intersecting signaling pathways (36), it is reasonable to expect that interventions targeting a single pathway would be incapable

of inhibiting migration in its entirety. Indeed, in previous reports reconstitution or overexpression of PTEN within cells resulted in only partial inhibition of cell migration (37, 38), suggesting that inhibition of cellular migration by PTEN does not occur in a dose-dependent manner. It is plausible that a threshold level of PTEN activity exists above which no further migration inhibition occurs despite increasing phosphatase activity.

Fibroblast migration and aggregation result in collagen secretion and extracellular matrix remodeling. Although this process is essential for normal wound healing, its dysregulation likely contributes to the excessive deposition of collagens that is characteristic of fibroproliferative disorders. In response to bFGF, PI3K is recruited to the cell membrane where it is activated (39). PI3K, in turn, phosphorylates PIP₂ at the D3 position of the inositol ring, creating the lipid second messenger PIP₃. PIP₃ subsequently binds to pleckstrin homology domains of multiple signaling molecules, including PKB/Akt, which results in Akt phosphorylation and activation (40). PIP₃ also activates the small Rho GTPases Rac and Cdc42, which results in cellular migration (41). PTEN counteracts the activities of PI3K by dephosphorylating PIP₃ and reversing Akt activation (28). Our finding that PGE₂ and butaprost both increase PTEN activity in a cell-free phosphatase assay conclusively demonstrates for the first time that PTEN is a target for EP2. However, we have neither addressed nor excluded the possibility that these EP2 ligands might concomitantly inhibit PI3K activity. It is certainly possible that, if coupled with a decrease in PI3K activity, the modest increases in PTEN activity observed after PGE₂ stimulation would produce a result consistent with our observations: that Akt phosphorylation would be decreased within intact cells, and migration would be inhibited.

Our data demonstrating that fibroblast migration is inhibited by PGE₂ agree with previous data (8). Kohyama and colleagues showed that fibroblast migration was also inhibited by forskolin and isoproterenol, both of which increase intracellular cyclic AMP (cAMP) (8). Among the EP receptors, only EP2 and EP4 exert their biologic activities through G_s-coupled activation of adenylate cyclase. Thus, the findings of Kohyama and coworkers suggested that either EP2 or EP4 might transmit migration-inhibitory signals from PGE₂. Using EP2-specific agonists as well as EP2-null fibroblasts, we now show that the EP2 receptor is responsible for the transduction of signals that results in the inhibition of fibroblast migration. That butaprost, an EP2-selective agonist, was able to induce PTEN activity to a much greater degree than PGE₂ further indicates that PGE₂ may be simultaneously signaling through other EP receptors in our system. For example, concomitant signaling through G_i-coupled EP3, which reduces intracellular cAMP, might have blunted the effect of PGE₂ signaling through G_s-coupled EP2. Therefore, PGE₂ analogs that selectively bind the EP2 receptor might be considerably more effective than PGE₂ itself (which might bind multiple receptors) in the treatment of fibroproliferative diseases.

In addition to migration, PGE₂ modulates a number of other fibroblast functions central to the fibrotic response, including proliferation (9), myofibroblast transdifferentiation (29), and collagen production (29,42). These observations suggest that a relative reduction in local PGE₂ production (or a relative inability to respond to locally available PGE₂) may be instrumental in the pathogenesis of fibroproliferative lung disease. Moreover, the EP2 receptor has been implicated in the inhibitory effects of PGE₂ on collagen production (29,43) and myofibroblast transdifferentiation (29). Whether inhibitory effects on fibroblast proliferation, collagen secretion, and myofibroblast transdifferentiation are also mediated through the activity of PTEN downstream of the EP2 receptor is currently unknown, but may be an area of significant importance for future study.

In our system, we have shown a critical role for PTEN in regulating fibroblast migration using *pten*-null cells. Because PTEN serves to counteract the effects of PI3K signaling, it is likely that baseline migration in *pten*-null cells is increased as a result of an imbalance between PI3K

and PTEN signaling. Further increases in fibroblast migration after the addition of bFGF suggests that PI3K activity is increased by bFGF, and is in agreement with previous reports (39). Most importantly, we found that in *pten*-null cells, EP2 stimulation was incapable of inhibiting fibroblast migration. Although it remains a possibility that the dysregulated migration observed in *pten*-null cells precludes regulation by any mechanism, our observation provides evidence that inhibition of fibroblast migration by EP2 stimulation requires the presence of functional PTEN within a cell.

Post-translational modifications are increasingly recognized as effective regulators of PTEN activity. In IMR-90 cells, increased PTEN phosphatase activity was not associated with increased protein expression; however, phosphorylation on tyrosine, serine, and threonine residues have also been shown to affect the activity of PTEN (24,34). Therefore, we assessed the phosphorylation of PTEN on the C-terminal residues S³⁸⁰, T³⁸², and T³⁸³, as well as on tyrosine residues in IMR-90 cells after EP2 stimulation. We observed that butaprost treatment resulted in a time-dependent decrease in tyrosine phosphorylation of PTEN that correlated temporally with the demonstrated increase in PTEN activity. These findings are in agreement with those of Lu and colleagues, who showed that decreasing tyrosine phosphorylation increases PTEN activity (24). Interestingly, we observed that PTEN activity, as measured by our cell-free phosphatase assay, returned to control levels 24 h after EP2 stimulation, although both tyrosine phosphorylation on PTEN and S⁴⁷³ phosphorylation on Akt remained low 24 h after stimulation. Thus, we conclude that other regulatory effects on both PTEN activity and migration contribute to our experimental findings. One family of protein tyrosine kinases that has been implicated in the phosphorylation of PTEN is the Src-family kinases (24), although others likely exist. Previous data provide clues to a link between PGE₂ and Src activity (7). Because Src activity has been linked to numerous cellular functions, including migration, it is plausible that Src-family kinases are affecting PTEN activity in our system. We have not yet addressed the role of Src-family kinases in mediating changes in PTEN phosphorylation and activity in fibroblasts after PGE₂ stimulation; however, this may be an important area of further investigation.

We observed no appreciable difference in phosphorylation of the C-terminal residues of PTEN. This appears to contrast with the data of Raftopoulou and colleagues, who noted that dephosphorylation on T³⁸³ was critical to inhibiting cell migration (34). However, because an antibody specific only to T³⁸³ phospho-PTEN is not available, we cannot exclude that this may also be occurring in our system. Furthermore, it is likely that PTEN inhibits migration through multiple mechanisms. Indeed, protein phosphatase effects of PTEN on focal adhesions to inhibit cell migration has been previously reported (37), thus demonstrating that inhibition of migration by PTEN may occur independent of the PI3K pathway. Our data support the hypothesis that lipid phosphatase activity of PTEN contributes to inhibition of fibroblast migration.

In summary, our data demonstrate a new role for PTEN in inhibiting fibroblast migration in response to PGE₂ through the EP2 receptor. Continued study of the mechanisms regulating fibroblast migration may provide insight into the development of novel treatments for disorders characterized by progressive fibroblast accumulation and fibrosis.

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Figure 1.

PGE₂ inhibits bFGF-induced fibroblast migration. (A) Migration of human lung fibroblasts in the presence or absence of bFGF, PGE₂, or butaprost. Results are expressed as the mean number of migrated cells per hpf $\pm$ SEM. Experiments were performed in triplicate wells, and the experiment was repeated twice with representative results shown (* $P < 0.001$). (B) The EP2 receptor is the predominant EP receptor expressed on IMR-90 cells. Bands corresponding to the EP1 and EP3 receptors were present but extremely faint. EP4 was undetectable. The results are representative of two separate experiments. (C) Migration of wild-type (C57Bl/6) (white bars) or EP2-null (black bars) murine lung fibroblasts in the presence or absence of bFGF, PGE₂, or butaprost. Results are expressed as the mean number of migrated cells per hpf $\pm$ SEM.

Experiments were performed in triplicate wells, and the experiment was repeated twice with representative results shown (* $P < 0.001$).

Figure 2.

PGE₂ and butaprost treatment of lung fibroblasts increases PTEN activity. (A) PTEN activity in IMR-90 cells increased 40% compared with control conditions by 6 h after PGE₂ treatment and continuing through 18 h (**P* < 0.001). PTEN activity returned to the control level by 24 h after treatment. Total PTEN levels were unchanged throughout the time course. (B) PTEN activity in IMR-90 cells was increased 3.5-fold after butaprost treatment as compared with control conditions (**P* < 0.001). PTEN activity returned to that of control by 24 h. Total PTEN levels were unchanged throughout the time course. (C) Increased PTEN activity is dependent on EP2 signaling. PTEN activity increases in a time-dependent fashion after butaprost treatment in C57Bl/6 cells (white bars), but not EP2-null cells (black bars). (D) Intracellular

production of cyclic AMP (cAMP) in IMR-90 cells after butaprost (*triangles*) or PGE₂ (*squares*) stimulation, reported in arbitrary units. The ratio of cAMP increase after butaprost as compared with PGE₂ is reported below the graph. The results are representative of two independent experiments. (*E*) Butaprost treatment resulted in a time-dependent diminution in S⁴⁷³ phospho-Akt. Control indicates IMR-90 cells in serum-free media alone. The data are representative of two sets of lysates prepared independently. Total Akt levels were unaffected. *Numbers above lanes* indicate relative density of phospho-Akt to Akt.

Figure 3.

PTEN activity is required for EP2-mediated inhibition of fibro-blast migration. Migration in wild-type (C57Bl/6) (*white bars*) murine embryonic fibroblasts and *pten*-null (*black bars*) embryonic fibroblasts. Baseline migration of *pten*-null fibroblasts was significantly greater than control fibroblasts. In the presence of bFGF, both control and *pten*-null fibroblasts demonstrated increased migration. Whereas bFGF-induced migration of wild-type fibroblasts was significantly inhibited by either butaprost or PGE₂ (**P* < 0.001), migration in *pten*-null cells could not be inhibited by either agent.

Figure 4.

Butaprost treatment of lung fibroblasts results in decreased PTEN tyrosine phosphorylation. (A) IMR-90 lung fibroblasts treated with butaprost (500 nM) were lysed, separated by electrophoresis, and were immunoblotted with antibodies against PTEN phosphorylated on S³⁸⁰ or S³⁸⁰T³⁸²T³⁸³, or total PTEN. No change in phosphorylation of these residues was observed after butaprost treatment. (B) IMR-90 lung fibroblasts treated with butaprost (500 nM) were lysed and PTEN was immunoprecipitated. Immunoprecipitates were separated by electrophoresis and immunoblotted with antibody against phosphotyrosine. Numbers above lanes indicate relative density of phosphotyrosine to total PTEN. Tyrosine phosphorylation of PTEN decreased over time after butaprost treatment. To ensure equal protein loading, blots were stripped and reprobed for PTEN.