Reversal of Myofibroblast Differentiation by Prostaglandin E₂

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Differentiation of fibroblasts into α -smooth muscle actin (SMA)-expressing myofibroblasts represents a critical step in the pathogenesis of fibrotic disorders, and is generally regarded as irreversible. Prostaglandin E₂ (PGE₂) has been shown to prevent multiple aspects of fibroblast activation, including the differentiation of fibroblasts to myofibroblasts. Here, we investigated its ability to reverse this differentiated phenotype. Fetal and adult lung fibroblasts were induced to differentiate into myofibroblasts by 24-hour culture with transforming growth factor (TGF)- β 1 or endothelin-1. Cells were then treated without or with PGE₂ for various intervals and assessed for α -SMA expression. In the absence of PGE₂ treatment, α -SMA expression induced by TGF- β 1 was persistent and stable for up to 8 days. By contrast, PGE₂ treatment effected a dose-dependent decrease in α -SMA and collagen I expression that was observed 2 days after PGE₂ addition, peaked at 3 days, and persisted through 8 days in culture. This effect was not explained by an increase in myofibroblast apoptosis, and indeed, reintroduction of TGF- β 1 2 days after addition of PGE₂ prompted dedifferentiated fibroblasts to re-express α -SMA, indicating redifferentiation to myofibroblasts. This effect of PGE₂ was associated with inhibition of focal adhesion kinase signaling, and a focal adhesion kinase inhibitor was also capable of reversing myofibroblast phenotype. These data unambiguously demonstrate reversal of established myofibroblast differentiation. Because many patients have established or even advanced fibrosis by the time they seek medical attention, this capacity of PGE₂ has the potential to be harnessed for therapy of late-stage fibrotic disorders.

Keywords: E prostanoid receptor; transforming growth factor- β 1; endothelin-1; α -smooth muscle actin; focal adhesion kinase

Pathologic scarring or fibrosis results in impaired organ function in diseases such as cirrhosis, diabetes, end-stage renal disease, scleroderma, and pulmonary fibrosis (1, 2). The accumulation of myofibroblasts within pathologic lesions is a pivotal feature of many fibrotic disorders (1, 2). Fibroblasts possess the potential to differentiate into myofibroblasts, which are distinguished from fibroblasts by their expression of contractile proteins, such as α -smooth muscle actin (α -SMA), and their exuberant production of extracellular matrix proteins, such as collagen I. This expression of α -SMA and increased extracellular matrix production endow myofibroblasts with the ability to participate in

CLINICAL RELEVANCE

The differentiation of fibroblasts to myofibroblasts is generally considered to be irreversible, and represents one of the challenges in treating patients with established fibrotic disease. This work demonstrates the ability of the antifibrotic mediator, prostaglandin E₂ (PGE₂), to dedifferentiate established myofibroblasts. These findings highlight the plasticity of myofibroblasts, and suggest a means by which PGE₂ has therapeutic potential even in late-stage fibrotic disorders.

wound contraction (3). Because the differentiation of fibroblasts to myofibroblasts is generally considered irreversible (4), resolution of normal wound repair is thought to require apoptosis of myofibroblasts (5). By contrast, pathologic fibrosis occurs when myofibroblasts fail to apoptose and instead accumulate and persist within tissues, contributing to progressive scarring. Indeed, idiopathic pulmonary fibrosis (IPF)—the most common and fatal type of lung fibrosis—is characterized by myofibroblast resistance to apoptosis (6–8).

A variety of profibrotic mediators, including transforming growth factor (TGF)- β 1 and endothelin-1, are potent inducers of fibroblast to myofibroblast differentiation (3, 9, 10), and inhibition of this process may serve as an effective means to prevent the progression of disease (11). Unfortunately, many patients with IPF reach clinical attention only after significant fibrosis has already occurred (12). For these patients, reversal of myofibroblast differentiation—were it possible—represents an attractive therapeutic approach to already established disease.

Prostaglandin E₂ (PGE₂) is a ubiquitous bioactive lipid mediator synthesized from arachidonic acid by alveolar epithelial cells (13), fibroblasts (14), and alveolar macrophages (15), which has been shown to inhibit fibroblast proliferation (16), collagen expression (16, 17), migration (18), and differentiation into myofibroblasts (17, 19). However, neither PGE₂ nor any other antifibrotic mediator has been conclusively shown to reverse the differentiated myofibroblast phenotype. In this study, we report that PGE₂ can indeed accomplish this. By demonstrating that the myofibroblast phenotype is more plastic than is generally appreciated, these findings suggest a new therapeutic paradigm that has the potential to reverse established fibrosis of the lung and potentially other organs.

MATERIALS AND METHODS

Cell Culture

IMR-90 fetal lung fibroblasts (American Tissue Culture Collection, Manassas, VA), CCL-210 adult lung fibroblasts (American Tissue Culture Collection), and fibroblasts grown from surgical lung biopsy specimens from patients with IPF were cultured in Dulbecco's modified Eagles medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT) and 100 U/ml penicillin/streptomycin at 37°C with 5% CO₂. Patient-derived cells were cultured from the outgrowth of surgical lung biopsy specimens from patients with IPF, as previously described

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(16), in studies approved by the University of Michigan institutional review board, with all patients providing informed consent. All experiments were performed in cells between passages 6 and 10. Cells were initially plated in six-well plates at 4×10^5 cells per well before being growth arrested in FBS-free Dulbecco's modified Eagles medium for 2 days. To induce myofibroblast differentiation, cells were pretreated with TGF- β 1 (2 ng/ml; R&D Systems, Minneapolis, MN) or endothelin-1 (100 ng/ml; EMD Millipore, Billerica, MA) for 24 hours. To investigate the ability of PGE₂ to reverse myofibroblast differentiation, medium was then removed and cells were subsequently treated for 1–8 days with PGE₂ (10–500 nM; Cayman Chemical, Ann Arbor, MI), aspirin (100 μ M; Cayman Chemical), the E prostanoïd (EP) 2 receptor agonist, butaprost free acid (500 nM; Cayman Chemical), the EP3 agonist, ONO-AE3-248 (100 nM; kind gift from Ono Pharmaceuticals, Osaka, Japan), the EP4 agonist, ONO-AE1-329 (100 nM; also a gift from Ono Pharmaceuticals), the adenylyl cyclase activator, forskolin (100 μ M; EMD Millipore), the focal adhesion kinase (FAK) inhibitor, PF573228 (10 μ M; Tocris Bioscience, Ellisville, MO), or vehicle control (DMSO).

Analysis of α -SMA, Collagen I, and Phosphorylated FAK

Levels of α -SMA, collagen I, and phosphorylated FAK protein were assayed by immunoblotting as previously described (16). Antibody concentrations were as follows: α -SMA (1:1,000; Dako, Carpinteria, CA); collagen I (1:500; CedarLane, ON, Canada); Tyr397-phosphorylated FAK (1:1,000; Cell Signaling, Beverly, MA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA); and α -tubulin (1:1,000, Sigma-Aldrich, St. Louis, MO). Densities of target protein bands were normalized to GAPDH or α -tubulin loading controls, and were expressed as a fraction of untreated control. The abundance and organization of α -SMA into stress fibers was assessed by immunofluorescence microscopy. Cells were cultured on glass slides, fixed and permeabilized with 3% paraformaldehyde, and incubated with α -SMA antibody (1:100). Bound α -SMA antibody was visualized after incubation with FITC-conjugated secondary antibody. Levels of α -SMA and collagen 1A1 mRNA were assayed by real-time RT-PCR on the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using specific α -SMA and collagen 1A1 primers and probes (Applied Biosystems). Primer and probes for β -actin, used as endogenous loading control, were as previously reported (16).

Cell Count and Apoptosis Assay

Cell count was performed on a hemocytometer. Apoptosis was assayed by presence of cleaved poly-ADP ribose polymerase (PARP) using immunoblotting, as described previously (6). Cells treated with Fas ligand (100 ng/ml; EMD Millipore) and cycloheximide (0.5 μ g/ml; Sigma-Aldrich) were used as a positive control.

Statistical Analysis

Data are expressed as means ($\pm$ SEM). Data were analyzed on GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA) using ANOVA or Student's *t* test where appropriate, with a *P* value less than 0.05 defined as statistically significant.

RESULTS

PGE₂ Reverses Myofibroblast Differentiation in Fetal Lung Fibroblasts

TGF- β 1 is well recognized to induce fibroblast-to-myofibroblast differentiation (3), with the myofibroblast phenotype persisting for several days after TGF- β 1 treatment (20). Treatment of IMR-90 fetal lung fibroblasts with TGF- β 1 for 1 day resulted in an increase in α -SMA expression, a marker of differentiated myofibroblasts, which persisted through 5 days (Figure 1A) and, in fact, for up to 8 days (data not shown), indicating that the effect of TGF- β 1 is long lasting. Although PGE₂ has been shown to prevent TGF- β 1-induced myofibroblast differentiation (19), the ability of PGE₂ to reverse myofibroblast differentiation has never been investigated. To examine this, IMR-90 cells were pretreated with TGF- β 1

(2 ng/ml) for 1 day to induce myofibroblast differentiation, after which TGF- β 1-containing medium was removed and cells were washed and then treated with PGE₂ (500 nM) or medium alone for 1–8 days. PGE₂ significantly attenuated α -SMA expression by Day 2. The reduction in α -SMA peaked at Day 3 and persisted through Day 5 in IMR-90 cells (Figure 1A) and up to Day 8 in CCL210 cells (Figure 1B). Long-lived reduction in α -SMA expression was likewise observed even when PGE₂ was removed after 24-hour treatment (Figure 1B). α -SMA expression was enhanced above baseline when myofibroblasts were treated with aspirin, a COX inhibitor that inhibits endogenous production of PGE₂ (Figure 1B). The decrease in α -SMA expression by PGE₂, assessed after 2 days of treatment, was dose dependent (Figure 1C). Immunofluorescence microscopy of α -SMA revealed that PGE₂ treatment resulted in a profound decrease in both total α -SMA as well as that organized into stress fibers in most of the cells in culture (Figure 1D).

PGE₂ signals by ligating one of four G protein-coupled EP receptors (21). The EP2 and EP4 receptors signal predominantly through G α_s , leading to activation of adenylyl cyclase and increased production of the second messenger cAMP (22). EP3 couples to G α_i , which inhibits adenylyl cyclase (22). The inhibition of fibroblast proliferation (16), collagen expression (16), and myofibroblast differentiation (19) by PGE₂ has been shown to occur via ligation of the EP2 receptor, which is the most abundantly expressed receptor in fibroblasts (16). To determine which receptor mediates the PGE₂ reduction of α -SMA in differentiated myofibroblasts, myofibroblasts were treated with EP2-, EP3-, and EP4-selective agonists. Agonists for either the EP2 or EP4 receptors recapitulated the actions of PGE₂ in reducing α -SMA, whereas an EP3 agonist did not (Figure 1E). Treatment with forskolin, a direct adenylyl cyclase activator, also attenuated α -SMA expression in myofibroblasts (Figure 1E). These results suggest that PGE₂ reversal of myofibroblast differentiation occurs via the EP2/EP4-cAMP signaling pathway.

To determine if the decrease in α -SMA protein expression was due to a decrease in mRNA levels, real-time RT-PCR for α -SMA was performed in cells treated for 2 days with PGE₂. α -SMA mRNA levels were significantly decreased by PGE₂ in a manner paralleling the effects on the protein (Figure 2A). To verify that the effect of PGE₂ represented a true reversal of myofibroblast differentiation, and was not limited to α -SMA, we also examined the expression of collagen I, the predominant matrix protein whose expression is up-regulated in myofibroblasts. As observed for α -SMA, TGF- β 1 pretreatment increased collagen I expression, whereas subsequent addition of PGE₂ reduced collagen I mRNA (Figure 2B) and protein expression (Figure 2C). In contrast to the time course for the reduction of α -SMA expression, reduction in collagen I expression was maximal at 1 day. This difference likely reflects the faster turnover of collagen I than of α -SMA protein (20, 23).

To determine if the ability of PGE₂ to reverse the myofibroblast phenotype was specific for TGF- β 1-stimulated differentiation, we used an alternative profibrotic mediator capable of promoting α -SMA expression in fibroblasts: endothelin-1 (24). PGE₂ treatment likewise led to decreased expression of α -SMA in fibroblasts pretreated with endothelin-1 (Figure 3A), demonstrating that the reversal of myofibroblast differentiation is not limited to a single differentiating agent.

In all of the previous experiments, the TGF- β 1-containing medium was removed before PGE₂ was added. Including a dose of TGF- β 1 in the medium concurrently with PGE₂ treatment failed to abrogate the ability of PGE₂ to reduce α -SMA expression, showing that PGE₂ is capable of reversing myofibroblast differentiation even in the continued presence of a profibrotic stimulus (Figure 3B). Together, these data show that PGE₂ can achieve robust and sustained reversal of myofibroblast differentiation in fetal lung fibroblasts.

Figure 1. Prostaglandin E₂ (PGE₂) reverses transforming growth factor (TGF)- β 1-induced myofibroblast differentiation. (A) IMR-90 cells were pretreated with TGF- β 1 (2 ng/ml) for 1 day to induce myofibroblast differentiation, after which medium was removed and cells were treated with or without PGE₂ (500 nM) for 1–5 days in serum-free medium. Lysates were collected at the indicated days after treatment and immunoblotted for α -smooth muscle actin (α -SMA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Densitometric values of α -SMA relative to GAPDH, normalized to the Day 1 no-PGE₂ control, are shown graphically ($n = 3$) with a representative immunoblot shown above. * $P < 0.05$ relative to no-PGE₂. (B) CCL-210 fibroblasts were pretreated with TGF- β 1 (2 ng/ml) for 1 day to induce myofibroblast differentiation. Cells were then treated with PGE₂ (500 nM) or aspirin (ASA) (100 μ M) for 1 day before being washed with serum-free medium. Cell lysates were collected at Days 5 and 8 and assayed for α -SMA expression by immunoblot. A representative blot of three experiments is shown. (C) IMR-90 cells were treated with varying doses of PGE₂ for 2 days after initial pretreatment with TGF- β 1. Expression of α -SMA relative to GAPDH, normalized to no-PGE₂ control, is shown ($n = 3$). (D) IMR-90 cells were treated with or without PGE₂ (500 nM) for 2 days after initial pretreatment with TGF- β 1. Cells were immunostained for α -SMA; a representative image is shown from three replicates. (E) IMR-90 cells differentiated into myofibroblasts with TGF- β 1 were treated with either PGE₂ (500 nM), the E prostanoic (EP) 2 agonist, butaprost free acid (500 nM), the EP3 agonist, ONO-AE3-248 (100 nM), the EP4 agonist, ONO-AE1-329 (100 nM), or the direct adenylyl cyclase activator, forskolin (100 μ M). Expression of α -SMA was assayed by immunoblot (shown above) with densitometry relative to α -tubulin and normalized to no-PGE₂ (shown below) ($n = 3$). Results are expressed as means ($\pm$ SEM). * $P < 0.05$ relative to TGF- β 1 pretreatment.

PGE₂ Reverses Myofibroblast Differentiation in Nonfibrotic and Fibrotic Adult Lung Fibroblasts

To confirm that the findings obtained with fetal lung fibroblasts were also applicable to adult cells, we differentiated primary adult lung fibroblasts to myofibroblasts with TGF- β 1 (2 ng/ml) for 1 day before addition of PGE₂. PGE₂ treatment of adult

cells resulted in a decrease in α -SMA expression at Treatment Day 2 that was even more pronounced than that observed in fetal lung fibroblasts (Figure 4A). The organization of α -SMA into stress fibers was also notably diminished in adult cells, as observed by immunofluorescence microscopy (Figure 4B). Fibroblasts derived from patients with IPF have been noted to

Figure 2. PGE₂ reduces α -SMA mRNA and collagen expression in differentiated myofibroblasts. IMR-90 cells were pretreated with or without TGF- β 1 (2 ng/ml) for 1 day to induce myofibroblast differentiation before they were treated with PGE₂ (500 nM) for 2 days. α -SMA (A) and collagen 1A1 (B) mRNA were assayed by real-time RT-PCR ($n = 6$). (C) Cells were pretreated with TGF- β 1 (2 ng/ml) before treatment with or without PGE₂ (500 nM) for 1–2 days. Collagen I expression was assayed by immunoblotting, with representative immunoblot shown. Densitometric values relative to GAPDH normalized to no-PGE₂ Day 1 control are shown below ($n = 3$). Results are expressed as means $\pm$ SEM. * $P < 0.05$ relative to no-PGE₂; # $P < 0.05$ relative to no-TGF- β 1 pretreatment.

exhibit a greater baseline expression of α -SMA than do non-fibrotic cells (25), and also exhibit a variable degree of resistance to the antifibrotic actions of PGE₂ (26). Indeed, we observed that cultured cells from patients with IPF exhibited expression of α -SMA in the absence of TGF- β 1 pretreatment, and augmented α -SMA expression in response to exogenous TGF- β 1 to only a minimal degree (Figure 4C). In one line (IPF 1), PGE₂ was able to diminish α -SMA expression to a degree comparable to that observed in nonfibrotic fibroblasts, whereas in another line (IPF 2), a moderate degree of resistance to PGE₂ reversal of the myofibroblast phenotype was observed (Figure 4C).

The Decrease in α -SMA Expression by PGE₂ Is Not Due To a Decrease in Cell Number or Apoptosis of Myofibroblasts

It has previously been shown that PGE₂ can induce apoptosis in lung fibroblasts (6), although its effects on myofibroblast survival are unknown. To determine if the PGE₂-mediated decrease in α -SMA and collagen I expression is due to an increase in myofibroblast apoptosis, we performed direct cell counting of cells after PGE₂ treatment and observed no change in cell number (Figure 5A). Apoptosis was more specifically evaluated by measuring cleaved PARP. As a positive control, cleaved PARP was observed in myofibroblasts treated with Fas ligand (100 ng/ml) and cycloheximide (0.5 μ g/ml) (6). By

Figure 3. PGE₂ reverses myofibroblast differentiation in the presence of various profibrotic stimuli. (A) IMR-90 cells were pretreated with endothelin-1 (100 ng/ml) for 1 day before treatment with or without PGE₂ (500 nM) for 2 days ($n = 5$). (B) After initial pretreatment with TGF- β 1 (2 ng/ml), cells were treated with or without PGE₂ in the presence of TGF- β 1 (2 ng/ml), and α -SMA was assessed by immunoblot ($n = 5$). For both (A) and (B), representative immunoblots are shown above, with densitometric values of α -SMA relative to GAPDH and normalized to no-PGE₂ treatment shown below. Results are expressed as means $\pm$ SEM. * $P < 0.05$ relative to no-PGE₂.

Figure 4. PGE₂ reverses myofibroblast differentiation in primary adult fibroblasts from nonfibrotic and IPF lung. Primary adult lung fibroblasts from nonfibrotic lungs were pretreated with TGF- β 1 (2 ng/ml) for 1 day before treatment with or without PGE₂ (500 nM) for 1–2 days. (A) Cell lysates were assayed for α -SMA expression by immunoblot, with representative immunoblot shown above, and densitometric values relative to GAPDH and normalized to no-PGE₂ Day 1 control shown below ($n = 3$). Results are expressed as means $\pm$ SEM. * $P < 0.05$ relative to no-PGE₂. (B) α -SMA stress fiber organization was visualized by immunofluorescence microscopy after 2 days of PGE₂ treatment, with a representative image of three independent experiments shown. Magnification, 20 $\times$. (C) Fibroblasts from two separate patients with IPF were pretreated in the presence or absence of TGF- β 1 (2 ng/ml) for 1 day before treatment with or without PGE₂ (500 nM) for 2 days. Expression of α -SMA was assayed by immunoblot.

contrast, PGE₂ treatment failed to increase cleaved PARP (Figure 5B). These data suggest that reduced α -SMA and collagen I expression by PGE₂ is not explained by an increase in myofibroblast apoptosis.

PGE₂ Reversal of Myofibroblast Differentiation Involves Inactivation of FAK

Activation of FAK has been shown to be critical for fibroblast differentiation into myofibroblasts (27). We have previously

shown that PGE₂ is capable of preventing TGF- β 1-induced activation of FAK in undifferentiated lung fibroblasts (28). Here, we sought to determine if PGE₂ is capable of reversing FAK activation in myofibroblasts, and if inhibition of FAK is sufficient to reverse myofibroblast differentiation. PGE₂ treatment of TGF- β 1-elicited myofibroblasts resulted in a decrease of Tyr397-phosphorylated catalytically active FAK (Figure 6A). Because we did not observe a qualitative difference between adult and fetal lung fibroblasts in FAK inhibition by PGE₂, data from both cell types were averaged together in densitometry

Figure 5. PGE₂ reduction of α -SMA is not due to increased cellular apoptosis. IMR-90 cells were pretreated with TGF- β 1 (2 ng/ml) for 1 day before subsequent treatment with PGE₂ (500 nM) for 2 days. Cell counts were performed by hemocytometer (A), and presence of cleaved poly-ADP ribose polymerase (PARP) was assayed by immunoblot (B). A representative immunoblot of three experiments is shown above, with densitometry of cleaved PARP relative to total PARP shown below. FasL (100 ng/ml) and cycloheximide (CHX; 0.5 μ g/ml) were administered as a positive control. Results are expressed as means $\pm$ SEM.

Figure 6. PGE₂ reverses myofibroblast differentiation via decreased focal adhesion kinase (FAK) activation. (A) Fibroblasts were pretreated with TGF-β1 (2 ng/ml) for 1 day to induce myofibroblast differentiation before being treated with or without PGE₂ (500 nM) for 1–2 days. Expression of active Tyr397-phosphorylated FAK was assayed by immunoblot. A representative immunoblot from IMR-90 cells is shown above, with densitometry of phosphorylated FAK relative to GAPDH shown below (combined result of IMR-90 [*n* = 3] and CCL-210 [*n* = 2] cells). **P* < 0.05. (B) TGF-β1-induced myofibroblasts were treated with PGE₂ for 15 minutes to 6 hours and expression of Tyr397-phosphorylated FAK was assayed by immunoblot. (C) TGF-β1-induced myofibroblasts of fetal and adult origin were treated with the FAK inhibitor, PF573228 (10 μM), for 2 days and expression of collagen I and α-SMA were assayed by immunoblot. A representative immunoblot from IMR-90 cells is shown, with mean densitometry of collagen I and α-SMA expression shown below (combined result of IMR-90 [*n* = 3] and CCL-210 [*n* = 2] cells). Results are expressed as means ± SEM. **P* < 0.05.

measurements, as indicated in the legend for Figure 6. The decrease in phosphorylated FAK was evident and appeared to be maximal by Day 1 of PGE₂ treatment, a time point which precedes its maximal effects on myofibroblast differentiation (see Figure 1). Subsequent time course experiments revealed that FAK dephosphorylation was in fact observed as quickly as 15–30 minutes after PGE₂ addition (Figure 6B), and persisted through Day 2. Differentiated myofibroblasts treated with the FAK inhibitor, PF573228, used at nontoxic concentrations shown to decrease active phosphorylated FAK (8), likewise exhibited decreased α-SMA and collagen I expression, suggesting that the inhibition of FAK is sufficient to reverse myofibroblast differentiation.

Readdition of TGF-β1 after PGE₂ Reversal Restores the Myofibroblast Phenotype

We next sought to determine if fibroblasts dedifferentiated from myofibroblasts by treatment with PGE₂ could be differentiated back into myofibroblasts upon readdition of TGF-β1. Myofibroblasts were treated with PGE₂ for 2 days to reverse differentiation. Medium was removed and cells were subsequently retreated with TGF-β1 for 24 hours, and α-SMA expression was assayed by immunoblotting. α-SMA expression (Figure 7A), as well as levels of phosphorylated FAK (Figure 7B), increased after readdition of TGF-β1. This ability of PGE₂-treated cells to re-express α-SMA upon restimulation with TGF-β1 further argues against apoptosis accounting for the diminished expression of this contractile protein.

DISCUSSION

Pathologic fibrosis can occur in virtually all organs, and, by some estimates, represents a predominant feature in up to 45% of chronic diseases worldwide (1). The activation and persistence of myofibroblasts are critical to the exuberant extracellular matrix deposition and scarring associated with these diseases (1, 2). Although antifibrotic mediators, such as PGE₂ (19), and blockade of profibrotic mediators, such as TGF-β1 and endothelin-1 (9, 24), can attenuate fibroblast activation and prevent their differentiation into myofibroblasts, the potential for reversal of established myofibroblast differentiation is poorly understood. Here, we show for the first time that PGE₂, which has previously been shown to inhibit nearly all aspects of fibroblast function, possesses the additional ability to reverse the established phenotype of differentiated myofibroblasts. Although mitogenic growth factors have been reported to reduce α-SMA expression in myofibroblasts, the experimental contexts for these published studies were quite limited (20, 29). Our data provide a more comprehensive examination of the capacity for PGE₂ in this regard. PGE₂ robustly and dose-dependently inhibited both α-SMA and collagen I expression, which was sustained through several days. These effects were observed when cells were differentiated with either TGF-β1 or endothelin-1, and were observed in both fetal lung cells as well as adult cells derived from both nonfibrotic and IPF lung. The reversal of myofibroblast differentiation was not attributable to apoptosis, as dedifferentiated fibroblasts could be differentiated back into myofibroblasts, and instead correlated with inactivation of FAK. These findings establish the ability of this lipid mediator to not only prevent, but also to reverse this important feature of established fibrosis, implying a possible therapeutic advantage of PGE₂ over other agents that are only capable of preventing myofibroblast differentiation, in the treatment of patients with established fibrotic disorders.

The differentiation of fibroblasts to myofibroblasts is generally regarded as irreversible (4, 5). Indeed, treatment of differentiated

Figure 7. Readdition of TGF- β 1 to PGE₂-dedifferentiated myofibroblasts restores the myofibroblast phenotype. IMR-90 cells differentiated into myofibroblasts with TGF- β 1 for 1 day and then dedifferentiated by treatment with PGE₂ (500 nM) for 2 days were once again treated with TGF- β 1 (2 ng/ml) for 1 day and α -SMA ($n = 3$) (A) and Tyr397-phosphorylated FAK (B) were assayed by immunoblot. Both a representative immunoblot of α -SMA (A) top and densitometric values of α -SMA relative to GAPDH normalized to no-PGE₂ control (A) bottom are shown. Results are expressed as means $\pm$ SEM. * $P < 0.05$.

myofibroblasts with a TGF- β 1 receptor inhibitor failed to reduce α -SMA expression over 72 hours (20). In our system, cells that were differentiated into myofibroblasts by TGF- β 1 maintained α -SMA expression for up to 5 days after TGF- β 1 was removed. Phenotypically, myofibroblasts differ from fibroblasts by their expression of α -SMA and its organization into cytoplasmic stress fibers, which provides the intracellular machinery necessary for contraction, and by their exaggerated capacity for production of extracellular matrix proteins that comprise scars. The ability of PGE₂ to reduce expression of α -SMA at both mRNA and protein levels, its organization into stress fibers, and collagen I mRNA and protein in cells differentiated by either TGF- β 1 or endothelin-1 strongly suggests the disruption of the phenotypic program of myofibroblasts, rather than an effect that could instead be explained merely by the inhibition of a single gene. Furthermore, these data suggest that differentiated myofibroblasts are more plastic than previously appreciated. Although we have previously shown that PGE₂ is also capable of inducing fibroblast apoptosis (6), apoptosis cannot explain the decreased expression of α -SMA and collagen I that we observed. First, no significant apoptosis was identified under the experimental conditions used. Second, the ability of these cells to differentiate back into myofibroblasts with the readdition of TGF- β 1 indicates their functional viability.

The precise molecular program that distinguishes myofibroblasts from fibroblasts is poorly understood. Previous studies

have implicated FAK (30) and Akt (27) signaling, as well as endoplasmic reticulum stress (31), in myofibroblast differentiation; in addition, an epigenetic signature distinct from that of fibroblasts has been observed in myofibroblasts (32). All of these mechanisms represent potential targets that might mediate phenotypic reversal by PGE₂; indeed, this prostanoid is known to inhibit FAK and Akt activation (6, 28), and to increase global DNA methylation in fibroblasts (33). Here, we observed that levels of Tyr397-phosphorylated FAK in myofibroblasts decreased within 15 minutes of PGE₂ treatment, and that inhibition of FAK was capable of reversing the myofibroblast phenotype. FAK is a critical signaling molecule for several receptors mediating cell adhesion, including α v β 5 integrin. Loss of cell contact (34) and treatment of corneal myofibroblasts with blocking antibodies to α v β 5 integrin (35) have been shown to decrease α -SMA stress fiber formation. Our data suggest that the inhibition of adhesion signaling by PGE₂ represents a critical mechanism for the reversal of myofibroblast differentiation. We do note, however, that treatment of myofibroblasts with a FAK inhibitor did not reverse α -SMA expression to the same degree as PGE₂, suggesting that PGE₂ may employ other mechanisms (e.g., activation of PTEN/inhibition of Akt [18], activation of the wnt/ β -catenin signaling pathways [36], and modification of gene-specific DNA methylation patterns [33]) that are responsible for reversal of myofibroblast differentiation.

Extracts from amniotic membrane stroma (37), fibroblast growth factor (29), platelet-derived growth factor (20), and serum (20) have previously been reported to reverse myofibroblast differentiation; however, in these studies, either the specific mediator was not identified or the mechanisms responsible for reversal of myofibroblast differentiation were not elucidated. Our studies more convincingly establish the ability of PGE₂ to reverse differentiation, as the effects of PGE₂ were independent of stimulus, encompassed phenotypic features other than α -SMA, and excluded a role for apoptosis. Although not considered in those studies, it is noteworthy that fibroblast growth factor, platelet-derived growth factor, and serum are capable of inducing cyclooxygenase-2 expression (38), suggesting the possibility that up-regulated PGE₂ production might have contributed to the reversal of differentiation exerted by these mitogens. It has been hypothesized that the ability of these mitogens to stimulate proliferation is critical for their reversal of myofibroblast differentiation (20); in this way too, PGE₂ differs from such agents, as it inhibits, rather than promotes, proliferation (16). This distinction further enhances its potential profile of therapeutic actions as compared with mitogenic factors that would promote fibroblast proliferation. Activation of peroxisome proliferator-activated receptor γ (PPAR γ) has also been reported to promote dedifferentiation of myofibroblasts (39). However, because PGE₂ is not a ligand for PPAR γ (40) and, in fact, inhibits expression of PPAR γ (41), this too is not a feasible mechanism for this action of PGE₂. Thus, our results highlight what is likely to be a new paradigm by which PGE₂ stimulates dedifferentiation.

Our findings using EP-selective agonists suggest that the reversal of myofibroblast differentiation by PGE₂ proceeds by the ligation of the EP4 and, especially, the EP2 receptors and generation of the second messenger cAMP. The participation of these pathways in the reversal of myofibroblast differentiation is not surprising, given that these same EP receptors have been previously shown to be responsible for PGE₂ prevention of fibroblast proliferation (16, 42), collagen expression (16, 42, 43), migration (18), and differentiation (19). It is thus also possible that other pharmacologic agents or endogenous mediators that activate cAMP signaling might be expected to share this action of PGE₂.

We also explored reversal of myofibroblast differentiation by PGE₂ in a limited number of human IPF cell lines. In contrast to the cell lines derived from nonfibrotic lungs, cell lines derived from patients with IPF exhibited variable resistance to PGE₂ reversal of myofibroblast differentiation. Variability in PGE₂ responsiveness is consistent with our previous studies on PGE₂ inhibition of collagen expression and fibroblast proliferation (26). In those studies involving fibroblasts, but not myofibroblasts, we identified several mechanisms that accounted for variable PGE₂ resistance, including decreased expression of EP2 (by DNA hypermethylation [44]) and decreased activation of the downstream signaling molecule, protein kinase A (26). Further studies would be needed to determine the extent and the mechanism(s) of resistance of IPF cells to PGE₂ reversal of myofibroblast differentiation.

The accumulation and persistent activation of myofibroblasts contributes to the excessive scarring associated with many fibrotic disorders, such as IPF. Indeed, the pathology of IPF is characterized by the accumulation of activated myofibroblasts clustered in fibroblastic foci (11, 12), which have additionally been shown to be resistant to apoptosis (7). Although blockade of profibrotic mediators, such as TGF- β 1, can prevent the development of fibrosis in animal models (45), and agents that accomplish this are under development as antifibrotic therapeutics, it is not clear that this strategy will be able to reverse established fibrotic disease. Because most patients with IPF already have extensive fibrosis by the time they come to clinical attention, the ability to reverse established disease offers much greater therapeutic promise. By virtue of its ability to reverse the myofibroblast phenotype, PGE₂ may offer such a possibility. Of note, the IPF lung contains reduced levels of PGE₂ compared with normal lung (46), a characteristic that may be explained by diminished cellular synthetic capacity for this prostanoid (14). PGE₂ supplementation in an effort to restore this deficient brake on fibrosis is therefore an attractive therapeutic strategy. Although administration of exogenous PGE₂ has been shown to prevent bleomycin-induced pulmonary fibrosis (47), it has not been able to ameliorate fibrosis when employed in a so-called therapeutic regimen begun during the fibrotic phase. This might be explained by down-regulated expression of the EP2 receptor, which has been described in fibroblasts from mice with bleomycin-induced fibrosis (48), as well as from some patients with IPF (26). However, such resistance can be overcome, at least *in vitro* (44), and we have shown here that PGE₂ can also reverse myofibroblast differentiation in some IPF cell lines. It remains to be determined if this can be accomplished *in vivo* in humans. Nevertheless, whether with PGE₂ or some alternative therapeutic candidate, our finding that myofibroblast differentiation is a reversible phenomenon offers the possibility of a new therapeutic approach for IPF as well as other fibrosing disorders.

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