The antifibrotic effects of plasminogen activation occur via prostaglandin E_2 synthesis in humans and mice

Kristy A. Bauman,[1] Scott H. Wettlaufer,[1] Katsuhide Okunishi,[1] Kevin M. Vannella,[2] Joshua S. Stoolman,[1] Steven K. Huang,[1] Anthony J. Courey,[1] Eric S. White,[1] Cory M. Hogaboam,[3] Richard H. Simon,[1] Galen B. Toews,[1] Thomas H. Sisson,[1] Bethany B. Moore,[1] and Marc Peters-Golden[1]

[1]Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, [2]Immunology Graduate Program, and [3]Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA.

Plasminogen activation to plasmin protects from lung fibrosis, but the mechanism underlying this antifibrotic effect remains unclear. We found that mice lacking plasminogen activation inhibitor–1 (PAI-1), which are protected from bleomycin-induced pulmonary fibrosis, exhibit lung overproduction of the antifibrotic lipid mediator prostaglandin E_2 (PGE_2). Plasminogen activation upregulated PGE_2 synthesis in alveolar epithelial cells, lung fibroblasts, and lung fibrocytes from saline- and bleomycin-treated mice, as well as in normal fetal and adult primary human lung fibroblasts. This response was exaggerated in cells from *Pai1*$^{-/-}$ mice. Although enhanced PGE_2 formation required the generation of plasmin, it was independent of proteinase-activated receptor 1 (PAR-1) and instead reflected proteolytic activation and release of HGF with subsequent induction of COX-2. That the HGF/COX-2/PGE_2 axis mediates in vivo protection from fibrosis in *Pai1*$^{-/-}$ mice was demonstrated by experiments showing that a selective inhibitor of the HGF receptor c-Met increased lung collagen to WT levels while reducing COX-2 protein and PGE_2 levels. Of clinical interest, fibroblasts from patients with idiopathic pulmonary fibrosis were found to be defective in their ability to induce COX-2 and, therefore, unable to upregulate PGE_2 synthesis in response to plasmin or HGF. These studies demonstrate crosstalk between plasminogen activation and PGE_2 generation in the lung and provide a mechanism for the well-known antifibrotic actions of the fibrinolytic pathway.

Introduction

In patients with acute and chronic fibrotic lung diseases, fibrin forms within the lung due to a leakage of plasma from damaged vasculature and activation of the coagulation cascade. The extravascular fibrin is not cleared in a timely fashion because of a marked increase in the expression of plasminogen activation inhibitor–1 (PAI-1) relative to urokinase-type plasminogen activator (uPA) (1–3). Transgenic animal experiments have firmly established a causal link between the level of PAI-1 and the severity of fibrosis. Specifically, mice with impaired systemic plasminogen activation to plasmin as the result of overexpression of a PAI-1 transgene develop a more exuberant fibrotic response following bleomycin injury than do littermate controls (4). Similarly, poor outcomes are noted in mice with a targeted deletion of the plasminogen gene (5). Conversely, mice with a targeted deletion of their *Pai1* gene are profoundly resistant to lung fibrosis in the bleomycin model, and they also have significantly improved survival (4, 6). Transgenic overexpression (7), adenoviral delivery (8), and aerosolization (9) of uPA all limit lung fibrosis and improve survival following lung injury. Thus, a robust body of evidence indicates that plasminogen activation protects from lung fibrosis. However, an explanatory mechanism for this protective effect is unclear. Although it was originally attributed to plasmin-mediated breakdown of fibrin, it has been shown that fibrosis is not substantially inhibited in mice genetically lacking fibrinogen (6, 10). More recently, the antifibrotic actions of plasminogen activation to plasmin have been ascribed to other mechanisms including the proteolytic release of HGF (6, 11).

Another well-known antifibrotic factor in the lung is PGE_2. PGs, including PGE_2, are generated via conversion of arachidonic acid to PGH_2 via COX-1 or COX-2 enzymes. Via E prostanoid receptor 2–mediated (EP2-mediated) increases in intracellular cyclic AMP, PGE_2 directly inhibits major pathobiologic functions of effector fibroblasts including chemotaxis, proliferation, collagen synthesis, and differentiation to myofibroblasts (12–15). Derangements of PG synthesis are present in fibrotic diseases in humans and animal models of pulmonary fibrosis. Reduced PGE_2 levels have been reported in bronchoalveolar lavage fluid and conditioned medium from alveolar macrophages of patients with idiopathic pulmonary fibrosis (IPF) (16, 17). Fibroblasts from IPF patients are unable to upregulate the COX-2 enzyme and are thereby deficient in PGE_2 production (18–20); it has recently been suggested that this defect may be the consequence of histone deacetylation of the *COX2* gene promoter (21). The relevance of such an impairment is suggested by the facts that pharmacologic (administration of indomethacin) (22) or genetic (gene deletion of *Cox2*) (23) reduction in PGE_2 synthesis in the lung as well as gene deletion of EP2 (24) augment bleomycin-induced fibrosis in mice. By contrast, protection against experimental fibrosis has been observed when endogenous PGE_2 is overproduced (21, 25, 26) or when

Authorship note: Kristy A. Bauman, Scott H. Wettlaufer, and Katsuhide Okunishi contributed equally to this work. Bethany B. Moore and Marc Peters-Golden contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J Clin Invest.* 2010;120(6):1950–1960. doi:10.1172/JCI38369.

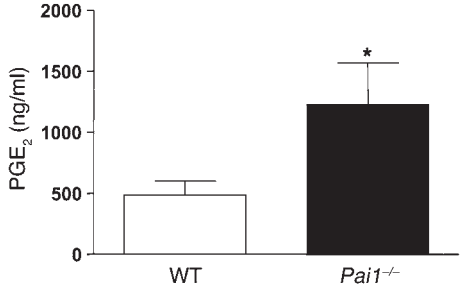

Figure 1
Bleomycin-treated *Pai1*$^{-/-}$ mice overproduce PGE_2. WT or *Pai1*$^{-/-}$ mice were injected with bleomycin on day 0. On day 21, lungs were removed and homogenized. Lipids were extracted using C18 cartridges, and levels of PGE_2 were measured by ELISA; $n = 5$, $^*P = 0.03$.

exogenous PGE is administered (27). Interestingly, a reduction in PGE_2 responsiveness mediated by a loss of the EP2 receptor has also been described in fibroblasts derived from bleomycin-treated mice (24) and IPF patients (28). Thus, diminished PGE_2 production and/or signaling characterize lung fibrosis and are likely to be pathophysiologically significant.

Here we hypothesized that the antifibrotic actions of plasminogen activation are mediated by the induction of PGE_2 synthesis. To date, there are no reports of cross-regulation between plasminogen activation and PGE_2 in the lung. Only a few examples of crosstalk between these two systems (in colon cancer, gastric fibroblasts, and osteoblasts) are noted in the literature (29–31), and no study has reported that plasminogen activation promotes PGE_2 synthesis in any organ or cell. Herein we demonstrate that (a) the plasminogen activation pathway enhances PGE_2 production in vivo and in relevant cell types in vitro; (b) PGE_2 synthesis accounts for direct inhibitory effects on fibroblasts of plasminogen activation to plasmin; (c) PGE_2 elaboration proceeds via a plasmin/HGF/COX-2 pathway; and (d) the HGF/COX-2/PGE_2 axis accounts for the protection against pulmonary fibrosis observed in *Pai1*$^{-/-}$ mice.

Results

Pai1$^{-/-}$ *mice exhibit increased production of* PGE_2 *in vivo*. We have previously reported that *Pai1*$^{-/-}$ mice are protected from bleomycin-induced lung fibrosis (4, 32). To determine whether this protected phenotype is associated with increased PGE_2 production, we measured PGE_2 levels in lung homogenates of WT or *Pai1*$^{-/-}$ mice during the fibrotic phase at day 21 after bleomycin treatment (33). Figure 1 demonstrates that the protected *Pai1*$^{-/-}$ mice produced significantly greater levels of PGE_2 in the lung than did WT mice. In order to determine the cellular source of increased PGE_2 production in the lung, we next analyzed the effects of plasminogen activation on 3 cell types known to be critical to the development of pulmonary fibrosis: fibroblasts, fibrocytes, and alveolar epithelial cells (AECs).

Plasminogen activation increases PGE_2 *secretion in murine lung fibroblasts*. Fibroblasts were isolated from lung explants harvested at day 14 from saline- or bleomycin-treated murine lungs and serum starved for 24 hours in serum-free medium (SFM) consisting of 0.1% BSA-DMEM. Thereafter, cells were exposed to 10 U/ml uPA, 45 mU/ml plasminogen, or the two in combination. After 24 hours, supernatants were collected, and PGE_2 levels were measured by ELISA. The addition of either uPA alone or plasminogen alone had no influence on the levels of PGE_2 produced by fibroblasts purified from either saline- (Figure 2A) or bleomycin-treated mice (data not shown). However, treatment with uPA plus plasminogen led to a significant increase in PGE_2 secretion in fibroblasts from both saline- and bleomycin-treated (Figure 2B) mice when compared with cells cultured in SFM alone.

PAI-1 inhibits uPA-mediated activation of plasminogen to plasmin. To determine whether endogenous capacity for plasminogen activation also regulates PGE_2 synthesis, we purified fibroblasts as above from WT or *Pai1*$^{-/-}$ mice and cultured the cells for 24 hours in the presence of SFM alone or SFM containing 10 U/ml uPA and 45 mU/ml plasminogen. Basal production of PGE_2 was higher in fibroblasts from *Pai1*$^{-/-}$ mice. In addition, the induction of PGE_2 synthesis in *Pai1*$^{-/-}$ fibroblasts by plasminogen activation was significantly greater than that in fibroblasts purified from WT mice

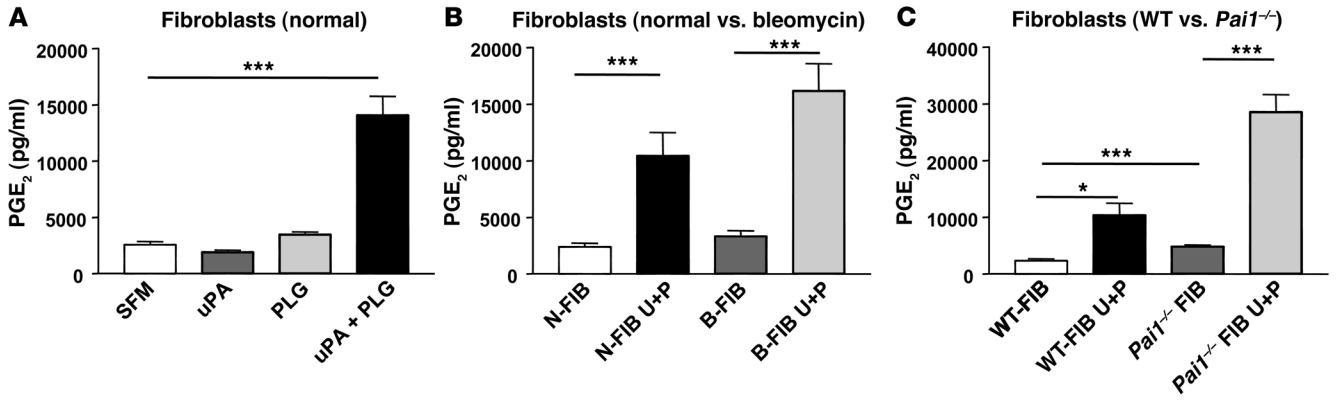

Figure 2
Plasminogen activation stimulates PGE_2 release in fibroblasts. (**A**) Fibroblasts from saline-treated mice were cultured at 5×10^5/ml and serum starved for 24 hours. Cells were then treated with SFM, 10 U/ml uPA, 45 mU/ml plasminogen (PLG), or uPA plus PLG for 24 hours. PGE_2 was then measured by ELISA in cell supernatants; $n = 5$, $^{***}P < 0.001$. (**B**) Mice were given i.t. saline or i.t. bleomycin on day 0. On day 14, lungs were harvested, minced, and cultured until day 28. Fibroblasts from normal and bleomycin-treated mice (N-FIB and B-FIB, **B**) were cultured in SFM or with uPA plus PLG (U+P) for 24 hours, and PGE_2 was measured; $n = 5$ or more in all groups, $^{***}P < 0.001$. (**C**) Fibroblasts were purified from WT or *Pai1*$^{-/-}$ mice and were treated with SFM or uPA plus PLG for 24 hours before culture supernatants were analyzed for PGE_2 production via ELISA; $n = 5$ or more in each group, $^*P < 0.05$, $^{***}P < 0.001$.

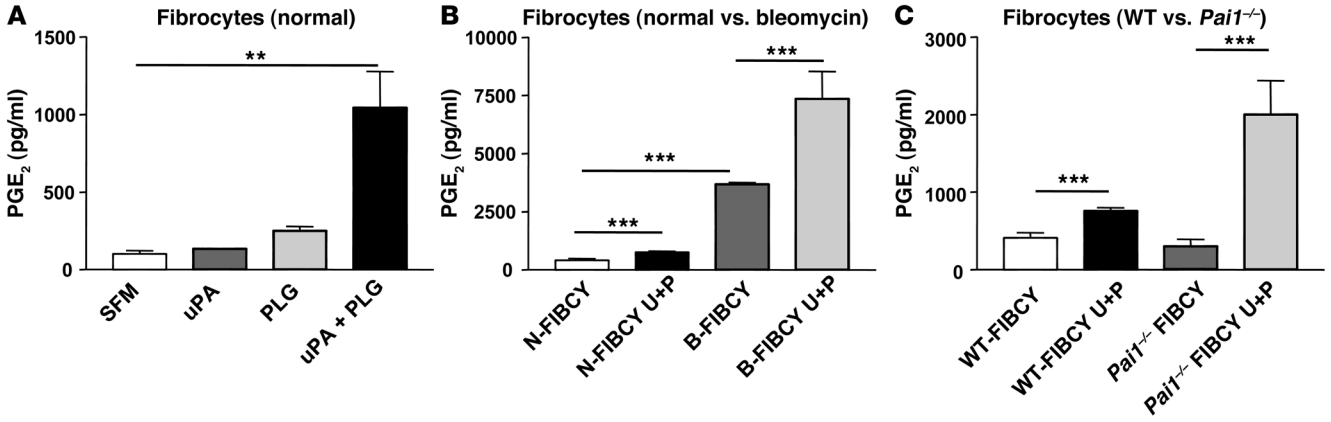

Figure 3

Plasminogen activation stimulates PGE_2 release in fibrocytes. (**A**) Fibrocytes from saline-treated mice were cultured at 5×10^5/ml and serum starved for 24 hours. Cells were then treated with SFM, 10 U/ml uPA, 45 mU/ml PLG, or uPA plus PLG for 24 hours. PGE_2 was then measured by ELISA in cell supernatants; $n = 3$, $^{**}P < 0.01$. (**B**) Mice were given i.t. saline or i.t. bleomycin on day 0. On day 14, lungs were harvested, minced, and cultured until day 28. Fibrocytes were then purified. Fibrocytes (FIBCY) from saline-treated, normal, and bleomycin-treated mice (**B**) were cultured in SFM or with uPA plus PLG for 24 hours, and PGE_2 was measured; $n = 5$, $^{***}P < 0.001$. (**C**) Fibrocytes were purified from WT or *Pai1*$^{-/-}$ mice and were treated with SFM or uPA plus PLG for 24 hours before culture supernatants were analyzed for PGE_2 production via ELISA; $n = 4$, $^{***}P < 0.001$.

(Figure 2C). These results suggest that the proteolytic function of uPA is important for the induction of PGE_2 generation.

Plasminogen activation increases PGE_2 secretion in murine fibrocytes. We next wished to extend these findings to fibrocytes, mesenchymal cells of bone marrow origin that contribute to pulmonary fibrogenesis (34, 35). The ability of these cells to elaborate PGE_2 has not previously been reported in any context. Fibrocytes were purified from the lungs of saline- or bleomycin-treated mice. As in fibroblasts, the addition of uPA alone or plasminogen alone had no effect on PGE_2 secretion (Figure 3A). Culture of fibrocytes with the combination of 10 U/ml uPA and 45 mU/ml plasminogen led to an increase in PGE_2 production in fibrocytes from both saline- and bleomycin-treated mice (Figure 3B). Fibrocytes purified from *Pai1*$^{-/-}$ mice showed enhanced secretion of PGE_2 compared with WT fibrocytes in the presence of uPA plus plasminogen (Figure 3C). It is also evident from these data that the capacity of fibrocytes to produce PGE_2 is substantially lower than that of fibroblasts.

Proteolytic actions of uPA and plasmin are responsible for promoting murine mesenchymal cell synthesis of PGE_2. The fact that uPA alone was incapable of stimulating PGE_2 synthesis suggests that this effect is not the result of uPA ligation of its receptor, uPAR, but rather is the result of its ability to convert plasminogen to plasmin. To confirm this, we cultured murine fibrocytes and fibroblasts with 50 mU/ml murine plasmin. Plasmin was indeed able to enhance PGE_2 secretion in both mesenchymal cell types isolated from saline- (Figure 4A) and bleomycin-treated (data not shown) lungs. To test whether the enzymatic activity of plasmin was necessary for PGE_2 generation, we examined the effects of its inhibitor, α2-antiplasmin. As shown in Figure 4B, addition of α2-antiplasmin significantly attenuated the ability of plasminogen activation to stimulate PGE_2 secretion. Similar results were noted in fibrocytes (data not shown).

Plasminogen activation leads to increased COX-2 production and decreased collagen synthesis in murine fibroblasts and fibrocytes. We next investigated whether COX-2 enzyme induction accounts for the ability of plasminogen activation to increase PGE_2 synthesis. Fibroblasts and fibrocytes were isolated from saline- or bleomycin-treated mice and cultured as above. After 24 hours, cell lysates were

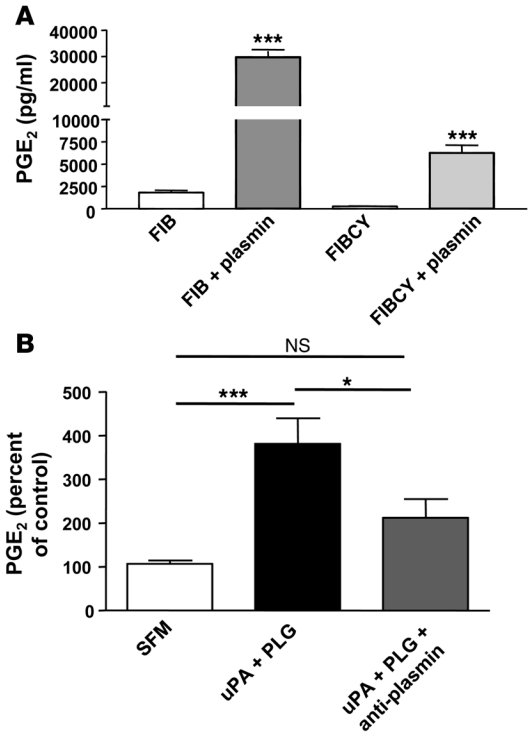

Figure 4

Induction of PGE_2 synthesis in mouse lung mesenchymal cells requires plasmin enzymatic activity. (**A**) Fibroblasts and fibrocytes were purified from saline-treated lungs, serum starved overnight, and cultured for 24 hours in SFM or with 50 mU/ml plasmin. PGE_2 was then measured in cell-free supernatants; $n = 3$, $^{***}P < 0.001$. (**B**) Fibroblasts from saline-treated mice were cultured in the presence of SFM, 10 U/ml uPA plus 45 mU/ml PLG, or uPA plus PLG plus 30 μg/ml α2-antiplasmin; $n = 7$ per group, $^{*}P < 0.05$, $^{***}P < 0.001$.

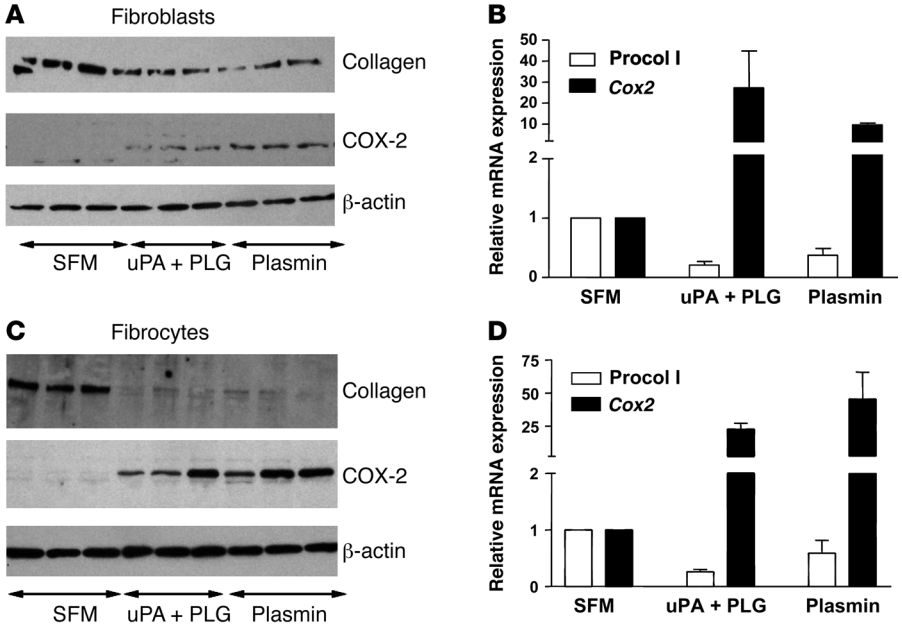

Figure 5
Plasminogen activation induces COX-2 and limits collagen I production in mouse lung mesenchymal cells. Fibroblasts (**A** and **B**) and fibrocytes (**C** and **D**) from saline-treated mice were cultured for 24 hours in the presence of SFM alone, 10 U/ml uPA plus 45 mU/ml PLG, or 50 mU/ml plasmin. Cell lysates were prepared and analyzed by Western blot for expression of collagen I and COX-2 (**A** and **C**). Each lane represents a unique culture. Data are representative of 2 independent experiments. In **B** and **D**, total RNA was made from cells cultured as above and analyzed for expression of *Cox2* and the α1 chain of procollagen I (Procol I) by real-time RT-PCR. Values were first normalized to expression of β-actin in each sample. Then, the average of the $n = 3$ SFM-treated cultures was normalized to 1 for each gene.

collected, and COX-2 protein expression was evaluated by Western blot and its mRNA examined by real-time RT-PCR (Figure 5). Provision of uPA plus plasminogen or of plasmin alone indeed led to increased COX-2 mRNA and protein expression as compared with cells cultured in SFM in both fibroblasts (Figure 5, A and B) and fibrocytes (Figure 5, C and D). Since autocrine expression of COX-2 and production of PGE_2 are well known to inhibit fibroblast expression of the important matrix protein collagen I (15, 36), we also examined levels of collagen I protein and the α1 chain of procollagen I mRNA in these cell lysates. A simultaneous reduction in (pro)collagen I mRNA and protein accompanied the induction of COX-2 elicited by plasminogen activation to plasmin in fibroblasts (Figure 5, A and B) as well as fibrocytes (Figure 5, C and D).

Plasminogen activation increases PGE_2 secretion and COX-2 expression in murine AECs. On a per-cell basis, AECs are the most prodigious producers of PGE_2 within the lung (37). AECs were isolated from saline- and bleomycin-treated mice and adhered to fibronectin-coated plates. After 3 days of adherence, cells were washed with PBS and serum starved for 24 hours in SFM. Then, medium was changed, and 10 U/ml uPA, 45 mU/ml plasminogen, or the combination of both was added to the wells. After 24 hours, the supernatants were collected, and PGE_2 levels were measured by ELISA. Similar to the results seen with mesenchymal cells, neither uPA nor plasminogen alone had a measurable effect, but the two together elicited a significant increase in PGE_2 production in both normal (Figure 6A) and fibrotic (Figure 6B) AECs as compared with SFM controls. The greater capacity for PGE_2 synthesis of AECs compared with mesenchymal cells is apparent. When cells were treated with 50 mU/ml plasmin alone, PGE_2 secretion increased to levels similar to those observed with addition of uPA plus plasminogen (data not shown). Both basal and plasminogen activation–stimulated PGE_2 production were significantly greater in *Pai1*$^{-/-}$ than WT cells (Figure 6C). We next treated AECs with SFM, uPA plus plasminogen, or plasmin as above and measured *Cox2* mRNA induction by real-time RT-PCR. Both reagent and enzymatically generated plasmin resulted in 5- to 7-fold increases in *Cox2* mRNA (Figure 6D).

Plasminogen activation promotes PGE_2 synthesis via COX-2 induction, with resultant inhibition of collagen expression in human IMR-90 fibroblasts. In conjunction with the results obtained in murine cells, we used a primary human fetal lung fibroblast cell line, IMR-90, to test the effect of plasminogen system components on human fibroblasts. Although uPA had no effect, plasminogen alone or plasmin enhanced PGE_2 synthesis in IMR-90 cells (Figure 7A). The efficacy of plasminogen alone in IMR-90 cells, which was not observed in murine cells, reflects the fact that these human cells endogenously produce and secrete more uPA than do murine fibroblasts (Figure 7B). Thus, the endogenous uPA can activate exogenously supplied plasminogen to plasmin. The effects of plasmin on PGE_2 paralleled its effects on COX-2 protein expression and were inversely related to expression of collagen I protein (Figure 7C). Importantly, treatment of IMR-90 cells with the COX-1/2 inhibitor indomethacin prevented inhibition of collagen I synthesis by plasminogen (Figure 7D), implying that plasmin's suppressive effect in fibroblasts was indeed dependent on its ability to enhance prostanoid generation.

Induction of COX-2 expression and PGE_2 synthesis is independent of PAR-1 signaling. Plasmin's effects could likewise be mediated via its activation of the G protein–coupled protease activated receptor PAR-1. Involvement of this receptor was explored using the specific PAR-1 agonist peptide TFLLRN and the antagonist peptide FLLRN (38, 39) (both at 100 μM). As shown in Figure 8, the agonist was unable to significantly enhance COX-2 expression or PGE_2 synthesis above control levels (Figure 8A). Similar results were obtained in murine lung fibroblasts and fibrocytes (data not shown). Importantly, the antagonist was unable to attenuate the effect of plasmin on induction of COX-2 or PGE_2 levels (Figure 8B). These results argue against the involvement of PAR-1 in both murine and human mesenchymal cells.

HGF activation by plasmin mediates upregulation of PGE_2 biosynthesis. Since plasmin's effects on COX-2/PGE_2 were independent of PAR-1, we utilized IMR-90 cells to test the alternative possibility that they were mediated by activation or release of a growth factor that was itself capable of inducing COX-2. HGF is produced as a biologically inactive precursor, pro-HGF, and can be sequestered either in

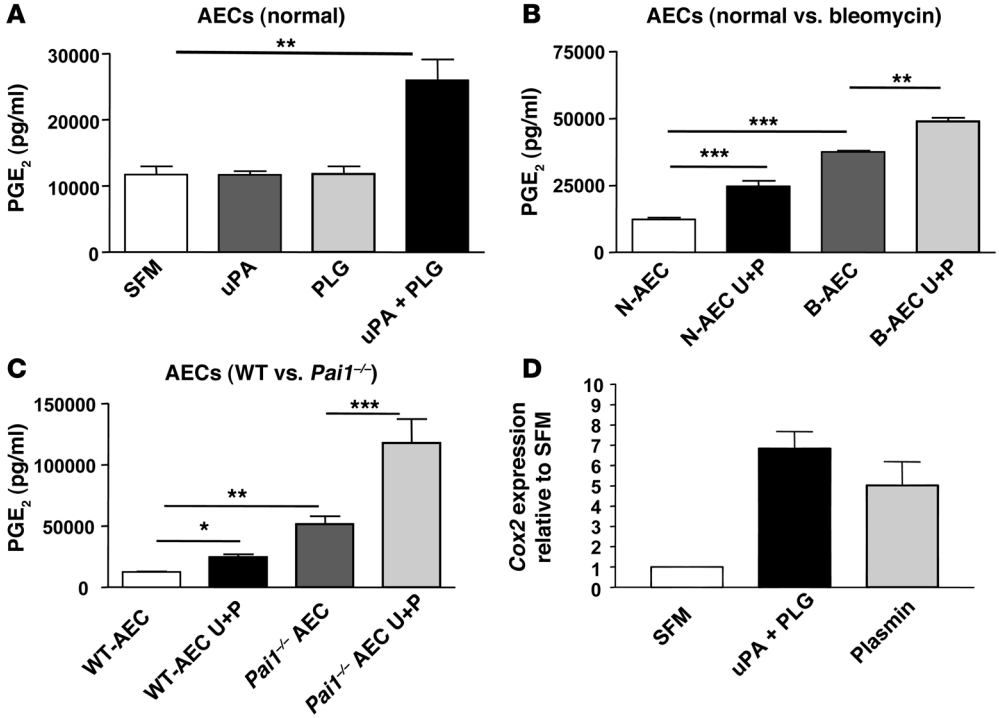

Figure 6

Plasminogen activation stimulates PGE_2 release in AECs. (**A**) AECs from saline-treated mice were cultured at 1.5×10^6/ml on fibronectin-coated plates and serum starved for 24 hours. Cells were then treated with SFM, 10 U/ml uPA, 45 mU/ml PLG, or uPA + PLG for 24 hours. PGE_2 was then measured by ELISA in cell supernatants; $n = 4$, $^{**}P < 0.01$. (**B**) Mice were given i.t. saline or i.t. bleomycin on day 0. On day 14, AECs were purified. AECs from saline-treated, normal and bleomycin-treated mice (N-AEC and B-AEC, **B**) were cultured in SFM or with uPA plus PLG for 24 hours, and PGE_2 was measured; $n = 3$ or more in all groups, $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) AECs were purified from WT or *Pai1*$^{-/-}$ mice and were treated with SFM or uPA plus PLG for 24 hours before culture supernatants were analyzed for PGE_2 production via ELISA; $n = 4$ or more in each group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**D**) AECs were purified from saline-treated mice and cultured at 1.5×10^6/ml on fibronectin-coated plates. Cells were serum starved overnight and then cultured in SFM, 10 U/ml uPA plus 45 mU/ml PLG, or with 50 mU/ml plasmin. Total RNA was prepared and analyzed for *Cox2* via real-time RT-PCR. Values for each sample were first normalized to β-actin, then the mean value for the SFM group was normalized to 1. $n = 2$ per group, representative of 2 experiments.

the extracellular matrix or on the cell membrane (40). Plasmin can cleave pro-HGF and release it from matrix (11, 41, 42), whereupon active HGF can interact with its receptor, c-Met, to oppose fibrogenesis. In renal (43) and bronchial (44) epithelial cells, HGF has been shown to induce COX-2 and increase PGE_2 generation, but such an action has not been reported in fibroblasts. Addition of plasmin to IMR-90 cell cultures caused a time-dependent increase in total HGF protein (Figure 9A); the rapid kinetics of this response (peak at 2 hours, with rapid decline thereafter) argue against an underlying transcriptional mechanism and instead suggest proteolytic activation and/or release from cells/matrix. We next incubated cells with HGF at 1 or 10 ng/ml (concentrations spanning the peak concentration of approximately 6 ng/ml measured in Figure 9A) and observed a dose-dependent induction of COX-2 protein (Figure 9B). A blocking antibody against the HGF receptor largely abrogated the ability of plasmin to increase PGE_2 production (Figure 9C), while a nonspecific IgG did not, implicating HGF in this action of plasmin. When IMR-90 cells were incubated with plasminogen and levels of HGF, PGE_2, and COX-2 were measured over a 24-hour period (Figure 9D), kinetic analysis showed that the rise in HGF preceded the increase in COX-2 expression, which in turn preceded the production of PGE_2. These results demonstrate that HGF, released from cells/matrix by plasmin, is instrumental in upregulating COX-2 and PGE_2, thereby contributing to the antifibrotic effect of plasminogen activation.

The HGF/COX-2/PGE_2 axis mediates protection against pulmonary fibrosis in Pai1$^{-/-}$ *mice in vivo*. To test the in vivo relevance of the HGF/COX-2/PGE_2 axis in mediating the antifibrotic actions of plasminogen activation, we evaluated the effects of a selective c-Met inhibitor, PHA-665752, on lung hydroxyproline accumulation in *Pai1*$^{-/-}$ mice. This inhibitor has previously been reported to exert optimal antitumor actions in mice in vivo at the dosage that we employed (45). Because prominent local irritant effects limit its repeated administration at a given site, we alternated its daily administration between i.v. and s.c. routes from days 10 to 20 after bleomycin treatment and harvested lungs for analysis at day 21 (see protocol in Figure 10A); control animals received vehicle alone. Administration of the c-Met inhibitor significantly increased collagen content in *Pai1*$^{-/-}$ mice (Figure 10B). Similar results were observed at day 14 (data not shown). To determine the impact of c-Met inhibition on the prostanoid synthetic pathway in the protected *Pai1*$^{-/-}$ mice, we quantified COX-2 protein (Figure 10C) and PGE_2 (Figure 10D) in lung homogenates. PHA-665752 significantly reduced COX-2 and PGE_2 in parallel with its enhancement of lung collagen. Together, these data indicate that the HGF/c-Met/COX-2/PGE_2 axis mediates the in vivo pro-

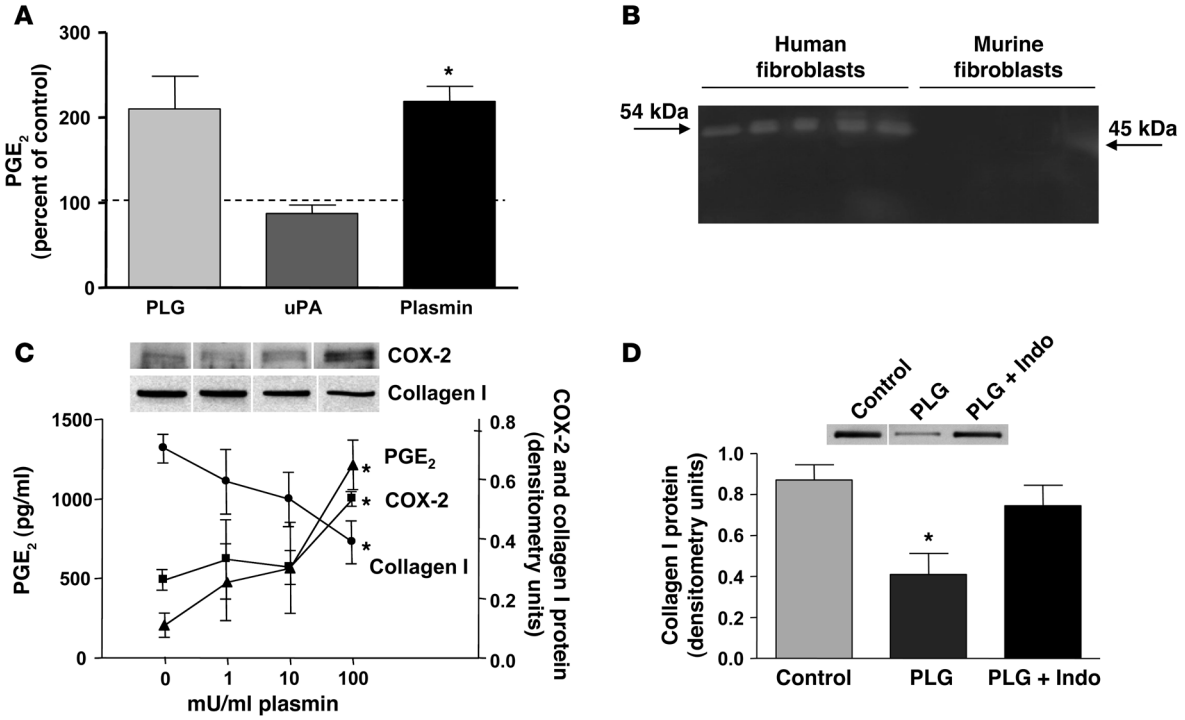

Figure 7

IMR-90 cell PGE_2 synthesis is regulated by plasminogen activation. (**A**) IMR-90 cells in SFM were treated with PLG (200 mU/ml), plasmin (100 mU/ml), or uPA (10 U/ml) for 18 hours. Medium was removed from the cells, and PGE_2 was measured by ELISA and is expressed relative to the control value measured in SFM alone; $n \geq 5$, $^*P < 0.05$ versus control. (**B**) Cell supernatants were collected from either saline-treated murine lung fibroblasts or human IMR-90 cells cultured at equal densities for 24 hours. Supernatants were then analyzed by zymography for the ability to activate PLG. A band of the appropriate size for human uPA (54 kDa) is readily distinguishable in the IMR-90 cell supernatants, but the lower-molecular-weight murine uPA band (45 kDa) is very faint in the murine lung fibroblasts; $n = 5$ per group. (**C**) IMR-90 cells in SFM were treated with 100 mU/ml plasmin for 18 hours. Media was harvested for PGE_2 determination (triangles), and lysates were harvested for collagen I protein expression (circles) or COX-2 protein expression (squares) as determined by immunoblot analysis and densitometry using α-tubulin as a loading control; $n = 3$, $^*P < 0.05$ versus control. Collagen I and COX-2 immunoblots are representative of experiments performed in triplicate. (**D**) IMR-90 cells were treated with SFM (control), PLG (100 mU/ml), or PLG plus indomethacin (Indo; 10 μM) for 18 hours, and collagen I was determined by immunoblot analysis and densitometry using α-tubulin as a loading control; $n = 3$, $^*P < 0.05$ versus control. Collagen I immunoblots are representative of experiments performed in triplicate. Blots in both **C** and **D** are composed from lanes that were run on the same gel but were noncontiguous.

tection against experimental pulmonary fibrosis associated with exaggerated plasminogen activation.

The ability of plasmin and HGF to induce PGE_2 secretion is defective in fibroblasts from patients with IPF. Previous studies have documented that lung fibroblasts from IPF patients manifest an inability to upregulate COX-2 in response to a variety of inducing agents (18, 23). We therefore wished to examine plasmin- and HGF-induced PGE_2 synthesis in fibroblasts from IPF patients exhibiting the characteristic histopathologic pattern of usual interstitial pneumonia (UIP) and utilized cells obtained from nonfibrotic lung resected from adult patients without IPF of a similar age for comparison. Normal adult lung fibroblasts exhibited an increase in PGE_2 production over baseline in response to both plasmin (Figure 11A) and HGF (Figure 11B), while fibroblasts from UIP lung were unable to augment PGE_2 synthesis in response to either stimulus. HGF release into the medium increased in response to plasmin in both normal and UIP fibroblasts, and no significant difference between them was evident (Figure 11C).

Discussion

Although research in the pathogenesis of pulmonary fibrosis has been dominated by studies investigating fibroblast activation signals, evidence indicates that tissue remodeling is also characterized by a relative deficiency in counterregulatory antifibrotic signals. Two such antifibrotic signals are PGE_2 and plasminogen activator activity. Each of these has been shown to be deficient in patients with IPF, and deficiency of each has been established to be pathogenically important in animal models of pulmonary fibrosis. Although PGE_2 has previously been shown to modulate expression of plasminogen activation system components such as PAI-1 (29–31), the influence of the plasminogen activation system on PGE_2 production has not been investigated previously. We found that plasminogen activation augmented PGE_2 secretion in 3 cell types known to be important players in the development of pulmonary fibrosis — fibroblasts, fibrocytes, and AECs. This action was seen in both normal and fibrotic cells in mice as well as in human adult and fetal lung fibroblasts. Increased PGE_2 generation was independent of uPA interaction with uPAR and was instead attributable to the enzymatic activities of uPA and of plasmin; however, PAR-1 was not the proteolytic target of plasmin. Rather, the operative mechanism involved the enzymatic release of HGF by plasmin and subsequent HGF-induced upregulation of COX-2. In vitro and in vivo experiments using *Pai1*$^{-/-}$ mice established that augmentation

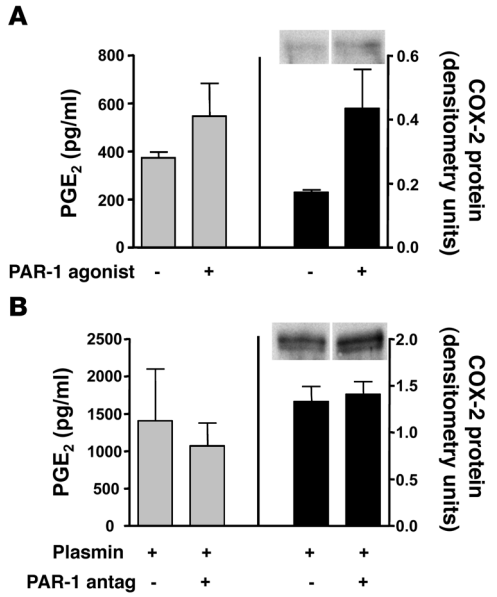

Figure 8
Lack of effect of PAR-1 agonist and antagonist peptides on PGE_2 production and COX-2 expression in IMR-90 cells. (**A**) IMR-90 cells in SFM were treated with the PAR-1 agonist peptide TFLLRN (100 μM) for 18 hours; $n = 3$. (**B**) IMR-90 cells in SFM were treated with plasmin (100 mU/ml) with and without the PAR-1 antagonist peptide FLLRN (antag; 100 μM) for 18 hours; $n = 3$. Where plasmin and FLLRN were coincubated, the antagonist was added 10 minutes before the plasmin. Lysates were harvested, and COX-2 protein expression was determined by immunoblot analysis and densitometry using α-tubulin as a loading control. Medium was removed from the cells for PGE_2 determination. COX-2 immunoblots in **A** and **B** are derived from the same experiments, so that differences in densitometric units accurately reflect differences between plasmin (**B**) and no-plasmin control (**A**) conditions. None of the comparisons shown represent statistically significant differences. Blots in both **A** and **B** are composed from two lanes that were run on the same gel but were noncontiguous.

of PGE_2 biosynthesis is also a consequence of exaggerated endogenous plasmin activity. Moreover, our data suggest that the HGF/COX-2/PGE_2 axis is critical for the antifibrotic actions of plasmin in vitro and in vivo. Finally, plasmin- and HGF-mediated PGE_2 synthesis was impaired in fibroblasts from IPF patients, indicating a defect in this newly identified form of antifibrotic crosstalk.

In murine fibroblasts and fibrocytes, stimulation with uPA plus plasminogen led to increased PGE_2 synthesis. Treatment with uPA or plasminogen alone was insufficient to produce this effect. In human cells, which as we demonstrate secrete higher levels of uPA constitutively, the addition of plasminogen alone was able to induce PGE_2 generation. Several lines of evidence argue that the ability of plasminogen activation to accomplish this was mediated by the enzymatic activity of plasmin rather than via ligation of the uPA receptor uPAR. First, treatment with uPA alone failed to promote PGE_2 production. Second, the ability of plasminogen activation to induce PGE_2 production was inhibited by the addition of α2-antiplasmin. Third, the genetic absence of PAI-1, which removes the major inhibitor of plasminogen activation, augmented PGE_2 synthesis in vitro and in vivo. Although PAR-1 is a potential proteolytic target for plasmin and its activation has been reported to be capable of COX-2 induction (39, 46), experiments with PAR-1 agonist and antagonist peptides failed to support a role for this G protein–coupled receptor in the enhancement of PGE_2 synthesis. This may be explained by the fact that although plasmin can activate PAR-1, thrombin is the principal protease for PAR-1 (47).

PGE_2 opposes fibroproliferative responses to lung injury by mechanisms that include inhibiting inflammatory and immune responses (48), promoting epithelial cell integrity (43, 49), and downregulating activation of a number of fibroblast functions including proliferation (15), migration (50, 51), myofibroblast differentiation (52), and collagen I accumulation (15). Although plasminogen activation shares with PGE_2 the ability to restrain fibrogenesis, its actions on fibroblast phenotypes are poorly characterized, but likely complex. Plasmin has been shown to promote fibroblast proliferation via its ability to activate the PAR-1 receptor (53), but also to promote lung fibroblast apoptosis (54). In this report, we demonstrate that generation of plasmin can limit collagen I synthesis. Inhibition of collagen expression was independent of PAR-1 but was prostanoid dependent, as judged by the ability of the COX-1/2 inhibitor indomethacin to abrogate this effect. While the contribution of other prostanoids to collagen inhibition cannot be excluded, PGE_2 is the most likely mediator of this effect, as it is the predominant prostanoid produced by both AECs and lung fibroblasts (26, 55). The net result of these actions of plasmin would be to limit fibrosis in vivo. In fact, our results demonstrating that *Pai1*$^{-/-}$ mice, which are protected from bleomycin-induced pulmonary fibrosis, have elevated levels of PGE_2 in the lung support this contention. Similarly, the fact that plasmin cannot induce the secretion of PGE_2 in fibroblasts from patients with IPF is also consistent with the known global defect in COX-2 upregulation in these cells (18, 23), a defect that may be explained by epigenetic silencing (21).

Plasmin can cleave a number of matrix proteins, including fibronectin, laminin, proteoglycans, and basement membrane (type IV) collagen, and is also known to activate a number of growth factors including TGF-β and HGF. Active HGF is an important effector of antifibrotic actions in vivo (56–58) and has been shown to specifically contribute to the antifibrotic influence of plasminogen activation (32). Exogenous administration of HGF (59) or *HGF* gene transfer (60, 61) has been shown to reduce the development of bleomycin-induced pulmonary fibrosis in vivo. HGF has been shown previously to suppress collagen I synthesis in fibroblasts (62), to augment collagenolytic activity in epithelial cells (57), and to induce COX-2 and PGE_2 synthesis in epithelial cells (44). Our results provide evidence that plasmin increases HGF (via some combination of proteolytic cleavage of pro-HGF and release from matrix sequestration; ref. 11) and this induces COX-2 synthesis. Importantly, the ability of an HGF receptor–blocking antibody to abrogate the effects of plasmin on COX-2/PGE_2 synthesis demonstrates that HGF is responsible for plasmin's effects on human lung fibroblast collagen synthesis. The inability of uPA to produce this same effect argues that plasmin-mediated proteolytic release of HGF from the matrix is critical in this process. Finally, the fact that a c-Met inhibitor abolished the protection against bleomycin-induced fibrosis observed in *Pai1*$^{-/-}$ mice in parallel with decreases in COX-2 protein and PGE_2 serves to implicate the HGF/COX-2/PGE_2 axis in the resistance of these plasmin-rich animals in vivo.

Transcription of the *HGF* gene is well known to be stimulated by substances that increase cyclic AMP, including PGE_2 (63). It is therefore likely that this ability of HGF to promote PGE_2 synthesis is part of a positive feedback loop that results in amplification of

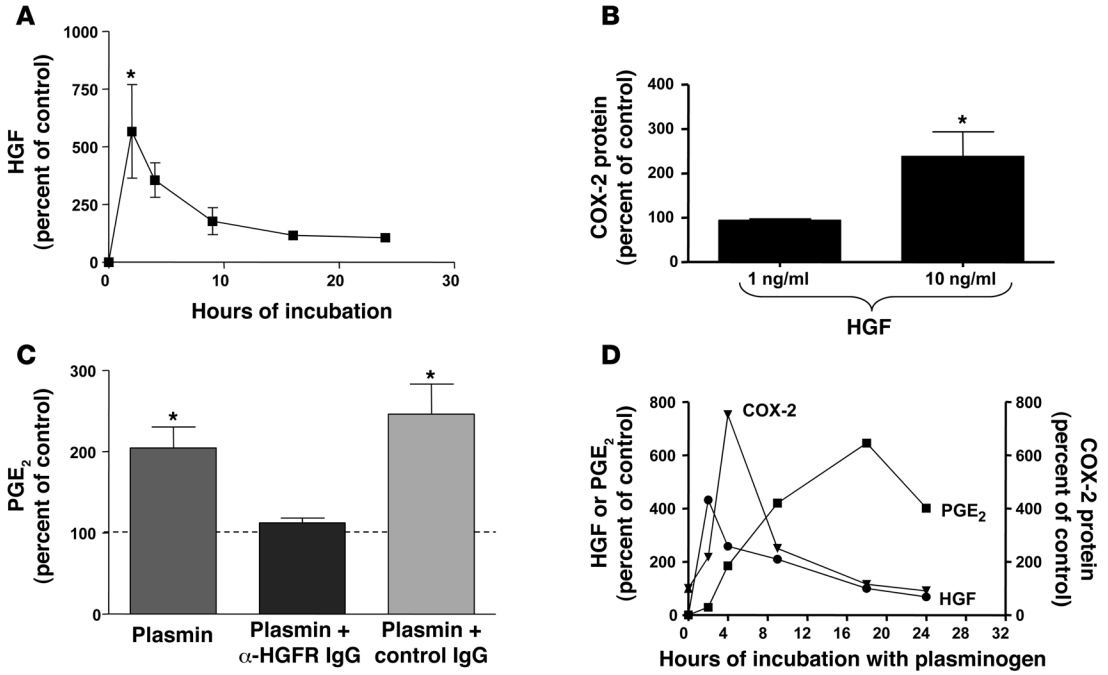

Figure 9

Plasmin induces IMR-90 cell COX-2 expression and PGE_2 synthesis in an HGF-dependent manner. (**A**) IMR-90 cells in SFM were treated with plasmin (50 mU/ml) for 2, 4, 9, 18, and 24 hours. Total HGF was determined by ELISA and normalized to levels measured in cells incubated with SFM alone; $n = 3$, $^*P < 0.05$ versus control. At 2 hours, the mean absolute concentration of HGF was 6.12 ng/ml. (**B**) IMR-90 cells in SFM were treated with HGF (1 or 10 ng/ml) for 18 hours. Lysates were harvested for COX-2 protein expression as determined by immunoblot analysis and densitometry using α-tubulin as a loading control; $n = 3$, $^*P = 0.05$ versus control (SFM alone). (**C**) IMR-90 cells in SFM were treated with plasmin (50 mU/ml), plasmin with an HGF receptor blocking antibody (20 μg/ml), or plasmin with a nonspecific IgG (control IgG; 20 μg/ml) for 18 hours ($n = 3$). PGE_2 was determined and normalized for cellular protein. $^*P < 0.05$ versus control (SFM alone). (**D**) IMR-90 cells in SFM were treated with PLG (200 mU/ml) for 2, 4, 9, 18, and 24 hours. Lysates were harvested for COX-2 protein expression (inverted triangles) as determined by immunoblot analysis and densitometry using α-tubulin as a loading control. Media was harvested, and PGE_2 (squares) and HGF (circles) were determined and normalized to cellular protein. Data are expressed relative to SFM control and are from 1 experiment representative of 2.

fibroblast HGF production. This, in turn, further contributes to an antifibrotic milieu by virtue of the ability of HGF to promote epithelial cell survival (64) and to inhibit epithelial-mesenchymal transdifferentiation (43, 65). It has long been known that plasminogen activation is antifibrotic but that its mechanisms do not necessarily involve fibrin degradation (6, 10). Our results provide new insight into potential mechanisms whereby plasminogen activation exerts its antifibrotic effects in vivo.

Methods

Animals. C57BL/6 mice were purchased from The Jackson Laboratory. Animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. *Pai1*$^{-/-}$ mice on a C57BL/6 background as described previously (66) were bred in-house.

Reagents. Murine uPA, plasmin, and plasminogen were purchased from Molecular Innovations. Human uPA was purchased from American Diagnostica, and human plasminogen, human plasmin, HGF (containing both active and pro-forms), and the nonselective COX inhibitor indomethacin were purchased from Sigma-Aldrich. Primary antibodies for immunoblot analysis were obtained from the following suppliers: anti–COX-2 from Cayman Chemical; anti-human and anti-mouse antibodies against type 1 collagen both from CedarLane Laboratories; anti–α-tubulin from Sigma-Aldrich; anti-uPA from Oxford Biomedical. Secondary anti-murine antibodies for Western blot analysis were purchased from Pierce Biotechnology and Cell Signaling Technology. Secondary anti-rabbit antibodies for Western blot were purchased from Cell Signaling Technology. Bleomycin was purchased from Sigma-Aldrich. PGE_2 was obtained from Cayman Chemicals and dissolved in DMSO. Blocking antibody against the HGF receptor c-Met was obtained from R&D Systems. PGE_2 ELISA kits were purchased from Assay Designs or Cayman Chemicals, and HGF ELISA kit was purchased from R&D Systems. The PAR-1 agonist TFLLRN and the PAR-1–blocking peptide FLLRN were purchased from AnaSpec. The His-tagged α2-antiplasmin was purchased from Molecular Innovations. The c-Met inhibitor PHA-665752 was purchased from Tocris Bioscience.

Bleomycin model of pulmonary fibrosis. Mice were anesthetized with intraperitoneal ketamine/xylazine, and the trachea was exposed by incision. Bleomycin was dissolved in PBS and instilled intratracheally (i.t.) using a 27-gauge needle at a dose of 0.00135 U/g mouse in a volume of 50 μl. In some experiments, WT and *Pai1*$^{-/-}$ mice were treated with the selective c-Met inhibitor PHA-665752 (45) (25 mg/kg) daily from days 10 to 20 after bleomycin treatment via i.v. or s.c. routes on alternate days; this dosage was selected because it was determined from dose-response experiments to exhibit optimal antitumor effects in mice in vivo (45). Lungs were harvested on day 21 after bleomycin treatment and analyzed for COX-2 protein by immunoblot analysis, PGE_2 by ELISA, and hydroxyproline content as previously described (24).

Murine fibroblast and fibrocyte purification. Fibroblasts and fibrocytes were isolated from lungs of mice on day 14 after saline or bleomycin treatment, as described (35). Murine lungs were perfused via the right ventricle with 5 ml 0.9% NaCl and removed under aseptic conditions. Lungs were

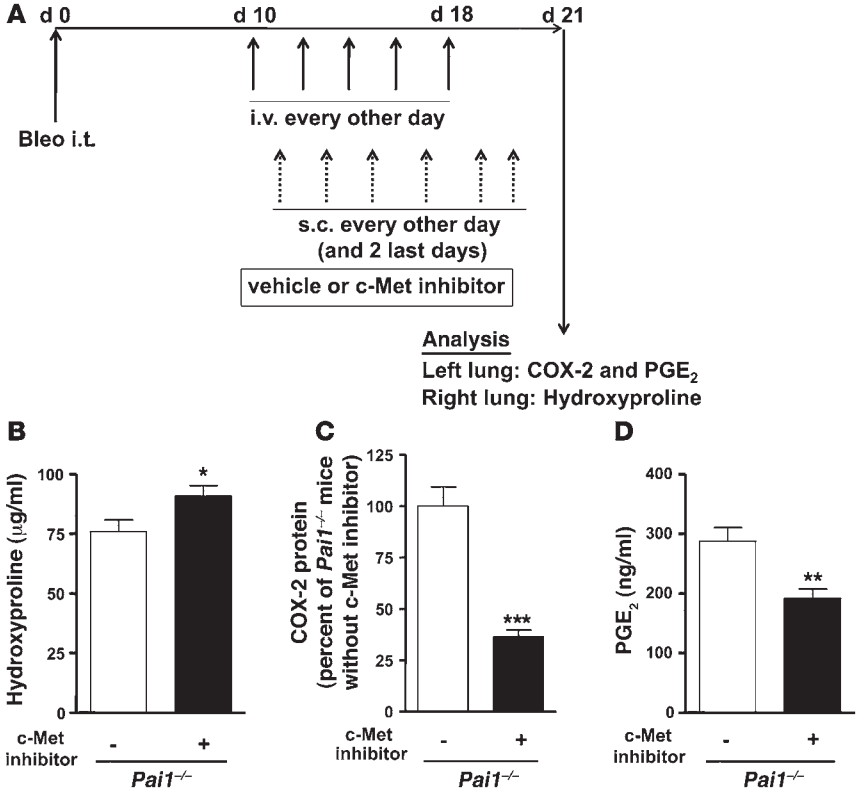

Figure 10
A c-Met inhibitor increases collagen deposition in the lungs of *Pai1*$^{-/-}$ mice in parallel with reductions in COX-2 expression and PGE_2 production. (**A**) Protocol. *Pai1*$^{-/-}$ mice were injected with bleomycin (Bleo) on day 0 and were treated with vehicle (L-lactate and 10% polyethylene glycol) alone (control, $n = 6$) or with the c-Met inhibitor PHA-665752 (25 mg/kg) ($n = 7$) in vehicle. On alternate days, administration was i.v. via tail vein or s.c., as shown. On day 21, lungs were harvested. (**B**) Collagen deposition in the right lung was determined by measuring hydroxyproline content. (**C**) COX-2 levels in left lung homogenates were determined by immunoblot analysis and densitometry. Data are expressed as percent of vehicle-treated mice. (**D**) PGE_2 levels in the lipid extracts from left lungs were measured by ELISA. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control. Similar results were obtained in a second experiment.

minced with scissors in DMEM containing 10% fetal calf serum. Lungs from a single animal were placed in 15 ml medium in tissue culture flasks. Mesenchymal cells were allowed to grow out of the minced tissue, and when cells reached 70% confluence, they were passaged following trypsinization. Cells were grown for 14 days (2–3 passages) before being used. To separate fibrocytes from fibroblasts, cells were incubated with anti-CD45 Abs coupled to magnetic beads (Miltenyi Biotec). Labeled cells were then sorted by binding the cell population to positive selection columns using a SuperMACS apparatus (Miltenyi Biotec) according to the manufacturer's instructions. Immunohistochemical staining or flow cytometry staining on this population confirmed that these fibrocytes were $CD45^+$collagen I^+. In contrast, the fibroblast population was $CD45^-$collagen I^+.

Murine AEC purification. Type II AECs were isolated from mice using the method of Corti and coworkers (67). Following anesthesia and heparinization, the mouse was exsanguinated and the pulmonary vasculature perfused. The trachea was cannulated, and the lungs were filled with 1–2 ml Dispase (BD Biosciences), followed by 1 ml of low-melting-point agarose. Lungs were then removed and placed in iced PBS to harden the

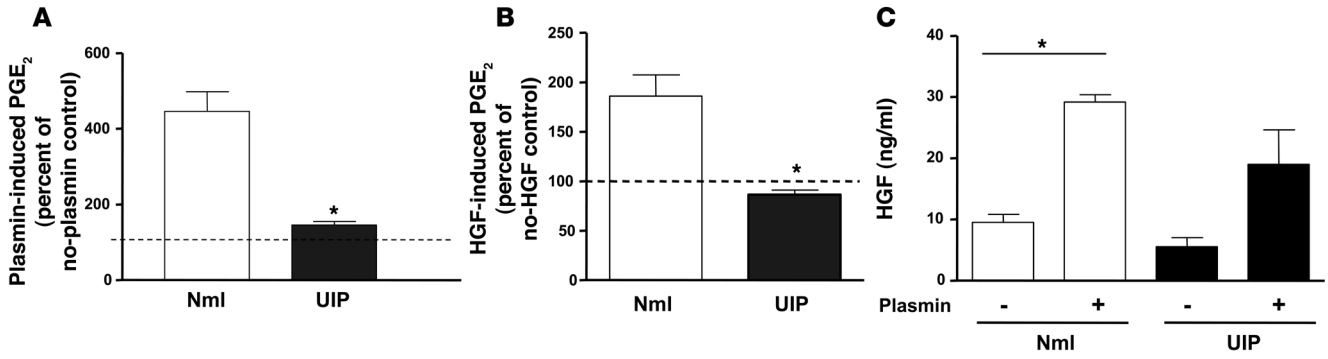

Figure 11
Plasmin and HGF are unable to upregulate PGE_2 production in fibroblasts from patients with IPF. Fibroblasts from IPF patients diagnosed with UIP or fibroblasts obtained from histologically normal (Nml) lung resections were cultured in SFM (control) with or without plasmin (100 mU/ml) (**A**) or HGF (10 ng/ml) (**B**) for 18 hours. Medium was removed, and PGE_2 levels were determined by ELISA. The SFM (control) sample for each cell line was normalized to 100% (dotted lines). Data are from cells derived from $n = 3$ patients per group. $^*P < 0.05$ versus Nml. (**C**) Fibroblasts from Nml and UIP lung were treated with SFM alone (control) or plasmin (100 mU/ml) for 2 hours (based on the kinetics of HGF release shown in Figure 9A), and HGF levels in the supernatants were determined by ELISA. Data are from cells derived from $n = 3$ patients per group; $^*P < 0.05$ versus control Nml without plasmin.

agarose. The lungs were placed in 2 ml Dispase and incubated for 45 minutes at 24°C. Subsequently the lung tissue was teased from the airways and minced in DMEM with 0.01% DNase. The lung mince was gently passed successively through 100-, 40-, and 25-μm nylon mesh filters. Bone marrow–derived cells were removed by magnetic depletion using anti-CD32 and anti-CD45 Abs. Mesenchymal cells were removed by overnight adherence in a petri dish. The nonadherent AECs were then plated at 500,000 cells/well on fibronectin-coated 24-well plates. Cells were maintained in DMEM with penicillin/streptomycin/amphotericin B and 10% fetal calf serum at 37°C in 5% CO_2. The final adherent population included only 4% nonepithelial cells at day 2 in culture by intermediate filament staining.

Human cell culture. IMR-90 fetal human lung fibroblasts were obtained from the Coriell Institute for Medical Research at passage 4. They were cultured in DMEM plus 10% FBS at 37°C in 5% CO_2 and used for experimentation at passage 6–9. As previously described (68), primary adult "normal" human lung fibroblasts were isolated from the margins of lung tissue resected from patients with suspected lung cancer that displayed normal lung histology, whereas IPF fibroblasts were cultured from lung biopsy specimens of patients diagnosed with IPF whose tissue histopathology showed UIP. Patients in the two groups were of similar age, and specimens from both groups were obtained with written informed consent under a protocol approved by the Institutional Review Board of the University of Michigan. Primary fibroblasts were cultured in the above medium and used at passage 7. For experimentation, cells were trypsinized, counted, and plated in Falcon 6-well plates (BD Biosciences) at 500,000–800,000 cells per well. They were allowed to adhere for 6–8 hours and then cultured in serum-free DMEM for 18–24 hours. At the time of experimental treatment, the cells were 60%–80% confluent.

ELISA determinations for PGE_2 and HGF. ELISA was used to quantify PGE_2 and HGF per the manufacturers' instructions in cell-free supernatants. In lung homogenates, lipids were extracted using C18 Sep-Pak cartridges (Waters) as previously described (22), and PGE_2 was then measured by ELISA in reconstituted lipid extracts.

HGF receptor blocking antibody. HGF receptor blocking antibody or nonspecific IgG (both at 20 μg/ml) were added to the medium 10 minutes prior to the addition of plasmin and allowed to incubate with the cells for 18 hours. The medium was then removed and assayed for levels of PGE_2.

Western blot. Murine cells were washed with ice-cold PBS and 200 μl cold lysis buffer (1% w/w Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.02 M EDTA, 0.05 M NaF, 0.002 M NA_3VO_4, and 1:100 dilution of Calbiochem Protease Cocktail Set II (Calbiochem-Novabiochem) was added to each sample. For human samples, the lysis buffer contained PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. 1× Protease inhibitor from Roche was added fresh each time along with a 1:100 dilution of 0.2 M sodium orthovanadate. Lysates were assayed for total protein concentration using the DC Protein Assay (Bio-Rad). For mouse cells, 4 μg of protein from each sample was then analyzed for expression of COX-2 or β-actin using methods that have been described previously (52). For human samples, 20–25 μg of protein was loaded prior to testing for collagen I, COX-2, or tubulin.

Zymography. Zymography was performed to determine the molecular size of plasminogen activators, using the method of Powell-Jones (69) with modifications. The cell supernatants were subjected to SDS-PAGE using 10% slab gels containing *a*-casein (7 mg/ml; Sigma-Aldrich) and Glu-plasminogen (20 mg/ml). The gel was washed in 1% Tween 80 for 1 hour at 37°C and incubated in 0.1% Tween 80 in PBS overnight at room temperature. The gel was then stained with Coomassie blue and destained in a solution of 10% acetic acid and 50% methanol. The molecular weights of the lytic bands were calculated by comparison to human and mouse uPA standards.

Statistics. Data are presented as mean ± SEM of values determined from the indicated number of experiments. Data analysis employed GraphPad Prism software, using Student's *t* test or ANOVA with Bonferroni correction, as appropriate, to determine significant differences between group means. In all instances, a *P* value less than 0.05 was considered significant.

Acknowledgments

The authors thank Bi Yu, Natalya Subbotina, and Carol Wilke for technical assistance. This work was supported by NIH grants HL087846 and AI065543, a grant from the Pulmonary Fibrosis Foundation, and a Career Investigator Award from the American Lung Association of Michigan (B.B. Moore); NIH grants HL058897 and HL094311 (to M. Peters-Golden) and HL078871 (to T.H. Sisson); and a Parker B. Frances Fellowship, an American Thoracic Society Career Development Award, and NIH grant HL094657 (to S.K. Huang).

Received for publication January 19, 2010, and accepted in revised form March 17, 2010.

Address correspondence to: Bethany B. Moore, 4053 BSRB, 109 Zina Pitcher Place, Ann Arbor, Michigan 48109, USA. Phone: 734.647.8378; Fax: 734.612.2331; E-mail: Bmoore@umich.edu. Or to: Marc Peters-Golden, 6301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, Michigan 48109, USA. Phone: 734.763.9077; Fax: 734.764.4556; E-mail: petersm@umich.edu.

1. Bertozzi P, et al. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N Engl J Med*. 1990;322(13):890–897.
2. Chapman HA, Allen CL, Stone OL. Abnormalities in pathways of alveolar fibrin turnover among patients with interstitial lung disease. *Am Rev Respir Dis*. 1986;133(3):437–443.
3. Idell S, et al. Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. *J Clin Invest*. 1989;84(2):695–705.
4. Eitzman DT, et al. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest*. 1996;97(1):232–237.
5. Swaisgood CM, French EL, Noga C, Simon RH, Ploplis VA. The development of bleomycin-induced pulmonary fibrosis in mice deficient for components of the fibrinolytic system. *Am J Pathol*. 2000;157(1):177–187.
6. Hattori N, et al. Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. *J Clin Invest*. 2000;106(11):1341–1350.
7. Sisson TH, Hanson KE, Subbotina N, Patwardhan A, Hattori N, Simon RH. Inducible lung-specific urokinase expression reduces fibrosis and mortality after lung injury in mice. *Am J Physiol Lung Cell Mol Physiol*. 2002;283(5):L1023–L1032.
8. Sisson TH, Hattori N, Xu Y, Simon RH. Treatment of bleomycin-induced pulmonary fibrosis by transfer of urokinase-type plasminogen activator genes. *Hum Gene Ther*. 1999;10(14):2315–2323.
9. Gunther A, et al. Prevention of bleomycin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med*. 2003;168(11):1358–1365.
10. Ploplis VA, et al. A total fibrinogen deficiency is compatible with the development of pulmonary fibrosis in mice. *Am J Pathol*. 2000;157(3):703–708.
11. Matsuoka H, Sisson TH, Nishiuma T, Simon RH. Plasminogen-mediated activation and release of hepatocyte growth factor from extracellular matrix. *Am J Respir Cell Mol Biol*. 2006;35(6):705–713.
12. Baum B, Moss S, Breul R, Crystal R. Effect of cyclic AMP on the intracellular degradation of newly synthesized collagen. *J Biol Chem*. 1980;255(7):2843–2847.
13. Goldstein R, Polgar P. The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts. *J Biol Chem*. 1982;257(15):8630–8633.
14. Korn J, Halushka P, Leroy E. Mononuclear cell modulation of connective tissue function: suppression of fibroblast growth by stimulation of endogenous prostaglandin production. *J Clin Invest*. 1980;65(2):543–554.
15. Huang S, Wettlaufer S, Hogaboam C, Aronoff D, Peters-Golden M. Prostaglandin E_2 inhibits collagen expression and proliferation in patient-derived normal lung fibroblasts via E prostanoid 2 receptor and cAMP signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L405–L413.
16. Ozaki T, Moriguchi H, Nakamura Y, Kamei T, Yasuoka S, Ogura T. Regulatory effects of prostaglandin E_2 on fibronectin release from human alveolar macrophages. *Am Rev Respir Dis*. 1990;

141(4 pt 1):965–969.
17. Borok Z, et al. Augmentation of functional prostaglandin E levels on the respiratory epithelial surface by aerosol administration of prostaglandin E. *Am Rev Respir Dis*. 1991;144(5):1080–1084.
18. Wilborn J, Crofford L, Burdick M, Kunkel S, Strieter R, Peters-Golden M. Cultured lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis have a diminished capacity to synthesize prostaglandin E_2 and to express cyclooxygenase-2. *J Clin Invest*. 1995;95(4):1861–1868.
19. Vancheri C, et al. Different expression of TNF-alpha receptors and prostaglandin E_2 production in normal and fibrotic lung fibroblasts: potential implications for the evolution of the inflammatory process. *Am J Respir Cell Mol Biol*. 2000;22(5):628–634.
20. McAnulty RJ, Hernandez-Rodriguez NA, Mutsaers S, Coker R, Laurent G. Indomethacin suppresses the anti-proliferative effects of transforming growth factor-beta isoforms on fibroblast cell cultures. *Biochem J*. 1997;321(pt 3):639–643.
21. Coward WR, Watts K, Feghali-Bostwick CA, Knox A, Pang L. Defective histone acetylation is responsible for the diminished expression of cyclooxygenase 2 in idiopathic pulmonary fibrosis. *Mol Cell Biol*. 2009;29(15):4325–4339.
22. Moore BB, et al. GM-CSF regulates bleomycin-induced pulmonary fibrosis via a prostaglandin-dependent mechanism. *J Immunol*. 2000;165(7):4032–4039.
23. Keerthisingam C, et al. Cyclooxygenase-2 deficiency results in a loss of the anti-proliferative response to transforming growth factor-β in human fibrotic lung fibroblasts and promotes bleomycin-induced fibrosis in mice. *Am J Pathol*. 2001;158(4):1411–1422.
24. Moore BB, et al. Bleomycin-induced E prostanoid receptor changes alter fibroblast responses to prostaglandin E_2. *J Immunol*. 2005;174(9):5644–5649.
25. Arras M, et al. IL-9 protects against bleomycin-induced lung injury: involvement of prostaglandins. *Am J Pathol*. 2005;166(1):107–115.
26. Moore BB, et al. Alveolar epithelial cell inhibition of fibroblast proliferation is regulated by MCP-1/CCR2 and mediated by PGE_2. *Am J Physiol Lung Cell Mol Physiol*. 2003;284(2):L342–L349.
27. Failla M, et al. 16,16-dimethyl prostaglandin E_2 efficacy on prevention and protection from bleomycin-induced lung injury and fibrosis. *Am J Respir Cell Mol Biol*. 2009;41(1):50–58.
28. Huang S, et al. Variable prostaglandin E_2 resistance in fibroblasts from patients with usual interstitial pneumonia. *Am J Respir Crit Care Med*. 2008;177(1):66–74.
29. Allan EH, Martin TJ. Prostaglandin E_2 regulates production of plasminogen activator isoenzymes, urokinase receptor, and plasminogen activator inhibitor-1 in primary cultures of rat calvarial osteoblasts. *J Cell Physiol*. 1995;165(3):521–529.
30. Iwamoto J, et al. Expression of urokinase-type plasminogen activator and its receptor in gastric fibroblasts and effects of nonsteroidal antiinflammatory drugs and prostaglandin. *Dig Dis Sci*. 2003;48(12):2247–2256.
31. Pai R, Nakamura T, Moon WS, Tarnawski AS. Prostaglandins promote colon cancer cell invasion; signaling by cross-talk between two distinct growth factor receptors. *FASEB J*. 2003;17(12):1640–1647.
32. Hattori N, et al. The plasminogen activation system reduces fibrosis in the lung by a hepatocyte growth factor-dependent mechanism. *Am J Pathol*. 2004;164(3):1091–1098.
33. Peters-Golden M, et al. Protection from pulmonary fibrosis in leukotriene-deficient mice. *Am J Respir Crit Care Med*. 2002;165(2):229–235.
34. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med*. 1994;1(1):71–81.
35. Moore BB, Murray L, Das A, Wilke CA, Herrygers AB, Toews GB. The role of CCL12 in the recruitment of fibrocytes and lung fibrosis. *Am J Respir Cell Mol Biol*. 2006;35(2):175–181.
36. Kolodsick JE, Peters-Golden M, Larios J, Toews GB, Thannickal VJ, Moore BB. Prostaglandin E_2 inhibits fibroblast to myofibroblast transition via E prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation. *Am J Respir Cell Mol Biol*. 2003;29(5):537–544.
37. Charbeneau RP, et al. Impaired synthesis of prostaglandin E_2 by lung fibroblasts and alveolar epithelial cells from GM-$CSF^{-/-}$ mice: implications for fibroproliferation. *Am J Physiol Lung Cell Mol Physiol*. 2003;284(6):L1103–L1111.
38. Jenkins RG, et al. Ligation of protease-activated receptor 1 enhances alpha(v)beta6 integrin-dependent TGF-beta activation and promotes acute lung injury. *J Clin Invest*. 2006;116(6):1606–1614.
39. Syeda F, et al. Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. *J Biol Chem*. 2006;281(17):11792-11804.
40. Lyon M, Deakin J, Mizuno K, Nakamura T, Gallagher J. Interaction of hepatocyte growth factor with heparin sulfate. Elucidation of the major heparan sulfate structural determinants. *J Biol Chem*. 1994;269(15):11216–11223.
41. Mizuno K, Nakamura T. Proteolytic activation of a single chain precursor of hepatocyte growth factor by extracellular serine-protease. *Biochem Biophys Res Commun*. 1992;189(3):1631–1638.
42. Shanmukhappa K, Matte U, Degen JL, Bezerra JA. Plasmin-mediated proteolysis is required for hepatocyte growth factor activation during liver repair. *J Biol Chem*. 2009;284(19):12917–12923.
43. Zhang A, Wang MH, Dong Z, Yang T. Prostaglandin E_2 is a potent inhibitor of epithelial-to-mesenchymal transition: interaction with hepatocyte growth factor. *Am J Physiol Renal Physiol*. 2006;291(6):F1323–F1331.
44. Lee YH, Suzuki YJ, Griffin AJ, Day RM. Hepatocyte growth factor regulates cyclooxygenase-2 expression via beta-catenin, AKT, and p42/p44 MAPK in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2008;294(4):L778–L786.
45. Christensen JG, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. *Cancer Res*. 2003;63(21):7345–7355.
46. Sokolova E, Grishina Z, Buhling F, Welte T, Reiser G. Protease-activated receptor-1 in human lung fibroblasts mediates a negative feedback downregulation via prostaglandin E_2. *Am J Physiol Lung Cell Mol Physiol*. 2005;288(5):L793–L802.
47. Trejo J. Protease-activated receptors: new concepts in regulation of G protein-coupled receptor signaling and trafficking. *J Pharmacol Exp Ther*. 2003;307(2):437–442.
48. Armstrong R. Investigation of the inhibitory effects of PGE_2 and selective EP agonists on chemotaxis of human neutrophils. *Br J Pharmacol*. 1995;116(7):2903–2908.
49. Savla U, Appel HJ, Sporn PH, Waters CM. Prostaglandin E_2 regulates wound closure in airway epithelium. *Am J Physiol Lung Cell Mol Physiol*. 2001;280(3):L421–L431.
50. Kohyama T, et al. Prostaglandin E_2 inhibits fibroblast chemotaxis. *Am J Physiol Lung Cell Mol Physiol*. 2001;281(5):L1257–L1263.
51. White ES, et al. Prostaglandin E_2 inhibits fibroblast migration by E-prostanoid 2 receptor-mediated increase in PTEN activity. *Am J Respir Cell Mol Biol*. 2005;32(2):135–141.
52. Thomas P, Peters-Golden M, White E, Thannickal V, Moore B. PGE_2 inhibition of TGF-beta1-induced myofibroblast differentiation is SMAD-independent but involves cell shape and adhesion-dependent signaling. *Am J Physiol Lung Cell Mol Physiol*. 2007;293(2):L417–L428.
53. Mandal SK, Rao LV, Tran TT, Pendurthi UR. A novel mechanism of plasmin-induced mitogenesis in fibroblasts. *J Thromb Haemost*. 2005;3(1):163–169.
54. Horowitz JC, Rogers DS, Simon RH, Sisson TH, Thannickal VJ. Plasminogen activation-induced pericellular fibronectin proteolysis promotes fibroblast apoptosis. *Am J Respir Cell Mol Biol*. 2007;38(1):78–87.
55. Lama V, Moore B, Christensen P, Toews G, Peters-Golden M. Prostaglandin E_2 synthesis and suppression of fibroblast proliferation by alveolar epithelial cells is cyclooxygenase-2-dependent. *Am J Respir Cell Mol Biol*. 2002;27(6):752–758.
56. Bogatkevich GS, et al. Down-regulation of collagen and connective tissue growth factor expression with hepatocyte growth factor in lung fibroblasts from white scleroderma patients via two signaling pathways. *Arthritis Rheum*. 2007;56(10):3468–3477.
57. Gong R, Rifai A, Tolbert EM, Centracchio JN, Dworkin LD. Hepatocyte growth factor modulates matrix metalloproteinases and plasminogen activator/plasmin proteolytic pathways in progressive renal interstitial fibrosis. *J Am Soc Nephrol*. 2003;14(12):3047–3060.
58. Marchand-Adam S, et al. Defect of pro-hepatocyte growth factor activation by fibroblasts in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2006;174(1):58–66.
59. Dohi M, Hasegawa T, Yamamoto K, Marshall BC. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. *Am J Respir Crit Care Med*. 2000;162(6):2302–2307.
60. Gazdhar A, et al. Gene transfer of hepatocyte growth factor by electroporation reduces bleomycin-induced lung fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L529–L536.
61. Watanabe M, et al. Hepatocyte growth factor gene transfer to alveolar septa for effective suppression of lung fibrosis. *Mol Ther*. 2005;12(1):58–67.
62. Sherriff-Tadano R, et al. Antifibrotic effects of hepatocyte growth factor on scleroderma fibroblasts and analysis of its mechanism. *Mod Rheumatol*. 2006;16(6):364–371.
63. Matsumoto-Taniura N, Matsumoto K, Nakamura T. Prostaglandin production in mouse mammary tumour cells confers invasive growth potential by inducing hepatocyte growth factor in stromal fibroblasts. *Br J Cancer*. 1999;81(2):194–202.
64. Fan S, et al. The cytokine hepatocyte growth factor/scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3′ kinase. *Oncogene*. 2000;19(18):2212–2223.
65. Shukla MN, Rose JL, Ray R, Lathrop KL, Ray A, Ray P. Hepatocyte growth factor inhibits epithelial to myofibroblast transition in lung cells via SMAD7. *Am J Respir Cell Mol Biol*. 2009;40(6):643–653.
66. Carmeliet P, et al. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J Clin Invest*. 1993;92(6):2746–2755.
67. Corti M, Brody AR, Harrison JH. Isolation and primary culture of murine alveolar type II cells. *Am J Respir Cell Mol Biol*. 1996;14(4):309–315.
68. Huang SK, et al. Variable prostaglandin E_2 resistance in fibroblasts from patients with usual interstitial pneumonia. *Am J Respir Crit Care Med*. 2008;177(1):66–74.
69. Powell-Jones CH. Rapid SDS-PAGE zymography of plasminogen activators. *Thromb Res*. 1988;49(2):299–302.