ORIGINAL RESEARCH

Activated Human Lung Fibroblasts Produce Extracellular Vesicles with Antifibrotic Prostaglandins

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Abstract

The differentiation of interstitial lung fibroblasts into contractile myofibroblasts that proliferate and secrete excessive extracellular matrix is critical for the pathogenesis of pulmonary fibrosis. Certain lipid signaling molecules, such as prostaglandins (PGs), can inhibit myofibroblast differentiation. However, the sources and delivery mechanisms of endogenous PGs are undefined. Activated primary human lung fibroblasts (HLFs) produce PGs such as PGE_2. We report that activation of primary HLFs with IL-1β inhibited transforming growth factor β–induced myofibroblast differentiation in both the IL-1β–treated cells themselves (autocrine signal) and adjacent naive HLFs in cocultures (paracrine signal). Additionally, we demonstrate for the first time that at least some of the antifibrotic effect of activated fibroblasts on nearby naive fibroblasts is carried by exosomes and other extracellular vesicles that contain several PGs, including high levels of the antifibrotic PGE_2. Thus, activated fibroblasts communicate with surrounding cells to limit myofibroblast differentiation and maintain homeostasis. This work opens the way for future research into extracellular vesicle–mediated intercellular signaling in the lung and may inform the development of novel therapies for fibrotic lung diseases.

Keywords: lung fibroblast; myofibroblast differentiation; prostaglandin; pulmonary fibrosis; resolution

Clinical Relevance

We demonstrate that fibroblasts produce extracellular vesicles containing antifibrotic prostaglandins that can functionally inhibit myofibroblast differentiation by themselves and neighboring fibroblasts. This may be an important pathway by which homeostasis is maintained, and loss of this pathway may be implicated in the development of lung fibrosis. Extracellular vesicles also represent a novel route for delivering targeted antifibrotic therapies.

Human lung fibroblasts (HLFs) are the most abundant cells in the pulmonary interstitium (1). These mesenchymal cells produce the extracellular matrix (ECM) that gives the lung its structural integrity, and are best known for their critical role in wound healing (2). In normal wound healing, fibroblasts, often under the direction of the master profibrotic cytokine transforming growth factor β (TGF-β), differentiate into contractile myofibroblasts characterized by the presence of cytoplasmic stress fibers and focal adhesion complexes, the expression of α-smooth muscle actin (α-SMA) and calponin, and enhanced

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synthesis of ECM proteins such as collagen and fibronectin (3). However, in pulmonary fibrosis, an aberrant, unchecked wound-healing response can result in excessive interstitial myofibroblast proliferation and ECM deposition, severely hindering gas exchange (4). Therefore, understanding the homeostatic mechanisms that regulate myofibroblast differentiation and ECM production is crucial for identifying novel strategies to prevent and treat pulmonary fibrosis.

In addition to their well-known roles in wound healing and fibrosis, other investigators and we have shown that HLFs also perform an innate immune function as sentinel cells that respond to inhaled pathogens or toxicants by producing inflammatory mediators (5). For instance, HLFs produce proinflammatory ILs such as IL-6 and IL-8 after exposure to IL-1β (6), cigarette smoke extract (6, 7), bradykinin (8), TNF (9), mast cells (10), and even red blood cells (11). Additionally, HLFs express abundant cyclooxygenase-2 (COX-2), the limiting inducible enzyme that catalyzes the production of prostaglandins (PGs) from arachidonic acid, in response to proinflammatory stimuli, and IL-1β is a particularly strong COX-2 inducer in HLFs (12). Taken together, these results have established HLFs and PGs as key amplifiers of the inflammatory response to injurious stimuli, with the potential to control the severity of inflammatory lung disease (13–15).

On the other hand, we have recently shown that HLFs may also play an active role in moderating the response to injurious stimuli (6). Primary HLFs activated with IL-1β produce proinflammatory mediators early in the response, but then undergo a temporally regulated switch to produce a wide array of antiinflammatory PGs, including PGE_2 and PGJ_2 (6). This novel finding is congruent with other recent work showing that many innate immune cells initially respond to injurious stimuli with proinflammatory signals, but subsequently switch to produce proresolving mediators that facilitate the resolution of acute inflammation and restoration to baseline (16). It also raises the possibility that activated fibroblasts produce antifibrotic mediators that can act on themselves or their neighbors to resist profibrotic stimuli and maintain normal homeostasis.

Here, we investigated whether the PGs produced by IL-1β–activated fibroblasts have functional antifibrotic activity. We show that preactivation of fibroblasts with IL-1β inhibits TGF-β–stimulated myofibroblast differentiation in both pretreated HLFs (autocrine signaling) and naive bystander HLFs in coculture (paracrine signaling), and that antifibrotic signals are carried in extracellular vesicles (EVs). Our work uncovers a previously unrecognized endogenous antifibrotic signaling circuit among activated lung fibroblasts, revealing a novel pathway that may be exploited to develop urgently needed new therapies for fibrotic lung diseases.

Methods

Details regarding the methods used in this work are provided in the data supplement. Briefly, primary HLF cultures were established from normal lung tissue from male and female donors as previously described (17), or donated for research through the NHLBI Molecular Atlas of Lung Development (RFA-HL-14-007). Informed consent was obtained from all donors, or next of kin when the donation was made for research, under approval of the University of Rochester Institutional Review Board. Fibroblast-fibroblast cocultures were established with or without IL-1β pretreatment, and myofibroblast differentiation was induced using TGF-β. Myofibroblast differentiation and ECM production were assessed by Western blot and/or immunofluorescence. EVs were quantified from conditioned culture media using nanoparticle tracking analysis, CD63 ELISA, and CD63 immunocapture. Exosomes and other EVs were isolated by ultracentrifugation or precipitated using ExoQuick-TC exosome precipitation solution (System Biosciences) and analyzed for eicosanoid content by mass spectrometry, or used as a cotreatment with TGF-β in culture. Key experiments were performed in up to five different HLF strains, as shown in the figure legends, and all experiments were done in at least three biological replicates. Strains 1–5 were derived from different donors without fibrosis, and the labels are consistent throughout the figures. Results are reported as the mean ± SEM. One-way ANOVA with Tukey's *post hoc* test or paired *t* test was performed using GraphPad Prism software (v.7). *P* values < 0.05 were considered significant.

Results

IL-1β Pre- or Cotreatment Inhibits TGF-β–induced Myofibroblast Differentiation and ECM Production

We recently reported that primary HLFs activated with IL-1β produce elevated levels of PGE_2, as well as several metabolites of PGD_2 that are ligands for the antiinflammatory and antifibrotic transcription factor PPARγ (6). Based on this finding, we wanted to determine whether these PGs were functional and could inhibit TGF-β–induced fibroblast-to-myofibroblast differentiation. We treated primary HLFs with IL-1β either 24 hours before treatment or as a cotreatment with TGF-β. IL-1β inhibited both myofibroblast differentiation (Figures 1A and 1B) and ECM production (Figures 1C and 1D), regardless of whether it was used as a pretreatment or cotreatment with TGF-β. Although some markers trended toward greater inhibition when IL-1β was used as a pretreatment rather than as a cotreatment, there were no significant differences in protein expression of α-SMA, calponin, collagen, or fibronectin between the two treatment regimens.

Conditioned Medium of IL-1β–treated HLFs Inhibits TGF-β–induced Myofibroblast Differentiation

Given that IL-1β–activated HLFs produce high levels of E-, D-, and J-series PGs and their metabolites (6), at least some of which are reported to be antifibrotic, we tested whether conditioned media of IL-1β–activated HLFs would inhibit TGF-β–induced myofibroblast differentiation of naive HLFs. Three strains of primary HLFs were treated as illustrated in Figure 2A. Donor HLFs were treated with IL-1β (1 ng/ml) for 24 hours, and conditioned media were then removed and used as a cotreatment with TGF-β on recipient or target HLFs of the same strain. Myofibroblast differentiation was assessed 72 hours after TGF-β treatment by Western blot (Figure 2B) and immunofluorescence (Figure 2C) for α-SMA expression. The conditioned media of IL-1β–treated, but not untreated, HLFs robustly inhibited TGF-β–induced expression of α-SMA.

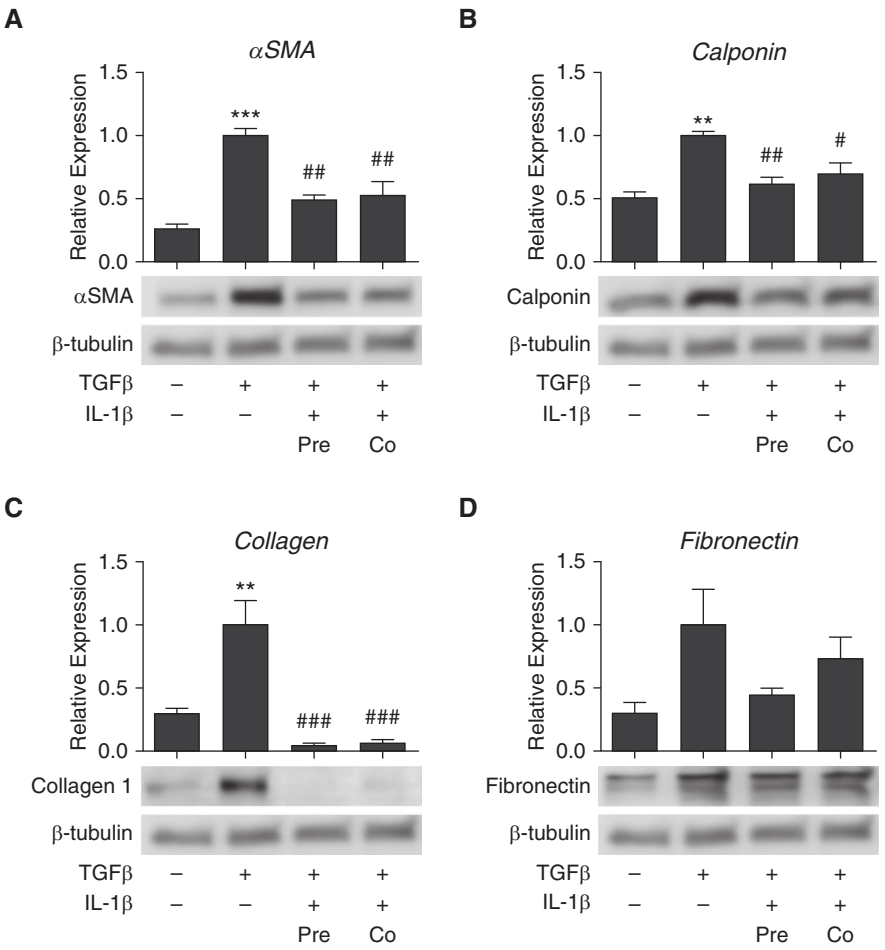

Figure 1. IL-1β pre- or cotreatment inhibits TGF-β–induced myofibroblast differentiation and extracellular matrix production. Primary HLFs were treated with TGF-β (0.5 ng/ml) alone, or 24 hours after pretreatment with IL-1β (1 ng/ml), or with IL-1β (1 ng/ml) as a cotreatment. (*A–D*) Cells were harvested 72 hours after TGF-β treatment, and expression of α-SMA (*A*), calponin (*B*), type I collagen (*C*), and fibronectin (*D*) was analyzed by Western blot and densitometric analysis. Data shown are mean ± SEM of $n = 3$ replicates per condition, protein expression relative to loading control, normalized to TGF-β1 alone. $^{**}P < 0.01$ and $^{***}P < 0.001$ by ANOVA, compared with untreated control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ by ANOVA, compared with TGF-β alone. Results shown are from strain 1; two other fibroblast strains were similar, data not shown. See Figure E1 for details on figure assembly. α-SMA = α−smooth muscle actin; Co = cotreatment; HLFs = human lung fibroblasts; Pre = pretreatment; TGF-β = transforming growth factor-β.

IL-1β–pretreated HLFs Inhibit TGF-β–induced Myofibroblast Differentiation and ECM Production in Direct Contact Coculture

Although both IL-1β cotreatment and conditioned medium from IL-1β–treated HLFs can inhibit myofibroblast differentiation, this does not exclude the possibility that IL-1β is exerting direct antifibrotic effects in this model (18). Therefore, we developed a direct-contact coculture model that would allow us to test our hypothesis in HLFs with the IL-1β removed. Primary HLFs were treated as outlined in Figure 3A. Separate populations, A and B, of the same HLF strain were established at equal densities in T25 flasks. One or both of these populations were pretreated with IL-1β for 24 hours before the coculture was established. After the 24-hour pretreatment, the HLFs were washed, detached from the flasks, and combined at a 1:1 ratio (i.e., of A to B populations) to establish direct-contact cocultures. Cocultures were then treated with TGF-β, and cells were harvested 72 hours later for Western blot analysis of markers of myofibroblast differentiation and ECM production.

When either half of the coculture (i.e., population A or B) was pretreated with IL-1β before the coculture was established, total TGF-β–induced α-SMA, calponin, collagen 1A, and fibronectin protein levels were significantly reduced compared with cocultures in which neither source population was pretreated with IL-1β (Figures 3B–3E). To our knowledge, this is the first demonstration that activated HLFs produce functional antifibrotic mediators that act in a paracrine fashion to inhibit myofibroblast differentiation by naive fibroblasts. Interestingly, IL-1β pretreatment of both populations (i.e., both A and B) did not result in significantly more inhibition of any of these markers than when just one population was pretreated. This suggests that when only one population was preactivated, it produced sufficient antifibrotic mediators to inhibit myofibroblast differentiation and ECM production by both the pretreated cells (i.e., autocrine signal) and nonpretreated cells (i.e., paracrine signal).

IL-1β–pretreated HLFs Inhibit TGF-β–induced Myofibroblast Differentiation in Noncontact Coculture in a Partially COX-2–Dependent Manner

To confirm that HLFs exert antifibrotic signals in a paracrine fashion, we used a noncontact coculture system as shown in Figure 4A. Target (or "bottom") fibroblasts were plated in standard 12-well plates, and donor (or "top") fibroblasts were plated separately in hanging inserts. Donor HLFs in hanging inserts were pretreated with IL-1β (1 ng/ml) for 24 hours, and then washed with PBS and transferred into wells containing previously plated target HLFs. The coculture was then treated with TGF-β (0.5 ng/ml) and 72 hours later, the target fibroblasts were assessed for expression of the myofibroblast differentiation marker α-SMA by Western blot (Figure 4B) or immunofluorescence (Figure 4C). In primary HLF strains from five different donors, the target HLFs showed markedly reduced TGF-β–induced α-SMA expression levels when cocultured with IL-1β–pretreated top HLFs as compared with when they were cocultured with unpretreated top HLFs, indicating that IL-1β–activated HLFs indeed produce soluble antifibrotic mediators. Because activation of HLFs with IL-1β causes rapid and strong upregulation of COX-2 (6), the

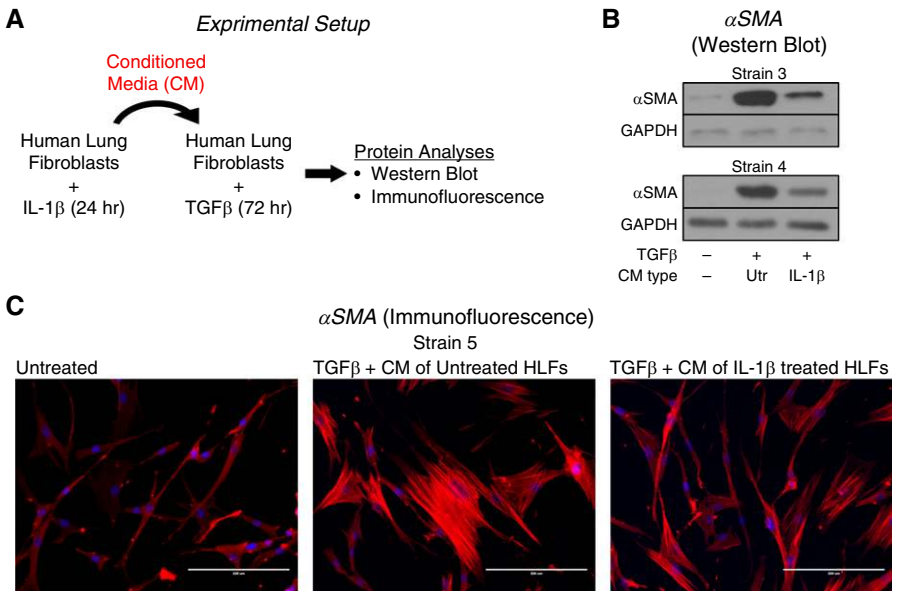

Figure 2. Conditioned medium of IL-1β–treated HLFs inhibits TGF-β–induced myofibroblast differentiation. Donor HLFs were untreated or treated for 24 hours with IL-1β (1 ng/ml) to generate conditioned media. (*A*) Recipient HLFs were then treated with TGF-β (0.5 ng/ml) plus the conditioned media of untreated or IL-1β–treated donor HLFs of the same strain. (*B* and *C*) Expression of α-SMA was determined 72 hours after TGF-β treatment by Western blot for HLF strains 3 and 4 (*B*) and by immunofluorescence for HLF strain 5 (*C*). Scale bars: 200 μm. Utr = untreated.

limiting enzyme in the production of antifibrotic PGs, we hypothesized that the inhibitory effect of coculture with IL-1β–treated HLFs is COX-2 dependent. Therefore, we repeated the noncontact coculture experiments, as depicted in Figure 4D, using the selective COX-2 inhibitor celecoxib as a cotreatment with IL-1β on the donor (i.e., top) HLFs in hanging inserts. Consistent with our previous results, TGF-β–induced myofibroblast differentiation was robustly inhibited when the donor HLFs were pretreated with IL-1β. However, inhibition was markedly attenuated when the donor HLFs were pretreated with both IL-1β and celecoxib (Figure 4E), indicating that the inhibitory effect of IL-1β–pretreated HLFs is indeed at least partly COX-2 dependent.

Conditioned Media of HLFs Contain PG-Containing EVs

To further investigate the mechanism of intercellular communication of antifibrotic signals between IL-1β–activated HLFs and naive bystander fibroblasts, we analyzed the conditioned media of untreated and IL-1β–activated HLFs for EV content using a NanoSight NS300 nanoparticle characterization instrument. Equal numbers of HLFs were plated in T75 flasks and allowed to adhere for 48 hours before treatment with IL-1β. Supernatants were collected for analysis 24 hours after IL-1β treatment. Overall, the conditioned media of both untreated and IL-1β–treated HLFs were similar with respect to total EV content and average particle size (Figure 5A). However, the size distribution of the particles differed depending on whether or not HLFs were treated with IL-1β, as shown in Figure 5B. Importantly, the conditioned media of both untreated and IL-1β–treated HLFs contained EVs 40–160 nm in diameter, the size range consistent with exosomes (Figure 5C). However, because the remaining experiments used EV preparations without further size fractionation, we refer to these preparations as EVs rather than exosomes.

Next, to determine whether EVs of IL-1β–treated HLFs contain antifibrotic PGs, we performed targeted eicosanomics by mass spectrometry. We collected EVs from four HLF strains, with and without IL-1β treatment, and analyzed the preparations by mass spectrometry for a targeted set of eicosanoids. As shown in Figure 5D, the concentrations of PGE_2 and 13,14 dh-15k-PGE_2 were significantly elevated in EVs from IL-1β–treated HLFs compared with untreated HLFs. The levels of PGA_2, PGJ_2, and $PGF_{2\alpha}$ and its stable metabolite 13,14 dh-15k-$PGF_{2\alpha}$ were increased with IL-1β pretreatment in EVs from some, but not all, of the HLF strains, but the absolute quantities were much lower and the overall results did not reach statistical significance (Figure 5D).

We further characterized these EVs by measuring levels of the hallmark exosome marker CD63 by ELISA, and by exosome capture flow cytometry (Figures E4A and E4B in the data supplement). Here, we isolated EVs using either the polymer precipitation method or the classically used method of ultracentrifugation (19). In either polymer-precipitated or ultracentrifugation-isolated EVs, we detected a significant population of $CD63^+$ EVs. These results show that HLFs are capable of producing $CD63^+$ EVs, which include the size range consistent with exosomes. Importantly, we can rule out the possibility that these EVs were derived from bovine serum in the culture medium, as CD63 was undetectable in medium without cells (Figures E4A and E4C). We also investigated whether EVs were the sole source of PGs produced by IL-1β–treated HLFs or the conditioned medium also contained soluble PGs. Because the most abundant PG identified by mass spectrometry was PGE_2, we focused our analyses on it. We measured PGE_2 levels in polymer-precipitated EVs, ultracentrifugation-isolated EVs, and the remaining soluble extracts from IL-1β–treated HLFs (Figure E4C). Interestingly, although PGE_2 was clearly packaged into EVs, it was also present at high levels in the EV-free fraction.

EVs from IL-1β–treated HLFs Inhibit TGF-β–induced Myofibroblast Differentiation and ECM Production

Exosomes and other small EVs have recently gained attention as an important means of intercellular communication (20, 21), and based on their PG content and presence in HLF conditioned media, we hypothesized that IL-1β–treated HLFs deliver antifibrotic signals via EVs. To test this hypothesis, we prepared EVs from conditioned media of untreated and IL-1β–treated HLFs using a polymer precipitation solution. Concentrated EVs were then resuspended in fresh culture medium and used as a cotreatment with TGF-β; after 72 hours, cells were harvested for determination of myofibroblast differentiation and ECM

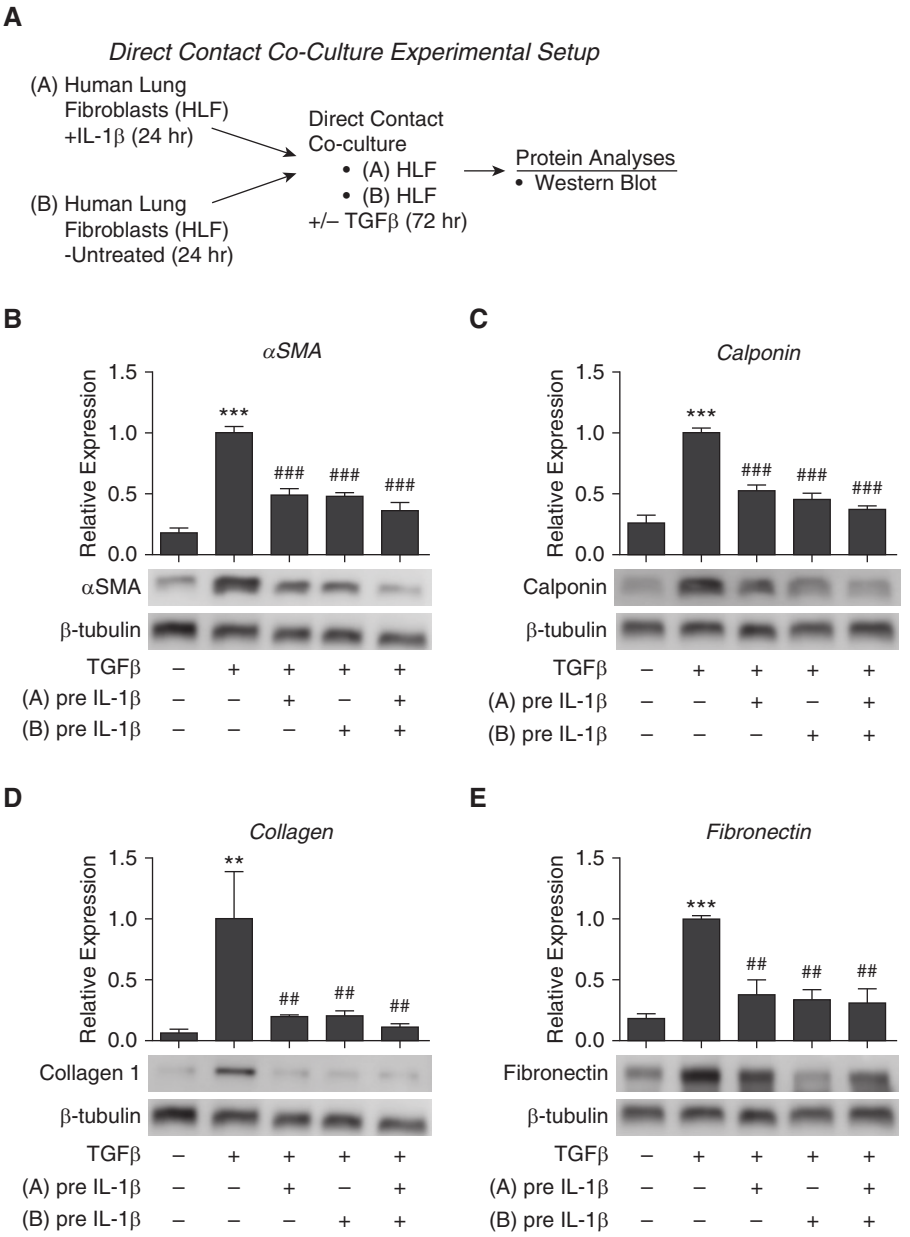

Figure 3. IL-1β–pretreated HLFs inhibit TGF-β–induced myofibroblast differentiation and extracellular matrix production in direct contact coculture. On Day 0, HLFs were plated at equal densities in T25 flasks and left untreated or treated with IL-1β (1 ng/ml). (*A*) After 24 hours, on Day 1, the conditioned media were removed by aspiration and the HLFs were washed with 1× PBS, removed from flasks by trypsinization, counted, resuspended in fresh culture medium, and mixed in 1:1 ratios to establish direct-contact cocultures of unpretreated/unpretreated, unpretreated/pretreated, or pretreated/pretreated HLFs. The cocultures were then treated with TGF-β (0.5 ng/ml). (*B–E*) Cells were harvested on Day 4, and expression of α-SMA (*B*), calponin (*C*), type I collagen (*D*), and fibronectin (*E*) was analyzed by Western blot and densitometric analysis. Data shown are mean ± SEM of $n = 3$ replicates per condition, protein expression relative to loading control, normalized to TGF-β with no IL-1β pretreatment. $^{**}P <$ 0.01 and $^{***}P < 0.001$ by ANOVA, compared with untreated control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ by ANOVA, compared with TGF-β with no IL-1β pretreatment. Data from HLF strain 4 are shown; strains 1 and 2 were similar. See Figures E2 and E3 for details on figure assembly.

production by Western blot. Importantly, the number of donor cells used for EV production was approximately equal to the number of recipient target cells treated (EVs isolated from a single well were used on a target well of the same size with similarly confluent cells). EVs from IL-1β–treated HLFs robustly inhibited TGF-β–induced expression of α-SMA, calponin, and collagen (Figure 6). Additionally, EVs from IL-1β–treated donor cells isolated by ultracentrifugation also blocked TGF-β–induced expression of α-SMA in donor cells (Figure E4D).

HLF strains from different donors pretreated with IL-β produced EVs with variable levels of D- and J-series PGs, but all of the strains produced >1,000 pg/ml PGE_2, which suggested that PGE_2, rather than D- and J-series PGs, is the key inhibitory factor. To confirm this hypothesis, we treated HLFs with TGF-β and EVs from fibroblasts activated in the presence or absence of the COX-2 inhibitor celecoxib. We also treated target fibroblasts with normally activated EVs in the presence of the PGE_2 receptor E prostanoid receptor 2 (EP2) antagonist AH6809. Interestingly, both celecoxib treatment of donor cells and EP2 antagonism of target cells reversed the antifibrotic effect of EVs, demonstrating that PGE_2 accounts for a major fraction of the antifibrotic properties of EVs from activated HLFs (Figure E6).

Discussion

A key event in the pathogenesis of fibrosis is the differentiation of HLFs into myofibroblasts, and TGF-β is a powerful agonist of this process (22). On the other hand, HLFs also act as innate immune sentinel cells that are capable of responding to proinflammatory stimuli, and production of proinflammatory mediators by stimulated fibroblasts has been long documented (8–11, 17). However, we recently reported that IL-1β–activated HLFs also produce PGs with PPARγ ligand activity, suggesting that HLFs may actually be able to moderate fibrosis (6). We therefore addressed the following questions: *1*) do HLFs activated with IL-1β resist TGF-β–induced myofibroblast differentiation and ECM production; *2*) do IL-1β–activated HLFs produce antifibrotic signals directed toward themselves (i.e., autocrine signals), nearby HLFs (i.e., paracrine signals), or both; *3*) are these signals mediated by COX-2–derived PGs; and *4*) how are these signals delivered?

In our model, IL-1β significantly provokes inflammatory activation of HLFs, inducing COX-2 expression and subsequent PG production. IL-1β is one of the most powerful proinflammatory

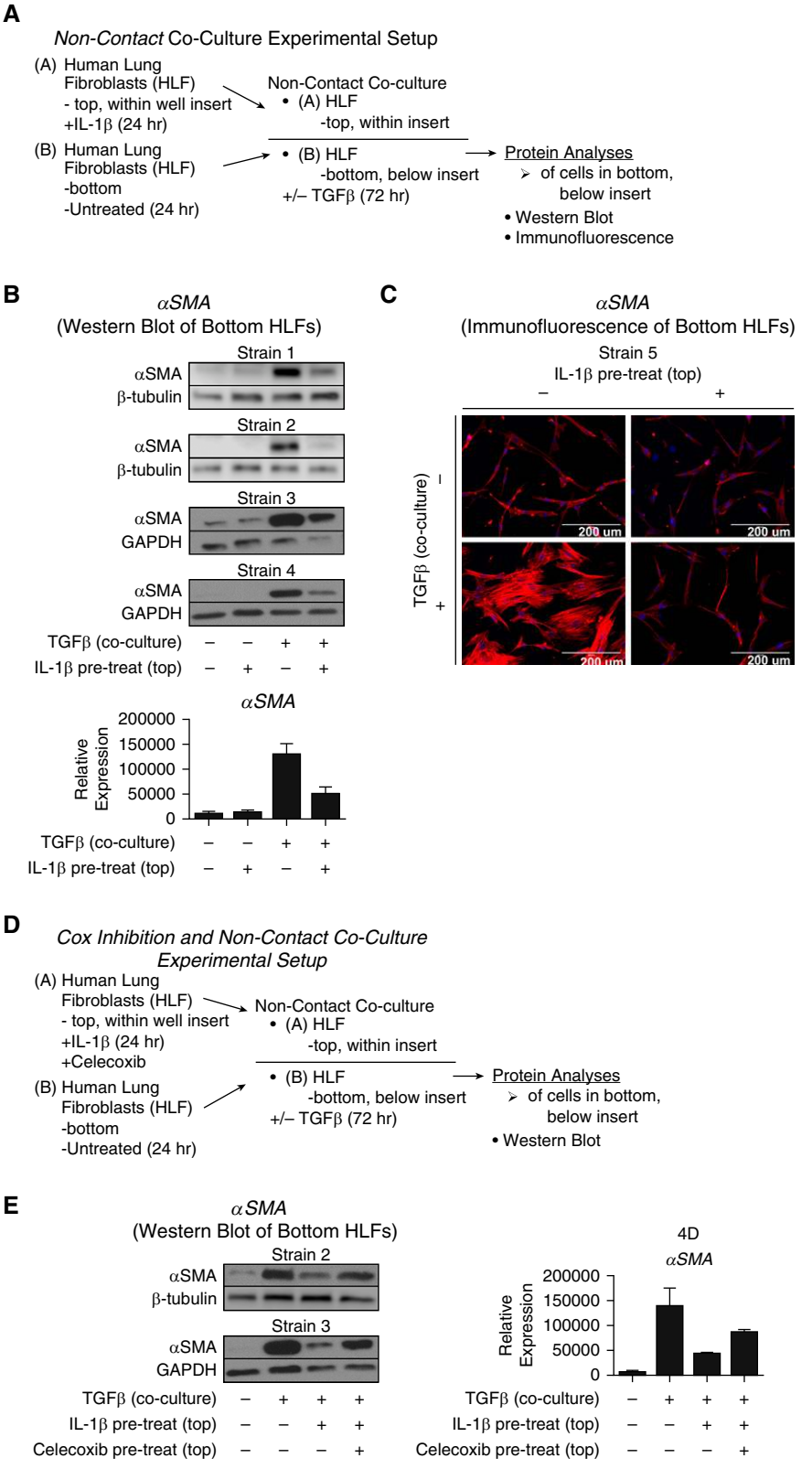

Figure 4. IL-1β–pretreated HLFs inhibit TGF-β–induced myofibroblast differentiation in noncontact coculture in a partially COX-2–dependent manner. (*A*) On Day 0, recipient (bottom) HLFs were plated in 12-well hard-bottom plates, and equal numbers of the same HLF strain were plated separately in hanging inserts. The donor (top) HLFs were left untreated or treated with IL-1β (1 ng/ml).

cytokines known, and affects nearly every cell type in the body (23). In the lung, IL-1β mediates the initiation and persistence of inflammation associated with cigarette smoking (24), chronic obstructive pulmonary disease (25), and viral infection (26), among other disorders, making it a highly biologically relevant cytokine for study. Its role in pulmonary fibrosis is less clear, and may depend on whether the underlying disease features a strong inflammatory component (e.g., sarcoid) or not (e.g., idiopathic pulmonary fibrosis). Recent studies have shown that IL-1β is antifibrotic in *in vitro* models using human lung (27), dermal (27), and cardiac (18) fibroblasts, in agreement with our results herein. Although these studies reported that IL-1β has antifibrotic activity, the mechanism(s) remained unclear. Here, we are the first to show that conditioned medium from IL-1β–treated fibroblasts contains antifibrotic properties, and that IL-1β induces an antifibrotic state in lung fibroblasts that persists after the IL-1β is removed. Our work is also the first to implicate COX-2 activation in the mechanism underlying the effects of IL-1β, and the first to report autocrine or paracrine antifibrotic signaling by fibroblasts. It is also important to emphasize that IL-1β is used here as a model stimulus, and other inflammatory stimuli, particularly those that upregulate COX-2 expression, may similarly prompt HLFs to engage in endogenous antifibrotic signaling, as illustrated in our recent study using cigarette smoke extract (6).

Further investigation indicated that the antifibrotic activity produced by activated HLFs could at least partially be explained by the production of EVs containing PGE_2. EVs are membrane-bound bodies that contain cytosol enclosed in a lipid bilayer. Exosomes were discovered and characterized over three decades ago; however, interest in exosomes as vehicles for intercellular communication has exploded in recent years, as exosomes are now known to be present in nearly every bodily fluid and compartment, to carry genetic messages between cells in mRNA and microRNA (28), and to carry cargo that can alter the phenotype of recipient cells (29). Previous work has shown that RBL-2H3 leukemia cell–derived exosomes carry PGs and phospholipases for transcellular communication (30). Interestingly, in RBL-2H3–derived

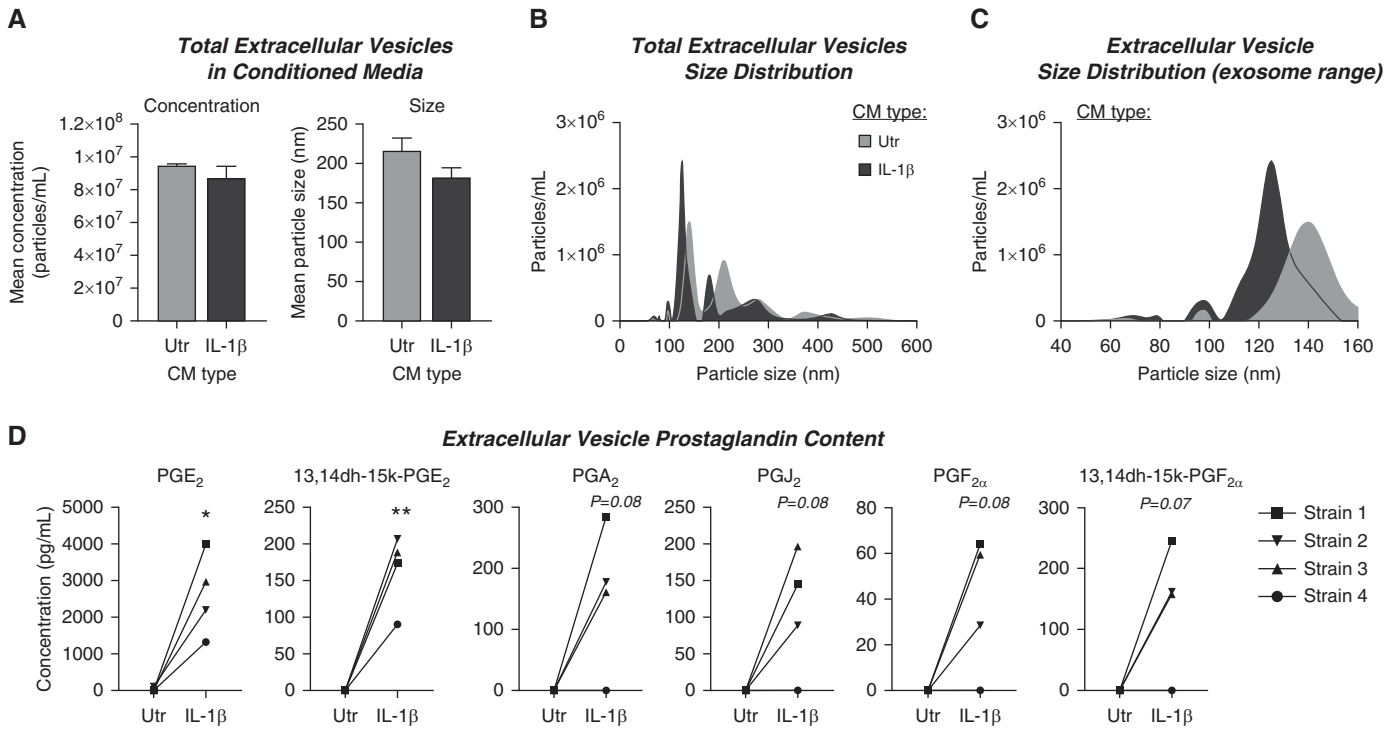

Figure 5. Conditioned media of HLFs contain extracellular vesicles (EVs), including prostaglandin-containing exosomes. (*A–C*) On Day 0, primary HLFs were plated in equal densities into two T75 flasks and allowed to adhere for 24 hours. On Day 1, HLFs were washed with 1× PBS and given fresh serum-free Eagle's minimum essential medium (MEM). On Day 2, HLFs were either Utr or treated with IL-1β (1 ng/ml), and supernatants were collected for analysis on Day 3. Fresh supernatants were immediately analyzed using the NanoSight NS300 nanoparticle characterization instrument and Nanoparticle Tracking Analysis software. Cell-free media were used as a blank control to determine background mediator levels. Data shown in *A* are mean ± SEM of $n = 3$ replicates per condition. Background subtraction was performed by subtracting the mean concentration at each particle size from the counts obtained on conditioned media. Data in *B* and *C* are mean of $n = 3$ replicates, background subtracted. Data in *C* are from the same experiment as the data in *B*, regraphed to highlight the contribution of the exosome size fraction (40–160 μm) to the total EV content. (*D*) To generate EV samples for eicosanomic analysis, each of four normal strains of primary HLFs was plated in two T75 flasks at equal densities on Day 0. On Day 1, cells were washed in 1× PBS and the media were changed to phenol red–free MEM; cultures were left Utr or treated with IL-1β (1 ng/ml). On Day 2, the supernatants were harvested and exosomes were precipitated from supernatants overnight using ExoQuick-TC Exosome Precipitation Solution. On Day 3, concentrated exosomes were resuspended in 1× PBS and immediately stored at −80°C for targeted lipidomic analysis by liquid chromatography–mass spectrometry as detailed in the data supplement. $^*P < 0.05$ and $^{**}P < 0.01$, compared with Utr, paired *t* test.

exosomes, numerous lipid-related proteins and bioactive lipid molecules are detectable. These exosomes could be efficiently taken up by target cells, and PGs such as PGE_2 could be delivered to influence target cell fate. This further supports our hypothesis that lipid mediators can be packaged by lung fibroblasts into EVs to influence lung physiology. However, until this report, there has been a paucity of data regarding EVs in the lung in general, and in fibrotic lung disease in particular. One group successfully isolated microRNAs from the exosomes of exhaled breath condensates, and proposed that this might be a novel method for detecting biomarker signatures of pulmonary diseases such as asthma (31). Although recent reports have demonstrated that cancer-associated fibroblasts produce exosomes/EVs that influence the tumor microenvironment (32), to our knowledge, neither the production of EVs by HLFs nor eicosanomic analysis of HLF-derived EVs has been previously reported. The fact that lung fibroblasts communicate via EVs and that EVs can contain antifibrotic signals, including PGE_2, holds intriguing therapeutic potential, as synthetic EVs might be able to efficiently target lung fibroblasts to deliver antifibrotic

Figure 4. (Continued). After 24 hours, on Day 1, the conditioned media were removed from the donor HLFs in hanging inserts, and the donor HLFs were washed with 1× PBS and transferred into the wells to rest in close apposition to the recipient HLFs, thus establishing a noncontact coculture. The cocultures were then treated with TGF-β (0.5 ng/ml). (*B* and *C*) Recipient (bottom) cells were harvested on Day 4, and expression of α-SMA was determined by Western blot for HLF strains 1–4 (*B*) and by immunofluorescence for HLF strain 5 (*C*). Scale bars: 200 μm. (*D*) To determine COX-2 dependence, on Day 0, recipient (bottom) HLFs were plated in 12-well hard-bottom plates, and equal numbers of the same HLF strain were plated separately in hanging inserts. The donor (top) HLFs were left untreated or treated with IL-1β (1 ng/ml) or IL-1β (1 ng/ml) plus celecoxib (10 μM). After 24 hours, on Day 1, the conditioned media were removed from the donor HLFs in hanging inserts, and the donor HLFs were washed with 1× PBS and transferred into the wells to rest in close apposition to the recipient HLFs, thus establishing a noncontact coculture. The cocultures were then treated with TGF-β (0.5 ng/ml). (*E*) Recipient (bottom) cells were harvested on Day 4 and expression of α-SMA was determined by Western blot.

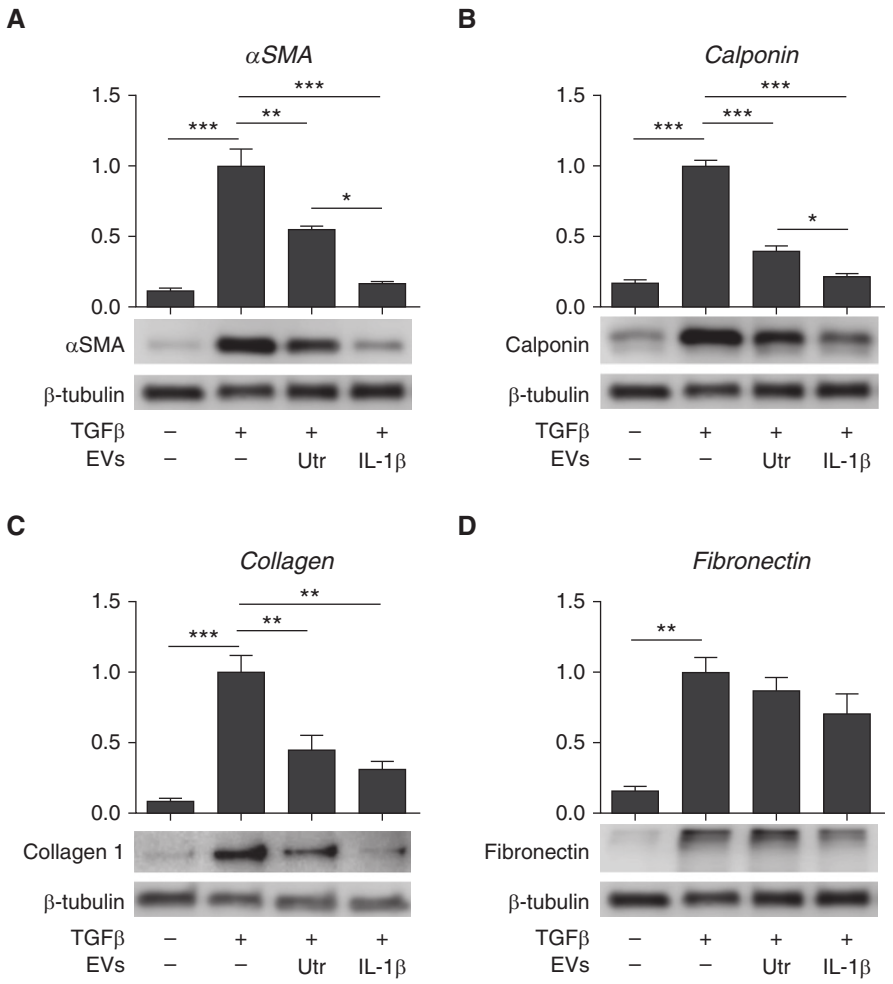

Figure 6. EVs of IL-1β–treated HLFs inhibit TGF-β–induced myofibroblast differentiation and extracellular matrix production. Donor primary HLFs were plated in two T75 flasks, and recipient HLFs of the same strain were plated in 12-well plates on Day 0. On Day 1, donor HLFs were left untreated or treated with IL-1β (1 ng/ml). On Day 2, the supernatants of donor HLFs were harvested and EVs were precipitated from supernatants overnight using ExoQuick-TC exosome precipitation solution. On Day 3, concentrated EVs were resuspended in fresh MEM, and recipient HLFs were treated with TGF-β (0.5 ng/ml) alone or in combination with the resuspended EVs from Utr or treated (IL-1β) donor HLFs. (*A–D*) On Day 6, recipient HLFs were harvested and expression of α-SMA (*A*), calponin (*B*), type I collagen (*C*), and fibronectin (*D*) was analyzed by Western blot and densitometric analysis. Data shown are mean ± SEM of $n = 3$ replicates per condition, protein expression relative to loading control, normalized to TGF-β alone. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by ANOVA. See Figure E5 for details on figure assembly.

therapeutics directly to these key effector cells (33–35).

EVs from activated HLF strains from four different donors contained large amounts of PGE_2 (>1,000 pg/ml in all strains) but only small and variable amounts of D- and J-series PGs, which are known ligands for the antifibrotic transcription factor PPARγ. Although we might have expected that HLF production of PPARγ ligands would contribute to the antifibrotic activity of fibroblast EVs, fibroblast strains that produced little or no PGJ_2 were nevertheless able to strongly suppress TGF-β–stimulated myofibroblast differentiation (e.g., strain 4 in Figures 2, 4, and 5). Therefore, despite the fact that EVs from activated fibroblasts contain some PPARγ ligands, the majority of the antifibrotic activity in the EVs can be attributed to PGE_2. We confirmed this by treating the donor cells with celecoxib and treating the target cells with an EP2 antagonist. PGE_2 has been studied extensively and has been shown to inhibit fibroblast proliferation and migration, myofibroblast differentiation, and ECM production, and to promote myofibroblast apoptosis (15, 36–38). We preciously reported that PGE_2 produced by lung epithelial cells has antifibrotic activity on lung fibroblasts (39); here, we show that HLFs themselves can produce PGE_2 with autocrine and paracrine antifibrotic activity.

Here, we found PGE_2 present in both the soluble fraction and the EV fraction. Furthermore, we show that PGE_2 in both conditioned medium and the EV fraction has antifibrotic activity. In the present study, we did not delineate the relative contribution of soluble PGE_2 compared with EV-associated PGE_2, and future studies are needed to elucidate this. Without minimizing the role of soluble PGE_2, the fact that activated HLFs package PGE_2 into EVs has important functional and therapeutic implications. Unlike soluble mediators, EVs can travel and serve as a novel reservoir for antifibrotic signals, and can be compartmentalized or sequestered to specifically and locally deliver signaling molecules and information to particular target cells, whereas a soluble fraction cannot (29, 30, 40). It is worth noting that PGE_2 signals through its cognate G protein–coupled receptor EP2 at the cell surface. Subra and colleagues suggested that PGE_2 associated with exosomes can be delivered to surface receptors such as EP2 when the membranes of the EV and target cell fuse (30). Exosomes may also have multiple interactions with the ECM (20, 41, 42). This interaction may increase the time exosomes spend at the cell surface to allow increased delivery and targeting of PGE_2 to its surface receptors. Thus, exosomes may help to concentrate PGE_2 on the surface for increased EP2 or other PGE_2 receptor activation. Another possibility is that selective interactions with the ECM may help guide exosomes/EVs to different recipient cell types. Future studies, perhaps in 3D culture systems, would be of interest to determine whether exosome/EV trafficking is selective or is guided to lung fibroblasts, epithelial cells, or other important lung cell types.

EVs prepared from untreated lung fibroblasts exhibited partial antifibrotic activity despite having very low levels of PGE_2 (compare Figure 5D with Figures 6A and 6B). In addition to lipids, exosomes are known to contain mRNA, microRNA, and

proteins, any of which could deliver antifibrotic signals (43–45). It is also possible that EV binding to the cell surface can alter TGF-β signaling. TGF-β signaling is regulated by many factors, and IL-1β reduces endogenous TGF-β expression in periodontal cells (46). Additionally, recent work has revealed that exosomes alter other cell compartments, such as the inflammasome (47, 48). Serum exosomes/EVs regulate inflammasome signaling in IL-1β–treated macrophages. Therefore, HLF EVs may disrupt or alter the inflammasome to reduce or limit IL-1β and/or TGF-β signaling. Further work to characterize exosome interactions and how they regulate or alter these pathways is warranted.

Previous studies have shown that lung fibroblasts serve as a source of PGs that could influence cell fate. Wilborn and colleagues initially highlighted that IL-1β–treated HLFs upregulate COX-2 and produce high levels of PGE_2 (49). Additional studies have shown that IL-1β–induced PGE_2 production can control HLF proliferation (50). These previous studies support our work presented here and the concept that HLFs can be an important source of antifibrotic PGs. Given that HLFs can account for the majority of interstitial tissue volume and contribute to normal physiological function and disease states (3), they are likely to be a critical endogenous source for lipid signaling molecules. Here, our studies show that fibroblasts are capable of producing PGs, especially PGE_2, in the nanomolar range, which has strong physiological effects at these levels (14). Currently, the relative importance *in vivo* of PG production by different lung cell types (e.g., fibroblasts, epithelial cells, monocyte/macrophages, and lymphocytes) is unknown. Future studies with cell-type–specific COX-2 or PGE synthase knockout mice may prove illuminating.

In summary, here we report the novel findings that activated primary HLFs produce PGE_2, which is partly carried in EVs and exerts antifibrotic effects in both an autocrine and paracrine fashion. Furthermore, we have identified EVs containing PGE_2 as the mechanism by which IL-1β exerts its antifibrotic effects. We have characterized, for the first time, EV production by HLFs, and in particular, the functional effects and PG content of HLF-derived exosomes. Our work opens the door for additional investigations into the content of HLF-derived exosomes, and may have major implications for the development of new therapeutics for fibrotic lung disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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