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Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal lung disease. A maladaptive epithelium due to chronic injury is a prominent feature and contributor to pathogenic cellular communication in IPF. Recent data highlight the concept of a “reprogrammed” lung epithelium as critical in the development of lung fibrosis. Extracellular vesicles (EVs) are potent mediator of cellular crosstalk, and recent evidence supports their role in lung pathologies such as IPF. Here, we demonstrate that syndecan-1 is overexpressed by the epithelium in the lungs of IPF patients and in murine models after bleomycin injury. Moreover, we find that syndecan-1 is a pro-fibrotic signal that alters alveolar type II (ATII) cell phenotypes by augmenting TGFβ and Wnt signaling among other pro-fibrotic pathways. Importantly, we demonstrate that syndecan-1 controls the packaging of several anti-fibrotic microRNAs into EVs that have broad effects over several fibrogenic signaling networks as a mechanism of regulating epithelial plasticity and pulmonary fibrosis. Collectively, our work reveals new insight into how EVs orchestrate cellular signals that promote lung fibrosis and demonstrate the importance of syndecan-1 in coordinating these programs.

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic and fatal lung disease. A maladaptive epithelium due to chronic injury is a prominent feature and contributor to pathogenic cellular communication in IPF. Recent data highlight the concept of a “reprogrammed” lung epithelium as critical in the development of lung fibrosis. Extracellular vesicles (EVs) are potent mediator of cellular crosstalk, and recent evidence supports their role in lung pathologies such as IPF. Here, we demonstrate that syndecan-1 is overexpressed by the epithelium in the lungs of IPF patients and in murine models after bleomycin injury. Moreover, we find that syndecan-1 is a pro-fibrotic signal that alters alveolar type II (ATII) cell phenotypes by augmenting TGFβ and Wnt signaling among other pro-fibrotic pathways. Importantly, we demonstrate that syndecan-1 controls the packaging of several anti-fibrotic microRNAs into EVs that have broad effects over several fibrogenic signaling networks as a mechanism of regulating epithelial plasticity and pulmonary fibrosis. Collectively, our work reveals new insight into how EVs orchestrate cellular signals that promote lung fibrosis and demonstrate the importance of syndecan-1 in coordinating these programs.
Introduction

Idiopathic pulmonary fibrosis (IPF), a progressive fibrotic disease of the lungs, has a substantial impact on patient mortality with a median survival of 2-3 years (1, 2). Although the abnormal fibroproliferation that occurs in IPF involves a complex interplay between parenchymal and immune cells, the epithelial barrier has a fundamental role particularly in early events that lead to lung fibrosis (3). Recently, senescence or depletion of alveolar epithelial cells has been implicated in the pathogenesis of lung fibrosis (4-9). In addition, several epithelial expressed genes have been found to confer a risk for the development of pulmonary fibrosis (e.g., MUC5B, SFTPC, SFTPA2, ABCA) further supporting dysfunctional alveolar epithelium as a sentinel event in the development of fibrosis (10).

Within the distal lung, alveolar type II (ATII) cells can undergo phenotypic changes and exhibit abnormalities referred to as “reprogramming” during fibrogenesis (11, 12). These alterations to the alveolar epithelium shift their normal reparative response to injury toward pathogenic consequences such as loss of epithelial integrity and excessive matrix deposition (13). The process of alveolar epithelial reprogramming is clearly multifactorial requiring alterations in several signaling pathways. One prominent example is TGF-β, a cytokine that is central to the fibrogenic process and can induce changes to the epithelial phenotype (14-17). Moreover, re-activated developmental pathways, such as Wnt signaling, can also affect alveolar epithelial plasticity in facilitating lung fibrosis (18-22).

Extracellular vesicles (EVs) are released from the cell and have the capacity to alter signaling networks in distal cells through intercellular delivery of various functional cargo (e.g., nucleic acids, proteins, lipids) (23, 24). Several subsets of EVs have been well-described with exosomes (40 – 150 nm diameter) and microvesicles (50 – 1000 nm diameter) being the predominant forms. Collectively, EVs play an important role in tissue homeostasis and alterations to their production (quantity and quality) occur during and contribute to various diseases. In pathological states, dysregulation of EV cargo can have profound effects in disease by
fundamentally altering the microenvironment and cellular phenotypes through disruption of normal homeostatic pathways while augmenting pathologic signals. To this end, several studies reported EVs having a functional role in pulmonary diseases such as acute lung injury, COPD, and lung cancer (25-31). Only recently have studies begun unraveling the role that EVs play in mediating fibroproliferation (32-37).

Syndecan-1 is a type I transmembrane protein with glycosaminoglycan side chains that is localized to the epithelium placing it in an ideal location to govern lung mucosal immune responses (38-43). The mechanisms by which syndecan-1 mediates its effects have revolved predominantly around its ability to act as a cell surface co-receptor or for the shed ectodomain to facilitate cell signals through presentation or sequestration of ligands with cognate receptors (44). Recently, Baetti et al. made a novel observation that syndecan-1 is found in exosomes and has a distinct role in biogenesis (45). Subsequent studies not only confirmed this seminal work but also demonstrated the ability of syndecan-1 to regulate the packaging of specific cargo within EVs (25, 46, 47).

Here, we identify a novel phenotype where syndecan-1 overexpression by the epithelium during lung fibrosis promotes fibroproliferation. Our studies demonstrate that syndecan-1 supports reprogramming of the alveolar epithelium toward a pro-fibrotic phenotype. Furthermore, we reveal that syndecan-1 controls alveolar epithelial plasticity by altering microRNA cargo within EVs that can govern networks of fibrogenic pathways such as TGFβ and Wnt signaling.
Results

Syndecan-1 is overexpressed by the alveolar epithelium in fibrotic lungs

To discern the importance of syndecan-1 in pulmonary fibrosis, we first evaluated the expression of syndecan-1 in interstitial lung disease (ILD) populations by mining the Lung Genomics Research Consortium (LGRC) transcriptomic database (http://www.lung-genomics.org) and found that syndecan-1 expression was significantly elevated in the lungs of ILD patients (Figure 1A). We then determined that syndecan-1 was overexpressed in IPF lungs and localized to the alveolar epithelium (Figure 1B-C). Next, we utilized a recently published single cell RNA-sequencing (scRNAseq) dataset (48) to further localize syndecan-1 expression in IPF versus normal lungs and found that syndecan-1 was significantly upregulated by ATII cells in fibrotic lungs (Figure 1D-E).

Bleomycin injury is a common murine model of lung fibrosis, so we evaluated syndecan-1 expression in mouse lungs after treatment. Similar to the findings in IPF lungs, syndecan-1 was overexpressed by the ATII cells in fibrotic region of bleomycin-injured mice (Figure 2A-B). We also evaluated syndecan-1 expression by scRNAseq analysis of bleomycin-injured mice and found augmented syndecan-1 expression by ATII cells in fibrotic lungs versus control (Figure 2C-D). Altogether, our data reveal that syndecan-1 is overexpressed by ATII cells in both human IPF disease and murine bleomycin-induced lung fibrosis.

Syndecan-1 overexpression is pro-fibrotic

The similarities in syndecan-1 overexpression between murine and human lung fibrosis suggest a conserved effect in lung fibrosis across species. Therefore, we injured wild-type (WT) and syndecan-1 deficient (Sdc1−/−) mice with bleomycin to determine the functional role of syndecan-1 during lung fibrosis. No appreciable difference are found in the uninjured lungs of WT and Sdc1−/− mice (Supplemental Figure 1) (38). WT mice exhibited significantly higher mortality (Figure 2E) and histologically developed more fibrosis in comparison to Sdc1−/− mice (Figure 2F-G).
Moreover, WT mice had higher lung collagen content (Figure 2H) and profibrotic gene expression (Supplemental Figure 2). Finally, we demonstrated syndecan-1 promotes fibroproliferation and expansion of the fibroblast compartment with more immunostaining of ASMA in lung histology from bleomycin-injured WT compared to Sdc1$^{-/}$ mice (Supplemental Figure 3). Additionally, scRNAseq analysis of fibroblasts in WT and Sdc1$^{-/}$ bleomycin-injured lungs demonstrated a 1.81-fold increased expression of Mki67 suggesting more proliferation of fibroblasts in WT compared to Sdc1$^{-/}$ conditions (FDR<0.05). These findings clarify the significance of syndecan-1 overexpression and implicate it in the pathogenesis of fibrosis in mouse and man.

Syndecan-1 promotes alveolar epithelial reprogramming toward a fibroproliferative phenotype

ATII cells, and more importantly their dysfunction, are an important driver of lung fibrosis (11-13). We postulated that aberrant syndecan-1 expression by ATII cells facilitates alterations to the phenotypes of these critical lung progenitors during lung fibrosis. Cultured cells can have altered behavior so we employed scRNAseq transcriptomic analysis of cells isolated from the lungs of control and bleomycin-injured WT and Sdc1$^{-/}$ mice to understand how syndecan-1 controlled ATII behavior in vivo. Visualization by t-SNE plots revealed WT and Sdc1$^{-/}$ ATII cells clustered together based on exposure condition (Figure 3A). Moreover, a shift of ATII cell populations was seen following bleomycin treatment in both genotypes indicating a strong transcriptional signal elicted by injury. However, when we evaluated the profile of differentially expressed genes based on the interaction between genotype and treatment, a different picture emerged wherein Sdc1$^{-/}$ ATII cells from bleomycin injured mice displayed transcriptional patterns more similar to untreated conditions (Figure 3B).

To further understand how these changes correspond to differences in cellular phenotype, we performed functional enrichment analysis using the KEGG database on differentially expressed genes between WT and Sdc1$^{-/}$ ATII cells. In contrast to downregulated genes, a
distinctive enrichment pattern emerged with a preponderance of profibrotic signaling pathways among genes significantly upregulated after bleomycin injury in WT ATII cells (Figure 3C and Supplemental Table 1). Additionally, a number of profibrotic mediators are released by the lung epithelium in IPF (12), and we found that most of these genes were significantly upregulated in WT ATII cells after bleomycin injury suggesting a dependence on syndecan-1 in their expression during fibrosis (Figure 3D).

These data suggest that syndecan-1 promotes ATII plasticity toward a fibrotic phenotype. To identify the regulatory signals modulated by syndecan-1 in mediating this effect, we performed causal network analysis using the set of differentially expressed genes following bleomycin injury between genotypes (49). This unbiased approach allows for statistical identification of upstream regulators as well as downstream consequences inferred from the overall changes found in the transcriptional responses. Interestingly, TGFβ was one of the most significant upstream regulators identified by this analysis (p < 10^-60; Figure 3E) further supporting a role for syndecan-1 in promoting fibrogenesis. In addition, gene product network analysis using TGFB as a seed constructed an expansive interactome with relationships to many differentially expressed genes, which had a predominance with higher expression in the WT condition (Supplemental Figure 4).

These results implicate TGFβ signaling as a dominate player in syndecan-1-dependent reshaping of ATII behavior during lung fibrosis.

We noted that β-catenin (CTNNB1) was a major downstream consequence identified in the causal network analysis (Figure 3E). Reactivation of the developmental Wnt/β-catenin pathway contributes to ATII cell reprogramming and development of lung fibrosis (18-22, 48, 50). Therefore, we performed a network analysis using β-catenin as a seed and identified an extensive array of differentially expressed genes that interacted with the Wnt/β-catenin signaling pathway (Figure 3F and Supplemental Figure 5). Additionally, most of these genes had higher expression in WT compared to Sdc1^-/- ATII cells indicating that syndecan-1 overexpression augments Wnt/β-catenin signaling in ATII cells during lung fibrosis. Cumulatively, our data support
the concept that syndecan-1 overexpression promotes lung fibrosis by facilitating a pathologic reprogramming of ATII cells in vivo through modulation of fibrogenic pathways such as TGFβ and Wnt/β-catenin.

**EVs isolated from fibrotic lungs promote lung fibrosis**

Recent studies have found that EVs carry pro-fibrotic mediators that have fibrogenic consequences in vitro (34-37). However, no studies to date have determined the in vivo effects of EVs in lung fibrosis. Because cultured cells lose the context of a diseased microenvironment (e.g., matrix, other cells, microbiome, etc), we chose to isolate EVs from the airspaces of fibrotic lungs (henceforth described as fibrotic EVs). EVs isolated from the airspaces originated primarily from epithelial cells, which is consistent with published results (Supplemental Figure 6) (29, 51). These fibrotic EVs were re-instilled into mice to test the functional consequences in lung fibrosis in vivo. No obvious effect on lung fibrosis was observed when fibrotic EVs isolated from bleomycin-injured mice were given to healthy mice (unpublished data). In contrast, when WT mice were injured with a low dose of bleomycin that induced only mild fibrosis, fibrotic EVs augmented lung fibrosis (Figure 4A-C). Similarly, mice treated with low-dose bleomycin and instilled with fibrotic EVs isolated from IPF lungs had less survival and trended toward more lung fibrosis in comparison to control EVs (Supplemental Figure 7). These results demonstrate that EVs in fibrotic lungs have the capacity to augment fibroproliferative signals.

To examine how fibrotic EVs functionally alter cellular signals, we evaluated murine lung epithelial cells treated with fibrotic EVs from bleomycin-injured lungs by RNAseq to identify the transcriptomic consequences on the cellular phenotype. A distinct change in the transcriptional architecture occurred with fibrotic EV treatment (Figure 4D and Supplemental Table 2). To understand the meaning of these changes, KEGG pathway enrichment analysis was performed using highly significantly upregulated and downregulated genes in fibrotic EV treated cells compared to control (Supplemental Table 2). Many of the significant pathways identified in the
KEGG analysis of upregulated genes mediate fibroproliferation (Figure 4E). Notably, TGFβ and Wnt signaling were identified indicating fibrotic EVs can augment the signaling adapters of these important fibrogenic pathways. Furthermore, we treated cells with fibrotic EVs from IPF lungs and demonstrated an upregulation of Wnt/β-catenin signaling, which provides functional data supporting the concept that EVs promote profibrotic signaling (Supplemental Figure 8).

**Syndecan-1 alters EV cargo to promote profibrotic reprogramming of epithelial cells**

After establishing that fibrotic EVs can modulate fibroproliferation, our next step was to determine if syndecan-1 overexpression facilitated the lung fibrotic phenotype through alterations in EV function. We incubated lung epithelial cells with fibrotic EVs from bleomycin-injured WT and Sdc1−/− mice to identify the syndecan-1-dependent transcriptomic changes in response to treatment (Figure 5A and Supplemental Table 3). KEGG pathway analysis of the differentially expressed genes between WT and Sdc1−/− fibrotic EV treated cells revealed several significantly enriched pathways (Figure 5B and Supplemental Table 3). We noted a high degree of similarity in these pathways when compared to the significant KEGG pathways determined from the transcriptomic differences in ATII cells from IPF and normal lungs (using the scRNAseq dataset from Reyfman et al. (48); Supplemental Table 3). Indeed, 6 of 11 KEGG pathways (FDR<0.0001) were identical (Figure 5B) suggesting that syndecan-1 regulates the ability of fibrotic EVs to alter epithelial cell plasticity similar to the changes found in IPF.

To further investigate syndecan-1 dependent effects on the ability of fibrotic EV to control cellular behavior, we evaluated individual genes that were highly significant in their differential expression between cells treated with WT and Sdc1−/− fibrotic EVs (Figure 5C and Supplemental Table 3). Interestingly, Muc5b was the most significant gene identified in our analysis with more expression in WT fibrotic EV treated conditions. Considering MUC5B is highly expressed in IPF lungs, and a promoter variant is the most predictive risk factor for the IPF (52), identification of this gene provide additional support for the importance of syndecan-1 in modulating the fibrogenic
properties of fibrotic EVs. Furthermore, several other genes were identified to be upregulated in the WT fibrotic EV condition that aligned with important fibrogenic pathways (e.g., TGFβ and Wnt) whereas cell cycle genes decreased suggesting impairment to cell proliferation. Together, these results indicate syndecan-1 facilitates the ability of fibrotic EVs to reprogram lung epithelial cells toward a profibrotic phenotype.

**Syndecan-1 controls packaging of anti-fibrotic microRNAs into EVs**

MicroRNAs (miRNAs) are important mediators of cellular signaling networks that can have distal effects via EV delivery (53). We have previously shown that syndecan-1 regulates miRNA packaging in EVs in lung cancer (25). Therefore, we postulated that syndecan-1 modulates cellular phenotypes during lung fibrogenesis by altering miRNA profiles in EVs. To test this hypothesis, we first performed miRNA-sequencing of fibrotic EVs isolated from bleomycin-injured WT and Sdc1−/− mice. We found that in comparison to the Sdc1−/− condition, fibrotic EVs from WT mice had significantly lower amounts of 5 miRNAs (miR-144-3p, miR-142a-3p, miR-142b, miR-503-3p, and miR-34b-5p) (Figure 6A). Because miR-142b does not have a human homolog, we excluded this miRNA from further analysis. Moreover, we found that miR-144-3p and miR-142-3p (the human homolog of murine miR-142a-3p) had a similar trend of decreased EV levels in IPF versus control (which is analogous to WT versus Sdc1−/− conditions, respectively) (Supplemental Figure 9).

We proceeded to identify the targets of these miRNAs in order to understand the effect of their changes in EV levels. Muc5b was the most significantly upregulated gene in lung epithelial cells treated with WT fibrotic EVs (Figure 5C and Supplemental Table 3). We found that Muc5b contained a miRNA-34-5p seed sequence in its 3'UTR region (Figure 6B). Furthermore, miR-34b-5p mimics reduced MUC5B protein expression (Figure 6C) and targeted the Muc5b 3'UTR in a dual-luciferase reporter assay (Figure 6D) confirming Muc5b as a target of miR-34b-5p. We evaluated the Muc5b mRNA levels and did not detect its presence in WT and Sdc1−/− EVs.
(unpublished data). Altogether, these findings indicate that the difference seen in Muc5b expression after EV treatment (Figure 5C and Supplemental Table 3) was due to miRNA suppression and not direct transfer of the Muc5b transcript.

Our data consistently pointed to syndecan-1-dependent augmentation of TGFβ signaling so we determined potential miRNA targets in this critical pro-fibrotic pathway. We found Tgfbr1 contained several miR-144-3p seed sequences in the 3’UTR region (Figure 6E). Transfection of cells with miR-144-3p mimics significantly downregulated TGFBR1 (Figure 6F) and could also suppress the Tgfbr1 3’UTR reporter (Figure 6G). For the differentially packaged miRNAs in EVs (Figure 6A), we mined available literature and curated all the miRNA targets that were identified experimentally by either 3’UTR suppression assays or pull-down of miRNA:mRNA complexes (e.g., HITS-CLIP (54)). These miRNAs suppressed numerous TGFβ signaling adaptors that spanned across both canonical and non-canonical pathways (Figure 6H and Supplemental Table 4). In addition to finding an extensive network of suppression, several miRNAs had identical targets (e.g., TGFBR1, SMAD4), which would allow synergistic regulation of these specific adaptors. Finally, we found that miR-144-3p and miR-142-3p could suppress TGFβ signal transduction in cells stimulated with TGF-β1 (Figure 6I and Supplemental Figure 10). These results functionally demonstrate that specific EV cargo, the loading of which are syndecan-1 dependent, can dampen TGFβ signaling and serve as a mechanism of regulating lung fibrosis.

In addition to TGFβ signaling, our results consistently identified alterations in Wnt signaling. Therefore, we constructed a miRNA regulatory network of experimentally validated targets that encompassed key components of the Wnt signaling pathway (Figure 6J and Supplemental Table 4). Further, we evaluated the ability of these miRNAs to suppress β-catenin signaling and observed that they effectively governed activation of the Wnt/β-catenin canonical pathway (Figure 6K). Moreover, we also found these miRNAs regulated other processes that mediate pro-fibrotic signals such as cellular senescence, ER stress, and Notch signaling (Supplemental Figure 11 and Supplemental Table 4). These findings support a general model
by which EVs, through miRNA cargo, exert an overarching regulatory control of multiple pro-fibrotic pathways in fibrogenic diseases such as IPF.

**Syndecan-1 regulation of EV cargo promotes lung fibrosis**

To elucidate the syndecan-1 dependent effects on the ability of fibrotic EVs in modulating lung fibrosis *in vivo*, we performed EV transfer experiments. Following bleomycin lung injury of WT mice, fibrotic EVs from the BAL of WT or Sdc1−/− conditions were delivered intratracheally. We found that mice treated with Sdc1−/− EVs had histologic evidence of reduced lung fibrosis compared to those receiving WT EVs (*Figure 7A-B*). Whole lung collagen content was also significantly reduced in Sdc1−/− EV treated mice as measured by hydroxyproline assay (*Figure 7C*). In contrast to the results with low dose bleomycin-injured mice (*Figure 4A-C*), fibrotic WT EVs did not augment fibrosis compared to saline control, which we attribute to the near maximal achievable fibrosis from the higher dose of bleomycin used in these experiments. These studies demonstrate that syndecan-1 has the capacity to drive lung fibrosis *in vivo* through the differential regulation of EV cargo.
Discussion

Reprogramming of the alveolar epithelium is an early event in a multistep process that culminates in the end-stage fibrosis of IPF (13). Our study provides important new information by revealing a syndecan-1-dependent mechanism where EVs modulate multiple signaling pathways that control epithelial plasticity and the development of lung fibrosis. Indeed, we demonstrate EVs from fibrotic lungs can augment profibrotic pathways and lung fibrosis. Moreover, syndecan-1 participates in this process by regulating anti-fibrotic microRNA cargo within the EVs. Interestingly, our data consistently showed a strong predilection for syndecan-1 in governing TGFβ and Wnt signaling among other important developmental pathways that are reactivated during fibrosis. Changes in the cellular response to biological signals is the basis of epithelial reprogramming, and our work builds upon this framework by providing evidence for syndecan-1-dependent EV regulation of pro-fibrotic signals that govern epithelial plasticity in lung fibrosis.

Having pleiotropic effects, TGFβ is a key pro-fibrotic cytokine that has well-known effects for stimulating extracellular matrix deposition (55, 56). Although activation of TGFβ is a major checkpoint for its function, our data suggest that EVs can modulate this pathway through delivery of microRNAs that suppress signaling adaptors. Not only could the constellation of microRNA targets affect the overall magnitude of response to the receptor-ligand interaction, but a shift in the overall cellular response between canonical (i.e., SMAD) and non-canonical (i.e., PI3K, p38, ERK) signaling could occur depending on the preferential suppression of signaling adaptors within each downstream signaling pathway. The same concept can be true for any of the other pathways (e.g., Wnt) that are regulated by the altered microRNAs and brings into focus how changes to the EV cargo could have dramatic effects on cellular phenotypes and lead to the epithelial reprogramming seen in lung fibrosis.

Syndecan-1 can regulate Wnt/β-catenin signaling in development and tumorigenesis by presenting Wnt ligands to the cognate receptor (57-59). This study provides a new level of understanding of how syndecan-1 controls Wnt signaling and the pathological consequences
associated with disturbances in this pathway. Wnt/β-catenin signaling, an important developmental pathway, is reactivated and pro-fibrotic in lung fibrosis (19-21). Furthermore, recent studies using scRNAseq approaches have reinforced the importance of aberrant Wnt activation in the ATII cells within the fibrotic lungs (48, 50). Wnt/β-catenin signaling is dysregulated in the aged lung and promotes senescence, and a pathogenic feature of reprogrammed fibrotic lung epithelial cells is cellular senescence (8, 9, 48, 60, 61). Altogether, these findings suggest that aberrant signaling through this pathway can promote lung fibrosis via initiation of cellular senescence. Our work further adds to this concept by demonstrating that EVs have regulatory roles by directly targeting cellular senescence and/or indirectly though Wnt/β-catenin signaling.

Although our results indicate syndecan-1 has the strongest effect on TGF and Wnt signaling, we also found effects on the unfolded protein response and developmental signals such as Notch and hedgehog, which are functional participants in pathogenic lung fibroproliferation (22). Moreover, Muc5b was identified as the most significant upregulated gene by an unbiased transcriptomic evaluation of cells treated with WT compared to Sdc1- EVs. This result is intriguing when considering a common promoter variant of MUC5B (rs35705950) is the strongest risk factor for the development of IPF (52). In fact, rs35705950 also is a risk factor for the development of lung fibrosis in patients with interstitial lung abnormalities and rheumatoid arthritis (62, 63). IPF patients have increased MUC5B expression independent of genetic risks (52), and increased Muc5b in the distal airways appears to impair mucociliary clearance as the mechanism of promoting fibrosis (64). Our findings uncover another regulatory step (i.e., EVs) governing its expression in fibrotic lungs.

The effects on profibrotic signaling pathways are mediated by microRNAs that are specifically packaged by syndecan-1 dependent mechanisms within EVs. By compiling the targets of these microRNAs (miR-144-3p, miR-142-3p, miR-34b-5p, miR-503-5p), we were able to demonstrate broad effects on multiple elements within each signaling pathway. The power of microRNAs to control signaling networks are their ability to simultaneously suppress multiple
targets (65). Indeed, most studies, ours included, typically find modest changes of any given microRNA. In contrast, target identification studies typically transfecct mimetics in vitro resulting in hundreds-fold overexpression of the microRNA and demonstrating regulation of a signaling pathway. These studies are necessary for validation of microRNA targets, but this level of overexpression is rarely seen in vivo and likely does not reflect the true mechanism of action (i.e., through profound suppression of a singular target). Instead, our findings build a model by which microRNAs govern cellular signaling through simultaneous suppression of multiple components of a given pathway (66-69). As such, modest changes in multiple signaling adaptors can synergy to modulate signal transduction. Moreover, pathological alterations in several microRNAs that have overlapping targets will further magnify this effect (70). The implication of our findings suggests that in contrast to a single therapeutic microRNA approach, which may have significant off-target effects, a better strategy may be to use combinatorial microRNAs that have overlapping targets thus allowing cooperative suppression of pathogenic pathways with lower dosing of any given microRNA.

The method by which microRNA cargo are packaged into EVs is still not fully elucidated. However, it is now evident that sorting of cargo into EVs is not a stochastic process, but instead, cellular mechanisms specifically facilitate loading (24). Indeed, certain microRNAs are found only in EVs but not in the cell of origin and vice versa indicating cellular processes specifically shuttle cargo into the EV (71-73). In fact, two-independent studies have identified miR-144-3p and miR-142-3p, both of which were identified in our study, to be preferentially loaded into EVs (73, 74). A general concept is that pathways mediating EV biogenesis also facilitate cargo loading (24). However, microRNAs may also be shuttled by RNA binding proteins into the EVs during biogenesis through specific binding motifs (71-73, 75-78). In this vein, our findings demonstrate that aberrant overexpression of syndecan-1 in lung fibrosis mediates its detrimental effects through the alteration of microRNA cargo loaded into EVs. It remains to be determined if this is from the ability of syndecan-1 to regulate EV biogenesis or through interactions with RNA binding
proteins. To date, efforts to target a single pathway in the pathogenesis of IPF have failed to yield successful therapies. In particular, targeting TGFβ and Wnt has been elusive and both pirfenidone and nintedanib, which are approved in IPF, have pleiotropic targets (2). These data support the concept that targeting upstream mechanisms that mediate TGFβ and Wnt effector functions may be a novel approach to drug development in IPF.

There are numerous other possible cargo that could also be adding to the fibrotic EV function (24). In fact, our data suggest that other unidentified factors exist. The miRNAs we identified had an overwhelming anti-fibrotic profile in the identified targets and do not explain the ability of fibrotic EVs to augment lung fibrosis in mice injured with low dose bleomycin. It is possible that miRNA targets that have yet to be identified could have pro-fibrotic consequences. However, the more plausible explanation is that fibrotic EVs also carry pro-fibrotic factors. In support of this, we recently reported that fibrotic EVs isolated from IPF lungs carried functional WNT5A (34), but additional protein cargo likely exist that drive fibrogenesis. Perhaps, other noncoding RNAs, such as lncRNAs, may be present that antagonize anti-fibrotic miRNAs resulting in a pro-fibrotic state. To further complicate things, lipids, which are EV constituents, can also mediate lung fibrosis (79). Future studies will require multi-omics approaches with integrated bioinformatic analyses to model the complicated interaction network of pro-fibrotic and anti-fibrotic signals in order to fully appreciate the complex means by which EVs moderate lung fibrosis.

Our results reveal a role by which EVs orchestrate the multiple profibrotic pathways that lead to pulmonary fibrosis and provide new insight into how these fibrogenic signals are modulated. Furthermore, we demonstrate that syndecan-1 has a key role in controlling the EV loading of anti-fibrotic microRNAs. Our findings support an integrated model by which EVs coordinate system-wide networks of cellular signals whose disruption leads to pathological states. Discovering mechanisms that control these global changes in biological networks could provide
novel therapeutic approaches to reverse pathological changes found in complex diseases such as lung fibrosis.
Methods

Cell line cultures and transfections

Mouse lung epithelial cells (MLE-12; originally provided by William Altemeier, MD, University of Washington), human bronchial epithelial cells (16HBE14o-; originally provided by Steve White, MD, University of Chicago), and human embryonic kidney cells (HEK293; ATCC) were propagated and maintained in 1X DMEM (Corning, Salt Lake City, UT) media supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin-streptomycin (Corning) and cultured in 5% CO₂ at 37°C. For EV treatment, EVs of a ratio of 10² particle per cell was used for all cell culture assays. Transfection of miRNAs and co-transfection of miRNAs and DNA plasmids were performed using lipofectamine RNAiMAX and lipofectamine 2000 (Life Technologies, Carlsbad, CA), respectively, per manufacture’s protocol.

Animals

C57BL/6 wild-type (WT) and syndecan-1 null (Sdc1⁻/⁻) age- and sex-matched at 8- to 12-week-old mice were maintained in a specific pathogen-free environment. All mice were from breeding of in-house colonies. For bleomycin lung injury experiment, mice were anesthetized by isoflurane inhalation, and bleomycin (0.25 – 0.75 units/kg, APP Pharmaceuticals, Schaumburg, IL) or sterile DPBS was intratracheally administered once. At day 21 after injury, bronchoalveolar lavage fluid (BALF) was collected for EV isolation, and lungs were harvested for histology, protein, RNA, and hydroxyproline assays. For all EV treatment experiments, EVs at 1x10¹⁰ particles diluted in 50 µL of sterile DPBS were administered intratracheally on every other day beginning at day 7 following bleomycin injury for a total of 6 doses.

Protein assays, histology, and immunostaining
Proteins were quantitated using bicinchoninic acid (BCA) protein assay (Pierce/Thermo Scientific) per manufacturer’s instruction. Western blot, dot blot and hydroxyproline assays were performed as previously published (43, 66).

Paraffin-embedded of 10% normal formalin buffer fixed human and mouse lung tissues were sectioned at 5-μm thickness and processed for histology studies. Hematoxylin and Eosin (H&E), Masson-Trichrome, Picro-Sirius red, and immunostaining were performed according to laboratory protocols as previously described (66, 80). All antibody clones are listed in Supplemental Table 5.

RNA isolation, quantitative reverse transcriptase, and polymerase chain reactions

TRIzol reagent (ThermoFisher Life Technology Inc) was used to isolate RNA from animal lung tissue and mammalian cells as previously described (66). Qiagen miRNeasy micro kit (Qiagen, Valencia, CA) was used for small RNA isolation and purification. All mRNA primers were purchased from Integrated DNA Technology (IDT) with detailed sequences provided in Supplemental Table 5. All miRNA primers were purchased from Qiagen. Quantitative PCR was performed using Applied Biosystems ViiA 7 Real-Time PCR (ThermoFisher Scientific, Canoga Park, CA).

Extracellular vesicle isolation and quantitation

EVs were isolated by ultrafiltration centrifugation, quantified by Nanoparticle Tracking Analysis (Nanosight 300; Malvern Panalytical, Westborough MA; Supplemental Figure 4), and further characterized by flow cytometry and western blotting as previously described (81), which demonstrated the EVs to be comprised largely by exosomes. In most instances, EVs were immediately processed for in vivo and in vitro applications. Excess EVs were aliquoted and stored in -80°C for future uses.
**Single cell RNA sequencing (scRNAseq)**

In scRNAseq experiments, freshly harvested WT and Sdc1\(^{-/-}\) mouse lungs (control and bleomycin treated) were enzymatically digested with elastase (Worthington Biochemical Corporation, Lakewood, NJ), and mechanically disrupted to generate single cell suspension per previously published protocol (50). Each mouse lung cells were hashed with mouse cell hashing antibodies (Totalseq\(^{TM}\), BioLegend, San Diego, CA) before pooling and sorting. Viable lung cell suspension (DAPI\(^{-}\)) was flow sorted for lung immune cells (CD45\(^{+}\)), endothelial cells (CD31\(^{+}\)), epithelial cells (CD326\(^{+}\)) and stromal cells (CD45\(^{-}\)CD31\(^{-}\)CD326\(^{-}\)). Equal proportions of all four cell types were pooled and subjected for single cell capture. Single cell capture and library construction using 10x Genomics Chromium system and 3’ library & gel bead kit v2 (10x Genomics, Pleasanton, CA) followed standard vendor guided protocol (82). Sequencing was performed using Illumina Novaseq 6000 (S2) system (Illumina, San Diego, CA) at UCLA Technology Center for Genomics & Bioinformatics (TCGB).

**RNAseq data analysis**

The detailed information for miRNA sequencing and data analysis was previously described by our group (25). The dataset of EV-miRNAs can be viewed through Gene Expression Omnibus (GEO) database with accession number GSE134948. In bulk mRNA sequencing data, Tophat2 (2.0.7) was applied to align sequencing reads to the reference mouse genome (GRCm38/mm10) (83). The relative gene expression RPKM and read counts were estimated using SAMMate (2.7.4) and Ensembl database (Mus Musculus.GRCm38.82) (84). Protein coding genes with at least 2 RPKM on average were used to perform the differential gene expression analysis using DESeq2 (85). To control for multiple testing, the \(p\)-values of multiple tests were adjusted using Benjamini-Hochberg’s method (86), and the significance level was designated as \(FDR < 0.01\). The mRNA dataset can be viewed through Gene Expression Omnibus (GEO) database with accession number GSE134741).
In scRNAseq analysis, CellRanger v3.0.2 software was used with the default settings for
de-multiplexing, aligning reads with STAR software to mm10 mouse reference genome, and
counting unique molecular identifiers (UMIs). Cell hashing data were de-multiplexed using CITE-
seq Count 1.4.1 (87). Downstream analysis was performed using the Seurat v2.3 R package (88)
and Partek Flow software (Partek, St. Louis, MO). Canonical correlation analysis (CCA) was
applied to integrate and combining data sets for unsupervised clustering. t-Distributed Stochastic
Neighbor Embedding (t-SNE) method was used for visualization of unsupervised clustering.
Differentially expressed genes between treatment group and control groups of each cell types
were identified using Seurat package, and significant differences were designated as an adjusted
\( p < 0.01 \). The dataset can be viewed through Gene Expression Omnibus (GEO) database with
accession number GSE131800).

For the bleomycin injury model in WT and Sdc1−/− ATII cells, ANOVA analysis was
performed to identify genes differentially expressed due to genotype by exposure (G x E)
interaction. Adjusted \( P \)-value (Benjamini-Hochberg) < 0.01 was used for significance threshold.
Upstream regulator and causal network analysis (Ingenuity software, Qiagen Bioinformatics,
Redwood City, CA) was applied to identify known transcriptional regulators by comparing the
direction of change observed in our differentially expressed genes (G x E interaction) and
expected effects based on prior knowledge (49). Functional enrichment analysis using KEGG
pathways were performed using Webgestaldt as previously described (89).

**Luciferase reporter assay**

The 3’UTR binding site of Tgfbr1 and Muc5b was functionally assessed using methods as
previously described (66).

**Statistics**
One-way and two-way ANOVA analysis was used for multiple comparisons. Student’s $t$-test (2-tailed) was used for two-group analysis. Survival comparison was performed with the log-rank and Gehan-Wilcoxon tests. All analysis was performed in GraphPad Prism 7.0. Results were presented as the mean ± SEM. $P$-value < 0.05 was considered statistical significance.

**Study Approvals**

Human lung tissue was collected at Cedars-Sinai Medical Center (CSMC; Los Angeles, CA) in accordance with guidelines and with approval from the CSMC Institutional Board Review Committee. Animal experiments were conducted in accordance with and approved by the Institution Animal Care and Use Committee (IACUC) at CSMC.
Author Contributions

TP, CY, LG, SAB, RB, DJ, PWN, CH, TJA, MK, BRS, and PC contributed to conceptual design of all experiments. TP, CY, LG, RB, FAV, SAB performed experiments. TP, CY, LG, RB, SAB, ND, MK, SAG, PC analyzed data. DH, CME, TX, DJ, TJA, CH, OB, MK, and BRS provided material and technical assistance. TP, CY, ND, SAB, PWN, MK, SAG, and PC were involved in manuscript writing.
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References


activator cAMP-response element-binding protein (CREB)-binding protein (CBP).


42. Gill SE, Nadler ST, Li Q, Frevert CW, Park PW, Chen P, et al. Shedding of Syndecan-1/CXCL1 Complexes by Matrix Metalloproteinase 7 Functions as an


Figure 1. Syndecan-1 is overexpressed by alveolar type II (ATII) epithelial cells in IPF lungs. (A) Transcriptomic data was derived from the Lung Genomic Research Consortium (LGRC) database (http://www.lung-genomics.org) was analyzed for syndecan-1 expression in the lungs from normal patients and those with interstitial lung disease. *p<0.05 by Student’s t-test. (B-C) Lung explants from normal and IPF patients were processed for (B) syndecan-1 immunohistochemical staining (brown); Scalebar = 200 µm and (C) immunofluorescent staining for epithelial cells (CDH1; green), myofibroblasts (ACTA2, green) and syndecan-1 (SDC1; red); Scalebar = 100 µm. (D-E) We analyzed a published whole lung scRNAseq dataset of control
(n=8) and IPF (n=8) lungs (48). (D) Using the University of California Santa Cruz (UCSC) cell browser (https://www.nupulmonary.org/resources/), cell populations were displayed in a t-SNE plots. The IPF ATII cell cluster was outlined by a solid line (bottom panel). (E) Syndecan-1 expression was determined in ATII cells in control and IPF lungs and displayed in a violin plot. Horizontal red dotted line is the mean value.
Figure 2. Syndecan-1 overexpression by the lung epithelium promotes fibrosis. WT and Sdc1<sup>-/-</sup> mice were injured with bleomycin (0.75 unit/kg) and sacrificed 21 days after injury. Lungs from WT mice were processed for (A) syndecan-1 immunohistochemical staining (brown); Scalebar = 200 µm and (B) immunofluorescent staining for epithelial cells (CDH1; green), myofibroblasts (ACTA2, green) and syndecan-1 (SDC1; red); Scalebar = 100 µm. (C) Lungs from WT control (n=3) and bleomycin-injured (n=3) mice were processed for scRNAseq, and t-SNE plots were generated by the Loupe Cell Browser (10x Genomics). Syndecan-1 expression in alveolar type II (ATII) cells in bleomycin-injured condition is outlined by a solid line. (D) Syndecan-1 expression was determined in ATII cells in control and bleomycin-injured lungs and displayed in a violin plot. Horizontal red dotted line is the mean value. (E) Survival of bleomycin-injured WT and Sdc1<sup>-/-</sup> mice (n=37 in each group); *p<0.05 by Log-rank (Mantel-Cox) survival analysis. (F) H&E staining and (G) Masson's trichrome staining of bleomycin-injured WT and Sdc1<sup>-/-</sup> mouse lungs; Scalebar = 200 µm. (H) Hydroxyproline content in lungs from bleomycin-injured WT and Sdc1<sup>-/-</sup> mouse lungs (n=8 in each group). *p<0.0001 by two-way ANOVA.
Figure 3. Syndecan-1 regulates alveolar epithelial reprogramming by facilitating TGFβ and Wnt signaling. Uninjured or bleomycin-injured (0.75 unit/kg; 21 days) WT and Sdc1−/− mice (n=3 per condition) were sacrificed and processed for scRNAseq of cells isolated from dissociated lungs. (A) t-SNE plots of alveolar type II (ATII) cells were generated by the Loupe Cell Browser (10x Genomics). (B) Hierarchical cluster analysis of differentially expressed genes (genotype x condition) in ATII cells between control and bleomycin-injured WT and Sdc1−/− mice. (C) Using genes significantly upregulated (FDR<0.01) in ATII cells of bleomycin-injured WT lungs relative to Sdc1−/− lungs, a KEGG pathway analysis was performed to determine the significantly enriched functional pathways represented by the geneset. Bars indicated number of genes in the pathway.
and color indicates FDR level of the KEGG analysis. (D) Heatmap of the expression (transcript counts) of epithelial-derived pro-fibrotic mediators that were significantly different in scRNAseq analysis of ATII cells from WT compared to Sdc1\(^{-}\) mice. (E) Cluster network analysis using Ingenuity Pathway Analysis of differentially expressed genes between ATII cells in WT and Sdc1\(^{-}\) mice identified TGFβ signaling as a highly significant upstream regulator \((p<10^{-60})\). (F) Wnt signaling interaction network using β-catenin (Ctnnb1) as a seed to visualize the connectivity of differentially expressed genes in ATII cells between WT and Sdc1\(^{-}\) mice. The majority of genes interacting with Wnt signaling were upregulated in WT conditions. Please refer to Supplemental Figure 5 for an annotated version with the gene symbols.
Figure 4. EVs within fibrotic lungs have pro-fibrotic properties. (A-C) WT mice were injured with low dose bleomycin (0.25 units/kg) and then given the respective treatments as indicated. Representative images of (A) H&E staining and (B) Sirius red staining (Scalebar = 200 µm) on day 21 after bleomycin. (C) Hydroxyproline content (n=6-8 in each group) in the lungs after bleomycin treatment *p<0.05; **p<0.005; ***p<0.0005 by one-way ANOVA analysis. (D-E) MLE-12 cells were treated with control and fibrotic EVs and processed for RNAseq. (D) Heatmap of differentially expressed genes in MLE-12 cells treated with fibrotic EVs compared to control. (E) Pro-fibrotic pathways identified by KEGG pathway analysis of upregulated mouse lung epithelial cells in fibrotic-EV treated condition compared to control. Bars indicated number of genes in the pathway and color indicates FDR level. Refer to Supplemental Table 2 for entire gene list and KEGG pathway analysis.
Figure 5. Syndecan-1 alters EV cargo to promote reprogramming of epithelial cells. MLE-12 cells were treated with fibrotic EVs isolated from WT and Sdc1−/− mice lungs injured with bleomycin. Cells were subsequently processed for RNAseq. (A) Heatmap of differentially expressed genes between MLE-12 cells treated with fibrotic EVs from bleomycin-injured WT and Sdc1−/− mice. (B) KEGG pathway analysis of differentially expressed genes in WT and Sdc1−/− fibrotic EV treated cells. Bars indicated number of genes in the pathway and color indicates FDR level (only highly significant pathways with FDR<0.0001 are presented). Furthermore, KEGG pathway analysis was performed for differentially expressed genes in alveolar type II (ATII) cells using a published dataset from a scRNAseq evaluation of IPF versus control lungs (48). The hashed filling pattern indicates the identical KEGG pathway identified between analysis of the two scRNAseq datasets (EV treated lung epithelial cells and ATII cells from IPF patients). Please refer to Supplemental Table 3 for the complete KEGG analysis and gene lists. (C) Volcano plot of the FDR level versus magnitude of differentially expressed genes in WT and Sdc1−/− fibrotic EV treated cells. Highly significant genes (FDR>0.0001) pertinent to lung fibrosis were identified and notated in the graph and color-coded into groups consistent with their effect.
Figure 6. Syndecan-1 controls packaging of anti-fibrotic microRNAs into EVs. WT and Sdc1-/- mice were injured with bleomycin (0.75 unit/kg) and sacrificed 21 days after injury. Fibrotic EVs were collected from the airspaces, and miRNAs were isolated for RNAseq. (A) Analysis of the miRNA levels within fibrotic EVs from bleomycin-injured WT and Sdc1-/-mice identified significant differences in 5 miRNAs. (B) Predicted miR-34b-5p binding site in the Muc5b 3'UTR region was identified with microrna.org. (C) Muc5b dot blot of MLE-12 cells transfected with control and miR-34b-5p mimics. (D) Luciferase activity of HEK cells transfected with a reporter plasmid containing
a chimeric firefly luciferase with the *Muc5b* 3’ UTR and either a control or miR-34b-5p. (E) Predicted miR-144-3p binding site in the *Tgfr1* 3’UTR region was identified with microrna.org. (F) TGFBR1 immunoblot of MLE-12 cells transfected with the control or miR-144-3p mimics. (G) Luciferase activity of HEK cells transfected with a reporter plasmid containing a chimeric firefly luciferase with the *Tgfr1* 3’ UTR and either a control or miR-144-3p. (H) TGFβ signaling regulatory network of experimentally identified targets of miR-34b-5p (*blue*), miR-503-5p (*purple*), miR-144-3p (*brick-red*), and miR-142-3p (*orange*). These curated targets were experimentally validated either by UTR analysis (close circle) or with pull-down methods (dotted circle). (I) Immunoblot for pSMAD2 and SMAD2 using MLE-12 cells transfected with control or miR-144-3p mimics followed by TGFβ stimulation. (J) Wnt signaling regulatory network of experimentally identified targets (labeling identical to panel (H)). (K) Luciferase activity of HEK cells co-transfected with a TopFlash reporter plasmid and miRNAs as labeled. *p<0.05; **p<0.005; ***p<0.0005 by one-way ANOVA analysis.
Figure 7. Syndecan-1 dependence of EVs in modulating lung fibrosis. WT mice were injured with bleomycin (0.75 unit/kg) and then intratracheally instilled with fibrotic EVs isolated from bleomycin-injured WT and Sdc1−/− mice. Representative images of (A) H&E and (B) Picro-sirius Red staining, and (C) Hydroxyproline content (n=12-15 per group) of lungs recovered from WT vs Sdc1−/− fibrotic EV treated mice; Scalebar = 200 µm. *p<0.05; **p<0.005; ***p<0.0005 by one-way ANOVA analysis.