Beyond Fibroblast Heterogeneity: What Single-Cell RNA Sequencing Tells Us

Fibroblasts are the primary cell type responsible for synthesis and remodeling of the extracellular matrix (ECM) in response to injury. Targeting fibroblasts represents a promising strategy for preventing, treating, and potentially reversing fibrotic lung diseases such as idiopathic pulmonary fibrosis (IPF). Lung fibroblasts display a considerable degree of heterogeneity in both phenotype and activity. This diversity is evidenced by the divergent and specific expression of cell-surface markers such as Thy-1, and differences in the cytoskeletal composition, lipid content, and cytokine profile of distinct fibroblast populations (1, 2). Fibroblasts isolated from the fibroblastic foci in IPF are morphologically and functionally distinct from fibroblasts isolated from normal lungs (3), suggesting that the selective expansion of specific fibroblast subsets is associated with the pathogenesis of IPF. Considerable effort has been expended to characterize lung fibroblast populations with the advent of new technologies, such as transgenic lineage tracing and single-cell RNA sequencing (RNA-seq). For example, a study using clonal cell-labeling strategies with multicolor reporters demonstrated a diversity of mesenchymal populations with different locations, patterns of migration, recruitment mechanisms, and lineage boundaries in the developing mouse lung (4). Single-cell and population-based RNA-seq identified a functionally distinct myofibrogenic mesenchymal cell type ($Axin2^{+}$) as well as a mesenchymal alveolar niche cell type ($Pdgfra^{+}Axin2^{+}$) that is critical for alveolar epithelial cell growth and self-renewal (5). Similarly, single-cell analyses revealed distinct mesenchymal niches that drive airway ($Lgr6^{+}$) and alveolar ($Lgr5^{+}$) differentiation (6). More recent transcriptomic analyses conducted at single-cell resolution identified a novel profibrotic mesenchymal population ($Pdgfrb^{hi}$) along with known mesenchymal cell subtypes in bleomycin injury-induced mouse lung fibrosis (7). Interestingly, studies of fibrosis in multiple organs with transgenic mouse reporter lines found that only a minority of collagen-producing cells coexpressed α-smooth muscle actin (α-SMA) in the fibrotic lung and kidney (8), suggesting that α-SMA is an inconsistent marker of activated fibroblasts or myofibroblasts.

In this issue of the *Journal*, Peyser and colleagues (pp. 74–85) report on single-cell RNA-seq analyses of lung fibroblast populations in normal, bleomycin-treated, and bleomycin + nintedanib–treated mice at Day 11 after bleomycin lung injury (9). Using independent computational approaches, the authors identified an activated fibroblast subset characterized by significant enrichment of 49 genes, including collagen genes (e.g., *Col5a2* and *Col1a1*) and myofibroblast signature genes (e.g., *Acta2* and *Tagln*). These 49 genes were classified into four groups: ECM, contractile genes, focal-adhesion genes, and genes involved in cell signaling. Compared with normal control fibroblasts, no single gene was specifically associated with the identified activated fibroblast population. The *Ltbp2* gene had the highest correlation to a fibroblast "activation" signature. It was expressed in 78% of activated fibroblasts, but also in 8% of nonactivated fibroblasts and 1.7% of nonfibroblasts. *Col5a2*, another gene with a strong correlation to a fibroblast activation signature, was expressed in 95.8% of activated fibroblasts, 41.6% of nonactivated fibroblasts, and 4.1% of other cell populations. The authors found that fibroblast numbers did not increase at Day 11 after bleomycin treatment as compared with saline treatment. In contrast, significant increases in the numbers of macrophages, proliferating myeloid cells, and dendritic cells were observed at Day 11. Nintedanib treatment reduced the number of activated fibroblasts and their expression of ECM genes, but did not reduce the total number of fibroblasts. Transforming growth factor β (TGF-β) signaling represents a core pathway of tissue fibrosis. This pathway mediates the fibrotic response through a number of mechanisms, including fibroblast activation. In this study, the authors failed to prove a correlation between expression of TGF-β signaling genes and fibroblast activation in bleomycin-induced mouse lung fibrosis. To investigate this further, the authors performed single-cell RNA-seq analyses on primary normal human and IPF lung fibroblasts cultured in the absence and presence of TGF-β. The results showed that normal lung fibroblasts treated by TGF-β did not cluster with IPF fibroblasts, suggesting that short-term TGF-β treatment does not induce an IPF phenotype *in vitro*. However, it is not known whether there are similarities in gene expression profiles between IPF lung fibroblasts and activated fibroblasts identified from bleomycin-treated mouse lungs. Consistent with previous findings (8), the authors found that *Acta2*, the gene encoding α-SMA protein, was expressed in only 63.6% of activated fibroblasts and 11% of nonactivated fibroblasts, again arguing against α-SMA as a single marker of fibroblast activation in lung fibrosis.

After epithelial injury, lung fibrosis is believed to progress as a consequence of dysregulated repair, in which resident cells in the lung, including epithelial cells, fibroblasts, macrophages, and endothelial cells along with myeloid cells, produce an array of cytokines and growth factors that stimulate fibroblast proliferation, differentiation, and matrix synthesis. The active form of TGF-β is a potent fibrogenic cytokine that is believed to play the most important role in the development

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of lung fibrosis. Contrary to this popular view, the work of Peyser and colleagues neither supports fibroblast proliferation as an early event in bleomycin-induced mouse lung fibrosis nor demonstrates a correlation of expression of TGF-β pathway genes with fibroblast activation. These findings suggest that, beyond expanding our knowledge of cellular identities, single-cell RNA-seq data challenge prevailing ideas about fibrosis development and progression in the lung, thus opening new paths for studying the mechanisms underlying the pathogenesis of pulmonary fibrosis.

A limitation of Peyser and colleagues's study is that the characterization of mouse lung fibroblast populations and identification of the activated fibroblast population were performed at a single time point, i.e., Day 11 after bleomycin treatment. The study could be strengthened by time-series experiments, particularly in the early stage of lung fibrosis, for a more rigorous test of the findings. Of importance, it is not known whether the activated, pathogenic fibroblast at Day 11 after bleomycin injury in a rodent resembles the pathogenic cell type in IPF, particularly considering that the former pathology resolves with time and the latter is a progressive disease (10). Additionally, it is unclear to what extent the activated fibroblast population identified in this study correlates with similar fibrogenic mesenchymal populations identified by other groups (5, 7), as well as the fibrogenic signature of human IPF fibroblasts. A comparison of transcriptional programs has shown correlations between the myofibrogenic $Axin2^{+}$ mesenchymal population and the $Pdgfrb^{hi}$ population (7). Altogether, the development of single-cell RNA-seq and computational methods provides unprecedented power for systematic investigations of cellular heterogeneity. Single-cell analyses have a great potential to help us better understand the complexity of fibrotic lung disease, which will benefit the development of more effective therapies. ■

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