REVIEW

Mechanisms of Lung Fibrosis Resolution

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Fibrogenesis involves a dynamic interplay between factors that promote the biosynthesis and deposition of extracellular matrix along with pathways that degrade the extracellular matrix and eliminate the primary effector cells. Opposing the often held perception that fibrotic tissue is permanent, animal studies and clinical data now demonstrate the highly plastic nature of organ fibrosis that can, under certain circumstances, regress. This review describes the current understanding of the mechanisms whereby the lung is known to resolve fibrosis focusing on degradation of the extracellular matrix, removal of myofibroblasts, and the role of inflammatory cells. Although there are significant gaps in understanding lung fibrosis resolution, accelerated improvements in biotechnology and bioinformatics are expected to improve the understanding of these mechanisms and have high potential to lead to novel and effective restorative therapies in the treatment not only of pulmonary fibrosis, but also of a wide-ranging spectrum of chronic disorders. (*Am J Pathol* 2016, 186: 1066–1077; <http://dx.doi.org/10.1016/j.ajpath.2016.01.018>)

Fibrogenesis in the lung involves a balance between factors that promote the biosynthesis and accumulation of extracellular matrix (ECM) components and the pathways that deactivate or eliminate the effector cells and remove the excess ECM. Ongoing basic and translational studies continue to improve our understanding of the cells, mediators, and pathways leading to the initiation and progression of fibrosis. Progress in these research efforts was highlighted by the 2014 Federal Drug Administration approval of pirfenidone and nintedanib for idiopathic pulmonary fibrosis (IPF), both of which significantly slow disease progression.^{1,2} However, novel therapies to promote the resolution of fibrosis have yet to be identified. Because most patients have a significant fibrotic burden at the time of clinical presentation, understanding the basic mechanisms mediating the lungs' capacity to resolve fibrosis is critically important, and may lead to novel therapeutic strategies to actually reverse established disease. Successful fibrosis resolution comprises identification and elimination of the fibroproliferative stimulus, clearance of transformed myofibroblasts, degradation and clearance of ECM, and reconstitution of damaged lung

epithelium and alveoli. This review will summarize current studies focused on the primary mechanisms of lung fibrosis resolution and the modifying roles of inflammatory cells that either facilitate or interfere with these processes.

Reversibility of Tissue Fibrosis

Fibrosis has traditionally been thought to be an irreversible process, the end stage of disordered wound healing, and thus refractory to therapeutic intervention beyond prevention of progressive scarring. However, a growing body of data now reveals the highly plastic nature of

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Figure 1 Conditional TGF α transgenic mice induce transgene expression following enteral administration of doxycycline (Dox). Pentachrome stains of subpleural regions in TGF α mice without Dox (A) after 42 days of Dox (B) and 42 days on Dox followed by 126 days off Dox (C). Right ventricular (RV) hypertrophy measured by comparing RV divided by left ventricle plus septal weight (RV/LV + S) at intervals on and off Dox (D). Reprinted from Hardie et al¹² with permission of the American Thoracic Society. * $P < 0.05$ versus control.

organ fibrosis that can regress in certain circumstances.³ In the kidney, established glomerulosclerosis is reversible, and the interstitial matrix can resolve.⁴ Studies in rodents demonstrate reversal of liver fibrosis in the experimental models of carbon tetrachloride or alcohol intoxication and bile duct ligation.⁵ Although fibrosis regresses in these models, it is not fully reversible in advanced disease. Prolonged damage in liver fibrosis models leads to increased acellular ECM deposition and a steep decline of potential reversibility. Factors associated with inefficient fibrolysis in the liver include advanced vascular remodeling with architectural distortion, extensive cross-linking of ECM components hindering proteolytic removal, and the disappearance of cells that digest and degrade scar tissue.⁶

Chronic infection with hepatitis C virus is a common cause of liver fibrosis, and the most compelling evidence that spontaneous resolution of fibrosis is possible in humans comes from studies examining the response to antiviral treatments. There is clear histological evidence from biopsy tissue of patients with mild-to-moderate fibrosis who are successfully treated for hepatitis C virus infection indicating resolution of the fibrosis seen in the index biopsy.⁷ Although fibrosis in the liver regresses, it is not fully reversible in patients with cirrhosis. However, even in extensive cirrhosis, the need for liver transplant is often not directly because of the fibrosis, but rather the sequelae of fibrosis causing portal hypertension, variceal bleeding, and ascites.⁷

Multiple animal models of lung fibrosis have been developed, primarily using acute injury or transgenic approaches.⁸ Although no animal model yet fully recapitulates the histological pattern seen in IPF or other

interstitial lung diseases, these models have generated important insights into the pathobiology of fibroproliferation. Fibrosis in most of these models is reversible, with complete or near complete restitution of lung architecture after the inciting fibrogenic agent is removed, such as after a single intratracheal administration of bleomycin. More durable pulmonary fibrosis is produced with intratracheal administration of high-dose amphibole asbestos fibers, intratracheal fluorescein isothiocyanate in susceptible mouse strains, and repetitive doses of systemic bleomycin.^{9–11} Similar to liver fibrosis, the degree of reversibility in the lung appears to be related to the duration or intensity of the fibrogenic trigger. Transgenic mice conditionally overexpressing transforming growth factor (TGF)- α in the lung epithelium develop a profound fibrotic burden leading to cachexia, secondary pulmonary hypertension, marked alterations in lung mechanics, and death. When the TGF- α expression is extinguished after 4 weeks of expression, there is complete resolution of histological fibrosis. However, mice exposed to longer periods of TGF- α expression show persistent subpleural fibrosis up to 126 days after discontinuation of TGF- α expression (Figure 1, A–C).¹² Despite this residual fibrosis, mice have complete or near complete recovery of physiological changes, including secondary pulmonary hypertension (Figure 1D). Similarly, a recently published model of sepsis-induced acute lung injury in baboons revealed biochemical and radiographic evidence of persistent pulmonary fibrosis in long-term survivors studied between 6 and 27 months after the acute episodes.¹³ However, only one of the six long-term survivors demonstrated static lung compliance values lower than

healthy control baboons. Together, these animal studies support the concepts that the functional impairments from even severe lung fibrosis can be recoverable and that residual fibrosis may have minimal physiological impact.

The reversibility of lung fibrosis in human disease is less clear and likely depends on the specific insult, age, sex, and other modifying conditions of the patient. Acute respiratory distress syndrome is characterized histologically, in its organizing phase, by fibroblast accumulation and deposition of ECM in the lung.¹⁴ Studies assessing long-term outcomes in acute respiratory distress syndrome survivors report persistent lung dysfunction and computed tomographic abnormalities in only subsets of subjects, suggesting significant fibrosis resolution for most patients.^{15,16}

In summary, animal models of fibrosis in the kidney, liver, and lung indicate the potential to reverse a substantial fibrotic burden and return to near predisease function after the fibrogenic insult is extinguished. The concept of fibrosis reversal in solid organs is supported in human disease, where liver cirrhosis caused by hepatitis infection demonstrates marked resolution after antiviral treatment. However, the lung lacks the regeneration capacity of the liver, and the potential to significantly reverse more extensive lung fibrosis, such as develops in advanced cystic fibrosis or IPF, is unknown.¹⁷ The emergence of new pharmacological treatments for these conditions targeting the underlying disease process, such as modulators and potentiators for cystic fibrosis, may provide insight into the extent of the lung's capacity to resolve extensive pulmonary fibrosis.

Degradation of the Extracellular Matrix

Composition of the Extracellular Matrix in Fibrosis

The ECM is a heterogeneous, highly dynamic, three-dimensional tissue providing structural support as well as spatial and temporal contextual cues to cells during embryonic development, wound repair, and tissue homeostasis.¹⁸ In the human lung, the ECM is composed primarily of type I and III collagen in approximately a 2:1 ratio, which provides tensile strength; elastin, which provides the structural framework and elasticity of the alveolar wall; and laminin and collagen IV, which constitute the bulk of the alveolar and airway basement membranes.^{18–20} This relationship changes in lung fibrosis. Studies examining ECM composition from patients with a variety of fibrotic diseases demonstrate an increase in total type I and III collagen in early lung fibrosis, with type III collagen predominant in the thickened alveolar septa and interstitium.²¹ In more established areas of scar formation, there was almost exclusive type I collagen, representing approximately 80% of the total collagen in IPF.^{21,22} Increases in other constituents of the ECM include types V, VI, and VII collagens, fibronectin, elastin, hyaluronan, and glycosaminoglycans.^{18,23,24}

Extracellular Pathways of ECM Degradation

Matrix degradation likely occurs through a multistep process that involves extracellular protease-mediated cleavage, recognition of fibrils by membrane-bound receptors, and cellular uptake with intracellular lysosomal degradation of cleaved matrix fragments.^{24,25} Several enzymatic families are known to degrade matrix in the lung through the extracellular pathway, including matrix metalloproteinases (MMPs), cysteine proteinases (cathepsins B and L), and serine proteinases (plasmin and plasminogen activator). The foremost proteolytic enzymes involved in extracellular cleavage are the MMPs. The MMPs are a family of zinc- and calcium-dependent endopeptidases produced by both connective tissue and inflammatory cells. Through recognition of specific cleavage sites, the MMPs degrade ECM, including fibrillar and nonfibrillar collagens and elastin.^{24,26} Primarily on the basis of *in vitro* degradation assays, the MMPs can be grouped according to the enzymatic substrate to include collagenases, gelatinases, metalloelastases, and stromelysins. The collagenases, which include MMP-1, MMP-8, and MMP-13, are believed to be central toward degrading fibrotic tissue owing to the high predominance of type I collagen in established areas of scar formation. Fragments of the cleaved collagen are hydrolyzed to gelatin, which is further degraded by the gelatinases, specifically MMP-2 and MMP-9. Metalloelastase (MMP-12) has potent elastin-degrading activity, and stromelysin (MMP-3) has a wide substrate profile with activity against collagens, laminin, and fibronectin.

Tissue inhibitors of metalloproteinases (TIMPs) are a family of four homologous members of soluble proteins that bind to active MMPs. The prevailing hypothesis for ECM turnover has been a balance in activity between extracellular MMP and the TIMP family. However, gene mutation studies do not support this paradigm and demonstrate contradictory roles for MMPs and TIMPs in lung fibrosis. For instance, studies in MMP-9 and MMP-12 knockout mice, administered bleomycin or other fibrogenic challenges, reveal either a profibrotic effect, an antifibrotic effect, or no effect on the development of lung fibrosis.²³ Loss of TIMPs would be expected to lead to enhanced matrix proteolysis; however, TIMP3 knockout mice demonstrated enhanced fibrosis after bleomycin associated with persistent neutrophil influx.²⁷ Mice lacking TIMP1 have similar severity of fibrosis but greater neutrophilic inflammation compared with wild-type mice after bleomycin injury.²⁸ These studies and others now demonstrate that the MMPs and TIMPs influence several cellular processes, including proliferation, survival, and inflammation, that directly or indirectly influence myofibroblast activation and ECM turnover.^{23,24,29,30}

Cross-linking of collagen and elastin are post-translational modifications of the ECM that can hinder fibrosis resolution by preventing extracellular proteolytic enzymes from accessing binding sites. One family of

Figure 2 Collagen internalization is mediated by macropinocytic, phagocytic, and endocytic pathways where fragments are ultimately degraded by the lysosome. Figure modified from McKleroy et al²⁴ with permission from the American Physiological Society. Mfge8, milk fat globule–epidermal growth factor 8; uPARAP, urokinase plasminogen activator receptor–associated protein.

enzymes mediating this cross-linking is lysyl oxidase and its homologues (lysyl oxidase–like enzymes 1 to 4).³¹ The lysyl oxidases catalyze the oxidation of primary amines, such as lysine residues into aldehydes. These aldehydes then react with other aldehydes or amines to cross-link collagen or elastin. Recent studies demonstrate that inhibition of lysyl oxidase–like-2 experimentally reduced myocardial fibrosis and also prevented and reversed bleomycin-induced lung fibrosis.^{32,33} Thus, although manipulating extracellular proteases may be premature because of the limited understanding of their precise function in ECM turnover, targeting pathways that allow better access of extracellular proteases to the ECM is promising. Monoclonal antibodies to lysyl oxidase–like-2 have been tested in phase 2 trials in IPF, and small-molecule lysyl oxidase–like-2 selective inhibitors have been developed and are expected to enter phase 1 trials for the treatment of pulmonary fibrotic disease.

Intracellular Pathways of ECM Degradation

The intracellular pathways of ECM degradation are comparatively less well studied than the extracellular degradation processes. Collagens that have been pre-cleaved by collagenases are internalized more efficiently than intact collagen, thereby allowing cells to clear and recycle matrix components.³⁴ The primary processes mediating intracellular collagen degradation include recognition and internalization of collagen fragments, where they are ultimately degraded into the lysosomal network by cysteine proteinases. Several pathways have been shown to internalize collagen fragments (Figure 2). The $\alpha_2\beta_1$ and $\alpha_3\beta_1$ bind collagen internalizing them for lysosomal degradation, and inhibition of these integrins reduces phagocytosis of collagen-coated beads.³⁵ A receptor-mediated endocytosis pathway of collagen uptake has been described for the transmembrane urokinase plasminogen activator receptor–associated protein. This

receptor, which is expressed predominantly in fibroblasts, can bind and internalize both fibrillar and nonfibrillar collagens to the lysosome for degradation before recycling to the plasma membrane.³⁶ Collagen IV and gelatin internalization were nearly ablated in urokinase plasminogen activator receptor–associated protein deficient mouse lung fibroblasts in an integrin-independent mechanism.³⁴ Compared with controls, mice deficient in urokinase plasminogen activator receptor–associated protein exhibit increased total lung hydroxyproline content 28 days after bleomycin injury than controls without alterations in inflammation, or rates of collagen synthesis.

The extracellular glycoprotein milk fat globule–epidermal growth factor 8 (Mfge8) is a soluble glycoprotein that mediates collagen uptake by binding collagen fragments for uptake by macrophages. Mice deficient in Mfge8 demonstrate greater total lung hydroxyproline content 28 days after bleomycin injury than controls. This increased fibrosis was not associated with increased inflammation, impaired apoptotic cell clearance, or changes in rates of collagen synthesis, but rather a defect in collagen turnover. Mfge8 knockout macrophages exhibited defective collagen uptake that could be rescued by recombinant Mfge8.³⁷ Mfge8 also negatively regulates inflammation and autoimmunity by facilitating the clearance of apoptotic cells.³⁸ This dual role suggests that the failure to resolve may be imprecise coordination of effectors, such as Mfge8, that bridge apoptosis and enzymatic ECM breakdown.

In summary, proteolytic degradation of the ECM is of vital importance in fibrosis resolution, but still relatively understudied. The specific extracellular proteinases essential to degrade lung ECM *in vivo* remain under investigation; and while interfering with intracellular pathways of ECM degradation, they lead to greater fibrosis experimentally. However, it remains unclear if defects in these pathways lead to progressive disease. Advances in gene deletion studies may allow future

Table 1 Factors and Mechanisms Affecting Lung Myofibroblast Dedifferentiation, Apoptosis, and Aging

Factors	Potential mechanisms	Experimental models	Reference
c-FLIP	Inhibits apoptosis via direct apoptosis inhibitor	Mouse and IPF myofibroblasts	Golan-Gersti et al ⁴⁸
FGF-1	Inhibit differentiation via blocking Smad2	Primary human fibroblasts	Ramos et al ⁴⁹
MyoD	Induces differentiation via TGF-β1	Normal human fibroblast	Hecker et al ⁴⁰
NFAs	Inhibit differentiation via PPAR-γ up-regulation	IPF/normal human fibroblasts, murine <i>in vivo</i>	Reddy et al ⁵⁰
Nox4-Nrf2	Inhibits apoptosis in aged cells via oxidant imbalance	Mouse and IPF myofibroblasts, murine <i>in vivo</i>	Hecker et al ⁵¹
Nrf2	Inhibits differentiation via antioxidative stress	IPF/normal human fibroblasts	Artaud-Macari et al ⁵²
PAI-1	Inhibits apoptosis in aged cells via TGF-β1 sensitivity	Murine fibroblasts and <i>in vivo</i>	Huang et al ⁵³
PGE2	Inhibit differentiation via FAK activation	Normal human fibroblasts	Garrison et al ⁵⁴
Rho/ROCK	Induces differentiation and survival via MKL1	IPF/normal human fibroblasts, murine <i>in vivo</i>	Zhou et al ⁴¹
SRF/MRTF	Induces differentiation and survival via XIAP and PAI-1	Normal fibroblasts, murine <i>in vivo</i>	Sisson et al ⁵⁵
Survivin	Inhibits apoptosis via direct apoptosis inhibitor	Normal human fibroblast	Horowitz et al ⁵⁶
TGF-β1	Inhibits apoptosis via PI3K signaling	IPF/normal human fibroblasts	Kulasekaran et al ⁵⁷
Thy-1	Induces differentiation if epigenetically silenced	Rat and human myofibroblasts	Sanders et al ⁵⁸
XIAP	Inhibits apoptosis via direct apoptosis inhibitor	IPF/normal human fibroblasts	Ajayi et al ⁵⁹

c-FLIP, c-Fas-like IL 1β-converting enzyme—inhibitory protein; FGF, fibroblast growth factor; IPF, idiopathic pulmonary fibrosis; MRTF, myocardin-related transcription factor; MyoD, myoblast determination protein 1; NFA, nitrated fatty acid; Nrf2, nuclear factor erythroid 2-related factor 2; PAI, plasminogen activator inhibitor; PGE, prostaglandin E; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROCK, Rho-associated kinases; SRF, serum response factor; TGF, transforming growth factor; XIAP, X-linked inhibitor of apoptosis.

studies to experimentally track production and alterations of each of the functionally relevant protease members and pathways *in vivo* during fibrosis resolution to determine their impact in spontaneous resolution.²³

Removal of Myofibroblasts in Lung Fibrosis

Impact of Myofibroblasts

Myofibroblasts are differentiated cells with features of both fibroblasts and smooth muscle cells, and are characterized by a spindle or stellate morphology with intracytoplasmic stress fibers, a contractile phenotype, and expression of α-smooth muscle actin (α-SMA).^{39,40} Myofibroblasts are essential in normal wound healing by facilitating wound closure and synthesis of ECM proteins.³ Termination of the reparative response that leads to normal wound healing includes the elimination of myofibroblasts from granulation tissue. In contrast, the persistence of myofibroblasts in injured tissues is associated with nonresolving and progressive fibrosis in the lung and other organs.^{39,41} Because myofibroblasts are the main effectors of fibrosis, successful resolution is dependent on inhibiting myofibroblast differentiation from precursor cells and eliminating active myofibroblasts. Myofibroblast differentiation is triggered by several mediators, including cytokines, growth factors, and ECM composition, as recently reviewed.³⁹ However, even if differentiation can be inhibited, persistent myofibroblasts can induce a feed-forward mechanism for maintaining the myofibroblast phenotype and inducing neighboring cells into fibrotic and dysfunctional cells through mechanotransduction-mediated up-regulation of profibrotic and prosurvival

genes.^{39,41,42} Therefore, elimination of embedded myofibroblasts is paramount to promoting resolution. There are three primary molecular mechanisms mediating myofibroblast removal: dedifferentiation, apoptosis, and senescence. A greater understanding of these mechanisms and determining how they may be manipulated has significant potential to lead to the development of novel antifibrotic therapies.

Role of Dedifferentiation in Removing Myofibroblasts

Cell dedifferentiation is the regression of a cell from a more specialized state back to an earlier developmental stage, a process that in the extreme case confers pluripotency. The mechanisms underlying cell-specific dedifferentiation have been indicated to be critical in developmental biology,⁴³ regenerative medicine,⁴⁴ and tumorigenesis.⁴⁵ Myofibroblast differentiation was previously considered irreversible. However, studies using Cre-loxP-based genetic labeling of myofibroblasts demonstrated that the myofibroblast phenotype is reversible in spontaneous recovery after experimental models of carbon tetrachloride— or alcohol-induced liver fibrosis.^{46,47} Factors and mechanisms have recently been identified that induce dedifferentiation of lung myofibroblasts *in vitro* (Table 1).^{40,41,48–59} MyoD is a master transcriptional regulator of myogenic differentiation and mediates TGF-β1–induced myofibroblast differentiation of human lung fibroblasts.^{40,60} Down-regulation of endogenous MyoD with RNA interference in myofibroblasts was associated with dedifferentiation and increased proliferation.⁴⁰

TGF-β1–stimulated normal human lung fibroblasts incubated with fibroblast growth factor-1 demonstrate reduced collagen gel contraction and a significant decrease

in the percentage of α -SMA-expressing myofibroblasts.⁴⁹ Dedifferentiation of myofibroblasts after fibroblast growth factor-1 administration was associated with reduced Smad2 phosphorylation. These findings support a direct dedifferentiation effect of fibroblast growth factor-1 by antagonizing TGF- β actions, an observation also observed in corneal myofibroblasts.⁶¹

Thy-1 is a cell surface glycoprotein present on normal lung fibroblasts but is epigenetically silenced in myofibroblasts within the fibroblastic foci of IPF via DNA hypermethylation and chromatin modifications.⁶² DNA methyltransferase inhibition and histone deacetylase inhibition restored Thy-1 expression in primary rat lung myofibroblasts and reduced α -SMA expression.⁵⁸

Prostaglandin E2 (PGE2) is an endogenously produced lipid mediator that inhibits multiple fibroblast functions, including proliferation, collagen production, and differentiation.³⁹ In fetal and adult lung fibroblasts, the addition of PGE2 reversed TGF- β 1 and endothelin-1-induced myofibroblast differentiation via inhibition of focal adhesion kinase activation.⁵⁴ This effect was not explained by apoptosis, and reintroduction of TGF- β 1 2 days after the addition of PGE2 prompted dedifferentiated fibroblasts to reexpress α -SMA, indicating redifferentiation to myofibroblasts. Furthermore, PGE2 was capable of directionally reversing more than half of the myofibroblast genes whose expression was altered by TGF- β 1, thereby demonstrating the plasticity of the myofibroblast phenotype at a transcriptomic level.⁶³ However, PGE2, when administered to mice 14 days after bleomycin, failed to provide significant therapeutic effects on lung function or fibrosis.⁶⁴

Nuclear factor erythroid 2-related factor 2 (NRF2) is regarded as a master regulator of cellular resistance to oxidants and regulates expression of genes, limiting the inflammatory and fibrotic responses.³⁹ In primary IPF and normal lung fibroblasts, siRNA activation of NRF2 by either Keap1 siRNA or sulforaphane induced myofibroblast dedifferentiation with down-regulation of α -SMA, along with reduced cell proliferation, migration, and contraction, and was associated with restored oxidant/antioxidant balance in the cells.⁵² NRF2 knockdown abolished Sulforaphane's dedifferentiation effects, suggesting that they were NRF2 mediated. However, these *in vitro* observations did not translate *in vivo* because sulforaphane administration was unsuccessful in altering bleomycin-induced pulmonary fibrosis in mice.⁵²

Endogenous nitrated fatty acids are potent agonists of peroxisome proliferator-activated receptor γ , which have been shown to exhibit antifibrotic activity in the lung through SMAD inhibition.^{65,66} Cultured IPF myofibroblasts treated with the most common nitrated fatty acids in human plasma, 10-nitro-oleic acid, increased peroxisome proliferator-activated receptor γ expression, while reversing myofibroblast differentiation, as assessed by α -SMA levels and contractility.⁵⁰ More important, intratracheal treatment of mice with 10-nitro-oleic acid for 7 successive days beginning 21 days after bleomycin injection reduced the total lung collagen content and α -SMA

protein levels. These findings are unique by supporting that nitrated fatty acids can reverse collagen deposition and myofibroblast differentiation both *in vitro* and *in vivo*.⁵⁰

Taken together, several candidate pathways have now been identified that induce dedifferentiation of lung myofibroblasts *in vitro*. Future preclinical studies will determine whether these pathways have the potential to be manipulated to induce myofibroblast dedifferentiation as a novel therapy for late-stage fibrotic disorders.

Role of Apoptosis in Removing Myofibroblasts

There are three pathways of cell death: apoptosis, autophagy, and necrosis. The particular mode of cell death may predominate depending on the cell type and the injury.⁶⁷ There is significant overlap and cross talk between different modes of cell death, and distinctions are not always clear; however, almost all of the accumulated data in lung fibrosis relate to apoptotic cell death.^{67,68}

Apoptosis is mediated by the concerted action of a set of cysteine proteases known as caspases. There are two major pathways of apoptosis, the death receptor and mitochondrial pathways, which converge to activate effector caspases, caspase-3 and caspase-7, which execute the apoptotic cell death program.^{67,68} Apoptosis has been shown to be the primary mechanism of myofibroblast elimination in granulation tissue during the resolution phase of normal wound repair.⁶⁹ Apoptosis has also been identified as a primary mechanism of myofibroblast removal in the bleomycin lung fibrosis model.^{55,70,71} An accumulating body of literature demonstrates that lung fibroblasts and myofibroblasts in both mouse fibrosis models and IPF acquire an apoptosis-resistant phenotype.^{41,48,55,59,70,71} Several mechanisms by which myofibroblasts evade apoptosis have been identified,⁷² and several common mechanistic themes have emerged. First, soluble profibrotic mediators that activate myofibroblasts, such as TGF- β 1, also promote resistance to apoptosis.⁵⁷ Second, prosurvival protein kinases, including phosphatidylinositol-3 kinases, which are activated by soluble fibrogenic mediators in normal lung fibroblasts, are expressed at higher levels in IPF fibroblastic foci.^{73,74} Third, endogenous inhibitors of apoptosis, such as Fas-like IL 1 β -converting enzyme—inhibitory protein and X-linked inhibitor of apoptosis, are induced by soluble profibrotic mediators and expressed at increased levels in fibrotic lung fibroblasts.^{48,56,59} Fourth, the composition and biomechanical properties of the ECM can modulate fibroblast resistance to apoptosis.^{41,75} Fifth, epigenetic alterations, such as methylation of the Thy-1 promoter in fibroblastic foci of IPF, have been linked to resistance to apoptosis.^{62,76}

Several recent murine studies demonstrate that pharmacological strategies to induce myofibroblast apoptosis after fibrosis were already established and can accelerate resolution.^{41,55,70,71} The histone deacetylase inhibitor

suberoylanilide hydroxamic acid induced apoptosis in the myofibroblasts of IPF patients through up-regulation of the proapoptotic gene *Bak* and down-regulation of the antiapoptotic gene *Bcl-xL* by specific histone modifications and DNA methylation changes.⁷¹ Furthermore, suberoylanilide hydroxamic acid reduced postinflammatory fibrosis in bleomycin-treated mice mediated, at least in part, by modulating *Bak* and *Bcl-xL* levels in the lungs.⁷¹ Targeting myofibroblast stress fiber formation through, either the Rho-associated kinase pathway or myocardin-related transcription factor/serum response factor signaling, induced preexisting lung myofibroblasts of IPF patients to undergo apoptosis.^{41,55} Specific pharmacological agents targeting these pathways also reduced postinflammatory lung fibrosis in bleomycin-treated mice.^{41,55} Pharmacological targeting inhibitor of apoptosis family proteins X-linked inhibitor of apoptosis and cellular IAP reduced postinflammatory lung fibrosis in bleomycin-treated mice while promoting fibroblast/myofibroblast apoptosis.⁷⁰

In summary, experimental evidence demonstrates that strategies that augment apoptosis in myofibroblasts hasten lung fibrosis resolution. These findings thereby support apoptosis of myofibroblasts as an important mechanism in normal lung fibrosis resolution. Combined with the growing evidence supporting myofibroblast resistance to apoptosis in IPF, these emerging approaches also have strong potential to lead to novel therapies to treat fibrotic lung disease.

Role of Senescence and Aging in Removing Myofibroblasts

Cellular senescence is an adaptive, stable form of cell cycle arrest associated with loss of proliferative activity, persistence of active metabolism, and resistance to apoptosis. Senescent cells are refractory to mitogenic stimulation by activating regulators of cell cycle progression, including p53, p21, and p16.^{77,78} Extrinsic senescence develops after cellular damage or stress and is hypothesized to be an important mechanism to constrain the malignant progression of tumor cells.⁷⁹ Replicative senescence is an intrinsic mechanism whereby there is a limitation in the number of times that cells can divide and is believed to contribute to aging-related pathologies by the acquisition of the senescence-associated secretory phenotype, which enables cells to secrete a variety of growth factors, cytokines, and proteases, which contribute to age-related tissue dysfunction.^{79,80}

One function of the senescence response is to limit the development of fibrosis during wound healing. In the carbon tetrachloride liver fibrosis model, myofibroblasts senesce during spontaneous resolution, and produce a gene expression profile consistent with cell cycle exit, down-regulated genes encoding ECM components, and up-regulated ECM-degrading enzymes, including MMPs.⁸¹ Senescent myofibroblasts also up-regulated genes predicted to enhance immune surveillance, where they were eventually cleared by natural killer

cells, thus removing the myofibroblasts and accelerating the resolution of fibrosis. When the injury was performed on mice deficient in the p53/p21 and p16 pathways, representing mice deficient in a senescence response, healing was accompanied by a marked increase in fibrosis.⁸¹ Similarly, in a mouse model of skin wounding, recruited myofibroblasts are driven into senescence at later stages of wound healing, whereupon they stopped proliferating and up-regulated expression of MMPs while also down-regulating expression of collagen and TGF- β .⁸² This myofibroblast senescence is triggered by the matricellular protein CCN1, which directly binds to integrin $\alpha_6\beta_1$ activating RAC1 to trigger accumulation of reactive oxygen species and damage response pathways inducing senescence.⁸³ In mice expressing a mutant CCN1 protein that is defective in integrin binding and hence in inducing senescence, the wounds were deficient in senescent cells and mice healed with significantly more fibrosis.⁸²

The role of senescence pathways in lung myofibroblasts is less understood. A transient senescence response was observed in fibroblasts from young mice with resolving bleomycin-induced fibrosis supporting a role for senescence in spontaneous lung fibrosis resolution.⁵¹ However, there are accumulating data supporting the fact that prolonged activation of senescent pathways contributes to persistent pulmonary fibrosis. Pulmonary fibrosis is classically, but not exclusively, a disease of aging. The incidence of IPF increases more than fivefold when comparing adults 45 to 54 years of age with those >75 years of age.⁸⁴ Recent reviews in aging biology have identified specific biological processes that are altered in aging, and several have been associated with human pulmonary fibrosis disorders, including genomic instability, telomere erosion, epigenetic changes, proteostasis, and cellular senescence.⁸⁵ Several investigators demonstrate that aged fibroblasts are profibrotic.^{53,86–89} Consistent findings from these studies indicate differences in the fibroblast response to injury as a primary mechanism for the greater fibrosis susceptibility among aged lungs. Compared with young mice, fibroblasts isolated from aged mice show reduced Thy-1 expression, increased TGF- β receptor levels, increased expression of matrix proteins when incubated with TGF- β , and a higher resistance to apoptosis.^{86,88,90} Senescent lung fibroblasts have been reported to resist apoptosis through the epigenetic mechanism of histone modification, resulting in up-regulation of the antiapoptotic gene, *Bcl-2*, and low expression of the proapoptotic gene, *Bax*.⁹¹

Although the aged lung is more susceptible to the onset of fibrosis, few studies in the lung or other organs specifically address the capacity for resolution of established fibrosis in aged models. Hecker et al⁵¹ demonstrated that bleomycin-induced fibrosis resolution was impaired in aged mice. In contrast to younger mice where the senescence response was transient, persistent fibrosis in the

aged mice was associated with a prolonged senescent and apoptosis-resistant phenotype of myofibroblasts. These cellular phenotypes were mediated by an imbalance between elevated expression of the reactive oxygen species—generating enzyme Nox4 and an impaired induction of the counterregulatory response from the antioxidant Nrf2. Genetic and pharmacological targeting of Nox4 in aged mice after fibrosis enhanced the capacity for fibrosis resolution, supporting the concept that restoration of Nox4-Nrf2 reduction-oxidation balance in myofibroblasts may be a therapeutic strategy to resolve persistent fibrosis or even reverse its progression.⁵¹

In summary, although transient senescence may play an important role in myofibroblast elimination in normal wound healing, prolonged senescence of myofibroblasts in the lung induces resistance to apoptosis and may be a primary mechanism of persistent fibrosis. Supporting this hypothesis, Baker et al,⁸⁰ using transgenic approaches in mice allowing for the inducible elimination of cells carrying the p16 senescence biomarker, demonstrated that life-long removal of senescent cells delays the onset of age-related phenotypes. Furthermore, late-life clearance attenuated the progression of already established age-related disorders, including sarcopenia, cataracts, and loss of adipose tissue.⁸⁰ Collectively, these studies support that senescent cells contribute to age-related phenotypes and elimination of senescent myofibroblasts may be an effective strategy to prevent or delay age-related pathologies, such as pulmonary fibrosis.

Role of Inflammatory Cells in Lung Fibrosis Resolution

Elements of the innate and adaptive immune system have been shown to be intricately involved in lung fibrosis resolution by clearing ECM fragments and removing apoptotic cells.^{24,92} The indispensable role of the immune system in resolving fibrosis is highlighted by studies from Wallach-Dayana et al,⁹³ who demonstrated myofibroblasts from mice receiving bleomycin-induced lung fibrosis resist lymphocytic Fas-induced apoptosis and survive longer when grafted into allogeneic mice. These findings support the importance of specific immune surveillance of lung myofibroblasts in the resolution of fibrosis.

The macrophage is the prototypical innate immune cell involved in chronic inflammation and fibrosis.⁹⁴ Macrophages are almost always found in close proximity with collagen-producing myofibroblasts, and are believed to contribute and sustain a fibroproliferative lung microenvironment through their expression of profibrotic genes.⁹² The presence of these alternatively activated macrophages has been linked to acute exacerbation of IPF patients consistent with the idea that macrophages can modulate the stability or progression of IPF.⁹⁵ When macrophages from the lungs of healthy controls or IPF patients were

cultured and stimulated with collagen monomers, the IPF-derived macrophages increased expression of profibrotic markers and cytokines, consistent with a classification of the alternatively activated state.⁹⁶

Although macrophages can clearly promote fibrogenesis, depletion of macrophages during the recovery phase of both toxic liver injury and the lung bleomycin fibrosis models delayed the resolution of fibrosis.^{97,98} These findings support that functionally distinct subpopulations of macrophages also play critical roles in recovery. Macrophages may enhance fibrosis resolution through several mechanisms: macrophages are the key sources of extracellular proteases facilitating ECM degradation; macrophages are primary cells involved in phagocytosis of cellular debris, collagen, and apoptotic cells; macrophages also recruit or activate other collagenase-producing cells, including T regulatory cells, which have been implicated in the suppression of fibrosis.⁹² The surface markers that differentiate fibrolytic macrophages and the specific precursor from which they develop have yet to be identified. However, the concept that subpopulations of macrophages can be manipulated to enhance lung fibrosis resolution was demonstrated by Redente et al,⁹⁹ where pulmonary delivery of tumor necrosis factor- α to mice with established bleomycin-induced fibrosis accelerated fibrosis resolution by directly down-regulating and reducing the number of alternatively activated macrophages.

Thus, although myofibroblasts may be master mediators of fibrosis through their synthesis of ECM components, macrophages are considered to play equally important roles by serving as the master regulators of both myofibroblast function and ECM degradation.⁹² Global depletion of macrophages is not a clinically feasible option to treat established and progressive disease because they are not a single rigid phenotype but pleomorphic populations. Accordingly, a key goal of future research will focus on identifying the specific macrophage subpopulations that facilitate resolution, whether these macrophage subpopulations represent a new class of recruited macrophages from the bone marrow, or if the same population of macrophages can adjust its phenotype. Identifying the signals that regulate macrophage phenotypic conversion, differentiation, or recruitment may provide methods to influence the beneficial aspects of inflammation to impede progression or reverse ongoing disease.⁹⁴

Conclusions and Future Directions

Although resolution of fibrotic remodeling occurs in normal wound healing, it remains unclear whether pulmonary fibrosis in clinical settings is reversible. Animal studies support the hypothesis that the lung has the capacity to effectively resolve a significant fibrotic burden and allow near complete predisease physiological

recovery after removal of the fibrogenic insult. This resolution occurs through a dynamic process intricately integrated with the immune system that involves degradation and removal of the ECM and elimination of activated myofibroblasts. Several key clinical questions remain regarding lung fibrosis resolution. First, in progressive fibrotic disorders, such as IPF, how much, if any, is progression secondary to dysregulation of the resolution pathways? The higher incidence of fibrosis in the aged population and recent studies reporting impaired lung fibrosis resolution in aged rodents have high potential to determine whether resolution pathways are altered and lead to ongoing disease. Second, can innate pathways be altered to accelerate resolution in progressive fibrosis to alter the balance of deposition versus degradation? Eradication of the inciting stimulus is the most efficacious means to promote resolution, but in many progressive disorders this is often not an option. Elimination of active myofibroblasts is likely the key component to successful resolution. Recent preclinical investigations support the concept that targeting intrinsic lung myofibroblast dedifferentiation, apoptosis, or senescence pathways effectively promotes resolution and may prove to be practical approaches for treatment of lung fibrosis.^{41,50,55,70,71}

There are clearly significant gaps in understanding lung fibrosis resolution likely reflecting the biological complexity and limitations in the animal models and basic scientific methods previously available to investigators. However, accelerated improvements in biotechnology and bioinformatics are revolutionizing biomedical research, and it is likely these new techniques and discoveries will continue to improve the understanding of the mechanisms of lung fibrosis resolution. Continued efforts focused on defining these mechanisms and determining their relevance in human disease is a critical barrier that must be crossed to lead to novel and effective restorative therapies in the treatment not only of pulmonary fibrosis, but also a wide-ranging spectrum of chronic disorders.

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