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New therapeutics based on emerging concepts in pulmonary fibrosis

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Abstract

Introduction: Fibrosis is an irreversible pathological endpoint in many chronic diseases, including pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) is a progressive and often fatal condition characterized by (myo)fibroblast proliferation and transformation in the lung, expansion of the extracellular matrix and extensive remodeling of the lung parenchyma. Recent evidence indicates that IPF prevalence and mortality rates are growing in the United States and elsewhere. Despite decades of research on the pathogenic mechanisms of pulmonary fibrosis, few therapeutics have succeeded in the clinic, and they have failed to improve IPF patient survival.

Areas Covered: Based on a literature search and our own results, we discuss the key cellular and molecular responses that contribute to (myo)fibroblast actions and pulmonary fibrosis pathogenesis; this includes signaling pathways in various cells that aberrantly and persistently activate (myo)fibroblasts in fibrotic lesions and promote scar tissue formation in the lung.

Expert Opinion: Lessons learned from recent failures and successes with new therapeutics point toward approaches that can target multiple pro-fibrotic processes in IPF. Advances in preclinical modeling and single-cell genomics will also accelerate novel discoveries for effective treatment of IPF.

Keywords

Heat shock protein 90; Idiopathic Pulmonary Fibrosis; Lung; Myofibroblasts; Wilms' tumor1

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1. INTRODUCTION

Fibrosis is a pathological endpoint in many chronic diseases associated with unresolved inflammation and tissue remodeling. Collagen and other extracellular matrix (ECM) components, such as fibronectin and elastin, are overproduced and accumulate, expanding the inflamed or damaged connective tissue. Fibrosis is a major cause of death through permanent scarring and functional impairment of such vital organs as the heart, intestine, kidney, liver, and lung [1]. Fibrosis has been shown to affect various organ systems in systemic auto-immune diseases, such as scleroderma and systemic lupus erythematosus and also monogenic disease such as cystic fibrosis (Figure 1). It is also a major pathological feature of cancers, regulating the invasion and metastasis of oncogenic cells in tumors. Clearly, developing effective therapeutics against tissue fibrosis is an urgent pursuit in diverse research areas. In the lung, profibrotic responses are essential to maintaining the barrier function during injury and establishing immunity against infectious pathogens, such as bacteria and helminths, but repetitive lung injury often, but not always, follows a sequence that results in persistent fibrosis and irreversible fibrotic damage. Fibroblast proliferation and differentiation into ECM-producing myofibroblasts initiate the fibrotic cascade. These mesenchymal cells dictate excessive ECM production and scar tissue formation in the lung. They resist apoptotic clearance to produce excessive ECM in fibrotic lesions. However, the precise molecular events that prompt myofibroblast accumulation in progressive fibrosis as opposed to wound-healing responses are not thoroughly understood. Some clinical evidence in mice and humans suggests that pulmonary fibrosis may resolve in the absence of injury or pro-fibrotic triggers [2]. In genetically susceptible individuals, mutations in telomerase (TERT, TERC, RTEL1), the surfactant proteins (SFTPB, SFTPC), and the MUC5B gene promoter trigger fibroproliferative processes and fibrotic remodeling via mechanisms that remain largely unknown [3].

For decades, fibrosis research has focused on the cellular and molecular triggers of fibroblast proliferation and transformation into ECM-producing myofibroblasts. In this review, we highlight emerging concepts on the cellular and molecular origins of fibrotic lesions in pulmonary fibrosis pathogenesis. In particular, we discuss the mesenchymal cell-specific transcription factors and heat shock proteins involved in myofibroblast activation. We review recent progress in targeting signaling pathway intermediates, which has opened new avenues to inhibiting profibrotic processes while simultaneously attenuating or reversing established, ongoing pulmonary fibrosis.

2. Current Evidence

2.1. Emerging cellular targets in pulmonary fibrosis

Myofibroblasts are responsible for ECM deposition in the lung and crucial to scar tissue formation because they produce and secrete excessive amounts of ECM proteins such as collagens, glycoproteins, and proteoglycans. In progressive fibrosis, activation of the contractile protein alpha-smooth muscle actin (α SMA) transforms fibroblasts into myofibroblasts. However, despite decades of research, the progenitor cells that undergo myofibroblast transformation to cause alveolar destruction in peripheral areas of the lung remain poorly defined [4, 5].

This subsection summarizes new findings on the cellular origins of myofibroblasts during lung fibrogenesis. Previous studies have defined five: (i) resident fibroblasts; (ii) hematopoietic fibroblasts, called fibrocytes; (iii) mesothelial cells lining subpleural areas; (iv) pericytes that tightly associate with the microvasculature; and (v) epithelial cells.

2.1.1. Resident fibroblasts.—These non-hematopoietic cells reside in various parenchymal areas of the lung. They produce matrix proteins and growth factors that provide the structural support essential during lung development and in maintaining mucosal barrier integrity and physiological functions. They are not terminally differentiated and differentiate into myofibroblasts during lung injury and repair [6, 7]. Studies from our group and several others emphasize their contribution to α SMA-expressing myofibroblasts that accumulate after fibrotic injury. Using FoxD1-Cre; -TdT-R; Col-GFP^{Tg} mice, Hung et al. showed that, following bleomycin injury, 55 percent of α SMA+ myofibroblasts are derived from collagen-1-(α)1-positive and PDGFR α -expressing resident fibroblasts [8]. A recent study to determine whether lipofibroblasts, a type of resident fibroblast that contains an excess of neutral lipids, can differentiate into α SMA-expressing myofibroblasts, found that they co-expressed α SMA during bleomycin-induced lung injury in Adrp^{Cre-ERT2/mT/mG} mice. Among lineage-labeled lipofibroblasts, 19 percent became α SMA-positive myofibroblasts during bleomycin-induced fibrosis [9]. Overall, the lung resident fibroblasts represent a heterogeneous population of cells expressing different network of genes that engage in paracrine and autocrine interactions in fibrosing lungs. Thus, therapies that modulate the activation and/or recruitment of resident fibroblasts could emerge as viable treatments for pulmonary fibrosis.

2.1.2. Hematopoietic fibroblasts.—Fibrocytes are unique mesenchymal progenitor cells derived from bone marrow that express a variety of cell surface markers related to leukocytes, hematopoietic progenitor cells, and fibroblasts [10, 11]. They were originally identified in tissues with active fibrosis and inflammation during fibroproliferative diseases like idiopathic pulmonary fibrosis (IPF)[12]. The surface markers they express vary with tissue and disease status, but they all express the common leukocyte antigen (CD45) and mesenchymal marker type-I collagen (Figure 2). Their pathogenic role in pulmonary fibrosis is well established in various experimental animal models and clinical samples of IPF [10, 13]. Serum amyloid P (SAP), an endogenous blood stream protein inhibits the differentiation of circulating monocytes into pro-fibrotic macrophages (M2) and fibrocytes which was found to be reduced in IPF patients[14, 15]. Recombinant SAP is currently under clinical trials for patients with IPF [16]. However, transformation of fibrocytes into α SMA-positive myofibroblasts is controversial, and recent studies reveal that it is limited. Hashimoto et al. used enhanced green fluorescent protein (EGFP)-chimeric mice and found that most GFP+ cells were negative for α SMA, suggesting that few fibrocytes become myofibroblasts during bleomycin-induced lung injury [17]. Using GFP-chimeric mice, we induced pulmonary fibrosis by overexpressing transforming growth factor alpha (TGF α) in airway club cells. Although fibrocytes accumulated in fibrotic lesions, the majority of them did not become α SMA-expressing myofibroblasts [18]. Note that in the pathogenesis of pulmonary fibrosis, fibrocyte infusion produces severe fibrotic lesions in part due to the paracrine effect on lung-resident (myo)fibroblasts. Fibrocytes infused into wild-type mice challenged with either

bleomycin or fluorescein isothiocyanate (FITC) exacerbated pulmonary fibrosis but had no effect on normal mice [19].

These *in vivo* findings suggest that fibrocytes alone cannot induce fibrosis but may play a pathogenic role in ongoing lung injury by secreting paracrine factors that stimulate resident fibroblast proliferation and/or transformation to myofibroblasts [18]. Fibrocytes have been shown to secrete many paracrine factors, including periostin, which is elevated during bleomycin-induced pulmonary fibrosis and IPF [20]. A recent study found that periostin prompts myofibroblast differentiation during bleomycin-induced lung fibrosis [21]. Further studies on the role of fibrocyte-secreted paracrine factors in fibroblast activation could inform new therapeutic strategies.

2.1.3. Mesothelial cells.—A single sheet of cuboid pleural mesothelial cells (PMCs) lines the lung. PMCs express both epithelial and mesenchymal cell-specific genes, such as calretinin, cytokeratin, collagen, desmin, and vimentin, but not smooth muscle actin. During embryonic lung development, they become mesenchymal cells in a process called the mesothelial-to-mesenchymal transition (MMT); as a result, mesothelium-derived myofibroblasts populate the perivascular and peribronchial areas of the adult lung [22]. Recent studies using cultured PMCs provide some evidence of MMT in the pathogenesis of pulmonary fibrosis. In particular, the TGF β 1/SMAD3 axis was implicated in MMT and myofibroblast accumulation in the parenchyma of TGF β 1-injured lungs [23]. Although these studies suggest that MMT contributes to pulmonary fibrosis, the lack of robust lineage-tracing models is a significant limitation [24–27].

More recent studies have shown that tamoxifen-dependent Cre recombinases, also known as CreERT2-driven recombination in Wilms tumor (WT1^{CreERT2}) mice is more reliable and reproducible than in WT1^{CreEGFP} reporter mice [13, 28–30]. Use of WT1^{CreERT2} mice enabled accurate labeling of the WT1-positive mesothelial cells lining embryonic lungs, later shown to engender the mesenchymal cells of the lung parenchyma [28, 29]. We demonstrated that WT1 is downregulated in the postnatal stages of lung development but upregulated in mesothelial cells in IPF and a mouse model of severe fibrotic lung disease [13]. Indeed, *in vivo*, postnatal mesothelial lung cells transform into myofibroblasts in TGF α /WT1^{CreERT2/mTmG} reporter mice during TGF α -induced pulmonary fibrosis. They are found in subpleural areas of fibrotic lungs but not in peribronchial or adventitial regions [31]. However, PMCs did not transform into myofibroblasts during single-dose bleomycin-induced injury or adeno TGF β 1-induced pulmonary fibrosis [28], perhaps because TGF β -driven signaling or other signaling events are less intense. Future studies of myofibroblast formation should be designed to elucidate both up- and downstream WT1 targets and possible crosstalk with the TGF β /SMAD pathway. Understanding the complex regulation of myofibroblast formation by TGF β -dependent and independent pathways in the pathogenesis of pulmonary fibrosis is essential to developing more efficacious therapeutics for IPF.

2.1.4. Pericytes.—Pericytes are mural cells that tightly associate with the microvasculature endothelium with the help of multiprotein complexes to strongly support microvascular homeostasis and angiogenesis [32, 33]. Pericytes express such markers as neural/glial antigen 2 (NG2), platelet-derived growth factor receptor beta (PDGFR- β),

cluster differentiation 90 (CD90), and CD146 [33]. Recent studies using a cell-specific lineage-tracing approach have shown that pericytes become myofibroblasts in vivo. Hung et al. used FoxD1-Cre mice to tag pericyte-like cells that co-express NG2. After bleomycin injury, FoxD1-progenitor cells proliferated in fibrosing lungs. These fate-mapped cells have been shown to express α SMA, suggesting a possible pericyte-to-myofibroblast transition, but the myofibroblasts originating from them do not comprise the majority of α SMA-positive cells [8]. In another study, human lung pericytes were grown on decellularized IPF matrix to demonstrate their transformation into myofibroblasts [34]. However, the nonspecific and continuous labeling in FoxD1-reporter mice is a major limitation in assessing the direct conversion of pericytes to myofibroblasts upon injury in adult lungs. The use of inducible Cre to trace the lineage of NG2-positive cells indicates that pericyte-like cells undergo proliferative expansion, but most do not become myofibroblasts during bleomycin-induced injury [35]. Overall, PCs are a potential source of myofibroblasts, but future studies must establish their percent contribution to the pathogenesis of fibrotic lung disease.

2.1.5. Epithelial cells.—The epithelial-to-mesenchymal transition (EMT) is a complex molecular process in which a polarized epithelial cell adopts a mesenchymal cell phenotype with enhanced migratory capacity, invasiveness, apoptosis resistance, and production of ECM components, such as collagen and fibronectin. EMT's role in cancer and fibrosis has been described in many organs. Previous studies using patient-derived samples and various in vivo models of pulmonary fibrosis demonstrated that EMT is an important cellular phenotypic change that contributes to the mesenchymal cell pool in the pathogenesis of fibrotic lung disease [36–39]. However, a recent lineage-tracing study performed in well-controlled, tamoxifen-inducible Sftpc-CreERT and Scgb1a1-CreERT reporter mice found no EMT during bleomycin-induced pulmonary fibrosis [35]. In the TGF α model, EMT's contribution was determined by generating a quadruple transgenic mouse in which airway epithelial cells were tagged permanently with activation of LacZ reporter gene, and TGF α was overexpressed using doxycycline (dox) to induce severe fibrotic lung disease. Note that the lung epithelial cells showed high LacZ activity and beta-galactosidase (β -gal) staining, but the mesenchymal cells of the fibrotic regions showed no staining, suggesting that airway epithelial cells do not transform to myofibroblasts [40].

Adult lungs are primarily comprised of the epithelial compartment, of which 90 percent consists of morphologically distinct epithelial cell types, including alveolar types I and II. Other studies indicate that activated alveolar epithelial cells produce collagen and secrete other factors in fibrotic lungs, but whether they have capacity to differentiate further into mesenchymal cells or lose their epithelial cell characteristics in the pathogenesis of pulmonary fibrosis remains unknown (PMID: 24508728). During pulmonary fibrosis, epithelial cells not only play a crucial role on their own but also secrete paracrine factors that may activate mesenchymal cells [41]. The EMT is a controversial mechanism that must be further investigated using appropriate epithelial cell reporter models. Understanding epithelial-mesenchymal crosstalk will clarify disease pathogenesis and lead to IPF therapies.

2.2 Emerging molecular targets in pulmonary fibrosis

Mechanistic studies have identified TGF β as a master regulator of profibrotic processes, including ECM production, inflammation, and myofibroblast formation, particularly the accumulation of these apoptosis-resistant cells in IPF. However, growing in vitro and in vivo evidence indicates that TGF β -independent pathways induce myofibroblast transformation and pulmonary fibrosis [42]. This subsection reviews research on emerging molecular targets of (myo)fibroblast activation in pulmonary fibrosis.

2.2.1. Fox proteins.—Fox proteins are a family of “winged-helix” DNA-binding domains (DBDs) containing transcription factors that regulate the expression of an array of genes involved in embryonic development and adult tissue homeostasis [43]. Several key subfamilies, such as FoxF1, FoxM1, and FoxO3, are strongly implicated in IPF pathogenesis [44–48]. FoxF1 is critical to regulating the transcription of genes involved in branching morphogenesis during lung development [49, 50]. In adult lungs, its expression is observed in bronchiolar smooth muscle cells, connective tissue fibroblasts, endothelial cells, and a subset of alveolar septal cells [50]. Although research has shown that its mRNA and protein levels are downregulated in IPF and a mouse model of bleomycin-induced pulmonary fibrosis, its loss has been associated with increased myofibroblast invasion and collagen production [45].

CDH2 and CDH11 are cadherin family proteins involved in cell-cell adhesion via a calcium-dependent signaling mechanism [51, 52]. A molecular switch from CDH2 to CDH11 expression was shown to determine TGF β fibroblast-to-myofibroblast conversion[51]. Black et al. demonstrated that FoxF1 directly binds to Cdh2 and Cdh11 promoters to activate CDH2 but repress CDH11 expression [45], and the loss of FoxF1 resulted in increased myofibroblast invasion and collagen production during bleomycin-induced pulmonary fibrosis [45]. Moreover, its overexpression was sufficient to attenuate both fibroproliferation and TGF β 1-driven Col1 expression [53]. Selectively deleting it in α SMA-expressing cells was sufficient to exacerbate bleomycin-induced pulmonary fibrosis [45].

FoxM1 and FoxO3a proteins act downstream of the phosphoinositol-3-kinase (PI3K)-AKT signalling cascade [54]. Their axis has been shown to regulate such biological processes as cellular metabolism, proliferation, differentiation, invasion, apoptosis, and DNA-damage response and is thus pivotal in over 20 types of human cancers as well as pulmonary fibrosis [54, 55]. FoxM1 regulates proliferation in many types of lung cells by altering expression of the cyclin genes, CCND1 and CCNB1 and serine/threonine-protein kinase (PLK1), involved in the G1/S and G2/M transitions[56] [48]. FoxM1 is elevated in fibroblasts isolated from IPF and the bleomycin model [48]. Several mitogens are implicated in its upregulation via the PI3K α /PDK1/AKT pathway [48], which a recent study associated with increased fibroproliferation and myofibroblast formation. In contrast, by downregulating FoxM1, FoxO3a has been shown to sensitize IPF fibroblasts to radiation-induced cell death [57]. Mesenchymal cell-specific deletion of the FoxM1 gene or inhibition of FoxM1 using siomycin A was shown to attenuate expression of several profibrotic genes, including α Sma, Col1 α 1, Tgf β 1, and connective tissue growth factor (Ctgf) in the bleomycin model [48]. FoxM1 has been shown to induce expression of the RAD51 and BRCA2 proteins essential

for DNA-damage repair after radiation-induced injury [57]. Importantly, FoxM1 levels correlate with IPF fibroblast resistance to the apoptosis after radiation-induced cell death [57]. In this model, FoxM1 was found upregulated in alveolar epithelial cells (AECs) and the conditional deletion of FoxM1 in AECs shown to attenuate radiation-induced pulmonary fibrosis [44]. Therefore, targeting FoxM1 specifically in fibroblasts could be a new therapeutic for the treatment of fibrotic diseases.

2.2.2. WT1.—Wilms tumor1 protein is a four zinc finger transcription factor that plays an essential role in the development of such critical organs as lung, heart, and kidney and regulates posttranscriptional modifications and RNA metabolism [58]. Germline mutations or loss of WT1 have been associated with severe developmental defects and embryonic lethality in mice [58, 59]. Although expressed at low levels in lung mesothelial cells of adult mice, it can be upregulated in both mesothelial and mesenchymal cells to cause severe fibrosis in the lung [13, 31, 60]. In gain-of-function studies in primary fibroblasts, we demonstrated that its overexpression alone was sufficient to induce fibroproliferation, myofibroblast formation, and ECM production [31]. Furthermore, its genetic loss markedly reduced expression of ECM genes, such as Col1 α and Col5 α , and proliferative genes, such as Grem1, Runx1, Wnt4, Igf1, Ccnb1, and E2f8.

Studies using α SMA reporter cells demonstrate that WT1 overexpression is sufficient to induce fibroblast-to-myofibroblast transformation, and chromatin immunoprecipitation experiments indicate that WT1 binds directly to the promoter DNA sequence of α SMA to induce myofibroblasts [31]. WT1 has been shown to maintain the mesenchymal cell phenotype by repressing epithelial genes, such as Snail (Snai1) and E-cadherin (Cdh1), during embryonic stem cell differentiation [61]. Note that haplo-insufficiency of WT1 was sufficient to attenuate fibroproliferation, myofibroblast accumulation, and collagen deposition in both TGF α - and bleomycin-induced pulmonary fibrosis in vivo [31]. Our new findings suggest that WT1-driven effects on fibroproliferation are non-cell autonomous and may involve paracrine factors secreted by WT1-expressing cells [31]. They highlight the need for more detailed investigation of the molecular mechanisms of WT1-driven fibroblast activation and pulmonary fibrosis and whether crosstalk between WT1 and the TGF β /SMAD pathway regulates them. By identifying WT1 as a positive regulator of fibroblast activation, they suggest a new target for treating fibrotic lung diseases and possibly regulating fibrosis in other organs.

2.2.3 Heat shock protein 90.—The molecular chaperone Hsp90 constitutes 1–2 percent of the total cellular protein pool and plays an essential role in protein folding while stabilizing a variety of client proteins involved in cellular signaling, transcription regulation, and translation [62]. Its molecular functions depend on its ATPase activity and complex formation with other proteins. In mammalian cells, Hsp90 α and Hsp90 β are the two major isoforms involved in Hsp90-driven functions [63]. They are 86 percent identical, with 93 percent sequence similarity and a characteristic functional MEEVD motif [63]. Both have an N-terminal ATPase catalytic center that binds ATP and assists in chaperoning [63].

Typically, more Hsp90 α than Hsp90 β is expressed, but in various disease conditions, both may be induced [64, 65]. Hsp90 β levels are elevated in gastrointestinal stromal tumors,

multiple myeloma, breast cancer, and neuroblastomas associated with poor survival [64, 66, 67]; comparative proteomic analysis shows a significant increase in both Hsp90 α and Hsp90 β with IPF as compared to healthy lungs [68]. Since many studies confirm the upregulation of Hsp90 expression and its ATPase activity in IPF and mouse models of pulmonary fibrosis [69–73], a recent study proposes a correlation between circulating levels of the Hsp90 α isoform and IPF severity[70].

Findings on how Hsp90 regulates signaling pathways to activate fibroblasts have provided new insights into the molecular mechanisms of severe fibrotic lung disease [70–74]. In particular, using tanespimycin (17-AAG) to block Hsp90 ATPase activity hampered the TGF β 1 signaling pathway by inducing proteasomal degradation of TGF β RII, an essential signaling receptor for the TGF β 1 ligand [72]. Similar findings in the tight-skin (Tsk-1) mouse model of dermal fibrosis show that administering alvespimycin (17-DMAG) blocked the TGF β 1 signaling pathway by decreasing SMAD2/3 phosphorylation [74]. Another study showed that Hsp90 forms a complex with TGF β RII to induce fibroblast activation. In human lung fibroblasts treated with exogenous human recombinant Hsp90 α , expression of α SMA and other ECM proteins, such as Col1 and fibronectin, increased [71], and a monoclonal anti-Hsp90 α antibody (1G6-D7) mitigated these effects by attenuating profibrotic signaling via the kinases ERK, Akt, and P38 [71]. We showed that Hsp90 β knockdown in fibroblasts attenuates their proliferation and formation of myofibroblasts, while loss of Hsp90 α had a limited or no effect on proliferation and ECM production but attenuated fibroblast migration [73]. Hsp90 inhibition with 17-AAG reduced migratory capacity, proliferation, and ECM production in fibroblasts[73]. New therapeutics inhibiting Hsp90 could target the profibrotic signaling pathways involved in fibroblast activation and pulmonary fibrosis.

2.3 New advances in inhibiting pulmonary fibrosis signaling pathways

Drugs approved for IPF delay loss of lung function but fail to stabilize or improve IPF patients lung function or survival. A plethora of mechanisms and pathways are involved in lung fibrosis, including repetitive epithelial cell injury, wound healing, epithelial-mesenchymal crosstalk, and lung fibrotic pathology. To improve clinical outcomes, researchers are pursuing alternative therapeutic approaches to fibrogenesis and its resolution, especially drugs targeting the signaling pathways that maintain the fibrotic networks. Many inhibitors target the downstream signaling molecules involved in fibrogenesis and lesion progression.

2.3.1 TGF β and Integrins.—The pathological role of TGF β as a fibrogenic agent is well documented [75–77]. Of its three forms, TGF β 1 is a potent fibrogenic factor secreted by immune and epithelial cells and fibroblasts. Published studies demonstrate that it promotes fibroblast-to-myofibroblast transformation and collagen deposition both *in vitro* and *in vivo* [78, 79]. However, in a phase 1 trial (NCT00125385), an antibody to the TGF β isoforms (GC1008) showed low efficacy. Targeting TGF β directly disturbs the other homeostatic effects it regulates in other organs, therefore, more targeted approach towards the molecular mechanisms that activates the latent TGF β might be advantageous in rescuing from fibrosis. Targeting the integrins or lysophosphatidic acid (LPA) or autotaxins might be a right approach in dealing with fibrosis.

Activation of latent TGF β is regulated by the integrin α v β 6, expressed more strongly on the epithelial cells of fibrotic than healthy lungs [78, 79]. In a TGF α transgenic fibrotic model, genetic deletion of β 6 slightly reduced fibrosis, indicating that other signaling mechanisms may act before the α v β 6-TGF β 1 pathway [79] as well as the complexity of fibrotic disease. However, preclinical primate studies with BG00011, a humanized monoclonal antibody against α v β 6 (formerly known as STX-100 and acquired by Biogen), decreased active TGF β 1 signaling in a dose-dependent manner as assessed by pSMAD2 expression in bronchoalveolar lavage fluid (BAL) cells. A phase 2A, randomized, placebo-controlled, dose-escalation study (NCT01371305) on 41 IPF subjects revealed an inverse dose-response relationship with pSMAD2 levels in BAL cells. The data on safety, tolerability, and dose selection led to a phase 2B study [80]. In a phase 1 investigation, another nonpeptidic α v β 6 inhibitor (GSK3008348; GlaxoSmithKline) showed good tolerability in healthy subjects, and its tolerability and safety in IPF patients is currently under investigation (NCT03069989).

2.3.2. T cells, innate lymphoid cells 2 and IL-13.—Uncontrolled inflammation and oxidative burst are known to cause progressive lung damage and thickening of peripheral areas of the lung [81]. Histological evaluations of fibrotic lungs revealed infiltration of mononuclear immune cells (T-cells, B-cells and macrophages) into the mature fibrotic lesions in IPF [82]. One of the challenges in studying the role of inflammation in IPF is to identify pathogenic inflammatory cells and their mediators involved in the maintenance of persistent inflammation and progressive fibrosis. Th2-cytokine polarized T cell responses have been shown to mediate tissue damage and fibrosis in IPF [83, 84]. In IPF patients, IL-13 levels are elevated and correlate inversely with lung function [85]. Also, IL-4 and IL-13 have been shown to contribute to inflammation and fibrosis in multiple animal models of pulmonary fibrosis including the bleomycin-induced pulmonary fibrosis [86–90]. IL-13 is shown to activate fibroblasts which participate in production and deposition of excess collagen and fibronectin in lung and other organs [91]. Recent studies suggest the innate lymphoid cell 2 (ILC2) are also important source for IL-13 production [92]. Tralokinumab, Lebrikizumab and SAR156597 were the antibody mediated targeting either IL-13 alone or IL-4 and IL-13 entered into clinical trials [93–95]. However, anti-IL-13 clinical trials were stopped due to lack of efficacy and could not meet their primary end points. This suggests that targeting the IL-13 alone may not be beneficial to the IPF patients.

2.3.3 Lipids.—Apart from proteins, some lipid-mediated signaling pathways are implicated in IPF. LPA levels are high in IPF patients' BAL, acting through receptors LPA_{1–6} [96]. Knockout studies on receptors LPA1 and 2 attenuated lung fibrosis [96, 97]. The LPA-LPA1 axis assists in epithelial cell apoptosis after lung injury while opposing apoptosis in fibroblast cells [97]. Xu MY et al. demonstrated that in epithelial cells, the LPA-LPA2 axis activates TGF β 1, mediated by α v β 6, through RhoA-facilitated contraction [98]. However, targeting the enzyme responsible for LPA formation would inhibit the pathogenesis the LPA-LPA1/LPA2 axes mediate.

In this context, Galapagos developed GLPG1690, a selective inhibitor of autotaxin, the phospholipase most responsible for excessive LPA formation in IPF lung tissues [99].

Deleting the autotaxin gene from bronchial epithelial cells and macrophages reduced fibrotic disease [99]. GLPG1690-treated IPF fibroblasts had a marked reduction in fibrotic mediators like CTGF, interleukin-6 (IL-6), and endothelin-1 (ET-1)[100]. Recently, a 12-week, randomized, double-blinded, placebo-controlled phase 2a study (NCT02738801) on 23 IPF patients found GLPG1690 to be well tolerated, and its pharmacokinetic and dynamic profiles were similar to those in healthy subjects as determined by a phase 1 study. LPA concentrations were reduced during the treatment period and reached baseline values without treatment. The mean decline in forced vital capacity (FVC) was consistent with three months of pooled data on the IPF medications nintedanib and pirfenidone [101].

In parallel, Bristol-Myers Squibb developed BMS-986020, a potent antagonist of the LPA1 receptor. Preclinical studies showed antifibrotic effects in, not only the lungs, but liver, kidney, eyes, and skin. It went through phase 1 trials, but although phase 2 (NCT01766817) was completed in 2016, the outcome has not yet been reported.

2.3.4. Cellular senescence and metabolism.—IPF is a disease of elderly population and multiple lines of evidence suggests aging associated factors such as cellular senescence, telomere attrition, and deregulated metabolism role in its pathogenesis [102]. Cellular senescence is well studied in the pathogenesis of IPF. AECs and fibroblasts are crucial cell types that undergo cellular senescence following injury in pulmonary fibrosis. Treatment with senolytic drugs that selectively eliminates senescent cells by inducing apoptosis shown to eliminate pro-fibrotic senescent fibroblasts in vitro and alveolar epithelial cells ex vivo [103–105]. In particular, a cocktail of dasatinib and quercetin shown to improve lung function during bleomycin-induced pulmonary fibrotic mice. However, visible fibrosis was not altered as evidenced by histopathological score [103]. Recent studies suggest that antioxidant and senolytic properties of metformin could be used to dampen bleomycin-induced pulmonary fibrosis [106, 107]. However, more work is needed to clarify whether use of senolytic drugs can selectively eliminate pathogenic cell types but not cells required for lung regeneration in fibrosis resolution.

2.3.5. Connective tissue growth factor (CTGF).—In IPF patients, high CTGF expression has been shown to promote ECM deposition, myofibroblast activation, cell adhesion, and invasion, the key processes of tissue remodeling and fibrosis [108, 109]. FibroGen identified pamrevlumab (FG-3019) as a candidate monoclonal antibody that efficiently reduces CTGF activity to restore tissue homeostasis. In an irradiated mouse model, FG-3019 treatment eight weeks after lung injury reduced lung density and maintained it for six months. Within two weeks of administration, it also decreased the infiltration of mast cells and macrophages[110]. These promising results led to a phase 1, nonrandomized, open-label, dose-escalation study (NCT00074698) performed on 21 IPF patients, who showed no adverse effects. In the phase 2A, open-label study (NCT01262001) with 89 participants, single doses of either 15mg/kg or 30mg/kg were administered intravenously to two cohorts every three weeks for 45 weeks. FVC declined on average 140 ml, but for 30 percent of patients, FVC increased. Note that this study is the first to show any improvement in pulmonary fibrosis, observing reduced reticular fibrosis and mild adverse effects [111]. Based on these results, a phase 2B, randomized, placebo-controlled study

(NCT01890265) was conducted on 103 participants, a subset of whom were treated with FDA-approved drugs. FibroGen announced positive and encouraging results, and combinatorial therapy with either of the FDA-approved drugs was deemed safe. A phase 3 study is planned to evaluate the drug's clinical potential for treating pulmonary fibrosis.

2.3.6. The PI3K/AKT/mTOR pathway.—One of the pathways dysregulated in IPF involves phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and mammalian target of rapamycin (mTOR) [112, 113]. It is associated with cell metabolism, growth, proliferation, differentiation, survival, and endoplasmic reticulum-related stress [113, 114]. Cell surface receptors, such as tyrosine kinase, activate p110, a catalytic unit of PI3K that converts phosphoinositide bisphosphate (PIP2) to phosphoinositide triphosphate (PIP3) to activate AKT and the pathway. In IPF tissues, expression of phosphatase and tensin homolog (PTEN) and caveolin-1 is suppressed in fibroblasts, and AKT activity is high, leading to fibroproliferation [115, 116]. PI3K p110 γ expression is also high, and in vitro inhibition studies reduced proliferation and α -SMA expression [112].

AKT effector molecules include BCL-2, Fox proteins, and mTOR. They suppress tuberous sclerosis complex (TSC1/2), which indirectly activates mTOR, a kinase that promotes fibroblast proliferation. Of its two complexes, mTORC1 and mTORC2, mTORC1 is sensitive to rapamycin and shows antiproliferative properties. Its phosphorylates, chiefly S6 kinase 1 (S6K1) and 4E-BP1, regulate protein translation. Our studies in TGF α mice showed that LY-2584702, an S6K inhibitor, decreased the subpleural fibrosis TGF α induces [117]. In IPF epithelial cells, phosphorylated S6K (p-S6K) levels are high, and Hippo/YAP signaling active. Expression of the active form of Yes-associated protein (YAP) in human bronchial epithelial cells showed that its stability requires high p-PI3K and p-S6K. Inhibiting p-S6K with temsirolimus blocked YAP entry into the nucleus, thereby suppressing YAP-mediated gene expression of CTGF, AXL, and AJUBA [118]. We propose that in IPF pathogenesis, the interaction of these two signaling pathways prompts cell proliferation and migration and prevents epithelial cell differentiation.

In the bleomycin model, rapid activation of PI3K/AKT and increased protein expression of hypoxia-inducible factor-1 α (HIF-1 α) and VEGF [119] contribute to fibroproliferation and collagen secretion. Studies on treatment with sirolimus, a rapamycin analogue in the bleomycin rat model, reduced IL-13, PDGF-A, and TGF β 1 levels and increased IFN- γ levels in BAL fluid. It also decreased hydroxyproline content [120]. A pilot study (NCT01462006) treated IPF patients with sirolimus and measured circulating fibrocytes since their number is correlated with disease progression. Fibrocytes are recruited into the lung based on CXCL12-CXCR4 chemistry, and mTOR signaling regulates their expression of receptors. Sirolimus significantly reduced the number of circulating fibrocytes, and safety profiles were acceptable [121]. A long-term study in IPF patients is planned.

Ompalisib (GSK2126458) is a highly selective dual inhibitor of PI3K and mTOR. Although originally developed in the oncology setting, it is now tested in IPF patients since the PI3K/mTOR pathway is extremely active in the fibrotic foci of their tissues. Treatment in precision-cut, IPF-tissue BAL cells showed dose-dependent reductions in p-AKT and

reduced proliferation of fibroblast cultures [122]. A phase 1B trial (NCT01725139) also showed p-AKT reduction in BAL, and the safety profile in IPF patients was very good [123].

The MAPK (mitogen-activated protein kinase) cascade is another major pathway deregulated in IPF. Our studies in a fibrotic mouse model demonstrate that targeting both the MAPK and PI3K pathways reduced fibrosis and collagen content more efficiently than targeting only one [124]. Thus, targeting several deregulated pathways in IPF may be advantageous.

2.3.7. Lysyl oxidase.—Excess deposition of extracellular matrix cells and tissue stiffening, largely by collagen I, are hallmarks of fibrosis. Two enzymes, transglutaminase 2 (TG2) and lysyl oxidase-like 2 (LOXL2), are primarily responsible for the formation and organization of collagen fibers. They have binding pockets for cytokines and integrins; the latter covalently links collagen and elastin fibers to maintain the tissue's mechanical force and modulate its stiffness. Serum levels in IPF patients are high and correlate with disease progression and mortality [125]. Immunohistological findings show increased staining for the enzyme in IPF explanted tissue compared to normal lung tissue [126]. Administration of murine monoclonal antibody against LOXL2 (AB0023) in the bleomycin model showed marked reduction in fibrosis, alveolar thickening, TGF β 1 and its signaling, ET-1, CXCL12, and LOXL2. It also reduced the number of fibrocytes in the lungs and the lymphocyte count in BAL as compared to vehicle-treated mice [126]. This study led to the discovery of simtuzumab, a humanized monoclonal antibody to LOXL2. Two phase 2 trials, RAINER (NCT01769196) and ATLAS (NCT01759511), were terminated due to its lack of efficacy. Pharmaxis and Synairgen developed a small-molecule LOXL2 antagonist, PXS-5382A that shown to reduce the fibrotic area as assessed by trichome staining in a murine model. A clinical trial is under way to establish its toxicity.

2.3.8. RhoA/ROCK.—Cytoskeletal elements are reorganized during wound healing. One of the major pathways in the context of actin assembly and actomyosin contraction is the rho-associated coiled-coil-forming protein kinase (RhoA/ROCK), which belongs to the serine-threonine kinase family. Its two isoforms, ROCK1 and ROCK2, are activated by rho GTPases residing downstream of many ligand/receptor pairs, such as LPA-LPA1, S1P-S1P1, thrombin-proteinase-activated receptor, and TGF β -RTK [127, 128]. ROCK activity is elevated in the fibrotic lesions of mice and IPF patients and involved in the profibrotic activation of fibroblasts and epithelial and endothelial cells at the injury site [129, 130]. ROCK inhibition studies with fasudil reduced the immune cells count in BAL fluid; production of the fibrotic markers TGF β 1, CTGF, α -SMA, plasminogen activator inhibitor-1 (PAI-1), and collagen; and the phosphorylated form of SMAD2/3 in the bleomycin model [131, 132]. In ROCK1/2 haplo-insufficient studies, Knipe et al. showed that both isoforms afforded comparable protection from bleomycin-induced injury [128]. Together, these preliminary studies show that inhibiting ROCK may benefit IPF patients. Consequently, Kadmon conducted a 24-week, phase 2, open-label trial with KD025, a selective inhibitor of ROCK2 in IPF patients formerly treated with pirfenidone or nintedanib (NCT02688647). The drug was well tolerated and improved lung function as measured by FVC with an absolute median difference of 127ml between the treated and nontreated groups.

3. Conclusion

Therapeutic inhibition of established and ongoing fibrosis remains a major challenge due to an incomplete understanding of cellular and molecular mechanisms that participate in the formation and maintenance of scar tissue. Recent advances have helped to characterize radiological and histological changes from the early to late stages of IPF. Fibrosis has been shown to begin in subpleural areas and progress into the parenchyma of the distal areas. Dysregulated growth-factor signaling in various lung cells (epithelial, mesothelial, endothelial, mesenchymal, macrophage) contributes to IPF. However, the roles molecular heterogeneity and cell-specific signaling pathways in non-mesenchymal cells play in myofibroblast activation remain unknown. A multipronged approach to disrupt abrupt signaling in various lung cells without interrupting regenerative processes could be a more viable strategy than targeting a single gene or enzyme. In support, the FDA-approved antifibrotic drugs pirfenidone and nintedanib target several pathways and gene networks.

Recent technological advances in identifying molecular signatures at the single-cell level will eventually enable us to map both cellular and molecular differences between IPF and normal lungs. Furthermore, use of systems biology tools will help us to identify an integrated antifibrotic strategy that will simultaneously target relevant pathways and/or gene networks to treat this highly complex and intransigent disease. Future research on the role of cell-specific transcription factors and other druggable targets in pulmonary fibrosis will enable evidence-based combination therapies can be selected for new clinical trials. We must also learn how to harness the beneficial mechanisms of lung development and inflammation, so damaged lung can be regenerated, the ultimate goal of fibrosis research in all organs.

4. Expert Opinion

Over the past two decades, many clinical trials on IPF failed to translate bench findings to the clinic. Despite appropriate study design and well-defined primary endpoints, mortality either increased or showed no change in the treatment arm compared to the placebo group. Studies on warfarin, ambrisentan, bosentan, macitentan, imatinib, corticosteroids, azathioprine, N-acetylcysteine, triple therapy (N-acetylcysteine, prednisolone, azathioprine), SAR156597, lebrikizumab, and carlumab are the best examples [95, 133–141]. These disappointments drive clinicians and researchers to improve their definitions of meaningful endpoints and disease parameters.

IPF is an epithelial-fibroblastic disorder, where fibroproliferation and myofibroblast formation and persistence cause excessive ECM deposition [2]. In late 2014, the FDA approved two IPF drugs, nintedanib and pirfenidone, under the brand names Ofev and Esbriet, respectively, which inhibit the key pathways of fibrosis, inflammation, and oxidation. Their exact mechanism of action is unclear, but they prolong IPF patients' survival.

Nintedanib (BIBF 1120) inhibits tyrosine kinases and targets VEGFR, PDGFR, FGFR, and TGF β -mediated signaling [142]. It also inhibits fibroblast-to-myofibroblast transformation and reduces fibrosis in bleomycin-and silica-induced models [143]. For approval, three

randomized, double-blinded, placebo-controlled clinical trials—TOMORROW (NCT00514683), INPULSIS-1 (NCT01335464), and INPULSIS-2 (NCT01335477)—were conducted. Nintedanib's effect on lung function decline was evaluated in 1,228 IPF patients at a dosage of 150mg twice a day for 52 weeks. The outcome was measured as reduction in decline of the lung's forced vital capacity (FVC) over a year as compared to placebo. All the three trials showed significant reduction in FVC decline, but only TOMORROW and INPULSIS-2 showed a significant delay of the first acute IPF exacerbation. Side effects included diarrhoea, nausea, and increases in liver enzymes, bilirubin, and bleeding due to VEGF blockage.

Pirfenidone is a pyridone molecule with as yet unexplained anti-inflammatory, anti-oxidative and antifibrotic properties. Recent studies have shown that it inhibits collagen fibril formation and/or the hedgehog signaling pathway by reducing the glioma-associated oncogene homolog protein (Gli 2) and its target genes [144, 145]. Results from a phase 1 trial (NCT02648048) with vismodegib, a molecule that like pirfenidone, inhibits the hedgehog pathway, have not been reported yet. However, in mouse lung fibroblasts, pirfenidone increased the expression of antifibrotic factors, such as nuclear factor-erythroid-related factor 2 (Nrf2), heme oxygenase-1 (Ho-1), and glutathione peroxidase 1 (Gpx1), to reduce oxidative stress [146]. The first trial on pirfenidone, conducted in Japan, established its feasibility for treating advanced pulmonary fibrotic patients, and a later study by Taniguchi et al. showed that it slowed the decline of lung vital capacity and improved patient survival. The results of the CAPACITY 004 and 006 and ASCEND clinical trials paved the way for its approval to treat IPF patients with adverse gastrointestinal events and photosensitivity. The long-term treatment study RECAP proved that the drug does not increase adverse events [147]. A trial of a therapy combining nintedanib with pirfenidone (INJOURNEY) showed acceptable adverse events and tolerability, but reports on lung function have not yet been analyzed [148].

IPF is difficult to treat because of the many distinct immunological and molecular mechanisms that initiate and maintain the progression of fibrotic lung diseases. Dysregulated signaling pathways and (myo)fibroblast activation are major contributors, and inhibiting a single pathway or growth factor in mouse models or human clinical trials has not been effective in attenuating established and ongoing lung fibrosis [1, 124, 149]. Recent success with nintedanib and pirfenidone, which target the various signaling pathways and cell types in IPF, has changed the landscape. Clinical trials are now dominated by combinatorial designs. Synergistic strategies that more completely arrest or reverse fibrosis are sorely needed and will require a better understanding of the molecular pathways that drive fibroblasts toward dysregulated matrix remodeling. We must also identify patient subtypes based on pathway-specific biomarkers and genetic phenotyping in clinical staging for these new combinatorial trials. Further, we must incorporate our new insights on lung development, regenerative medicine, and the beneficial aspects of inflammation, so we can regrow damaged tissue to duplicate the normal architecture of the lung.

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Article highlights

- Lung resident fibroblasts contribute to the major pool of myofibroblast in fibrotic lung lesions.
- Fibrocytes secrete paracrine factors and induce lung resident fibroblast activation.
- Transcription factors such as FoxF1, FoxM1 and WT1 play an important role in fibroproliferation and myofibroblast transformation.
- Hsp90 is a positive regulator of fibroblast activation and inhibition of Hsp90 ATPase activity attenuates pulmonary fibrosis.
- TGF β 1 is a major fibrogenic factor and improvement in strategies to inhibit TGF β 1-driven signalling may help to combat with fibrosis.
- Infiltration of immune cells and excessive Th2 cytokine production contributes to pulmonary fibrosis.
- Inhibition of multiple signalling pathways that are heightened in IPF lungs such as RhoA, ROCK, MAPK, PI3K, AKT and mTOR pathways may offer a therapeutic advantage with unclear mechanism of action.
- Elucidating the underlying mechanisms of the ECM clearance and the regenerative capacity in lungs are of interest to improve fibrosis resolution.
- Combinatorial therapeutic strategies are urgently needed that not only delay the progression but also reverse established and ongoing fibrosis in lungs.

Figure 1: Tissue fibrosis and associated diseases

Tissue fibrosis is a major cause of death worldwide due to organ failure as several chronic diseases are associated with severe fibrosis leading to organ failure due to impairment in the regulation of inflammation, metabolic pathways and infections. Monogenic mutations could lead to malformed proteins which can manifest into impaired tissue remodeling and fibrosis in chronic diseases such as Cystic Fibrosis (CF), Duchenne Muscular Dystrophy (DMD) and familial interstitial lung diseases (fILD).

Figure 2: The cellular pathway of fibrocyte-driven (myo)fibroblast activation in severe fibrotic lung disease.

Fibrocytes originate from monocyte progenitors and migrate to fibrotic lesions of injured lungs to secrete paracrine factors that can cause fibroproliferation and fibroblast-to-myofibroblast (FMT) transformation in the pathogenesis of pulmonary fibrosis.