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REVIEW

Pathogenesis of fibrosis in interstitial lung disease

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Purpose of review
Pulmonary fibrosis is a chronic and progressive lung disease involving unclear pathological mechanisms. The present review presents and discusses the major and recent advances in our knowledge of the pathogenesis of lung fibrosis.

Recent findings
The past months have deepened our understanding on the cellular actors of fibrosis with a better characterization of the abnormal lung epithelial cells observed during lung fibrosis. Better insight has been gained into fibroblast biology and the role of immune cells during fibrosis. Mechanistically, senescence appears as a key driver of the fibrotic process. Extracellular vesicles have been discovered as participating in the impaired cellular cross-talk during fibrosis and deeper understanding has been made on developmental signaling in lung fibrosis.

Summary
This review emphasizes the contribution of different cell types and mechanisms during pulmonary fibrosis, highlights new insights for identification of potential therapeutic strategies, and underlines where future research is needed to answer remaining open questions.

Keywords
epithelium, extracellular vesicles, fibroblasts, pulmonary fibrosis, senescence

INTRODUCTION

Pulmonary fibrosis is a dysregulated wound healing process in which injured epithelium does not heal adequately. Research in the field has impressively expanded during the last decade with significant acceleration of scientific reports in the late 2000s (Fig. 1). The current model to approach pulmonary fibrosis pathobiology starts in the distal lung epithelium [1]. Pathologic lung epithelium (caused by genetic, environmental exposure, aging, etc.) promotes chronic activation of immune cells and fibroblasts, key cells producing extracellular matrix (ECM) components. Impaired cellular cross-talk is a seminal mechanism during fibrosis. Experimental models including classical rodent models and more recent *ex-vivo* models based on patient-derived samples, significantly increased pulmonary fibrosis understanding [2,3]. This review discusses the most relevant advances made in the field over the last two years from burning topics in the community. Keywords corresponding to the selected articles are shown in Fig. 2.

LUNG EPITHELIUM: WHY IS IT OUT OF CONTROL?

Epithelial cells represent the first line of defense to protect the lung from external insults. Default in lung epithelium is actively investigated as it appears to drive physiological wound healing toward an uncontrolled fibrotic process.

Distal epithelium is mainly composed of alveolar epithelial type I (ATI) cells which can be regenerated by differentiating alveolar epithelial type II (ATII) cells upon injury. ATII/ATI transition has recently been proposed as a two-step mechanism involving a TGF-β1-dependent increased expression of Keratin-8 [4▪▪], a component of intermediate filament in epithelial cells often associated with metastatic cells; and a decrease in TGF-β signaling and subsequent *Krt8*. Accumulation of KRT8 positive cells, along with the well-known aberrant production of TGF-β1, is consistent with impaired ATII/ATI transition in which ATII cells are locked into a transitional state

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KEY POINTS

- Keratin-8 is expressed and characterizes the abnormal and activated ATII cells.
- Fibroblasts have major transcriptional changes during fibrosis and participate to immune cell interaction via the PD-L1 pathway.
- Unbiased approaches identify immune cells as critical in fibrosis.
- Senescence and aging represent a major driver of the fibrotic process.
- Extracellular vesicles are novel players in pulmonary fibrosis.

explaining their accumulation in idiopathic pulmonary fibrosis (IPF) lung. Further investigation is required to assess the translation of these findings to human IPF. A better understanding of this KRT8-positive population might hold promise to develop strategies to redirect inefficient repair observed in IPF toward a functional regeneration.

Endoplasmic reticulum stress is a common feature of enhanced proliferation in cells thanks to increased protein synthesis and turnover. Endoplasmic reticulum stress is closely linked to the unfolded protein response regulated by signaling such as the ubiquitin system. Deletion of the E3 ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4-2) in lung epithelium triggers lung fibrosis with distal airway remodeling [5]. Fibrotic tissue of Nedd4–2$^{-/-}$ mice share common pattern with IPF lung tissue. Same effect was observed upon deletion of *Grp78* (endoplasmic reticulum chaperone)

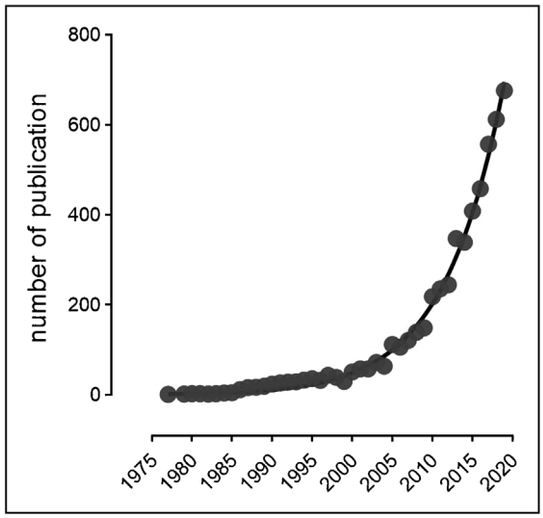

FIGURE 1. Publication number per year with the keywords 'Lung + fibrosis + IPF.' Data source: PubMed.

in cells expressing the *Sftpc* gene (encoding surfactant protein C, classical ATII marker) along with apoptosis and senescence in ATII cells [6]. *Grp78* deficiency reduces proliferation and colony-forming efficiency in ATII cells. Inhibiting endoplasmic reticulum stress with tauroursodeoxycholic acid restores the effects of *Grp78* deficiency with decreased fibrotic markers in mouse tissue as well as in lung slices generated from IPF patients. Presence of spliced Xpb1, marker of endoplasmic reticulum stress, correlates with increased *Muc5b* in mouse distal airway [7]. Given that the gain of function rs35705950 *MUC5B* polymorphism represents the strongest genetic factor associated with IPF, the potential of endoplasmic reticulum stress inhibition needs to be further investigated.

Significant transcriptomic changes were observed in cells of BAL from IPF patients, with a signature associated with disease mortality [8]. This signature revealed enrichment for classical genes expressed by airway basal cells which were not found in lavage from patients with chronic obstructive pulmonary disease (COPD) or sarcoidosis. Basal cells constitute a progenitor reservoir within the lung capable to give rise to all types of pulmonary epithelial cells. Although their exact role is unclear, they emerge as critical contributors in pulmonary fibrosis. Further work is warranted to uncover whether airway basal cells might have potential to promote an efficient repair in IPF.

FIBROBLASTS AND EXTRACELLULAR MATRIX: A CONNECTED COUPLE

Fibroblasts are seminal cells in fibrosis, responsible for ECM production. Activated fibroblasts undergo reprogramming with substantial transcriptional changes. Forkhead box F1 (FOXF1) belongs to a family of transcription factor regulating cell growth, organ development, and repair. FOXF1 is decreased in IPF fibroblasts and its genetic deletion in α-SMA-expressing cells worsens bleomycin-induced fibrosis in mice [9]. FOXF1 loss favors myofibroblast activation by repressing the adhesion molecule *CDH2* and enhanced *CDH11* (also known as osteoblast-cadherin). FOXF1^{neg}/CDH11^{pos} fibroblasts have increased migratory and invasiveness properties. Similarly, FoxO3, another Forkhead box family member, is downregulated in fibrotic fibroblasts [10]. *FoxO3*$^{-/-}$ mice are more susceptible to bleomycin-induced fibrosis. FoxO3 activation blocks fibrogenesis after bleomycin *in vivo* and hampers ECM production by IPF fibroblasts. A transcriptomic profiling of lung fibroblasts isolated from mice challenged with either bleomycin or silica identifies additional major hubs of transcription factors [11]. One of them relies on the transcription factor sterol regulatory element-

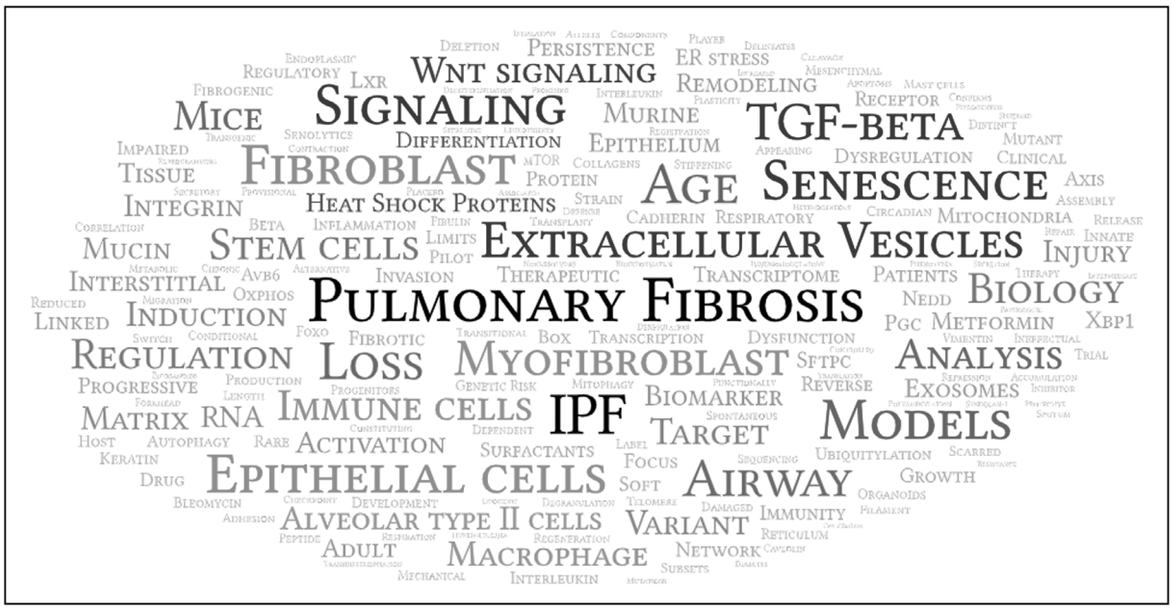

FIGURE 2. Word-could generated with the title of the reviewed references. Cellular components are highlighted in green and fibrosis mechanisms indicated in blue.

binding protein 1 (SREBP-1c), activated by liver X receptor (LXR). Its expression decreases during pulmonary fibrosis and LXR agonists, resulting in SREBP-1c activation, diminish fibroblast activation, and fibrosis progression in fibrotic mice.

During IPF, fibroblasts expressing Wilms' tumor 1, a classical transcription factor in mesothelial cells, accumulate within the lung [12]. Wilms' tumor 1 binds the α-SMA promoter to induce myofibroblast transformation. The adenovirus-mediated transfer of *Wt1* gene induces fibrosis and $Wt1^{+/-}$ mice exhibit noticeable decreased fibrosis upon bleomycin or TGF-α expression. *Wt1* is expressed in pleural mesothelial cells during development. In line with the typical subpleural onset of fibrotic lesions observed in IPF patients, we and others have demonstrated the role of mesothelial cells in IPF, which activate and migrate from the pleura toward the lung parenchyma to promote fibrosis [13–16]. Altogether, these data highlight the need for deeper investigation focusing on Wilms' tumor 1 as a potential therapeutic target in IPF.

Recent literature emphasizes mitochondria disorders in IPF fibroblasts. Defective peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARγCoA1), a regulator of mitochondria biogenesis already unraveled in ATII cells during fibrosis, is observed in lung fibroblasts in mice challenged with bleomycin as well as in IPF [17]. In the bleomycin model, PPARγCoA1 expression return to normal prior to fibrosis resolution in young mice whereas it does not recover in old mice in which fibrosis does not resolve.

Transcriptome changes are often associated with dysregulation of the cellular machinery. CFIm25, a regulator of alternative polyadenylation, is downregulated in IPF [18]. In normal fibroblast, CFIm25 silencing affects key profibrotic signaling [(TGF-β, wingless/integrase-1 (Wnt)] and its deletion in *Col1a1*-expressing cells worsens bleomycin lung toxicity. Consequently, CFIm25 overexpression in IPF fibroblasts results in decreased expression of profibrotic markers. These findings need to be further investigated in different cell types and to understand potential side-effects which might be driven by alternative polyadenylation activation.

Interactions between fibroblasts and ECM are key to promote their activation. Hyaluronan and fibrinogen are localized at the interplay between myofibroblasts and damaged alveolar epithelium, suggesting their importance in driving fibrosis progression [19]. More research is needed to clarify whether these two ECM components might represent therapeutic targets in IPF. Integrins, such as αvβ3, are key players in cell to ECM interactions. Engagement of αvβ3 with ECM promotes fibroblast contraction and stiffness, contributing to fibrosis [20]. αvβ3 is enhanced upon loss of the cell surface protein Thy-1. Interestingly, *Thy-1* downregulation is associated with acute lung injury and aging.

Vimentin is another key component promoting fibroblast invasive properties. Withaferin A, a pleiotropic inhibitor impacting vimentin intermediate filaments assembly, nuclear factor Kappa B, and heat shock protein HSP90, with known anticancer activity, counteracts invasiveness and promotes autophagy in

IPF fibroblasts [21]. Withaferin A hampers bleomycin-induced fibrosis in mice and decreases fibroblast invasion capacity.

Expression of caveolin-1, a scaffolding protein in the plasma membrane with seminal role in cellular trafficking, is attenuated in IPF and its loss correlates with disease progression in animal models. Small peptides mimicking caveolin-1 hampers fibrosis in experimental rodent models with reduced ECM and fibrosis markers and increased ATII survival [22▪]. These findings have been confirmed using lung tissue from patients with end-stage IPF, in which caveolin peptide attenuates fibrosis hallmarks.

Fibroblast activation is believed to be driven by soluble factors. IL-11 is over-produced in the lung of IPF patients via its secretion by fibroblasts [23▪]. IL-11 activates fibroblasts resulting in increased α-SMA, collagen, and TGF-β signaling. IL-11 overexpression results in spontaneous fibrosis, whereas anti-IL-11 or genetic deletion of IL-11 receptor protected from bleomycin-induced lung injury.

Resistance to apoptosis via senescence is belived to drive fibroblasts accumulation in IPF. The silencing of MyoD, a dysregulated transcription factors in fibrotic fibroblasts, restores myofibroblast susceptibility to apoptosis [24]. The inhibition of MyoD restores fibrosis resolution in aged mice subjected to bleomycin. Further work is required to deeply understand the precise mechanism regulating the fate of senescent cells in normal conditions.

UNRAVELING THE ROLE OF IMMUNITY IN THE GAME

The importance of immunity in fibrogenesis remains unclear and the working model of IPF pathobiology was not always supportive for a role of the immune compartment in IPF. Transcriptomic analyses all share the mutual point of showing an immune cell signature in IPF [25,26▪,27,28]. Their activation is observed in dense fibrotic areas and not in normal tissue of the lung of IPF patients [25] suggesting their role at sites where active fibrosis occurs.

In IPF fibroblasts, the immune checkpoint protein programmed cell death ligand 1 (PD-L1) is upregulated in invasive fibroblasts over noninvasive fibroblasts and its expression is necessary to induce fibrosis in severe combined immuno deficient mice [29▪]. TGF-β induces PD-L1 expression in fibroblasts and its secretion via extracellular vesicles [30]. Consequently, PD-L1 siRNA decreases TGF-β-driven induction of fibrosis markers. The link between PD-L1 and fibroblast activation/invasiveness suggests that pathological fibroblasts need to evade the immune system to promote fibrosis progression.

In IPF, programmed cell death protein 1 (PD-1) is overexpressed by $CD4^{+}$ positive T cells [31]. These $PD\text{-}1^{high}/CD4^{pos}$ T cells exhibit enhanced TGF-β expression consistent with a Th17 subtype phenotype and promote ECM production when co-cultured with lung fibroblasts. PD-1 regulates signal transducer and activator of transcription 3 and their blockade hampers collagen production *in vivo*. The potential for using PD-1 inhibitor has been confirmed by challenging PD-1 knockout mice to bleomycin. This important study paves the way to propose immune checkpoint inhibitors, already developed and major treatments in the cancer field, to mitigate pulmonary fibrosis development.

Cells belonging to innate immunity are also actively investigated in the field. Mast cells accumulate in IPF lung and correlate with disease severity [32]. Mechanical stretch, as occurring in IPF lung, promotes their degranulation. The use of the mast cell stabilizer cromoglycate diminished pulmonary fibrosis induced by TGF-β overexpression. Macrophages seem also to be aberrantly activated and exhibit pathological mitochondria during IPF with production of mitochondrial ROS [33]. How ROS contribute to increase pulmonary fibrosis is still unclear and warrants further investigation. A subset of macrophages was identified based on the transferrin receptor CD71 expression [34]. $CD71^{neg}$ macrophages have upregulated fibrotic genes and correlate with worse survival in IPF.

THE RISE OF AGING

Genetic studies on large cohort of patients with IPF and control patients have linked variants of senescence genes with increased IPF risk [35]. Mutations of the telomere-related gene regulator of telomere elongation helicase 1 (*RTEL1*) have been identified in patients with IPF [36]. RTEL1 staining in IPF lung shows expression in epithelial and immune cells but not in fibroblastic foci.

In parallel, aged ATII cells have lower *Sftpc* expression along with increased WNT/β-catenin activity [37]. Chronic WNT/β-catenin activation induces senescence in ATII cells along with a profibrotic phenotype accompanied by *Krt8* expression. In fibroblasts, senescence is believed to explain apoptosis resistance in pulmonary fibrosis. Senescence also induces paracrine secretion of several factors known as senescence-associated secretory phenotype (SASP). Leukotrienes are aberrantly produced by senescent IPF fibroblasts and promote fibrosis after bleomycin exposure in mice [38]. Bone-marrow-derived mesenchymal stem cells isolated from IPF patients also exhibit senescence along with significant DNA damage as well as mitochondrial and

cellular dysfunction [39]. These studies warrant further investigation on senescence and the secretion of SASP components to understand their role, cellular origin, and identify potential targets in pulmonary fibrosis.

To induce the clearance of senescent cells, the senolytic drug combination dasatinib (multiple tyrosine kinases inhibitor) and quercetin (antioxidant) has been investigated in a phase I study in IPF patients [40▪▪]. These drugs, already showing encouraging data in the bleomycin model, may alleviate physical dysfunction in patients with IPF. However, whether dasatinib-quercetin combination impacts SASP components production remains unknown.

mTOR is a key cellular signaling able to drive senescence. A phase I study has investigated a phosphoinositide 3-Kinases/mTOR inhibitor in patients with IPF, showing acceptable tolerability and target engagement [41]. mTOR activation is tightly regulated with the cell bioenergetic sensor and metabolic regulator AMPK pathway. AMPK signaling is downregulated in IPF fibroblasts, along with mTOR activation [42▪]. The AMPK activator Metformin, widely used for treatment of type 2 diabetes, inhibits myofibroblast differentiation and decreases hydroxyproline accumulation in the lung of mice exposed to bleomycin. Further research demonstrates that Metformin drives myofibroblasts to transdifferentiate and acquire a lipofibroblast phenotype [43]. A post-hoc analysis to evaluate the effect of Metformin in IPF patients was carried out but failed to show any effect of Metformin on clinically relevant outcomes [44].

Altogether, senescence appears to play a significant role in IPF. Inhibition strategies need to be further investigated to be proposed as potential therapeutic option. Senescence phenotype has been observed in many cell types, mainly fibroblasts and epithelial cells, within IPF lung. As senescence can spread via the secretion of SASP component, a better understanding of the underlying biology during IPF is needed. Whether senescence starts in a specific cell type and subsequently contaminates neighboring cells remains to be explored.

EXTRACELLULAR VESICLES, NEW PLAYERS IN THE FIELD

Extracellular vesicles, including exosomes, are lipidic structures released by all cells as an intercellular communication system. Extracellular vesicles abberently accumulate in BAL from IPF patients [45▪▪]. Interestingly, BAL-extracellular vesicles from IPF patients harbor increased levels of WNT5A, a known profibrotic molecule expressed by fibroblasts. Extracellular vesicle secretion seems also altered in sputum collected in patients with IPF [46]. Analysis of sputum-derived extracellular vesicles revealed specific miRNA in patients with IPF compared with healthy donors. Syndecan-1 is overexpressed in the lung of IPF patients, mainly in epithelial cells, and has a key role in controlling miRNA cargo in extracellular vesicles during pulmonary fibrosis [47]. *Sdc1*$^{-/-}$ *m*ice produce extracellular vesicles with an altered miRNA cargo compared with wildtype mice, in line with the secretion upon *Sdc1* overexpression of profibrotic extracellular vesicles promoting TGF-β and Wnt signaling in receiving cells. Although significant advances have been made, more work is needed to decipher how extracellular vesicles contribute to fibrosis and whether they can serve as potential biomarker. Identification of relevant cellular cross-talk appears as a central prerequisite to better evaluate whether inhibition of extracellular vesicle secretion may represent a therapeutic strategy in IPF.

Extracellular vesicles are also studied as vector for potential therapeutic approaches. In the bleomycin fibrosis model, nebulization of extracellular vesicles produced from mesenchymal stem cells impairs fibrosis and promotes resolution with decreased collagen accumulation and (myo)fibroblast differentiation [48].

DEVELOPMENTAL SIGNALING, WHEN REACTIVATION OCCURS IN ADULTHOOD

Reactivation of developmental signaling pathways is a major hallmark of IPF. A role of the matricellular ECM component Fibulin-1c was described in pulmonary fibrosis [49]. Mice with Fibulin-1c deletion are protected from bleomycin-induced lung toxicity. Fibulin-1c interacts with the latent TGF-β binding protein to induce TGF-β activation. We have recently characterized the role of Tripartite Motif Containing 33 (TRIM33), an E3 ubiquitin ligase facilitating Smad4 nuclear export. TRIM33 deletion exaggerates fibrosis induced by bleomycin exposure [50]. Together, identifying targets to control TGF-β signaling remains an active area of investigation. The rational for a total inhibition of TGF-β signaling raises questions. Most likely, TGF-β will need to be tightly regulated and mechanisms explaining why this signaling is out of control remain to be clearly identified.

Recently, the Hippo – yes-associated protein (YAP)/Tafazzin (TAZ) signaling has gained significant attention in IPF. Epithelial cells exhibit increased YAP activity during IPF [51]. Overexpression of YAP in bronchial epithelial cells promotes cell proliferation and migration, in line with abnormal cellular activation/differentiation observed in ATII during IPF. TAZ inhibition blocks ATII/ATI transition, consistent with the hypothesis that YAP/TAZ signaling is important to regenerate the

lung epithelium after injury [52▪]. In fibroblast, YAP binds the Twist1 promoter enhancing proliferation, migration, and ECM components production [53]. Transfer of shRNA targeting YAP1 ameliorates fibrosis after bleomycin exposure in mice. The growing body of literature focusing on YAP/TAZ pathway shows many aspects of the signaling in epithelial or fibroblast cells. Better understanding is warranted to propose YAP/TAZ inhibition as a potential therapeutic approach for IPF.

CONCLUSION

Recent literature has deepened our understanding about the mechanisms involved in pulmonary fibrosis. The ATII cell transitional state has been better defined and factors involved in fibroblast activation might hold promise to serve as therapeutic options. The role of immune cells has been further investigated. The senescence field moved forward with early human testing of potential therapies. Extracellular vesicles were identified as key players in inter-cellular cross-talk and means to regulate developmental signaling are still actively researched. Further investigations are needed to understand whether this knowledge can translate into new therapeutic strategies to control pulmonary fibrosis progression.

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Conflicts of interest

O.B., P.S.B., G.B., and F.G. have nothing to disclose. P.B. reports personal fees for advisory board work and travel support for meeting attendance from Roche and Novartis, personal fees for advisory board work and reimbursement of meeting registration from Boehringer, personal fees for advisory board work from TEVA and AstraZeneca, travel support for meeting attendance from Chiesi, reimbursement of meeting registration from Stallergene, all outside the submitted work.

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