Mesenchymal cells in the Lung: Evolving concepts and their role in fibrosis

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Keywords:
Mesenchymal cells
Pulmonary fibrosis
Myofibroblast
Differentiation
Fibrosis resolution

A B S T R A C T

Mesenchymal cells in the lung are crucial during development, but also contribute to the pathogenesis of fibrotic disorders, including idiopathic pulmonary fibrosis (IPF), the most common and deadly form of fibrotic interstitial lung diseases. Originally thought to behave as supporting cells for the lung epithelium and endothelium with a singular function of producing basement membrane, mesenchymal cells encompass a variety of cell types, including resident fibroblasts, lipofibroblasts, myofibroblasts, smooth muscle cells, and pericytes, which all occupy different anatomic locations and exhibit diverse homeostatic functions in the lung. During injury, each of these subtypes demonstrate remarkable plasticity and undergo varying capacity to proliferate and differentiate into activated myofibroblasts. Therefore, these cells secrete high levels of extracellular matrix (ECM) proteins and inflammatory cytokines, which contribute to tissue repair, or in pathologic situations, scarring and fibrosis. Whereas epithelial damage is considered the initial trigger that leads to lung injury, lung mesenchymal cells are recognized as the ultimate effector of fibrosis and attempts to better understand the different functions and actions of each mesenchymal cell subtype will lead to a better understanding of why fibrosis develops and how to better target it for future therapy. This review summarizes current findings related to various lung mesenchymal cells as well as signaling pathways, and their contribution to the pathogenesis of pulmonary fibrosis.

1. Mesenchymal cells and mesenchymal cell heterogeneity

Mesenchymal cells refer to the broad category of stromal cells predominantly responsible for synthesis of connective tissue (Andrzejewska et al., 2019; Gottipamula et al., 2018; Spees et al., 2016). Derived from the mesoderm, mesenchymal stem cells or stromal cells have the capacity to differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes, depending on the type of terminally differentiated tissue (Andrzejewska et al., 2019; Gottipamula et al., 2018; Spees et al., 2016). In the lung, these mesenchymal cells are often referred as fibroblasts or myofibroblasts, and they provide structural support and survival cues to the adjacent bronchial and alveolar epithelium (Fang et al., 2019; Sveiven and Nordgren, 2020; Raslan and Yoon, 2020). Mesenchymal cells play key roles in lung growth, alveolar differentiation, and development (Nasri et al., 2021; McCulley et al., 2015; Mizikova et al., 2020). Recent studies, however, suggest that "lung fibroblasts" or "lung mesenchymal cells" are themselves quite diverse, with a variety of different functions and gene expression signatures that vary by location (Liu et al., 2021; Danopoulos et al., 2020; Narvaez del Pilar et al., 2022; Sountoulidis et al., 2022) (Table 1). During lung development, multipotent mesenchymal progenitors that are characterized as Gli1+ Wnt2+ Isl1+, give rise to multiple mesenchymal cell lineages in the adult lung, including alveolar fibroblasts, smooth muscle cells, and pericytes, which are adventitial fibroblasts that closely associate with blood vessels (Peng et al., 2013). Studies have also identified lung mesenchymal cell progenitors as FoxD1 positive, which give rise to collagen-expressing mesenchymal cells associated with blood vessels and airway epithelium (Barron et al., 2016). With the exception of smooth muscle cells, which are important structural components of the airways and the pulmonary vasculature, and whose transcriptome and tissue distribution are well documented (Amrani and Panettieri, 2003; Townsley, 2012; Lam et al., 2019; Gorr et al., 2020; Saygin et al., 2020), the precise function, tissue localization, and transcriptomic profiling of many lung mesenchymal subtypes are just beginning to be elucidated.

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Table 1
Lung mesenchymal cell heterogeneity. Mesenchymal cells residing in different lung locations and expressing distinct marker genes. CTHRC1 marks a population of alveolar fibroblasts implicated in collagen production and pathogenic remodeling of fibrotic mouse and human lungs.

Location	Marker	Reference
Airways	AXIN2, PDGFRB	(Zepp et al., 2017)
	LGR6, αSMA	(Lee et al., 2017)
	ACTA2, TAGLN	(Xie et al., 2018)
Alveoli	AXIN2, WNT2, PDGFRA	(Zepp et al., 2017)
	COL1A1, COL13A1, COL14A1	(Xie et al., 2018)
	LGR5	(Lee et al., 2017)
	COL13A1, PLIN2, LPL	(Park et al., 2019)
	CTHRC1, COL1A1 (Pathogenic fibroblasts)	(Tsukui et al., 2020)
Blood vessels	FOXD1	(Hung et al., 2013)
	NG2, FOXJ1	(Rock Jason et al., 2011)
	Gli1	(Kramann et al., 2015)

Single-cell RNA-sequencing and lineage tracing studies in mice have now been able to shed greater light on the heterogeneity of mouse mesenchymal cells or fibroblasts in healthy and fibrotic lungs. For example, Zepp and colleagues were among the first to identify two transcriptional and spatially distinct mouse mesenchymal lineages – one in the alveolar niche and the other associated with the airway. (Zepp et al., 2017). Although both mesenchymal lineages were defined as Axin2+, alveolar mesenchymal cells expressed the markers Wnt2 and Pdgfra, whereas those located in the airways were enriched for Pdgfrb. Pdgfra was previously identified as a marker of progenitor cells of the lung mesenchyme with an important role during lung development and fibrosis (Liu et al., 2021; Boström et al., 1996; Li et al., 2018; Lindahl et al., 1997; Biasin et al., 2020), suggesting that distinct progenitors may give rise to mesenchymal cells in the alveoli versus the airway.

Work by Lee and colleagues (Lee et al., 2017) identified Lgr5^{+} and Lgr6^{+} mesenchymal cells in the mouse lung, that were transcriptionally similar to the Axin2-expressing cells identified by Zepp and colleagues, and that shared a similar distinction between mesenchymal cells in the airways versus alveolar space. Lgr6 mainly marked a population of mesenchymal cells surrounding conducting airways, with limited expression among mesenchymal cells in the alveolar space. Lgr6 positive cells nearby conducting airways also express α-smooth muscle actin (αSMA), a marker of smooth muscle cells, thus are thought to serve as myofibroblast progenitors, whereas those located in the alveolar space were negative for this marker. Similarly, alveolar mesenchymal cells identified by Zepp and colleagues as Axin2+, Wnt2+ Pdgfra+, were found by Lee and coworkers to also exclusively express Lgr5. The Lgr5+ alveolar mesenchymal cells were found to support alveolar differentiation and appear to be distinct from other Pdgfra^{+} progenitor cells, which are important for AT2 self-renewal and differentiation (Lee et al., 2017). Axin2+ Pdgfra+ alveolar mesenchymal cells are located in close proximity to alveolar epithelial cells, and similarly to Lgr5+ cells, they can support alveolar differentiation (Zepp et al., 2017), whereas Axin2+ Pdgfra- cells, which were found to be located near the conducting airways, mainly contributed to fibrogenic myofibroblasts. Xie and colleagues also described two mouse lung mesenchymal populations expressing myofibroblast (*Acta2*, *Tagln*), and matrix -derived (*Col13a1*, *Col14a1*) markers, and whose transcriptional signatures largely overlap with those of Axin2+ Pdgfra- and Axin2+ Pdgfra+ mesenchymal progenitors respectively (Xie et al., 2018). All together, these analyses revealed the presence of multiple mesenchymal cell populations in airway *vs* alveolar compartments that express different markers and are derived from different lineages. Whether these populations of mesenchymal cells possess distinct functions and contribute to divergent pathology remains to be determined, but the fact that they are found in anatomically distinct locations suggest that their functions may indeed be unique and differ during both homeostasis and during repair and regeneration (Fig. 1).

Mesenchymal cells by nature secrete collagen and maintain extracellular matrix (ECM) and this function is also often used to better understand the functional heterogeneity of these cells. Using the Col1a1-GFP transgenic mice, we previously showed that Col1a1+ mesenchymal cells represent approximately 5 % of the whole lung cells, and they are distributed in different lung locations, including peribronchial regions and alveoli nearby large vessels and capillaries (Ligresti et al., 2019). Using the same collagen-GFP mouse model, Tsukui and colleagues performed scRNA-seq together with immunostaining analysis to characterize collagen-expressing cells in normal and fibrotic mouse lungs (Tsukui et al., 2020). These analyses confirmed the presence of multiple subpopulations of Col1a1-expressing cells with distinct transcriptional signatures and unique anatomic locations (Tsukui et al., 2020). Col1a1+ cells were mainly located in the alveolar space, adventitial cuffs surrounding large airways, and arteries in bronchovascular bundles. Interestingly, Col1a1+ located nearby airways were also enriched for the myofibroblast/SMCs progenitor markers Lrg6 and Axin2 similarly to those reported by Lee and coworkers (Lee et al., 2017; Tsukui et al., 2020), suggesting that airway injury and repair is also characterized by mesenchymal cells that either acquire or differentiate into high-collagen producing and secreting cells. Collagen-I is not the only matrix protein synthesized by lung mesenchymal cells as Col1a1+ cells in the alveolar space also expressed at high levels Col13a1 and Col14a1.

One particular subpopulation of Col1a1+ cells found almost exclusively in fibrotic lungs is characterized by the expression of collagen triple helix repeat containing 1 (*Cthrc1*); these cells were responsible for some of the highest levels of collagens suggesting that Cthrc1 + cells may be key culprits in the exuberant collagen production characteristic of fibrosis.

Finally, lipofibroblasts are mesenchymal cells enriched in lipid vesicles that were previously described in mouse lungs (Liu et al., 2021; Park et al., 2019; McGowan and Torday, 1997; Opitz et al., 2017; Riccetti et al., 2020). They are involved in multiple important functions in the lung, such as surfactant synthesis and vitamin A storage (McGowan and Torday, 1997; Opitz et al., 2017; Riccetti et al., 2020,; Dirami et al., 2004). A study by Ntokou and colleagues reported that during lung maturation the large majority of lipofibroblasts derived from Pdgfra+ progenitor cells (Ntokou et al., 2015). A recent paper by Li and colleagues, however, indicated that only 5% of lung lipofibroblasts were derived from Pdgfra + progenitors (Li et al., 2018), and other studies report that lipofibroblasts may be derived from Tbx4- and FGF10-expressing progenitors that give rise to pathogenic myofibroblasts in the adult lung following injury (El Agha et al., 2014; Zhang et al., 2013). A subpopulation of Col13a1+ cells also express *Plin2* and *Lpl*, markers now identified as lipofibroblasts. The expression of Col13a1+ in lipofibroblasts indicates the potential contribution of this type of collagen in matrix remodeling.

2. Mesenchymal cells in lung injury and repair

As opposed to other organs, the amount of connective tissue in the lung must remain minimal and restrained to allow for optimal gas exchange and diffusion of oxygen from the alveoli to the capillaries. However, by virtue of its exposure to the environment, the lung is also susceptible to damage and injury, and mesenchymal cells are necessary for wound healing and repair. For the rest of this review unless otherwise stated, we will limit our discussion of mesenchymal cells to fibroblasts and myofibroblasts predominantly in the lung parenchyma and their roles in contributing to alveolar repair and lung fibrosis. A review of mesenchymal cells that contribute to airway fibrosis, as can be observed in asthma, COPD, bronchiectasis, and constrictive and obliterative bronchiolitis is beyond the scope of this review and are reviewed elsewhere (Nasri et al., 2021; Bonner, 2010). Nonetheless, many of the same concepts and mechanisms that lead to mesenchymal cell activation

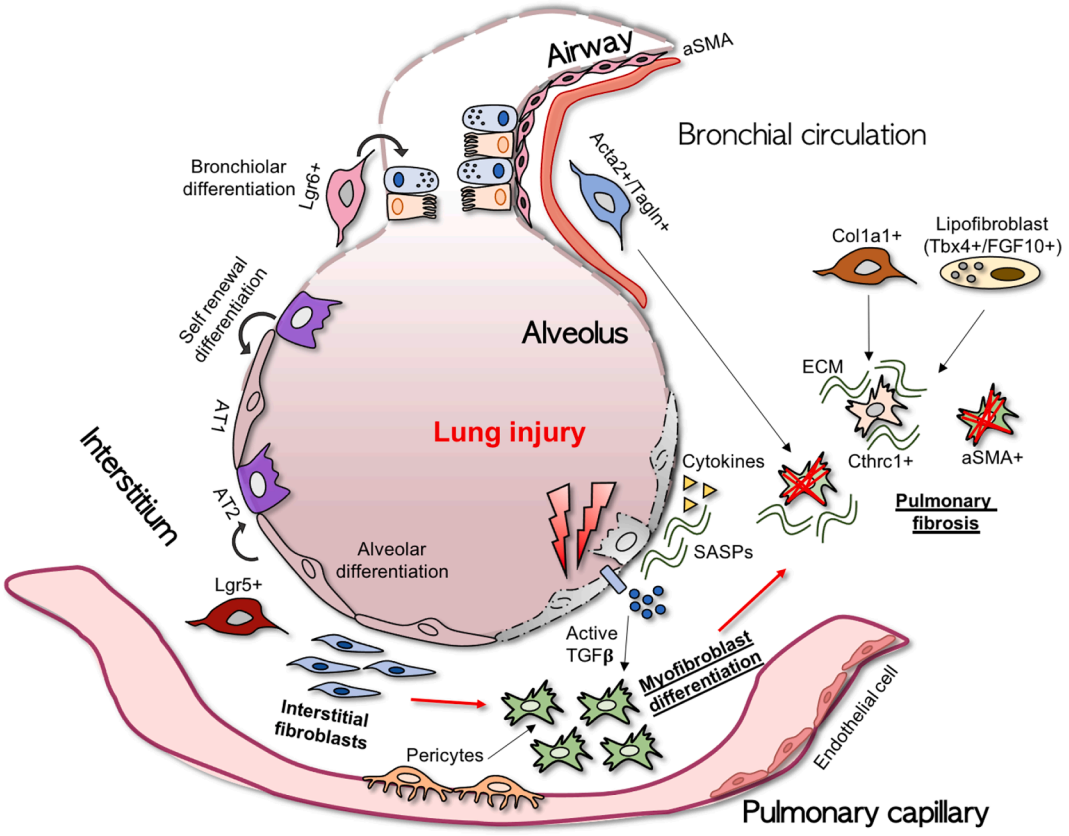

Fig. 1. Lung mesenchymal cell spatial distribution and differentiation in response to injury. Distinct lung mesenchymal cells reside in different locations and give rise to activated myofibroblasts in response to injury.

and fibrosis of the parenchyma can be seen in airway and vascular compartments of the lung.

Multiple environmental insults, from infection to chemicals and toxins to pollutants, contribute to lung inflammation and injury (Eckhardt and Wu, 2021; Wong et al., 2016; Peters et al., 2021). If the injury is severe enough, mesenchymal cells play crucial roles in the repair process. Myofibroblasts, by virtue of their contractile properties and ability to secrete large amount of ECM, are the major mesenchymal cell type that are activated during lung development and repair following injury (Hinz et al., 2007; Kim and Vu, 2006; Betensley et al., 2016; Darby et al., 2016). Inherent in its name, "myofibroblast" is often defined as fibroblast with contractile properties *via* its expression of smooth muscle markers, including alpha-smooth muscle actin (αSMA) or smooth muscle 22α (SM22α), and are capable of mechanically alter the surrounding tissue (Hinz et al., 2007; Darby et al., 2016; An et al., 2017). Myofibroblasts often accumulate in high numbers especially in pulmonary fibrosis or in other mouse models of lung injury (Mei et al., 2021; Moore and Hogaboam, 2008; Moss et al., 2022; Liu et al., 2017). In the prototypical fibrotic lung disease idiopathic pulmonary fibrosis (IPF), αSMA-expressing cells are often observed in fibroblastic foci (FF), which are areas of active ECM matrix deposition considered the leading edge of fibrosis (Betensley et al., 2016; Bartis et al., 2014; Jones et al., 2016). Based on these findings, αSMA-expressing myofibroblasts are often believed to be the major source of collagen in fibrotic lungs and understanding what activates them and how they are regulated is a major focus of research in lung injury and repair (Fig. 1).

3. Signaling pathways implicated in myofibroblast differentiation

Myofibroblasts are traditionally considered "activated" fibroblasts derived from the differentiation of quiescent fibroblasts via the activation of multiple signaling pathways and transcriptional regulators (Fortier et al., 2021; Froidure et al., 2020; Gibb et al., 2020). Transforming growth factor (TGFβ) is considered one of the most potent and prototypical mediators that promotes myofibroblast differentiation, enhances contractility and ECM protein production (Fortier et al., 2021; Froidure et al., 2020).

The role of TGFβ and the downstream signaling pathways that promote myofibroblast activation have been extensively described in other reviews (Chanda et al., 2019; Frangogiannis, 2020; Piersma et al., 2015). TGFβ is known to signal through both SMAD-dependent and SMAD-independent pathways and all these pathways are known to play an important role in pulmonary fibrosis (Chanda et al., 2019). Other mediators known to stimulate myofibroblast differentiation, including lysophosphatidic acid (LPA), have also been shown to act, at least partially, via TGFβ and downstream SMAD signaling (Cabello-Verrugio et al., 2011; Jeon et al., 2008). The mechanoregulators yes-activated protein (YAP) and tafazzin (TAZ) are known to alter myofibroblast activity by modulating TGFβ signaling, as YAP- and TAZ-deficient lung fibroblasts have been shown to be less responsive to TGFβ and exhibit reduced contractile forces and ECM protein deposition (Tschumperlin et al., 2018). Because TGFβ is secreted in latent form in the ECM, cleavage and activation of TGFβ by integrins is known to play an important role in regulating myofibroblasts (Nishimura, 2009). Indeed, integrin-mediated activation of latent TGFβ to its active form was demonstrated to be a critical step in the initiation and maintenance of this signaling pathway (Nishimura, 2009), and integrin β6 knockout mice exhibit reduced fibrosis following bleomycin treatment due to a reduction of the biologically active TGFβ (Horan et al., 2008).

Additional signaling pathways, including activation of Notch, have been implicated in the fibrogenic activation of lung mesenchymal cells during fibrosis (Chanda et al., 2019). Notch pathway was found to be activated and required for lung myofibroblast differentiation both *in*

vitro and *in vivo* (Liu et al., 2009), and the Notch ligand Jagged is critical for the induction of genes associated with cell contractility and ECM protein deposition (Yin et al., 2018). Members of the p38 MAPK family have also been shown to participate in the activation of lung fibroblast during fibrosis (Khalil et al., 2005), and lungs from IPF patients exhibit increased phosphorylation and activation of p38 MAPK in mesenchymal cells (Yoshida et al., 2002); implicating this protein kinase in the pathogenesis of IPF. Of the four isoforms (α, β, γ, and δ) of p38, p38α is the most studied, and its fibrogenic function, as well as crosstalk with other pro-fibrotic signaling pathways, including TGFβ pathway, have been extensively investigated in the pathogenesis of pulmonary fibrosis (Yoshida et al., 2002; Kasuya et al., 2021). Indeed, several studies have reported that multiple p38 inhibitors can block lung fibroblast activation *in vitro* (Paw et al., 2021), and attenuate bleomycin-induced pulmonary fibrosis (Matsuoka et al., 2002). Finally, pirfenidone, an approved drug for the treatment of IPF, was shown to act as a small molecule inhibitor of the gamma isoform of p38 (Moran, 2011), further corroborating the involvement of this signaling in the pathogenesis of pulmonary fibrosis.

The Wnt signaling pathway is also a well-known initiator and stimulator of myofibroblasts and mesenchymal cells (Hu et al., 2021; Shi et al., 2022; Koopmans et al., 2016; Baarsma and Konigshoff, 2017; Carthy et al., 2011). Wnt proteins are secreted ligands that interact with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP5/6) (Raslan and Yoon, 2020; Hu et al., 2021; Liu et al., 2022). In lung mesenchymal cells, Wnt signaling induces a cascade of intracellular signaling that ultimately results in stabilization of the transcription factor β-catenin (Hu et al., 2021; Shi et al., 2022). Stabilized β-catenin translocates into the nucleus to promote the transcription of numerous myofibroblast-related inflammatory and pro-fibrotic mediators (Carthy et al., 2011; Guo et al., 2022; Akhmetshina et al., 2012; Cao et al., 2018). TGFβ stimulates Wnt signaling in a p38-dependent manner by decreasing the expression of the Wnt antagonist Dickkopf-1 (DKK-1) (Akhmetshina et al., 2012; Dees et al., 2021). Consistent with these findings, elevation of DKK-1 ameliorates lung fibrosis induced by constitutively active TGFβ (Akhmetshina et al., 2012; Ren et al., 2013; Piersma et al., 2015). These findings demonstrate the crosstalk between Wnt and other pro-fibrotic signaling pathways in promoting lung fibroblast activation and fibrosis (Fig. 2). Other transcription factors such as KLF4 serve as a brake on myofibroblast activation in part through its inhibition of Wnt signaling (Evans et al., 2010; Lin et al., 2017). Decreased expression of KLF4 has been observed in human IPF lungs, and transgenic mice overexpressing KLF4 exhibited attenuated fibrotic responses following bleomycin challenge (Lin et al., 2017), supporting an anti-fibrotic function for this transcription factor. A recent study by Chandran and coworkers, however, reported mesenchymal cell-type-specific roles of KLF4 during myofibroblast differentiation and lung fibrogenesis (Chandran et al., 2021). Indeed, loss of KLF4 in PDGFR-β+ mesenchymal cells mitigated lung collagen deposition and fibrosis in mice, while its ablation in αSMA+ cells promoted pro-fibrotic macrophage recruitment and exacerbated lung fibrosis in these animals. These findings demonstrated that KLF4 exhibits divergent cell-type-specific effects by promoting or inhibiting the fibrogenic activation of lung mesenchymal cells during fibrosis, highlighting the importance of dissecting different signaling pathways during the fibrogenic activation of various lung mesenchymal cell lineages.

In addition to Smad and Wnt/b-catenin, other transcription factors have also been shown to promote myofibroblast activation in lung mesenchymal cells (Moss et al., 2022; Froidure et al., 2020; Sun et al., 2021; Inui et al., 2021). The forkhead-box family of transcription factors have recently been identified as important drivers of myofibroblasts (Li et al., 2019; Balli et al., 2013). Penke and colleagues showed that the cell cycle regulator FOXM1 is upregulated in IPF lung fibroblasts and in those from fibrotic mouse lungs (Penke et al., 2018). In addition to promoting proliferation, FOXM1 augments the expression of αSMA and ECM protein synthesis in mesenchymal cells. Genetic ablation of FOXM1 in fibroblasts or its pharmacological inhibition by Siomycin A attenuated bleomycin-induced pulmonary fibrosis, implicating FOXM1 as a

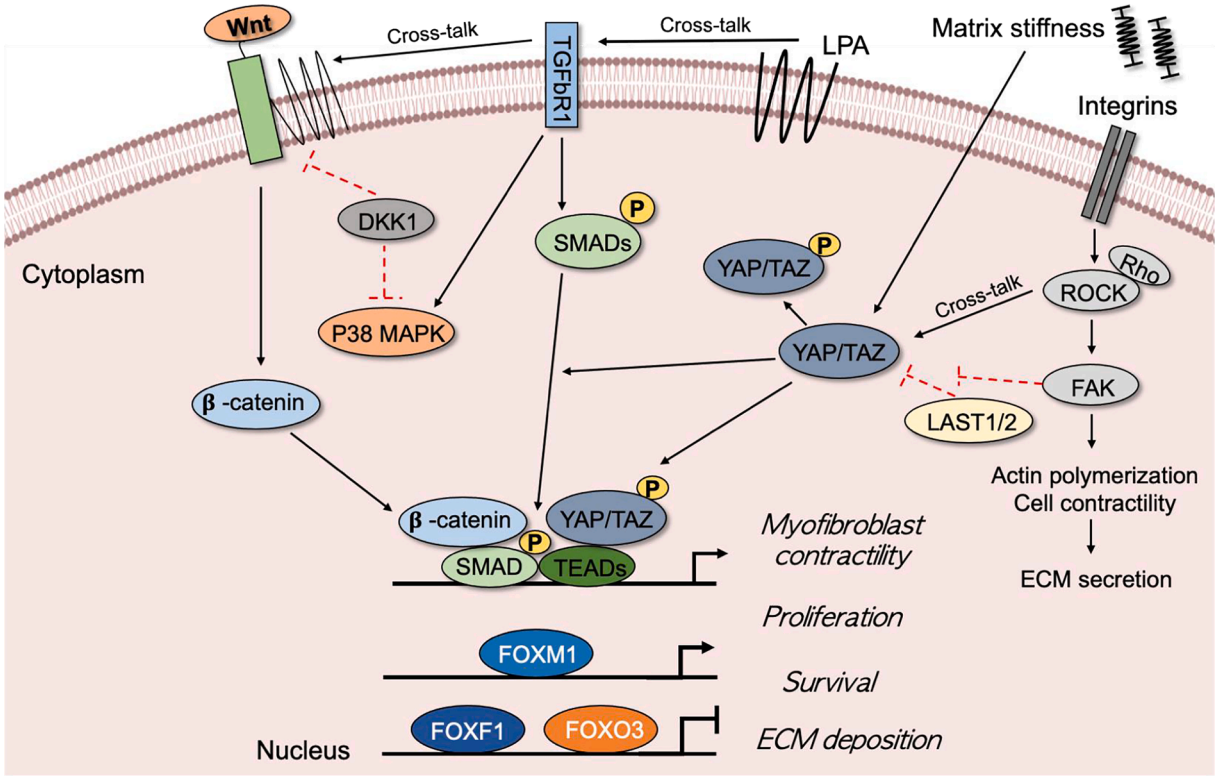

Fig. 2. Representative signaling pathways implicated in myofibroblast activation. Activation of multiple signaling pathways, including TGFβ, Wnt, p38 MAPK, and integrins, leads to myofibroblast differentiation *via* distinct transcriptional regulators. *Figure modified using BioRENDER.com.*

driver of lung mesenchymal cell activation beyond its canonical function as cell cycle regulator.

While FOXM1 has been shown to promote myofibroblast activation, other FOX family members, such as FOXF1 and FOXO3, were shown to be repressed in activated lung fibroblasts, and their repression is critical to supporting and maintaining lung mesenchymal cells in an activated fibrogenic state (Black et al., 2018; Melboucy-Belkhir et al., 2014; Al-Tamari et al., 2017; Nho et al., 2011). Reduced expression of FOXF1 was shown in human IPF lung biopsies, and transgenic mice with fibroblast-specific inactivation of FOXF1 exhibited exacerbated lung collagen secretion and fibrosis (Black et al., 2018). Similarly, the expression and activity of FOXO3 were also found to be attenuated in IPF-derived fibroblasts, and ablation of this transcription mediator in mouse lung mesenchymal cells *in vivo* enhanced susceptibility to bleomycin-induced lung fibrosis, impaired lung function, and increased mortality (Al-Tamari et al., 2017). All together these studies implicate FOX family members as critical downstream regulators of pro-and anti-mesenchymal cell activation, and their pharmacological inhibition or reconstitution may serve as novel treatment strategy for fibrotic disorders **(**Fig. 2**)**.

4. Contribution of the mechanical microenvironment to lung mesenchymal cell activation

The behavior and function of mesenchymal cells are influenced not just by soluble signals and mediators, but also by the mechanical properties of the microenvironment, including the stiffness of the surrounding extracellular matrix (ECM) (Tschumperlin et al., 2018; Marinkovic et al., 2013). Fibrosis is characterized by progressive tissue stiffening resulting from collagen deposition, which has been shown to be an independent factor in activating myofibroblasts and promoting disease progression (Ligresti et al., 2019; Haak et al., 2019; Jones et al., 2021). This results in a positive feedback loop whereby stiffness leads to myofibroblast activation, which leads to further ECM deposition causing an even stiffer ECM environment. Stiff matrices have been shown to increase proliferation, apoptosis resistance, and enhanced contractility of mesenchymal cells (Darby et al., 2014; Goffin et al., 2006; Aarabi et al., 2007; Nho et al., 2022). Pathological fibroblasts isolated from IPF lungs maintain an activated state when cultured on stiff matrices *in vitro* but become more quiescent when cultured on physiologically compliant matrices, indicating the dynamic nature of these cells when cultured in different microenvironmental conditions (Nho et al., 2022; Marinković et al., 2013; Nemeth et al., 2020). Increased matrix-stiffness was associated with suppression of PGE_2 and elevation of TGFβ signaling in lung mesenchymal cells (Marinković et al., 2013; Berhan et al., 2020; Giménez et al., 2017), providing a mechanism by which stiffness affects fibroblast activity.

Numerous membrane receptors have been shown to sense and respond to alterations of the lung mechanical environment, and among them, integrins have been extensively studied for their capacity to convert mechanical signals from the surrounding ECM into intracellular cytoskeleton changes (Kechagia et al., 2019; Marchioni et al., 2021; Tschumperlin, 2015). External forces applied to cells lead to the clustering of integrins and the formation of adhesion complexes, which coordinate multiple cellular behaviors, such as cell growth and migration (Kechagia et al., 2019; Marchioni et al., 2021; Tschumperlin, 2015; Deng et al., 2020). Once engaged with the ECM, integrins activate the downstream effectors Rho kinase (ROCK) and focal adhesion kinase (FAK) to promote actin polymerization and cell contractility (Marchioni et al., 2021; Deng et al., 2020; Sun et al., 2016; Keely, 2011). Targeting inhibition of these integrin-mediated downstream signaling *in vitro* was also shown to block mechanical stretch-induced TGFβ activation (Brown and Marshall, 2019; Munger and Sheppard, 2011). In addition to their role in cell contractility, ROCK and FAK are also potent stimulators of ECM protein secretion (Bonnans et al., 2014), and their pharmacological inhibition attenuated collagen deposition and lung fibrosis in mouse models *in vivo* (Htwe et al., 2017; Lagares et al., 2012).

Whereas integrin-matrix interactions are critical for initiating mechanosensing, other transcription factors and nuclear signaling are ultimately required to transduce mechanical signals into activation of mesenchymal cells. Of these, the hippo signaling effectors YAP and TAZ are the most studied (Dupont et al., 2011; Cai et al., 2021; Wada et al., 2011). As previously mentioned, YAP and TAZ are transcriptional co-factors that translocate from the cytoplasm into the nucleus and stimulate the transcription of many myofibroblast-related genes (Dupont et al., 2011; Cai et al., 2021; Wada et al., 2011). They are directly phosphorylated by the kinases LATS1 and LATS2, leading to their cytoplasmic sequestration and subsequent degradation (Pocaterra et al., 2020; Moya and Halder, 2019; Heng et al., 2021).YAP and TAZ lack DNA-binding domains, but they can indirectly engage with DNA through their interactions with the co-factors TEADs, as well as with other transcription factors including SMAD and β-catenin (Moya and Halder, 2019; Heng et al., 2021). The link between mechanotransduction and YAP/TAZ activation is felt to occur through ROCK, which is a key upstream regulator of YAP and TAZ activation. The efficacy of ROCK inhibitors in fibrosis may thus be a consequence of reduced YAP/TAZ activation (Zhou et al., 2013; Shimizu et al., 2001; Wu et al., 2021) **(**Fig. 2**)**.

The importance of mechanoresponses in the activation of mesenchymal cells have sparked interest in targeting mechanosensing pathways such as YAP/TAZ as a strategy to limit fibrosis (Tschumperlin et al., 2018). Given the widespread expression of YAP/TAZ in multiple cell types and their critical role in lung epithelial regeneration following lung injury (LaCanna et al., 2019; Hicks-Berthet et al., 2021; Gokey et al., 2021), a selective strategy that blocks YAP/TAZ only in mesenchymal cells would be ideal. Haak and colleagues have utilized the Gαs-coupled dopamine receptor D1 (DRD1) as a selective target, as it is mainly expressed in lung mesenchymal cells compared to other cell types in the lung (Haak et al., 2019). Small molecule inhibitor of DRD1 selectively inhibited YAP/TAZ function in mesenchymal cells and attenuated pro-fibrotic gene expression and collagen secretion both *in vitro* and *in vivo*. This work also revealed inhibition of DOPA decarboxylase (DDC), the enzyme responsible for the biosynthesis of dopamine, in IPF lungs fibrosis, and DCC expression inversely correlates with disease severity, consistent with an endogenous protective role for dopamine signaling in the lung, which fails during the progression of fibrosis. Pharmacologically activation of dopamine signaling in fibrotic lungs may be a suitable strategy to selectively block pathogenic mechanosignals in mesenchymal cells without compromising important regenerative functions in other cell types.

The effect of mechanotransduction on myofibroblast and mesenchymal cell activation are felt to occur not just at the transcriptional level, but also at the level of chromatin modification. Matrix stiffness has been shown to directly affect chromatin organization and leads to increased histone 3 (H3) and H4 acetylation (Killaars et al., 2020; Killaars et al., 2019), facilitating gene transcription. The mechanism by which this occurs is not completely known but is felt to occur through actin filament–mediated sequestration of histone deacetylases (HDACs), suggesting that cell cytoskeleton can directly alter gene expression by regulating the cytoplasmic retention of epigenetic regulators (Killaars et al., 2020). Recent studies in our laboratory demonstrated that freshly isolated lung mesenchymal cells cultured on pathologically stiff substrates resulted in increased global H3K9 methylation along with cell contractility. The increase in H3K9 methylation was a result of increased CBX5/G9a activity and pharmacological inhibition of CBX5/G9a attenuated matrix stiffness–induced global H3K9 methylation (Ligresti et al., 2019). Other studies suggest that matrix stiffness can lead to altered DNA methylation patterns in lung mesenchymal cells (Koh et al., 2016). Finally, matrix stiffness has been shown to impact chromatin accessibility in lung mesenchymal cells (Jones et al., 2021). Using assay for transposase-accessible chromatin (ATAC) followed by next-generation sequencing, we showed that freshly isolated lung

mesenchymal cells cultured on stiff matrices exhibited increased chromatin accessibility in genomic regions associated with matrix and contractile genes, including C*ol1a1* and *Acta2,* respectively, demonstrating that matrix stiffness can directly orchestrate chromatin remodeling (Jones et al., 2021). From this analysis, we further identified zinc finger protein 416 (ZNF416), as modulating chromatin accessibility in the setting of mechanical stiffness. Overexpression of ZNF416 in normal lung fibroblasts led to the increased expression of genes implicated in cell proliferation and ECM remodeling, such as *PDGFB*, *ACTA2*, and *LOXL2*, while enhancing cell contractile and the deposition of ECM proteins, including collagen I and fibronectin, demonstrating for the first time that ZNF416 is a critical mechanoregulator of fibroblast activation.

5. Mesenchymal cells in the pathogenesis of pulmonary fibrosis and IPF

Although activation of mesenchymal cells is critical for normal wound repair and regeneration, the persistence of these cells at sites of injury and their exuberant production of ECM are thought to be a major driver of pathogenic fibrosis. Of the many types and causes of lung fibrosis, IPF is unfortunately-one of the most common and prototypical of the fibrotic lung diseases that results in progressive scarring, impaired gas exchange, and ultimately death (Mei et al., 2021; Upagupta et al., 2018; Wolters et al., 2014). Lung fibrosis in these individuals is considered progressive and the cause is unknown. As opposed to other fibrotic conditions that are often driven by excessive inflammation (Moss et al., 2022; Wynn and Ramalingam, 2012), there is a relative paucity of inflammation in IPF, and patients do not respond to typical anti-inflammatory medications (Wynn and Ramalingam, 2012; Glass et al., 2022). Instead, IPF is characterized by the accumulation of collagen-producing mesenchymal cells and myofibroblasts that result in excessive matrix accumulation and alveolar destruction (Mei et al., 2021; Upagupta et al., 2018; Wolters et al., 2014). The mesenchymal cells, and particularly activated myofibroblasts, are thus considered the major targets of treatment for IPF.

After normal wound repair, myofibroblasts are felt to vanish after the injury resolves (Darby et al., 2016; Glasser et al., 2016; Yang et al., 2014), however the persistence of these cells in IPF lungs is believed to be the major contributor factor to the progression of this disease (Betensley et al., 2016; Mei et al., 2021; Glass et al., 2022). Numerous studies employing lineage tracing and conditional gene deletion strategies in mice, have supported the concept that mesenchymal cells are crucial for orchestrating lung ECM remodeling in fibrotic lungs (Liu et al., 2021; Zepp et al., 2017; Sun et al., 2021; Inui et al., 2021; Barros et al., 2019). In the remaining sections, we will explore what is known about the origins of mesenchymal cells, their activation, and persistence of these cells in IPF.

5.1. Origin of myofibroblast in IPF

As we had previously discussed, myofibroblasts often arise from the differentiation of existing fibroblasts, driven predominantly by signals such as TGFβ and Wnt/β-catenin, to name a few. The role of epithelial-mesenchymal transition has been controversial, but many fate-mapping and animal model studies have shown that epithelial-mesenchymal transition is not a significant contributor to the myofibroblast population in the fibrotic lung (Hill et al., 2019; Rock Jason et al., 2011; Hung, 2020). Recent single cell transcriptomic studies have further shed light on the cellular heterogeneity that contribute to myofibroblasts (Liu et al., 2021; Danopoulos et al., 2020; Narvaez del Pilar et al., 2022; Sountoulidis et al., 2022). Just as there are multiple mesenchymal subtypes in the normal lung, studies on IPF and mouse models of fibrosis have also identified multiple mesenchymal cell populations that are responsible for myofibroblasts. Most notably, based on various mouse model of lung fibrosis in combination with fate mapping approaches, numerous laboratories have shown that myofibroblasts can originate from multiple resident lung cells of the mesenchymal lineage, including pericytes, lipofibroblasts, adventitial fibroblasts, and alveolar fibroblasts (Rock Jason et al., 2011; Hung, 2020; Mack and Yanagita, 2015). Data from single-cell transcriptomic and histological analysis of IPF lungs, however, indicate that many of the pathogenic myofibroblasts originate from pre-existing myofibroblasts that become more activated and acquire discrete pathogenic features, such as increased contractility and collagen secretion (Adams et al., 2020), rather than the *trans*-differentiation of fibroblasts to myofibroblasts.

Early fate mapping studies using mesenchymal stem cell markers have been useful in determining how mesenchymal cells become activated and expand in mouse models of fibrosis (Sveiven and Nordgren, 2020; Sountoulidis et al., 2022; Riccetti et al., 2020,; Moss et al., 2022; Rawlins, 2008). For example, mesenchymal cells that expand in fibrotic mouse lungs, are often found to express markers of adventitial fibroblasts such as Foxd1 and Gli1, suggesting that these cells may derived from mesenchymal cells that normally reside in the perivasculature niche (Hung et al., 2013; Kramann et al., 2015; Cassandras et al., 2020). In a seminal study in mice, Rock and colleagues also demonstrated that expansion of mesenchymal cells is closely associated with blood vessels, and these cells express the pericyte markers NG2 and FoxJ1 (Rock Jason et al., 2011). Although these proliferative cells were identified in fibrotic regions, they did not increase α-SMA expression, nor were shown to express collagen genes, suggesting that lung injury may trigger different local insults leading to distinctive mesenchymal transcriptional responses within different anatomic locations. The intimate association of mesenchymal cells with blood vessels in the bronchial and alveolar regions raises many questions as to the involvement of the pulmonary vasculature in myofibroblast activation and, more broadly, in the pathogenesis of IPF. We have recently found that aged mice exhibited vascular rarefaction following bleomycin-induced lung injury and this abnormality was associated with perivascular expansion of collage-I-expressing mesenchymal cells and with persistent fibrosis (Caporarello et al., 2020). Furthermore, Biasin and colleagues (Biasin et al., 2020) recently reported the expansion of resident αSMA + cells in areas of the lung exhibiting extensive vascular remodeling, further suggesting a direct implication of the lung vascular compartment in mesenchymal cell activation. These vascular-related findings along with the increasing knowledge of the mesenchymal cell implications in the pathogenesis of pulmonary fibrosis, will lead to novel research opportunities to investigate the crosstalk between lung mesenchymal cells and blood vessel endothelial cells, and, more broadly, its contribution to the pathogenesis of IPF.

Several other studies have attempted to address the origin of pathogenic fibroblasts in the lung during fibrosis. For example, it was shown in mice that the Pdgfra+ mesenchymal lineage contributes to the appearance of myofibroblasts during lung development, and pathogenic myofibroblasts during lung fibrogenesis (Li et al., 2018). These Pdgfra+ mesenchymal cells, however, are not the only source of pathogenic myofibroblasts. Indeed, in a study by El Agha and colleagues it was shown that Plin2-lineaged lipofibroblasts can also give rise to myofibroblasts (El Agha et al., 2017), and complementary studies by Zepp and colleagues demonstrated that Axin2+ and Wnt2+ mesenchymal cells, which reside in close proximity to airways and alveoli, can also give rise to αSMA+ myofibroblasts under pathogenic conditions, with the Axin2 lineage contributing to nearly 50% of the total myofibroblasts (Zepp et al., 2017; El Agha et al., 2017). Intriguingly, the capacity of these Axin2+ cells to generate myofibroblasts upon injury was accompanied by their inability to support alveolar regeneration and AT2 cell maintenance (Zepp et al., 2017), suggesting a link between lack of regeneration and the acquisition of pathogenic myofibroblastic features.

These studies in mice, of course, do not necessarily indicate whether the same pathologic process occurs in humans with fibrosis. Since fibrosis is ultimately the consequence of exuberant collagen production, studies in humans have focused less on the origin of different myofibroblasts and more on those cells responsible for generating collagen.

Starting in mice and then extending to humans, Tsukui and colleagues identified a unique population of CTHRC1-expressing mesenchymal cells in fibrotic mouse and human lungs that exhibits high expression of collagens and other ECM components (Tsukui et al., 2020). CTHRC1 encodes for a secreted protein previously implicated in cell migration and TGFβ signaling (Mei et al., 2020). The authors also showed that these collagen-producing cells likely derived from a population of alveolar fibroblasts, corroborating previous studies implicating Pdgfra + alveolar fibroblasts as source of collagen producing cells in response to bleomycin-induced lung fibrosis (Biasin et al., 2020). These latter findings also support the notion that ECM production is not exclusive to αSMA+ cells but other mesenchymal cells of the Pdgfra+ lineage, which poorly expressed contractile genes, can be the pathogenic source of ECM proteins in the fibrotic lung. In support of this hypothesis Tsukui and colleagues also showed that the Acta2 was strongly expressed in smooth muscle cells and pericytes that exhibited low collagen expression, yet further demonstrating that Acta2 alone does not define all cells responsible for ECM production in the fibrotic lung. The fact that CTHRC1+ cells are found in the lungs of both patients with IPF and scleroderma suggests that there may be overlapping mechanisms in different fibrotic disorders and that the appearance and aberrant activation of these cells may be linked to the progressive nature of these diseases.

In human IPF lungs, distinct COL1+ and αSMA+ cell populations coexist, and they are both increased in number, suggesting that lung mesenchymal cell populations may transit between different cellular states. αSMA+ myofibroblasts may also exhibit different functions than those that are purely collagen-producing. This latter hypothesis is supported by a recent study in mice showing that myofibroblast lineage acted as drivers of airway and alveolar regeneration following injury (Zepp et al., 2017). Biasin and colleagues (Biasin et al., 2020) also observed the expansion of αSMA+ myofibroblasts in lung areas with extensive vascular remodeling, suggesting a possible contribution of myofibroblasts in lung vascular repair in response to injury.

Single cell RNA-seq analysis of human IPF lungs have recently provided new insights into the heterogeneity of mesenchymal cells in the pathogenesis of IPF, specifically, in those sub-populations of cells expressing high levels of ECM genes. In addition to the appearance of mesenchymal cells that express high levels of collagen, other transcriptionally distinct mesenchymal cell subtypes were also identified. Similarly to fibrotic mouse lungs, human IPF derived lungs exhibit distinct cell subtypes residing different regions of the lung, such as myofibroblasts in subepithelial regions around airways and areas of active remodeling. Other myofibroblasts are often found restricted to the subpleural or parenchymal regions (Liu et al., 2021; Tsukui et al., 2020; Habermann et al., 2020;6(28):eaba1972-eaba.), further implicating the existence of multiple distinct mesenchymal lineages in fibrotic human lungs with potential distinct contributions to disease progression. More recently a study by Liu and colleagues identified orthologous subpopulations with conserved transcriptional signatures in murine and human lung mesenchymal cells (Liu et al., 2021). They reported increased expression of αSMA in multiple mesenchymal subtypes in fibrotic mouse and IPF lungs with some exhibiting increase expression of collagen genes and other not. All these subpopulations, however, maintained relatively unchanged transcriptional signatures, suggesting that activated mesenchymal cells do not fully *trans*-differentiate into myofibroblasts but rather they acquire pathogenic features, such as increased contractility and collagen secretion (Liu et al., 2021) (Fig. 1). These new studies continue to push our understanding of the heterogeneity of mesenchymal cells and their potential contribution to myofibroblasts in the IPF lung.

Although many of the insights from lineage tracing studies and the functions of different mesenchymal cell populations have been extended from mouse to human studies, it is important to recognize that mouse models of fibrosis and human disease have clear differences. The dynamics of lung injury and subsequent remodeling in mice, via bleomycin or other agents, are difficult to study in humans. Mouse models of fibrosis are often self-resolving and thus, do not approximate the progressive nature observed in IPF. Intratracheal administration or inhalation of a single dose of bleomycin is the most common animal model of fibrosis and has provided critical insights into the pathogenesis of lung injury, but the fact that in this model fibrosis resolves, limits some of its utility as a model of IPF. Pathologically, it does not recapitulate the same findings as often seen in IPF, including fibroblastic foci and microscopic honeycombing. Studies using repetitive dosing of with bleomycin (Redente et al., 2020), or instillation of bleomycin in aged mice (Hecker et al., 2014;6(231):231ra47.; Caporarello et al., 2020) (discussed below) have been offered as alternative models that have shown to persist and thus, to be potentially more relevant to human disease. Future studies aimed at further exploring the underlying molecular mechanisms regulating the persistence of different mesenchymal cell populations and their aberrant activation using chronic disease models could offer alternative therapeutic strategies to reverse fibrotic lung diseases.

5.2. *Signals that contribute to myofibroblast activation in IPF*

During lung fibrosis multiple aberrant signaling pathways in bystander cells have been hypothesized to activate and sustain a complex cellular interplay leading to the activation and expansion of pathogenic mesenchymal cells (Moss et al., 2022; Chanda et al., 2019; Sun et al., 2021; Inui et al., 2021), including cellular senescence, telomere shortening, oxidative stress, and mitochondrial dysfunction (Piñeiro-Hermida et al., 2020; Otoupalova et al., 2020; Parimon et al., 2021; Bueno et al., 2020). Pathogenic signals from the lung parenchyma were proposed to be involved in all IPF development phases leading to epithelial dysfunction and ultimately persistent fibroblast activation and ECM protein accumulation (Mei et al., 2021; Moss et al., 2022). Repeated injuries and failure to proper repair have been hypothesized as putative mechanisms leading to lung epithelial dysfunction, chronic release of inflammatory mediators, and myofibroblast propagations in IPF lungs (Mei et al., 2021; Moss et al., 2022). Furthermore, in IPF lungs aberrant epithelial cells were also found to surround active fibrogenic regions, such as fibroblastic foci, and express markers of cellular senescence such as p21 and p16 (Sun et al., 2021; Jablonski et al., 2017; Jiang et al., 2017), thus they may contribute to perpetuate myofibroblast activation. In fact, senescent type-II epithelial cells (ATII) can secrete large amounts of inflammatory mediators as part of their senescence-associated secretory phenotype (SASP), which can lead to activation of myofibroblasts (Mora et al., 2017; Hernandez-Gonzalez et al., 2021). In addition, aberrant epithelial cells can also release other fibrogenic mediators, including TGFβ and connective tissue growth factor (CTGF) (Inui et al., 2021; Hewlett et al., 2018; Pan et al., 2001; Sakai and Tager, 2013), which further enhance inflammation and ECM protein deposition by lung mesenchymal cells (Inui et al., 2021; Hewlett et al., 2018; Sakai and Tager, 2013; Blobe et al., 2000).

A seminal study by Yang and coworkers reported that following TGFβ stimulation, mouse alveolar epithelial cells cultured *in vitro* were able to activate lung fibroblasts through production of CTGF (Yang et al., 2014). Intriguingly, cytoskeleton dynamics appear to be involved in this paracrine activation as the expression of CTGF was dramatically reduced by the inhibition of RhoA, a small GTPase and modulator of actomyosin contractility (Yang et al., 2014). Mice with lung epithelial deletion of CTGF exhibited attenuated fibrotic responses *in vivo*, further demonstrating injury-mediated epithelial activation of CTGF as a driver of lung mesenchymal cell activation and fibrosis. In a more recent study, Yao and colleagues performed an RNA sequencing analysis on human lung fibroblasts that have been exposed to conditioned media from human ATII cells undergoing oncogene-induced epithelial-mesenchymal transition and identified multiple differentially expressed genes that have been implicated in fibroblast migration and extracellular matrix secretion (Yao et al., 2021). The authors also found that the large majority of differently expressed genes in these aberrant ATII cells are regulated by

zinc finger E-box-binding homeobox 1 (ZEB1). Among them was tissue plasminogen activator (tPA), an enzyme involved in fibrin remodeling, and whose function has been previously implicated in lung fibrosis (Yao et al., 2021). Increased nuclear localization of ZEB1 has been found among alveolar epithelium most adjacent to areas of abundant ECM protein deposition. The crosstalk between epithelial cells and fibroblasts occurs both ways, and fibrogenic mesenchymal cells have also been shown to promote epithelial cell dysfunction. Human alveolar epithelial cells exposed to conditioned medium of IPF-derived fibroblasts exhibited exaggerated injury responses and dysfunctional epithelialization (Conforti et al., 2020). In addition, paracrine signaling from IPF-derived lung fibroblasts was also shown to promote RAS activation in ATII cells, suggesting that aberrant bidirectional epithelial-mesenchymal crosstalk in fibrotic lungs is crucial to initiate and propagate self-sustaining profibrotic signals (Yao et al., 2021; Yao et al., 2019).

Secretome analysis of IPF-derived lung fibroblasts identified the matricellular protein secreted protein acidic and rich in cysteine (SPARC) as the most abundant protein secreted by these diseased cells. SPARC was shown to promote pathological activation of ATII cells, and this effect was mediated by integrins and resulted in via epithelial barrier deterioration, further supporting the contribution of IPF-derived fibroblast paracrine signals to alveolar epithelial dysfunction (Conforti et al., 2020).

The crosstalk between epithelial and mesenchymal cells have also contributed to increased WNT/β-catenin signaling in IPF (Chanda et al., 2019; Hu et al., 2021; Baarsma and Konigshoff, 2017; Königshoff et al., 2008; Shi et al., 2017; Hu et al., 2020). Previous studies using human lung samples have shown increased levels of β-catenin in bronchial epithelial cells and hyperplastic ATII cells of IPF lungs (Pan et al., 2001). *In vitro* studies using human lung-derived cell lines have shown that β-catenin-mediated expression and secretion of Wnt mediators, including Wnt1, Wnt7b, and Wnt3a (Chanda et al., 2019; Königshoff et al., 2008; Chilosi et al., 2003; Königshoff and Eickelberg, 2010), promoted the activation of human lung fibroblasts (Hu et al., 2021; Königshoff et al., 2008; Königshoff and Eickelberg, 2010). Furthermore, nuclear β-catenin accumulation was also demonstrated in fibroblastic foci in IPF lungs and was often associated with bronchiolar lesions (Chilosi et al., 2003). Pharmacological inhibition of WNT/β-catenin pathway thus has therapeutic promise because of its ability to inhibit fibrogenic Wnt signaling in both fibroblasts and epithelial cells (Hu et al., 2021; Wang et al., 2014; Henderson et al., 2010; Ulsamer et al., 2012). Other molecules such as extracellular matrix metalloproteinase inducer (EMMPRIN), a glycosylated transmembrane protein induced by TGFβ in alveolar epithelial cells, is highly upregulated in fibroblastic foci of IPF lungs and was shown to activate WNT/β-catenin signaling in human lung fibroblasts *in vitro* (Hasaneen et al., 2016;17:17-.). Finally, WNT/β-catenin and TGFβ both induce epithelial-mesenchymal transition (EMT) promoting a mesenchymal phenotype in epithelial cells, potentially contributing to further fibrosis (Zhou et al., 2012).

Given the capacity of lung endothelial cells to influence mesenchymal cell activation (Cao et al., 2016), and the recent advances concerning transcriptional heterogeneity of the pulmonary vasculature (Gillich et al., 2020), the ability of endothelial cells to stimulate mesenchymal cells and contribute to lung fibrosis is also being recognized. We recently showed that aging-associated vascular dysfunction facilitates sustained fibroblast activation and lung fibrosis in mice (Caporarello et al., 2020; Caporarello et al., 2022). Given that vascular regeneration is crucial to lung repair and fibrosis resolution (Caporarello et al., 2020), it is likely that dysregulation of paracrine signals from the surrounding vascular compartment may be directly involved in sustaining mesenchymal cell activation and fibrosis. In this regard, we recently discovered that dysregulation or loss of eNOS, an enzyme that produces soluble nitric oxide (NO), in mouse lung endothelial cells with aging perpetuated lung fibroblast activation and impaired lung fibrosis resolution (Caporarello et al., 2020), revealing an important function for lung endothelium-derived NO in promoting fibroblast quiescence.

Previous studies showed that endothelial-derived NO can bind to and activate the receptor soluble guanylate cyclase (sGC) in smooth muscle cells resulting in cell relaxation (Kollau et al., 2018). Our study also demonstrated that pharmacological activation of sGC signaling pathways in human lung fibroblasts attenuated TGFβ responses and promoted quiescence in these cells, demonstrating that the eNOS/sGC signaling pathway regulates fibrogenic responses beyond its known function as modulator of smooth muscle cell contractility (Caporarello et al., 2020).

In another study, Martin and colleagues showed that genetic overexpression of the endothelial transcription factor sterol regulatory element-binding protein 2 (SREBP2) in human lung endothelial cells promoted endothelial-to-mesenchymal transition (EndMT) and contributed to lung fibrosis in mice (Martin et al., 2021). Lineage tracing studies in combination with imaging analysis, however, demonstrated that these dysfunctional endothelial cells do not fully differentiate into mesenchymal cells, thus they do not represent a major source of myofibroblasts (Martin et al., 2021). Instead, these altered endothelial cells can release many inflammatory mediators, including cytokines with profibrotic factors, thus exacerbating lung fibrogenesis (Martin et al., 2021).Together, these studies support the concept that lung endothelial cells restrain fibroblast activation under physiological conditions and that dysfunctional endothelial cells lose this homeostatic function.

5.3. *Mechanisms implicated in myofibroblast persistence in IPF lungs*

Although mesenchymal cells are needed to orchestrate normal tissue repair following injury (Hinz et al., 2007; Darby et al., 2016), part of the pathology of fibrosis includes not only their activation, but also their persistence and resistance to apoptosis even after the injurious insult(s) have resolved (Mei et al., 2021; Moss et al., 2022; Wolters et al., 2014; Hanson et al., 2019; Kasam et al., 2019). Numerous studies, in fact, suggest that the aberrant activation of survival pathways in myofibroblasts during the pathogenesis of pulmonary fibrosis is implicated in their apoptosis resistance (Hanson et al., 2019; Kasam et al., 2019). Increased apoptosis resistance of pathogenic myofibroblasts can result in the continuous and irreversible expansion of scar tissue in the lung (Mei et al., 2021; Moss et al., 2022; Upagupta et al., 2018; Kulkarni et al., 2016). Early studies reported that several apoptosis-associated proteins, including X-linked inhibitor of apoptosis protein (XIAP), Bid, Blc-2, and cellular FLICE inhibitory protein (c-FLIP), were shown to be dysregulated in scar-forming mesenchymal cells (Golan-Gerstl et al., 2012; Tanaka et al., 2002). Other studies have shown that fibroblasts isolated from IPF lungs exhibit resistance to Fas-mediated apoptosis (Hanson et al., 2019; Huang et al., 2013; Im et al., 2016). In support of this hypothesis, a recent study from Redente and colleagues demonstrated that Fas is necessary for lung fibroblast apoptosis during fibrosis resolution following a single dose of bleomycin, and that loss of Fas signaling in mesenchymal cells was responsible for their apoptosis resistance, persistent activation, and pathogenic ECM secretion (Redente et al., 2020). We additionally showed that overexpression of protooncogene serine/threonine-protein kinase-1 (PIM1), a protein kinase implicated in inflammation and senescence (Jin et al., 2014; Tursynbay et al., 2016), in normal lung fibroblasts induced BAD phosphorylation and inhibited caspase-3 activation induced by FAS-activating antibodies (Pham et al., 2022), suggesting that a pathogenic cross-talk between PIM-1-induced inflammation and Fas signaling may be implicated in the apoptosis evasion of pathogen lung fibroblasts.

Intratracheal instillation of a single dose bleomycin in mice is classically used as a model of lung injury and fibrosis that eventually improves or resolves over time. As such, it can be used to study lung mesenchymal cell activation not only in the early phases of fibrosis (Liu et al., 2017; Williamson et al., 2015), but also provide insights into the mechanisms underlying fibrosis resolution (Moss et al., 2022; Froidure et al., 2020; Inui et al., 2021; Glasser et al., 2016). Interestingly, although bleomycin-induced fibrosis spontaneously resolves (Tashiro

et al., 2015; Redente et al., 2011) in young mice, aged mice do not demonstrate the same degree of resolution and often develop persistent fibrosis. The association between IPF and aging has led to several studies to identify age-related mechanisms that contribute to the diminished capacity for fibrosis resolution (Parimon et al., 2021; Murtha et al., 2019; Selman et al., 2016; Venosa, 2020). Cellular senescence, a hallmark of aging (Lopez-Otin et al., 2013), was shown to contribute to the development of pulmonary fibrosis (Parimon et al., 2021; Hernandez-Gonzalez et al., 2021; Schafer et al., 2017), and apoptosis evasion has been shown to play a role in the accumulation of senescent myofibroblasts and mesenchymal cells during fibrosis progression (Parimon et al., 2021; Hinz and Lagares, 2020; Merkt et al., 2021). Interestingly, senescence among mesenchymal cells does not lead to their diminished activity. Rather, as a result of senescence-associated secretory phenotype, they often promote inflammation, generate more matrix proteins, and demonstrate apoptosis resistance to result in persistent fibrogenesis. These apoptosis-resistant senescent mesenchymal cells secret large amount of inflammatory and pro-fibrotic mediators such as IL-6, IL-8, IL-1β and TNFα to enhance lung mesenchymal cell activation (Parimon et al., 2021; Hernandez-Gonzalez et al., 2021; Burton and Faragher, 2015; Kumari and Jat, 2021). This effect was shown to be attenuated by senolytics, a class of drugs that selectively clear senescent cells (van Deursen, 2019; Zhu et al., 2015; Zhang et al., 2022; Hickson et al., 2019). Whereas different cell types, including epithelial and endothelial cells, can become senescent in aging and during the progression of lung fibrosis (Parimon et al., 2021; Hernandez-Gonzalez et al., 2021; Venosa, 2020), accumulation of senescent myofibroblasts contributed to the non-resolving lung fibrosis in aged mice (Kato et al., 2020). Pathogenic myofibroblast accumulation in fibrotic lungs of aged mice is largely due to alterations in the cellular redox balance resulting from the elevation of the reactive oxygen species-generating enzyme NADPH oxidase-4 (NOX4), and deteriorated antioxidant responses with aging (Bernard and Thannickal, 2020; Phan et al., 2021). High levels of NOX4 in fibroblastic foci in IPF lungs, along with reduced expression of the antioxidant response factor NRF2 (NFE2-related factor 2), were linked to persistent fibroblast activation, senescence, and apoptosis-resistance phenotype (Hecker et al., 2014;6(231):231ra47.). Interestingly, studies performed using genetic or pharmacological inhibition of Nox4 in mice, showed that genetic or pharmacological inhibition of Nox4 stimulated mitochondrial oxidative phosphorylation and increased mitochondrial biogenesis in lung fibroblasts (Bernard et al., 2017). The authors also demonstrated that this effect is due to increased Nrf2 expression and is independent of PGC1α (Bernard et al., 2017), suggesting additional mechanisms controlling mitochondrial biogenesis and metabolism in lung fibroblasts. Since metabolism is essential to many cellular processes, including proliferation, differentiation, and stress responses (Wang and Lei, 2018), several studies have attempted to connect this process to persistent fibroblast activation during lung fibrosis.

The AMP-activated protein kinase (AMPK) is a metabolic sensor of intracellular adenosine nucleotides; it is activated by decreased levels of ATP and increased levels of ADP or AMP and promotes the switch from anabolic to catabolic pathways, to generate more ATP (Mihaylova and Shaw, 2011). AMPK activity was shown to be deficient in IPF-derived lung fibroblasts, along with other metabolic pathways, including autophagy and mitochondrial oxidative phosphorylation (Rangarajan et al., 2018). Pharmacological activation of AMPK with Metformin, an AMPK activator widely used to treat complications of type 2 diabetes, accelerated the resolution of experimentally induced lung fibrosis by enhancing autophagy and promoting mitochondrial biogenesis, and ultimately myofibroblast apoptosis (Rangarajan et al., 2018). The anti-fibrotic effect of metformin was also confirmed in a recent study by Kheirollahi and colleagues (Kheirollahi et al., 2019), in which the authors demonstrated that metformin accelerates lung fibrosis resolution by inducing myofibroblast-to-lipofibroblast differentiation through the upregulation of BMP2 and the activation of PPARγ (Kheirollahi et al., 2019).

These studies suggest that metabolic perturbations and impaired mitochondrial oxidative phosphorylation are important drivers of persistent fibroblast activation. When oxygen is not limited, cellular metabolism largely relies on mitochondrial oxidative phosphorylation to generate energy, however, under oxygen deprivation, energetic needs are often achieved *via* glycolysis (Yadav et al., 2020). In some circumstances, however, glycolysis is engaged even when oxygen is abundant, a phenomenon described as "Warburg effect" (Koppenol et al., 2011; Potter et al., 2016), which has been previously shown to drive cancer cell grow and metastasize (Lu et al., 2015; Vaupel and Multhoff, 2021). Augmented aerobic glycolysis was found to be required for the initiation and sustained activation of lung fibroblasts (Xie et al., 2015), and TGFβ was shown to facilitate the glycolytic switch of lung fibroblasts (Bernard et al., 2015; Yin et al., 2019), suggesting an important role for metabolic reprogramming in myofibroblast activation and persistence during lung fibrosis. Several studies have also shown that lactate dehydrogenase 5 (LDH5), a glycolytic enzyme responsible for generation of lactate, was elevated in IPF-derived mesenchymal cells, and increased lactate production was shown to be elevated in IPF-derived fibroblasts and to potentiate their activation in response to fibrogenic stimuli (Rangarajan et al., 2018; Xie et al., 2015; Kottmann et al., 2012).

While much we have learned about the transcriptional mechanisms leading to myofibroblast activation during the acute phase of lung fibrosis, very little is known in regard to the contribution of transcription factors to myofibroblast persistence in the context of sustained fibrosis. In this regard, our group has recently identified the nuclear factor of activated T cells-1 (NFATc1) as a putative mediator of the sustained transcriptional program of pathogenic myofibroblasts during lung fibrosis in aged mice (Pham et al., 2022). NFATc1 is a transcription factor that was previously implicated in T cell activation and exhaustion (Gabriel et al., 2016; Martinez et al., 2015; Xu et al., 2019). Our study demonstrated that about a third of all differentially expressed genes in fibrotic aged lung fibroblasts following bleomycin challenge contained binding motifs for NFATc1 and, among them, were numerous pro-survival and pro-fibrotic genes. Intriguingly, another member of the NFAT family, NFATc2, was shown to promote lung fibroblast proliferation in response to hypoxia (Senavirathna et al., 2018), supporting the engagement of multiple NFAT transcription factors in perpetuating lung fibroblast activation in response to various fibrogenic cues.

Since myofibroblasts in IPF lung are resistant to apoptosis, many researchers have considered whether "de-differentiating" them to a more quiescent phenotype would serve a more tractable therapeutic approach. Several studies support myofibroblast de-differentiation as one of the intrinsic mechanisms underlying lung fibrosis resolution following lung injury (Fortier et al., 2021; Glasser et al., 2016; Kato et al., 2020), and loss of pro-regenerative and anti-fibrotic pathways in pathogenic lung mesenchymal cells has been associated with fibrosis propagation (Balestrini and Niklason, 2015; Hagood et al., 2005). For example, the peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathway, which plays a crucial role in adipocyte biology, has been largely involved in lung myofibroblast de-differentiation both *in vivo* and *in vitro* (El Agha et al., 2017), and PPARγ agonists, including rosiglitazone, have been shown to attenuate TGFβ signaling in IPF-derived fibroblasts, as well as, protect mice from developing lung fibrosis (Burgess et al., 2005; Avouac et al., 2017; Derrett-Smith et al., 2021; Genovese et al., 2005).

Epigenetic repression of PPARγ gene was proposed as a potential mechanism leading to the inhibition of this pathway in activated mesenchymal cells (Wei et al., 2022), and our recent studies suggest that the CBX5/G9a epigenetic pathways is implicated in this repressive mechanism (Ligresti et al., 2019). These latter findings suggest that an epigenetic intervention to increase PPARγ gene expression in pathogenic lung mesenchymal cells may synergistically potentiate the anti-fibrotic effect of rosiglitazone and other PPARγ agonists. IPF is also associated with decreased production of prostaglandin E_2 (PGE_2), one of the most

potent anti-fibrotic mediators that is capable of both inhibiting and reversing myofibroblast differentiation. PTGS2, which synthesizes PGE_2, was shown to be epigenetically repressed in IPF (Evans et al., 2016; Gabasa et al., 2013). Exogenous PGE_2 was shown to reverse myofibroblast activation and pro-fibrotic gene expression *via* inhibition of focal adhesion kinase (FAK) activation (Thomas et al., 2007; Garrison et al., 2013), thereby demonstrating the transcriptional plasticity of the myofibroblast phenotype. PGE_2 signals through G-protein coupled receptors to generate cAMP, and drugs that lead to sustained levels of cAMP, such as phosphodiesterase inhibitors, have recently been proposed as therapy for IPF (Nunez et al., 2020). This may be a more attractive strategy than providing exogenous PGE_2 as IPF fibroblasts have been shown to be resistant to PGE_2, in part through epigenetic loss of the PGE_2 receptor, PTGER2.

Using a Col1α1-GFP transgenic mouse model we compared the transcriptional signatures of FACS sorted GFP + lung mesenchymal cells at different time points post bleomycin challenge, including peak of fibrosis (14 days post-bleomycin) and during the early phase of fibrosis resolution (30 days post-bleomycin) (Tan et al., 2021). This study led to the discovery that mesenchymal cells are remarkably plastic, exhibiting transient activation during the peak of fibrosis followed by a return to baseline during the early resolution phase post injury. The return to baseline during the resolution phase was associated with the re-expression of several anti-fibrotic genes that were inhibited during the early fibrotic phase, further supporting the self-resolving nature of this model, and the implication of anti-fibrotic mediators in this process.

Mesenchymal cell plasticity may be considered a self-protective feature that ensures the return of activated cells to quiescence, however, recent evidence, including those from our group, suggest that this capacity may deteriorate with aging (Pham et al., 2022), leading to their persistent activation in fibrotic lungs. Transcriptional analysis of lung fibroblasts isolated from fibrotic aged mouse lungs showed that, in contrast to young ones, these cells failed to return to a quiescent state, exhibiting elevated expression of pro-fibrotic and pro-survival genes, which largely overlapped with those enriched in young fibroblasts at the peak of fibrosis (Day 14 post bleomycin administration). These findings led to the hypothesis that aging may perpetuate fibrogenic gene signatures that are typically associated with the acute fibrotic phase after bleomycin injury. Several inflammatory-associated pathways were also enriched in aged lung fibroblasts, including IL-6, STAT3, and the apoptosis inhibitor PIM1, which is consistent with previous observations demonstrating an increased low-grade inflammation with aging, which may contribute to the incapacity of myofibroblasts to return to quiescence. The use of newer animal models of lung fibrosis, including repetitive injury by bleomycin, may allow us to better understand why fibrosis might not resolve and why myofibroblasts persist in progressive fibrosis (Fig. 3).

5.4. *Epigenetic changes in lung mesenchymal cells associated with IPF*

Much of the discussion to this point has emphasized the factors that lead to myofibroblast and mesenchymal cell activation and the mechanisms for their persistence during fibrosis. Although microenvironmental factors such as matrix stiffness, extracellular matrix composition, soluble mediators, and adjacent cell signaling all affect the activity and function of fibroblasts and myofibroblasts in the local milieu, a striking feature found in many studies of IPF is the persistence of a pro-fibrotic phenotype even when cells are isolated and studied *ex vivo* (Helling and Yang, 2015). The persistence of this activated, myofibroblast-like phenotype from mesenchymal cells, especially in the IPF lung, support the hypothesis that these cells possess epigenetic changes. Unlike genetic alterations, epigenetic changes do not involve changes to the DNA sequence itself, but they affect DNA accessibility to either promote or inhibit gene transcription (Berger et al., 2009; Weinhold, 2006).

Epigenetics encompasses three main modifications: DNA methylation, histone modifications, and non-coding RNAs. Given that the large majority of epigenetic investigations in the lung fibrosis field have been directed toward the implications of DNA methylation and histone modification, in this review we focus only on these two epigenetic mechanisms.

DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto cytosines that precede a guanine nucleotide or CpG islands, and it is usually associated with gene repression and silencing, whereas demethylation is linked to increased gene expression (Moore et al., 2013; Bommarito et al., 2019; Dhar et al., 2021). Over the years, a number of genes have been shown to be hypermethylated in

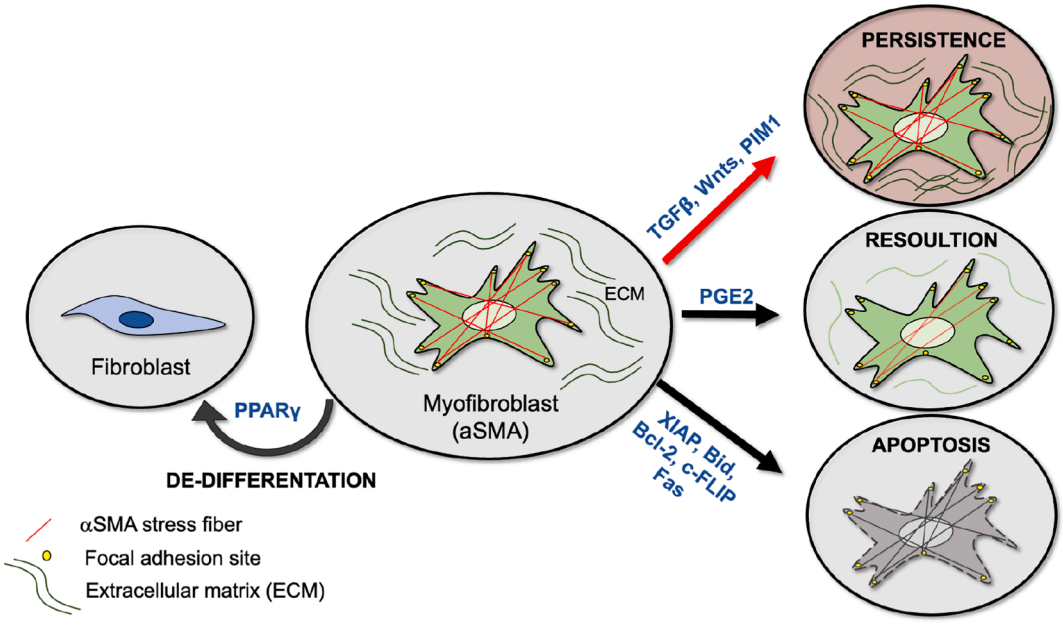

Fig. 3. Persistence and reversibility of lung myofibroblast activation. Myofibroblast differentiation is induced and maintained by several fibrotic mediators such as TGFβ, Wnts, and PIM1. Several signaling pathways, including PPARγ and PGE2, have been proposed to promote myofibroblast de-differentiation and lung fibrosis resolution post lung injury. Apoptosis mediators, such as Bcl-2 ans FAS, have also been shown to promote fibrosis resolution, and failure in activating these pathways results in persistent fibrosis.

fibroblasts from IPF lungs, including those encoding for Thy-1 (Sanders et al., 2008; Robinson et al., 2012), p14 (Cisneros et al., 2012), BMPER (Huan et al., 2015), COX-2 (Evans et al., 2016), and PTGER2 (Huang et al., 2010), contributing to their decreased expression (Zhang et al., 2017; Zhou et al., 2020). Thy-1 is a cell-surface glycoprotein largely expressed in cells of mesenchymal origin, including resident lung fibroblasts, and one of the first genes shown to be differentially methylated in fibrotic lung fibroblasts. Loss of Thy-1 expression in fibroblasts was shown to correlate with lung fibrogenesis, and DNA methylation appears to be the main driver of its gene repression (Sanders et al., 2008; Robinson et al., 2012). Given the anti-fibrotic function of Thy-1 (Hagood et al., 2005; Zhou et al., 2020; Sanders et al., 2007), these studies suggest that DNA methylation-mediated gene silencing may be directly implicated in the transition from quiescence to activated fibroblasts (Fig. 4). *In vitro* studies showed that lung fibroblasts exposed to the methylation inhibitor 5-azacytidine (5-aza) exhibited increased expression of Thy-1 (Sanders et al., 2008), and blocked the expression of collagen-I and other pro-fibrotic mediators, further supporting the hypothesis that epigenetic mechanisms may be responsible for the pathogenic transition of mesenchymal cells during the onset of fibrosis.

As previously mentioned, PGE_2 is a potent inhibitor of fibroblast activation and collagen deposition, and fibroblasts from the lungs of IPF patients and mouse models of fibrosis exhibited increased DNA methylation at the promoter region of PTGER2, which encodes for the E prostanoid-2 receptor of PGE_2. This modification was associated with the reduced expression of PTGER2 and increased resistance to prostaglandin E_2 (PGE_2) (Huang et al., 2010). Other studies reported that PTGS2, which encodes for prostaglandin-endoperoxide synthase 2 (COX-2), the enzyme responsible for the generation of PGE_2, was also methylated in pathogenic fibroblasts, and treatment of these diseased cells with inhibitors of DNA methylation restored PTGS2 gene expression, and increased sensitivity to FasL-induced apoptosis (Evans et al., 2016).

Not only have candidate genes been shown to be differentially methylated in IPF, but global DNA methylation patterns are also altered in lung fibroblasts isolated from IPF lungs compared to healthy lungs (Huang et al., 2010). Some of these differences in methylation were observed in genes implicated in extracellular matrix production and proliferation, but also in novel genes associated with organ morphogenesis and potassium ion channels, which may also be important in the fibrotic process. Among these genes are *CDKN2B*, *KCNMB1*, *CARD10*, and *MGMT*. Intriguingly, marked variability in DNA methylation was observed in different IPF cell lines, suggesting that differences in DNA methylation may ultimately contribute to the heterogeneity among mesenchymal cells. DNA methylation is a dynamic process and is promoted by specific DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B (Moore et al., 2013; Bommarito et al., 2019; Dhar et al., 2021). DNA methylation is reversible, and removal of methyl groups is mainly achieved by demethylases known as ten eleven translocases (TETs) (Moore et al., 2013; Bommarito et al., 2019; Dhar et al., 2021). Recent studies showed that there is an increased expression of DNMTs, specifically DNMT3a and DNMT3b, but not DNMT1 in IPF lung tissues (Sanders et al., 2012; Avci et al., 2022; Bartczak et al., 2020), suggesting that multiple DNMTs can act on different genes to promote their repression. In addition, another study showed that TGFβ increased the protein levels of DNMT1 and DNMT3a in lung fibroblasts without altering their transcript levels (Koh et al., 2016), implicating post-translational modifications as potential mechanisms leading to DNMT accumulation and chromatin modifications.

Histone modifications, including methylation and acetylation, have also been implicated in mesenchymal cell activation and matrix remodeling during the initiation and progression of pulmonary fibrosis (Helling and Yang, 2015). Histone methylation is regulated by a dynamic interplay between histone methyltransferases (HMTs) and histone demethylases (HDMs), where HMTs add methyl groups at lysine and arginine residues on the H3 and H4 histones, and HDMs enzymatically remove methyl groups from these residues (Bannister and Kouzarides, 2011; Mandumpala et al., 2022). Dysregulation of the balance between these epigenetic modifications has been found to contribute to the activation of mesenchymal cells (Huang et al., 2013). Xiao and colleagues showed that Enhancer of zeste homolog 2 (EZH2), a histone methyltransferase involved in the trimethylation of histone H3 at lysine

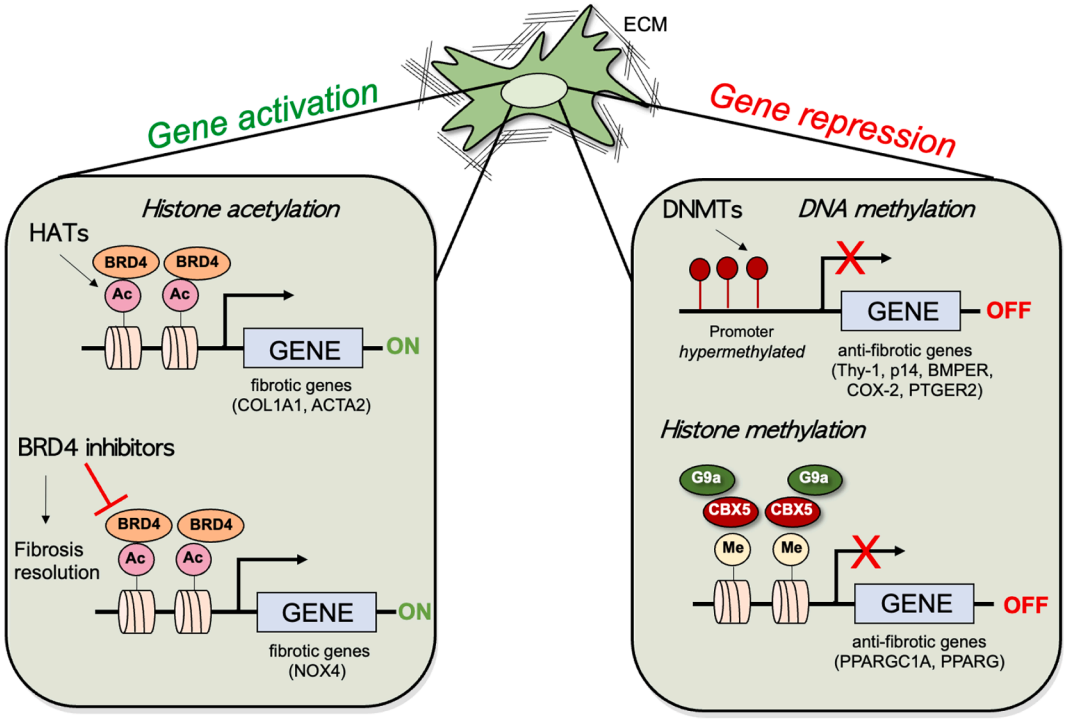

Fig. 4. Epigenetic mechanisms involved in lung fibroblast activation. Epigenetic mechanisms (DNA methylation or histone methylation/acetylation) are implicated in fibroblast activation by promoting or inhibiting the transcription of pro-fibrotic (ACTA2, COL1A1) or anti-fibrotic genes (PPARG, PPARGC1A).

27, was upregulated in activated fibroblasts in response to TGFβ *in vitro,* and its enzymatic attenuation by a specific inhibitor, blocked TGFβ fibrogenic effects and ameliorated lung fibrosis in mice (Xiao et al., 2016). We showed that the histone methyltransferase G9a (EHMT2), a histone methyltransferase involved in the mono- and di-methylation of histone H3 at lysine 9, in concert with the epigenetic reader chromobox homolog 5 (CBX5, also known as HP1α), is essential to the initiation and maintenance of fibroblast activation through epigenetic repression of peroxisomal and mitochondrial genes (Ligresti et al., 2019). This latter study also led to the discovery that epigenetic inhibition of the mitochondrial regulator PGC1α (encoded by *PPARGC1A*) is essential to the activation and metabolic reprogramming of IPF-derived fibroblasts (Caporarello et al., 2019), and restoration of PGC1α in these cells, reduced their contractility and collagen secretion. In a different study, we demonstrated that inhibition of G9a in human lung fibroblasts *in vitro* promoted the elevation of CEBPA, a transcription factor and PGC1α co-activator (Ligresti et al., 2019), whose function is critical in the regulation of energy homeostasis and lipid storage (Cheng et al., 2018). These findings suggest that epigenetic mechanisms controlling mesenchymal cell metabolism may be coupled to their sustained contractile and synthetic activation during pulmonary fibrosis. Furthermore, G9a deletion in collagen-expressing mesenchymal cells resulted in decreased collagen production and attenuated bleomycin-induced fibrosis (Ligresti et al., 2019). Intriguingly, we found that CBX5 was elevated in IPF-derived lung fibroblasts and that CBX5 silencing blocked the transcription of pro-fibrotic genes in these cells, while promoting the elevation of anti-fibrotic genes (Ligresti et al., 2019), suggesting that CBX5 epigenetically sustains the fibrogenic state of diseased fibroblasts *ex vivo* **(**Fig. 4**)**.

Histone acetylation, which is tightly regulated by the interplay between histone acetyltransferase (HATs) and histone deacetylase (HDACs), has also been shown to be critical in the pathogenic conversion of lung mesenchymal cells during lung fibrosis (Li et al., 2017; Hu et al., 2017). A large body of literature reported the role of HDACs in this pathogenic conversion and several HDAC inhibitors have been shown to epigenetically reprogram activated mesenchymal cells *in vitro* and block lung fibrosis *in vivo* (Korfei et al., 2022; Lyu et al., 2019; Sanders et al., 2014). Given the role of HDACs in inhibiting gene transcription by deacetylating histone tails and promoting chromatin compaction (Seto and Yoshida, 2014;6(4):a018713-a.; Li et al., 2020), the antifibrotic effects of HDAC inhibitors, may be, at least in part, due to the upregulation of anti-fibrotic genes. In support of this hypothesis, we recently found that the HDAC inhibitor pracinostat potently blocked lung fibroblast activation *in vitro*, and this effect was primarily mediated by HDAC7 and associated with the upregulation of ant-fibrotic genes, including *PPARGC1A*, *DACH1* and *ARHGAP12* (Jones et al., 2019). These findings further magnify the interplay between histone methylation and acetylation in the transcriptional repression of anti-fibrotic mediators to facilitate myofibroblast accumulation and fibrosis progression. Furthermore, HDACs and DNMTs have been shown to interact with the epigenetic reader CBX5 to maximize their repressive functions (Lan and Shi, 2009; Aygün et al., 2013), demonstrating how epigenetic silencing of endogenous anti-fibrotic genes contributes to the transition of mesenchymal cells to an activated, fibrogenic state.

Whereas epigenetic readers, such as CBX5, are important mediators of chromatin compaction and anti-fibrotic gene silencing, others were shown to be essential in promoting chromatin accessibility and facilitate the transcription of genes important in fibrosis, including those encoding for collagens and contractile genes. For example, the epigenetic reader bromodomain-containing protein 4 (BRD4), a bromodomain and extra-terminal (BET) family member that binds to acetylated histone lysine and promotes gene transcription, was found to be essential for the transcription of pro-fibrotic genes in pathogenic lung fibroblasts *in vitro* and *in vivo* (Bernau et al., 2022; Stock et al., 2019). Inhibition of BRD4 using the specific inhibitor JQ1, attenuated the transcription of pro-fibrotic genes in IPF-derived lung fibroblasts (Stock et al., 2019; Tang et al., 2013), suggesting that BRD4-mediated transcription is central in promoting long lasting fibrogenic effects **(**Fig. 4**)**. One of the first evidence linking epigenetic mechanisms to persistent lung fibrosis in a preclinical mouse model was recently reported in a paper by Sanders and colleagues, in which the contribution of BRD4 to persistent lung fibrogenesis in aged mice was investigated (Sanders et al., 2020). Using a newly developed BRD4 inhibitor (OTX015) the authors showed that targeting this epigenetic reader reversed established age-associated lung fibrosis, and this effect was accompanied by a marked reduction of Nox4 expression in mesenchymal cells (Sanders et al., 2020). Mechanistically, targeting Brd4 partially inhibited the nuclear translocation and DNA recruitment of the acetyltransferases p300 to Nox4 gene promoter, leading to Nox4 gene repression. Further analysis revealed that Nox4 transcription was dependent on H4K16 acetylation at the Nox4 promoter, demonstrating that acetylation marks are maintained and passed through cell generations to ensure sustained transcriptional activation of pro-fibrotic mediators. These appealing observations support previous *in vitro* observations reported in a study by Stock and colleagues, in which the anti-fibrotic effect of the BRD4 inhibitor JQ1 was tested in fibroblasts isolated form IPF lungs (Stock et al., 2019). This study showed that JQ1 reversed TGFβ-mediated NOX4 upregulation and attenuated ROS production and consequently myofibroblast differentiation.

In the light of evolving molecular insights into the role of metabolic reprogramming in fibrosis (Henderson and O'Reilly, 2021; Selvarajah et al., 2021;14(697):eaay1027.), more studies are required to deepen understanding how epigenetic mechanisms are implicated in this process. Furthermore, metabolic intermediates, which are produced during energy generation and biosynthesis, are utilize as substrates and co-factors for a variety of epigenome-modifying enzymes (Nieborak and Schneider, 2018; Paro et al., 2021), allowing metabolism to directly transfer environmental changes to the chromatin.

Finally, several studies have also demonstrated that epigenetic-mediated pathways contribute to apoptosis resistance of lung mesenchymal cells (Huang et al., 2013; Bartczak et al., 2020; Sanders et al., 2014; Bulvik et al., 2020). For example, HDAC-mediated gene silencing was reported to be implicated in the apoptosis resistance of IPF-derived lung fibroblasts (Bulvik et al., 2020). Fibroblasts from IPF patients exhibit decreased histone acetylation and increased methylation of the Fas gene, which is consistent with its constrained expression in IPF lungs (Huang et al., 2013). Pharmacological inhibition of HDACs restored Fas expression in IPF fibroblasts and sensitized them to apoptosis. An epigenetic bases for the apoptosis resistance of IPF-derived fibroblasts was further reported in a study, in which the authors demonstrated that inhibition of histone deacetylation using SAHA, a potent inhibitor of class I and II histone deacetylases, induced apoptosis of IPF-derived lung fibroblasts (Huang et al., 2013; Sanders et al., 2014). SAHA treatment was shown to upregulate the pro-apoptotic gene Bak while down-regulating the anti-apoptotic gene Bcl-xL in these diseased cells, suggesting that an epigenetic intervention may serve as a strategy to overcome apoptosis resistance and promote fibrosis resolution.

All together, these data support the notion that epigenetic mechanisms are inextricably linked to the pathogenic and metabolic conversion of mesenchymal cells, and they are critically important to initiate, and perhaps sustain, the fibrogenic sequel of lung mesenchymal cell activation. Given that the large majority of these studies do not consider the heterogeneity of lung mesenchymal cells, future investigations using single-cell epigenomic tools, including single cell ATAC-seq and single cell DNA methylation, will be necessary to elucidate the impact of epigenetic mechanisms on specific chromatin decoration patterns in different mesenchymal cell types.

6. Final remarks

Over the last two decades much has been learned about the genesis and differentiation of myofibroblasts during lung fibrosis, and multiple cellular and molecular mechanisms have been implicated in the

pathogenesis of this disease. Mesenchymal cells in the lung are now recognized to be heterogeneous, with different cellular trajectories and transcriptional profiles that are engaged during the differentiation of myofibroblasts. Studies based on IPF lungs also identified multiple mesenchymal cell populations that expressed the same markers identified in fibrotic mouse lungs, suggesting that signaling pathways responsible for the myofibroblasts appearance may be conserved between mice and humans. While epigenetic- and stiffness-related mechanisms are important during the activation of lung mesenchymal upon injury, how these mechanisms are involved in the persistent activation of these cells, and whether they are responsible for the mesenchymal cell heterogenicity, is not fully understood. Future studies aimed at understanding these important aspects of the lung mesenchymal cell biology are critical for the identification of novel therapeutic approaches for the management of lung fibrotic disorders.

Funding

Supported by NIH HL142596 (G.L.), HL158733 (G.L.), HL127203 (S. K.H.).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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