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**Lineage tracing of alveolar fibroblasts during delayed  
resolution of pulmonary fibrosis in aged mice**

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“Lineage tracing of alveolar fibroblasts during delayed resolution of pulmonary fibrosis in aged mice”

In the Institute for Lung Health (ILH)

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## Abbreviations and Acronyms

3D: Three-dimensional

4-OHT: 4-Hydroxytamoxifen

A2M: Alpha-2-macroglobulin

*Acta2*: Actin Alpha 2, Smooth Muscle

*Adamts4*: ADAM metalloproteinase with thrombospondin type 1 motif 4

AECs: Alveolar epithelial cells

*Ager*: Advanced glycosylation end-product specific receptor

Alv: Alveolar organoids

AM: Alveolar macrophage

AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase

APOE: Apolipoprotein E

ApoptoticF: Apoptotic fibroblasts

ASMCs: Airway smooth muscle cells

AXIN2: Axis inhibition protein 2

BAL: Bronchoalveolar lavage

BALO: Bronchioalveolar lung organoid

BASCs: Bronchioalveolar stem cells

Bleo: Bleomycin

BMP: Bone morphogenetic protein

CCR7: C-C motif chemokine receptor 7

CD20: Cluster of differentiation 20

CD31: Cluster of differentiation 31

CD45: Cluster of differentiation 45

*CEBPB*: CCAAT Enhancer Binding Protein Beta

CFE: Colony-forming efficiency

CO<sub>2</sub>: Carbon dioxide

Col13a1: Collagen type XIII alpha 1 chain

*COL1A1*: Collagen type I alpha 1 Chain

CTGF: Connective tissue growth factor

*CTHRC1*: Collagen triple helix repeat containing 1

CXCL12: C-X-C motif chemokine ligand 12  
CXCR4: C-X-C motif chemokine receptor 4  
DAPI: 4',6-diamidino-2-phenylindole  
DEGs: Differentially expressed genes  
DMEM: Dulbecco's Modified Eagle's Medium  
*d.p.i.*: Days post instillation  
ECM: Extracellular matrix  
ECs: Endothelial cells  
EGF: Epidermal growth factor  
EMT: Epithelial-to-mesenchymal transition  
EndoMT: Endothelial-to-mesenchymal transition  
FACS: Fluorescence-Activated Cell Sorting  
FBS: Fetal Bovine Serum  
FGF: Fibroblast growth factor  
FGF10: Fibroblast growth factor 10  
FGFR: Fibroblast growth factor receptor  
FGFR2b: Fibroblast growth factor receptor 2b  
FVC: Forced vital capacity  
FOXD1: Forkhead box D1  
FOXJ1: Forkhead box J1  
FSP1: Fibroblast-specific protein 1  
FST: Follistatin  
GEO: Gene expression omnibus  
GO: Gene ontology  
GPR40: G-protein-coupled receptor 40  
Gyg: Glycogenin  
h: Human  
HBSS: Hank's balanced salt solution  
HEPES: N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)  
H&E: Hematoxylin/ eosin  
HLCA: Human lung cell atlas



HO-1: Heme oxygenase-1

*HOPX*: HOP Homeobox

*Hprt*: Hypoxanthine guanine phosphoribosyl transferase

*i.p.*: Intraperitoneally

*i.t.*: intratracheal

IAAPs: injury-associated alveolar progenitors

IF: Immunofluorescence

IFN $\gamma$ : Interferon gamma

ILD: Interstitial lung disease

IM: Interstitial macrophage

*INMT*: Indolethylamine N-Methyltransferase

IPF: Idiopathic pulmonary fibrosis

ITS: Insulin/Transferrin/Selenium

JAK: Janus kinase

KEGG: Kyoto Encyclopaedia of Genes and Genomes

Krt5: Keratin 5

LIF: Lipofibroblast

*LIMCH1*: LIM and Calponin Homology Domains 1

LPA: Lysophosphatidic acid

LPAR: Lysophosphatidic acid receptor

m: Mouse

Macf1: Microtubule actin cross-linking factor 1

MTC: Masson's Trichrome

MEM: Minimum essential medium

Mfap4: Microfibril associated protein 4

Min: Minutes

ml: Milliliters

MMT: Mesothelial-to-mesenchymal transition

mTOR: Mammalian target of rapamycin

MyoFBs: Myofibroblasts

MYH11: Myosin heavy chain 11-positive

NG2: Neural/glial antigen 2  
 nm: Nanometers  
 NOX4: NADPH oxidase 4  
 NPNT: Nephronectin  
 ns: Not significant  
 NSIP: Non-specific interstitial pneumonia  
 PBS: Phosphate buffer saline  
 PBGD: Porphobilinogen Deaminase  
 PCLS: precision-cut lung slice  
 PD-L1: Programmed cell death 1 ligand 1  
 PDE4B: Phosphodiesterase 4B  
 PDGFRA: Platelet derived growth factor receptor alpha  
 PDGFR $\beta$ : Platelet-derived growth factor receptor  $\beta$   
 PECAM-1: Platelet and endothelial cell adhesion molecule 1  
 Pen/Strep: Penicillin/Streptomycin  
 Pen/Strep/L-Glu: Penicillin/streptomycin/L-glutamine  
 PFA: Paraformaldehyde  
 PI3K: Phosphatidylinositol 3-kinase  
 PLIN2: Perilipin 2  
 PMCs: Pleural mesothelial cells  
 PPAR: Peroxisome proliferator-activated receptor  
 PPARG: Peroxisome proliferator activated receptor gamma  
 ProlifF: Proliferating fibroblasts  
 qPCR: Quantitative-real time PCR  
 rMCs: Resident mesenchymal cells  
 Robo2: Roundabout guidance receptor 2  
 ROCK2: Rho associated coiled-coil containing protein kinase 2  
 RSMCs: Repair-supportive mesenchymal cells  
 RNA: Ribonucleic acid  
 RT: Room temperature  
 rTIMP-3: Recombinant tissue inhibitor of metalloproteinases

RTKs: Receptor tyrosine kinases  
Runx1: Runt-related transcription factor 1  
Sal: Saline  
*Sca-1*: Stem cell antigen 1  
scRNA-seq: Single-cell RNA sequencing  
Scube2: Signal peptide-CUB-EGF domain-containing protein 2  
SEM: Standard error of the mean  
*Sfrp1*: Secreted frizzled-related protein 1  
*SFTPC*: Surfactant protein C  
siRNA: Small interfering RNA  
SMC: Smooth muscle cells  
SPF: Specific pathogen-free  
*SPP1*: Secreted phosphoprotein 1  
T2D: Type 2 diabetes  
Tam: Tamoxifen  
TASCs: Type 2-associated stromal cells  
TAZ: Transcriptional coactivator with PDZ-binding motif  
*Tcf21*: Transcription factor 21  
tdTom: tdTomato  
TG: Transgenic  
TGF $\beta$ 1: Transforming growth factor  $\beta$ 1.  
TNF $\alpha$ : Tumor necrosis factor-alpha  
UIP: Usual Interstitial Pneumonia  
VCAN: Versican  
VE-cadherin: Vascular endothelial cadherin  
Veh: Vehicle  
vWF: Von willebrand factor  
WNT: Wiggless-related integration site  
WT: Wild-type  
*Wt1*: Wilms tumor 1  
YAP: Yes-associated protein

μl: Microliters

μm: Micrometers

°C: Celsius

## 1. Introduction

### 1.1 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is an idiopathic form of usual interstitial pneumonia (UIP) and remains one of the most aggressive and lethal forms of interstitial lung disease (ILD). The median survival time after diagnosis is 2-3 years (Gross and Hunninghake, 2001; Günther et al., 2012; Selman et al., 2001). Current anti-fibrotic therapies are not capable of halting the progression of this disease; rather, they only slightly delay disease progression and lead to mild improvement in the quality of life of affected patients. Such therapies are also very broad, and not only inhibit profibrotic signaling (such as transforming growth factor beta or TGF $\beta$  pathway) but also benign, repair-promoting signaling pathways (such as fibroblast growth factor receptor 2b (FGFR2b) signaling) (El Agha et al., 2018). This is mainly attributed to a gap in our knowledge regarding the precise identity of both culprit and proregenerative cells in the fibrotic lung and the associated molecular mechanisms. The latter likely need to be manipulated in a cell type-specific manner to efficiently reverse aberrant parenchymal remodeling and promote regeneration (Zabihi et al., 2024).

Idiopathic pulmonary fibrosis is characterized by loss of alveolar epithelial type 1/2 cells (AEC1s/ AEC2s) and replacement of the intricate alveolar network by fibrous scars populated by myofibroblasts (MyoFBs) and the associated extracellular matrix (ECM) components including collagen, laminin, and fibronectin. This also creates a chronic inflammatory environment together with macrophages and other immune cells. While balanced activation of MyoFBs is central to physiological wound healing, persistent activation of these cells lead to aberrant wound healing process and the accompanying fibrogenesis in the lung can lead to progressive loss of lung structure and function, and consequently respiratory failure (**Figure 1**) (Zabihi et al., 2024). During the healing process, both immune cells and fibroblasts become activated. These activated fibroblasts secrete ECM components at the site of injury, and orchestrate matrix remodeling, which facilitates wound closure. Under physiological conditions, these fibroblasts are subsequently removed through apoptotic clearance, thereby re-establishing tissue

homeostasis. In contrast, MyoFBs exhibit resistance to apoptosis, resulting in persistent ECM accumulation and progressive lung fibrosis (Phan, 2012; Zabihi et al., 2024).

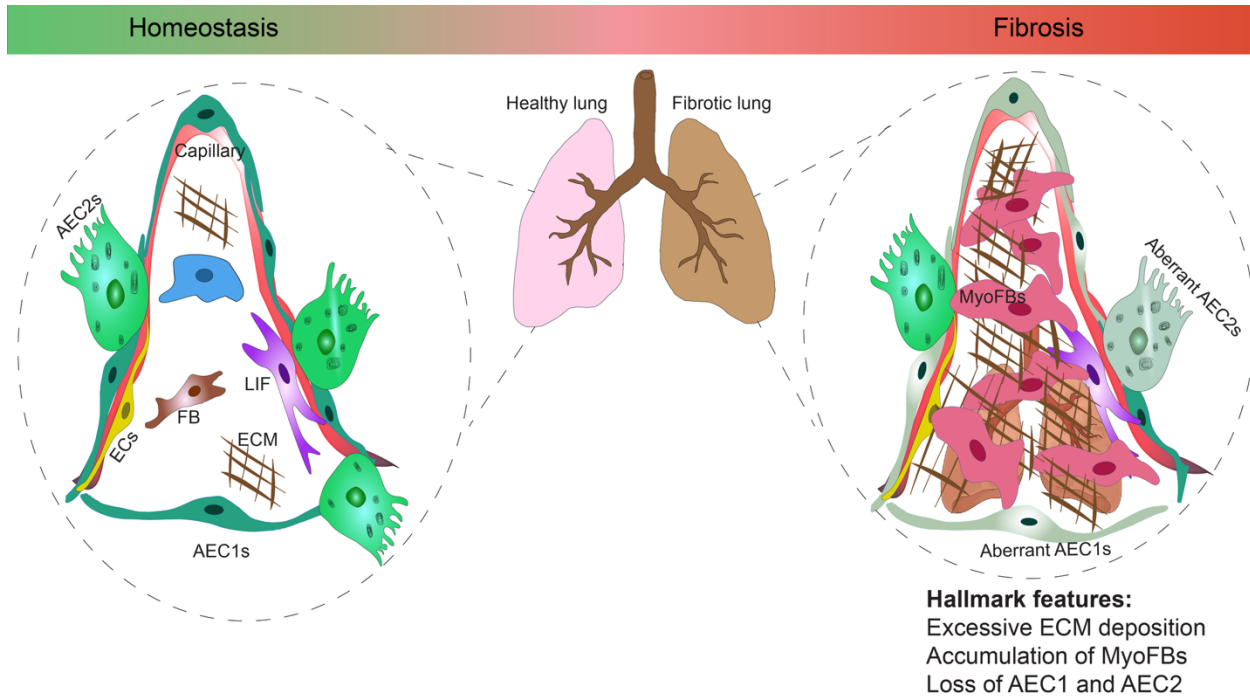
Early studies documented MyoFBs as modified fibroblasts in granulation tissue and their role was linked to wound contraction and healing (Gabbiani et al., 1971). It was established that such spindle and stellate-shaped cells with cytoplasmic stress fibers had similar features to smooth muscle cells (SMC). Indeed, MyoFBs express alpha-smooth muscle actin (ACTA2) and type I collagen, which distinguishes them from normal fibroblasts (Hinz et al., 2007; Zhang et al., 1994). MyoFBs are the main regulatory cells that secrete and deposit excessive ECM. Yet, the expression of *Acta2* is often transient and therefore not consistently expressed by MyoFBs and their derivatives in the fibrotic lung. Another issue is that *Acta2* expression does not always correlate with collagen deposition (Biasin et al., 2020; Tsukui et al., 2020; Zabihi et al., 2024).

## **1.2 Signaling pathways involved in the pathogenesis of lung fibrosis**

Basic and translational research, particularly using mouse models, has shown that developmental signaling pathways such as TGF $\beta$ , wingless-related integration site (WNT), hedgehog, and Hippo, to name a few, play a critical role in the emergence and activation of MyoFBs during the development of pulmonary fibrosis (Burgoyne and Königshoff, 2018; Chanda et al., 2019; Kendall and Feghali-Bostwick, 2014; Zabihi et al., 2024). Indeed, TGF $\beta$ 1 is widely regarded as the master regulator of fibrogenesis as it activates fibroblasts, promotes MyoFBs differentiation, and induces ECM deposition (Coker et al., 2001; Hinz and Gabbiani, 2010; Kasai et al., 2005). TGF $\beta$ 1 becomes activated during injury and acts as a potent pro-fibrotic mediator and drives the transition of resident fibroblasts to MyoFBs. TGF $\beta$ 1 is believed to be mainly produced by alveolar macrophages (AMs) and AEC2s. Apart from its direct effect on mesenchymal cells, it also inhibits alveolar epithelial cell proliferation. Although WNT signaling is associated with AEC2s-mediated regeneration (Nabhan et al., 2018; Zacharias et al., 2018), WNT signaling is mainly regarded as profibrotic, and WNT inhibition attenuates fibrosis in animal models (Henderson et al., 2010; Königshoff et al., 2009). WNT is also required for TGF $\beta$ -mediated fibrogenesis (Akhmetshina et al., 2012). Another example is Yes-

associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) that are the effectors of the hippo pathway. It has been shown that YAP and TAZ promote MyoFBs differentiation and their targeting reverses lung fibrosis (Haak et al., 2019; Link et al., 2022; Liu et al., 2015). On the other hand, bone morphogenetic protein (BMP) signaling is regarded as mostly anti-fibrotic signaling. BMP2, BMP4, and BMP7 are among the most studied BMP ligands in the context of lung fibrosis. BMP4 suppresses ECM production by human fibroblasts, and overexpression of BMP7 inhibits TGF $\beta$ -induced collagen production by MyoFBs (Pegorier et al., 2010). BMP2 inhibits TGF $\beta$ 1 action via peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) activation in SMC (Calvier et al., 2017) and induces lipogenic differentiation in fibroblasts derived from IPF lungs (Kheirollahi et al., 2019). Finally, fibroblast growth factor (FGF) signaling is generally anti-fibrotic (such as FGF7 and FGF10 signaling through the epithelial III-b isoform of FGFR2; FGFR2b), while few ligands (such as FGF2) signaling through the mesenchymal form (FGFR2c) can be profibrotic (El Agha et al., 2017; Gupte et al., 2009; MacKenzie et al., 2015, 2015; Zabihi et al., 2024).

To date, there is no curative therapy for IPF. However, active research in the fields of lineage tracing, multi-omics, and mouse and humanized lung injury models have expanded our understanding regarding the cellular origin of MyoFBs and shed light on novel therapeutic avenues that hold promise for future exploitation to treat patients (Zabihi et al., 2024).



**Figure 1. Cellular changes in the alveolar niche during homeostasis and fibrosis.**

The maintenance of alveolar structure and function in healthy lung tissue relies on dynamic crosstalk between epithelial and mesenchymal cell populations. However, repetitive epithelial injury disrupts epithelial–mesenchymal interaction and leads to lung fibrosis. This triggers fibroblast activation and massive recruitment of MyoFBs, which accumulate in the alveolar niche. MyoFBs secrete excessive amounts of ECM proteins leading to abnormal matrix stiffening, distortion of alveolar architecture, and progressive scarring. AEC1s/ AEC2s: Alveolar epithelial type 1/2 cells; ECs: Endothelial cells; ECM: Extracellular matrix; LIF: Lipofibroblast; FB: Fibroblasts; MyoFBs: Myofibroblasts.

### 1.3 Current and novel therapeutic approaches for Idiopathic pulmonary fibrosis

Three compounds are currently approved by the FDA to treat IPF: pirfenidone (Azuma et al., 2005; King et al., 2014; Noble et al., 2011; Raghu et al., 1999; Taniguchi et al., 2010), nintedanib (Hostettler et al., 2014; Richeldi et al., 2014) and nerandomilast (Herrmann et al., 2022; Richeldi et al., 2025, 2022). Pirfenidone is an oral drug with anti-inflammatory, antioxidative, and anti-proliferative properties and therefore slows fibrogenesis through modulation of collagen generation, reduction of TGF $\beta$ 1-induced fibroblast activation, and circulating tumor necrosis factor-alpha (TNF $\alpha$ ). Nintedanib is an inhibitor of multiple



receptor tyrosine kinases (RTKs) including vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR). Nintedanib therefore targets downstream signaling pathways related to the proliferation, migration, and differentiation of mesenchymal cells and MyoFBs. However, such scattergun approach might have consequences related to shutting down signaling pathways that are potentially beneficial for lung regeneration such as FGFR2b signaling (El Agha et al., 2018). Unfortunately, these medications do not cure or completely halt IPF progression, even display tolerability and side effects. Prolonged consumption of these two drugs may cause resistance and ultimately lung failure. Lung transplantation remains another option for these patients, but the availability of donor lungs is obviously limited (Liu et al., 2017). Nerandomilast (BI 1015550) is a novel, oral drug and selective phosphodiesterase 4B (PDE4B) inhibitor with antifibrotic and immunomodulatory mechanisms (Richeldi et al., 2025). PDE4 enzymes are responsible for hydrolyzing the second messengers, cyclic adenosine monophosphate (cAMP) and cGMP, that regulate inflammation and fibroblast activity. Roflumilast, a non-selective PDE4 inhibitor, is clinically limited by gastrointestinal adverse effects. (Kolb et al., 2023). In contrast, nerandomilast is selective inhibition of PDE4B and improved tolerability. Recent clinical trial on nerandomilast showed a slight decline in forced vital capacity (FVC) in IPF patients over 52 weeks, indicating that disease progression was attenuated. Patients treated with PDE4B inhibitor, either alone or in combination with existing antifibrotic drugs such as nintedanib or pirfenidone, showed improved maintenance of lung function compared to placebo group (Richeldi et al., 2025).

There have been initial concerns regarding drug–drug interactions between pirfenidone and nintedanib affecting pharmacokinetics and bioavailability. However, nintedanib with add-on pirfenidone or pirfenidone with add-on nintedanib did not show additional side effects compared to monotherapy. Yet, it is still unclear whether combination therapy confers a benefit in terms of outcomes (Flaherty et al., 2018; Huh et al., 2021; Vancheri et al., 2018). Another combination therapy is pirfenidone with add-on N-acetylcysteine. While such treatment did not alter the tolerability profile of pirfenidone, it did not seem to be beneficial for patients (Behr et al., 2016). Similarly, sildenafil add-on to anti-fibrotic

treatment did not improve clinical outcomes (Kang and Song, 2021; Kolb et al., 2018; Zabihi et al., 2024). On the other hand, preclinical assessment of combination therapy with IFN $\gamma$  and pirfenidone appears to be promising. IFN $\gamma$  exerts an anti-fibrotic effect by inhibiting MyoFBs differentiation (Vu et al., 2019). It remains to be seen whether such combination would benefit IPF patients (Zabihi et al., 2024).

#### **1.4 Bleomycin-induced pulmonary fibrosis model and the impact of aging on fibrogenesis**

Bleomycin-induced lung injury is the most widely used and well-characterized experimental model for investigating the development and resolution of pulmonary fibrosis. Bleomycin, a chemotherapeutic antibiotic, was identified as a pro-fibrotic agent when patients with Hodgkin's lymphoma and testicular cancer developed lung fibrosis following administration (Hollander et al., 2016; Udupa et al., 2019). Bleomycin acts by causing single and double-strand DNA breaks in tumor cells, thereby disrupting the cell cycle leading to apoptosis (Moore and Hogaboam, 2008). The first animal model of bleomycin-induced interstitial lung fibrosis was established in 1974 through repeated intraperitoneal injections in rats (Adamson and Bowden, 1974). Bleomycin administration in rodents leads to acute epithelial injury, followed by inflammation, fibroblast activation, ECM deposition, and ultimately fibrosis. The model goes through acute inflammation (days 1–7), fibrogenesis (days 7–28), and resolution (days 42–63) phases, making it a valuable tool for studying fibrogenesis, cellular dynamics and signaling pathways (Izbicki et al., 2002). However, this model has been criticized for its inability to fully recapitulate fibrosis in human including honeycomb cyst or fibroblastic foci formation, age and gender dependency, the presence of inflammation/acute lung injury preceding the fibrotic phase, rapid progression of the disease and auto resolution mechanism, where it is irreversible in human (Moeller et al., 2008). Despite these limitations, the bleomycin-induced lung fibrosis model remains the most cost-effective, reproducible and widely used model of IPF, providing critical insights into IPF pathogenesis and potential therapeutic strategies.

As mentioned above, age and gender can influence the level of fibrosis progression. Aged male mice are more prone to fibrosis with a greater mortality and fibrotic changes

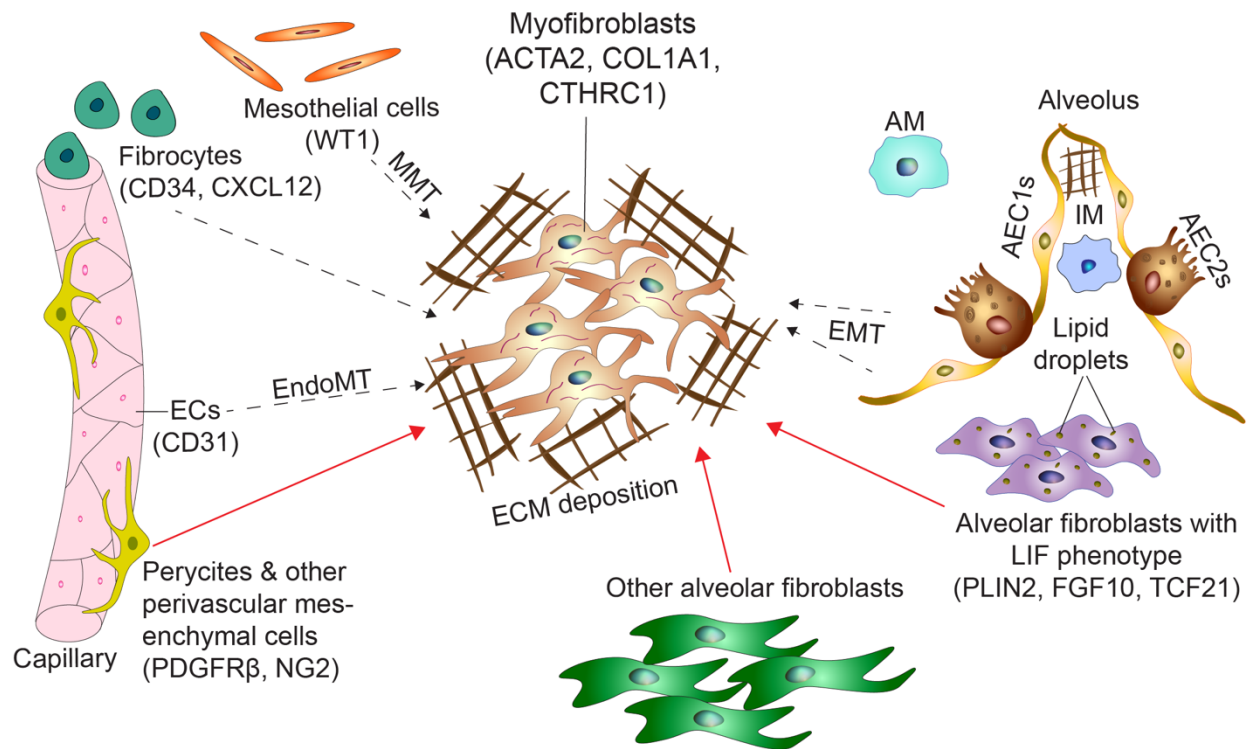
compare to young male mice. Moreover, female mice showed less fibrosis and mortality than male mice (Redente et al., 2011). Furthermore, most of the studies focused on young mice, which is a limitation since bleomycin-induced pulmonary fibrosis in young mice spontaneously resolves, failing to replicate the persistent fibrosis observed in human IPF patients (Hecker et al., 2009). Since IPF is an age-associated disease, studying fibrosis in aged mice may provide more clinically relevant insights. Aged mice exhibit delayed fibrosis resolution and prolonged transitional states, making them a better model for understanding differentiation trajectories during both fibrosis formation and delayed resolution (Redente et al., 2011; Sueblinvong et al., 2012). A recent study showed impaired resolution and altered mesenchymal niche activity in aged bleomycin-treated mice lungs, and the mechanisms of the LIF-to-MyoFB switch remain largely conserved between young and old mice (Lingampally et al., 2024).

Aging, environmental exposures and genetic factors are associated with an increased risk of developing IPF (García-Sancho et al., 2011; Selman et al., 2016; Taskar and Coultas, 2006). IPF is an age-related disease and typically manifests in middle-aged adults, suggesting a strong link between aging and increased incidence and prevalence (Raghu et al., 2006). Hallmarks of aging including genomic instability, telomere shortening, cellular senescence, mitochondrial dysfunction, and elevated reactive oxygen species (ROS) have been implicated in IPF pathogenesis (Thannickal, 2013). Notably, NADPH oxidase 4 (NOX4), an enzyme involved in ROS generation and regulation, is elevated in IPF lungs, and its inhibition has been shown to attenuate lung fibrosis (Hecker et al., 2009). Further refinement of the LIF population enriched with stem cell antigen 1 (*Sca-1*)/*Fgf10* double-positive cells suggested a higher potential for this subpopulation to support AEC2s growth in alveolar organoids (Taghizadeh et al., 2021). However, aging impairs the niche activity of rMCs due to increased *Nox4* expression, which can be reversed by NOX4 inhibition. Of note, young AEC2s formed fewer and smaller organoids when co-cultured with aged rMCs, whereas aged AEC2s cells showed no significant change regardless of whether they were co-cultured with young or aged rMCs (Chanda et al., 2021). Moreover, lipid scarcity in aging lungs has been implicated in reduced

AEC2s renewal capacity, and lipid replenishment has been shown to restore this activity in alveolar organoids (Liang et al., 2024).

### **1.5 Cellular sources of myofibroblasts in lung fibrosis**

Understanding the cellular origin and related signaling pathways that control the emergence, maintenance and function of MyoFBs may help develop novel anti-fibrotic therapies. The origin of MyoFBs in lung fibrosis continues to be debated although mounting evidence indicates that they mainly derive from alveolar fibroblasts such as LIFs. Other mesenchymal subsets such as adventitial fibroblasts and pericytes, in addition to epithelial, endothelial, and bone marrow-derived cells also contribute to the fibrogenic milieu (**Figure 2**) (Zabihi et al., 2024). With the advent of the single-cell and spatial omics, the key will be to integrate the wealth of knowledge acquired through multiple independent studies to select the most relevant pathological mechanisms that hold promise for therapeutic intervention. Correcting aberrant cellular and molecular mechanisms and differentiation trajectories to promote fibrosis resolution, such as by MyoFBs apoptosis and deactivation through redifferentiation to LIFs, is a promising therapeutic strategy. While novel therapeutic targets currently under investigation hold great promise to treat IPF, the holy grail remains achieving targeted therapy where culprit cells are selectively targeted and proregenerative cells are nurtured to overcome remodeling and promote lung regeneration, thus reversing pulmonary fibrosis (Zabihi et al., 2024).



**Figure 2. Model for the cellular origin of MyoFBs in the fibrotic lung.** Recent evidence indicates that resident populations of mesenchymal cells are the major contributors to MyoFBs accumulation through direct transdifferentiation (red arrows). Other putative differentiation events are marked by dashed lines and likely represent minor contributors to MyoFBs pool. AM: Alveolar macrophage; AEC1s/ AEC2s: Alveolar epithelial type 1/2 cells; CD31: Cluster of differentiation 31; CD34: Cluster of differentiation 34; COL1A1: Collagen type I alpha 1; CTHRC1: Collagen triple helix repeat containing 1; CXCL12: C-X-C motif chemokine ligand 12; ECs: Endothelial cells; ECM: Extracellular matrix; EMT: Epithelial-to-mesenchymal transition; EndoMT: Endothelial-to-mesenchymal transition; FGF10: Fibroblast growth factor 10; IM: Interstitial macrophage; LIF: Lipofibroblast; MMT: Mesothelial-to-mesenchymal transition; MyoFBs: Myofibroblasts; NG2: Neural/glial antigen 2;  $\beta$ : Platelet-derived growth factor receptor  $\beta$ ; PLIN2: Perilipin 2; TCF21: Transcription factor 21; WT1: Wilms tumor 1 (Zabihi et al., 2024).

### **1.5.1 Lipofibroblasts as a source and fate for myofibroblasts in the fibrotic lung**

There is a great body of literature suggesting that MyoFBs have a mesenchymal origin (El Agha et al., 2017; Hoyles et al., 2011; Kleaveland et al., 2014; Kramann et al., 2015; Rock et al., 2011; Tsukui et al., 2015; Xie et al., 2016). LIFs are resident alveolar fibroblasts that are enriched with neutral lipid droplets and are important for AEC2s maintenance in the alveolar compartment of the lung. They provide adjacent AEC2s with triglycerides to be incorporated in the process of surfactant production (McGowan and Torday, 1997; Tahedl et al., 2014). LIFs are also niche cells for AEC2s stem cells and are therefore important for their maintenance and self-renewal (Barkauskas et al., 2013; Chung et al., 2018; Taghizadeh et al., 2021). Being in close proximity to AEC2s renders LIFs susceptible to profibrotic cytokines produced by AEC2s and immune cells, and therefore a likely contributor to the MyoFBs pool in the fibrotic lung. Previously, it had been shown that fetal rat lung fibroblasts lose their lipogenic phenotype and acquire a MyoFBs-like phenotype when exposed to hyperoxia (Rehan and Torday, 2003). Human embryonic lung fibroblasts follow a similar trend when exposed to nicotine (Rehan et al., 2007). LIFs had been known to express the adipocyte marker adipose differentiation-related protein (ADRP or perilipin 2 [PLIN2]), which is important for lipid uptake and trafficking (Schultz et al., 2002), and PPAR $\gamma$  (Rehan and Torday, 2012; Varisco et al., 2012), the master regulator of adipogenesis (Varisco et al., 2012). FGF10 and transcription factor 21 (TCF21) are other known markers for LIFs (Al Alam et al., 2015; El Agha et al., 2014; Park et al., 2019). Lineage tracing of PLIN2<sup>+</sup> cells revealed a significant contribution to MyoFBs in bleomycin-induced pulmonary fibrosis. Despite the massive apoptosis of MyoFBs, they differentiate back to LIFs during fibrosis resolution (El Agha et al., 2017). Examination of FGF10<sup>+</sup> cells using an enhancer trap approach showed a similar result. Bulk gene expression analysis on homogenates prepared from lung explants revealed downregulation of adipogenic genes in parallel to upregulation of MyoFBs associated genes. The same study showed that pre-existing SMCs are not a major source of MyoFBs in the fibrotic lung. However, the cells that acquire *Acta2* expression during fibrogenesis, are contributed massively to lipofibroblasts (El Agha et al., 2017; Lingampally et al., 2024; Zabihi et al., 2024).

With the advent of single-cell RNA sequencing (scRNA-seq), a more comprehensive transcriptomic profile has been established for LIFs, and the list of marker genes has been refined to include in addition to the already known markers (*Tcf21*, *Plin2*, *Fgf10*, and *G0s2*), new markers such as *Limch1* (LIM and calponin homolog domains 1), *Gyg* (glycogenin), *Macf1* (microtubule actin cross-linking factor 1), *Mfap4* (microfibril associated protein 4), *Npnt* (nephronectin), *Wnt2*, *Col13a1* (collagen type XIII alpha 1 chain), and *Inmt* (indolethylamine N-methyltransferase). In humans, this list includes *TCF21*, *LIMCH1*, *A2M* (alpha-2-macroglobulin), *RGCC* (regulator of cell cycle), and genes related to lipid/retinoic acid processing and/or storage (Liu et al., 2020). LIFs were detected in both donor and IPF lungs (El Agha et al., 2017; Liu et al., 2020; Schipke et al., 2021) with collagen and ECM-related genes being among the top differentially regulated genes in these cells in IPF lungs compared with donor lungs (Liu et al., 2020). Another study showed that human LIFs can be identified by the expression of *APOE* (apolipoprotein E), *PLIN2*, and *FST* (follistatin) (Travaglini et al., 2020; Zabihi et al., 2024).

More recently, lineage tracing of *TCF21*<sup>+</sup> cells also revealed that LIFs are significant contributors to MyoFBs formation in the context of bleomycin-induced pulmonary fibrosis, and that they pass through a transitional state characterized by the expression of secreted frizzled-related protein 1 (*Sfrp1*) (Mayr et al., 2024). *SFRP1* inhibits lung fibroblast invasion and *TGFβ1* inhibits its expression to promote the progression of these transitional fibroblasts into collagen triple helix repeat containing 1 (*CTHRC1*)<sup>+</sup> MyoFBs. The same study identified adventitial fibroblasts and, to a less extent, peribronchial fibroblasts, are other potential sources of MyoFBs in the fibrotic lung. Importantly, it also showed that MyoFBs undergo deactivation via differentiation into LIFs, peribronchial fibroblasts, and pericytes during fibrosis resolution (Mayr et al., 2024). Another recent study utilized AEC1 ablation or butylated hydroxytoluene (BHT; 3,5-di-tert-4-butylhydroxytoluene)-induced lung fibrosis and subsequent resolution to show that alveolar fibroblasts characterized by the expression of *Tcf21* enter a transitional state marked by the expression of *Sfrp1* that precedes the emergence of *ACTA2*<sup>+</sup> MyoFBs (Konkimalla et al., 2023). The authors identified another subset of transitional alveolar

fibroblasts marked by the expression of *Runx1*. RUNX1+ ACTA2+ cells return to a normal alveolar fibroblast phenotype during fibrosis resolution (Konkimalla et al., 2023), thus agreeing with the model that MyoFBs reacquire their initial AEC2s-supportive phenotype that resembles LIFs during resolution (El Agha et al., 2017; Kheirollahi et al., 2019).

Another line of evidence supporting the LIF-to- MyoFBs axis during fibrosis development is the finding that alveolar fibroblasts expressing signal peptide, CUB domain, EGF-Like 2 (*scube-2*), a population that expresses typical LIF markers such as *Inmt*, *Npnt*, *Tcf21*, and *Wnt2*, have been shown to contribute to MyoFBs formation (Tsukui et al., 2024). Along the way, these cells transit through an inflammatory state before giving rise to MyoFBs (Tsukui et al., 2024). The same group had previously established CTHRC1 as a marker of MyoFBs in the fibrotic lung and used computational analysis to demonstrate that alveolar fibroblasts give rise to MyoFBs in the fibrotic lung (Tsukui et al., 2020). Interestingly, the same study used Col1a1<sup>GFP</sup> mice and showed that both alveolar fibroblasts and adventitial fibroblasts upregulate *Cthrc1* when transferred into bleomycin-treated lungs (Tsukui et al., 2020).

Platelet-derived growth factor receptor alpha (PDGFR $\alpha$ ) is a marker of alveolar fibroblasts including LIFs, also termed type 2-associated stromal cells (TASCs) (Barkauskas et al., 2013; Chung et al., 2018). *Pdgfra*+ rMCs can be a niche for AEC2 progenitor cells. Lineage tracing combined with scRNA-seq of PDGFR $\alpha$ + cells in normal and fibrotic lungs showed that LIFs acquire a reversible fibrogenic profile with an attenuation of lipogenic pathways in the fibrotic lung (Trempe et al., 2023). These findings agree with the reversible LIF-MyoFB-LIF switch previously described before (El Agha et al., 2017; Lingampally et al., 2020).

Currently, the precise fate of MyoFBs during the resolution of pulmonary fibrosis is still debatable. It has been suggested that pathological MyoFBs undergo apoptotic clearance during the resolution of lung fibrosis, and that such event is critical for the removal of collagen-rich scars and restoration of normal lung architecture and function (Pham et al., 2022; Rangarajan et al., 2018; Redente et al., 2020). On the other hand, MyoFBs



deactivation/dedifferentiation represents an alternative route for fibrosis resolution. The latter mechanism involves MyoFBs undergoing fate switching and “returning” to a proregenerative LIF phenotype, which supports the regrowth of healthy alveolar structures populated by AEC1s and AEC2s (El Agha et al., 2017; Kheirollahi et al., 2019). Other fates following deactivation/dedifferentiation are also plausible but await further investigation. Of note, both resolution routes (apoptosis and redifferentiation into LIFs) can be potentiated via treatment with the first-line antidiabetic compound, metformin (Glucophage), which accelerates the resolution of lung fibrosis (Kheirollahi et al., 2019; Rangarajan et al., 2018). While metformin inhibits collagen production (Kheirollahi et al., 2019; Rangarajan et al., 2018) and induces apoptosis (Rangarajan et al., 2018) via an adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)-mediated mechanism, redifferentiation into the LIF phenotype in response to metformin treatment is largely AMPK-independent, and involves the secretion of BMP2, leading to PPAR $\gamma$  activation and subsequent lipogenic differentiation and facilitate lipid droplet formation (Kheirollahi et al., 2019). The molecular mechanisms controlling the apoptosis vs. redifferentiation balance remain to be seen, but they likely involve signals coming from regenerating AEC2s and the surrounding niche, thus dictating fate decision-making in these MyoFBs (Zabihi et al., 2024).

To sum up, there is accumulating evidence implicating LIFs, or more generally alveolar fibroblasts, in fibrogenesis at least by serving as a major cell of origin of pathological MyoFBs that feature in fibrotic lung disease (**Figure 2**). Future research aimed at maintaining these cells in their AEC2s-supportive state and preventing their transdifferentiation into MyoFBs, and/or promoting the redifferentiation of MyoFBs back to LIFs represents a logical and promising therapeutic strategy (Zabihi et al., 2024).

## **1.5.2 Other sources**

### **1.5.2.1 Pericytes and other perivascular cells**

Pericytes are another potential contributor to the MyoFBs pool in the fibrotic lung (**Figure 2**). Pericytes are mesenchymal perivascular cells wrapped around capillaries. They share the basement membrane and closely interact with endothelial cells in the vascular niche

and control tight junctions. Pericytes also support angiogenesis and vascular development (Bergers and Song, 2005). Pericytes are identified by the expression of neural/glial antigen 2 (NG2) (also known as chondroitin sulfate proteoglycan 4 or CSPG4) and platelet-derived growth factor receptor  $\beta$  (PDGFR $\beta$ ) (Winkler et al., 2010). However, since these markers are also expressed by other mesenchymal subsets such as SMCs and fibroblasts, they are not unique pericyte markers. Of note, PDGFR $\beta$  has been shown to mark MyoFBs progenitors in fibrosis of multiple organs including the lung (Henderson et al., 2013). Co-localization of NG2 and ACTA2 has been demonstrated in human IPF lung sections (Sava et al., 2017).

Pericytes were suggested as a source of MyoFBs in bleomycin-induced pulmonary fibrosis in mice (Hung et al., 2013). The evidence was based on the finding that forkhead box D1 (FOXD1)+ mesenchymal progenitors labeled during embryonic development give rise to pericytes postnatally, and that the latter cells expand and produce collagen, along with another independent population of resident fibroblasts (Hung et al., 2013). Lineage tracing of NG2+ and forkhead box J1 (FOXJ1)+ cells showed increased proliferation of these cells in addition to localizing to fibrotic regions; however, it was reported that they express only low levels of ACTA2 (Rock et al., 2011). Moreover, myosin heavy chain 11-positive (MYH11+) cells, a population that include pericytes in addition to SMCs, were proposed to contribute to MyoFBs formation in the fibrotic mouse lung (Hannan et al., 2021). Another study utilized an intratracheal adoptive cell transfer method to show that resident fibroblasts in alveolar walls have the highest profibrotic potential compared to NG2+ pericytes or epithelial cells, thus downplaying the potential of pericytes to give rise to MyoFBs in bleomycin-induced pulmonary fibrosis (Tsukui et al., 2024).

Taken together, pericytes remain an enigmatic and heterogeneous population of mesenchymal cells. The key will be to generate novel research tools allowing deep characterization of these cells at the transcriptomic and functional levels and dissecting them from the rest of the mesenchymal landscape. Their contribution to the MyoFBs pool seems very likely but awaits further confirmatory investigations using better research tools (Zabihi et al., 2024).

### 1.5.2.2 Epithelial cells

Epithelial cells such as AEC2s have been proposed to give rise to MyoFBs by undergoing epithelial-to-mesenchymal transition (EMT) (**Figure 2**). The latter is a dynamic mechanism that was first described by Greenburg and Hay (Greenburg and Hay, 1982) who showed that polarized epithelial cells can differentiate into mesenchymal cells, often fibroblasts and MyoFBs (Willis et al., 2005). Similar to MyoFBs, exaggerated or uncontrolled EMT may lead to tissue fibrosis (Kalluri and Weinberg, 2009). EMT is characterized by downregulation of epithelial markers (such as E-cadherin) and upregulation of mesenchymal markers such as fibroblast-specific protein 1 (FSP1), ACTA2, collagen 1, vimentin, and fibronectin (Gabbiani, 2003; Nieto, 2011; Nieto et al., 2016). Signaling factors including FGF, hepatocyte growth factor (HGF), WNT/ $\beta$ -catenin, epidermal growth factor (EGF), and TGF $\beta$  family can initiate EMT (Hofman and Vouret-Craviari, 2012; Yilmaz and Christofori, 2009). It has been suggested that mesenchymal cells recruited to the site of injury in the context of IPF might stimulate AEC2s to undergo EMT and acquire a fibrogenic nature (Willis, 2006). Most of the experimental evidence for the involvement of EMT in MyoFBs formation stems from the finding that treatment of primary AEC2s or AEC2s-like cell lines with TGF $\beta$  leads to an EMT phenotype *in vitro* (Jayachandran et al., 2009; Maniscalco et al., 1994; Willis et al., 2005; Zabihi et al., 2024). Pulmonary fibrosis induced by overexpression of TGF $\beta$ 1 via adenoviral delivery also shows signs of EMT (Kim et al., 2006). However, *in vivo* lineage tracing of AEC2s in the bleomycin model of lung fibrosis did not show signs of EMT, suggesting that this phenomenon is not very prominent in the fibrotic lung, at least in the bleomycin model in mice (Rock et al., 2011; Zabihi et al., 2024).

### 1.5.2.3 Endothelial cells

It has been reported that endothelial cells also have the potential to undergo a phenotype transition similar to EMT with common characteristics, known as endothelial-to-mesenchymal transition (EndoMT) (**Figure 2**). EndoMT was first proposed by Karasek (Karasek, 2007) who reported that endothelial cells transform into MyoFBs and that this transition is controlled by TGF $\beta$  signaling. In this transition, endothelial cells lose their

typical markers such as cluster of differentiation 31 (CD31)/platelet and endothelial cell adhesion molecule 1 (PECAM-1), tyrosine kinase with immunoglobulin like and EGF like domains 1 (Tie1), tyrosine kinase with immunoglobulin like and EGF like domains 2 (Tie2), vascular endothelial cadherin (VE-cadherin), and von Willebrand factor (vWF), and acquire mesenchymal markers including ACTA2, N-cadherin, collagen 1, and vimentin. Endothelial cells also produce profibrotic molecules that recruit immune cells and contribute to inflammation and fibrosis (Hashimoto et al., 2010; Zeisberg et al., 2008, 2007; Zabihi et al., 2024).

Fate tracing of *Tie2*<sup>+</sup> endothelial cells in bleomycin-induced pulmonary fibrosis revealed co-expression of collagen 1 and a so-called transitional population of *Tie2*/vimentin-double positive cells, suggesting the differentiation of endothelial cells into mesenchymal cells (Jia et al., 2021). Another recent study suggested the presence of endothelial cell-like MyoFBs that are present in the healthy lung, and that these cells contribute to the MyoFBs pool in the fibrotic lung (Wu et al., 2023; Zabihi et al., 2024).

#### **1.5.2.4 Bone marrow-derived mesenchymal precursors: Fibrocytes**

Fibrocytes are spindle-shaped fibroblast-like cells that are derived from hematopoietic precursors and express both stromal and myeloid markers such as collagen 1, cluster of differentiation 34 (CD34), and cluster of differentiation 45 (CD45) (Bucala et al., 1994) (**Figure 2**). These circulating connective tissue cells have been described in human fibrotic lung diseases and cancer as expressing C-X-C motif chemokine receptor 4 (CXCR4), C-C motif chemokine receptor 7 (CCR7), and C-C motif chemokine receptor 2 (CCR2) (Bucala, 2015). Fibrocytes can potentially activate fibroblasts and participate in inflammation leading to ECM remodeling and fibrosis. However, fate mapping of these cells is relatively challenging due to the lack of fibrocyte-specific markers. Moreover, deletion of *Col1a1* gene in the hematopoietic lineage including fibrocytes did not hinder lung fibrosis development and collagen deposition, thus adding to the controversy regarding direct transdifferentiation of fibrocytes into MyoFBs (Kleaveland et al., 2014; Zabihi et al., 2024).

In another study, fibrocytes were detected in IPF but not donor lungs and their abundance positively correlated with the number of fibroblastic foci (Andersson-Sjöland et al., 2008). Flow cytometry-b. The fibrocyte chemoattractant CXCL12 was again found to be elevated in the plasma of IPF patients and was detectable in the bronchoalveolar lavage (BAL) fluid of 40% of patients but not in donors (Andersson-Sjöland et al., 2008). Flow cytometry-based quantification of fibrocyte counts also suggested that their abundance correlates with poor prognosis (Moeller et al., 2009; Zabihi et al., 2024).

#### **1.5.2.5 Pleural mesothelial cells**

The pleura is composed of flattened epithelial cells, called mesothelial cells, originating from the embryonic mesoderm. Pleural mesothelial cells (PMCs) have been shown to acquire a MyoFBs-like phenotype when treated with TGF $\beta$ 1 *in vitro*, a process referred to as mesothelial-to-mesenchymal transition (MMT) (Nasreen et al., 2009) (**Figure 2**).

Wilms tumor 1 (*Wt1*) is highly expressed by mesothelial cells during embryonic lung development but is significantly suppressed postnatally in mice. While the extent of migration of embryonic PMCs into the lung parenchyma highly depends on the experimental conditions and time of labeling during embryonic lung development (Dixit et al., 2013; El Agha, 2019; Lüdtke et al., 2019; Moiseenko et al., 2017; Que et al., 2008), a contribution of these cells to MyoFBs has been demonstrated in the fibrotic lung postnatally (von Gise et al., 2016). Loss of *Wt1* in PMCs was suggested to contribute to their differentiation into MyoFBs via MMT therefore contributing to lung fibrosis (Karki et al., 2014); however, this can be rescued by carbon monoxide or induction of heme oxygenase-1 (HO-1) expression (Zolak et al., 2013). Given that lung fibrosis is widely regarded to initiate in the lung periphery, it is plausible that the pleura is involved in fibrogenesis and possibly through providing MyoFBs progenitors (Zabihi et al., 2024).

## 1.6 Lung organoid models derived from adult mouse stem cells

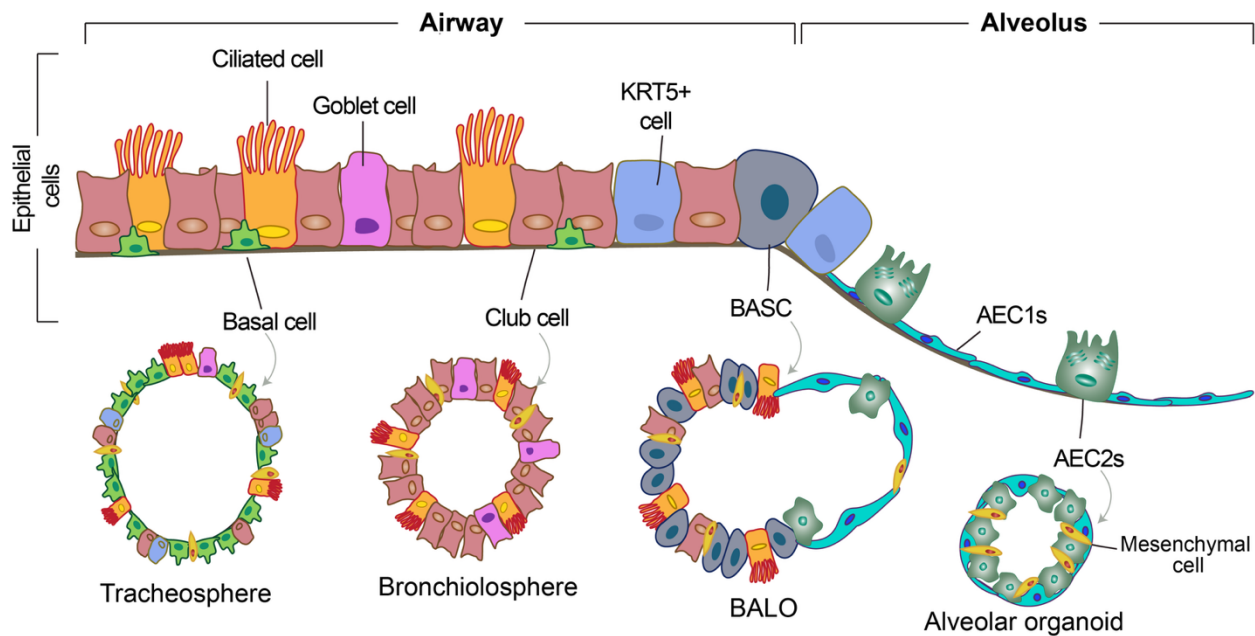
Organoids are three-dimensional (3D) structures that are representative of the structure, cellular morphology, and certain functional features of the tissue or organ of interest. In recent years, murine and human organoids have emerged as powerful tools for studying organ development, cellular interaction, drug screening, and regenerative mechanisms. Due to their unlimited capacity to proliferate and differentiate, adult stem and progenitor cells have been widely used to develop organoids for research purposes (Lancaster and Knoblich, 2014; Zhao et al., 2022).

In the context of pulmonary research, several murine organoid systems have been developed to create airway and/or alveolar structures resembling various aspects of lung organogenesis, homeostasis, and regeneration (Vazquez-Armendariz and Herold, 2021) (**Figure 3**). For instance, tracheal organoids, or tracheospheres, with a visible lumen can be grown from basal stem cells even in the absence of stromal cells or other non-basal cells (Rock et al., 2009). Airway organoids, or bronchiolospheres, can be generated by co-culturing club cells with airway smooth muscle cells (ASMCs) or repair-supportive mesenchymal cells (RSMCs), thus mimicking the intercellular communication that occurs during airway epithelial regeneration in vivo following naphthalene injury (Chu et al., 2022; Lee et al., 2017; Moiseenko et al., 2020; Vazquez-Armendariz et al., 2021). Such bronchiolospheres can also be generated by culturing club cells with [CD45<sup>+</sup> CD31<sup>+</sup> EpCAM<sup>+</sup> Sca-1<sup>+</sup>] (non-leukocytic, non-endothelial, non-epithelial, stem cell antigen-1-expressing) rMCs (Vazquez-Armendariz et al., 2021). The most comprehensive system so far is the bronchioalveolar lung organoid (BALO) model, which relies on co-culturing bronchioalveolar stem cells (BASCs) and rMCs, resulting in a highly sophisticated mimicry of the bronchioalveolar region of the mature mouse lung (Lee et al., 2017; Vazquez-Armendariz et al., 2020).

AEC2s represent a stem cell population in the alveolar compartment of the lung (Barkauskas et al., 2013). These cells self-renew and regenerate this compartment by differentiating into AEC1s that are responsible for gas exchange, eventually via intermediate cell types (Choi et al., 2020; Kobayashi et al., 2020; Strunz et al., 2020).

Although the heterogeneous nature of AEC2s was not obvious initially, emerging research suggests that there are indeed AEC2 subsets that display varying stem-cell features. For example, WNT-responsive AEC2s (expressing axis inhibition protein 2 or AXIN2), termed alveolar epithelial progenitors (AEPs), have been shown to represent a stable lineage during homeostasis, and are capable of rapid expansion following acute lung injury (Zacharias et al., 2018). Another subpopulation of AEC2s is enriched in programmed cell death 1 ligand 1 (PD-L1 or CD274) expression and was dubbed “injury-associated alveolar progenitors” (IAAPs). These cells replenish the mature AEC2 pool upon alveolar injury (Ahmadvand et al., 2021; Lv et al., 2022).

Isolation of AEC2s by FACS for alveolar organoid has so far mostly relied on the use of genetically modified mice (*Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup>) that allow labeling of surfactant protein C-positive (SFTPC+) AEC2s. Such cells can also be filtered through the additional use of LysoTracker, a dye that stains lysosomes and therefore detects lamellar bodies that are abundant in AEC2s (Van der Velden et al., 2013). A population of rMCs that expresses *FGF10* and contains neutral lipids (therefore possessing a lipofibroblast-like phenotype) promotes the formation of AEC2-derived alveolar organoids containing AEC2s and AEC1s (Quantius et al., 2016; Taghizadeh et al., 2021). Alveolar organoids had been initially described by co-culturing lineage-labeled AEC2s with PDGFR $\alpha$ -expressing mesenchymal cells, also termed type 2-associated stromal cells (TASCs), that display lipofibroblast characteristics (Barkauskas et al., 2013, Kobayashi et al., 2020). The co-culture assay using Sca-1+ mesenchymal cells and lung epithelial progenitor cells had already been developed (McQualter et al., 2010).



**Figure 3. Murine lung organoid models generated from epithelial progenitor cells.**

Multiple murine organoid models have been established to reconstruct airway and alveolar structures that mimic key features of lung development, maintenance, and repair. Basal cells generate tracheospheres containing basal, ciliated, and secretory cells. Club cells form bronchiolospheres with ciliated cells and different types of mesenchymal cells. BASCs, ciliated cells, and club cells are co-cultured with mesenchymal cells and give rise to BALO. Co-culture of AEC2s with  $\text{Pdgfra}^+/\text{Sca1}^+$  rMCs produce alveolar organoids containing AEC1s and AEC2s. BASCs: Bronchioalveolar stem cells; AEC1s/ AEC2s: Alveolar epithelial type 1/2 cells; BALO: Bronchioalveolar lung organoid



## **2. Objectives**

This study is divided into two parts, emphasizing the following topics:

### **2.1 Generation of alveolar organoids from wild-type (WT) mice**

In recent years, lung organoids have emerged as a powerful tool to study stem cell self-renewal and reorganization *ex vivo*. Alveolar organoids have particularly taken center stage due to their usefulness in 1) defining subsets of AEC2s with enhanced stemness/progenitor capabilities, and 2) testing the AEC2-supportive ability of mesenchymal cell subsets. One bottleneck has been the requirement of transgenic mouse lines that allow isolation of AEC2s with high viability and purity. To overcome that, a new protocol was proposed to serve as a resource for the lung stem-cell research community interested in AEC2 self-renewal and differentiation, and interaction with mesenchymal niche cells.

**The objectives of this part are the following:**

- 1) Overcome the need for transgenic mice for the generation of alveolar organoids
- 2) Establish a robust and reproducible protocol for the generation of alveolar organoids
- 3) Obtain a pure culture of alveolar organoids that is devoid of bronchiolar and bronchioalveolar organoids

### **2.2 Investigating the origin and fate of myofibroblasts in bleomycin-induced lung fibrosis in aged mice**

IPF is the most common and aggressive form of ILD. Despite extensive research on the pathomechanisms of fibrogenesis, less is known about fibrosis resolution. To date, the therapeutic options for affected patients remain very limited. Recent scRNA-seq studies have highlighted the role of LIF-to-MyoFBs differentiation during fibrosis formation. However, these studies are performed on young mice, while bleomycin-induced fibrosis resolves spontaneously and fails to mimic the chronic progression of IPF.

Given this background, 52–to-56-week-old female *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>fllox</sup>* mice were used to model fibrosis with delayed resolution, mimicking human IPF pathology. Single-cell RNA sequencing on lineage-labeled *Fgf10*<sup>+</sup> cells was performed following bleomycin-

induced lung injury to investigate cell heterogeneity and fate trajectories. The expression data were validated by in situ analysis on lung sections and the selected candidate pathway was functionally validated in ex vivo models.

**The objectives of this part are the following:**

- 1) Characterize the fate of *Fgf10*<sup>+</sup> AFs during lung fibrosis development and resolution in aged mice
- 2) Identify the pathways mediating delayed fibrosis resolution in aged mice
- 3) Assessing the therapeutic potential of candidate pathways using ex vivo models
- 4) Translating the findings to the human context using patient-derived material

### 3. Material and methods

#### 3.1 Animal experiments

All animal studies and experiments were conducted in compliance with the approved protocols by the animal ethics committee of Justus-Liebig University Giessen and the local authorities (Regierungspraesidium Giessen, permit numbers: G76/2020–No. 1026\_GP, G57/2021, 757\_M and 825\_M). All mice were kept under specific pathogen-free (SPF) conditions with unrestricted access to food and water. The room was maintained under controlled conditions, with a 12-hour light/dark cycle, a constant temperature of 22 °C, and humidity levels ranging between 40% and 70%. Eight-to-twelve-week-old WT mice with a C57BL/6 genetic background were obtained from Charles River (#632). *Sftpc*<sup>tm1(Cre/ERT2,rtTA)Hap</sup> (or simply *Sftpc*<sup>Cre-ERT2</sup>) mice were previously described (Chapman et al., 2011), and Gt(ROSA)26Sor<sup>tm9(CAG-tdTomato)Hze</sup> (or simply *tdTomato*<sup>flox</sup>) mice were purchased from the Jackson Laboratory (stock number 007905). The two lines were bred to generate *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice. To lineage label *Fgf10*<sup>+</sup> cells, 52-to-56-week-old (aged) female *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice were used. These mice were previously generated and validated in our lab (Chu et al., 2021).

#### 3.2 Tamoxifen and bleomycin instillation

To label *Fgf10*<sup>+</sup> cells, mice were Intraperitoneally (*i.p.*) injected with tamoxifen (Sigma-Aldrich, T5648-5G) reconstituted in corn oil (Sigma-Aldrich, C8267) at a dose of 0.1 mg/g body weight. Mice received three successive injections on days -13, -11 and -9 *prior* to intratracheal (*i.t.*) instillation (150 µl) of either saline or bleomycin (1.5-2 U.kg<sup>-1</sup> body weight; Bleomedac, PNZ-02411351) using a micro-sprayer (Penn-Century, Inc.). Lungs were collected on days 14, 30 and 60 *post* bleomycin injury for further analysis. *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice were injected with tamoxifen intraperitoneally three times at a dose of 0.25 mg/g body weight per injection.

### 3.3 Genotyping

Mice ear biopsies were digested in 200  $\mu$ l Isopropanol digestion buffer containing 2  $\mu$ l proteinase K at 56°C, 500 rpm on a thermomixer comfort overnight. The next day, the samples were centrifuged for 10 min at 14,000 rpm and the supernatant was mixed with Isopropanol by inverting the tubes to precipitate DNA. Next, PCR was performed to amplify DNA using specific primers (**Table 1**). The PCR products were then analyzed using a QIAxcel capillary gel electrophoresis instrument (Qiagen, 9002123).

**Table 1. Primer sequences and expected band size for genotyping**

| Mouse line                      | Expected band size |         | Primer sequence (Mutant)                                                    | Primer sequence (WT)                                                      |
|---------------------------------|--------------------|---------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------|
|                                 | WT                 | Mutant  |                                                                             |                                                                           |
| <i>Fgf10<sup>Cre-ERT2</sup></i> | 580 bp             | 251 bp  | (Fwd) AAC ACC TCT<br>GCT CAC TTC CTC<br>(Rv) AGG GTC CAC CTT<br>CCG CTT TT  | (Fwd) GCA GGC AAA<br>TGT ATG TGG CA<br>(Rv) TGC TTG CGT<br>GTC TTA CTG CT |
| <i>Sftpc<sup>Cre-ERT2</sup></i> | 500 bp             | 1000 bp | (Fwd) CCC AGT CCC<br>TCT CTG AAT TTG<br>(Rv) GTT TCT ACC GAC<br>CCT GTG AAG | CAT CGC TCG ACC<br>AGT TTA GTT A                                          |
| <i>tdTomato<sup>flox</sup></i>  | 297 bp             | 189 bp  | (Fwd) CTG TTC CTG<br>TAC GGC ATG G<br>(Rv) GGC ATT AAA GCA<br>GCG TAT CC    | (Fwd) CCG AAA ATC<br>TGT GGG AAG TC<br>(Rv) AAG GGA GCT<br>GCA GTG GAG TA |

### 3.4 Lung dissociation for organoid generation and Fluorescence-Activated Cell Sorting

Lungs from WT and *Sftpc<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* (TG) mice were collected for single-cell suspension preparation. Mice were sacrificed by cervical dislocation under isoflurane anesthesia. The lungs were flushed via transcardiac perfusion using 20 mL of Hank's Balanced Salt Solution (HBSS; Thermo Fisher Scientific, 14175095). Digestion was

performed by intratracheal injection of 2 mL sterile dispase (5 U/mL; BD Biosciences, 354235) and incubation in 3 mL of dispase solution (5 U/mL) at room temperature (RT) for 40 min to allow enzymatic separation of distal epithelial cells. After digestion, the lung tissue was dissociated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5% HEPES and 0.01% DNase I in MACS C-tubes, using the gentle MACS dissociator. The lung homogenates were passed through 100  $\mu$ m and 40  $\mu$ m cell strainers (Sarstedt, 83.3945.100, 83.3945.040 respectively). The cell suspension was centrifuged and incubated with an antibody cocktail containing biotin rat anti-mouse CD45 (BD Biosciences, 553078), CD16/32 (BD Biosciences, 553143), and CD31 (BD Biosciences, 553371) at 37 °C for 30 min to deplete hematopoietic and endothelial cells. After centrifugation, cells were resuspended in DMEM containing Dynabeads Biotin Binder (Invitrogen, 11047) for 30 min at RT on a rotator. For magnetic separation, cells were transferred to a magnetic rack for 10 min at RT. Cell suspensions were then blocked with gamma globulins (10% Gamunex; Grifols), re-suspended in MASC buffer (1 $\times$  PBS, 0.5% Fetal Bovine Serum (FBS) (Gibco, 10270-106), 2mM UltraPure EDTA (Invitrogen, 15575-038) and stained with anti-mouse CD31 (Alexa Fluor 488, Biolegend, 102514, 1:50), CD45 (FITC, BD Biosciences, 553080, 1:50), CD326 (APC/Cy7, Biolegend, 118218, 1:50), Sca-1 (Pacific Blue, Biolegend, 108120, 1:50), and Podoplanin antibodies (APC, Biolegend, 127410, 1:50) for 15 min at 4 °C. Next, CD45<sup>-</sup> CD31<sup>-</sup> EpCAM<sup>-</sup> Sca-1<sup>+</sup> rMCs and CD45<sup>-</sup> CD31<sup>-</sup> EpCAM<sup>low</sup> AECs were isolated by FACS. At least 40,000 rMCs and 350,000 AECs from one WT mouse and 250,000 tdTomato<sup>+</sup> (tdTom<sup>+</sup>) cells from one TG mouse can be sorted. To get a higher yield of rMCs, WT lungs can be separately digested with 0.5% collagenase type IV (Gibco, 17104-019) as previously described (Moiseenko et al., 2020). FACS Aria III cell sorter (BD Biosciences) with 85  $\mu$ m nozzle was used for sorting and measurements.

### 3.5 Lung dissociation for single-cell RNA sequencing

To isolate tdTom<sup>+</sup> cells for scRNA-seq, lungs from *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice were chopped into small pieces and digested using 0.5% collagenase type IV in HBSS for 30 min on a rotator at 37 °C. After digestion, lung homogenates were passed through 70  $\mu$ m and 40  $\mu$ m cell strainers to obtain single-cell suspensions. After centrifugation, the pellet

was resuspended in an antibody cocktail containing anti-mouse CD31 (AF488-conjugated), CD45 (AF488-conjugated) and CD326 (EpCAM, APC/Cy7-conjugated) for 20 min on ice in the dark. After washing, cells were stained with SyTOX (1:1000, Invitrogen, S34862) to exclude dead cells and sort live cells for scRNA-seq. Data were analysed using FlowJo software (FlowJo LLC, version 10.10.0).

### 3.6 Alveolar organoid generation and analysis

To generate alveolar organoids, 17,000 rMCs and 5,000 AECs from either WT or *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice were flow-sorted, mixed, and resuspended in 50  $\mu$ L of culture medium containing minimum essential medium (MEM) alpha (Gibco, 41061029), 10% FBS, 1% L-glutamine–penicillin–streptomycin (Sigma-Aldrich, G1146), 1% insulin/transferrin/selenium (ITS) (Gibco, 41400-045), and 0.0002% heparin (Stem Cell Technologies, 07980). Next, 50  $\mu$ L of cold growth factor-reduced Phenol Red-free Matrigel (Corning, 356231, Corning) was mixed at a 1:1 ratio with the cell suspension, transferred to a 24-well plate on a 0.4  $\mu$ m cell culture insert (Millipore, PICM01250) and incubated at 37 °C for 15 min for polymerization. After incubation, 350  $\mu$ L of medium was added to the lower chamber and incubated at 37 °C with 5% CO<sub>2</sub>. The medium was changed every 2 days. To test the effect of Interleukin-1 beta (IL-1 $\beta$ ) on alveolar organoid formation, recombinant murine IL-1 $\beta$  (PeproTech, 211-11B-10UG) was added to the organoid medium at a concentration of 20 ng/mL. For fibrotic induction on alveolar organoid, the formed WT alveolar organoids were treated with 5 ng/mL rTGF $\beta$ 1 (R&D system, 240-B) for three days, followed by 300 ng/mL recombinant protein TIMP-3 (rTIMP-3) (R&D system, 973-TM-010) treatment for another three days. The alveolar organoids were used for quantitative-real time PCR (qPCR) and immunofluorescence (IF). Cultures were imaged using EVOS M7000 (Thermo Fisher, AMF7000). The number and area of alveolar organoids were measured by ImageJ software (v2.14.0/1.54f). The colony-forming efficiency (CFE) was determined by dividing the number of counted alveolar organoids by the initial number of seeded AECs.

### **3.7 Induction of Cre activity in ex vivo alveolar organoid cultures**

To induce Cre-mediated recombination ex vivo, organoid medium containing 1  $\mu$ M of 4-Hydroxytamoxifen (4-OHT) (Merck, H6278) was added to the organoid cultures on day 1. After 24 h, the organoid medium containing 4-OHT was removed, and the organoid inserts were washed twice with 1 $\times$  PBS. Then, the inserts were placed on a new 24-well plate, and alveolar organoids were cultured in organoid medium until day 16.

### **3.8 Whole-mount immunofluorescence staining of alveolar organoids**

Cultured alveolar organoids were fixed with 4% paraformaldehyde (PFA) (Merck, 104005) for 20 min at RT, then washed three times with 1 $\times$  PBS. Fixed alveolar organoids were blocked and permeabilized with blocking buffer (5% serum, 0.5% Triton-X-100 in 1 $\times$  PBS) overnight at 4  $^{\circ}$ C. The serum was chosen from the same species as the host of the secondary antibody. For IF, alveolar organoids were incubated with primary antibodies (Rabbit  $\alpha$ -Pro-SFTPC (Seven Hills Bioreagents, WRAB-9337, 1:800), Mouse  $\alpha$ -HOP (SantaCruz, sc-398703, 1:150), Rat  $\alpha$ -KRT8 (Developmental Studies Hybridoma Bank (DSHB), TROMA-I-c, 1:200)) Mouse/Rat anti-Rage (R&D Systems, MAB1179, 1:200) in a blocking buffer overnight at 4  $^{\circ}$ C. The next day, washing buffer (2% serum, 0.3% Triton-X-100 in 1 $\times$  PBS) was added 3 times for 10–15 min, and secondary antibodies were added overnight at 4  $^{\circ}$ C. To stain nuclei, the NucBlue™ Fixed Cell ReadyProbes™ Reagent (Thermo Fisher, R37606) was utilized. Lastly, stained alveolar organoids were washed three times with washing buffer for 10–15 min. The Matrigel containing the organoids was cut out of the insert with a scalpel. The sample was placed on a glass slide and slide was mounted by ProLong™ Glass Antifade Mountant (Thermo Fisher Scientific, P36984) and drying for 1 day at 4  $^{\circ}$ C in the dark before proceeding imaging.

### **3.9 Hematoxylin and eosin staining and fibrosis area quantification**

Mouse lung perfusion was done using HBSS. Once the harvested, lungs were fixed with 4% PFA and sequentially dehydrated through a series of ethanol concentrations. The lungs were then embedded in paraffin blocks and cut into 5- $\mu$ m-thick sections using a microtome (Leica, RM2255). Before staining, paraffin sections were deparaffinized with

Xylol and sequentially rehydrated. The sections were stained with hematoxylin (Roth, T865.2) for 2 min, and washed with running water for 10 min. The slides were stained with eosin (Thermo Fisher Scientific, 6766007) for 1 min. The stained slides were dehydrated and mounted. The damaged area was quantified by Orbit image analysis software (V3.64, Idorsia Pharmaceuticals Ltd).

### **3.10 Picro Sirius Red and Masson's Trichrome staining**

Five- $\mu$ m-thick paraffin sections were deparaffinized and rehydrated. For Picro Sirius Red staining (Abcam, ab150681), an adequate amount of dye was applied to cover the tissue and incubated for 1h at RT. The dye was washed in two changes of 1% acetic acid. Masson's Trichrome staining (MTC) (Abcam, ab150686) was carried out according to the manufacturer's instructions. Stained sections were dehydrated, mounted and finally imaged.

### **3.11 In situ hybridization, immunofluorescence and quantification**

Five- $\mu$ m-thick paraffin sections from mice or human transplanted lungs were deparaffinized in two changes of Xylol and rehydrated through a series of ethanol concentrations. RNAscope was performed according to the manufacturer's instructions (ACD, Doc. No. 323100-USM) using mouse probes for *Fgf10* (Mm-Fgf10, 446371-C1), *Inmt* (Mm-Inmt, 486371-C1), *Tcf21* (Mm-Tcf21, 508661-C1), *Robo2* (Mm-Robo2, 475961-C2), *Cthrc1* (Mm-Cthrc1, 413341-C1) and *Adamts4* (Mm-Adamts4, 537341-C2) or human probes for *CTHRC1* (Hs-Cthrc1, 413331-C1), *ADAMTS4* (Hs-ADAMTS4, 497161-C3), *INMT* (Hs-INMT, 459961-C1) and *ROBO2* (Hs-ROBO2, 502341-C2). Anti-tdTomato antibody (Sicgen, AB8181-200, 1:250) was used to boost the tdTomato fluorescent signal that was diminished after antigen retrieval. Immunostaining on PCLS was performed as previously described (Khadim et al., 2025). For IF staining on paraffin sections, the samples were blocked with 5% Bovin Serum Albumin (BSA) (Jackson ImmunoResearch, 001-000-162) for 1h at RT, followed by incubation with primary antibodies (ADAMTS4, Thermo Fisher Scientific, PA1-1749A) overnight at 4 °C. The next day, samples were washed three times with PBS, then incubated with secondary



antibodies for 1h at RT. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, D1306) and slides were mounted by Fluoromount-G® (Southern Biotech, 0100-01). Fluorescent images were acquired using a Leica DM550 B fluorescence microscope equipped with LAS X software (version 3.5; Leica Microsystems). Images were quantified using ImageJ software.

### **3.12 Human-derived lung tissue**

Fresh human lung tissues from non-IPF donors used for precision-cut lung slice (PCLS) cultures were obtained from Hannover Medical School (MHH, Hannover, Germany) in compliance with 'The code of ethics of the world medical association' (Approval by the Ethics Committee of MHH renewed on 2015/04/22; approval number 2701-2015) and those from IPF patients from the UGMLC Giessen Biobank, member of the DZL Platform Biobanking (Approval by the Ethics Committee of Justus-Liebig-University Giessen; approval number AZ 58/15). Formalin-fixed, paraffin-embedded human donor and IPF lung sections used for in situ hybridization and fresh material used for RNA extraction followed by qPCR were obtained from the UGMLC Giessen Biobank (Approval by the Ethics Committee of Justus-Liebig-University Giessen; approval number AZ 58/15). The use of human samples was in accordance with the Declaration of Helsinki and written informed consent was obtained from all participants or their next of kin.

### **3.13 Generation of human and mouse PCLS and treatments**

Fresh human lung specimens were obtained from IPF patients that underwent lung transplantation. Human lung tissues were inflated with 2% UltraPure low melting agarose (Invitrogen, 16520-100) and cut into 300-µm-thick slices using a vibratome. The resulting hPCLS were cultured in DMEM/F-12, GlutaMAX™ medium (Thermo Fisher Scientific, 31331028) supplemented with 10% FBS, 1% penicillin-streptomycin (Thermo Fisher Scientific, 15070063) and 0.5% amphotericin (Sigma, A2942). Cultures were treated with vehicle, 300 ng/mL rTIMP-3 or 500 ng/mL versican (VCAN) (Abbexa, abx069660) in medium containing 0.1% FBS for three days. Mouse PCLS preparation was carried out as described above at a thickness of 200 µm. WT mPCLS were treated with vehicle or 5

ng/mL rTGFβ1 for five days followed by treatment with vehicle, rTGFβ1 + Vehicle or rTGFβ1 + rTIMP-3/VCAN for three days. PCLS obtained from aged Bleo d14 *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* mice were treated with vehicle or 500 ng/mL VCAN for three days. The samples were collected for RNA extraction, qPCR and staining.

### 3.14 RNA extraction and gene expression analysis

RNA was extracted from human and mouse PCLS using Trizol (Thermo Fisher Scientific, 10296028) and RNeasy mini kit (Qiagen, 74106). RNA was extracted from alveolar organoid cultures using the RNeasy micro kit (Qiagen, 74004). cDNA synthesis was performed using the Quantitect reverse transcription kit (Qiagen, 205311) according to the manufacturer's instructions. qPCR was performed using PowerUp SYBR green master mix (Thermo Fisher Scientific, A25742) and a QuantStudio™ 3 Real-Time PCR System machine (Applied Biosystems™). Gene expression levels in alveolar organoids were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and presented as fold change relative to day 8 of culture. Gene expression levels in mouse and human PCLS were normalized to Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and *GAPDH* respectively. Primer sequences are shown in Table 2.

**Table 2. Primer sequences used for qPCR**

| Gene           | Forward primer (5' - 3')         | Reverse primer (5' - 3')         |
|----------------|----------------------------------|----------------------------------|
| <i>hACTA2</i>  | CTG TTC CAG CCA TCC TTC AT       | TCA TGA TGC TGT TGT AGG<br>TGG T |
| <i>hCEBPB</i>  | AGA AGA CCG TGG ACA AGC<br>ACA G | CTC CAG GAC CTT GTG CTG<br>CGT   |
| <i>hCOL1A1</i> | ATG TTC AGC TTT GTG GAC<br>CTC   | CTG TAC GCA GGT GAT TGG TG       |
| <i>hCTHRC1</i> | CAG GAC CTC TTC CCA TTG<br>AAG C | GCAACA TCC ACT AAT CCA GCA<br>CC |
| <i>hHOPX</i>   | ATT CCA CCA CGC TGT GCC<br>TCA T | AGT CTG TGA CGG ATC TGC<br>ACT C |

|                 |                                     |                                   |
|-----------------|-------------------------------------|-----------------------------------|
| <i>hINMT</i>    | ATT CGC CTG TGA GCT GGA<br>AGG A    | AGG TGG ACA TCG CAC TTG<br>AGC A  |
| <i>hLIMCH1</i>  | CTG GAG CTG AAA CAA GAC<br>AAC GG   | GTC CTC TTG ACT ATT CGG TTC<br>CG |
| <i>hNPNT</i>    | CAG GTG CAT GAA CAC TTA<br>CGG C    | CAC AGC CAT ACT GAC AGT<br>TTG CC |
| <i>hPBGD</i>    | TGT CTG GTA ACG GCAATG CG           | CCC ACG CGA ATC ACT CTC AT        |
| <i>hPDGFRA</i>  | CCA CCT GAG TGA GAT TGT             | TCT TCA GGA AGT CCA GGT           |
| <i>hPLIN2</i>   | TCAGCT CCA TTC TAC TGT TCA<br>CC    | CCT GAA TTT TCT GAT TGG CAC<br>CC |
| <i>hPPARG</i>   | TCT GCAAAC ATATCA CAA GAA<br>ATG AC | ATA TCA AAG GAG TGG GAG<br>TGG    |
| <i>hSFTPC</i>   | GTC CTC ATC GTC GTG GTG<br>ATT G    | AGA AGG TGG CAG TGG TAA<br>CCA G  |
| <i>hSPP1</i>    | CGA GGT GAT AGT GTG GTT<br>TAT GG   | GCA CCA TTC AAC TCC TCG<br>CTT TC |
| <i>hTCF21</i>   | CAC TTG AGG CAG ATC CTG<br>GCT A    | CGG TCA CCA CTT CTT TCA<br>GGT C  |
| <i>hADAMTS4</i> | TCA CTG ACT TCC TGG ACA<br>ATG GC   | GGT CAG CAT CAT AGT CCT<br>TGC C  |
| <i>mGapdh</i>   | CAT CAC TGC CAC CCA GAA<br>GAC TG   | ATG CCA GTG AGC TTC CCG<br>TTC AG |
| <i>mActa2</i>   | ACT CTC TTC CAG CCA TCT<br>TTC A    | ATA GGT GGT TTC GTG GAT GC        |
| <i>mKrt8</i>    | TGG AAG GAC TGA CCG ACG<br>AGA T    | GGC ACG AAC TTC AGC GAT<br>GAT G  |
| <i>mAger</i>    | GCC ACT GGA ATT GTC GAT<br>GAG G    | GCT GTG AGT TCA GAG GCA<br>GGAT   |
| <i>mCol1a1</i>  | CCA AGA AGA CAT CCC TGA<br>AGT CA   | TGC ACG TCA TCG CAC ACA           |

|                 |                                   |                                    |
|-----------------|-----------------------------------|------------------------------------|
| <i>mCthrc1</i>  | TG TTCAGGACCTCTTCCCATCG           | GCCACATCTACCAATCCAGCAC             |
| <i>mHprt</i>    | CCT AAG ATG AGC GCA AGT<br>TGAA   | CCA CAG GAC TAG AAC ACC<br>TGC TAA |
| <i>mSftpc</i>   | GGT CCT GAT GGA GAG TCC<br>AC     | GAT GAG AAG GCG TTT GAG GT         |
| <i>mAdamts4</i> | GAA CGG TGG CAA GTA TTG<br>TGA GG | TTC GGT GGT TGT AGG CAG<br>CAC A   |

### 3.15 Bulk RNA sequencing

Bulk RNA sequencing was carried out according to standard procedures. For genome-wide analysis of gene expression, RNA sequencing libraries from polyadenylated mRNA were generated and sequenced using Illumina NovaSeq 6000 at the Institute for Lung Health (ILH)-Genomics and Bioinformatics at the Justus-Liebig University Giessen (Germany).

### 3.16 Single-cell RNA sequencing

To explore transcriptional changes specific to mesenchymal cells in the aged *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* animals, scRNA-seq was conducted on tdTom+ cells extracted from the lungs of saline or bleomycin-challenged mice. Cells were flow-sorted, and libraries were prepared using the Chromium Next GEM Single-Cell 3' Reagent Kit v3.1 from 10x Genomics. Sequencing data underwent processing through a previously established pipeline (Khadim et al., 2025), which in brief involved excluding cells with fewer than 1,200 detected counts or more than 40,000 counts, as well as those with over 20% mitochondrial gene expression. Genes detected in fewer than 20 cells were filtered out to ensure high-quality read alignment and gene quantification. Following the pre-processing of individual samples, data integration was performed using the Harmony algorithm, with batch correction applied through BBKNN (Batch Balanced K-Nearest Neighbors) and highly variable genes selected using SeuratV3 flavour.

Differentially expressed genes (DEGs) were identified using the presto package in Seurat, with a minimum log-fold change threshold of 0.5 and a detection rate of at least 50%. To compare differences between clusters and samples, differential expression analysis was performed using the *FindMarkers* function, followed by filtering and visualization of significant genes through a volcano plot using tidyplots (Engler, 2024) or with the pheatmap package. RNA velocity analysis was conducted to map transcriptional dynamics and cell state predictions were explored using scVelo package, selecting 500 highly variable genes and computing first- and second-order moments with 20 principal components and 30 nearest neighbours. Latent cell velocity and inferred differentiation trajectories were further inferred using CellRank (Weiler et al., 2024), employing standard settings and vignette, enabling probabilistic modelling of cell-fate transitions. To gain functional insights, cluster markers were mapped to ENTREZ gene identifiers and annotated using the org.Mm.eg.db database. Pathway enrichment analyses were conducted using Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases to identify enriched biological processes and signalling pathways, with results visualized using ggplot2 and dplyr.

Stromal cells from healthy, NSIP, IPF, and ILD donor lungs were obtained from the Human Lung Cell Atlas (HLCA) (Sikkema et al., 2023). Differential expression analysis was conducted by comparing IPF, NSIP and ILD to healthy stromal cells. DEGs were calculated by comparing each disease category against 'normal' (healthy stromal cells) using the Wilcoxon rank-sum test. Ensembl IDs mapped to the gene symbols were incorporated into the final data frame, and genes with infinite avg\_log2FC values were excluded. Key transcriptional changes associated with the AF1 signature were analysed using the clusterProfiler and fgsea packages (Bioinformatic analysis was done by Prof. Marek Bartkuhn and Dr. Ali Khadim).

### **3.17 Data availability**

Cluster quantification data, analysis code, DEGs, gene signatures used for GSEA, or scoring is available upon request. Raw and processed data will be uploaded on Gene Expression Omnibus (GEO).

### **3.18 Microscopy images and quantification**

Imaging was carried out using a Leica DM550 B fluorescence microscope or an SP8 confocal microscope (Leica Microsystems) controlled by LAS X software (version 3.5.7). For capturing the confocal z-stacks, we used 20x0.75NA air and 63xW 1.2NA objectives, while the scan settings were kept the same across all acquisitions. The acquired confocal images were cleared computationally, using the Lightning module of LAS X software. Subsequently, for the maximum intensity projection and quantification of the z-stack, we used the ImageJ/Fiji software (Schindelin et al., 2012). With Fiji, every projected image was thresholded using the default integrated algorithm. Then, the area occupied by the positive signal (of each marker) as well as the total organoid area were measured. The final area ratio of each marker was calculated by dividing the marker's area by the organoid's area.

### **3.19 Statistical analysis and figure preparation**

All data are presented as mean  $\pm$  standard error of the mean (SEM). Student's t test (unpaired, two tailed) was used to compare the means of two groups and One-way ANOVA was used to compare more than two groups with a single variable. The differences in the means were considered statistically significant when the p value was less than 0.05. Quantitative data were assembled and analyzed using GraphPad Prism software (GraphPad Software, version 10.4.1 (532)). The number of biological replicates (depicted as n) is indicated in the corresponding figure legends. Figures were created and assembled using Adobe Illustrator (Adobe).

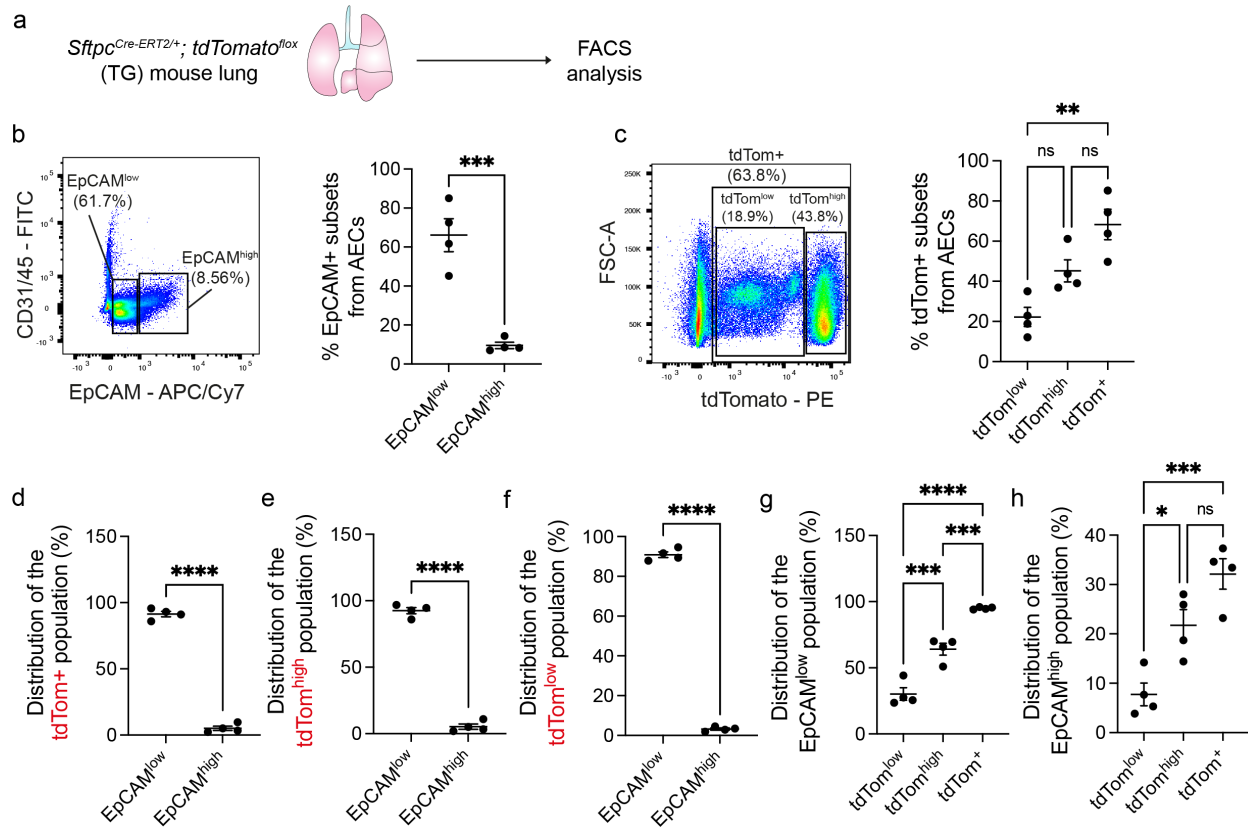
## 4. Results

### 4.1 PART I. Generation of alveolar organoids from wild-type mice

#### 4.1.1 AEC2s can be isolated from WT mouse lungs by sorting CD45<sup>+</sup> CD31<sup>+</sup> EpCAM<sup>low</sup> cells

To show a proof of concept that alveolar organoids can be generated from AECs that are FACS sorted from WT lungs, we first carried out EpCAM staining on AEC cell suspensions prepared from *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> lungs (**Figure 4a**). We identified EpCAM<sup>low</sup> (66.1% ± 8.4% of AECs) and EpCAM<sup>high</sup> (9.6% ± 1.6% of AECs) populations (**Figure 4b**). We have previously demonstrated that the EpCAM<sup>low</sup> population is enriched with AEC2s (Quantius et al., 2016), while the EpCAM<sup>high</sup> cell population is enriched with airway cells such as ciliated and secretory cells (Quantius et al., 2016) as well as bronchioalveolar stem cells (BASCs) (Vazquez-Armendariz et al., 2020). Moreover, tdTom<sup>low</sup> (22.2% ± 4.8% of AECs) and tdTom<sup>high</sup> (45.2% ± 5.5% of AECs) corresponding to injury-activated alveolar progenitors (IAAPs) and mature AEC2s, respectively, were also identified (**Figure 4c**) as previously described (Ahmadvand et al., 2021). IAAPs have been shown to be inefficient in generating alveolar organoids (Ahmadvand et al., 2021). Total tdTom<sup>+</sup> cells accounted for around two-thirds of the AEC preparation (**Figure 4c**), similar to EpCAM<sup>low</sup> cells (**Figure 4b**).

Gating for total tdTom<sup>+</sup> cells (tdTom<sup>low</sup> and tdTom<sup>high</sup>) showed that 91.4% ± 2.1% of these cells were contained in the EpCAM<sup>low</sup> population compared to traces in EpCAM<sup>high</sup> cells (**Figure 4d**). Gating for tdTom<sup>high</sup> and tdTom<sup>low</sup> populations showed similar results (**Figure 4e, f**). Further analysis of the EpCAM<sup>low</sup> population showed that 95% ± 0.42% of these cells were tdTom<sup>+</sup>, with the majority being tdTom<sup>high</sup> (64.1% ± 4.5%) and a minor proportion of tdTom<sup>low</sup> (30.2% ± 4.7%) (**Figure 4g**). On the other hand, the EpCAM<sup>high</sup> population showed lower proportions of tdTom<sup>+</sup> cells (**Figure 4h**). Therefore, the EpCAM<sup>low</sup> population was confirmed to be enriched with AEC2s.



**Figure 4. Gating strategy to identify AEC2s within the EpCAM<sup>low</sup> cell population.** a) Experimental design for FACS analysis from TG mouse lungs. b, c) Gating strategy for identifying EpCAM<sup>low</sup>, EpCAM<sup>high</sup>, tdTom<sup>low</sup>, and tdTom<sup>high</sup> populations with the corresponding quantification. (d–f) Distribution of tdTom<sup>+</sup>, tdTom<sup>high</sup>, and tdTom<sup>low</sup> cells among EpCAM<sup>low</sup> and EpCAM<sup>high</sup> populations. g, h) Distribution of EpCAM<sup>low</sup> and EpCAM<sup>high</sup> cells among tdTom<sup>low</sup>, tdTom<sup>high</sup>, and tdTom<sup>+</sup> populations. Each dot represents one lung; \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001; ns: not significant. AECs: alveolar epithelial cells; TG: transgenic.

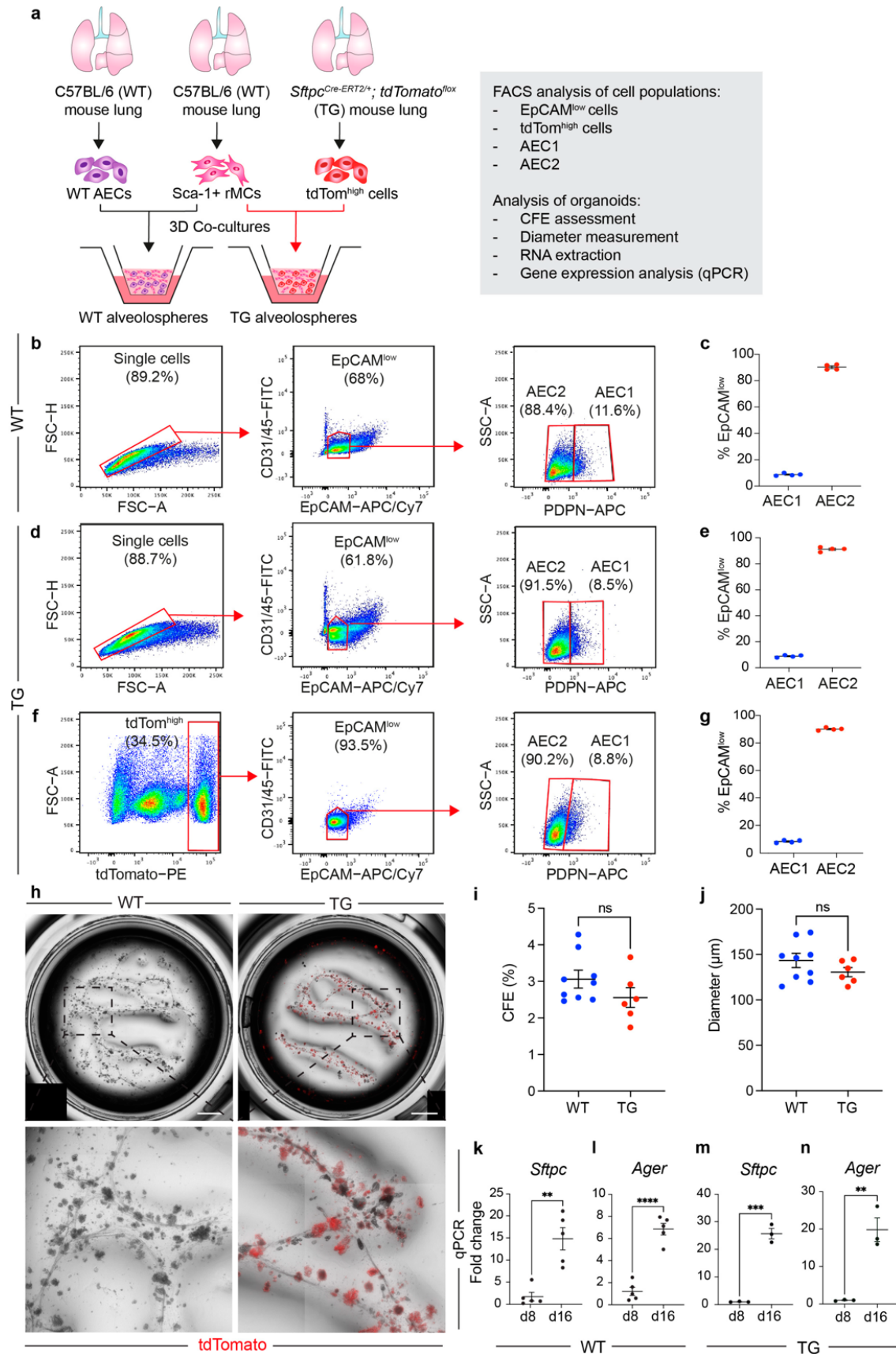
Next, rMCs were co-cultured with either AECs isolated from WT lungs (hereafter referred to as the WT approach) or tdTom<sup>+</sup> cells from *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> lungs (hereafter referred to as the transgenic (TG) approach) (**Figure 5a**). WT AECs were sorted from single-cell suspensions based on CD45<sup>−</sup> CD31<sup>−</sup> EpCAM<sup>low</sup> surface marker expression (**Figure 5b**). The majority of isolated cells were identified as CD45<sup>−</sup> CD31<sup>−</sup> EpCAM<sup>low</sup> PDPN<sup>−</sup> AEC2s (90.2% ± 1.01% from the parent EpCAM<sup>low</sup> gate), while only 8.8% ± 0.47% was CD45<sup>−</sup> CD31<sup>−</sup> EpCAM<sup>low</sup> PDPN<sup>+</sup> AEC1s (**Figure 5c**). Lungs



from *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> mice were similarly processed for FACS sorting. Staining for EpCAM and PDPN revealed comparable proportions of AEC2s and AEC1s within the EpCAM<sup>low</sup> population similar to the WT approach (**Figure 5d, e**). Since IAAPs are quiescent in the absence of injury and therefore do not robustly generate alveolar organoids (Ahmadvand et al., 2021), tdTom<sup>high</sup> cells were initially selected for alveolar organoid generation (**Figure 5f**). As shown in (**Figure 4e**), 92.5% ± 2.4% of tdTom<sup>high</sup> cells are found in the EpCAM<sup>low</sup> gate. Staining for PDPN revealed that 90.1% ± 0.5% of tdTom<sup>high</sup> EpCAM<sup>low</sup> cells were AEC2s, while only 8.46% ± 0.45% were AEC1s (**Figure 5g**), resembling the data attained with WT EpCAM<sup>low</sup> cells.

#### **4.1.2 The EpCAM<sup>low</sup> population robustly generates alveolar organoids, and it is comparable with sorted lineage-traced AEC2s**

The data described above clearly show that gating for CD45<sup>-</sup> CD31<sup>-</sup> EpCAM<sup>low</sup> cells in WT lungs allows for the sorting of an AEC population that predominantly consists of AEC2s. The finding that lineage-traced AEC2s essentially belong to the EpCAM<sup>low</sup> population (**Figure 4d–f**) strongly supports this conclusion. We therefore tested the ability of EpCAM<sup>low</sup> cells to generate alveolar organoids (WT approach) and compared the results to the TG approach. As expected, all alveolar organoids in the TG approach cells were tdTom<sup>+</sup> (**Figure 5h**). Both approaches were similarly efficient in generating alveolar organoids (WT CFE 3.14% ± 0.23% vs. TG CFE 2.55% ± 0.27%) (**Figure 5i**) with comparable alveolar organoid diameter (139.37 µm ± 6.86 µm for WT vs. 130.57 µm ± 5.01 µm for TG) (**Figure 5j**). Gene expression analysis was then carried out on WT and TG alveolar organoids on day 8 (when AECs start to form cysts) and day 16 (when alveolar organoids acquire a complex morphology and contain differentiated AEC2s and AEC1s). The results showed significant upregulation of the AEC2 marker *Sftpc* (**Figure 5k, m**) and the AEC1 marker *Ager* (**Figure 5l, n**) in both conditions.



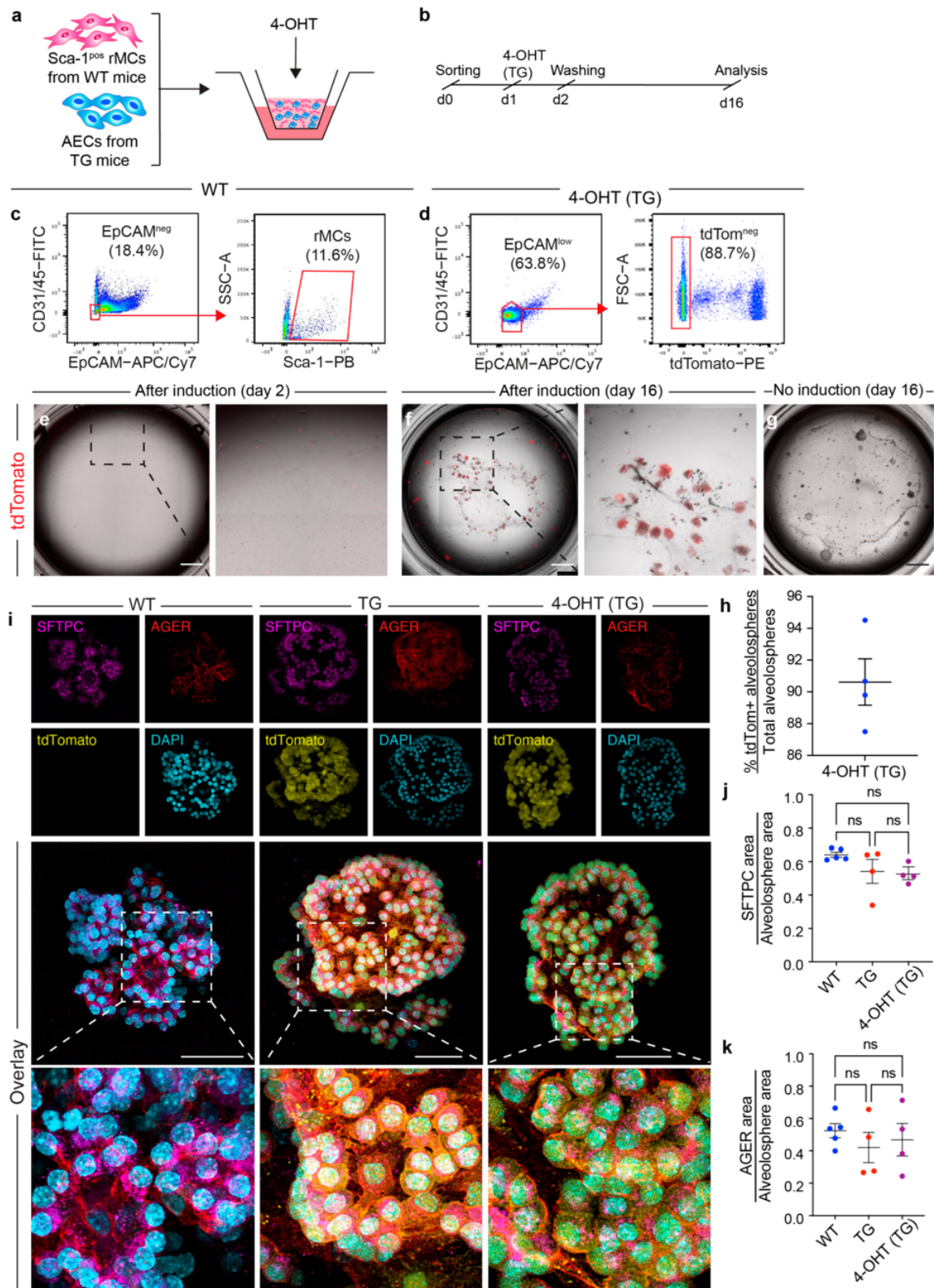
**Figure 5. Generation of alveolar organoids from WT and TG mouse lungs.** a) Experimental design for co-culturing WT or TG AEC2s with rMCs and subsequent analysis. b) Gating strategy for sorting AEC2s (EpCAM<sup>low</sup>) from WT mouse lungs. c) Quantification of AEC1s and AEC2s in the EpCAM<sup>low</sup> population. d) Gating strategy for identifying AEC2s (EpCAM<sup>low</sup>) in TG mouse lungs. e) Quantification of AEC1s and AEC2s in the EpCAM<sup>low</sup> population. f) Gating strategy to select EpCAM<sup>low</sup> cells from the tdTom<sup>high</sup> population. g) Quantification of AEC1s and AEC2s in the EpCAM<sup>low</sup> fraction of the tdTom<sup>high</sup> population. h) Tile scans of representative wells for WT and TG alveolar organoid cultures. (i, j) Quantification of CFE and alveolar organoid diameter. (k–n) qPCR for alveolar epithelial marker genes on days 8 and 16 of culture. (c,e,g) Each dot represents one lung; (i, j) Each dot represents one culture well; (k–n) Each dot represents two pooled culture wells. \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001; ns: not significant. AECs: alveolar epithelial cells; CFE: colony-forming efficiency; rMCs: resident mesenchymal cells; TG: transgenic; WT: wild-type. Scale bars: 1 mm.

#### 4.1.3 WT alveolar organoids derive from isolated AEC2s

To validate that the organoids indeed derive from AEC2s, the CD45<sup>–</sup> CD31<sup>–</sup> EpCAM<sup>low</sup> tdTom<sup>–</sup> AEC population was isolated from uninduced *Sftpc*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flox</sup> lungs and co-cultured with WT rMCs. Cre-mediated recombination was induced ex vivo by exposure to the active metabolite of tamoxifen, 4-OHT, between days 1 and 2 (**Figure 6a–d**). By day 2, cultures revealed single tdTom<sup>+</sup> cells (**Figure 6e**), with most alveolar organoids (90.61% ± 1.45%) being tdTom<sup>+</sup> on day 16 (**Figure 6f, h**). In the absence of 4-OHT, organoids were negative for tdTom (**Figure 6g**). These data strengthen the notion that the EpCAM<sup>low</sup> population indeed enriches for AEC2s that are capable of ex vivo expansion and subsequent alveolar organoid formation.

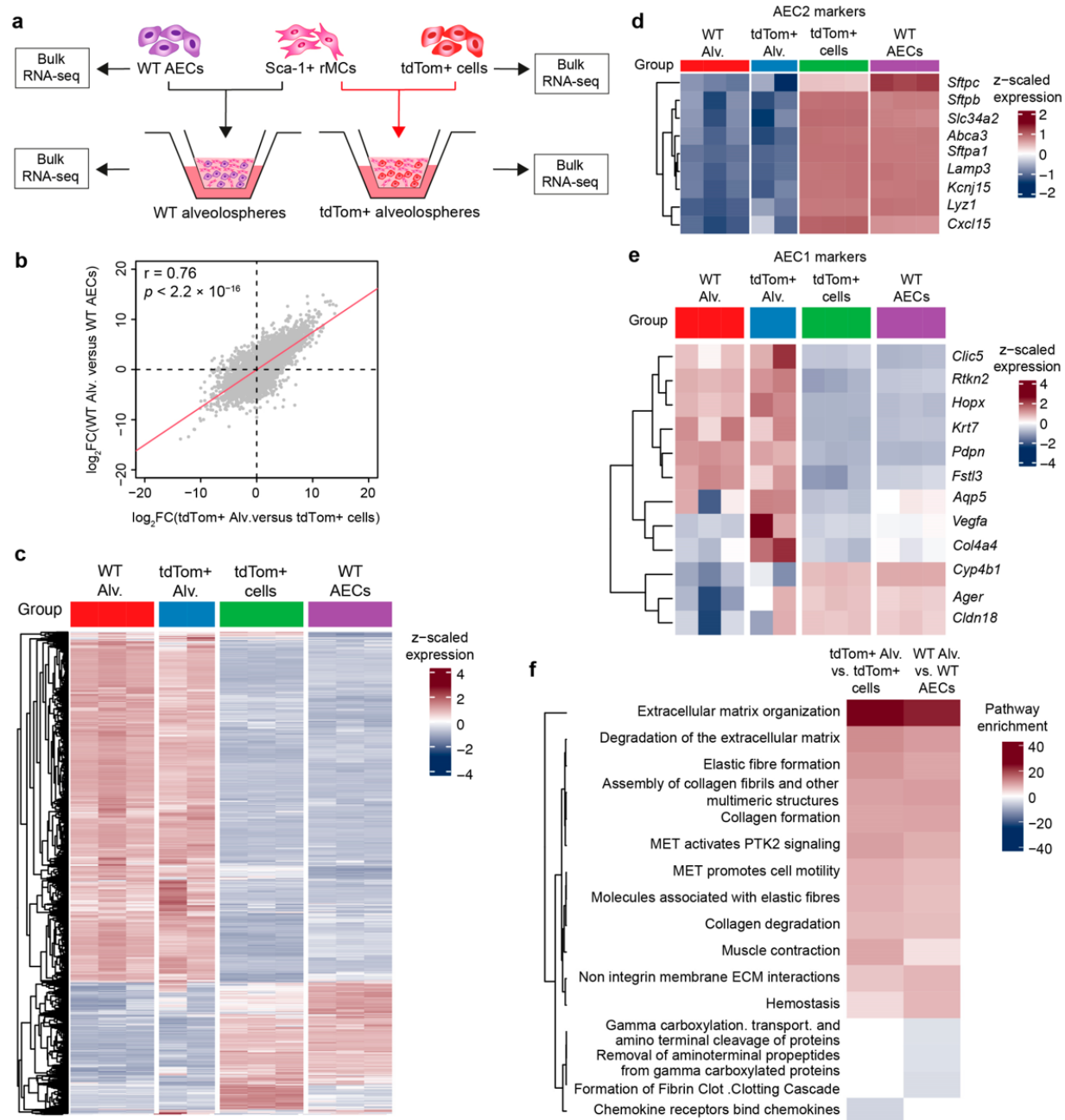
Alveolar organoids generated using the WT, TG, and 4-OHT (TG) approaches were then subjected to immunofluorescence for SFTPC and AGER, followed by whole-mount confocal imaging (**Figure 6i**). The data revealed similar ratios relative to alveolar organoid area among the three groups for SFTPC (WT 0.64 ± 0.01 vs. TG 0.54 ± 0.07 vs. 4-OHT (TG) 0.52 ± 0.02) (**Figure 6j**) and AGER (WT 0.52 ± 0.04 vs. TG 0.42 ± 0.09 vs. 4-OHT

(TG)  $0.46 \pm 0.1$ ) (**Figure 6k**), without apparent differences in terms of overall morphology, suggesting that the cellular composition within these conditions is highly comparable.



**Figure 6. Alveolar organoids derive from SFTPC+ AEC2s and contain both AEC1s and AEC2s.** a, b) Experimental design for co-culturing uninduced TG AEC2s with WT rMCs and ex vivo induction with 4-OHT. c, d) Gating strategy for sorting WT rMCs and tdTom<sup>+</sup> EpCAM<sup>low</sup> cells from uninduced TG mouse lungs. e, f) Tile scans of representative wells on days 2 and 16. The dashed boxes are magnified in the right panels. The tdTom signal is shown in red. g) Tile scan of a day 16 culture well in the absence of 4-OHT. h) Quantification of the percentage of tdTom<sup>+</sup> alveolar organoids. i) Representative images of the maximum intensity projection images of individual organoids from the indicated conditions stained for SFTPC and AGER. Note the presence of the tdTom<sup>+</sup> signal in the TG and 4-OHT (TG) conditions but not the WT condition. j, k) Quantification of the ratio of the SFTPC and AGER signals to alveolar organoid area. (h) Each dot represents one culture well; (j, k) Each dot represents one organoid. 4-OHT: 4-hydroxytamoxifen; AECs: alveolar epithelial cells; ns: not significant; rMCs: resident mesenchymal cells; TG: transgenic; WT: wild-type. Scale bars: (e–g) 1 mm; (i) 50  $\mu$ m.

To further demonstrate that the alveolar organoids generated using the WT approach are comparable to those generated using the TG approach, we carried out RNA-seq on isolated WT AECs, tdTom<sup>+</sup> cells, and the corresponding organoids (**Figure 7a**). Notably, the same gene sets are modulated during organoid formation regardless of whether the starting material is tdTom<sup>+</sup> cells or WT AECs (**Figure 7b, c**). Moreover, a comparison of AEC2 and AEC1 markers showed no difference between the two groups (**Figure 7d, e**), confirming that the cell population isolated using our WT EpCAM<sup>low</sup> approach is very similar to the tdTom<sup>+</sup> population isolated from transgenic animals. We also found that the AEC1 signature is upregulated in alveolar organoids compared with isolated AEC2s, while the AEC2 signature is diluted in alveolar organoids (**Figure 7d, e**), which is expected given the emergence of AEC1s and the abundance of mesenchymal cells in the alveolar organoids but not in purified AEC2s. Pathway analysis of the top genes differentially regulated between alveolar organoids and AEC2s was related to ECM organization, elastic fibers, collagen biosynthesis, and cell motility (**Figure 7f**).



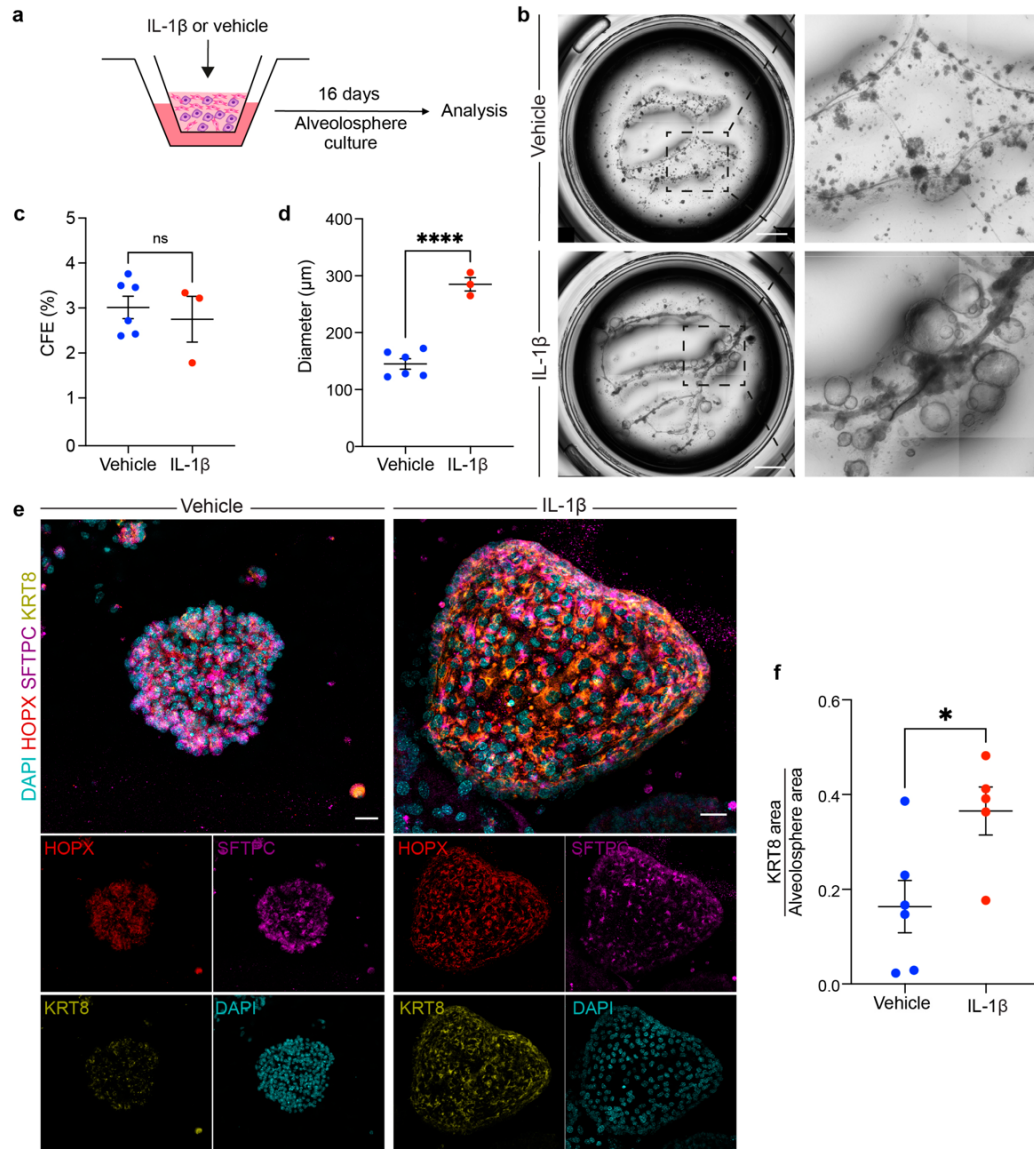
**Figure 7. Resemblance between the WT and TG approaches at the transcriptomic level.** a) Experimental design. b) Scatter plot showing the correlation between the WT and TG approaches. c) Heatmap showing the top 2000 differentially expressed genes between alveolar organoids and sorted cells. d, e) Heatmaps showing selected AEC2 and AEC1 marker genes in various samples. f) Reactome pathway analysis of the top differentially expressed genes in alveolar organoids versus sorted cells. Pathway enrichment was calculated by multiplying  $-\log_{10}(\text{p value})$  by +1 (in case of upregulation)



or -1 (in case of downregulation). Alv.: alveolar organoids; rMCs: resident mesenchymal cells; TG: transgenic; WT: wild-type

#### **4.1.4 Stimulation with recombinant murine IL-1 $\beta$ alters alveolar organoid formation**

It has been reported that IL-1 $\beta$  treatment leads to enlarged alveolar organoids that feature accumulation of the KRT8<sup>+</sup> intermediate cell state (Choi et al., 2020); therefore, recombinant IL-1 $\beta$  was added to WT cultures as another proof of concept that such organoids behave similarly to those obtained using the TG approach (**Figure 8a**). After 16 days of culture, organoids clearly showed a cyst-like morphology rather than a typical alveolar organoid morphology in the IL-1 $\beta$  group (**Figure 8b**), which was also reflected by the quantification of organoid diameter ( $285.08 \mu\text{m} \pm 11.6 \mu\text{m}$  vs.  $145.04 \mu\text{m} \pm 9.16 \mu\text{m}$ ) (**Figure 8d**). Analysis of the CFE did not reveal a difference between the two groups (**Figure 8c**). Notably, IF showed a significant increase in the coverage of the KRT8 signal relative to the overall alveolar organoid area upon IL-1 $\beta$  treatment ( $0.36 \pm 0.05$  vs.  $0.16 \pm 0.05$ ) (**Figure 8e, f**). These data indicate that our WT approach can recapitulate previous findings attained using the TG approach.



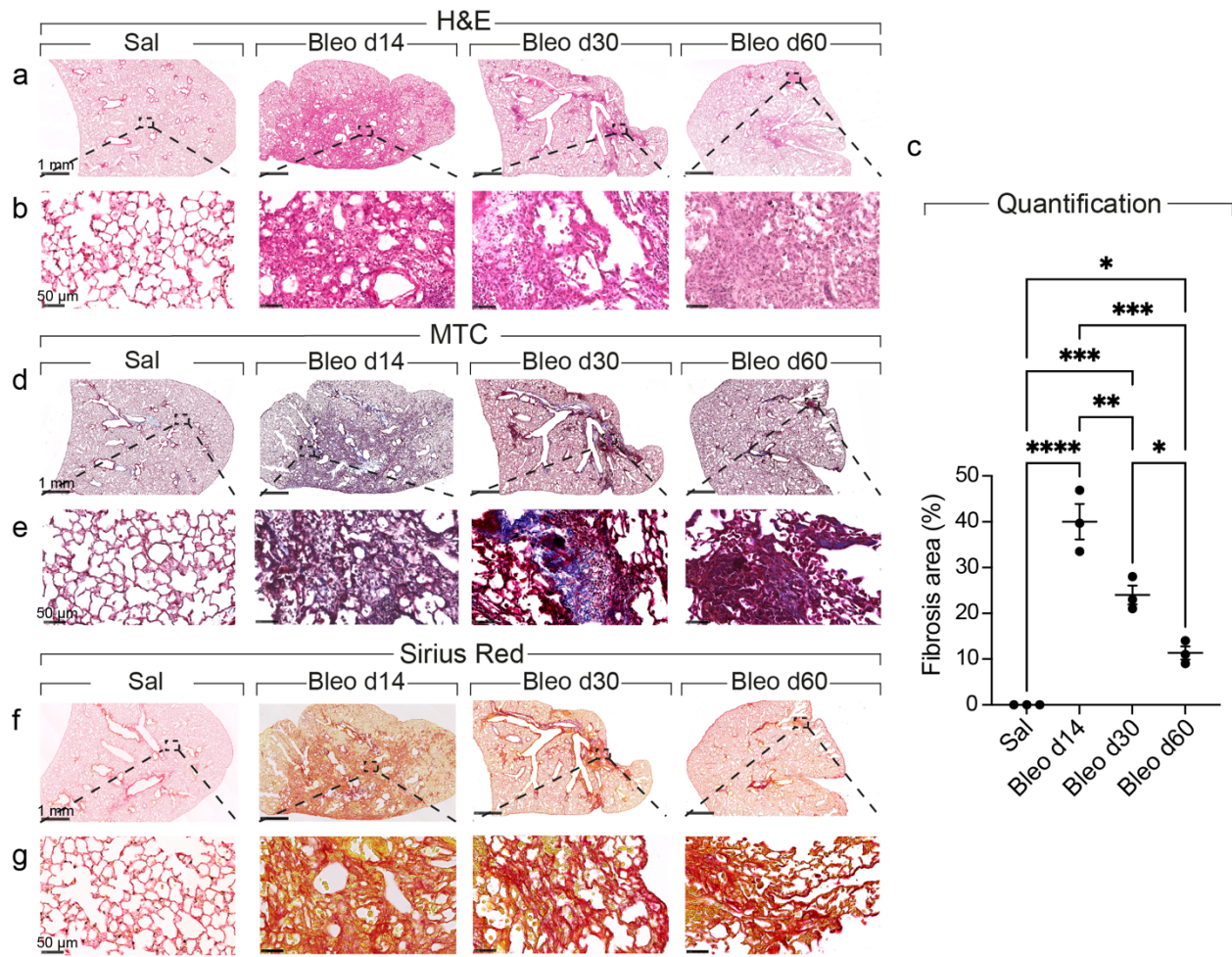
**Figure 8. Treatment with recombinant IL-1 $\beta$  impacts alveolar organoid morphology and differentiation.** a) Experimental design for co-culturing WT AEC2s with WT rMCs in the presence or absence of IL-1 $\beta$ . b) Tile scans of representative wells on day 16. The dashed boxes are magnified in the right panels. c, d) Quantification of CFE and alveolar organoid diameter. e) Representative maximum intensity projection images of individual organoids from the indicated conditions stained for HOPX, SFTPC, and KRT8. f) Quantification of the ratio of the KRT8 signal to alveolar organoid area. (c,d) Each dot represents one culture well; (f) each dot represents one organoid. \*  $p < 0.05$ ; \*\*\*\*  $p < 0.0001$ ; ns: not significant. CFE: colony-forming efficiency. Scale bars: (b) 1 mm; (e) 50  $\mu$ m.



## 4.2 PART II. Investigating the origin and fate of myofibroblasts in bleomycin-induced lung fibrosis in aged mice

### 4.2.1 Aged mice demonstrate incomplete fibrosis resolution following bleomycin injury

Female *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* mice at 52-to-56 week of age were administered three intraperitoneal (*i.p.*) of tamoxifen followed by a single *i.t.* instillation of bleomycin (Bleo) or Saline (Sal) (**Figure 9a**). This experimental approach enabled the labeling of pre-existing *Fgf10*<sup>+</sup> cells, allowing us to track their fate during fibrosis formation and resolution. Lungs were harvested at day 14 (d14) (corresponding to peak of fibrosis) and days 30 (d30) and 60 (d60) (corresponding to early and late fibrosis resolution, respectively) after the Bleo challenge (**Figure 9a**). Hematoxylin and eosin (H&E) staining confirmed the development of pulmonary fibrosis by d14 where 40% ± 3.8% of the tissue was fibrotic (**Figure 9a-c**). Despite structural improvements from Bleo d30 till Bleo d60, persistent damage was still observed at these timepoints (24% ± 2.08% and 11.3% ± 1.4% respectively) (**Figure 9a-c**). Masson's Trichrome and Picro Sirius Red staining showed increased collagen deposition at Bleo d14, and to a less extent at Bleo d30 and Bleo d60 (**Figure 9d-g**).

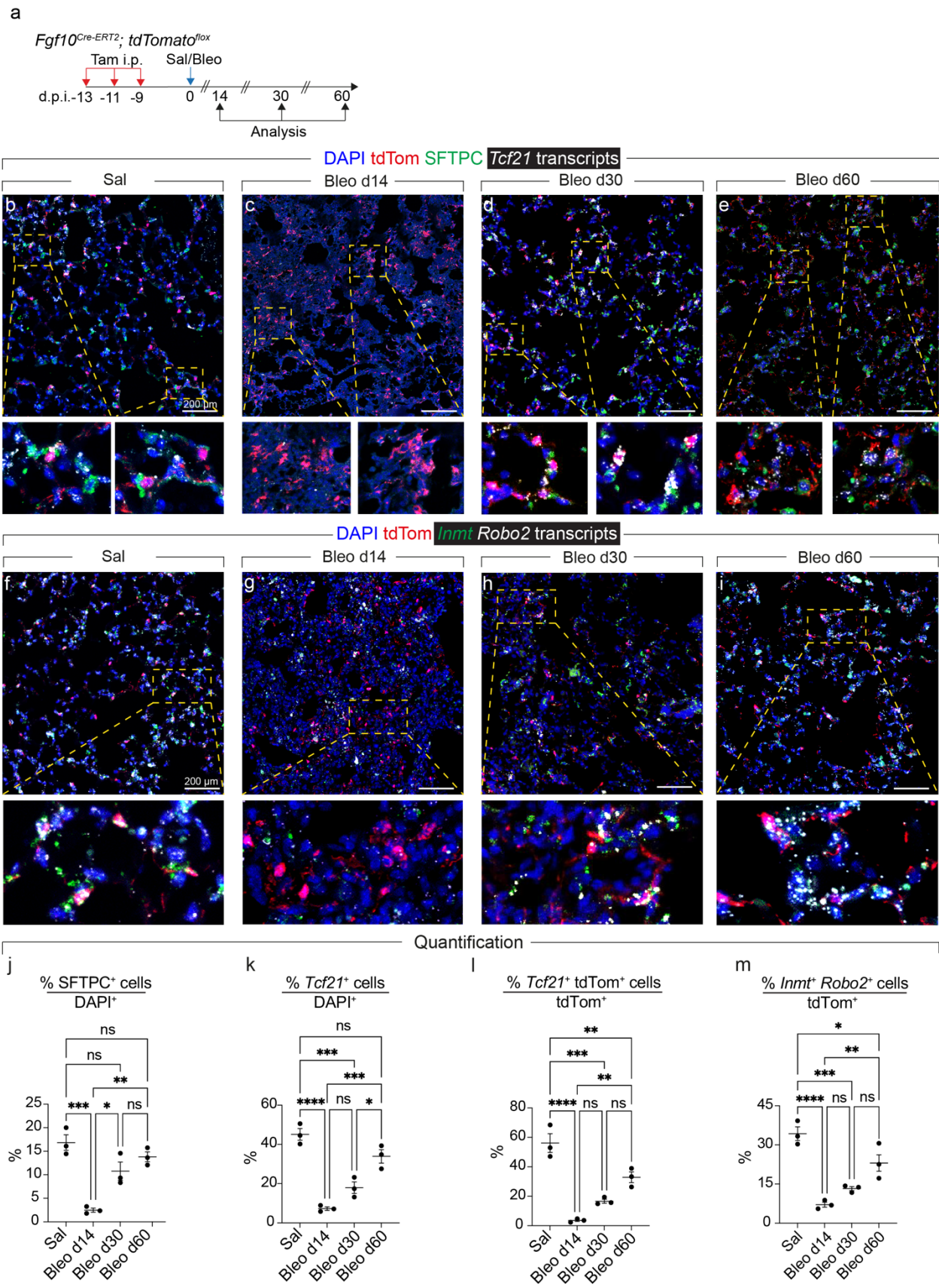


**Figure 9. Fibrosis formation and collagen deposition were confirmed at the peak of the injury.** a) Representative hematoxylin and eosin (H&E) staining of lung sections showed lung fibrosis formation following bleomycin instillation at day 14 with partial resolution observed on days 30 and 60 post injury. b) The black boxes are magnified in the lower panels. c) Quantification of lung damage in H&E staining. d) Representative Masson's Trichrome (MTC) staining of collagen in lung sections from saline- and bleomycin-treated groups. More abundant collagen is accumulated in alveolar space. Collagen fibers are stained blue. e) The black boxes are magnified in lower panels. f) Representative Picro Sirius Red staining of collagen in saline- and bleomycin-treated groups. g) The black boxes are magnified in the lower panels where collagen content is detected in red. n = 3 per group. Each dot represents one lung. Scale bars: (a, d, f) 1 mm. (b, e, g) 50 μm. Data are presented as mean ± SEM. Statistical analysis was performed

using Ordinary one-way ANOVA with Tukey's multiple comparisons test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ .

#### **4.2.2 Loss of LIFs signature and AEC2s cells at the peak of the injury and rehabilitating of the cells during fibrosis resolution**

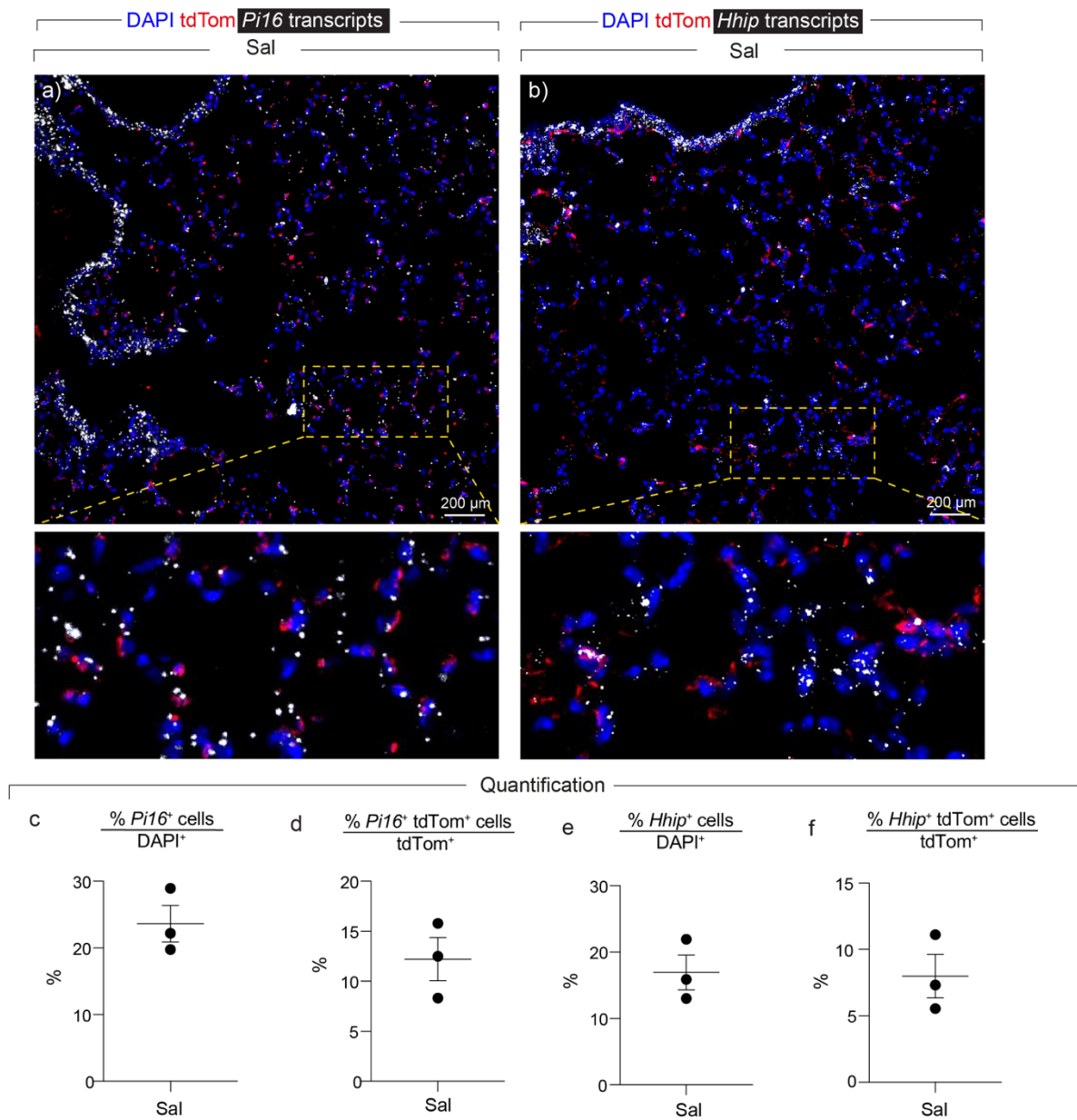
We then assessed the status of LIF markers in Sal and Bleo lungs using RNAscope combined with IF for SFTPC+ cells (**Figure 10**). In Sal lungs,  $56.1\% \pm 6.3\%$  of tdTom+ cells also expressed *Tcf21* and these cells were observed adjacent to SFTPC+ cells (AEC2s) (**Figure 10b, k, l**). Following bleomycin injury (Bleo d14), tdTom/*Tcf21* double positive cells and AEC2s were dramatically lost in fibrotic regions (**Figure 10c, j-l**). Similarly, tdTom/*Inmt/Robo2* triple-positive cells were decreased from  $34.1\% \pm 2.6\%$  in Sal to  $7.09\% \pm 0.9\%$  in Bleo d14 (**Figure 10f, g, m**). These results suggest that tdTom+ cells lose their LIF profile at the peak of fibrosis. Conversely, during early (Bleo d30) and late (Bleo d60) fibrosis resolution, the number of AEC2s and the expression of LIF markers in lineage-labeled cells were largely, yet incompletely, restored (**Figure 9c, Figure 10d, e, h, i**). RNAscope analysis of *Pi16* and *Hhip* in saline-treated control lungs revealed that the *Fgf10*+ lineage-labeled lipofibroblast population is heterogeneous (**Figure 11**). Subsets of these cells expressed *Pi16* or *Hhip*, suggesting the presence of distinct fibroblast subtypes within the *Fgf10*+ lineage under homeostatic conditions (**Figure 11a-c**).



**Figure 10. Characterization of lipogenic markers in naïve and fibrotic aged lungs.**

a) Timeline and schematic of the experimental design. Female 52-to-56-week-old *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* mice were subjected to three successive doses of tamoxifen before saline or bleomycin instillation to lineage trace *Fgf10*<sup>+</sup> cells at distinct timepoints. Control lungs were harvested 14 days after saline treatment and bleomycin-treated lungs were collected at days 14, 30 and 60 upon bleomycin administration. b-e) Representative in situ hybridization for *Tcf21* mRNA (in grey) combined with immunofluorescence staining against pro-SFTPC (in green). The dashed boxes are magnified in the lower panels. f-i) Representative in situ hybridization for *Robo2* (in grey) and *Inmt* mRNA (in green). The dashed boxes are magnified in the lower panels. j-m) Quantification of RNAscope and immunofluorescence shown in b-i. The red signal represents the endogenous tdTom expression. Scale bars 200  $\mu$ m; n = 3 per group. Each dot represents one lung. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using Ordinary one-way ANOVA with Tukey's multiple comparisons test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ; ns: not significant. *i.p.*: intraperitoneal injection; Tam: Tamoxifen; *d.p.i.*: days post instillation; Sal: Saline; Bleo: Bleomycin; *Tcf21*: Transcription factor 21; tdTom: tdTomato; *Robo2*: Roundabout guidance receptor 2; *Inmt*: Indolethylamine N-Methyltransferase.





**Figure 11. Identification of adventitial and peribronchial fibroblasts in untreated lungs.** a) Representative in situ hybridization for *Pi16* mRNA (in grey) as adventitial marker. b) Representative in situ hybridization for *Hhip* mRNA (in grey) as peribronchial marker. The dashed boxes are magnified in the lower panels. c-g) Quantification of RNAscope shown in a, b. The red signal represents the endogenous tdTom expression. Scale bars 200  $\mu$ m; n = 3 per group. Each dot represents one lung. Data are presented as mean  $\pm$  SEM. Tam: Tamoxifen; Sal: Saline; *Pi16*: Peptidase Inhibitor 16; *Hhip*: Hedgehog Interacting Protein.

#### 4.2.3 Interconversion of *Fgf10*<sup>+</sup> cells between the LIF and MyoFB states during fibrosis formation and resolution

To characterize lineage-labeled *Fgf10*<sup>+</sup> (tdTom<sup>+</sup>) cells and precisely determine their fate during fibrosis development and resolution, tdTom<sup>+</sup> cells were FACS-sorted from Sal, Bleo d14, Bleo d30 and Bleo d60 lungs (**Figure 12a, b**). FACS analysis demonstrated significant abundance of tdTom<sup>+</sup> cells in Bleo d14 lungs and sustained presence of these cells during resolution (Bleo d30 and d60) (**Figure 12c**). Next, sorted tdTom<sup>+</sup> cells were loaded for scRNA-seq. Integrative UMAPs showed that tdTom<sup>+</sup> cells in Sal predominantly consist of alveolar fibroblasts 1 (AF1; around 55%) and AF2 (around 36%) along with peribronchial fibroblasts (PeriFBs; around 6.6%) and traces of myofibroblasts (MyoFBs; around 2.6%) (**Figure 12d, e**). Importantly, the analysis also showed that the MyoFB cluster peaked at Bleo d14 (7.3% of total cells) with partial regression during early (5.3% at Bleo d30) and late (3.9% at Bleo d60) resolution (**Figure 12d, e**). Interestingly, the AF1 cluster expanded at the expense of AF2 during resolution (**Figure 12d, e**).

We then subclustered the MyoFB cluster to gain insights into the heterogeneity of these pathological cells and the transcriptomic changes occurring during fibrogenesis and recovery (**Figure 12f**). The analysis revealed seven subclusters including *Cthrc1*<sup>high</sup>, *Cthrc1*<sup>low</sup>, *Gli1*<sup>high</sup>, *Prolif*<sup>high</sup>, *LIF*<sup>high</sup>, *AF2*<sup>high</sup> and a very minor SMC subcluster (**Figure 12f, g**). This was confirmed by applying a MyoFB and LIF score reflecting the expression of the top 485 DEGs for the respective populations (**Figure 12h, i**). Analyzing individual timepoints showed the presence of almost only *LIF*<sup>high</sup> and *Cthrc1*<sup>low</sup> MyoFBs in Sal, while *Cthrc1*<sup>high</sup> and *Gli1*<sup>high</sup> MyoFBs were predominant in Bleo d14 before regressing through resolution (**Figure 12g, j**). The *AF2*<sup>high</sup> subcluster was prominent in Bleo d30 (**Figure 12g, j**). In Bleo d60, the *LIF*<sup>high</sup> cluster represented the majority of the remaining cells (**Figure 12g, j**), confirming our previous finding that MyoFB-to-LIF transition marks fibrosis resolution (El Agha et al., 2017b). A heatmap of the top regulated genes in these subclusters shows top markers such as *Cxcl10* (Pro-inflammatory cytokine) in Sal, *Cthrc1*, *Penk*, and *Gli1* (ECM and MyoFB markers) in Bleo d14, *Ly6a* (AF2 marker) in Bleo d30 and *Wnt4*, *Inmt*, and *Ces1d* (LIF markers) in Bleo d60 (**Figure 12k**). Contrasting *Cthrc1*<sup>high</sup> versus *LIF*<sup>high</sup> MyoFBs identified the top markers for the respective subclusters

**(Figure 12l).** A similar analysis was conducted on the AF1 cluster and the analysis revealed ten subclusters including two *LIF<sup>high</sup>*, *LIF<sup>int</sup>*, two *LIF<sup>low</sup>*, *AF2<sup>high</sup>*, *AF2<sup>low</sup>*, *LIF<sup>Adams4</sup>*, Apoptotic fibroblasts (*ApoptoticF*) and Proliferative fibroblasts (*ProlifF*) **(Figure 12m-q)**. The emerging picture is that the *LIF* signature is dominant in Sal, lost during fibrogenesis and re-emerges during late resolution (Bleo d60). Similarly to MyoFB subclustering, an *AF2* signature emerges during early resolution (Bleo d30) **(Figure 12q)**. A heatmap of the top regulated genes in these subclusters showed top markers such as *Polr2l* (mRNA transcription) in Sal, *Mki67* and *Top2a* (Proliferation) in Bleo d14, *Ly6a* (*AF2* marker) in Bleo d30, and *Il6* (Pro-inflammatory cytokine) in Bleo d60 **(Figure 12r)**. Contrasting *LIF<sup>high</sup>* versus *LIF<sup>int</sup>* identified the top markers for the respective subclusters such as *Inmt* and *Npnt* for *LIF<sup>high</sup>* and *Gdf15* and *Cdkn1a* for *LIF<sup>int</sup>* **(Figure 12s)**.



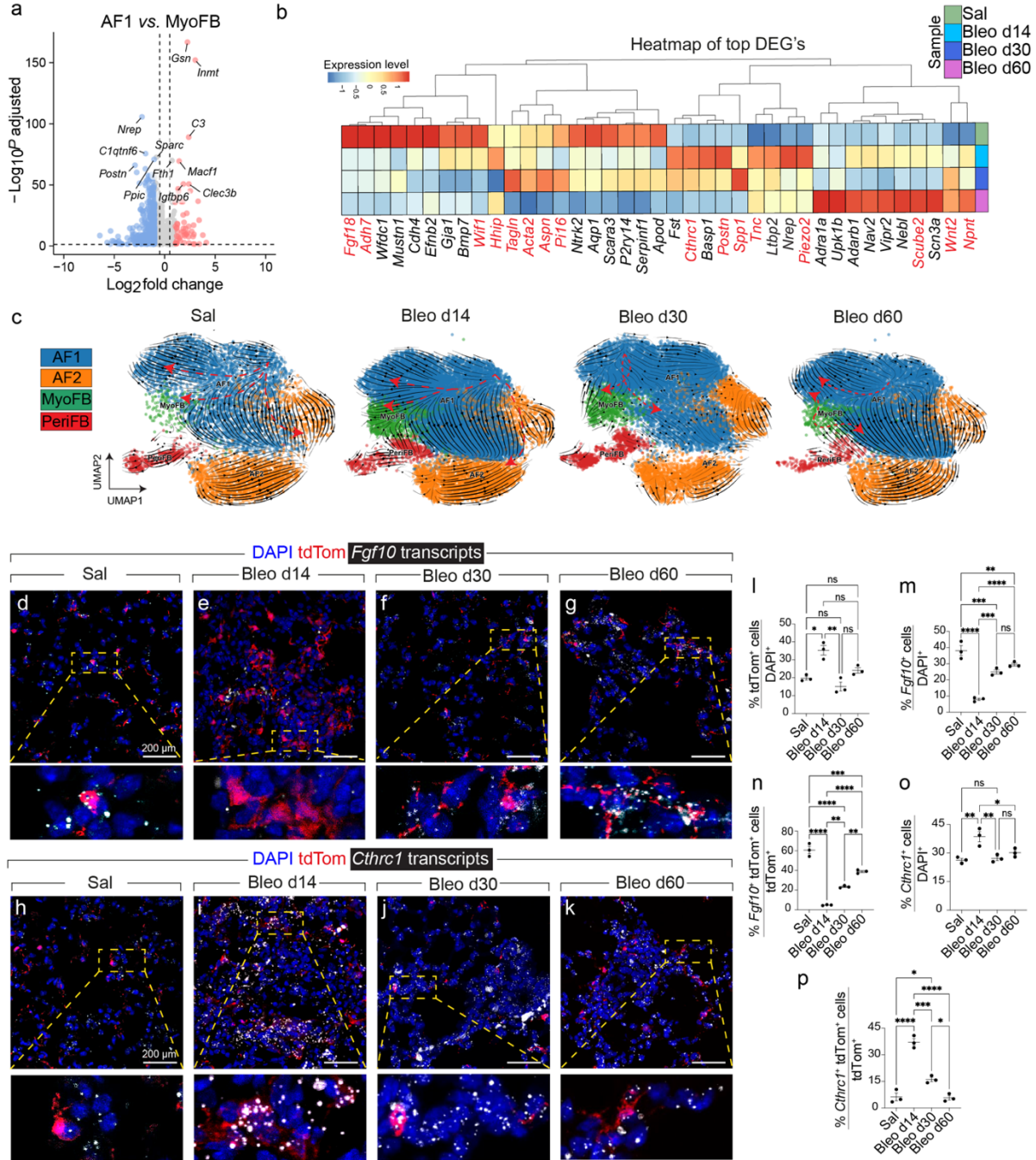


**Figure 12. Lineage tracing of *Fgf10*<sup>+</sup> cells revealed their heterogeneity in normal and fibrotic mouse lungs.**

a) Timeline and schematic of the experimental design. b) Gating strategy for isolating tdTom<sup>+</sup> cells from mesenchymal population for single cell RNA sequencing (scRNA-seq). c) Quantification of sorted lineage-labeled cells from mesenchymal population in saline and experimental groups showing accumulation of tdTom<sup>+</sup> cells after the injury in fibrotic lungs. n = 4 per group. Each dot represents one lung. d) Integrative Uniform Manifold Approximation and Projection (UMAP) plot of lineage-labeled *Fgf10*<sup>+</sup> cells isolated from Sal, Bleo d14, Bleo d30 and Bleo d60, showing different clusters containing AF1, AF2, MyoFB and PeriFB. The MyoFB and AF1 clusters are marked with dashes. e) Frequency of clusters in each condition and UMAP plots for individual timepoints highlighting MyoFB cluster. f) Integrative UMAP of MyoFB subclusters. g) UMAP plots of MyoFB subclusters in individual timepoints. h, i) UMAP plots showing the score of *LIF* and *MyoFB* signatures. j) Frequency of clusters in each condition. k) Heatmap of top regulated genes in each sample. l) Volcano plot showing top markers between *Cthrc1*<sup>high</sup> versus *LIF*<sup>high</sup> MyoFBs. m) Integrative UMAP of AF1 subclusters. n) UMAP plots of AF1 subclusters in individual timepoints. o, p) UMAP plots showing the score of *LIF* and *MyoFB* signatures. q) Frequency of clusters in each condition. r) Heatmap of top regulated genes. s) Volcano plot showing top markers between *LIF*<sup>high</sup> versus *LIF*<sup>low</sup> subclusters. *i.p.*: Intraperitoneal injection; Tam: Tamoxifen; *d.p.i.*: days post instillation; Sal: Saline; Bleo: Bleomycin; MyoFB: Myofibroblasts; PeriFB: Peribronchial fibroblasts; AF1: Alveolar fibroblasts 1; AF2: Alveolar fibroblasts 2; SMC: Smooth muscle cells; ProlifF: Proliferating fibroblasts; ApoptoticF: Apoptotic fibroblasts.

Further comparison of AF1s with MyoFBs revealed *Gsn*, *Inmt*, *C3*, *Igf1bp6*, *Macf1* and *Clec3b* as top DEGs in AF1s and *Nrep*, *Postn*, *C1qtnf6* and *Ppic* in MyoFBs (**Figure 13a**). A heatmap showing bulk analysis of top DEGs in Sal, Bleo d14, Bleo d30, and Bleo d60 is shown in **Figure 13b**. The data showed a general switch to MyoFBs during fibrogenesis and to LIFs during resolution. To gain further insights into differentiation trajectories, RNA velocity was carried out. The results showed clear transition from LIFs (AF1) to MyoFBs at Bleo d14 and to a lesser extent d30, while the reverse trajectory was observed at Bleo d60 (**Figure 13c**). Next, RNAscope and immunofluorescence were carried out and the

data revealed that tdTom<sup>+</sup> cells were massively present to the fibrotic regions in Bleo d14 lungs ( $35.1\% \pm 2.7\%$ ) compared to normal regions of Sal lungs ( $19.77\% \pm 1.02\%$ ) (**Figure 13d, e, l**). While the majority of tdTom<sup>+</sup> cells were enriched with *Fgf10* expression in Sal ( $60.77\% \pm 3.78\%$ ) (**Figure 13d, n**), only  $6.3\% \pm 2.15\%$  of tdTom<sup>+</sup> cells expressed *Cthrc1* (**Figure 13h, p**). At the peak of the injury, these tdTom<sup>+</sup> cells lose *Fgf10* expression (**Figure 13e, n**) and express *Cthrc1* (MyoFB marker) ( $37.1\% \pm 2\%$ ) (**Figure 13i, p**). With the start of resolution, these tdTom<sup>+</sup> cells start reacquiring *Fgf10* expression and by Bleo d60,  $38.8\% \pm 1.04\%$  of them express *Fgf10* (**Figure 13f, g, n**). In contrast, only  $5.7\% \pm 1.24\%$  *Cthrc1*/tdTom double-positive cells were detected in the damaged regions of Bleo d60 (**Figure 13k, p**).



**Figure 13. Contribution of AF1 cluster to MyoFBs during fibrogenesis and reverse trajectory during resolution.** a) Volcano plot showing top markers between AF1 and MyoFB. b) Heatmap of top DEGs in each condition. c) RNA velocity analysis of experimental conditions showing LIF-MyoFB-LIF reversible switch during fibrogenesis and resolution. d-g) Representative in situ hybridization for *Fgf10* mRNA (in grey) in saline and bleomycin-treated lungs. Low and high magnification. The dashed boxes are

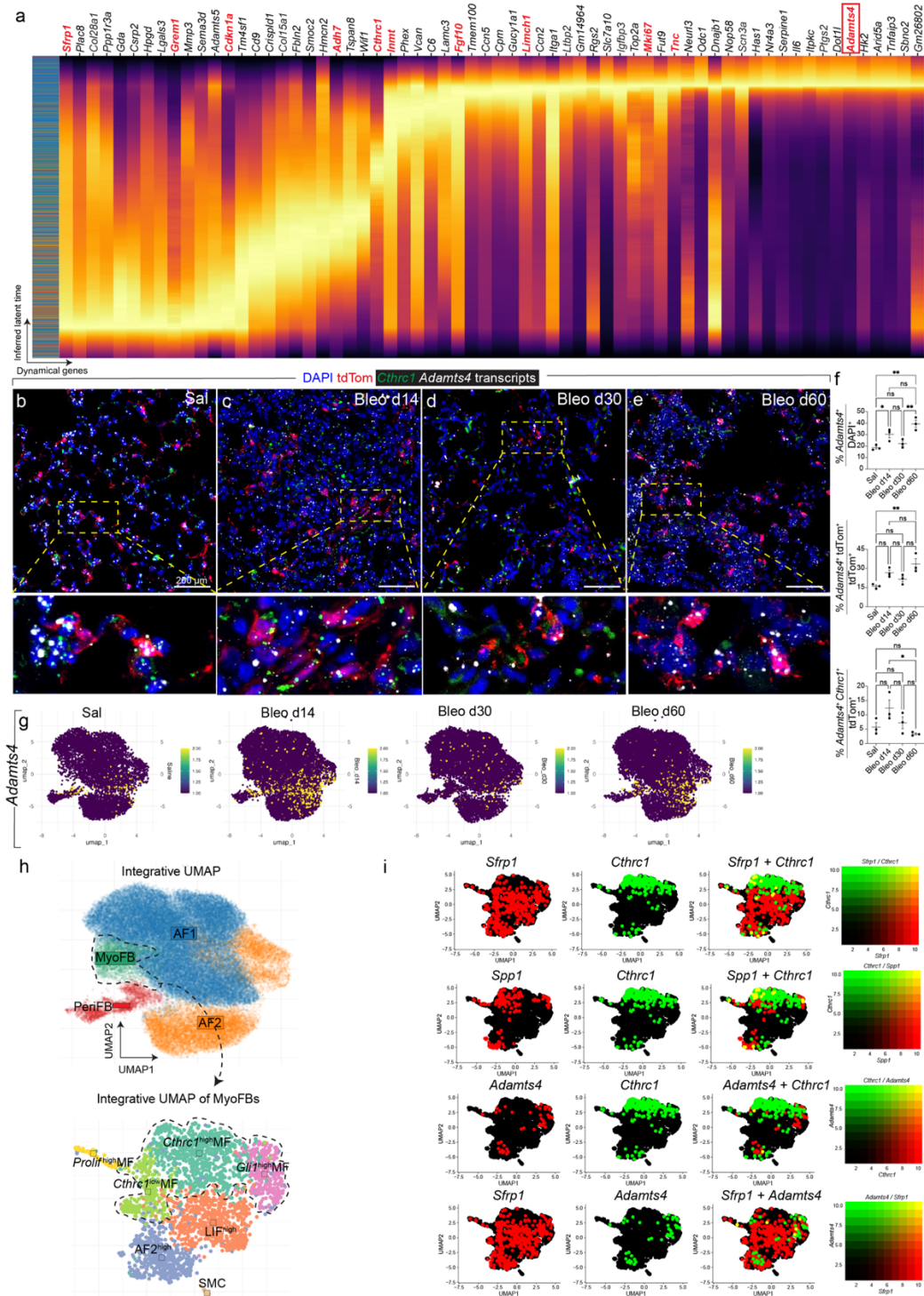
magnified. Scale bars 200  $\mu$ m. h-k) Representative in situ hybridization for *Cthrc1* mRNA (in grey) in saline and experimental groups. *Cthrc1* expression is as expected upregulated in response to injury at Bleo d14 but in less extent in Bleo d60. Of note, tdTom<sup>+</sup> cells are highly abundant in fibrotic regions at Bleo d14. The dashed boxes are magnified. The red signal represents the endogenous tdTom expression. Scale bars 200  $\mu$ m. l-p) Quantification of cell clusters. n = 3 per group. Each dot represents one lung. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using Ordinary one-way ANOVA with Tukey's multiple comparisons test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ; ns: not significant. Sal: Saline; Bleo: Bleomycin; MyoFBs: Myofibroblasts; PeriFB: Peribronchial fibroblasts; AF1: Alveolar fibroblasts 1; AF2: Alveolar fibroblasts 2.

#### **4.2.4 Persistent expression of *Adamts4* in AF clusters during late fibrosis resolution**

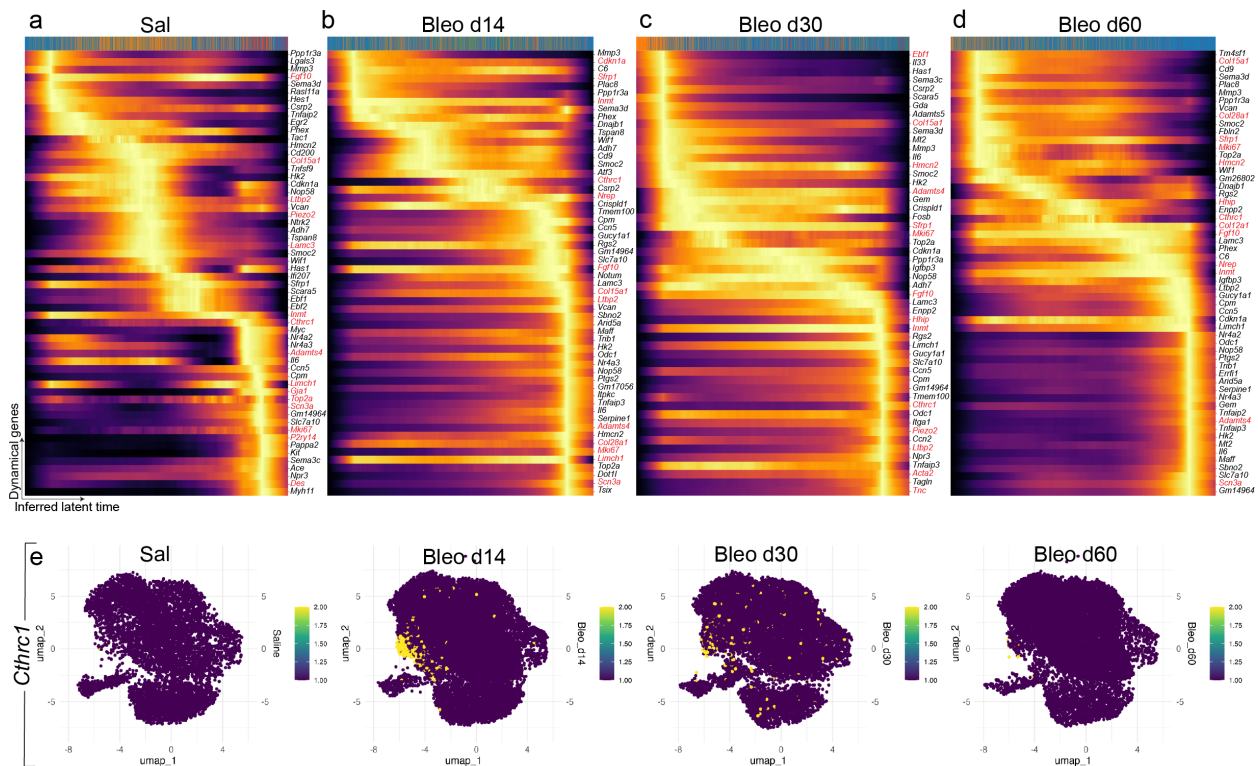
We then carried out RNA cell velocity with latent inferred time to further investigate differentiation trajectories, and the results showed a trajectory from a transitional state (*Sfrp1*<sup>+</sup>) to MyoFB/proliferative state and then LIFs (**Figure 14a and Figure 15a-d**). Interestingly, ADAM metalloproteinase with thrombospondin type 1 motif 4 (*Adamts4*) was among the top genes at the end of this fate trajectory (**Figure 14a and Figure 15a-d**). ADAMTS4 was previously shown to mediate exuberant fibroblast activity leading to exaggerated immunopathology and lung damage after influenza virus infection (Boyd et al., 2020) with one of its major substrates being versican (VCAN), which is an important immunomodulator that guides cell proliferation, migration and differentiation during inflammation. ADAMTS4 is involved in maintaining neutrophil infiltration and therefore sustaining an inflammatory milieu in the lung. We therefore hypothesized that the ADAMTS4/VCAN axis is involved in delayed resolution of pulmonary fibrosis. We then investigated the expression of *Adamts4* by RNAscope and found that it is indeed upregulated in tdTom<sup>+</sup> cells at d60 even in the absence of *Cthrc1* expression (**Figure 14b-f**). Examination of the UMAP plots revealed upregulation of *Adamts4* in AF1/2s at d14, which was persistent at d60 (**Figure 14g**). We also analyzed dual gene expression in the MyoFB subclusters and the results showed that while *Cthrc1* showed occasional co-expression with *Sfrp1* and *Spp1*, an overlap was minimal with *Adamts4*, which



occasionally overlapped with *Sfrp1* (**Figure 14h-i**). Since the MyoFB cluster is largely cleared by d60, these data support the finding that *Adamts4* is largely expressed in AFs during delayed resolution at d60 (**Figure 14g, 15e**).



**Figure 14. Persistent expression of *Adamts4* in AFs clusters during late fibrosis resolution.** a) Integrative RNA cell velocity with latent inferred time of top DEGs. b-e) Representative in situ hybridization for *Cthrc1* mRNA (in green) and *Adamts4* mRNA (in grey) in saline and bleomycin-treated lungs. The dashed boxes are magnified. Scale bars 200  $\mu$ m. f) Quantification of cell populations. n = 3 per group. Each dot represents one lung. g) UMAP plot of *Adamts4* gene in each condition in integrative analysis showing persistence of expression in alveolar fibroblasts cluster in Bleo d60. h) Integrative UMAP of MyoFBs subcluster. i) UMAP plot of dual gene analysis for *Sfrp1*, *Spp1*, *Adamts4* and *Cthrc1* in MyoFBs subclusters. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using Ordinary one-way ANOVA with Tukey's multiple comparisons test. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; ns: not significant. Sal: Saline; Bleo: Bleomycin; MyoFB: Myofibroblasts; PeriFB: Peribronchial fibroblasts; AF1: Alveolar fibroblasts 1; AF2: Alveolar fibroblasts 2; *Sfrp1*: Secreted frizzled related protein 1; *Cthrc1*: Collagen triple helix repeat containing 1; *Spp1*: Secreted phosphoprotein 1; *Adamts4*: ADAM metalloproteinase with thrombospondin type 1 motif 4.



**Figure 15. *Cthrc1*+ MyoFB cluster is decreased during late fibrosis resolution.** a-d) RNA cell velocity with latent inferred time. e) Feature plots showing *Cthrc1* expression in

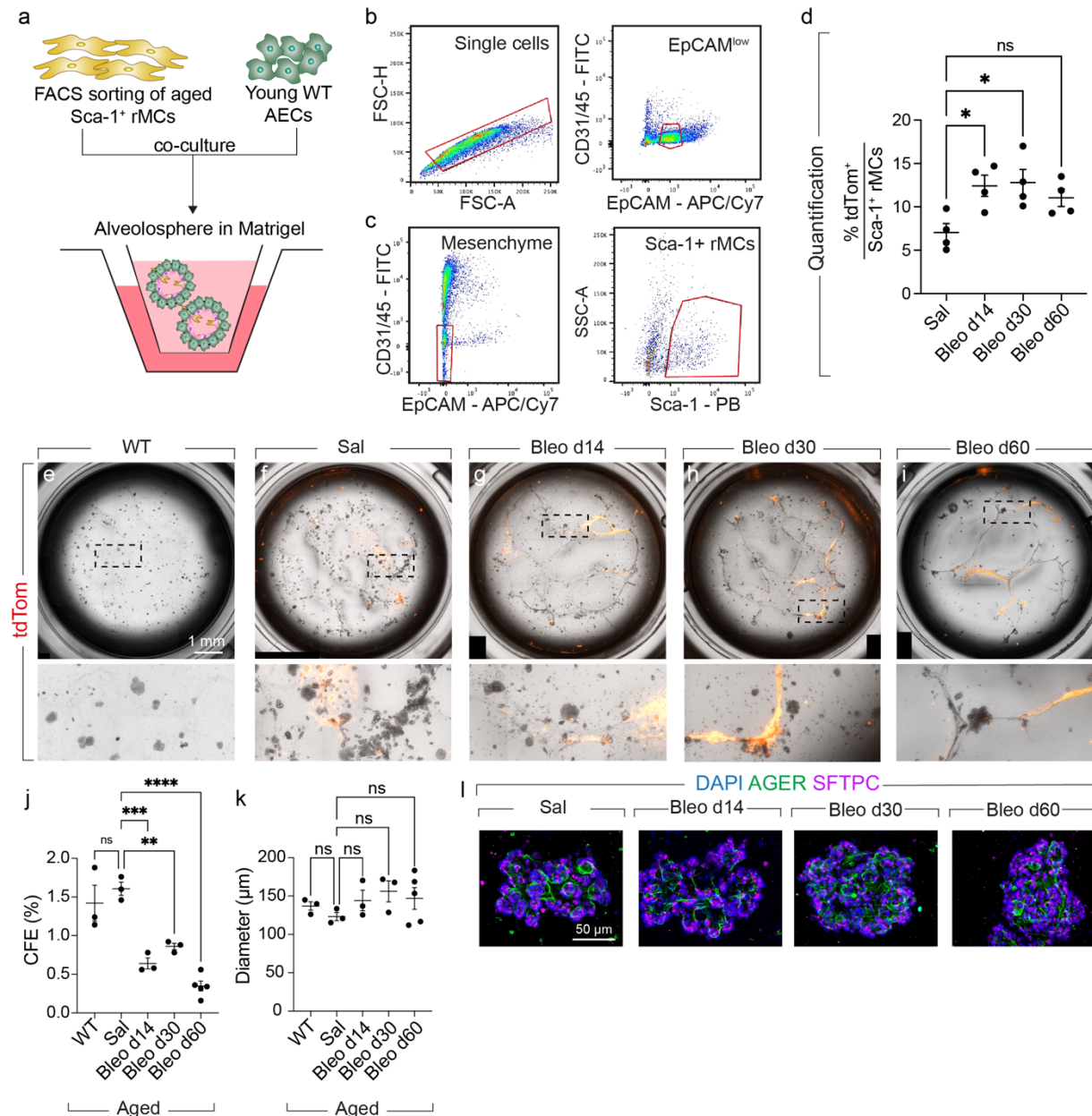
saline and bleomycin-treated lungs. *Cthrc1* expression is abundant in MyoFB at the peak of fibrosis (Bleo d14) and the expression being reduced during resolution (Bleo d60).

#### **4.2.5 Functional validation of mesenchymal cells niche activity for AEC2s maturation and self-organization in an alveolar organoid model.**

The niche activity of mesenchymal cells for AEC2s growth was evaluated using the alveolar organoid assay (**Figure 16a**). For this purpose, EpCAM<sup>low</sup> population which is enriched with AEC2s (Zabihi et al., 2024) was sorted from young and uninjured WT mice (**Figure 16b**) and were co-cultured in Matrigel with Sca-1+ rMCs (CD31/CD45/EpCAM triple-negative mesenchymal cells and positive for Sca-1) obtained from aged mice (**Figure 16c**). The FACS analysis showed that tdTom+ cells overlapped ( $7.04\% \pm 1.04\%$ ) with mesenchymal cells in saline that increased slightly after Bleo injury (Bleo d14:  $12.44\% \pm 1.21\%$ , Bleo d30:  $12.8\% \pm 1.51\%$ , Bleo d60  $11.05\% \pm 1\%$ ) (**Figure 16d**).

To examine the functionality of these formed alveolar organoids, efficiency of the cultures and alveolar organoids size were compared between mesenchymal cells isolated from aged WT and saline-treated mice, which serve as controls for bleomycin-treated mice (**Figure 16e, f**). Both groups displayed comparable CFE (aged WT:  $1.42\% \pm 0.23\%$ ; sal:  $1.61\% \pm 0.08\%$ ) and diameters (aged WT:  $137 \mu\text{m} \pm 5.5$ ; sal:  $123 \mu\text{m} \pm 5.3$ ) (**Figure 16j, k**). In contrast, mesenchymal cells isolated from Bleo d14 mice exhibited significantly reduced niche activity compared to sal and WT controls (**Figure 16g, j**). However, the size of the alveolar organoids (**Figure 16k**), and the mesenchymal cells support for the maturation/ differentiation of AEC2s (SFTPC+) to AEC1s (AGER+) were not impacted between groups (**Figure 16l**). Of note, niche activity of Sca1+ rMCs was not reversed during the resolution phase of pulmonary fibrosis. In aged lungs, Sca1<sup>+</sup> rMCs failed to regain their supportive capacity and remained in a dysfunctional state, unable to sustain the self-renewal and differentiation of AEC2s in the alveolar organoid assay (**Figure 16h–j**). These findings demonstrate that both bleomycin injury and aging compromise mesenchymal niche functionality, thereby impairing alveolar organoid growth.



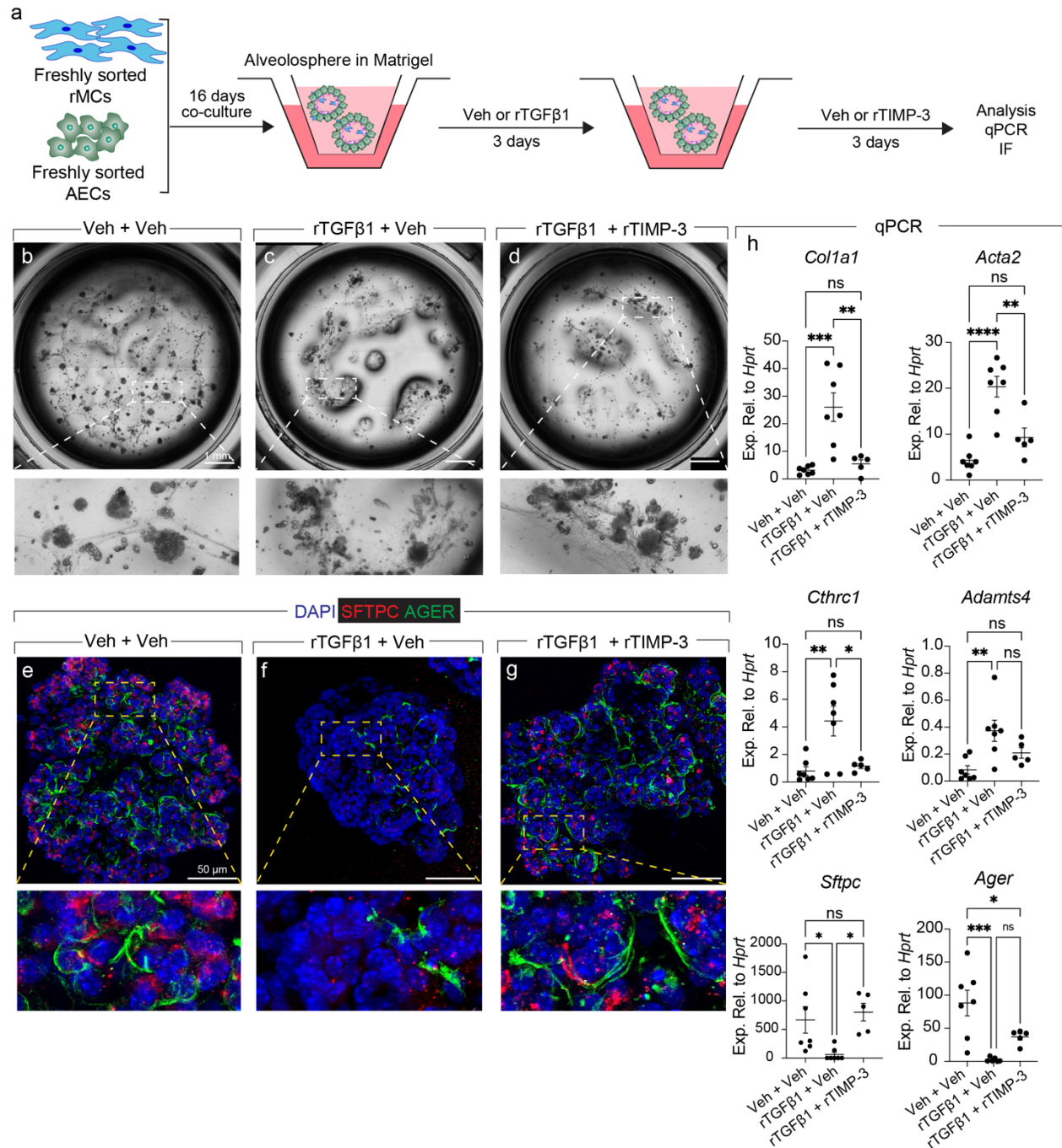


**Figure 16. Aged Sca-1<sup>+</sup> rMCs impaired AEC2 differentiation in alveolar organoids after bleomycin injury.** a) Experimental design for generating murine alveolar organoids by co-culturing sorted Sca-1<sup>+</sup> rMCs from old *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flax</sup> mouse and AEC2s from young uninjured WT mouse in Matrigel. b) Gating strategy for isolating EpCAM<sup>low</sup> population (enriched with AEC2s) from young WT mouse lungs. c) Gating strategy for isolating Sca-1<sup>+</sup> rMCs from aged *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flax</sup> mouse lungs. d) Quantification of tdTom<sup>+</sup> cells from mesenchymal population in aged *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>flax</sup> mice. Each dot represents one lung. n=4. e) Bright-field image of formed

alveolar organoids by mixing Sca-1+ rMCs from an uninjured one-year-old WT mouse and young AEC2s. f-i) Tile scan images of representative wells showing formed alveolar organoids from saline- and bleomycin-induced lungs. The dashed boxes are magnified in the lower panels. j, k) Quantification of CFE and size ( $\mu\text{m}$ ) of alveolar organoids. Each dot represents one culture well. n=3; n=5 for Bleo d60. l) Confocal images of alveolar organoids stained for AGER and SFTPC. Scale bars: 50  $\mu\text{m}$  (e-i) 1 mm; (l) 50  $\mu\text{m}$ ; ns: not significant; rMCs: resident mesenchymal cells; AEC2s: Alveolar Epithelial cells type 2; CFE: Colony-Forming Efficiency; WT: wild-type. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ .

#### 4.2.6 Inhibition of *ADAMTS4* attenuates fibrogenesis ex vivo

To show a proof of concept that ADAMTS4 inhibition promotes fibrosis resolution, we carried out alveolar organoid assays by co-culturing WT AECs with Sca-1+ rMCs from young WT mice (**Figure 17a**). Following sixteen days of culture, organoids were treated with recombinant rTGF $\beta$ 1 or vehicle (Veh) for three days followed by treatment with Veh or recombinant tissue inhibitor of metalloproteinase 3 (rTIMP-3) for three days (**Figure 17b-d**). TIMP-3 is a known inhibitor of ADAMTS4 activity (Masahide Kashiwagi et al., 2001; Wayne et al., 2007) although it also targets other metalloproteinases. IF and qPCR analysis showed that rTIMP3 treatment preserved organoid morphology with the presence of AEC2s (SFTPC+) and AEC1s (AGER+) cells (**Figure 17e-g**), attenuated *Col1a1*, *Acta2*, *Adamts4* and *Cthrc1* expression and restored the expression of *Sftpc* and *Ager* markers (**Figure 17h**).



**Figure 17. Inhibiting *ADAMTS4* mitigates fibrosis induction in murine alveolar organoids.** a) Schematic of the experimental design. Alveolar organoids are generated by mixing rMCs and AECs. The formed alveolar organoids are treated with 5ng/mL rTGFβ1 for three days to induce fibrosis, followed by rTIMP-3 treatment for three days. b-d) Tile scans of representative wells from different experimental groups. The dashed boxes are magnified in the lower panels. Each dot represents one culture well. Scale bars

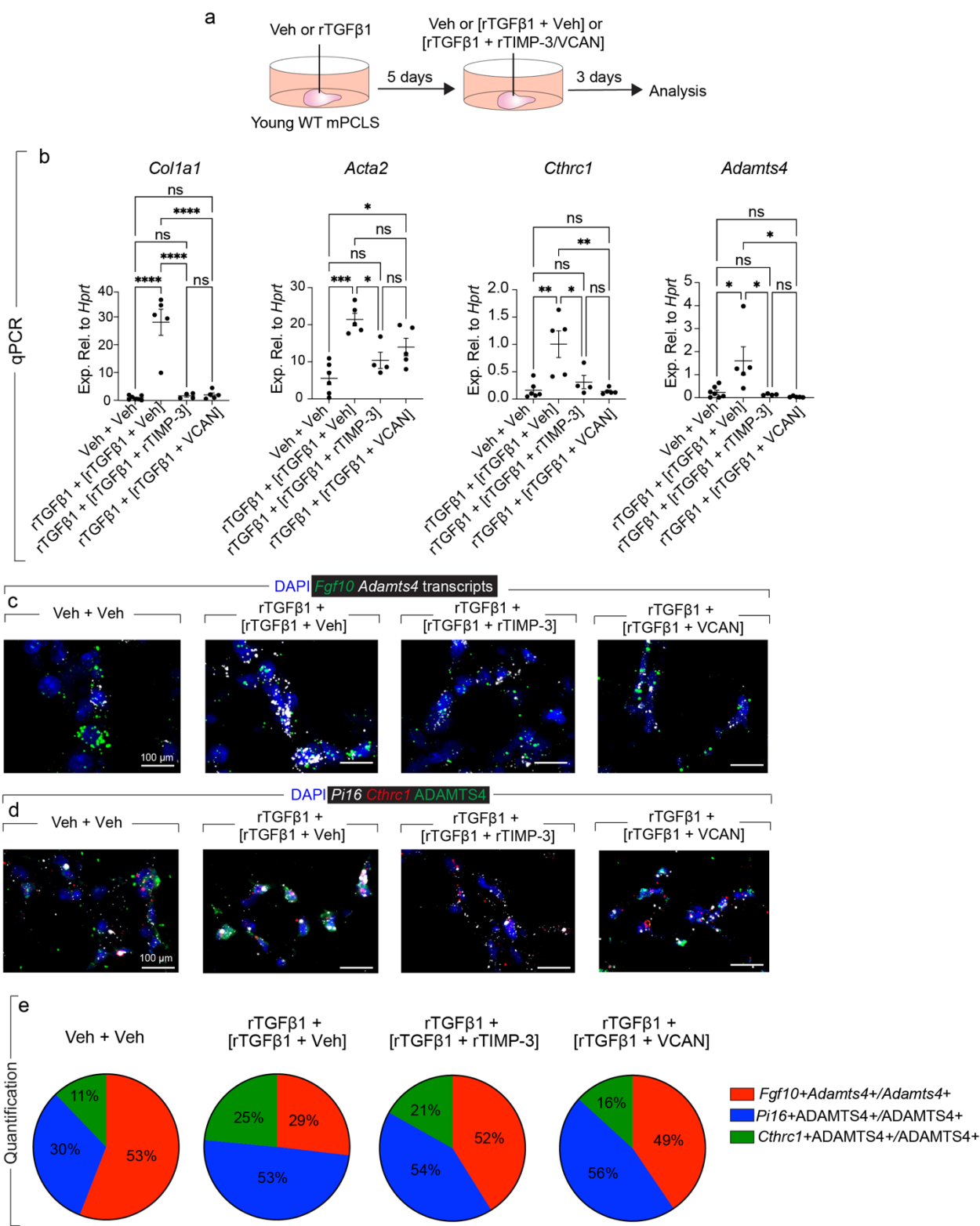
1 mm. e-g) Representative confocal images of alveolar organoids stained for AGER (in green) and SFTPC (in red). Scale bars 50  $\mu$ m. h) Quantitative PCR analysis for *Col1a1*, *Acta2*, *Cthrc1*, *Adamts4*, *Sftpc* and *Ager*. n = 7 for Veh + Veh; n = 7 for rTGF $\beta$ 1 + Veh; n = 5 for rTGF $\beta$ 1 + rTIMP-3. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons for more than two groups. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ; ns: not significant. AECs: Alveolar epithelial cells; rMCs: Resident mesenchymal cells; rTIMP-3: recombinant tissue inhibitor of metalloproteinases; rTGF $\beta$ 1: recombinant transforming growth factor beta 1; Veh: Vehicle; WT: wild-type.

#### 4.2.7 ADAMTS4 as a potential therapeutic target in lung fibrosis

As another layer of validation, PCLS from WT mice were generated and subjected to vehicle or rTGF $\beta$ 1 treatment for five days. Since fibrogenesis is reversible in the PCLS model if rTGF $\beta$ 1 is removed from the culture medium, PCLS were further treated with rTGF $\beta$ 1 in the presence of vehicle or rTIMP-3 or Versican (VCAN) (**Figure 18a**). Consistent with the observations in the organoid experiments, rTIMP3 and VCAN treatment significantly attenuated rTGF $\beta$ 1-induced fibrogenesis as evidenced by the downregulation of key profibrotic markers, including *Col1a1*, *Acta2*, *Cthrc1*, and *Adamts4* expression (**Figure 18b**). Moreover, RNAscope analysis of *Fgf10*, *Pi16* and *Cthrc1*, combined with *Adamts4* mRNA or protein staining, revealed that under homeostatic conditions, *Adamts4* is predominantly expressed in alveolar (*Fgf10*+) and adventitial (*Pi16*+) fibroblasts (AFs). Following TGF $\beta$ 1 stimulation and fibrosis induction, the number of *Fgf10* + fibroblast cells was decreased, but *Adamts4* expression was still enriched in both *Fgf10* + and *Pi16* + fibroblast subsets, indicating a shift toward a profibrotic phenotype. Notably, treatment with rTIMP-3 or VCAN attenuated this profibrotic transition by reducing *Adamts4* + cells and partially restoring the *Fgf10* + fibroblast population (**Figure 18 c-d**).

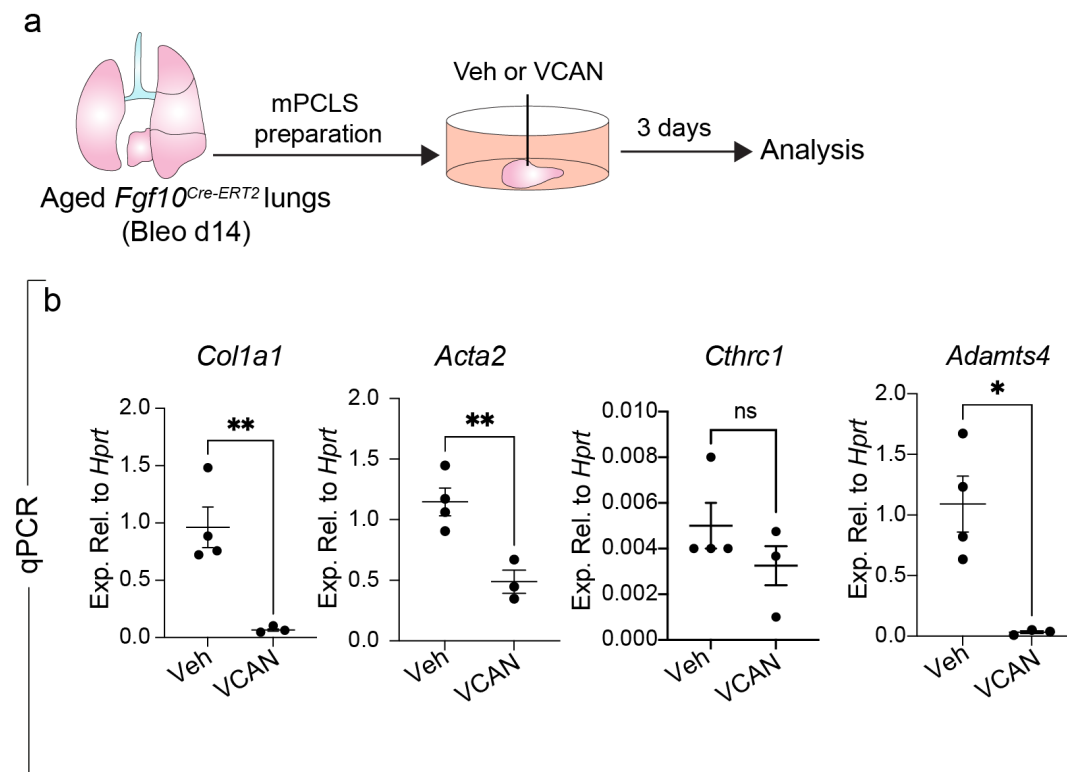
In another set of experiments, PCLS from aged Bleo d14 *Fgf10*<sup>Cre-ERT2/+</sup>; *tdTomato*<sup>fllox</sup> mice were cultured ex vivo for three days while being treated with VCAN or Veh, and similar

results were obtained as with the rTIMP3 experiments where the expression of myogenic genes and *Adamts4* decreased (**Figure 19a, b**).





**Figure 18. ADAMTS4 inhibition attenuated fibrogenesis in murine PCLS.** a) Experimental design. b) Quantitative PCR analysis for indicated genes (n = 4 for Veh; n = 3 for VCAN). c) Representative in situ hybridization for *Fgf10* mRNA (in green) and *Adamts4* mRNA (in grey). d) Representative in situ hybridization for *Pi16* (in grey), *Cthrc1* mRNA (in red) combined with IF staining for ADAMTS4 (in green). e) Quantification of RNAscope and IF shown in c-d. Scale bars 100  $\mu$ m and 200 $\mu$ m. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons for more than two groups. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ; ns: not significant. mPCLS: Mouse precision-cut lung slices; rTIMP-3: recombinant tissue inhibitor of metalloproteinases; rTGF $\beta$ 1: recombinant transforming growth factor beta 1; VCAN: Versican; Veh: Vehicle; WT: wild-type.



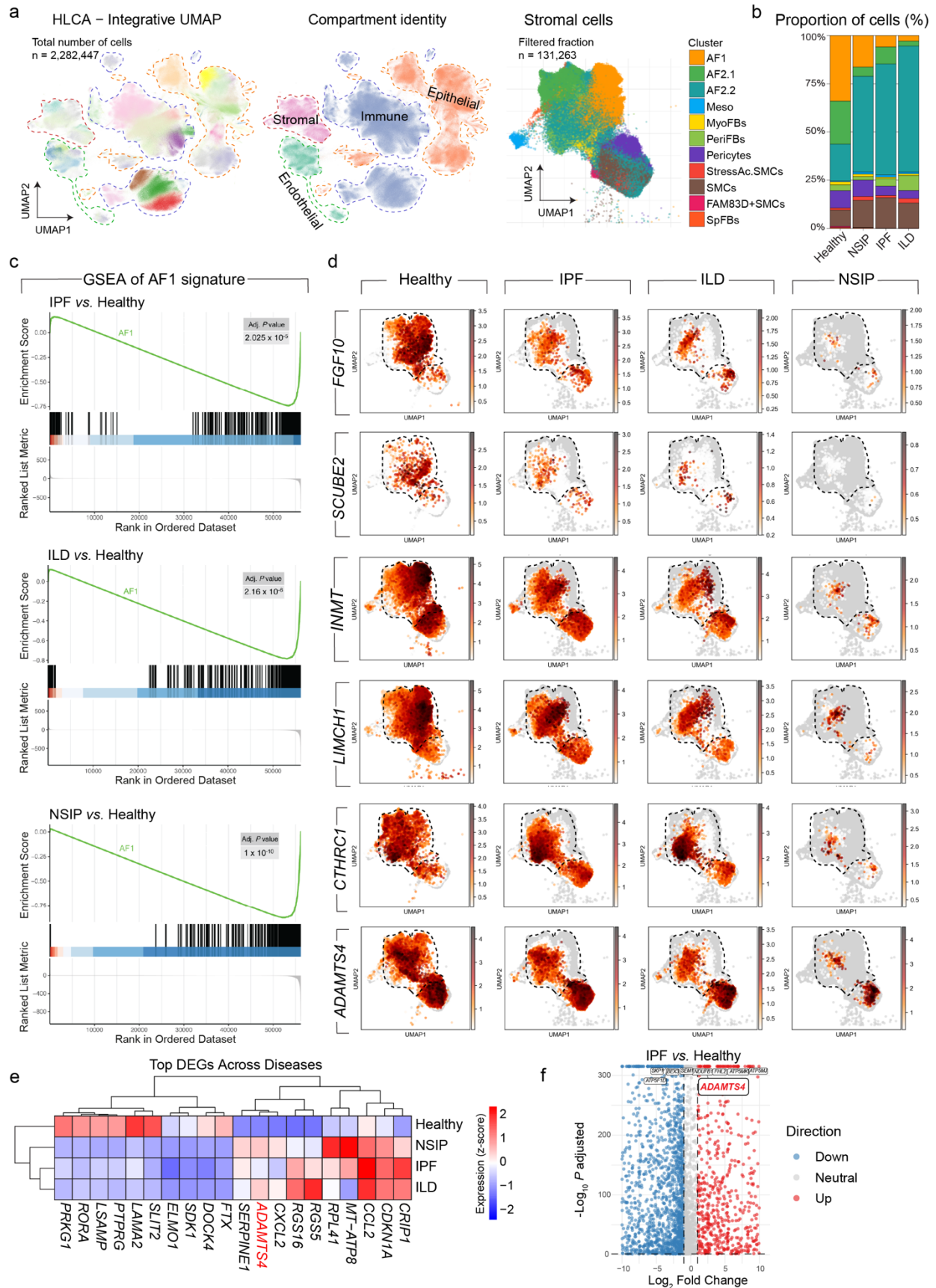
**Figure 19. VCAN modulates cultured mPCLS derived from 14-day bleomycin-treated lungs**

a) Experimental design. b) Quantitative PCR analysis for indicated genes (n = 4 for Veh; n = 3 for VCAN). Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using student's t test (unpaired, two-tailed). \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; ns: not significant. VCAN: Versican; Veh: Vehicle; WT: wild-type; Bleo: Bleomycin; *Col1a1*: Collagen Type I

Alpha 1 Chain; *Cthrc1*: Collagen Triple Helix Repeat Containing 1; *Acta2*: Actin Alpha 2, Smooth Muscle; *Adamts4*: ADAM metalloproteinase with thrombospondin type 1 motif 4.

#### 4.2.8 Accumulation of *ADAMTS4*<sup>+</sup> cells in human lung fibrosis

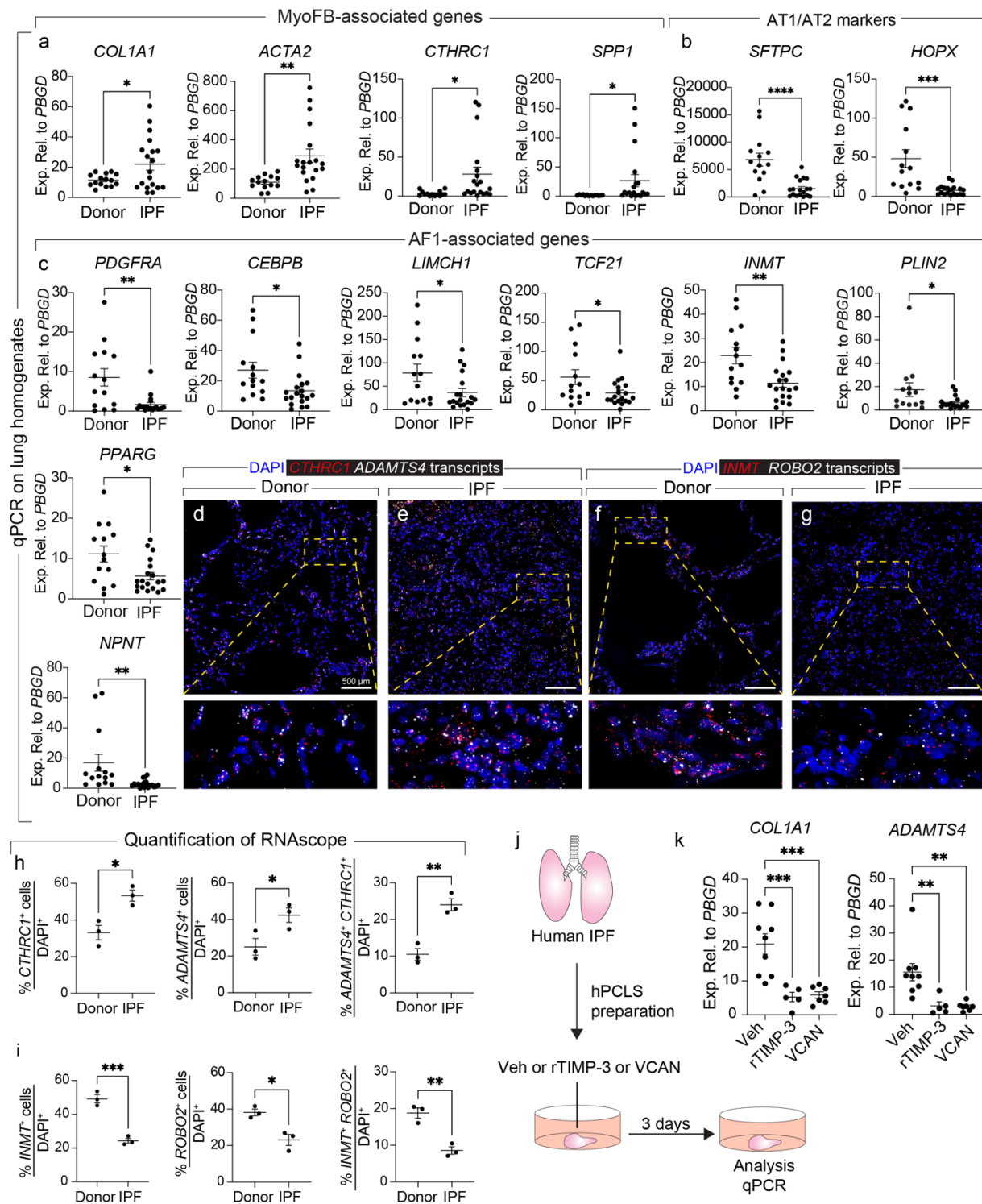
To validate our findings in the human context, we datamined the human lung cell atlas (HLCA) by filtering for the stromal fraction in healthy, IPF, ILD and non-specific interstitial pneumonia (NSIP) while preserving the original cluster annotation (**Figure 20a**). Of note, cluster frequency analysis revealed significant loss of the AF1 cluster in IPF, ILD and NSIP compared to the healthy state (**Figure 20b**). We also carried out GSEA and the results showed significant downregulation of the *AF1* signature in all fibrotic disease states (**Figure 20c**). UMAP plots also showed downregulation of AF1 markers *FGF10*, *SCUBE2*, *INMT* and *LIMCH1* in parallel to upregulation of *CTHRC1* and *ADAMTS4* (**Figure 20d**). Importantly, interrogating the top 10 DEGs in an unbiased manner revealed that *ADAMTS4* is among the top genes that are upregulated in NSIP, IPF and ILD compared the healthy state (**Figure 20e**). We also generated a volcano plot comparing IPF versus healthy, and *ADAMTS4* was among the top upregulated genes in IPF (**Figure 20f**). These data indicate that our findings using mouse-derived samples potentially possess strong clinical relevance in humans with pulmonary fibrosis.





**Figure 20. Increased expression of *ADAMTS4* in human fibrotic disease lungs.** a) Integrative UMAP of healthy, IPF, ILD and NSIP from HLCA by filtering the stromal fraction. b) Frequency of clusters in each condition. c) GSEA analysis of AF1 markers in IPF, ILD and NSIP samples versus healthy. d) UMAP plots of *FGF10*, *SCUBE2*, *INMT*, *LIMCH1*, *CTHRC1* and *ADAMTS4* genes in healthy, IPF, ILD and SNIP samples. e) Heatmap of top DEGs across diseases. f) Volcano plot showing top upregulated genes in IPF versus healthy. ILD: Interstitial lung disease; IPF: Idiopathic pulmonary fibrosis; NSIP: Non-specific interstitial pneumonia.

We also obtained lung explant tissues from IPF and non-IPF donors and carried out qPCR. The results showed significant upregulation of the MyoFB-associated genes *COL1A1*, *ACTA2*, *CTHRC1*, and *SPP1* and downregulation of *SFTPC* and *HOPX* (as expected), but also significant downregulation of AF1-associated genes *PDGFRA*, *CEBPB*, *LIMCH1*, *TCF21*, *INMT*, *PLIN2*, *PPARG*, and *NPNT* (**Figure 21a-c**). We also carried out RNAscope for *CTHRC1* and *ADAMTS4* on donor and IPF lung tissues and the data showed an increase in the abundance of *CTHRC1*<sup>+</sup>, *ADAMTS4*<sup>+</sup> and double-positive cells in IPF lungs in parallel to a decrease in the abundance of cells expressing the AF1 markers *INMT* and *ROBO2* (**Figure 21d-i**). Finally, we generated PCLS from fresh IPF lung explants and treated them with rTIMP-3 or VCAN for three days to functionally validate our findings regarding *ADAMTS4* (**Figure 21j**). Gene expression analysis revealed a significant downregulation of *COL1A1* and *ADAMTS4* expression upon treatment (**Figure 21k**). Collectively, the data suggest that *ADAMTS4* represents a potential therapeutic target in human lung fibrosis.



**Figure 21. Characterization of lipogenic and myogenic markers in human lungs.** a-c) Quantitative PCR analysis of lung homogenates from donor and IPF lung samples. n = 14 for donors and n = 19 for IPF. d, e) Representative in situ hybridization for *CTHRC1* (in red) and *ADAMTS4* mRNA (in grey) in donor and IPF samples. The dashed yellow

boxes are magnified in the lower panels. Scale bars 500  $\mu\text{m}$ . f, g) Representative in situ hybridization for *INMT* (in red) and *ROBO2* mRNA (in grey) in donor and IPF samples. The dashed yellow boxes are magnified in the lower panels. Scale bars 500  $\mu\text{m}$ . h, i) Quantification of cell populations.  $n = 3$  per group. j) Human IPF PCLS were treated with rTIMP-3, VCAN or vehicle for 3 days. k) qPCR analysis on freshly IPF hPCLS showing downregulation of *COL1A1* and *ADAMTS4* after rTIMP-3 or VCAN treatment ( $n = 9$  for Veh;  $n = 5$  for rTIMP-3;  $n = 7$  for VCAN). Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using student's t test (unpaired, two-tailed) or one-way ANOVA with multiple comparisons for more than two groups. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ; hPCLS: Human precision-cut lung slices; IPF: idiopathic pulmonary fibrosis; rTIMP-3: recombinant tissue inhibitor of metalloproteinases; VCAN: Versican; Veh: Vehicle.

## 5. Discussion

### 5.1 PART I. Generation of alveolar organoids from wild-type mice

Over the past decade, lung organoids have emerged as important models to investigate morphogenic and regenerative aspects of lung biology and disease such as assessing epithelial stem/progenitor cell self-renewal, self-organization, and differentiation, as well as the supportive potential (or niche activity) of corresponding mesenchymal cells. These organoid systems mimic various anatomical regions of the mammalian lung and are therefore dubbed tracheospheres, bronchiolospheres, bronchioalveolar organoids, and alveolar organoids (Evans and Lee, 2020; Liberti and Morrissey, 2021; van der Vaart and Clevers, 2021; Vazquez-Armendariz et al., 2022; Vazquez-Armendariz and Herold, 2021). As with any culture assay, there is a strong need for standardization of the operating procedure to maximize data quality, robustness, and reproducibility (Zabihi et al., 2024).

One of the pioneering studies in the field is that of McQualter et al., who demonstrated the ability of lung EpCAM<sup>+</sup> cells to form airway, alveolar, and mixed organoids when co-cultured with EpCAM<sup>-</sup> Sca-1<sup>+</sup> mesenchymal cells in Matrigel (McQualter et al., 2010). The generation of organoids from murine and human AEC2s was later described, where the co-culture of AEC2s with PDGFR $\alpha$ <sup>+</sup> mesenchymal cells or TASCs, a population that includes lipofibroblasts, led to the formation of alveolar organoids containing AEC2s and AEC1s (Barkauskas et al., 2013; Chung et al., 2018). Alveolar organoid cultures were also used to characterize the WNT-responsive fraction of AEC2s in the murine lung and investigate alveolar niche interactions. Such an AEC2 subset displayed superior alveolar organoid formation capacity and responds to WNT and FGF stimulation (Frank et al., 2016; Nabhan et al., 2018; Zacharias et al., 2018). Another AEC2 subset that acts as a quiescent repertoire for mature AEC2s is the IAAPs, which do not show robust alveolar organoid formation capacity but are important for replenishing the bona fide AEC2 pool (Ahmadvand et al., 2022, 2021; Chong et al., 2023; Lv et al., 2022).

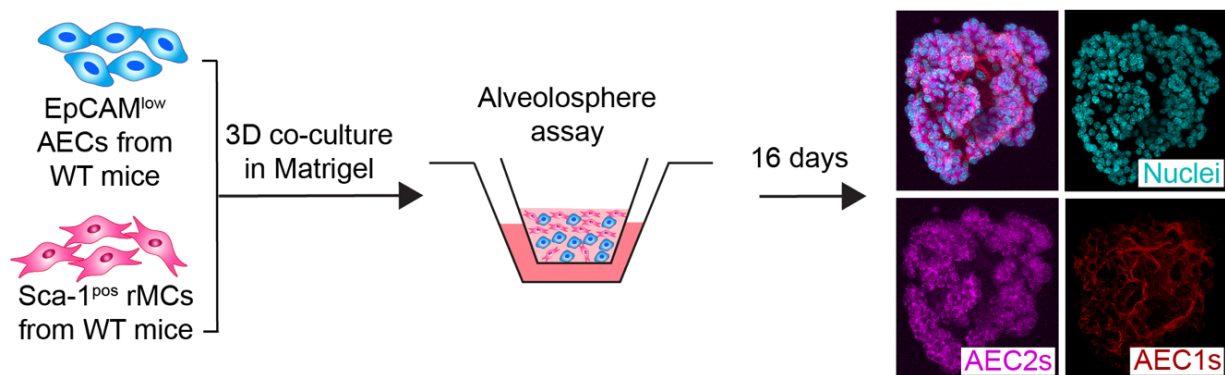
WNT and FGF signaling have been established as critical determinants of the AEC2 cell fate by independent studies (Ahmadvand et al., 2022; Brownfield et al., 2022; Hein et al.,

2022; Lee et al., 2017; Liberti et al., 2021; Yuan et al., 2019). The mesenchymal niche can be fractionated into AXIN2<sup>+</sup> (a WNT-responsive population enriched with Fgf7 expression) and FGF10<sup>+</sup> subsets. Both fractions show lipofibroblast features including proximity to AEC2s and neutral lipid content (Taghizadeh et al., 2021; Zepp et al., 2017). How distinct these two subsets are, especially in terms of in vivo function during homeostasis, repair, and regeneration, remains to be seen. A third niche population is represented by WNT ligand-producing leucine-rich repeat-containing G protein-coupled receptor-5-positive (LGR5<sup>+</sup>) fibroblasts that have shown the ability to support alveolar organoid formation (Lee et al., 2017). In our study, the bulk rMC population was used to achieve a simple and universal protocol to generate alveolar organoids from WT mice without the need for transgenic animals or discriminating between rMC subsets. **Table 3** highlights the main features of some of the published protocols to generate murine alveolar organoids (Zabihi et al., 2024). The emerging picture is that (1) most of the studies listed relied on the use of transgenic mice to generate alveolar organoids, (2) a starting material of 5000 AEC2s combined with mesenchymal cells at a ratio of 1:10 and later mixed with Matrigel (1:1) is the most used approach, and (3) the data show varying CFE values even when using similar protocols (Zabihi et al., 2024).

The effect of IL-1 $\beta$  on alveolar organoid formation using this WT approach was also evaluated. The accumulation of KRT8<sup>+</sup> transitional cells was evident based on whole-mount immunofluorescence data. The involvement of these and other transitional AEC2 populations in various respiratory diseases has gained tremendous interest in recent years (Kobayashi et al., 2020; Strunz et al., 2020) and further research is warranted to fully understand this phenomenon, decipher how to convert such dysplastic events into healthy scarless regeneration, and ultimately reconstitute the alveolar stem cell niche and restore optimal gas exchange in the diseased lung (Zabihi et al., 2024).

The proposed protocol thus allows robust and reproducible generation of murine alveolar organoids using the EpCAM<sup>low</sup> gating strategy, which represents a refinement of previous EpCAM gating strategies and avoids lumping EpCAM<sup>high</sup> with EpCAM<sup>low</sup> cells. As club cells and BASCs are not found in the EpCAM<sup>low</sup> fraction (Quantius et al., 2016; Vazquez-

Armendariz et al., 2020), our approach minimizes contamination with bronchiolar and bronchioalveolar organoids, allowing to predominantly obtain alveolar organoids. We believe that this protocol represents a step toward the standardization of this important research tool (**Figure 22**). Transcriptomic and functional analyses revealed enrichment of pathways involved in surfactant metabolism, epithelial regeneration, and ECM organization, reflecting a physiologically relevant alveolar environment. Overall, the optimized WT alveolar organoids system provides a standardized and accessible platform for studying alveolar development/regeneration, drug screening, epithelial–mesenchymal interactions, and lung disease mechanisms.



**Figure 22. Schematic figure for alveolar organoid formation from WT mice.** Murine alveolar organoids are generated by co-culturing Sca-1<sup>+</sup> rMCs and EpCAM<sup>low</sup> AECs population. The formed alveolar organoid after 16 days was used for IF staining to show the presence of SFTPC (AEC2s) and AGER (AEC1s) positive cells within the alveolar organoids.

**Table 3. Comparison between various murine alveolar organoid protocols**

| Study                   | Mouse strain                                                        | Epithelial cells                             | Mesenchymal cells                                      | Epi: Mes ratio | Set up                 | CFE        | Research question                |
|-------------------------|---------------------------------------------------------------------|----------------------------------------------|--------------------------------------------------------|----------------|------------------------|------------|----------------------------------|
| Barkauskas et al., 2013 | <i>Sftpc<sup>C</sup><sub>re-ERT2</sub>; tdTomato<sup>flox</sup></i> | 5 x 10 <sup>3</sup> SFTPC <sup>+</sup> cells | 1 x 10 <sup>5</sup> PDGFRα <sup>high</sup> cells (also | 1:20           | ALI in Matrigel (mixed | 2.3 ± 0.3% | Self-renewal and differentiation |

|                    |                                                                                                                                                                                             |                                                       |                                                             |      |                                                                                  |                                                                                                                                  |                                                                                         |
|--------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|-------------------------------------------------------------|------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
|                    | and<br><i>Pdgfr</i><br>$\alpha^{H2B-}$<br><i>GFP</i>                                                                                                                                        |                                                       | shown to be<br>LipidTOX+)                                   |      | 1:1) for<br>16-17<br>days                                                        |                                                                                                                                  | tion of<br>AEC2s                                                                        |
| Frank et al., 2016 | <i>Sftpc</i> <sup>C</sup><br><i>re-ERT2</i> ;<br><i>EYFP</i> <sup>f</sup><br><i>lox</i> and<br><i>Axin2</i><br><i>Cre-</i><br><i>ERT2</i> ;<br><i>tdTom</i><br><i>ato</i> <sup>flox</sup>   | 5 x 10 <sup>3</sup><br>EYFP+<br>or<br>tdTom+<br>cells | 5 x 10 <sup>4</sup><br>primary<br>adult lung<br>fibroblasts | 1:10 | ALI in<br>Matrigel<br>(mixed<br>1:1<br>with<br>MTEC-<br>SAGM<br>) for 14<br>days | ~1.7<br>%<br>with<br>total<br>AEC2<br>s<br>versu<br>s<br>~2.6<br>%<br>with<br>AXIN<br>2+<br>AEC2<br>s<br>before<br>passa<br>ging | Comparis<br>on<br>between<br>bulk and<br>WNT-<br>responsiv<br>e AEC2s.                  |
| Lee et al., 2017   | <i>Sftpc</i> <sup>C</sup><br><i>re-ERT2</i> ;<br><i>tdTom</i><br><i>ato</i> <sup>flox</sup> ,<br><i>Lgr5</i> <sup>Cr</sup><br><i>e-ERT2</i> ;<br><i>tdTom</i><br><i>ato</i> <sup>flox</sup> | 0.5-1 x<br>10 <sup>4</sup><br>SFTPC+<br>cells         | 0.5-1 x 10 <sup>5</sup><br>LGR5+ or<br>LGR6+ cells          | 1:10 | ALI in<br>Matrigel<br>(mixed<br>1:1) for<br>14<br>days                           | ~4.8<br>%<br>with<br>LGR5<br>+ cells<br>versu<br>s<br>~2.5<br>%                                                                  | Comparis<br>on<br>between<br>LGR5+<br>and<br>LGR6+<br>cells in<br>terms of<br>supportin |

|                   |                                                                                                                                                                                          |                                                  |                                        |      |                                                        |                                                                                      |                                                                                                                                                                     |
|-------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------|----------------------------------------|------|--------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                   | and<br><i>Lgr6<sup>E</sup></i><br><i>GFP-</i><br><i>Cre-</i><br><i>ERT2.</i> ;<br><i>tdTom</i><br><i>ato<sup>fllox</sup></i>                                                             |                                                  |                                        |      |                                                        | with<br>LGR6<br>+ cells<br>before<br>passa<br>ging                                   | g<br>bronchial,<br>alveolar,<br>and<br>bronchioa<br>lveolar<br>organoids                                                                                            |
| Lee et al., 2017  | <i>Scgb1a1<sup>Cre-</sup></i><br><i>ERT.</i> ;<br><i>YFP<sup>fllo</sup></i><br><i>x</i> and<br><i>Lgr5<sup>Cre</sup></i><br><i>re-ERT2.</i> ;<br><i>EYFP<sup>f</sup></i><br><i>lox *</i> | 0.5-1 x 10 <sup>4</sup><br>SCGB1<br>A1+<br>cells | 0.5-1 x 10 <sup>5</sup><br>LGR5+ cells | 1:10 | ALI in<br>Matrigel<br>(mixed<br>1:1) for<br>14<br>days | ~2.6<br>%<br>with<br>most<br>organ<br>oids<br>being<br>alveol<br>ar<br>organ<br>oids | Comparis<br>on<br>between<br>LGR5+<br>and<br>LGR6+<br>cells in<br>terms of<br>supportin<br>g<br>bronchial,<br>alveolar,<br>and<br>bronchioa<br>lveolar<br>organoids |
| Zepp et al., 2017 | <i>Sftpc<sup>C</sup></i><br><i>re-ERT2.</i> ;<br><i>EYFP<sup>f</sup></i><br><i>lox</i> ;<br><i>Axin2</i><br><i>Cre-</i><br><i>ERT2.</i> ;<br><i>EYFP<sup>f</sup></i>                     | 5 x 10 <sup>3</sup><br>SFTPC+<br>cells           | 5 x 10 <sup>4</sup>                    | 1:10 | ALI in<br>Matrigel<br>(mixed<br>1:1) for<br>21<br>days | Betwe<br>en<br>~1.2<br>% and<br>~6.2<br>%                                            | Comparis<br>on<br>between<br>the<br>alveolar<br>organoid-<br>supportiv<br>e ability of                                                                              |



|                          |                                                                                                                                                           |                                        |                                   |      |                                                            |                                                                                                      |                                                                                                                                       |
|--------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|-----------------------------------|------|------------------------------------------------------------|------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
|                          | <i>lox</i> ,<br><i>Wnt2<sup>C</sup></i><br><i>re-ERT2</i> ;<br><i>EYFP<sup>f</sup></i><br><i>lox</i> and<br><i>Pdgfr</i><br>$\alpha^{H2B-}$<br><i>GFP</i> |                                        |                                   |      |                                                            |                                                                                                      | various<br>mesench<br>ymal<br>subsets                                                                                                 |
| Chung<br>et al.,<br>2018 | <i>Sftpc<sup>C</sup></i><br><i>re-ERT2</i> ;<br><i>tdTom</i><br><i>ato<sup>flox</sup></i><br>and<br><i>Pdgfr</i><br>$\alpha^{H2B-}$<br><i>GFP</i>         | 5 x 10 <sup>3</sup><br>SFTPC+<br>cells | 5 x 10 <sup>4</sup>               | 1:10 | ALI in<br>Matrig<br>el<br>(mixed<br>1:1) for<br>14<br>days | Up to<br>~9%                                                                                         | Investigat<br>ing<br>BMP/SM<br>AD<br>signaling<br>in AEC2<br>renewal<br>and<br>differentia<br>tion                                    |
| Choi et<br>al., 2020     | <i>Sftpc<sup>C</sup></i><br><i>re-ERT2</i> ;<br><i>tdTom</i><br><i>ato<sup>flox</sup></i>                                                                 | 5 x 10 <sup>3</sup><br>SFTPC+<br>cells | Cultured<br>lung stromal<br>cells | 1:5  | ALI in<br>Matrig<br>el<br>(mixed<br>1:1) for<br>14<br>days | Betwe<br>en<br>~1.6<br>%<br>(witho<br>ut IL-<br>1 $\beta$ )<br>and<br>~3%<br>(with<br>IL-1 $\beta$ ) | Investigat<br>e the<br>effect of<br>IL-1 $\beta$<br>stimulatio<br>n on<br>AEC2<br>self-<br>renewal,<br>transition<br>al state,<br>and |

|                               |                                                                                             |                                                                                                                                                                                                                                                                         |                                                                                                                      |      |                                                              |                                                                                         |                                                                                 |
|-------------------------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|------|--------------------------------------------------------------|-----------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
|                               |                                                                                             |                                                                                                                                                                                                                                                                         |                                                                                                                      |      |                                                              |                                                                                         | differentia<br>tion                                                             |
| Ahmadv<br>and et<br>al., 2021 | <i>Sftpc</i> <sup>C</sup><br><i>re-ERT2</i> ;<br><i>tdTom</i><br><i>ato</i> <sup>flox</sup> | 1 x 10 <sup>3</sup><br>SFTPC+<br>cells                                                                                                                                                                                                                                  | 2 x 10 <sup>4</sup> rMCs<br>(EpCAM-<br>CD31-<br>CD45- Sca-<br>1+) from<br>C57BL/6<br>lungs                           | 1:20 | ALI in<br>Matrig<br>el<br>(mixed<br>1:1) for<br>14<br>days   | ~1.2<br>%                                                                               | Assess<br>the ability<br>of AEC2<br>subsets<br>to form<br>alveolar<br>organoids |
| Chanda<br>et al.,<br>2021     | C57B<br>L/6                                                                                 | 5 x 10 <sup>3</sup><br>AEC2<br>isolated<br><i>via</i><br><i>enzymat</i><br><i>ic</i><br><i>digestio</i><br><i>n</i><br>(Dispase<br>II), cell-<br>specific<br>antibody<br>labeling<br>(biotinyl<br>ated Ter-<br>119,<br>CD104,<br>CD16/32<br>,<br>CD45,<br>CD31),<br>and | 1 x 10 <sup>5</sup> lung<br>mesenchym<br>al stromal<br>cells (L-<br>MSCs)<br>isolated by<br>differential<br>adhesion | 1:20 | ALI in<br>Matrig<br>el<br>(mixed<br>1:1) for<br>9-12<br>days | Not<br>directl<br>y<br>stated<br>by<br>can<br>be<br>estim<br>ated<br>to be<br>~0.6<br>% | Assess<br>the effect<br>of aging<br>on<br>alveolar<br>organoid<br>formation     |

|                         |                                                                                                                                                                                    |                                                     |                                                                                                                  |      |                                                                   |                         |                                                                       |
|-------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------|------------------------------------------------------------------------------------------------------------------|------|-------------------------------------------------------------------|-------------------------|-----------------------------------------------------------------------|
|                         |                                                                                                                                                                                    | <i>magnetic separation (Anti-Biotin MicroBeads)</i> |                                                                                                                  |      |                                                                   |                         |                                                                       |
| Liberti et al., 2021    | <i>Sftpc<sup>C</sup><sub>re-ERT2</sub>; tdTomato<sup>flox</sup></i> and <i>Sftpc<sup>C</sup><sub>re-ERT2</sub>; Fgfr2<sup>b<sup>flox/flox</sup></sup>; tdTomato<sup>flox</sup></i> | 5 x 10 <sup>3</sup> SFTPC+ cells                    | 5 x 10 <sup>4</sup> primary adult lung fibroblasts                                                               | 1:10 | ALI in 1:1 mixture of MTEC-SAGM: Matrigel (mixed 1:1) for 14 days | Between ~9% and ~11%    | Assessing the role of FGFR2b signaling on alveolar organoid formation |
| Taghizadeh et al., 2021 | <i>Sftpc<sup>C</sup><sub>re-ERT2</sub>; tdTomato<sup>flox</sup></i> , Fgf10 <sup>-lacZ</sup> , ob/ob and C57BL/6                                                                   | 5 x 10 <sup>3</sup> EpCAM + Lysotracker+ tdTom+     | 5 x 10 <sup>4</sup> rMCs (EpCAM-CD31-CD45- Sca-1+) or EpCAM-CD31-CD45- Sca-1- cells; LipidTOX+ rMCs or LipidTOX- | 1:10 | ALI in Matrigel (mixed 1:1) for 14 days                           | Between ~2.2% and ~6.2% | Comparison of niche activity between various mesenchymal subsets      |

|  |  |  |                                                                                    |  |  |  |  |
|--|--|--|------------------------------------------------------------------------------------|--|--|--|--|
|  |  |  | rMCs;<br>FGF10+<br>rMCs/FGF1<br>0- rMCs;<br>ob/ob rMCs<br>from<br>C57BL/6<br>lungs |  |  |  |  |
|--|--|--|------------------------------------------------------------------------------------|--|--|--|--|

## 5.2 PART II. Investigating the origin and fate of myofibroblasts in bleomycin-induced lung fibrosis in aged mice

### 5.2.1 Mesenchymal progenitors expressing FGF10 as the source of LIF

Ever since lipid-droplet-containing fibroblasts were reported in the alveolar interstitium of neonatal rat lungs (Vaccaro and Brody, 1978), there has been controversy regarding the function or even existence of these elusive cells, which were later dubbed “LIFs”. Apart from their involvement in assisting AEC2s with pulmonary surfactant production, LIFs are widely regarded as niche cells for AEC2s (Barkauskas et al., 2013) as they produce important growth factors such as FGF10 that is important for AEC2s self-renewal and maintenance (Brownfield et al., 2022; Liberti et al., 2021). Of note, while LIFs were sometimes claimed to only exist in rodent lungs (Tahedl et al., 2014), their presence was later confirmed in the human lung (Schipke et al., 2021). Despite that, the term “LIFs” is still often ignored in single-cell atlases of the healthy and fibrotic lung where the corresponding cells are generally assigned the AF label. Earlier work had demonstrated that fibroblast-derived radiolabeled lipids that are encapsulated by PLIN2 are taken up by AEC2s (Schultz et al., 2002). Due to their strong expression of *Plin2*, the latter had been used as a marker of LIFs. We previously demonstrated that LIFs are a source of MyoFBs in the fibrotic lungs of young mice (El Agha et al., 2017). In that study, we lineage-traced *Plin2*<sup>+</sup> cells in the context of bleomycin-induced pulmonary fibrosis and subsequent resolution. However, the advent of single-cell transcriptomics allowed the identification of more reliable markers of LIFs thus enabling to carry out lineage tracing and the

downstream analysis in a more precise manner. *FGF10* is a reliable marker of AF1s as evident from single-cell atlases and multiple other studies, and the *LIF* signature is enriched in the AF1 fraction of AFs.

Fibroblast growth factor 10 has a critical role during lung development, maintaining homeostasis, mediating injury repair and regulating branching morphogenesis (Jones et al., 2020; Yuan et al., 2018). FGF10 dysregulation has been reported in IPF and is inversely correlated with disease progression. FGF10 is highly expressed in early-stage IPF to promote tissue repair, whereas its expression is reduced in end-stage disease (El Agha et al., 2017). On the other hand, overexpression of *Fgf10* attenuates lung fibrosis severity in bleomycin-treated mice (Gupte et al., 2009). Our group was the first to demonstrate, using lineage tracing, that *Fgf10* expressing cells constitute a pool of mesenchymal progenitors in the embryonic and postnatal lung (El Agha et al., 2014). During alveologenesis, *Fgf10*<sup>+</sup> cells give rise predominantly lipofibroblasts rather than alveolar myofibroblasts, and during adult life, they represent a subset of resident mesenchymal cells that may play a crucial role in regeneration following injury. Lineage tracing of *Fgf10*<sup>+</sup> cells during embryonic development further indicated that these cells serve as progenitors for lipofibroblast and vascular/airway smooth muscle cells (El Agha et al., 2014). In the current study, *Fgf10*<sup>+</sup> lineage-labeled cells represent a heterogeneous population that contributes to multiple mesenchymal lineages in the lung, including alveolar fibroblasts in the alveolar region, adventitial fibroblasts surrounding blood vessels, peribronchial fibroblasts in the bronchiolar region and airway/vascular smooth muscle cells during homeostasis and bleomycin injury. In our fibrosis model, bleomycin injury leads to a loss of *Fgf10*<sup>+</sup> lineage-labeled cells and their associated markers, including *Inmt*, *Robo2*, and *Tcf21*, indicating a transient loss of LIF characteristics during active fibrosis. During fibrosis resolution, both the number of AEC2s and the expression of LIF markers are partially restored, suggesting incomplete but ongoing regeneration of the alveolar niche.

### 5.2.2 Evidence of LIF-MyoFB-LIF reversible switch during fibrosis formation and resolution

Idiopathic pulmonary fibrosis is characterized by accumulation of MyoFBs and ECM components, which leads to severe distortion and scarring of the gas exchange units of the lung, the alveoli, and ultimately respiratory failure. Fibrosis-associated MyoFBs are therefore widely regarded as the culprits that compromise the architectural makeup of the lung in fibrotic disease. During the past decade, the cellular source of MyoFBs has been intensely investigated. The contribution of lung mesenchymal cell subsets to MyoFBs is still controversial. The rationale for such studies is that identifying the origin of these cells might help identify novel therapeutic targets and candidates to treat IPF. Recent advances in basic and translational research employing lineage tracing and multi-omics approaches have helped address the identity of MyoFBs precursors, highlight the underlying heterogeneity, and to a less extent investigate MyoFBs fate during fibrosis resolution. MyoFBs are involved in wound healing and activated cells at the site of the injury deposit ECM and regulates matrix remodeling and finally undergo apoptotic clearance and reconstitute normal tissue homeostasis. However, resistance of MyoFBs for apoptotic clearance can eventually lead to lung scarring (Zabihi et al., 2024)

In this study, we utilized our novel *Fgf10<sup>Cre-ERT2</sup>* knock-in line (Chu et al., 2021) to lineage-trace pre-existing *Fgf10*<sup>+</sup> cells in the context of pulmonary fibrosis. Our data clearly demonstrate that the *LIF<sup>high</sup>* fraction of *Fgf10*<sup>+</sup> cells contributes to MyoFBs formation during fibrosis development. These data are not only in line with our previous work using young mice (El Agha et al., 2017), but also with more recent literature implicating AFs as a major source of MyoFBs in the fibrotic lung (Fang et al., 2025; Tsukui et al., 2024). During resolution, MyoFBs undergo apoptotic clearance, deactivation or transitioning back to native state. The conversion of MyoFBs into AFs with a *LIF* signature is in line with our previously published data involving spontaneous or enhanced (via metformin treatment) resolution in young mice (El Agha et al., 2017; Kheirollahi et al., 2019) as well as other recent studies (Konkimalla et al., 2023; Mayr et al., 2024; Trempus et al., 2023). We also previously demonstrated downregulation of *PLIN2*, *CEBPA*, and *PPARG* in lung homogenates from IPF lung tissues compared to donors indicating reduced LIF

abundance/differentiation (El Agha et al., 2017). In the current work, we expanded the LIF gene panel to other marker genes shown to constitute a signature for LIFs according to single-cell atlases, and the data showed significant downregulation at the lung homogenate level as well as downregulation of the *LIF* signature in the stromal fraction of various fibrotic lung diseases contained in the HLCA.

Studies have shown that older mice are more susceptible than younger mice to pro-fibrotic stimuli such as bleomycin. Since bleomycin-induced pulmonary fibrosis spontaneously resolves in young mice while human IPF is progressive/irreversible and is aged-related disease, we utilized old mice in the current study to create a state of delayed fibrosis resolution that we believe better mimics the clinical setting. Altogether, our aged fibrosis mouse model and human-derived data strongly indicate that LIFs are indeed impaired in pulmonary fibrosis.

While scRNA-seq is a powerful tool for identifying distinct cellular subpopulations, our approach would be strengthened by analyzing multiple time points during fibrosis progression and resolution. In particular, it would be interesting to investigate the fate of *Adamts4*<sup>+</sup> AFs cells after late resolution (Bleo day 60) to determine whether they still persist in the lung tissue and contribute to ongoing damage or instead undergo apoptotic clearance. *Adamts4*<sup>+</sup> AFs population is associated with delayed fibrosis resolution in our aged mice. In the future, combining at least two different driver lines would be essential to improve labeling of LIFs that give rise to *Cthrc1*<sup>+</sup> MyoFBs and *Adamts4*<sup>+</sup> cells. For example, the *Fgf10* promoter could be used to drive Cre-ERT2 expression in combination with *Cthrc1*<sup>Dre-ERT2</sup> or *Adamts4*<sup>Dre-ERT2</sup> mice. Combining Cre and Dre recombinases with a suitable fluorescent reporter line would allow precise targeting *Cthrc1*<sup>+</sup> MyoFBs and *Adamts4*<sup>+</sup> AFs originating from *Fgf10*-lineage labeled cells for single-cell transcriptomic analysis during fibrosis and resolution.

### **5.2.3 ADAMTS4 inhibition mitigates fibrogenesis ex vivo**

During homeostasis, the balance between ECM synthesis and degradation is crucial for maintaining proper lung physiology and architecture. However, such ECM homeostasis

is disrupted during lung fibrosis. Matrix metalloproteinases (MMPs) play pivotal roles in regulating ECM turnover and fibrosis progression. MMPs are a family of zinc-dependent proteases responsible for collagen turnover. MMPs exhibit both pro-fibrotic (for e.g., MMP-1, MMP-7, and MMP-9) and anti-fibrotic (e.g., MMP-2 and MMP-3) activities. Elevated levels of MMP-1, MMP-7, and MMP-9 have been observed in the blood serum and lung tissue of IPF patients, with MMP-7 considered a potential diagnostic biomarker (Chuliá-Peris et al., 2022; Pardo et al., 2016; Rosas et al., 2008). ADAMTS4 is a member of ADAMTS family and is a metalloproteinase containing thrombospondin-like motifs. It is known to interact with ECM proteins and cleave proteoglycans such as aggrecan, VCAN, and brevican, in addition to contributing to collagen remodeling (Seals and Courtneidge, 2003). ADAMTS4 has been implicated in lethal immunopathology following influenza virus injury (Boyd et al., 2020) and a novel biomarker in adult cardiac injury (Khanam et al., 2022). Moreover, targeting ADAMTS4 inhibition by a small molecule improved heart function, reduced collagen content and prevent cardiac fibrosis (Vistnes et al., 2023). VCAN is a major substrate for ADAMTS4, and it is a component of the provisional matrix that facilitates recruitment of inflammatory cells such as leukocytes (Boyd et al., 2020; Wight, 2017). TIMP-3 is a broad-spectrum endogenous inhibitor that regulates multiple MMPs as well as members of the ADAM and ADAMTS families. Unlike other TIMPs, it is tightly bound to the extracellular matrix, allowing localized regulation of protease activity. Notably, TIMP-3 is considered one of the most potent inhibitors of ADAMTS4. (Hashimoto et al., 2001; M. Kashiwagi et al., 2001). *Timp-3* expression is significantly reduced during fibrosis formation in young mice but recovers during fibrosis resolution. *Timp-3* knockout mice exhibit persistent inflammation and fibrosis, resembling the pathology observed in aged mice. This effect is attributed to sustained MMP activity, increased neutrophil infiltration, and impaired anti-inflammatory responses. Interestingly, MMP-7 activity is elevated in *Timp3* knockout mice, while *Mmp7* knockout mice demonstrate impaired neutrophil migration and attenuated fibrosis after bleomycin injury (Gill et al., 2010; Zuo et al., 2002). Importantly, datamining of the HLCA revealed that *ADAMTS4* is among the top upregulated genes in IPF, ILD, and NSIP compared to the healthy state. These data strongly suggest that the relentless course of fibrogenesis seen in human pulmonary



fibrosis may be due to continued inflammation and remodeling mediated by ADAMTS4 and VCAN degradation.

In the future, it will be important to employ small molecule inhibitors to selectively inhibit *ADAMTS4* in the context of fibrogenesis and resolution using murine and humanized models of pulmonary fibrosis. Since TIMP-3 is known to inhibit multiple MMPs, it would be valuable to examine its potential role in modulating *Adamts4* expression by assessing ADAMTS4 metallopeptidase enzyme activity. Such analyses could provide insight into whether TIMP-3 contributes to ECM remodeling during fibrosis by directly influencing ADAMTS4-mediated proteolytic processes.

#### **5.2.4 Recent advances in therapeutic approaches for Idiopathic pulmonary fibrosis**

Current therapeutic strategies for IPF rely on the antifibrotic agents pirfenidone and nintedanib, both of which have been approved for clinical use. Although these treatments can delay disease progression, they fail to halt or reverse fibrosis. Nerandomilast (BI 1015550) is a recently FDA-approved oral PDE4B inhibitor for the treatment of IPF. This dual anti-inflammatory and antifibrotic drug can slow down disease progression and preserve lung function in IPF patients (Richeldi et al., 2022, 2025). Most of the therapeutic strategies under development shifted toward developing novel molecular targets and combination therapies and target profibrotic pathways such as connective tissue growth factor (CTGF), lysophosphatidic acid receptor (LPAR), TGF $\beta$ 1, Janus kinase 1/2/3 (JAK1/2/3), Rho associated coiled-coil containing protein kinase 2 (ROCK2), Src/Bcr-Abl, and others. **Table 4** summarizes some of these novel therapeutic candidates and targets (Zabihi et al., 2024).

**Table 4. Novel therapeutic approaches in IPF** (Modified from Zabihi et al., 2024)

| <b>Candidate</b>                         | <b>Mechanism of action</b>                                                                                     | <b>Phase</b> | <b>Clinical trial ID</b> | <b>References</b>                                                              |
|------------------------------------------|----------------------------------------------------------------------------------------------------------------|--------------|--------------------------|--------------------------------------------------------------------------------|
| <b>Recombinant human PTX-2 (PRM-151)</b> | Inhibitor of monocyte differentiation into proinflammatory macrophages and fibrocytes, and production of TGFβ1 | Phase 3      | NCT04552899              | (Blink et al., 2016; Raghu et al., 2022, 2019, 2018; Tzouvelekis et al., 2019) |
| <b>Treprostinil</b>                      | Activates prostaglandin E <sub>2</sub> receptor 2, prostaglandin D <sub>2</sub> receptor 1, and PPARs          | Phase 3      | NCT04708782              | (Kolb et al., 2022; Nathan et al., 2022, 2021; Waxman et al., 2021)            |
| <b>Pamrevlumab (FG-3019)</b>             | Monoclonal anti-CTGF antibodies                                                                                | Phase 2      | NCT01890265              | (Leask, 2019; Richeldi et al., 2020)                                           |
| <b>BMS-986020</b>                        | Lysophosphatidic acid receptor antagonist                                                                      | Phase 2      | NCT01766817              | (Decato et al., 2022; Palmer et al., 2018)                                     |
| <b>Rituximab</b>                         | Monoclonal anti-CD20 antibodies                                                                                | Phase 2      | NCT01969409              | (Kulkarni et al., 2021; Román et al., 2023)                                    |
| <b>PLN-74809</b>                         | Dual αvβ6/αvβ1 integrin inhibitor                                                                              | Phase 2      | NCT06097260              | (Decaris et al., 2021; Lefebvre et al., 2020)                                  |
| <b>PBI-4050</b>                          | Activates GPR40 and suppresses GPR84                                                                           | Phase 2      | NCT02538536              | (Khalil et al., 2019)                                                          |
| <b>Jaktinib hydrochloride</b>            | JAK1/2/3 inhibitor                                                                                             | Phase 2      | NCT04312594              | (Gu et al., 2023; Milara et al., 2018b, 2018a)                                 |
| <b>RXC007</b>                            | ROCK2 inhibitor                                                                                                | Phase 2      | NCT05570058              | (Gambardella et al., 2022)                                                     |

|                                |                       |           |             |                                                                                                                                                                                     |
|--------------------------------|-----------------------|-----------|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>TD139</b>                   | Galectin-3 inhibitor  | Phase 1/2 | NCT02257177 | (Hirani et al., 2021)                                                                                                                                                               |
| <b>Saracatinib</b>             | Src/Bcr-Abl inhibitor | Phase 1/2 | NCT04598919 | (Ahangari et al., 2022; Jaeger et al., 2022)                                                                                                                                        |
| <b>TRK-250</b>                 | TGF $\beta$ 1 siRNA   | Phase 1   | NCT03727802 | (“A Phase I Study of TRK-250, a Novel siRNA-Based Oligonucleotide, in Patients with Idiopathic Pulmonary Fibrosis   Journal of Aerosol Medicine and Pulmonary Drug Delivery,” n.d.) |
| <b>GSK2126458 (omipalisib)</b> | PI3K/mTOR inhibitor   | Phase 1   | NCT01725139 | (Lukey et al., 2019)                                                                                                                                                                |

One limitation is that these approaches predominantly target a single aberrant pathway while fibrogenesis is multifactorial, and it is highly unlikely that there is a “silver bullet” that can cure such complex disease. Moreover, there is a lack of precision in targeting MyoFBs despite the latter being regarded as the effector cells in fibrotic disease not only in the lung but in other organs. With the advent of single-cell omics and the accompanying gain of knowledge regarding cellular heterogeneity and signaling profiles, the aim remains achieving selective targeting of culprit cells, such as MyoFBs, to induce their clearance and deactivation as a strategy to treat lung fibrosis (Zabihi et al., 2024).

Apart from such compounds, there are also attempts to carry out cell therapy including the use of lung spheroid stem cells and MSCs. On the other hand, drug repositioning or repurposing has gained increasing interest in recent years and drugs with proven safety profiles, such as metformin, have shown therapeutic potential in preclinical studies (Cheng et al., 2021; Kheirollahi et al., 2019; Rangarajan et al., 2018; Sato et al., 2016;

Zabihi et al., 2024), with the rationale being in this particular case the association of lung fibrosis with metabolic disorders such as type 2 diabetes (T2D), and the observation that AMPK is inactivated in IPF lungs. Interestingly, a recent study reported that inhaled liposomes containing nintedanib and metformin successfully alleviated bleomycin-induced pulmonary fibrosis in mice (Li et al., 2023). While there has been skepticism regarding the anti-fibrotic effect of metformin in IPF, such concerns were based on post hoc analysis of the placebo arms of other clinical trials where many donors were receiving metformin as T2D therapy (Spagnolo et al., 2018; Tzouvelekis et al., 2018). The lack of significant difference in terms of outcomes between patients receiving metformin or not is confounded by at least pre-existing T2D, which itself can act as a risk factor for IPF (García-Sancho Figueroa et al., 2010). Another retrospective study reported a beneficial effect of metformin in reducing all-cause mortality and hospitalization in IPF (Teague et al., 2022). Therefore, it is important to design clinical trials specifically dedicated to testing the ability of metformin to reverse or at least halt fibrosis in IPF patients. Finally, there is an urgent need to improve our understanding of IPF pathophysiology at the cellular and molecular levels, particularly regarding MyoFBs origin and fate, to identify promising therapeutic windows to potentially treat affected patients (Zabihi et al., 2024).

#### **5.2.5 Modeling early fibrosis and drug screening using human and murine PCLS**

3D co-culture systems display cell-cell and cell-matrix interactions that are lacking in conventional 2D monolayer cultures. These 3D models mimic the lung microenvironment and enable the assessment of cellular structure and function during homeostatic and pathological conditions. In line with this approach, PCLS obtained from ex vivo lung explants are the most widely employed lung model to recapitulate native lung architecture, tissue stiffness and resident cell populations, making them highly suitable for translational research and regenerative therapeutics (Morin et al., 2013; Lehmann et al., 2024). PCLS contain nearly all cell types present in the lung including immune cells, endothelial cells, epithelial cells beside mesenchymal cells (Liu et al., 2019). Since PCLS are provided from end-stage IPF tissue, they represent a well-established model to investigate the effects of potential therapeutic compounds for the treatment of IPF patients.

PCLS derived from IPF patients treated with inhibitors of TGF $\beta$  regulators, dual  $\alpha\beta 6$  and  $\alpha\beta 1$  integrin inhibitors, exhibited reduced expression of *COL1A1* and *SERPINE1*, accompanied by attenuated fibrogenesis (Decaris et al., 2021). These findings provide preclinical support for ongoing Phase II clinical trials investigating the efficacy of the dual  $\alpha\beta 6/\alpha\beta 1$  integrin inhibitor PLN-74809 in patients with fibrotic lung disease (NCT04396756 and NCT04072315) (Decaris et al., 2021). Similarly, metformin as an antidiabetic and AMPK activator has a dual anti-fibrotic and lipogenic effect in explanted lung tissue. Treatment of IPF-derived hPCLS with metformin attenuated fibrosis by reducing collagen deposition and improved lung structure through MyoFB-to-LIF transition and increased lipid-droplet accumulation in fibroblasts. These findings underscore metformin as a promising and well-tolerated therapeutic candidate for therapies aimed at accelerating fibrosis resolution (Kheirollahi et al., 2019). In line with this, our data using rTIMP-3 and VCAN in the context of IPF hPCLS demonstrated a significant reduction in *COL1A1* and *ADAMTS4* expression following treatment, further indicating that ADAMTS4 represents a promising therapeutic target in human lung fibrosis.

Since IPF lung explants are typically obtained from patients with end-stage disease, they provide limited insight into the mechanisms driving disease initiation. This leads to an alternative model that more accurately reflects the early stages of fibrosis and enables the evaluation of potential therapeutic interventions at a stage when treatment may be most effective (Lang et al., 2023; Melo-Narvaez et al., 2025). In this regard, fibrotic cocktail containing TGF $\beta$ , PDGF-AB, TNF $\alpha$ , and lysophosphatidic acid (LPA) was proposed as an appropriate model of early fibrosis using human PCLS derived from donors without IPF. Treatment of hPCLS with these fibrotic components resulted in upregulation of pro-fibrotic and pro-inflammatory markers without affecting cell viability, reflecting features of alveolar injury and depletion of progenitor populations in the alveolar epithelium (Alsafadi et al., 2017). Due to the scarcity of human lung explants, murine PCLS were employed to test the effect of rTIMP-3 and VCAN on fibrotic remodeling, where it showed to mitigate rTGF $\beta 1$ -induced fibrogenesis, as indicated by the

downregulation of profibrotic markers. RNAscope analysis revealed that, under homeostatic conditions, ADAMTS4 expression is primarily localized to alveolar (*Fgf10*<sup>+</sup>) and adventitial (*Pi16*<sup>+</sup>) fibroblasts. Upon TGFβ1 stimulation, these fibroblast subsets underwent a profibrotic transition characterized by elevated ADAMTS4 and *Cthrc1* expression and reduced *Fgf10*<sup>+</sup> fibroblast numbers.

Cultured PCLS from bleomycin mice showed increased expression of fibrosis-related genes, similar to the in vivo bleomycin model (Cedilak et al., 2019). Moreover, treatment of PCLS obtained from saline or bleomycin-challenged mouse lungs with nintedanib showed increased *Sftpc* expression in fibrotic PCLS, whereas pirfenidone treatment did not have similar effect (Lehmann et al., 2018). Moreover, PCLS provided from healthy donors and treated with mentioned fibrotic cocktail to model early fibrosis, followed by nintedanib treatment restored AEC2s, representing a relevant feature of the anti-fibrotic mechanisms of nintedanib (Lehmann et al., 2018). In the current study, 14 days after bleomycin administration, mPCLS obtained from injured mice were treated with VCAN for three days confirmed attenuation of fibrogenesis.

PCLS retrain structure of the lung and resident cell populations, but they are limited by the absence of a recruitable immune system, restricting their ability to fully model immune responses. The future of these models will focus on enhancing their complexity to better mimic lung physiology (Nizamoglu et al., 2023). Heterogeneity across lung sections and between diseased samples introduces variability that requires precise analysis. The model is further constrained by its limited viability (up to two weeks), lack of breathing-associated mechanical forces, and challenges in translating drug dosing and administration routes to in vivo conditions (Kolanko et al., 2024). Despite the limited accessibility of lung tissue, particularly from patients, PCLS constitute a powerful ex vivo model for studying early innate immune signals and drug effects on resident lung cells.

### **5.2.6 Modeling fibrotic phenotype in lung organoids**

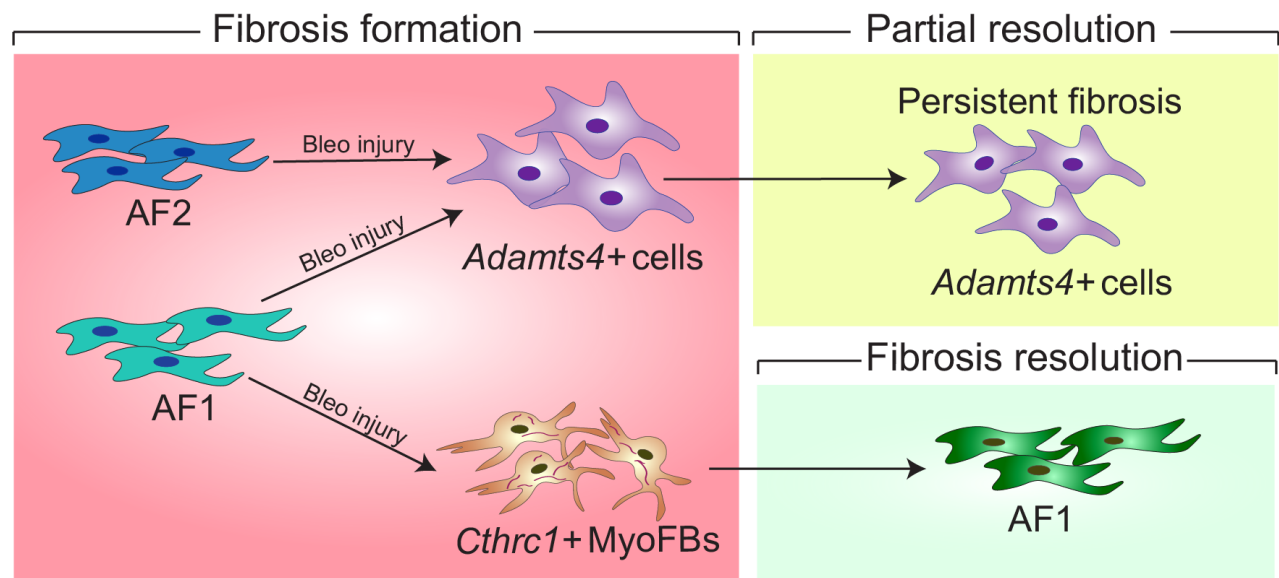
Lung organoids are particularly valuable for modeling epithelial-mesenchymal interactions that occur during homeostasis and disease. Significant advances in organoid technology have enabled the development of structures that closely recapitulate the

cellular and architectural complexity of the lung. Overall, lung organoid systems provide important insights into physiologically relevant molecular mechanisms of fibrosis, which are otherwise difficult to investigate in vivo. Importantly, patient-derived lung organoids could be used for high throughput drug screening and accelerate the development of personalized therapies for IPF (Vazquez-Armendariz et al., 2022). For instance, iPSC-derived AEC2 (iAEC2) exposed to fibrotic cocktail which has previously been shown to induce early fibrotic changes and epithelial phenotypic alterations. Treatment of hPCLS with the anti-fibrotic drugs, nintedanib and pirfenidone, reduced ECM deposition and pro-fibrotic mediator expression, although they did not fully reverse epithelial reprogramming (Ptasinski et al., 2023).

In our model, we employ murine WT alveolar organoids, which enable the assessment of morphological changes such as organoid size and density, providing a physiologically relevant platform to study fibrogenesis. Following TGF $\beta$ 1 treatment, the organoids became smaller and more condensed with accumulation of activated MyoFBs, modeling key aspects of human IPF. Inhibition of ADAMTS4 activity using rTIMP-3 attenuated fibrogenesis and maintained organoid morphology. rTIMP-3 treatment on fibrotic organoids restored the expression of epithelial markers, *Sftpc* and *Ager*, while reducing the expression of fibrotic and myofibroblast-associated genes, indicating a partial reversal of TGF $\beta$ 1-induced fibrotic remodeling within the organoid model. Collectively, these findings demonstrate that this system is well suited for studying fibrotic reprogramming and holds significant potential for application in antifibrotic drug discovery. Recently, an advanced BALOs model was developed by microinjecting ECs into differentiated BALOs, resulting in the integration of an endothelial compartment that aligned with AECs and exhibited microvascular characteristics (Ament et al., 2025). As the lack of vascularization remains one of the major limitations of organoid systems, incorporating FACS-sorted lung ECs into alveolar organoids represents a significant step toward creating more physiologically relevant and complex lung models in the future.

Loss of the rMC-AEC2s niche is a key hallmark of the aging lung (Chanda et al., 2021). Several studies have confirmed the loss of niche activity in aged mesenchymal cells

required to support lung organoid growth. However, differences in culture efficiency (CFE and size) may result from the use of varying protocols and sorting assays for AEC2s isolation (Chanda et al., 2021; Lingampally et al., 2025; Sinha and Lowell, 2016). In our model, aged alveolar organoids were generated by co-culturing rMCs from old *Fgf10<sup>Cre-ERT2/+</sup>* mice with young WT AEC2s demonstrated that the niche activity of Sca1+ rMCs is significantly impaired with age and cannot be restored during early (Bleo d30) and late (Bleo d60) fibrosis resolution. One possible explanation for this loss of supportive capacity is the persistent expression of *Adamts4* in mesenchymal cells isolated from Bleo d60 mice, which may limit their ability to support AEC2 growth and differentiation in the organoid model. To overcome this limitation and restore mesenchymal niche function, sorted Bleo d60 rMCs can be pre-treated with rTIMP-3 to inhibit *Adamts4* activity prior to be co-cultured with young AEC2s. Such approach would demonstrate whether ADAMTS4 inhibition reactivates the supportive potential of aged mesenchymal cells and therefore enhances alveolar organoid formation.



**Figure 23. Model of LIF-to-MyoFB reversible switch during fibrosis formation and persistent of *Adamts4*<sup>+</sup> cells during fibrosis resolution.** Lineage tracing of *Fgf10*<sup>+</sup> lipofibroblasts revealed that AFs give rise to *Cthrc1*<sup>+</sup> MyoFBs during fibrogenesis and to *Adamts4*<sup>+</sup> cells that persist during the delayed resolution phase.



## 6. Summary

Organoid models have become an integral part of the research methodology in the lung field. Distinct models of lung organoids mimicking various anatomical regions of mature lungs. In the distal lung, AEC2s represent a stem cell population that is engaged in regenerative mechanisms in response to various insults. These cells self-renew and give rise to AEC1s that carry out gas exchange. Multiple experimental protocols allowing the generation of alveolar organoids from murine lungs have been described. Among the drawbacks have been the requirement of transgenic mice allowing the isolation of AEC2s with high viability and purity, and the occasional emergence of bronchiolar and bronchioalveolar organoids. Here, we provide a refined gating strategy and an optimized protocol for the generation of alveolar organoids from wild-type mice. Our approach not only overcomes the need for transgenic mice to generate such organoids but also yields a pure culture of alveolar organoids that is devoid of bronchiolar and bronchioalveolar organoids. Our protocol contributes to the standardization of this important research tool.

IPF is an aging-associated, progressive and lethal lung disease that affects older adults. While lung fibrosis resolves in younger mice, aged mice developed non-resolving fibrosis in response to bleomycin-induced injury. In this study, we employed 52–to-56-week-old female *Fgf10<sup>Cre-ERT2/+</sup>; tdTomato<sup>flox</sup>* mice to establish a pulmonary fibrosis model with delayed resolution and recapitulate pathological features of IPF. Histological analyses and single-cell transcriptomics on sorted lineage-labeled cells were performed at the peak of fibrosis (Bleo d14) as well as early (Bleo d30) and late (Bleo d60) resolution. The lineage-tracing approach identified *Fgf10*+ AFs with *LIF* signature as a cellular source of not only MyoFBs that arise during fibrogenesis but also *Adamts4*+ cells that persist during delayed fibrosis resolution. Datamining and ex vivo therapeutic interventions indicated strong clinical relevance for our findings and highlighted ADAMTS4 as a promising therapeutic target in pulmonary fibrosis. Functional intervention using ex vivo assays revealed strong clinical relevance. Our study provides valuable insights into the cellular origins and trajectories of AFs and MyoFBs and sets the stage for identifying further targets for future interventions.

## 7. Zusammenfassung

Organoid-Modelle sind zu einem integralen Bestandteil der Forschung im Lungenbereich geworden. Es existieren verschiedene Modelle von Lungenorganoiden, welche unterschiedliche anatomische Regionen der ausgereiften Lunge nachbilden. In der distalen Lunge stellen alveoläre Typ-2-Epithelzellen (AEC2-Zellen) eine Stammzellpopulation dar, die bei verschiedenen Schädigungen an regenerativen Mechanismen beteiligt ist. Diese Zellen selbst erneuern sich und differenzieren zu alveolären Typ-1-Epithelzellen (AEC1-Zellen), welche den Gasaustausch übernehmen. Es wurden mehrere experimentelle Protokolle beschrieben, die die Erzeugung von Alveolarorganoiden aus Mauslungen ermöglichen. Zu den Nachteilen gehörten bislang unter anderem die Notwendigkeit transgener Mäuse, um AEC2-Zellen mit hoher Vitalität und Reinheit zu isolieren, sowie das gelegentliche Auftreten bronchiolärer und bronchioalveolärer Organoiden. Hier stellen wir eine verfeinerte Gating-Strategie und ein optimiertes Protokoll zur Erzeugung alveolärer Organoiden aus Wildtyp-Mäusen vor. Unser Ansatz eliminiert nicht nur die Abhängigkeit von transgenen Mäusen, sondern liefert auch eine reine Kultur alveolärer Organoiden, welche frei von bronchiolären und bronchioalveolären Organoiden ist. Unser Protokoll trägt zur Standardisierung dieses wichtigen Forschungsinstruments bei.

Die idiopathische Lungenfibrose (IPF) ist eine altersassoziierte, progrediente und tödliche Lungenerkrankung, welche vorwiegend ältere Menschen betrifft. Während eine Lungenfibrose bei jungen Mäusen oftmals abheilt, entwickelten gealterte Mäuse nach Bleomycin-induzierter Schädigung eine sich nicht zurückbildende Fibrose. In dieser Studie verwendeten wir 52–56 Wochen alte weibliche *Fgf10<sup>Cre-ERT2/+</sup>;tdTomato<sup>flox</sup>*-Mäuse, um ein Lungenfibrosemodell mit verzögerter Rückbildung zu erstellen, welches die pathologischen Merkmale der IPF nachbildet. Histologische Analysen und Einzelzell-Transkriptomik an sortierten, lineage-markierten Zellen wurden in der Phase der maximalen Fibroseausprägung (Bleo d14) sowie zu einem frühen (Bleo d30) und späten (Bleo d60) Zeitpunkt der Auflösung durchgeführt. Mittels Lineage-Tracing konnten *Fgf10*+ alveoläre Fibroblasten (AFs) mit *LIF*-Signatur als Ursprung sowohl der in der Fibrogenese gebildeten Myofibroblasten (MyoFBs) als auch der während der verzögerten

Fibroseauflösung fortbestehenden *Adamts4*+ Zellen identifiziert werden. Datenanalysen und ex vivo-therapeutische Interventionen wiesen auf eine hohe klinische Relevanz unserer Befunde hin und hoben ADAMTS4 als vielversprechendes therapeutisches Ziel bei pulmonaler Fibrose hervor. Funktionelle Interventionen in ex vivo-Assays untermauerten diese klinische Relevanz. Unsere Studie liefert wertvolle Einblicke in die zellulären Ursprünge und Entwicklungsverläufe von AFs und MyoFBs und schafft die Grundlage für die Identifizierung weiterer Zielstrukturen für zukünftige Interventionen.

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