



The evolution of *in vitro* models of lung fibrosis: promising prospects for drug discovery

Emanuel Kolanko^{1,5}, Anna Cargnoni^{2,5}, Andrea Papait^{3,4}, Antonietta Rosa Silini², Piotr Czekaj¹ and Ornella Parolini^{3,4}

¹Department of Cytophysiology, Katowice Medical University of Silesia in Katowice, Katowice, Poland. ²Fondazione Poliambulanza Istituto Ospedaliero, Centro di Ricerca E. Menni, Brescia, Italy. ³Università Cattolica del Sacro Cuore, Department Life Sciences and Public Health, Roma, Italy. ⁴Fondazione Policlinico Universitario “A. Gemelli” IRCCS, Roma, Italy. ⁵These authors contributed equally.

Corresponding author: Ornella Parolini (ornella.parolini@unicatt.it)



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***In vitro* lung fibrosis models have progressively improved to represent tools closely mimicking pathological processes and allow advancements of personalised medicine and enhance comprehension of pathogenic molecular mechanisms.** <https://bit.ly/40P8gqw>

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Abstract

Lung fibrosis is a complex process, with unknown underlying mechanisms, involving various triggers, diseases and stimuli. Different cell types (epithelial cells, endothelial cells, fibroblasts and macrophages) interact dynamically through multiple signalling pathways, including biochemical/molecular and mechanical signals, such as stiffness, affecting cell function and differentiation. Idiopathic pulmonary fibrosis (IPF) is the most common fibrosing interstitial lung disease (fILD), characterised by a notably high mortality. Unfortunately, effective treatments for advanced fILD, and especially IPF and non-IPF progressive fibrosing phenotype ILD, are still lacking. The development of pharmacological therapies faces challenges due to limited knowledge of fibrosis pathogenesis and the absence of pre-clinical models accurately representing the complex features of the disease. To address these challenges, new model systems have been developed to enhance the translatability of preclinical drug testing and bridge the gap to human clinical trials. The use of two- and three-dimensional *in vitro* cultures derived from healthy or diseased individuals allows for a better understanding of the underlying mechanisms responsible for lung fibrosis. Additionally, microfluidics systems, which replicate the respiratory system's physiology *ex vivo*, offer promising opportunities for the development of effective therapies, especially for IPF.

Introduction

Fibrosing interstitial lung diseases (fILDs) encompass a wide range of conditions with various causes, including distinct primary diseases, environmental exposures, drugs and irradiation. Some fILDs are linked to connective tissue diseases, while others are characterised as interstitial pneumonias with unidentifiable cause. fILDs affect a significant number of patients worldwide, with a prevalence ranging from 6.3 to 71 per 100 000 people [1]. Among patients with fILD, only 13–40% have a non-IPF progressive fibrosing phenotype, and the rest are idiopathic pulmonary fibrosis (IPF), which is the most severe ILD with high mortality and a life expectancy of 2–3 years after diagnosis [2, 3]. IPF exhibits a progressive phenotype and is often considered as the “prototype” fILD, serving as a reference point for many models of pulmonary fibrosis. Many clinical trials have tested pharmacological therapies for lung fibrosis. Unfortunately, most were ineffective or even harmful. Currently, there are only two drugs approved in lung fibrosis treatment: nintedanib and pirfenidone. Both are recognised for their effectiveness in managing IPF, with nintedanib also being used for the treatment of progressive fILDs, such as those associated with scleroderma. Nonetheless, these drugs can only partially delay disease progression and improve the patients' quality of life [4, 5]. In cases where the disease has advanced significantly, lung transplantation remains the sole curative option [6]. Therefore, there is an urgent need to develop effective therapies for IPF and other fILDs that do not respond to conventional therapies.



This requires a better understanding of the pathogenic mechanisms involved in fibrosis and the availability of models that closely mimic the disease.

When modelling pulmonary fibrosis, it is important to consider that the fiLDs are not solely determined by individual pathogenic processes taken *per se*, but rather are the result of a complex interplay of multiple processes that mutually influence each other (figure 1). Pulmonary wound repair is a very dynamic process characterised by injury, inflammation and repair phases. Although not all fiLDs fit the three-phase wound-healing paradigm, it is useful to take into account the following sequence of events during fibrosis model construction [7].

Lung injury often starts with damage to epithelial and endothelial cells, that initiates the clotting cascade, resulting in the temporary occlusion of injured vessels with platelets and fibrin-rich thrombi. This process progresses rapidly to a phase of vasodilation with increased endothelial permeability, which allows the recruitment of leukocytes to the site of injury [8]. Matrix metalloproteinases (MMPs) play a key role in allowing cellular access to and from the site of injury during inflammation [9, 10]. The closing phase consists of cellular reorganisation guided by a fibrin-rich scaffold formation, wound contraction, closure and re-epithelialisation [11] (figure 1).

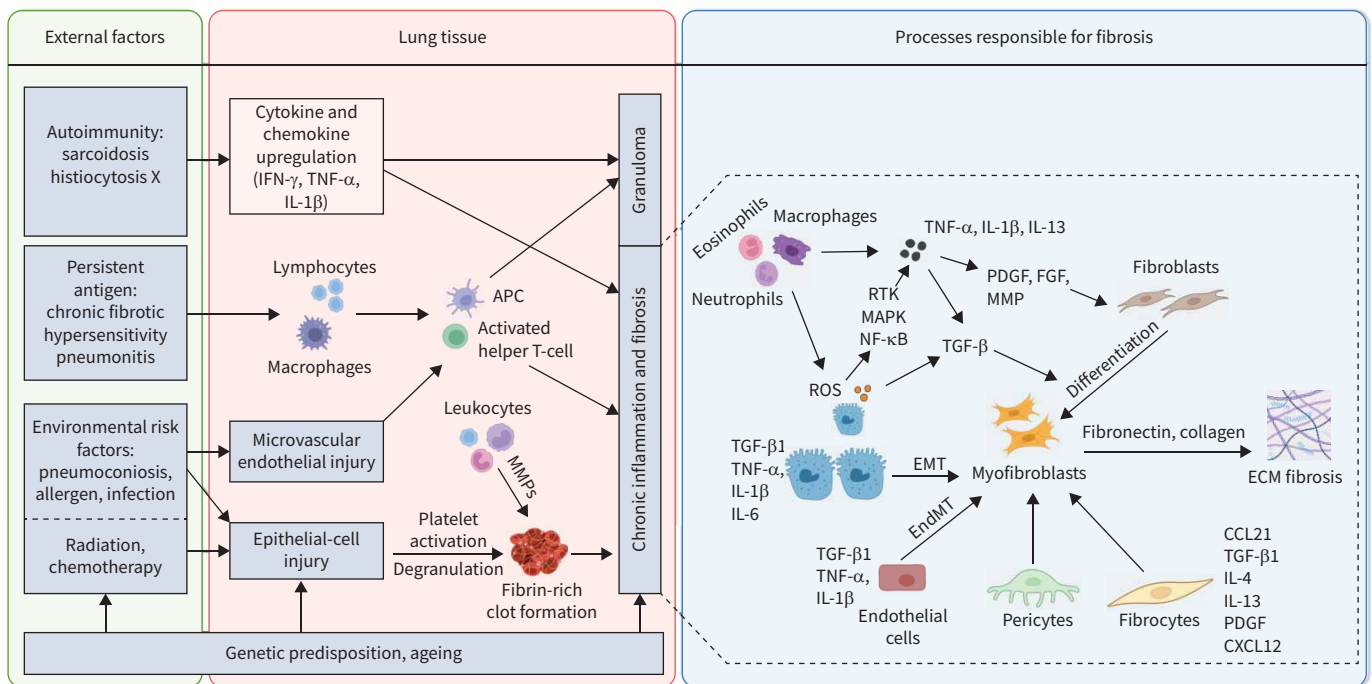


FIGURE 1 The cascade of events leading to pulmonary fibrosis that represents a response of the human organism to lung damage. The initiating factor is often injury to the lung epithelium caused by irritants such as environmental particles, allergens, infectious agents, chemotherapy, radiotherapy or an unknown factor. Prolonged exposure to the damaging factor, as well as numerous interactions between the patient’s immune system cells, lead to the development of a chronic inflammatory state and fibrosis. The degree of inflammatory process progression is also influenced by genetic predisposition and the patient’s age. At the beginning of the inflammatory process, immune cells including neutrophils, eosinophils, macrophages, T-cells and B-cells infiltrate the lung tissue. Activated neutrophils, as the inflammatory process progresses, increase the production of reactive oxygen species (ROS). ROS-dependent activation of receptor tyrosine kinase (RTK), mitogen-activated protein kinase (MAPK) and NF-κB pathways occurs, along with increased production of transforming growth factor (TGF)-β. TGF-β, through the recruitment of leukocytes, acts as the main profibrotic factor and leads to the further secretion of a cascade of other profibrotic cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-13. These activated leukocytes can further damage epithelial cells. In the next stage, stimulated by TNF-α and IL-1β, there is an increased expression of TGF-β and platelet-derived growth factor (PDGF), which, in turn, increases collagen production through the proliferation of fibroblasts and myofibroblasts recruited at the site of inflammation. As pulmonary fibrosis progresses, the pool of fibroblasts and myofibroblasts expands. This occurs mainly through the recruitment and differentiation of circulating fibrocytes, the activation and proliferation of pericytes, the transition from an epithelial to a mesenchymal state (EMT), and the transition from an endothelial to a mesenchymal state (EndMT). IFN: interferon; APC: antigen-presenting cell; FGF: fibroblast growth factor; MMP: matrix metalloproteinase. Figure designed using Biorender.com.

Pulmonary wound repair can be dysregulated by persistent irritants and genetic predispositions, leading to the progressive accumulation of extracellular matrix in most fibrotic conditions [8]. Persistent lung microinjury activates alveolar epithelial cells, resulting in excessive secretion of inflammatory mediators such as platelet-derived growth factor (PDGF), tumour necrosis factor (TNF)- α , angiotensin and chemokines [12], inducing the recruitment of inflammatory cells such as macrophages, dendritic cells, eosinophils, T-cells and B-cells [13, 14]. Specific immune cell subpopulations, such as the newly described macrophage/monocyte cell subtype called SatM recruited in lungs through CXCL12 derived from apoptotic epithelial cells [15] could play a significant role in the fibrosis process. Additionally, several cytokines participate in wound healing and fibrotic responses [13], for example, the permanent overproduction of profibrotic interleukin (IL)-13 and transforming growth factor (TGF)- β 1 can switch a controlled healing response into a pathogenic fibrotic response (figure 1).

To date, there are several animal models of pulmonary fibrosis available. These models encompass those induced by damaging agents such as chemicals, profibrotic cytokines, radiation, silica and asbestos. Additionally, there are transgenic models in which specific gene mutations associated with ILD, such as a mutant surfactant protein C gene (SFTPC) in alveolar type II (AT2) cells [16], are triggered to induce the development of pulmonary fibrosis. In addition, the bleomycin-induced pulmonary fibrosis model is widely used and well characterised [17]. This model has proven valuable in the exploration of novel pathogenic mechanisms that may have relevance to human disease. Notably, recent research has demonstrated the pivotal role of RNA-binding motif protein 7 (RBM7) in fibrosis onset [18]. Nevertheless, these models present notable limitations [19], possibly for the fact that all animal models of IPF are artificially induced using single agents or target a single cell type, while it is well known that the pathogenesis is multifactorial and still remains elusive.

In this context, *in vitro* models, despite being a simplified representation of actual diseased tissue, can be valuable because they allow for the identification of specific cellular and molecular mechanisms that trigger and induce disease progression.

Nonetheless, as they continue to evolve, *in vitro* models are increasingly approaching the complexity of *in vivo* systems. For example, lung organoids derived from induced pluripotent stem cells (iPSCs) obtained from patients enable monitoring of the alterations acquired by the different cell populations during the disease progression. This approach aids in pinpointing the specific states of maturation where issues may arise. Moreover, the use of human material, with a personalised medicine perspective, enables the screening of drugs, including off-label drugs, for drug repurposing. In recent years, *in vitro* models have been developed to improve preclinical drug testing and bridge the translational gap to human clinical trials. These techniques offer advanced imaging and analysis of disease mechanisms, allowing for drug discovery and validation in a personalised manner (figure 2 and table 1).

This is a comprehensive overview of the various *in vitro* models that are accessible for studying the molecular mechanisms underlying fibrosis pathogenesis, as well as their applications in drug screening. The review begins by examining simpler models and subsequently delves into the advancements in biotechnology that have facilitated the development of more physiologically relevant representations, ultimately improving our understanding of pulmonary fibrosis (PF). Furthermore, the review outlines the advantages and disadvantages of each model, providing valuable insights into their appropriateness for specific research objectives.

Two-dimensional *in vitro* models

The most frequently used *in vitro* models to study pulmonary fibrosis are two-dimensional (2D) cell cultures. These usually comprise cells that contribute to pulmonary fibrosis pathogenesis, such as fibroblasts, alveolar epithelial cells, endothelial cells and macrophages that are derived from healthy or diseased subjects (human or animal) (figure 2 and table 1).

Fibroblasts

Fibroblasts play a central role in the fibrotic process. During prolonged tissue injury and chronic inflammation, as a consequence of signals driven by cytokines, chemokines and growth factors, fibroblasts proliferate and differentiate toward myofibroblasts displaying exaggerated extracellular matrix (ECM) production, migration and contractile activity [99]. *In vitro* studies have utilised primary lung fibroblasts from healthy [20, 21] and IPF patients [22, 23], as well as immortalised cell lines [21, 24, 25]. Recently, a fibroblast reporter cell line (10-4ABFP) with the promoter of the myofibroblast marker α -smooth muscle actin (SMA) was developed for studying pulmonary fibrosis progression [26]. Many *in vitro* studies have been aimed to investigate the effects of profibrotic or antifibrotic molecules on the phenotype and functions

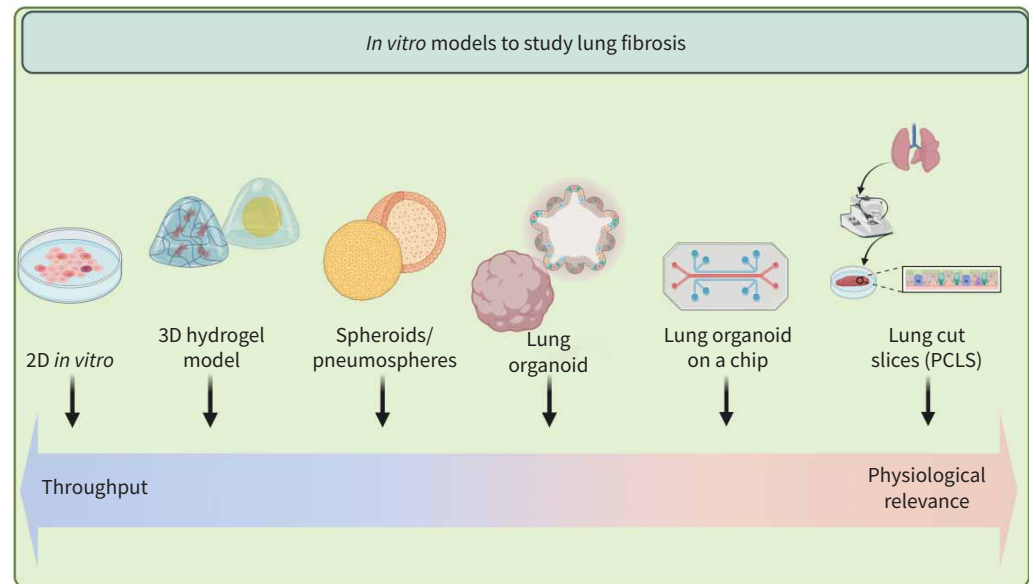


FIGURE 2 The evolution of *in vitro* models for studying pulmonary fibrosis involves the progression towards increasing biological complexity, starting from two-dimensional (2D) models and transitioning to early three-dimensional (3D) models such as spheroids. This complexity is further enhanced through the use of organoids, finally leading to the development of more advanced microfluidic approaches. Conversely, there is a trade-off between the ability to conduct large-scale studies with low costs, which may lack the ability to accurately reproduce the complexity of real biological systems, and the more sophisticated models that closely resemble biological systems, but are currently limited in their utilisation due to the complexity of use and the cost-related constraints. PCLS: precision-cut lung slices. Figure designed using Biorender.com.

of cultured fibroblasts. For instance, profibrotic molecules such as IL-6 and fibroblast growth factors (FGF)9 and FGF18 were found to confer apoptosis resistance [100, 101] and enhanced migration/invasion capacities to IPF-derived fibroblasts [101]. Contrarily, antifibrotic molecules, such as prostaglandin (PG)E2 and phosphodiesterase inhibitors, have been shown to stimulate fibroblast apoptosis [102], regulate the TGF- β 1-induced switch to myofibroblasts [21, 24], and suppress their motility [25]. The expression of α -SMA in myofibroblasts facilitates both their migration and contraction, thereby contributing to tissue stiffness and organ dysfunction. Recent studies [103, 104] have suggested that in addition to TGF- β 1, stiffness plays a significant role in promoting the activation of fibroblasts into myofibroblasts, *e.g.* phosphodiesterase (PDE)4 inhibitors were found to also counteract fibroblast-mediated *in vitro* contraction of collagen gels [25]. Furthermore, another study has reported that PDE4 inhibitors can counteract lung fibrosis by suppressing TNF- α production from alveolar macrophages of patients with IPF [105]. Results from these *in vitro* investigations have supported the potential application of PDE4 inhibitors in the treatment of pulmonary fibrosis. Phase III clinical trials are presently underway to assess the effectiveness, safety, and tolerance of a novel PDE4 inhibitor (BI 1015550) in patients with IPF (clinicaltrials.gov NCT05321069) and progressive fibrosing interstitial lung diseases (clinicaltrials.gov NCT05321082).

Studies conducted on human lung fibroblasts exposed to bleomycin or TGF- β have also highlighted the significance of the PI3K/Akt pathway in the differentiation of fibroblasts into myofibroblasts [27]. Indeed, inhibition of the PI3K/Akt pathway has proven to be effective in preventing myofibroblast formation [106]. However, despite these promising findings, clinical trials that investigate a specific PI3K/AKT inhibitor (LY294002) or other isoform-specific PI3K inhibitors [40] have yet to be initiated.

Moreover, research conducted with fibroblasts derived from IPF patients has revealed the crucial role played by epigenetic histone modifications in determining fibroblast resistance to apoptosis. Histone deacetylase (HDAC) inhibitors have been shown to restore the susceptibility of these cells to apoptosis [107]. Additionally, a recent work has demonstrated the therapeutic benefits of HDAC inhibitors in a bleomycin-induced pulmonary fibrosis model [28]. It is worth noting that while class I and pan-HDAC inhibitors are United States Food and Drug Administration-approved for cancer treatment, they are associated with various side-effects, possibly due to their impact on multiple cellular pathways [108].

TABLE 1 Summary of *in vitro* models to study lung fibrosis

Culture system	Cell type	Reference	Purpose	Advantages	Disadvantages
Two-dimensional <i>in vitro</i> models					
Fibroblasts	Primary human lung fibroblasts from healthy donors	[20, 21]	To investigate fibroblast apoptosis resistance, the switch toward myofibroblasts, the migration/invasion ability, the impact of tissue stiffness on their differentiation, the pathways involved in the differentiation of fibroblasts into myofibroblasts, and their epigenetic alterations To investigate the effects of profibrotic or antifibrotic molecules	Useful to understand the mechanisms underlying fibrosis development which involve the specific cell type (<i>i.e.</i> fibroblast to myofibroblast differentiation, EMT process)	Lack of both the variety of cell types and the ECM environment which characterise lung tissue These monocellular models are unable to replicate important signals arising from cell–cell and cell–matrix interactions occurring in the native tissue
	Primary human lung fibroblasts from IPF donors	[22, 23]			
	Immortalised fibroblast cell lines	[21, 24, 25]			
	Fibroblast reporter cell line (10–4ABFP)	[26]			
	Bleomycin-challenged fibroblasts TGF- β -challenged fibroblasts	[27, 28] [27]			
Alveolar epithelial cells	Alveolar epithelial cells from IPF patients	[29, 30]	To investigate alveolar epithelial cell proliferation, differentiation ability, secreted factors, EMT, the pathways involved in EMT, genetic and transcriptional profile and epithelial barrier properties		
	Alveolar epithelial cells from CC chemokine receptor 2 (CCR2) ^{-/-} mice	[31]			
	Mouse primary tracheal and bronchial epithelial cells and normal human lung fibroblasts	[32]			
	Rat type II alveolar epithelial cell line (RLE-6TN)	[33]			
	A549 cells (a human AT2 cell line)	[34]			
	TGF- β -challenged primary AT2 cells	[35]			
	RAS-inducible AT2 cell model (AT2ER:KRASV12)	[36]			
	Epithelial-like lung cell line: H441 cells (SALI culture)	[37, 38]			
	iAT2 cells	[39]			
	Cytokine-challenged iAT2 cells	[40]			
Co-culture models	IPF fibroblasts+A549 cells (in transwell)	[41]	To study the interactions between the different cell types involved in fibrogenesis	They allow the study of cell–cell interactions, either <i>via</i> a direct cell–cell contact and <i>via</i> secreted factors with paracrine actions	They still oversimplify the lung structure and environment
	Activated macrophages+lung fibroblasts (WI-38)	[42, 43]			
	Alveolar epithelial cells (A549), fibroblasts (MRC-5), and macrophages (differentiated THP-1) TGF- β -challenged	[44]			
	AT2 from IPF treated with conditioned media generated from fibroblasts	[45]			
	AT2 treated with senolytics in three-dimensional <i>ex vivo</i> lung tissue cultures	[46]			

Continued

TABLE 1 Continued

Culture system	Cell type	Reference	Purpose	Advantages	Disadvantages
Three-dimensional <i>in vitro</i> models					
Cells seeded on matrices and hydrogels	Lung fibroblast-embedded collagen matrix	[47, 48]	To investigate the impact of mechanical stimuli/stiffness to promote a fibrotic response (fibrogenic phenotype)	Adjusting matrix stiffness provides the ability to replicate both healthy and fibrotic microenvironments effectively. They allow for the study of cell migration, cellular traction and integrin adhesions in all three spatial planes	In many cases, it is not suitable for studying intercellular interactions
	IPF lung fibroblasts on polyacrylamide gels	[49]			
	Human lung fibroblasts on soft matrices, such as collagen hydrogels	[50]			
	Alveolar epithelial cell and lung fibroblast embedded in poly(ethylene glycol)-norbornene	[51]			
	Primary murine AT2 cells coated on hydrogel microspheres embedded within a hydrogel with primary murine fibroblasts	[51]			
Spheroids	iPSCs from dermal and lung fibroblasts and PBMCs derived from IPF patients embedded on polyacrylamide hydrogel	[52]	To reproduce patient's specific lung tissue complexity in terms of cells and ECM	Cells and extracellular matrices derive from a specific patient lung biopsy Potential to develop a personalised medicine	They frequently lack the presence of progenitor cells with a consequent strong difficulty in sustaining long-term three-dimensional culture (presence of hypoxia and necrosis in the spheroid core)
	Decellularised human lung scaffold	[53–56]			
	Human lung fibroblasts (IMR-90), bronchial epithelial cells (BEAS-2B) and macrophages (differentiated THP-1)	[57]			
	Heterogeneous primary cells derived from IPF patients' lung biopsies (pulmospheres)	[58]			
Pulmospheres derived from transbronchial biopsies collected from patients with non-IPF	[59]				

Continued

TABLE 1 Continued

Culture system	Cell type	Reference	Purpose	Advantages	Disadvantages	
Lung organoids	Progenitor cells	[60–62]	To develop clonal spheres known as bronchospheres, bronchioalveolar spheres or alveolospheres	Progenitor cells sourced from both donors and patients are applicable Well suited for examining mesenchymal–epithelial interactions High-throughput analyses can be conducted	Absence of vascularisation Absence of immune cell presence	
	Progenitor cells	[63]	To develop alveolospheres starting from AT2 alveolar cells			
	iPSC differentiated towards Nkx2.1+Sox9+	[64–67]	Differentiation of iPSC by a cocktail of factors and inhibitors			
		Bud tip progenitors	[68]	To generate foregut spheroids by culture in serum-free condition with exogenous administration of a cocktail of factors and inhibitors		
		iPSC differentiated towards lung epithelium cells	[69]	Organoid model of cystic fibrosis		
		iPSC carrying a homozygous deletion of F508 in the CFTR gene differentiated towards respiratory epithelial cells	[70, 71]	Cystic fibrosis organoid model		
		Fetal lung fibroblasts or iPSC-derived mesenchymal cells	[72]	Organoids originated in bioreactor with the addition of exogenous TGF- β 1, leading to a progressive scar phenotype marked by increased α -SMA and collagen-I expression		
		Alveolar epithelial cells derived from iPSC+primary human lung fibroblasts	[73]	IPF model of organoid using bleomycin		
		hPSCs (both ESCs and iPSCs)	[73–76]	Lung bud organoids employed to model fibrotic ILDs and viral infections, HPS, interstitial pneumonia and at least one ILD		
		Human embryonic stem cells	[73, 74]	IPF organoid model by introducing mutations in HPS1, HPS2 and HPS4 through CRISPR/Cas9 gene editing		
		Epithelial (A549), endothelial, and fibroblast cell lines	[77]	Fibrotic alveolar lung organoid model		
		Human alveolar epithelial type two cells (hAEC2)	[78]	Organoid derived from the transdifferentiation of AT2 in metaplastic KRT5 ⁺ basal cells		
		iPSCs carrying a disease-associated variant exclusive to AEC2 (SFTPC173T)	[79]	Generation of lung organoid model of ILD		
	Respiratory airways secretory cells	[80]	Generation of respiratory bronchioles organoids			

Continued

TABLE 1 Continued

Culture system	Cell type	Reference	Purpose	Advantages	Disadvantages
Microfluidic systems					
Organ-on-a-chip	Human lung endothelial and epithelial cells and human fibroblasts seeded on opposite sides of the membrane	[81]	Lung fibrosis pathogenesis investigation	Cost-effective Can replicate the biochemical microenvironment of the lung	Specialised equipment is needed for both culturing and analysis Experimental throughput is limited
	Human lung fibroblasts and immune cells	[82]	Investigation of mechanical strain contribution to the development of lung fibrosis	Allows for the incorporation of vascular and immune cells	
	Human endothelial cells, human lung fibroblasts	[83–88]	Testing influence of nanomaterials on human lung microtissue		
	Human endothelial cells, human lung fibroblasts	[89]	Testing lung fibrosis model starting from ECM from healthy or IPF lungs		
	Human lung fibroblasts TGF- β 1 challenged	[90–92]	Validation organ-on-a-chip for studying the efficacy of antifibrotic drugs, <i>i.e.</i> testing new antifibrotic drugs KD025 and BMS-986020		
Ex vivo model					
PCLS	PCLS derived from IPF patients	[93–96]	To test innovative molecules to target pathway involved in fibrogenesis and to evaluate the impact of AT2 cells on fibrosis progression	They retain the different lung cell types, the original lung architecture, microenvironment and metabolic functions Patient-derived PCLS can be employed for drug testing	Long-term culture can be challenging Analyses related to cell migration are constrained
	PCLS obtained from end-stage IPF and nonspecific interstitial pneumonia patients	[94]			
	PCLS from patients without fILD/IPF treated with a profibrotic cocktail	[97, 98]			
	Murine PCLS (from bleomycin-challenged mice)	[98]			
<p>IPF: idiopathic pulmonary fibrosis; TGF: transforming growth factor; EMT: epithelial–mesenchymal transition; ECM: extracellular matrix; AT2: alveolar type II cell; SALI: surfactant air–liquid interface; iAT2: induced pluripotent stem cell-derived AT2; iPSC: induced pluripotent stem cell; PBMC: peripheral blood mononuclear cell; CFTR: cystic fibrosis transmembrane conductance regulator; SMA: smooth muscle actin; ILD: interstitial lung disease; HPS: Hermansky–Pudlak syndrome; KRT: keratin; PCLS: precision-cut lung slices; fILD: fibrosing ILD.</p>					

Alveolar epithelial cell cultures

Alveolar epithelial cell cultures are important in understanding the initiation and progression of pulmonary fibrosis, especially IPF. Dysfunctional AT2 cells contribute to lung fibrosis due to their impaired cell renewal and their inability to differentiate into alveolar type 1 cells with respiratory functions [29]. Additionally, once activated, they secrete profibrotic mediators while showing an impaired secretion of factors that inhibit fibroproliferation, such as PGE2 and bone morphogenetic protein (BMP) [30, 31, 32, 109]. Moreover, AT2 dysfunction promotes epithelial–mesenchymal transition (EMT) processes and disrupts repair mechanisms [33]. Although submerged 2D cultures of A549 cells, a human AT2 cell line, do not fully replicate the pulmonary epithelium, they are used to demonstrate TGF- β -induced EMT characterised by downregulation of epithelial markers (E-cad, CK19), increased fibronectin and vimentin expression and increased collagen production [34]. Inhibition of TGF- β -induced EMT, collagen production and α -SMA expression have been observed in primary AT2 cells following treatment with resolvin-D1 [35].

In a more recent investigation [36], primary human AT2 cells and a RAS-inducible AT2 cell model (AT2ER:KRASV12) were employed to provide empirical evidence that supports the notion that in the context of IPF, EMT is a consequence of a profibrotic microenvironment that is modulated by the transcriptional regulator ZEB1. This was shown to occur through the activation of EGFR–RAS–ERK pathways. More specifically, ZEB1 disrupts paracrine communication between epithelial and mesenchymal cells by increasing the expression of tissue plasminogen activator. This, in turn, promotes TGF- β -induced α -SMA expression in human lung fibroblasts. While investigations into the role of EMT in IPF remain ongoing, the identification of potential EMT therapeutic targets is still elusive.

Additionally, another noteworthy model for studying human alveolar epithelium is the surfactant air–liquid interface (SALI) culture of H441 cells. Research has demonstrated that these cells, when cultured under specific conditions, exhibit a genetic and transcriptional profile akin to human alveolar cells in primary culture. They also express tight junction markers and develop multiple stratified monolayers with functional barrier properties [37, 38]. Notably, SALI cultures employing H441 cells with a knockout of surfactant protein (SP)B exhibit increased barrier permeability, while the cell phenotype remained unaffected, suggesting the potential utility of the SALI model as a disease model for SPB deficiency [37].

To overcome the reduced availability of patient samples, and the loss of phenotypic and functional features of primary human AT2 cells when cultured *in vitro*, AT2-like cells were derived from iPSCs.

To this regard, efforts have been made to develop culture protocols that enable the expansion of induced (i)AT2 cells. These cells cultured in three-dimensional (3D) Matrigel to generate alveolospheres proliferate while retaining their ability to produce SPC [39].

To investigate the dysfunction of alveolar epithelium in the context of IPF, a study has devised an air–liquid interface model derived from iPSCs. When this model is exposed to a cytokine cocktail that mimics the profibrotic milieu found in IPF lungs, it recapitulates both the impaired AT2 differentiation observed in IPF, and the shift toward an airway epithelial-like phenotype that is a characteristic feature of IPF-associated bronchiolisation of the lung epithelium [40].

Co-culture models

Co-culture models are used to study the interactions between different cell types. The Transwell system has been used to investigate the response of IPF fibroblasts to alveolar epithelial injury. Specifically, this model underlined the aberrant response of IPF fibroblasts to epithelial injury induced by mechanically scratching a cell monolayer constituted of A549 cells. Indeed, IPF fibroblasts showed impaired migration, increased secretion of profibrotic growth factors (basic FGF and PDGF-AA) and decreased production of hepatocyte growth factor (which has antifibrotic activity). Conversely, the presence of IPF fibroblasts triggered a transient EMT process in alveolar epithelial A549 cells [41].

Co-culture systems have also been used to investigate the role of alveolar macrophages on lung fibroblast functions. Activated macrophages (M1 by lipopolysaccharide and interferon- γ and M2 by IL-4), have contrasting effects on lung fibroblasts. Alternatively activated macrophages (M2) promote profibrogenic factors and collagen production, while classically activated (M1) increase fibrinolytic factors [42]. One study demonstrated that CD206⁺ macrophages downregulated collagen, α -SMA and TGF- β expression in lung fibroblasts through a paracrine mechanism involving IL-6 [43]. A recent co-culture system including alveolar epithelial cells (A549), fibroblasts (MRC-5) and macrophages (differentiated THP-1), was used to assess the fibrogenic potential of aerosolised quartz silica particles [44]. TGF- β treatment of this co-culture system induced a profibrotic response, including increased α -SMA and ECM protein expression, tissue

contraction, loss of barrier integrity and increased release of pro-inflammatory cytokines. This system shows promise for evaluating the fibrotic potential of exogenous substances.

Cellular senescence has garnered significant attention in recent decades due to its pivotal role in the pathogenesis of lung fibrosis. In this context, a recent investigation utilised a model of normal fibroblasts cultured in the presence of conditioned medium derived from *in vitro* cultures of AT2 cells obtained from individuals with IPF. This study revealed that senescent AT2 cells possess the capacity to stimulate collagen production in normal fibroblasts, primarily through the senescence-associated secretion of cytokines, chemokines, and matrix metalloproteinases regulated by NF- κ B [45]. Notably, when *ex vivo* 3D lung tissue cultures derived from healthy or bleomycin-challenged mice were treated with senolytic compounds, senescent AT2 cells were eliminated from the culture through induction of apoptosis. Consequently, this treatment led to a reduction in the release of senescence-associated factors such as MMP12, Serpine1 and Spp1, as well as a decrease in ECM components [46]. These studies collectively suggest that therapeutic strategies aimed at mitigating cellular senescence may offer novel avenues for the treatment of IPF.

3D *in vitro* models

Cells seeded on matrices and hydrogels

3D models (figure 2 and table 1) are usually obtained by seeding pulmonary cells onto a 3D structure mimicking the native lung ECM. Most 3D culture systems use collagen type-I as it is the predominant ECM protein in fibroblastic foci. ASMANI *et al.* [47] developed a microtissue array to model the progressive stiffening and contraction of alveolar tissue. This array was composed of lung fibroblasts embedded in a collagen matrix, suspended over multiple flexible micropillars. In presence of TGF- β 1, microtissue fibroblasts showed increased expression of α -SMA and procollagen, associated with increased contractile force. Preventive treatments with antifibrotic molecules (pirfenidone and nintedanib) have been shown to reduce tissue contraction and counteract tissue stiffening and the decline in alveolar compliance. Interestingly, the development of a 3D multicomponent hydrogel that better mimics the alveolar interstitial matrices (composed of both fibrous ECM proteins and amorphous hydrogel rich in glycosaminoglycans), demonstrated that myofibroblast differentiation did not correlate with hydrogel stiffness (*i.e.* matrix cross-linking), but rather with matrix fibre density [48].

Another study, which involved culturing lung fibroblasts derived from individuals with IPF on polyacrylamide gels with stiffness levels ranging from 1 to 20 kPa, revealed that there was an increase in the expression of α 6-integrin that was dependent on matrix stiffness. This upregulation was found to be contingent on the ρ -associated coiled-coil kinase (ROCK)-mediated activation of the c-Fos/c-Jun transcription complex [49]. Additionally, lung myofibroblasts cultured on rigid matrices exhibited heightened migratory activity. Intriguingly, the authors observed that inhibiting α 6-integrin conferred protection against experimentally induced lung fibrosis [49]. Building on the findings of this study and others conducted *in vivo* [110, 111], a phase 2b clinical trial (SPIRIT study, clinicaltrials.gov NCT03573505) was carried out in IPF patients using an anti- α v β 6 monoclonal antibody (BG00011). Unfortunately, patients who received BG00011 exhibited exacerbated fibrosis and experienced serious adverse events [112].

Prostanoids (such as PGE2) and cyclo-oxygenase (COX)2, the enzyme responsible for PGE2 synthesis, are significantly reduced in the lungs of individuals with IPF when compared to healthy controls [113]. A recent study, in line with a prior investigation [114], confirmed a direct correlation between matrix stiffness and the modulation of the PGE2–COX2 axis. Specifically, the culture of human lung fibroblasts on soft matrices, such as collagen hydrogels, promoted the expression of enzymes involved in eicosanoid biosynthesis (COX2, PTGES and cytosolic phospholipase A2), in comparison to fibroblasts cultured on rigid plastic plates [50]. These findings suggest that the increased stiffness characteristic of fibrotic regions may inhibit the expression of eicosanoids, which in turn contributes to the promotion of fibrosis.

More recently, a 3D lung model reproducing lung architecture and mechanics in fibrotic conditions was developed using poly(ethylene glycol)-norbornene to study alveolar epithelial cell and lung fibroblast interactions [51]. Cell-degradable hydrogel microspheres, replicating the shape and size of human alveoli, were coated with primary murine AT2 cells. These microspheres were magnetically aggregated to form an acinar structure and subsequently embedded within a hydrogel that had a stiffness of healthy or fibrotic lung tissue, and populated with primary murine fibroblasts. Viability of both cell types was maintained for ≥ 3 weeks post-embedding. The presence of alveolar epithelial cells promoted an early activation of fibroblasts to α -SMA⁺ myofibroblasts which was amplified by the additional presence of stiff hydrogel that

mimicked the fibrotic ECM microenvironment. These findings suggest that the combination of both cellular and mechanical stimuli is critical to promote a fibrotic response.

To develop a 3D model able to reproduce a progressive fibrosis model that does not require exogenous profibrotic inducers such as TGF- β to promote a fibrotic response, VUJAYARAJ *et al.* [52] generated iPSCs from dermal and lung fibroblasts and peripheral blood mononuclear cells (PBMCs) derived from IPF patients, differentiated them into different cell types critical for modelling fibrosis, and cultured them on a stiff polyacrylamide hydrogel to mimic fibrotic ECM. >90% of the differentiated cells were mesenchymal-like cells (expressing either mesenchymal and stemness markers), and a small portion (6.7%) expressed epithelial cell markers and immune cell markers (5%) in addition. The mesenchymal-like cells cultured on hydrogel formed dense scar-like aggregates that progressively enlarged assuming a phenotype of induced fibroblast activation, characterised by increased expression of collagen-I, α -SMA and TGF- β . Furthermore, with the progression of the induced fibroblast activation phenotype, the stiffness and the levels of cytokines/chemokines (monocyte chemoattractant protein-1, CXCL12, vascular endothelial growth factor-A, IL-6, IL-8 and colony stimulating factor 3) increased. The authors proposed this model as a platform to screen potential modulators of fibrosis. Finally, recent advances in lung bioengineering allowed for the study of lung repair and regeneration *ex vivo* using acellular human-derived lung tissue scaffolds. These scaffolds can be processed into thin slices or ECM extracts to study cell behaviour and cell–extracellular environment interactions. These models provide new insights into the role of the matrix and the extracellular environment in IPF [53]. For example, acellular lung scaffolds derived from adult or neonatal human lungs unsuitable for transplantation [54], and from patients with different lung chronic diseases such as COPD [55] and IPF [56], have revealed that both age and the specific type of disease affect the survival, adhesion and proliferation of cells seeded onto these scaffolds.

Spheroids

Spheroids are self-assembled 3D tissue models, *i.e.* cell aggregates composed of adult tissue cells. Lung spheroids are often used to reproduce 3D organ complexity for *in vitro* drug or toxicology tests (figure 2 and table 1). A recent study used spheroids composed of human lung fibroblasts (IMR-90), bronchial epithelial cells (BEAS-2B), and macrophages (differentiated THP-1) to evaluate the profibrotic activity of carbon nanotubes [57]. After 2 days from seeding, epithelial cells were closely located to fibroblast cells in the spheroid centre, while THP-1 macrophages were mostly found at the periphery. After 4 days, the microtissue formed a structure resembling a single round or multilobed spheroid, which was maintained for up to 7 days. Exposure to nanomaterials reduced microtissue viability and, interestingly, induced the expression of genes involved in acute inflammation (cytokines IL-1 β , TNF- α and IL-6) and ECM remodelling (collagens, decorin and MMPs).

Another study developed spheroids, known as pulmospheres, composed of heterogeneous primary cells derived from lung biopsies of IPF patients to test novel IPF therapies [58]. Pulmospheres are 3D structures composed of various cells present in the original biopsy, including AT2 cells, endothelial cells, vascular smooth muscle cells, myofibroblasts, and macrophages. These cells are embedded in patients' native lung ECM proteins, such as collagen type I, type IV and fibronectin. IPF pulmospheres, with respect to those from nonfibrotic subjects, are characterised by increased numbers of macrophages, endothelial cells and AT2 cells, as well as a 10-fold increase of α -SMA-positive cells, which contributes to their increased invasiveness. Interestingly, pulmosphere invasiveness was altered in presence of antifibrotic molecules, such as pirfenidone and nintedanib. In this context, a very recent study [59] found that pulmospheres derived from transbronchial biopsies of individuals with non-IPF ILDs exhibit a higher degree of invasiveness when compared to control samples. This level of invasiveness was found to correlate with a decline in forced vital capacity within 6–12 months post-biopsy. Additionally, the administration of nintedanib reduced the invasiveness of these pulmospheres, while pirfenidone had a more modest effect. The authors suggested that the 3D pulmosphere model could potentially serve as a valuable platform for the development of personalised therapies for ILDs and potentially other chronic lung conditions.

Lung organoids

Organoids represent a major step forward in disease modelling by allowing the understanding the molecular interactions within complex structures and representing a viable alternative for high-throughput drug screening [115, 116] (figure 2 and table 1). Organoids are the result of a process of self-organisation starting from a unicellular suspension within a mixture of different ECM proteins mimicking the extracellular support normally present *in vivo* [60]. They reproduce *in vitro* the high structural complexity with cytoarchitecture, morphological and functional profiles that closely mimic those present *in vivo* [60]. The first lung organoids were obtained from progenitor cells [60–62, 117]. Depending on the tissue of origin, progenitor cells isolated from specific lung biopsies (apical or distal) can develop clonal spheres

known as bronchospheres, bronchioalveolar spheres or alveolospheres. For example, alveolospheres are obtained starting from AT2 cells, selected as CD45⁻CD31⁻Epcam⁺HTII⁺ cells [63]. Currently, the formation of alveolospheres and bronchioalveolar spheres necessitates co-culture with other cell types. They have been successfully generated by combining alveolar or small airway epithelial progenitor cells with stromal components, such as fibroblasts or mesenchymal cells, suggesting that the factors released by stromal cells facilitate cell differentiation, maturation and the self-organisation of pulmonary epithelial organoids [61]. Unfortunately, the limited availability of human samples and the low yield of stem cells from tissue dissociation limit the application of lung organoid models obtained from transient lung progenitor cells. In part, these limitations have been bypassed by the use of iPSCs, which provide the unparalleled opportunity to study to which extent genetic mutations present in the patient's cells contribute to pathogenesis [118].

The generation of lung organoids from iPSCs leverages the evolutionary processes that occur during embryogenesis, particularly the understanding that the lung epithelium originates from the endoderm germ layer. Through the modulation of signalling pathways, the endoderm undergoes developmental processes that lead to the formation of the bronchial tree and to subsequent alveoli formation within the lungs. By replicating and manipulating signalling pathways, researchers can guide iPSCs towards differentiating into lung organoids, mimicking the developmental processes observed in embryogenesis [64, 65]. Notably, by stimulating the WNT and FGF signalling pathways while inhibiting the BMP/TGF- β pathways, the differentiation of iPSC-derived endoderm cultures can be directed towards a SOX2⁺ anterior foregut fate. Furthermore, the concurrent administration of a sonic hedgehog activator and retinoic acid enhances the expression of markers indicative of lung development, resulting in the emergence of Nkx2.1⁺Sox9⁺ cells. These cells, when seeded as a single-cell suspension within the ECM and expanded in a medium containing FGF10, lead to the formation of 3D lung organoids. These organoids consist of both P63⁺ basal cells and FOXJ1⁺ ciliated epithelial cells, reflecting the structural and cellular composition of the lung [64, 66, 67].

Alternatively, organoids can be generated from a progenitor population residing at the tips of the developing epithelium, known as "bud tip progenitors". These organoids have been obtained starting from foregut spheroids cultured in serum-free medium with the exogenous administration of FGF7, a GSK3 β inhibitor and all-trans retinoic acid. After 22 days of culture, the resulting organoids are enriched in SOX2⁺Sox9⁺ID2⁺Nkx2.1⁺ cells. These cells exhibit a transcriptional profile similar to that of human fetal bud tip progenitors able to give rise to both proximal airway and alveolar cells [68]. The latter type of organoids is particularly effective for studying the mechanisms involved in epithelial cell fate commitment during lung development. The possibility to obtain iPSCs from patient cells and to differentiate them into the major components of lung tissue allows the use of organoids to investigate how organs change with the disease and how they might respond to potential therapies. The earliest studies using iPSCs for modelling lung fibrosis were published in 2012 by WONG *et al.* [69]. The authors derived lung epithelium cells from iPSCs of patients with cystic fibrosis, with the aim to study the therapeutic effects of an analogue of the molecule VX-809 (C18). In a phase 2 trial, they demonstrated that C18 facilitates membrane translocation of F508del⁻ cystic fibrosis transmembrane conductance regulator (CFTR) partially rescuing the trafficking defect [69, 70]. Other groups generated lung organoids from iPSCs derived from cystic fibrosis patient fibroblasts. These organoids had fibrotic characteristics *in vitro*, which were removed by the CRISPR-mediated repair of CFTR mutation [71]. WILKINSON *et al.* [72] generated organoids mimicking IPF. The authors created a cohesive organoid by utilising collagen-functionalised alginate beads and human fibroblasts within a rotational bioreactor, resulting in structures that closely resembled the natural lung. As an example, they induced an IPF-like phenotype in organoids derived from fetal lung fibroblasts or iPSC-derived mesenchymal cells through the addition of exogenous TGF- β 1, leading to a progressive scar phenotype marked by increased α -SMA and collagen-I expression.

In a separate study, bleomycin was used to induce the IPF phenotype in fibroblast-dependent alveolar organoids, composed of human pluripotent stem cell-derived alveolar epithelial cells and primary human lung fibroblasts. This induction led to AT2 senescence, fibroblast activation mediated by epithelial cells, abnormal differentiation states in alveolar epithelial cells and an accumulation of ECM [73].

Organoids can also be directly generated by differentiating human pluripotent stem cells (either embryonic stem cells or iPSC), into 3D lung organoids that undergo a process resembling branching morphogenesis, known as lung bud organoids. These specialised structures find utility in modelling fibrotic ILDs and viral infections [74, 75]. Notably, these organoid models are characterised by the presence of mesenchymal components, rendering them particularly suitable for the study of conditions such as Hermansky-Pudlak syndrome interstitial pneumonia (HPSIP) [76]. In a separate investigation, an organoid model for IPF was engineered by introducing mutations in HPS1, HPS2 and HPS4 through CRISPR/Cas9 gene editing

applied to human embryonic stem cells. These mutations closely mirror those observed in Hermansky–Pudlak syndrome, a condition associated with a high incidence of HPSIP [73, 119]. These mutations induced an *in vitro* phenotype that correlates with the clinical incidence of HPSIP and led to an increased expression of IL-11, suggesting a novel role for this cytokine in the induction of fibrosis in organoids with HPS mutations [75].

Furthermore, others established an alternative fibrotic alveolar lung organoid model by inducing fibrosis in a mixed culture of epithelial (A549), endothelial and fibroblast cell lines that grew as organoids. Following this induction, the organoids exhibited increased fibroblast proliferation and a reduction in the lumen, which could be partially mitigated by treatment with the antifibrotic ρ -kinase inhibitor fasudil [77]. Additionally, another group used organoids to demonstrate the transdifferentiation of human alveolar epithelial type two cells (hAEC2) into metaplastic keratin (KRT)5⁺ basal cells under the influence of factors such as TGF- β signalling or CTHRC1^{hi} profibrotic mesenchyme. Since hAEC2 with basal cell characteristics are found in IPF lungs, advancing research on organoids could offer deeper insights into IPF pathogenesis [78]. Others have developed another model for studying AEC2 dysfunction which is implicated in the pathogenesis of adult and paediatric ILD. This model was created using patient-specific iPSCs carrying a disease-associated variant exclusive to AEC2 (SFTPCI73T). The study revealed that mutant iAEC2s accumulate significant amounts of misprocessed and mistrafficked pro-SFTPC protein, mirroring *in vivo* changes. Consequently, iAEC2s serve as a patient-specific pre-clinical platform for modelling the epithelial-intrinsic dysfunction at the onset of ILD [79].

Moreover, novel organoids models have been developed to mimic the structure of human respiratory bronchioles. These bronchioles house a unique secretory cell population that differs from cells in the larger proximal airways. The creation of these organoids has revealed that these respiratory airways secretory cells function as unidirectional progenitors for AT2 cells, which play a crucial role in maintaining and regenerating the alveolar niche. The authors have reported that this differentiation process is associated with WNT and Notch signalling pathways. In COPD, respiratory airways secretory cells exhibit transcriptional alterations corresponding to abnormal AT2 cell states. This phenomenon is linked to smoking exposure in both humans and ferrets. These findings identify a distinct progenitor cell type in a region of the human lung that is not found in mice, and it plays a critical role in maintaining the gas-exchange compartment while being altered in chronic lung diseases [80].

These models, among others, illustrate how the use of organoids can approach the structural complexity of interactions typically found at the organism level, although they are still in their early stages in some respects. The ability to use patient-derived material and to differentiate iPSCs into the target cells of interest provides insights into the impact of alterations at the single-cell level. This opens up significant possibilities for the discovery of new molecules and drug screening, including off-label drug repurposing.

Microfluidic systems

Organ-on-a-chip

Organ-on-a-chip is an innovative technology that mimics miniature organ models on chips which act as mini-bioreactors (figure 2 and table 1). These microfluidic systems allow for highly advanced modelling of lung diseases, particularly pulmonary fibrosis. By using longitudinally separated chambers filled with fluid or gas, it is possible to simultaneously conduct interconnected cell cultures. In the case of pulmonary fibrosis models, devices consist of channels lined with lung epithelial cells and a porous membrane that separates the airway and vascular compartments. Endothelial cells can be seeded on the opposite side of the membrane, representing the pulmonary vasculature [81]. Furthermore, the proposed model accurately replicated fibroblast behaviour and the drugs tested, pirfenidone and nintedanib, were effective in reducing fibroblast activation, confirming their potential use as antifibrotic agents in pulmonary fibrosis treatment [81]. Fibroblasts and immune cells can also be added to create a more complex microenvironment. The use of elastic materials allows for the simulation of physiological processes such as respiration, blood circulation or immune cell interactions. Currently, microfluidic systems are revolutionising research on pulmonary fibrosis and are promising tools for the development of new therapies [82].

In order to create robust organ-on-a-chip models, appropriate materials for the device's framework and suitable membranes on which cells are cultured must be carefully selected. The most commonly used materials for creating microfluidic devices are polydimethylsiloxane (PDMS), polycarbonate, polyester, hydrogels, synthetic polymers and hybrid materials such as silk fibroin-based materials. PDMS is the most commonly used due to its biocompatibility, optical transparency and ease of fabrication. PDMS has been used to create a lung-on-a-chip device that mimics the alveolar–capillary interface, enabling researchers to study gas exchange and lung inflammation [83, 84]. Polycarbonate is a thermoplastic polymer widely used

in the medical device industry due to its high strength, durability and biocompatibility. However, polycarbonate is not as elastic as PDMS, which can cause issues with cell attachment and deformation of the membrane [85]. New types of biological membranes made from lung ECM proteins, including collagen and elastin, have recently been developed. These membranes are biodegradable and stretchable, forming an array of alveoli with dimensions similar to those found *in vivo* [86]. Another class of materials with great potential in the development of lung-on-a-chip systems are hydrogels. Polyethylene glycol hydrogels can be modified to incorporate cell adhesion peptides to promote cell attachment and growth [87]. In addition to the selection of suitable materials, the choice of appropriate technologies for the fabrication of individual compartments is also crucial. For example, photolithography is commonly used to create microvasculature, while 3D printing is used to create the airway layer [82, 83].

In organ-on-a-chip models, a microporous polymer membrane acts as a barrier between the epithelial and endothelial cells, thus ensuring proper function. It must facilitate nutrient exchange and protein signal interactions between epithelial and endothelial cells while also preventing cell leakage from either side [88].

So far, successful models of lung fibrosis have been developed for studying disease pathogenesis. One study coated the device with ECM from healthy or IPF lungs. Lung epithelial cells and fibroblasts were seeded on opposite sides of the membrane. The study found that IPF ECM promoted the differentiation of fibroblasts into myofibroblasts, which are key contributors to fibrosis [89]. Another study utilised a lung-on-a-chip to investigate the effect of cyclic mechanical strain on lung fibrosis. The study found that this process increased the expression of profibrotic genes and promoted the differentiation of fibroblasts into myofibroblasts. These findings suggest that mechanical strain may contribute to the development of lung fibrosis [83].

There are high expectations for lungs-on-a-chip as a potential replacement for animal testing in the preclinical evaluation of new drugs for pulmonary fibrosis. Indeed, this tool combines the advantages of both 2D and 3D *in vitro* models, and additionally allows for the replication of physiological conditions impacting tissues in the body [90]. A recent study using this model has shown the ability of KD025 (a selective ROCK2 inhibitor) and BMS-986020 (a lysophosphatidic acid receptor 1 antagonist) in inhibiting the expression of α -SMA stress fibres and cytosolic procollagen [91], thus suggesting their antifibrotic property. It's worth noting that KD025 is currently under clinical trial investigation (clinicaltrials.gov NCT02688647).

In another study based on airway-on-a-chip model evaluated the ability of a novel drug delivery system to penetrate the mucus barrier in the airway and reach the target cells [92]. Nevertheless, microfluidic systems still require many improvements to become a viable commercial alternative to animal models. For example, the lung-on-a-chip model has lower throughput and scalability compared to 2D cell culture, which restricts its use in the early stages of drug development. Additionally, there is insufficient data to develop acceptance criteria for regulatory agencies [120].

The range of possibilities for the use of a lung-on-a-chip is much broader. Some authors used this model to study the lung toxicity and profibrotic activity of foreign material exposure, such as environmental pollutants [121] and nanoparticles including multiwalled carbon nanotubes and asbestos fibres [57].

By providing a more accurate representation of the *in vivo* microenvironment of lung tissue, these models can help to identify and test potential therapeutic agents more efficiently than traditional methods. Further research is needed to optimise the technology and to validate its use in clinical trials.

***Ex vivo* lung models**

Human lung *ex vivo* models retain native lung architecture maintaining ECM composition, stiffness and lung resident cell populations and thus are optimal models for drug discovery. The most used *ex vivo* lung model is represented by the precision-cut lung slices (PCLS) (figure 2 and table 1).

PCLS are usually prepared from fresh human or animal lung tissues filled with low melting point agarose and cut into 300–1000- μ m thick slices which are subsequently cultured *in vitro*. In PCLS, cell viability is maintained for 5–7 days [122]. In one study, PCLS derived from IPF patients were employed to investigate the functional relationship between PI3K/mTOR signalling and matrix deposition in IPF [93]. The application of a PI3K/mTOR inhibitor, specifically an analogue of GSK2126458, led to a reduction in the release of the human procollagen 1 amino-terminal peptide (P1NP) in IPF tissue slices. It's worth noting that the safety of GSK2126458 in IPF patients was demonstrated in a phase 1 clinical trial (clinicaltrials.gov NCT01725139) [123]. Further clinical trials are necessary to evaluate the clinical effectiveness of GSK2126458 in mitigating IPF.

Pre-clinical findings from multiple *in vivo* models [124] have indicated that caveolin-1 could serve as a promising target for IPF and other progressive ILDs. Interestingly, caveolin-1 derived peptides, such as CSP7 and LTI-03, have demonstrated the ability to inhibit the expression of profibrotic proteins like collagen 1A1, fibronectin and α -SMA [124], as well as enhance the survival of alveolar epithelial cells [94], in PCLS obtained from end-stage IPF and nonspecific interstitial pneumonia patients. Based on these promising findings, a phase 1 study established the tolerability of aerosolised CSP7 dry powder in healthy subjects (clinicaltrials.gov NCT04233814), and another clinical trial is ongoing (clinicaltrials.gov NCT05954988) to determine the optimal dose, safety, and tolerability of LTI-03 administered *via* inhalation to IPF patients.

Cultures of PCLS derived from fibrotic regions of human IPF lung tissues have also proven valuable in assessing the potential utility of metformin for IPF treatment. A study reported that metformin, a commonly used antidiabetic drug, when administered to IPF-derived PCLS, resulted in improved lung structure, reduced collagen deposition, and increased lipid-droplet accumulation in fibroblasts [95]. This suggests that metformin promotes lipogenic differentiation in myofibroblasts. In conjunction with *in vitro* models employing primary human IPF lung fibroblasts, the authors observed that lipogenic transdifferentiation of fibroblasts involves the upregulation of BMP2 and activation of peroxisome proliferator-activated receptor- γ . A recent retrospective study aimed at evaluating the clinical benefits of metformin in 2200 IPF patients with type 2 diabetes found that metformin therapy was associated with a significant reduction in all-cause mortality. However, the authors recommended further investigation through randomised clinical trials before considering the implementation of metformin in the clinical management of IPF [125].

A recent study [96] applied PCLS from IPF patients to evaluate the impact of AT2 cells on fibrosis progression. Inhibition of Notch1 signalling in human IPF PCLS improved the differentiation of AT2, leading to the restoration of SPB production and a reduction in collagen deposition.

Since PCLS are often prepared from IPF explants which typically represent end-stage disease, a study developed a model of human PCLS to characterise early fibrosis-like changes [97]. Specifically, human PCLS from patients without fILD/IPF were treated with a profibrotic cocktail composed of TGF- β , PDGF-AB, TNF- α and lysophosphatidic acid to induce fibrosis. This model is used not only to study the mechanisms leading to fibrosis but also for drug testing. For example, LEHMANN *et al.* [98] used this model to determine the mechanism of action of nintedanib and pirfenidone, and specifically to identify which type of cells and proteins can represent a target for these two drugs in order to select the most responsive patients and to develop new drugs. Interestingly, by using murine PCLS (from bleomycin-challenged mice) or human PCLS (with early fibrosis induced by the fibrotic cocktail), the authors found that, in contrast to pirfenidone, nintedanib improves AT2 functions in terms of SPC secretion, and this activity could contribute to antifibrotic effect of this drug.

Advantages and limitations of *in vitro* lung fibrosis models

One major challenge in the study lung fibrosis *in vitro* is represented by the development of relevant models to study pathogenesis and to develop effective therapies for human pulmonary fibrosis. Until now, drugs that demonstrated benefit in reducing lung fibrosis in pre-clinical animal models have often failed in human clinical trials. Thus, recent efforts are directed on developing alternative *in vitro* models able to predict the effects of a substance on the whole organism.

The simplest *in vitro* models are represented by fibroblasts or alveolar epithelial cells in 2D cultures, which have been useful to understand mechanisms underlying fibrosis development, such as fibroblast to myofibroblast differentiation [21] and EMT [35, 126].

However, monocellular culture models lack the variety of cell types and the ECM environment which contribute to lung homeostasis and function. Thus, they oversimplify the lung tissue and are unable to replicate important signals arising from cell–cell and cell–matrix interactions occurring in the native tissue [127]. Moreover, static 2D systems are not able to mimic the dynamic lung mechanical stress that occurs during inspiration/expiration cycles.

3D *in vitro* models replicate native lung tissue architecture with greater accuracy. They can be created using scaffolds (such as hydrogels) or can form through self-assembled structures often referred to as spheroids. 3D models allow for the study of cell migration, cellular traction and integrin adhesions in all three spatial planes. However, regardless of the type of cells (cell lines or patient-derived cells) used, they frequently lack the presence of progenitor cells [128], thus resulting in a greater difficulty, with respect to

2D culture systems, in sustaining long-term 3D culture and in generating complex, multicellular structures due to hypoxia and necrosis in the spheroid core [129].

These limitations are overcome with the use of organoids obtained from iPSCs derived from patients. In addition, iPSC-derived organoids maintain the mutations and aberrations present in the patient even if the retro-differentiation process applied to generate iPSCs can reverse the age-related epigenetic changes which are important risk factors in lung fibrosis development [130]. Furthermore, once established, the iPSC-derived organoid model can be scaled up, allowing high-throughput screening of drugs, off-label drugs and genomic variations [131]. However, at the same time, this model does not reproduce all cell–cell interactions present in the native tissue [131, 132]. For example, in many organoids the role of the nervous system, which is one of the main signalling pathways within a tissue microenvironment, is completely overlooked [131, 132]. In addition, organoids often lack vascularisation [133], which significantly limits their *in vitro* survival and their progression to more advanced stages of differentiation that would better mimic the patient's situation [134].

Microfluidics enable the creation of organ-on-a-chip models that better mimic vascularisation and cell-cell interactions mediated by fluids (blood, lymph, etc.). However, due to engineering limitations, the number of tubes used to mimic vessels in such systems is extremely fewer than in real tissues, thus reducing the actual impact of fluid-mediated parameters (including perfusion, shear and mechanical stress) on cells present in the system [135, 136].

The *in vitro* model that better retains the native lung architecture maintaining ECM composition, stiffness and viable lung resident cell populations seems to be PCLS. However, this model also has limitations, such as the low maintenance of tissue integrity during long-term culture which prevents the study of fibrogenic and fibrolytic events or molecules occurring over weeks, as observed in *in vivo* models. Furthermore, PCLS are static, although strategies have been developed to mimic breathing-related stress. In addition, cell trafficking from the blood into the lungs, and *vice versa*, cannot be assessed.

Conclusions and questions for future research

- The biological complexity of the fibrotic process, together with its clinical characteristics (age-related disease, diagnosed in advanced stages and highly variable progression from patient to patient), challenge the translation from bench to bedside.
- The current direction in determining therapeutic approaches for diseases is personalised medicine, where a specific drug is selected or developed for an individual patient or a selected group of patients.
- The evolution of *in vitro* models is leading toward the creation of devices that closely replicate pathological processes in patients. A prime example is the application of organoid technology in new therapies for patients affected by cystic fibrosis with rare mutations, demonstrating the feasibility of this approach and enabling the effective implementation of personalised medicine.
- To achieve *in vitro* models that are more representative of human physiology/pathology, it is essential to enhance model complexity by incorporating the nervous system and vascularisation, and addressing the absence of the immune component, as these elements play a relevant role in many diseases.
- The development of new FILD models will provide a better understanding of the pathogenetic mechanisms of pulmonary fibrosis and discover new therapeutic targets for the treatment of pulmonary fibrotic diseases.

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