# Alveolar Progenitor Cells in Lung Damage and Regeneration in Pulmonary Fibrosis

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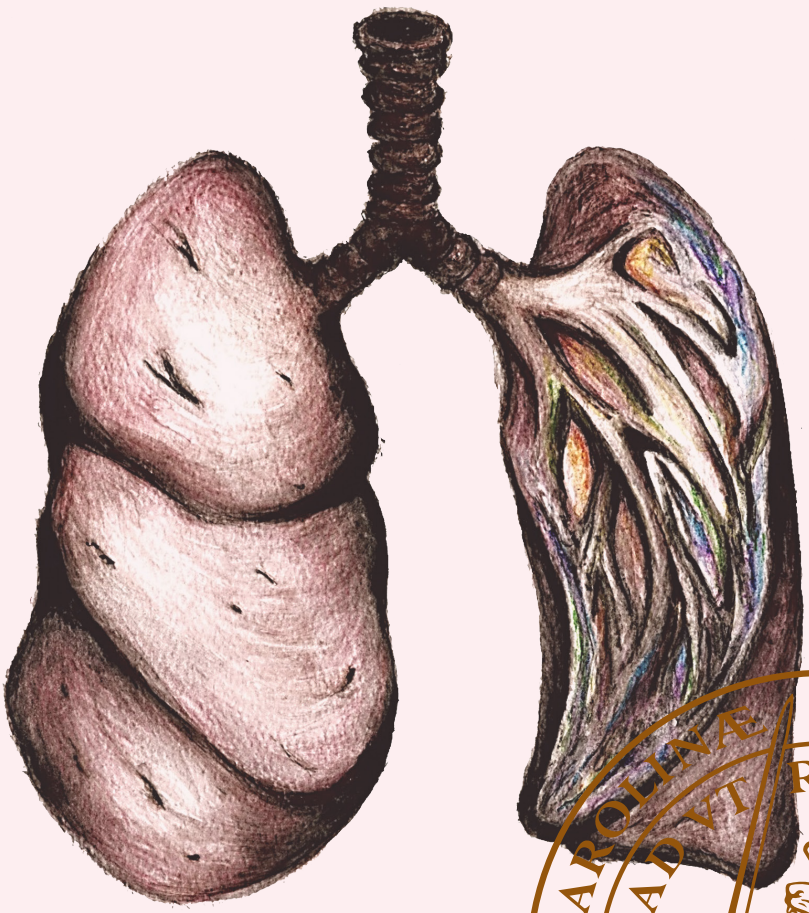

# Alveolar Progenitor Cells in Lung Damage and Regeneration in Pulmonary Fibrosis

# Alveolar Progenitor Cells in Lung Damage and Regeneration in Pulmonary Fibrosis

Doctoral thesis

Victoria Ptasinski

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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**Abstract:**

The lung is a vital organ in the body enabling us to breathe. During chronic lung diseases, the function of the lung is impacted by damage to the tissue structure. Idiopathic pulmonary fibrosis (IPF) is a devastating chronic lung disease in which aberrant tissue remodelling occurs in the distal regions of the lung. A contributing factor to the development of IPF is repeated injury to the epithelial cells of the alveoli. The aim of this thesis was to identify the contribution of dysregulated molecular pathways and alveolar cell populations to the pathogenesis of IPF and lay the ground for future design of novel anti-fibrotic therapeutics. In the work included in this thesis, selected markers proposed to be involved in the development of pulmonary fibrosis were studied, and findings from animal models of pulmonary fibrosis were linked to human lung tissue from IPF patients. Further, a novel in vitro model with human alveolar epithelial cells differentiated from induced pluripotent stem cells was developed. This model was applied to study the induction of recently described fibrotic, aberrant epithelial phenotypes found in the lungs of IPF patients. In addition, technical advances were developed related to isolation of lung epithelial cells from mouse lung tissue and visualisation of whole mouse lung by light-sheet fluorescence microscopy. The findings presented in this doctoral thesis contribute to increased understanding of the pathophysiology of IPF which will enable development of novel anti-fibrotic therapeutics, and provide valuable technical contributions to the broader field of lung research.



**Keywords:** Idiopathic pulmonary fibrosis, alveolar epithelium, aberrant basaloid cells, bleomycin model, induced pluripotent stem cells

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II. Hani N. Alsafadi, John Stegmayr, Victoria Ptasinski, Iran Silva, Margareta Mittendorfer, Lynne A. Murray, Darcy E. Wagner. **Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations.** *Stem Cell Reports.* 2022; 17(12): 2718-2731.

III. Victoria Ptasinski, Susan J. Monkley, Karolina Öst, Markus Tammia, Hani N. Alsafadi, Catherine Overed-Sayer, Petra Hazon, Darcy E. Wagner*, Lynne A. Murray*. **Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids.** *Life Science Alliance.* 2023; 6(8):e202201853.

IV. Hani N. Alsafadi, Victoria Ptasinski, Iran A. N. Silva, Isabel Tamargo, Emil Rehnberg, Thomas Volckaert, Botilda Lindberg, Annika Borde, Darcy E. Wagner, John Stegmayr. **inFLATION: infusion of Fluorescently Labeled Antibodies in Tissue of Intact OrgaNs for visualization of the rodent lung using light-sheet fluorescence microscopy.** *Manuscript in preparation for submission.*

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# List of papers related to the thesis

I. Victoria A. Ptasinski, John Stegmayr, Maria G. Belvisi, Darcy E. Wagner, Lynne A. Murray. **Targeting Alveolar Repair in Idiopathic Pulmonary Fibrosis.** *American Journal of Respiratory Cell and Molecular Biology.* 2021; 65(4): 347-365.

# Preface

In our daily lives, we are constantly experiencing events which are harmful to us in different ways – many of us can recognise the itch from a paper cut in the skin, or remember the pain from a broken leg. Months later, the cut in the skin is just a memory, and the leg is ready for long walks again. Yet how does the skin or the bone know how to repair themselves? What triggers them to start repairing, and how do they know when to stop? While these answers are not clear to my brain, thankfully my skin and bones seem to have these mechanisms figured out.

Although my doctoral thesis is not describing the skin or the bones, the same questions can be asked about another of our vital organs – the lung. In fact, I am certain that many more people asked themselves the same questions during the years I performed work for this thesis, which happened to coincide with the outbreak of the COVID-19 pandemic. COVID-19 is an example of an acute lung disease, which is apparent to us within days or weeks from initiation. Normally however, the lung is a "silent" organ – though we take breaths every day, we do not notice the early signs of injury in our lungs which may lead to the development of severe illnesses later in life. Patients diagnosed with chronic lung diseases are often identified once they are advanced in their disease, at which point it is too late to prevent it from progressing further. But why does the diseased lung not seem to know how to repair itself? Or does it know, but is just failing to do so effectively under some circumstances?

My doctoral thesis is based on four separate studies, each related to the topic of lung injury and repair. The studies are mostly focused on idiopathic pulmonary fibrosis (IPF), which is an incurable lung disease with rapid onset and fatal outcome. However, the techniques described in the included studies are also applicable to the wider field of lung biology. In all of us, the lung lies close to our hearts, and I hope you will find enjoyment in reading my doctoral thesis.

Victoria Ptasinski

# Key abbreviations

| | |
|---|---|
| IPF | Idiopathic pulmonary fibrosis |
| AEC1 | Alveolar epithelial type 1 cell |
| AEC2 | Alveolar epithelial type 2 cell |
| SP-C | Surfactant protein C |
| KRT | Keratin |
| ESC | Embryonic stem cell |
| iPSC | Induced pluripotent stem cell |
| iAEC2 | iPSC-derived alveolar epithelial type 2 cell |
| ECM | Extracellular matrix |
| FC | Fibrosis cocktail |
| TGF-β | Transforming growth factor β |
| 3DLD | 3D-printed lobe divider |
| LSFM | Light-sheet fluorescence microscopy |
| RNAseq | RNA sequencing |
| scRNAseq | single-cell RNA sequencing |

# Populärvetenskaplig sammanfattning

Vi kan andas genom våra lungor. Dessa är fyllda med strukturer som liknar små ballonger som fylls med luft under andning. När dessa små lungballonger blir skadade kan vi inte längre andas in tillräckliga mängder av syre vilket leder till att vi blir sjuka. Idiopatisk lungfibros (IPF) är en sjukdom som påverkar de små ballong-liknande strukturerna i lungorna som ansvarar för andningen. När sjukdomen förvärras kan inte lungorna längre fungera ordentligt. Detta leder till döden inom i genomsnitt 2-3 år efter diagnos eftersom det inte finns några botemedel mot denna sjukdom. Orsaken till IPF är inte känd men tros vara orsakad av skador på cellerna, kroppens allra minsta byggstenar, i lungan som ansvarar för andningen.

I min doktorsavhandling studerade jag möjliga orsaker till skadorna som drabbar cellerna i lungan vid sjukdomar som IPF. Den främsta anledningen till valet av detta ämne är att det idag inte finns några läkemedel som kan rädda patienterna från att dö av denna sjukdom. I studierna som ingår i denna avhandling undersöktes olika typer av förändringar i lungan för att hitta möjliga orsaker till IPF som kan behandlas. För att kunna studera skadan som cellerna i lungan utsätts för hos patienter med IPF utvecklades ett nytt experimentellt modellsystem baserat på stamceller. Den nya modellen kan i framtiden användas för att testa nya läkemedel för behandling av IPF. Dessutom utvecklades förbättringar av flera experimentella tekniker som ofta används i laboratoriet och som kommer att kunna förenkla framtida studier av lungan.

Resultaten av forskningen som presenteras i denna avhandling kommer att möjliggöra upptäckt och utveckling av nya läkemedel mot IPF, vilket i framtiden kommer att göra det möjligt att bota sjukdomen.

# Streszczenie popularnonaukowe

Idiopatyczne włóknienie płuc (angielski skrót IPF) należy do nieuleczalnych chorób płuc. Choroba atakuje pęcherzyki płucne, które odpowiadają za wymianę tlenu przy oddychaniu. Przy IPF pęcherzyki twardnieją, przy czym występuje zaburzenie wymiany tlenu. Płuca tracą swoją elastyczność, z efektem czego nie mogą prawidłowo pracować. Ostatecznie prowadzi to do niedotlenienia organizmu i śmierci w ciągu przeciętnie 2-3 lat po ustaleniu diagnozy. Przyczyna IPF nie jest znana, lecz są przypuszczenia że jest to skutek wielokrotnego uszkodzenia i nieprawidłowej regeneracji pęcherzyków płucnych.

Badania naukowe uwzględnione w mojej pracy doktorskiej miały na celu ustalenie przyczyny zaburzonej regeneracji płuc podczas chronicznych chorób takich jak IPF. Głównym powodem tej tematyki to brak leków które zapobiegają lub hamują niszczenie pęcherzyków płucnych. Wnioski z przeprowadzonych badań pozwolą na wynalezienie nowych leków przeciw IPF, które w przyszłości umożliwią uleczenie choroby.

# Popular science summary

Our lungs enable us to breathe through their small, balloon-like structures which are filled with air during breathing. In situations when those small balloons become injured, we cannot breathe and become sick. Idiopathic pulmonary fibrosis (IPF) is a disease occurring in these small balloon-like structures of the lungs responsible for breathing. As the disease becomes worse, the lungs become unable to expand and cannot work properly. This leads to death within an average of 2-3 years after diagnosis as there are no cures available for this disease. The cause of IPF is not known, but is believed to be the result of damage to the cells lining the balloon-like structures in the lung responsible for the breathing.

In my doctoral thesis, I studied the possible causes for the injury occurring in the lung cells during diseases such as IPF. The main reason for this topic is that there are no drugs available today which can rescue the patients from dying of this disease. In this thesis, changes in the lung were studied which are linked with the lung injuries seen in IPF. In addition, a new way of studying the damage which the lung cells are undergoing in patients with IPF was developed. The method, which is based on stem cells, can be used for testing of new drugs for treatment of IPF. Also, improvements of other experimental methods which are often used in the laboratory in studies of the lung were developed.

The results presented in this thesis will provide necessary tools for the discovery and development of new drugs against IPF, which in the future will make it possible to cure the disease.

# Abstract

The lung is a vital organ in the body enabling us to breathe. During chronic lung diseases, the function of the lung is impacted by damage to the tissue structure. Idiopathic pulmonary fibrosis (IPF) is a devastating chronic lung disease in which aberrant tissue remodelling occurs in the distal regions of the lung. A contributing factor to the development of IPF is repeated injury to the epithelial cells of the alveoli. The aim of this thesis was to identify the contribution of dysregulated molecular pathways and alveolar cell populations to the pathogenesis of IPF and lay the ground for future design of novel anti-fibrotic therapeutics. In the work included in this thesis, selected markers proposed to be involved in the development of pulmonary fibrosis were studied, and findings from animal models of pulmonary fibrosis were linked to human lung tissue from IPF patients. Further, a novel *in vitro* model with human alveolar epithelial cells differentiated from induced pluripotent stem cells was developed. This model was applied to study the induction of recently described fibrotic, aberrant epithelial phenotypes found in the lungs of IPF patients. In addition, technical advances were developed related to isolation of lung epithelial cells from mouse lung tissue and visualisation of whole mouse lung by light-sheet fluorescence microscopy. The findings presented in this doctoral thesis contribute to increased understanding of the pathophysiology of IPF which will enable development of novel anti-fibrotic therapeutics, and provide valuable technical contributions to the broader field of lung research.

# Introduction

## Overview of lung development

The lung is necessary for survival of many species, including humans. Our lungs enable us to live through breathing which is the act of oxygen delivery and waste removal from the blood. This vital task is performed through harmonised function of the many different, intricate structures of the lung. These lung structures start to develop already around the 4$^{th}$ week of human gestation and go through several stages prior to forming a functional, mature lung (1, 2) (**Figure 1**). The developmental process of the lung initiates from one of the three germ layers in the embryo called the endoderm. At this stage, a part of the endodermal layer forms the anterior foregut endoderm which starts budding out, a process termed branching morphogenesis. Two lung buds are formed through evagination of the endoderm. This initial stage extends until around the 7$^{th}$ week of human gestation. In the next developmental stage, which extends until around the 16$^{th}$ week of development in humans, further branching of the two lung buds forms smaller airways. This is termed the pseudoglandular stage. During this phase, the cell types lining the surfaces of the airways start to develop. The formation of the initial structures which enable gas exchange is initiated during the canalicular and saccular stages, occurring from around the 16$^{th}$ week of development and until just after birth. During this phase, specialised cells which later play important roles in the gas exchange process differentiate and are clearly distinguishable from other cells. The last stage of lung development, termed the alveolar stage, is initiated after birth in humans. At this stage, the structures and cell types necessary for the gas exchange process develop and mature fully. During the early years of life and until adolescence, the lung continues to expand in size as the thorax grows.

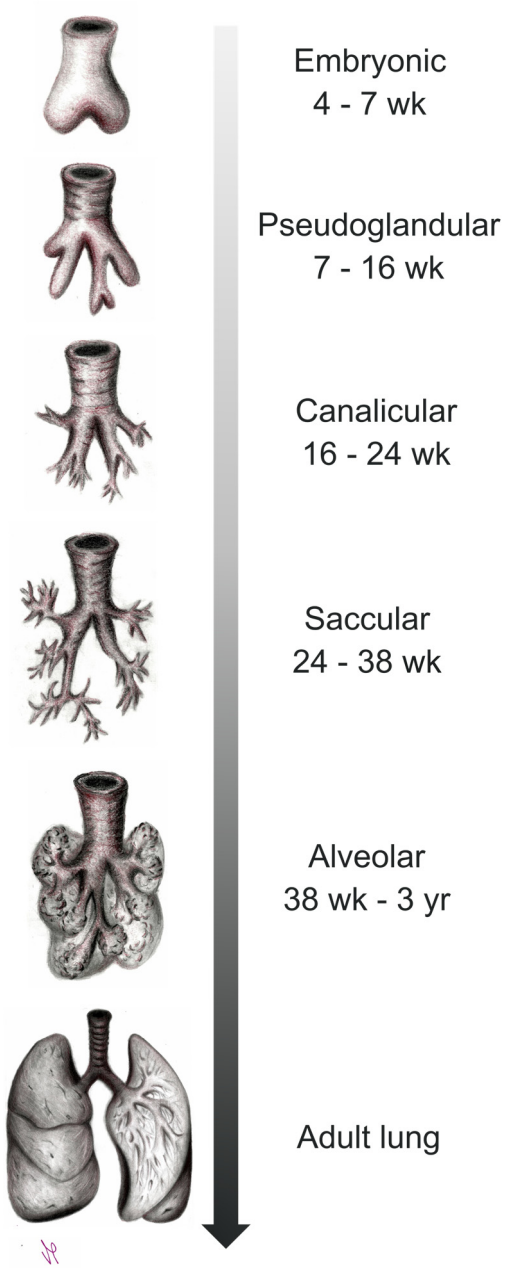



**Figure 1:** The different stages of human lung development. Illustrations by Victoria Ptasinski.

# The anatomical structure of the adult lung

The structure of the adult lung in mammals can be compared to a tree which is positioned upside down in our chests (**Figure 2**).

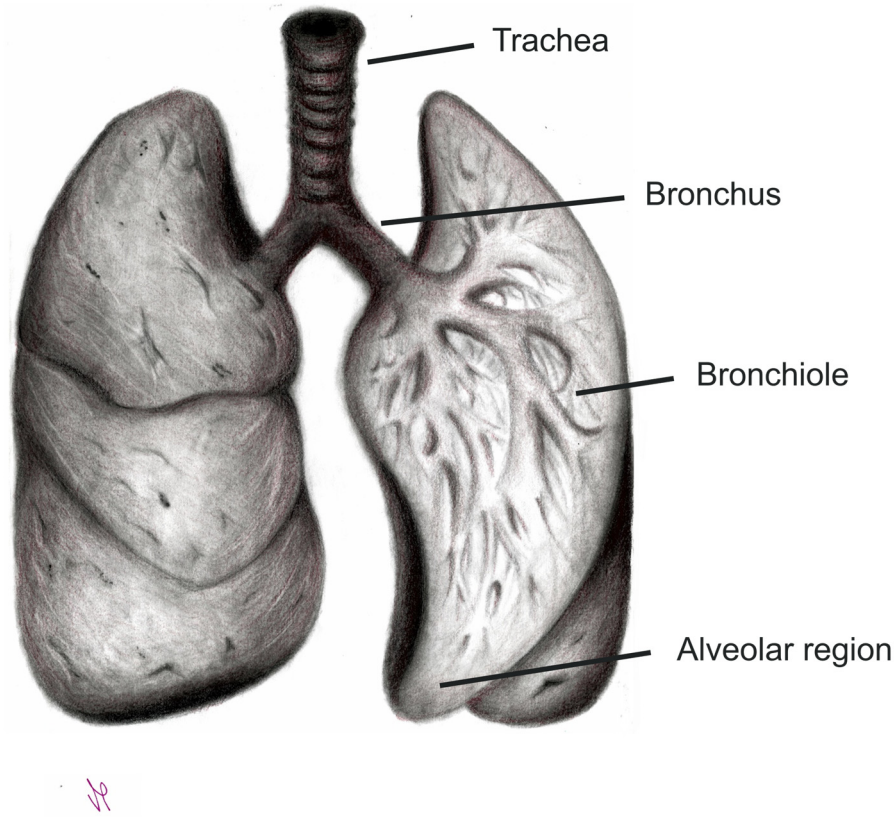



**Figure 2**: The proximo-distal structures of the human lung. Illustrations by Victoria Ptasinski.

When we take a breath through our nose or mouth, the air travels to reach the main airway of the lung, called the trachea. From there, the air is transported down into the different lobes of the lung through the bronchi which are branching out from the trachea (3). Both the bronchi and trachea are surrounded by cartilage rings to maintain patent airways. The human lung consists of in total 5 lobes – the right lung consists of 3 and the left lung consists of 2 (4). Inside the human lung lobes, the air travels further down through bronchioles, which are airway structures smaller than the bronchi. These branch out for as many as 23 branchings to form a complex transport

network for the air inside the lung (5). In contrast to the bronchi, bronchioles do not have cartilage rings, causing them to be more elastic than the trachea and bronchi (3). The human bronchioles are further divided into two subcategories – the conducting bronchioles, which are responsible for effective transport of the incoming air further down into the lung, and the respiratory bronchioles (3). In this most distal part of the human lung, the respiratory bronchioles branch into the alveolar ducts, from which the alveolar sacs are budding like grapes on a vine (5). The alveolar sacs contain balloon-shaped structures surrounded by capillaries, termed alveoli, in which the gas exchange occurs (6).

Although the overall anatomical structure is generally conserved among mammals due to the important functions each of the structures have in the breathing process, there are some differences. In some of the included studies in this thesis, mouse lungs are used for experiments due to the fact that they are more readily available. In contrast to human bronchi, the mouse bronchi do not contain any cartilage rings (7). Moving down to the lung lobes in mice, their right lung is composed of 4 lobes instead of 3 as in humans, while their left lung is not divided at all in contrast to the human lung (8, 9). Moreover, respiratory bronchioles are lacking in the smaller mouse lung (8, 10). Instead, the mouse alveoli branch out directly from the conducting bronchioles through transitional areas called the bronchioalveolar duct junctions (9, 10) (**Figure 3**).

In addition to the airways and alveolar structures, the lung contains a large network of blood vessels and capillaries enabling the blood to be transported for the gas exchange (5). As the blood becomes oxygenated and releases the waste products from used air back into the alveolus, these waste products travel the same way back out from the lung and into the mouth and nose, where we exhale the old air and take a new breath. The whole lung is enclosed in the pleura, which forms a sac holding all of the structures of the lungs together inside the thorax (11). The innermost layer of the pleura encompassing the outside of the lung is called the visceral pleura, and the outer layer attached to the thoracic wall is called the parietal pleura. The pleurae produce fluid filling the cavity between them, which lubricates the outside of the lung and enables it to expand freely during inhalation and prevent it from collapsing during expiration (11).

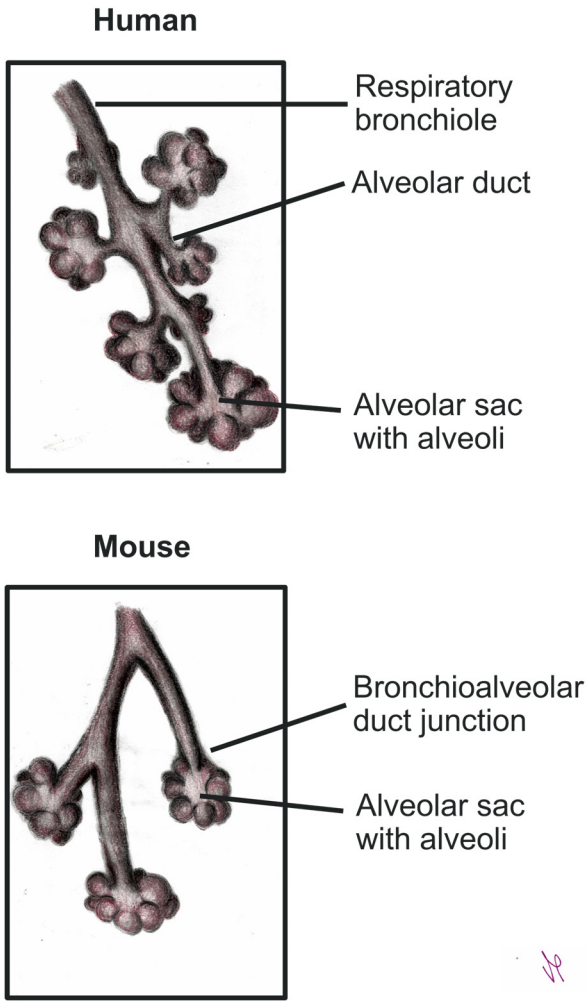



**Figure 3:** The structural differences between the human and the mouse lung in the alveolar region. Illustrations by Victoria Ptasinski.

## Cell types of the lung

With the development of advanced techniques allowing for discovery of novel cell types in the lung, it is known today that the lung is a complex organ harbouring more than 40 different cell types (12). The cell types are associated with different locations of the lung and have distinct functions. The physiological function of each different lung cell type under homeostatic conditions is of importance to understand, as these are often perturbed in the diseased lung.

*Epithelial cells*

The epithelial cells form the outer layer in the different regions of the lung and are the first barrier being exposed to the external environment. The structure of the lung epithelium and the cell types vary along the proximal-distal axis (**Figure 4**). The trachea and bronchi are lined by an epithelial layer forming a pseudostratified structure, meaning closely packed cells appearing to be growing in different layers. This epithelial layer is mainly composed of basal cells, secretory cells and ciliated cells, but also of less prevalent cell types including the pulmonary neuroendocrine cells, tuft cells and pulmonary ionocytes (7, 13, 14). The bronchioles are less complex in their cellular composition, having a cuboidal epithelium consisting mainly of secretory and ciliated cells (14). In the mouse, only the trachea is lined by a similar pseudostratified epithelium as found in the human bronchi (7, 14). Hence, the mouse trachea is more similar to human bronchi in terms of cellular composition. The bronchi in mice are instead lined by the simpler, cuboidal epithelium which does not contain the basal cells (7).

The lung has an ability to maintain the integrity and function of the epithelium through resident progenitor cells. The basal cells are the progenitor cells of the bronchial epithelium, with the ability to differentiate into secretory and ciliated cells under homeostatic conditions and following injury (15, 16). The progenitor cell capacity of the basal cells has been functionally demonstrated in animal models of lung regeneration (15) and in air-liquid interface culture of basal cells *in vitro* (17). Also, studies of the human bronchial epithelium have identified the occurrence of similar mutations of mitochondrial DNA (which are passed on through cell division) among basal cells, secretory cells and ciliated cells, which suggests that these cells are descendants of the basal cells also in the human lung (16). Recent studies of the mucociliary epithelium development in *Xenopus laevis* have suggested that the differentiation trajectories of basal, multiciliated and goblet cells separate earlier during embryonic development (18), indicating differences in the basal cell differentiation potential in development and in the adult lung. Structurally, the basal cells form the basal layer of the pseudostratified epithelium in the airway. As they have this important function, they are characterised by high expression of structural proteins such as keratins (KRT) (19, 20).

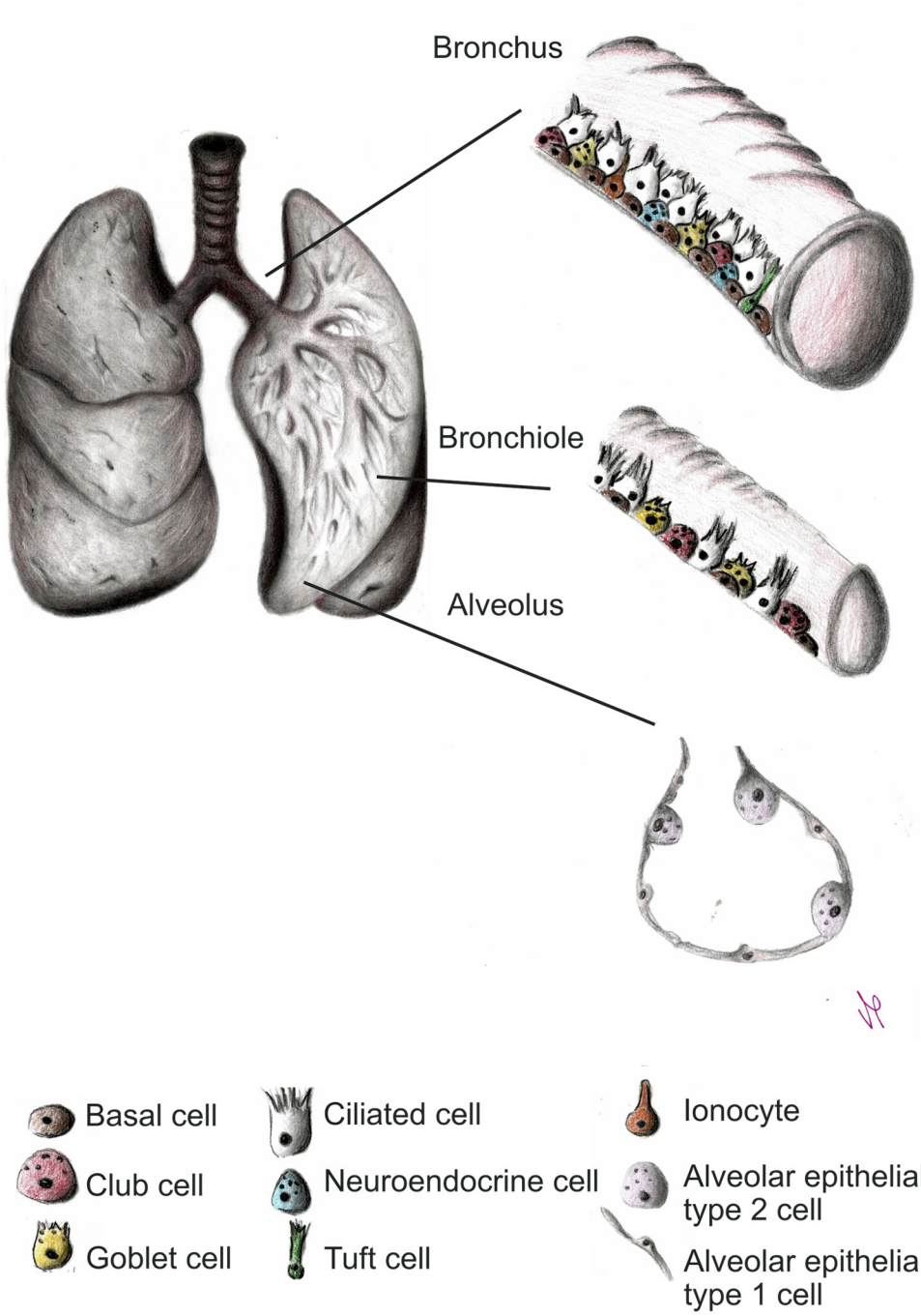



**Figure 4:** The variation in epithelial cell types along the proximo-distal axis in the human lung. Illustrations by Victoria Ptasinski.

The secretory cells include the club cells and goblet cells, and their function is to secrete factors from their granules which in different ways protect the lung from potentially harmful agents (21, 22). Club cells produce secretoglobins, which protect the epithelium from infections and oxidative stress (21). Goblet cells secrete mucus containing various types of mucins, which clear the airways of particles and infectious agents (22).

The physical clearance of the airways is enabled by the ciliated cells. These cells transport the mucus out of the airways by coordinated beating of their cilia, which face most outwards inside of the airways (23). Impaired ciliary beating or affected length or number of cilia are believed to be contributors to chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) (24).

The pulmonary neuroendocrine cells are innervated epithelial cells, which upon damage of the epithelium release neuropeptides which affect both the resident cells in the lung and immune cells (25). They are therefore considered as communicators connecting the immune and nervous systems in the lung. Similarly, the tuft cells are chemosensory cells and have both neuronal and immunological functions (26).

The less prevalent cells of the pseudostratified epithelium are not as well characterised in terms of function, partially due to the fact that some of them have only recently been discovered to be present in the lung (27, 28). The pulmonary ionocytes are suggested to be derived from the basal cells (27, 28). As they have a high expression of ion channels, including the cystic fibrosis transmembrane conductance regulator (CFTR), it has been suggested that they regulate the transport of ions and are involved in the pathogenesis of diseases such as cystic fibrosis where mutations of the CFTR gene are present (27, 28). However, the definitive function of the pulmonary ionocytes in the lung or their possible contribution to cystic fibrosis is still not known.

The epithelium in the alveoli is markedly different in structure and cell types compared to the airways. The alveoli contain alveolar epithelial type 1 cells (AEC1), which are thin cells forming the alveolar wall through which gases can pass during gas exchange (29). To prevent the delicate alveoli from collapsing during breathing, the alveolar epithelial type 2 cells (AEC2) secrete surfactant which helps in maintaining the surface tension inside the alveolus (30, 31). The AEC2 also have the important role of being the progenitor cells of the alveolar epithelium, as they are able to differentiate into AEC1 and self-renew under homeostatic conditions and during injury (32-34). The mouse lung harbours an additional, specific subpopulation of

cells with regenerative capacity called bronchioalveolar stem cells (BASC), which are able to differentiate into cells of both bronchial and alveolar origin (35, 36). Thus far, equivalents of BASC have not been identified in the human lung.

*Mesenchymal cells*

The mesenchymal cell layer lies underneath the epithelium and provides it with structural support together with the extracellular matrix (37). Although the exact cellular composition of the mesenchyme is not fully established, several main groups of cell types have been identified by independent single-cell studies; fibroblasts, endothelial cells, smooth muscle cells, pericytes, and lymphatic cells (12, 38-40).

The fibroblasts are cells with migratory capacity which can be present in different locations of the lung, such as around the airways, around the vasculature or around the alveolar walls (41). Their primary function is to produce components of extracellular matrix, such as collagen, to structurally support the epithelial layer (41). Moreover, due to their close localisation to the epithelial cells, there is extensive cross-talk between the fibroblasts and the epithelial cells throughout development and in the adult lung (42). For example, lung fibroblasts communicate with the lung epithelial cells to support their differentiation by secretion of growth factors during development (43) and from birth, they coordinate the wound healing process (44).

The endothelial cells line the inside of the blood vessels and their main role is to maintain the barrier through which nutrients and components necessary to cells are passed (45). Blood vessels and airways are surrounded by smooth muscle cells, which have a contractile function enabling constriction and dilation of airways and vessels in response to stimuli (46). The walls of the capillaries, which are the smallest blood vessels present in the lung in the alveoli, are also built up of a cell type called the pericytes (47). Pericytes have important functions involved in regulating the vascular homeostasis by signalling to the endothelial cells (48).

Lymphatic cells form a parallel network of lymphatic vessels surrounding the airways, vasculature and around the pleura (49). The lymphatic system is important for the ability to transport antigens and immune cells from the lung to the lymph nodes, which enables the activation of the immune system during respiratory infections (50).

*Immune cells*

The lung is constantly exposed to the external environment, which is a source of both harmful and non-harmful agents. Therefore, the lung must maintain a balance between tolerance and immune activation. This is the task of the immune cells present in the lung (51). Although a detailed description of the immune system and its components in the lung is outside the scope of this thesis, some populations of immune cells are believed to be particularly involved in the pathogenesis of chronic lung diseases which will be discussed in later sections. This includes the lung-resident macrophage types called alveolar macrophages and interstitial macrophages, which arise from precursors present already in the yolk sac and the liver during foetal development (52). The alveolar macrophages reside in the alveolar spaces in the lung and are thus the first line of defence against pathogens and harmful agents. They have the important role of secreting anti-inflammatory agents regulating other resident immune cells present in this region (53, 54) as well as the alveolar epithelium (55, 56). Upon infection, the alveolar macrophages contribute to clearing of the inhaled pathogens by phagocytosis (57) in addition to maintaining controlled activation of the immune system. Interstitial macrophages reside within the alveolar tissue (58). Although their function is less characterised than that of the alveolar macrophages, they are considered to play an important role in regulating the immune response by secretion of cytokines (59). They are however considered to be less engaged in phagocyting pathogens upon infection, compared with the alveolar macrophages (60) but more capable of presenting antigens (61).

## The extracellular matrix (ECM)

All structural cell types in the lung are attached to the extracellular matrix (ECM) through different types of adhesion receptors, which enables communication between the cells and their surrounding microenvironment (62). The ECM provides structural support to the cells but also functions as their niche of growth factors which are able to guide the cells in their growth dynamics (63). Although the focus in this thesis lies mostly on the behaviour of the cells in the lung, the ECM is able to instruct the cells to induce behaviours such as phenotypic changes and migration (64). Therefore, a brief description of the main constituents of the ECM is covered in this section.

The human ECM is predicted to be built up of around 300 different proteins which form the core structures (65). These can be further divided into two main parts; the basement membranes, which lie directly under the epithelial

and endothelial layers, and the interstitial matrices which are attached to the basement membrane, which form mesh-like structures (63). Collagen is a large constituent of the total protein content in the lung and is a fibrillar type of protein with low elasticity, which is responsible for maintenance of the structural architecture of the ECM (63). The predominant types of collagen present in the alveolar region of the lung are type I and III (66). Together with a highly elastic ECM constituent termed elastin, the collagens form a meshwork capable of expansion and recoil during breathing (63). Other constituents of the interstitial ECM are different members of glycoproteins, including fibronectin and tenascin C (67). The glycoproteins are built up of a core protein with attached polysaccharides or glycosaminoglycans which make them hydrophilic (63). This property enables the glycoproteins to form hydrogel-like structures, which provide elasticity to the ECM. In several chronic lung diseases, the components of the ECM are abnormally altered which affects physical properties such as stiffness and elasticity (68-70). This impacts the capacity of the lung to expand and recoil, thereby impacting our ability to breathe. Moreover, re-cellularisation experiments of diseased mouse lung ECM have demonstrated that the disease-related changes in composition and mechanical properties of the ECM impact the proliferation and viability of repopulating epithelial cells (71). In addition, epithelial and mesenchymal cells isolated from fibrotic lungs often have increased expression of adhesion molecules including integrins, cadherins and actins which connect the cells with each other and to the ECM (72, 73). This indicates that the cells alter their responses to the changing ECM properties, which impacts their own function and that of the cells around in disease.

## Idiopathic pulmonary fibrosis (IPF)

IPF is the most devastating disease of the chronic lung disease group called the interstitial lung diseases, which is a subclass characterised by inflammation and aberrant remodelling of the ECM in the distal regions of the lung. Patients diagnosed with IPF are predominantly middle-aged or elderly (with exception for familial IPF, which may occur earlier) and more frequently male (74). The term “idiopathic” in IPF refers to the unknown disease aetiology. There are proposed risk factors predisposing certain individuals to developing IPF, such as family history of IPF, tobacco smoking, gastroesophageal reflux and chronic viral infections (75). IPF is considered a rare lung disease and the adjusted prevalence is varying

dependent on geographical regions (0.57-4.51 per 10 000 in the Asia-Pacific regions, 0.33-2.51 in Europe, and 2.40-2.98 in North America) (76). The global incidence rate is currently estimated to be 0.09-1.30 per 10 000 (76). Even though IPF is rare, the outcome for patients is poor and the median survival is estimated to be only 2-3 years after diagnosis (77).

## Diagnosis

Patients with suspected IPF often present symptoms such as shortness of breath and cough of unknown cause (78). The diagnosis of IPF is primarily based on chest scans using high-resolution computed tomography (HRCT) showing patterns associated with usual interstitial pneumonia (UIP) (79). UIP is defined by the radiographic presence of areas of opacification and reticulation in the lung together with clustered airspaces of a cystic appearance and thick walls (termed honeycombing) and distortions of the airway walls (80). These structural alterations are often heterogenous throughout the lung, meaning that they appear in a patch-like manner interspersed with areas with normal lung structure (**Figure 5**).

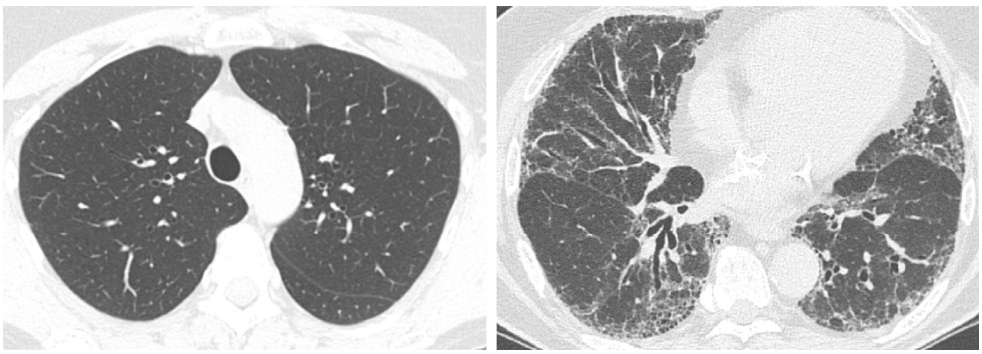


**Figure 5:** High-resolution computed tomography (HRCT) scan comparing the appearance of a healthy lung (left) and of usual intersitial pneumonia with regions of honeycombing (right), typical for patients with IPF. Images obtained from Hobbs and colleagues (81) with permission. Original images 1b (left) and 9d (right) published in: Hobbs S, Chung J H, Leb J, et al. "Practical Imaging Interpretation in Patients Suspected of Having Idiopathic Pulmonary Fibrosis: Official Recommendations from the Radiology Working Group of the Pulmonary Fibrosis Foundation". Radiology: Cardiothoracic Imaging, 2021. Published online February 25, 2021. Vol. 3 No. 1. Doi: 10.1148/ryct.2021200279. © Radiological Society of North America.

For patients to receive a diagnosis of IPF, all known causes of interstitial lung diseases such as environmental exposures, toxicity of drugs, connective tissue diseases or infections must have been excluded (79). Although global guidelines and recommendations for diagnosis and clinical management of IPF have been developed (79, 80), it is still challenging to diagnose IPF in

practice. Patients may initially receive alternative diagnoses which delays the time until appropriate treatment is given.

## Histopathology

In the remodelled areas of the IPF lung, aberrant appearance (hyperplasia) of the alveolar epithelial cells and the bronchiolar epithelium is seen (80) (**Figure 6**). Areas with cells structurally similar to airways appear in the alveolar region and form honeycombing structures, which are characteristic for IPF. The alveolar spaces become clogged with excessively produced ECM consisting mainly of collagen (80), which limits the space available for gas exchange. The excessive ECM production causes stiffness of the lung leading to impaired capacity to expand and recoil during breathing. The ECM is produced in characteristic structures called fibroblastic foci, where activated proliferative fibroblasts and myofibroblasts are found (80).

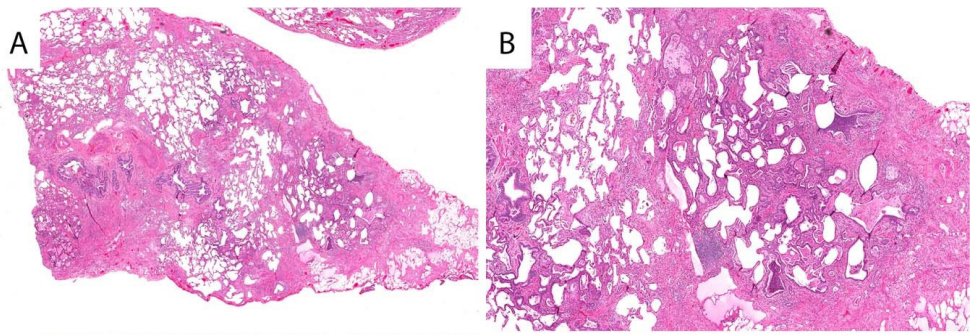



**Figure 6:** Histopathology of the IPF lung. The diseased lung has patchy areas of dense tissue regions (A), with clustered airspaces with thick walls forming the honeycombing regions (B, higher magnification of A). Reused from original figure by Smith (82). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 3A and 3B published in: Smith M L. "The histologic diagnosis of usual interstitial pneumonia of idiopathic pulmonary fibrosis. Where we are and where we need to go". Modern Pathology, 2022, Vol. 35, p.8-14. The original figure contains 2 additional panels.

## Clinical management

Historically, IPF was assumed to be an inflammatory disease and anti-inflammatory treatment was therefore commonly prescribed for IPF patients (83). These therapeutic approaches were however not effective. The available treatments for IPF patients today are the two drugs nintedanib and pirfenidone, which act on targets primarily found on mesenchymal cells and in the vasculature (84, 85). These drugs are effective in slowing down the disease progression but are not curative, and their use has been associated

with extensive side effects (86, 87). Although numerous clinical trials assessing alternative therapies for IPF are presently ongoing (88), the development of novel and effective therapies for IPF remains a challenge largely due to the initiating factors of IPF still not being known.

# Dysregulation of the alveolar epithelium in IPF

Although the mesenchymal cells are certainly playing an important role in the pathogenesis of IPF, recent work has suggested that the alveolar epithelium is the initial site of injury leading to the development of IPF. Moreover, studies of biomarkers in serum samples from individuals with IPF have shown that epithelial cell-associated biomarkers are correlated with severity of disease and predictive of mortality (89). Interestingly, modulation of the processes contributing to the abnormal behaviour of the alveolar epithelial cells has been shown to be sufficient to slow or reverse pulmonary fibrosis in animal models (90-92). A significant portion of the work included in this thesis is focused around these processes, which are outlined in this section.

## Cellular processes

### *Cell death*

The alveolar epithelial cells are gradually lost in IPF and functional re-epithelialisation of the fibrotic areas is hampered. A contributing cause to the loss of alveolar epithelial cells in IPF is cell death by activation of a cellular process termed apoptosis (88, 93). Apoptosis is a programmed form of cell death which is naturally triggered intrinsically by the cell itself when it cannot maintain homeostasis. This may occur when there is a deficit of necessary growth factors, when organelles are malfunctioning due to stress or when the DNA is damaged (94). Apoptosis may also occur as a response to certain signalling pathways which activate the death receptors (94). One of the major inducers of apoptosis through this pathway is tumour necrosis factor alpha (TNF-α), which is strongly expressed in alveolar epithelial cells in IPF (95). Activation of apoptosis by the cell leads to release of caspases, which are responsible for degradation of organelles and DNA eventually leading to disruption of the cell membrane integrity (94). Identification of active apoptosis in cells is commonly performed by detection of caspase 3

and 7, as well as intracellular markers responsible for initiation of mitochondrial degradation including BCL2 and BAX (94). These markers have been described to be elevated in hyperplastic alveolar epithelial cells in the fibrotic lung (96), indicating that apoptosis is an active process in alveolar epithelial cells in IPF which may be contributing to the failed re-epithelialisation.

*Senescence*

Senescence is a natural process occurring during development (97, 98) as well as in adulthood (99, 100). As senescent cells naturally accumulate with age (101) and IPF is mostly occurring in the elderly, senescence is believed to be a contributing factor the development of IPF. Senescence is however a challenging process to define experimentally because there is no defined expression profile which accurately defines senescent cells. However, numerous markers collectively termed as the senescence-associated secretory phenotype (SASP) have been described to be indicative of senescence (102). Increased expression of senescence-associated markers have been observed in fibrotic alveolar epithelial cells, including the cell cycle arrest proteins p21 and p53 (103). These observations were additionally confirmed in recent single-cell sequencing studies of alveolar epithelial cells isolated from individuals with IPF, in which the *p53* transcription factor was predicted to be a driver of the aberrant phenotypes occurring in these cells (104). Deletion of senescence-associated markers in animal models have been shown to reduce the severity of experimentally induced pulmonary fibrosis (90), indicating that the senescence-associated markers are attractive targets as novel anti-fibrotic agents. This has also been suggested by studies examining senolytic (anti-senescence) treatment of fibrotic, primary mouse alveolar epithelial cells *in vitro*, which lead to reduced expression of fibrosis-associated genes and increases in expression of epithelial proteins (105).

*Stress of the endoplasmic reticulum*

Stress of the endoplasmic reticulum (ER) is triggered when the cell accumulates abnormal amounts of misfolded proteins (106). Although markers indicative of activated ER stress are frequently observed in alveolar epithelial cells in IPF (107), it is not presently established whether this is a contributing factor to the disease or a consequence of other processes related to IPF. ER stress has been observed in alveolar epithelial cells with mutations in surfactant genes leading to aberrant folding and secretion of the surfactant proteins including surfactant protein C (SP-C) (108). Mutations in surfactant

proteins have also been observed in cases of familial IPF (109-113). Studies in animal models have also shown that ER stress due to some surfactant mutations may be sufficient alone to induce spontaneous pulmonary fibrosis (114) or cause increased susceptibility to fibrosis-inducing agents (115).

ER stress may also be induced by ineffective induction of another process termed autophagy, during which misfolded proteins and organelles of the cell are transported for degradation to structures called lysosomes (116). Although this is a normal process necessary for the appropriate function of the cells, excessive activation of this process in alveolar epithelial cells leads to induction of apoptosis (116). On the other hand, insufficient activation of autophagy leads to aberrant accumulation of dysfunctional organelles. Accumulation of particularly mitochondria is a feature especially linked to aging and has been observed in individuals with IPF (117).

*Impaired maintenance and repair of DNA*

Maintenance of the DNA integrity is of importance to ensure appropriate function of the cell. The chromosomes are protected through caps located at the tips of the chromosomal arms, the telomeres (118). Shortened telomeres have been reported in fibrotic alveolar epithelial cells (119). This effect is often a consequence of mutations in the enzyme responsible for the maintenance of the telomeres called telomerase (TERT). Mutations in telomerase have been identified in both familial and sporadic cases of IPF, indicating that environmental factors may also induce these mutations in individuals which do not have genetic predispositions (120, 121). Mutations contributing to shortened telomeres have also been identified in the telomere-capping shelterin component *Trf1*. In mice with AEC2-specific deletion of *Trf1*, spontaneous development of pulmonary fibrosis was observed after several months (122, 123) suggesting that loss of telomeres is a contributing factor to the development of IPF over time.

## Aberrant epithelial phenotypes

In IPF, alveolar epithelial cells often acquire non-epithelial markers (38-40, 104), a phenomenon termed epithelial reprogramming. This includes induced expression of mesenchymal markers, a process also termed epithelial-to-mesenchymal transition (EMT). One of the main pro-fibrotic signalling pathways which is described to contribute to the EMT process is the transforming growth factor β (TGF-β) pathway. Interestingly, during late lung development, active TGF-β stimulation is of importance for the

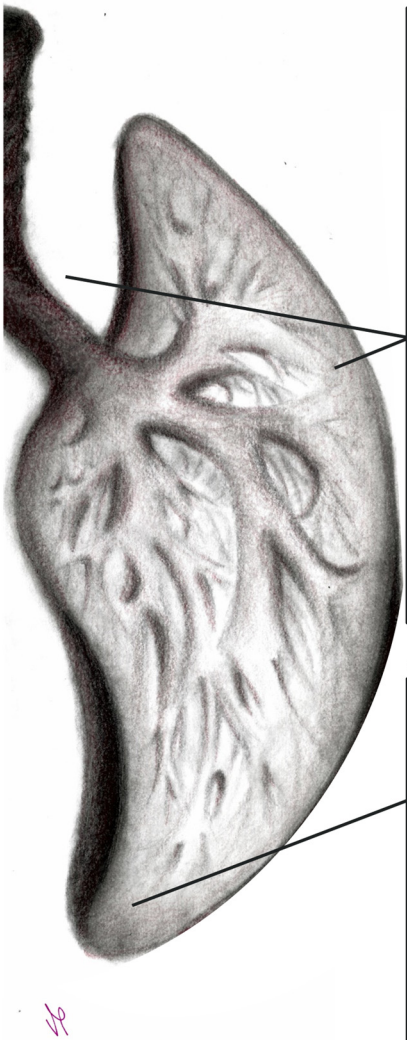
alveolarisation process in which AEC2 transdifferentiate into AEC1 (1, 124). However, TGF-β stimulation of primary AEC2 from adult rats and mice *in vitro* has been demonstrated to induce expression of the mesenchyme-associated markers α smooth muscle actin (α-SMA), collagen type 1 and vimentin (125). Similarly, expression of these mesenchymal markers has been observed in the alveolar epithelium in patients with IPF (126). Moreover, recent single-cell sequencing studies have been able to confirm that specific subpopulations of epithelial cells with aberrant, transitional phenotypes are induced in IPF (38, 39). These aberrant states are characterised by expression of the basal epithelial cell-associated markers keratin (KRT) 17 and 8 simultaneously with expression of mesenchymal markers and senescence-associated markers (38, 39). Studies including experiments performed *in vitro* and *in vivo* in mouse models of IPF have suggested that these aberrant transitional states might be AEC2 diverted from their trajectory towards AEC1 differentiation in IPF, leading to impaired alveolar repair (127-129). Other studies have suggested that the *KRT17*$^{+}$*/KRT5*$^{-}$ phenotype, also termed the aberrant basaloid cell phenotype, is derived from a population of airway epithelial cells which potentially migrate into the distal lung epithelium upon injury (130-132). However, the functional contribution of the epithelial aberrant transitional phenotypes to the pathogenesis of IPF is still to be elucidated. The ability to more accurately describe the origin and role of these aberrant transitional epithelial cells in lung fibrosis is dependent on the development of representable experimental models of epithelial reprogramming. This aspect is of importance for the ability to develop novel therapeutics for chronic lung diseases such as IPF, which is currently an incurable and devastating condition.

## Epithelial repair of lung injury

When the lung becomes injured through exposure to harmful agents, the epithelial cell types of the lung become activated to repair the injury. Just until recently, recovery of the injured human adult lung was mostly believed to be based on compensatory adaptation of the remaining healthy lung tissue by for example dilatation of the existing alveoli, rather than formation and growth of new lung tissue (133). However, studies of other larger animals have identified that compensatory growth of the lung occurs over months to years of time after removal of lung tissue (a procedure termed pneumonectomy, performed in humans for example during resection of

cancer). The compensatory lung growth process involves formation of new alveoli and size expansion of the lung (134, 135). Interestingly, long-term studies support that compensatory lung growth may occur in a similar manner in the adult human lung after pneumonectomy during a process taking several years (136). This observation was made in a relatively young patient (33 years) indicating that the lung's capacity to grow is retained to some extent even after the alveolarisation stage is completed in adolescence. While pneumonectomy causes a relatively major loss of lung tissue, there are other examples of less extensive injuries in which the adult human lung is able to recover. These include respiratory viral infections caused by for example influenza viruses (137) and SARS-CoV-2 (138). However, while most patients recover from severe viral infections caused by SARS-CoV-2, some patients experience respiratory problems for years after their infection (139). For other lung diseases of chronic nature such as IPF, the endogenous capacity of the lung to repair seems to be completely lost which leads to fatal outcomes for patients. These cases demonstrate that several aspects of adult human lung repair are incompletely understood and subsequently, inadequately managed clinically. Such aspects include the precise point in adult life at which the lung loses the capability to compensate injury, and whether all kinds of lung injuries are possible to repair.

Knowledge about how the endogenous repair mechanisms of epithelial cells are affected in lung injury may lead to a potential cure for chronic lung diseases such as IPF. As smaller animals have the capacity to grow lung tissue following injury quicker than larger animals, they are attractive model organisms for modelling regeneration after various types of lung injury. This ability has provided knowledge about putative regenerative subpopulations of lung epithelial cells activated under different injurious conditions in the lung (88, 140-142) (**Figure 7**).



**Airway region**

| Cell | Markers |
|---|---|
| Basal cell | $Krt5^+$, $Krt14^+$, $p63^+$, $Ngfr^+$, $Pdpn^+$ |
| | $Krt5^+$, $Krt14^-$ |
| | $Krt5^-$, $Krt14^+$ |
| BESC | $p63^+$, $Cc10^+$, $Sox2^+$ |
| Club cells | $H2\text{-}K1^+$ |
| Club-like cells | $Scgb1a1^+$, $Cyp2f2^+$ |
| v-CC | $Upk3a^+$ |
| DASC | $p63^+$, $Krt5^+$ |
| LNEP | $\alpha6\beta4^+$, $Cc10^-$ |
| RAS | $SCGB3A2^+$ |

**Alveolar region**

| Cell | Markers |
|---|---|
| AEP | $\alpha6\beta4^+$, $Sp\text{-}C^-$ |
| BASC | $Sp\text{-}C^+$, $Cc10^+$ |
| AEC2 | $Sp\text{-}C^+$, $Krt8^+$ |
| | $Sp\text{-}C^+$, $Cdh1^-$ |
| | $Sp\text{-}C^+$, $Cd44^+$ |
| | $Sp\text{-}C^+$, $Sca\text{-}1^+$ |
| | $Sp\text{-}C^+$, $Axin2^+$, $Tm4sf1^+$ |
| AEC1 | $Hopx^+$, $Igfbp2^-$ |

**Figure 7:** Proposed localisation of putative epithelial progenitor cell subpopulations in the lung. Cell population markers are adapted from Ptasinski and colleagues (88), Basil and colleagues (140), Cole and colleagues (141), and Rawlins and colleagues (142). BESC = bronchial epithelial stem cell; v-CC = variant club cell; DASC = distal airway stem cell; LNEP = lineage-negative epithelial progenitor cell; RAS = respiratory airway secretory; AEP = alveolar epithelial progenitor cell; BASC = bronchioalveolar stem cell; AEC1 = alveolar epithelial type 1 cell; AEC2 = alveolar epithelial type 2 cell. Illustrations by Victoria Ptasinski.

In addition to subpopulations of airway epithelial cells which predominantly contribute to repair of the airway epithelium upon injury (141, 142), observations in animal models have demonstrated that certain populations of airway epithelial cells may also contribute to alveolar repair (35, 36, 92, 129, 140, 143-148) (**Table 1**). The mouse-specific bronchioalveolar progenitor cell BASC has been reported to become activated upon lung injury by bleomycin and naphthalene and able to generate AEC2 in addition to other types of airway epithelial cells (35, 36). Further, studies have reported involvement of subpopulations of club cells which expand upon induced lung injury, able to generate alveolar epithelial cells (129). However, the same subpopulation is also proposed to contribute to the induction of transitional alveolar epithelial cell types defined by high expression of KRT8 (129) which are suggested to be hindering normal alveolar repair under fibrotic conditions in the lung (127). Moreover, several studies suggest that the AEC2 population actually consists of several subpopulations which are induced during different types of lung injury (34, 149-153). These subpopulations are particularly responsive to different cues in their microenvironment, such as local alterations in the oxygen level (152), or to particular signalling pathways which are also active during lung development (151, 153). In addition, subpopulations of AEC1 with potentially different repair capacity have also been identified (154, 155). However, as the AEC1 subpopulations have been identified by their expression of the marker *Hopx*, which is dysregulated in both AEC1 and AEC2 in IPF (156), the true plasticity of this population under fibrotic conditions is yet to be determined.

**Table 1:** Putative reparative epithelial progenitor cell populations contributing to alveolar repair in various types of lung injury. Based on Table 2 from Ptasinski and colleagues (88) and markers described by Basil and colleagues (140).

| Cell type | Species | Experimental model | Markers | Daughter cell type | References |
|---|---|---|---|---|---|
| AEC2 | Mouse<br>Human | Homeostatic conditions, BLM<br>*In vitro* organoid assay | Sp-C+ | AEC1, AEC2 | (34) |
| AEC2 | Rat | Hyperoxia | Sp-C+, Cdh1- | AEC2 | (152) |
| AEC2 | Mouse | Homeostatic conditions | Sp-C+, Cd44+ | AEC1, AEC2 | (149) |
| AEC2 | Mouse | *P.aeruginosa*-induced pneumonia | Sp-C+, Sca-1+, α6β4- | AEC1 | (150) |
| Wnt-responsive AEC2 | Mouse<br>Human | H1N1 influenza virus infection<br>*In vitro* organoid assay | Sp-C+, Axin2+, Tm4sf1+ | Wnt-responsive AEC2, AEC1, AEC2 | (151, 153) |
| AEC1 | Mouse | PNX | Hopx+, Igfbp2- | AEC1, AEC2 | (154, 155) |
| AEP | Mouse | BLM | α6β4+, Sp-C-, Cc10- | AEP, AEC2, secretory cells | (143) |
| Transitional alveolar epithelial cell | Mouse | BLM, H1N1 influenza virus infection, LPS | Krt8+ | AEC1 | (127, 129) |
| BASC | Mouse | Naphthalene, BLM | Sp-C+, Cc10+ | BASC, AEC1, AEC2, secretory cells, ciliated cells | (34-36) |
| BESC | Mouse | BLM | p63+, Scgb1a1+, Sox2+ | AEC1, AEC2 | (92) |
| DASC | Mouse | H1N1 influenza virus infection | p63+, Krt5+ | DASC, AEC1, AEC2, secretory cells | (146, 148) |
| LNEP | Mouse | H1N1 influenza virus infection | α6β4+, Sp-C-, Cc10- | LNEP, AEC2, secretory cells | (147) |
| v-CC | Mouse | Naphthalene, BLM | Upk3a+ | AEC1, AEC2, secretory cells | (144) |
| Club-cell derived epithelial progenitor cell | Mouse | BLM | H2-K1+ | AEC1, AEC2, airway cells | (145) |
| RAS | Ferret<br>Human | Cigarette smoke exposure<br>COPD | SCGB3A2+ | AEC2 | (140) |

Abbreviations: AEC2 = alveolar epithelial type 2 cell; AEC1 = alveolar epithelial type 1 cell; BLM = bleomycin; *P. aeruginosa* = *Pseudomonas aeruginosa*; Wnt = Wingless-related integration site; H1N1 = hemagglutinin type 1 and neuraminidase type 1; PNX = pneumonectomy; AEP = alveolar epithelial progenitor cell; LPS = bacterial lipopolysaccharide; BASC = bronchioalveolar stem cell; BESC = bronchial epithelial stem cell; DASC = distal airway stem cell; LNEP = lineage-negative epithelial progenitor cell; v-CC = variant club cell; RAS = respiratory airway secretory; COPD = chronic obstructive pulmonary disease.

Although the studies performed in animal models have contributed extensively to our knowledge about potential disease-specific subpopulations in the lung, similar knowledge about the human lung is more limited. When I began my work for this thesis, single-cell datasets from IPF lungs had just been published describing the phenotypes of specific subpopulations found in the fibrotic lungs (38-40). However, due to the unknown causes of the aberrant epithelial phenotypes present in IPF, there was a significant lack of human models in which these fibrotic subpopulations could be modelled and studied. In recent years, significant technical progress has been made in this field. In particular, the interest increased to create models in which it would be possible to study factors inducing the aberrant fibrotic phenotypes in human lung epithelial cells, an aspect which normally cannot be studied in primary cells isolated from the already diseased IPF lung. Therefore, heavy focus was directed towards development of models based on human stem cell-derived lung epithelial cells.

The overarching definition of a stem cell includes the capacity to both self-renew and differentiate into other cell types. Classically, stem cells have been categorised into a hierarchy according to their differentiation potency, ranging from the most versatile totipotent stem cells found during early embryonic development and down to the terminally differentiated cells (**Figure 8**) (157). According to this hierarchy, totipotent stem cells are able to form all cell types of the embryo and the extra-embryonic structures, such as the placenta (158). Pluripotent stem cells are the next type of cells in the stem cell hierarchy, which have the ability to form all cell types of the embryo but not any of the extra-embryonic cell types (158). During embryonic development, stem cells specialise and subsequently lose potency (159). Multipotent stem cells are able to form cell types of specific lineages, oligopotent stem cells are able to form several cell types and, lastly, unipotent stem cells are only able to form a specific cell type in addition to their self-renewal (158). The latter is often also termed progenitor cell, to distinguish these from the more potent stem cells. Progenitor cells are often found locally in different organs of the adult human body to maintain normal cell turnover and allow for timely repair in response to injury (159).

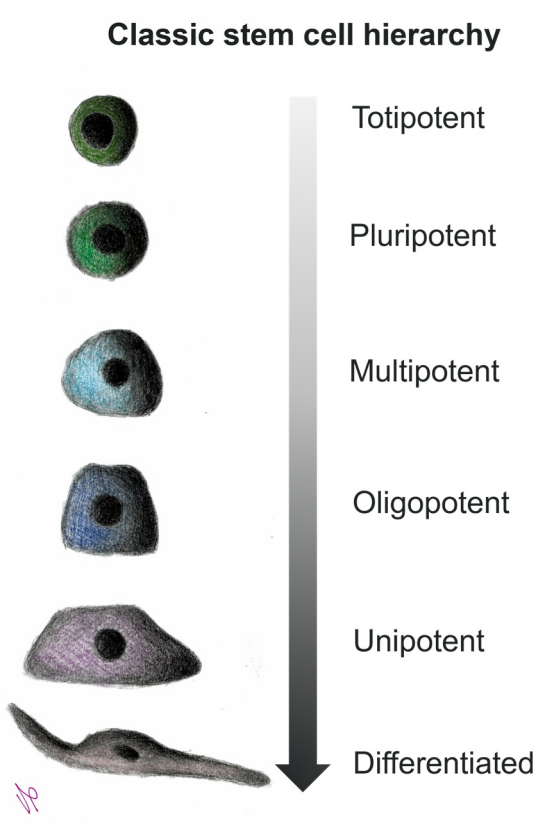



**Figure 8:** The classic stem cell hierarchy showing different types of stem cell populations and their differentiation potential. Illustrations by Victoria Ptasinski.

Pluripotent stem cells have attracted particular attention in the context of regenerative medicine due to their extensive differentiation potential. Experimentally, embryonic stem cells (ESC) are regarded as the golden standard of pluripotent stem cells and have been used since the 1980s, when the first mouse and human cell lines were derived (160, 161). In practice, embryonic stem cells are isolated at the pre-implantation stage of the blastocyst from the inner cell mass (160-162). The toolbox to obtain pluripotent stem cells was further expanded in 2006, when the capacity to induce pluripotent stem cells (iPSC) from somatic cells was first described (163, 164). In the context of modelling the aberrant epithelial phenotypes arising in pulmonary fibrosis, the majority of the recently developed human pluripotent stem cell-derived models have included derived lung epithelial cells cultured as organoids with supporting mesenchymal cells of different kinds (**Table 2**).

**Table 2:** Human pluripotent stem cell-derived organoid models of lung epithelial cells used in modelling of aberrant phenotypes arising in IPF.

| Year | Cell type | Mesenchymal cells | Key findings related to IPF | Time of induction | References |
|---|---|---|---|---|---|
| 2017 | ESC/iPSC-derived mix of lung mesenchymal and epithelial cells | Yes (co-derived from ESC/iPSC) | Modelling of ECM expression associated with HPSIP and IPF caused by genetic mutations | Day 15 of differentiation | (165) |
| 2017 | iPSC-derived mix of lung epithelial cells | Yes (foetal lung fibroblasts) | Modelling of contraction and fibrotic gene and protein expression by TGF-β stimulation | 8 days after TGF-β stimulation | (166) |
| 2019 | ESC-derived mix of lung mesenchymal and epithelial cells | Yes (co-derived from ESC) | Modelling of ECM expression associated with HPSIP and IPF caused by genetic mutations and identification of IL11 as a driver of fibrotic changes | Day 25 of differentiation for mutated organoids + 15 days after IL11 stimulation | (167) |
| 2019 | iPSC-derived AEC2 | Yes (foetal lung fibroblasts) | Modelling of aberrant lamellar bodies and SP-C secretion associated with HPSIP and IPF caused by genetic mutations | Day 35 of differentiation for mutated organoids | (168) |
| 2021 | ESC/iPSC-derived mix of lung mesenchymal and epithelial cells | Yes (co-derived from ESC/iPSC) | Induction of fibrotic gene and protein expression by TGF-β stimulation + treatment with anti-fibrotic drugs | 3 days after TGF-β stimulation | (169) |
| 2021 | iPSC-derived AEC2 | No | Modelling of impaired autophagy and aberrant SP-C trafficking and processing associated with *SFTPC* mutations in HPSIP and IPF | Day 35 of differentiation for mutated organoids | (170) |
| 2021 | iPSC-derived AEC2 | Yes (foetal lung fibroblasts) | Modelling of mitochondrial dysfunction and aberrant lamellar bodies associated with HPSIP and IPF caused by genetic mutations | Day 35 of differentiation for mutated organoids | (171) |
| 2021 | iPSC-derived AEC2 | Yes (foetal lung fibroblasts) | Induction of epithelial-mediated contraction, senescence and KRT17 expression by bleomycin | 6 days after bleomycin administration | (172) |
| 2021 | iPSC-derived mix of lung mesenchymal, immune and epithelial cells | Yes (co-derived from iPSC) | Induction of fibrotic gene and protein expression by TGF-β stimulation + treatment with anti-fibrotic drugs | 3 days after TGF-β stimulation | (173) |
| 2022 | ESC-derived mix of lung mesenchymal, immune and epithelial cells | Yes (co-derived from ESC) | Induction of fibrotic gene expression and morphological densening by bleomycin | 2 days after bleomycin administration | (174) |
| 2022 | ESC-derived mix of lung epithelial cells | No | Modelling DNA damage by bleomycin | 3 days after bleomycin administration | (175) |

Abbreviations: ESC = embryonic stem cell; iPSC = induced pluripotent stem cell; ECM = extracellular matrix; HPSIP = Hermansky-Pudlak Syndrome Interstitial Pneumonia; IPF = idiopathic pulmonary fibrosis; AEC2 = alveolar epithelial type 2 cells; IL11 = interleukin 11; SP-C/*SFTPC* = surfactant protein C; TGF-β = transforming growth factor β; KRT = keratin.

The ability to experimentally induce pluripotency in what has historically been regarded as terminally differentiated cells has sparked debate over whether the classically adopted stem cell hierarchy is in fact absolute. In recent years, this discussion has been extended to also cover other cells present *in vivo* which have historically been regarded as having limited differentiation potential. Studies utilising single-cell based analyses of primary human and mouse lung epithelial cells under injury and disease conditions have provided examples of how the classically regarded stem cell potential of the primary AEC2 population is in fact redefined in the adult body (**Figure 9**) (127-129). Classically, the AEC2 is regarded as a unipotent cell type which self-renews and differentiates into the AEC1 under homeostatic conditions and during lung injury (34). However, recent evidence has challenged that unipotency is the only capacity of the AEC2 by demonstrating alternative differentiation paths into intermediate states characterised by high expression of KRT8 upon various types of lung injury or diseases (127-129). In particular, primary human AEC2 (hAEC2) in organoid co-cultures with adult human lung mesenchyme (AHLM) during 7 days and onwards have been demonstrated to acquire different alveolar-basal intermediate (ABI) states, similar to the IPF-associated cell phenotypes including the $KRT17^{+}/KRT5^{-}$ aberrant basaloid cell state and metaplastic $KRT5^{+}$ basal cell state (128). Thus, these recent studies have demonstrated that the classically adopted cell identities and differentiation potentials may be altered in the adult lung during diseases such as pulmonary fibrosis, and to an extent not observed in the healthy adult lung. However, aspects which are still not entirely clear from the aforementioned stem-cell derived organoid studies include which types of mediators that have the ability to induce the complex aberrant fibrotic phenotypes, and hence can be considered as potential therapeutic targets for IPF.

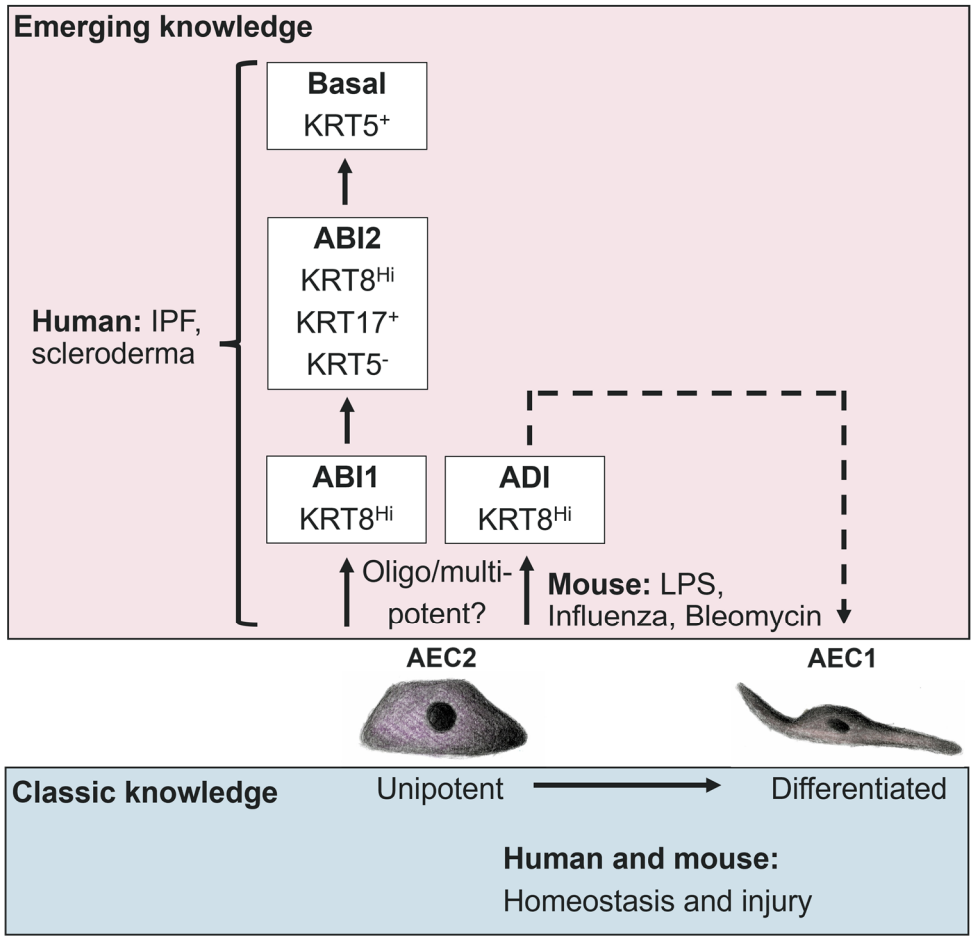



**Figure 9:** Emerging differentiation potential of the AEC2 under injury conditions in the adult lung. IPF = idiopathic pulmonary fibrosis; KRT = keratin; ABI = alveolar-basal intermediate; ADI = alveolar differentiation intermediate; LPS = lipopolysaccharide; AEC1 = alveolar epithelial type 1 cell; AEC2 = alveolar epithelial type 2 cell. Dashed line = impaired differentiation. Illustrations by Victoria Ptasinski.

# Research aims of doctoral thesis

The aim of this doctoral thesis was to identify dysregulated alveolar epithelial cell populations and molecular pathways in IPF which are amenable to pharmaceutical intervention. This was addressed by the following sub-aims:

I. Pharmacological modulation of known pro-fibrotic pathways to identify targets promoting endogenous lung regeneration under fibrotic conditions.

II. Development of relevant experimental systems to model induction of aberrantly activated alveolar epithelial cell populations in IPF.

III. Evaluation of molecular mechanisms contributing to the aberrantly activated alveolar epithelial cell populations in IPF.

# Methodology

Studying pathology and molecular changes in patient-derived material is possible even for rare diseases such as IPF. Patient samples are important sources of material for studies of potential biomarkers which can be correlated with disease severity or progression. However, there are a number of critical aspects which are not equally suitable to study using patient-derived material. As lung tissue obtained from IPF patients is resectioned from lungs with end-stage disease, these samples are no longer suitable for study of early inducers of IPF. Effects of potential inducers can however be studied using *in vivo* models of pulmonary fibrosis. Further, simplified *ex vivo* and *in vitro* models of the lung enable study of specific cell types and mechanisms in a more isolated setting than the human body or an animal. Such models allow for experimental modulation of disease-relevant mechanisms in a controlled manner, which is not possible in human subjects and often challenging in animals. Importantly, models resembling the disease aspects of IPF can also be applied for pre-clinical testing of novel anti-fibrotic drugs.

## *In vivo* models

Animal models enable study of the interactions of all cell types and structural components present in the lung during the initiation and progression of pulmonary fibrosis (176). Although spontaneous pulmonary fibrosis has been observed in other large species in addition to humans, including cats (177), dogs (178) and horses (179), the initiating factors of disease in these species are poorly characterised similarly to human IPF. Moreover, large animals are less manageable to utilise for experimental studies as they require larger space for housing. Instead, small rodents such as mouse are commonly used for this purpose. A common way of inducing lung fibrosis *in vivo* is by administration of various lung irritants which have been observed to lead to development of interstitial lung disease in man. Examples of such irritants are silica and asbestos, which are environmental causes of interstitial lung

disease in humans (180, 181). Other strategies to induce experimental pulmonary fibrosis are based on clinical observations of drug-induced interstitial lung disease following for example treatment with the anti-neoplastic drug bleomycin (182, 183), or after irradiation of the chest during treatment of thoracic and breast malignancies (184). Increased knowledge around mechanisms contributing to propagation of pulmonary fibrosis has led to the development of other *in vivo* models based on overexpression of cytokines and mediators such as TGF-β, or genetic mutations associated with familial IPF (185).

### The bleomycin model of pulmonary fibrosis in mouse

The bleomycin model was one of the first lung fibrosis models to be developed and is still widely used as it resembles many of the pathological features seen in IPF (176, 186). In **Paper I** and **Paper IV** included in this thesis, the bleomycin model was utilised using mouse as the model organism. Bleomycin administration results in an initial inflammatory stage, involving alveolar epithelial cell damage, inflammatory cell recruitment, and pro-inflammatory mediator release (186, 187) (**Figure 10**). Following the initial inflammatory stage, a sub-acute stage develops involving pro-fibrotic cytokine expression as well as fibroblast proliferation and differentiation around the sites of injury (186, 187). The final stage is characterised by increased collagen deposition and development of patchy fibrotic areas in the lung (186, 187).

Alike in the bleomycin model, inflammatory cell infiltration in the alveolar region is also seen in the human IPF lung (188, 189). Therefore, IPF has historically been regarded to be an inflammatory disease, and corticosteroids were used for treatment. However, this treatment strategy was shown to be ineffective and corticosteroids are no longer prescribed for this purpose (190). Instead, the focus shifted towards development of drugs mainly suppressing the excessive fibroblast proliferation and myofibroblast activation, which is another pathological feature shared between human IPF and the bleomycin model. Mesenchymal cells and structures are the targets of the currently used IPF drugs in the clinic, nintedanib and pirfenidone. Although treatment with these drugs slows down the progression of IPF, none of these treatments are curative indicating that IPF is driven by other factors. Despite the fact that the efficacy of drugs in the bleomycin model does not always translate to human IPF, the bleomycin model is still regarded as the golden standard for efficacy testing of anti-fibrotic drugs in pre-clinical development.

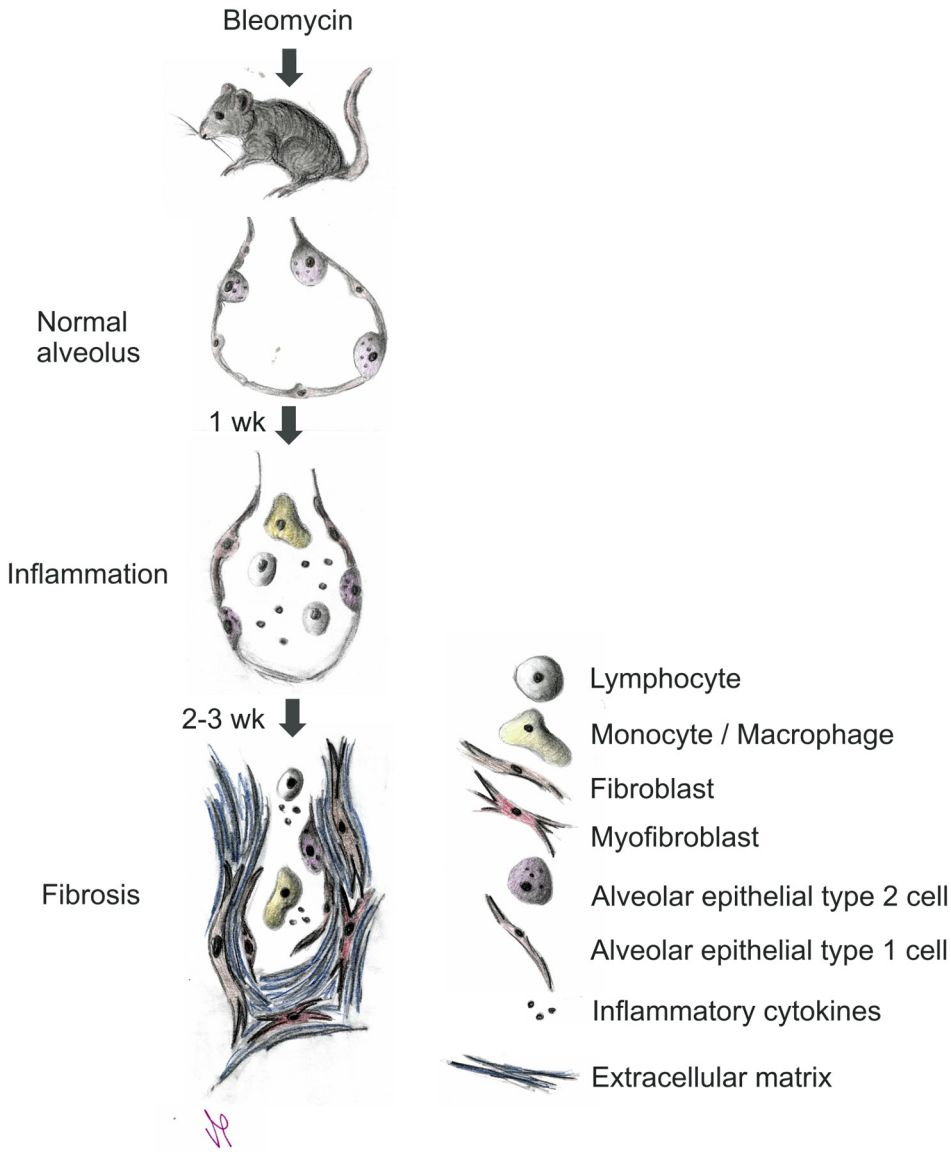



**Figure 10:** The different stages of bleomycin-induced pulmonary fibrosis in mouse. Illustrations by Victoria Ptasinski.

Readouts to assess the extent of induced fibrosis in the lung following bleomycin administration include histopathological assessment of the lung tissue structure. Gross morphology is commonly evaluated by performing haematoxylin and eosin (H&E) staining of lung tissue sections. Haematoxylin stains the basophilic structures, such as for example the nucleic acid material found in cell nuclei (191). Eosin counterstains the basic structures by staining the acidic structures, such as proteins in the cytoplasm of the cells (191). Through this, it is possible to distinguish the morphology of cells and their organisation in the tissue section. As collagen deposition is a hallmark of pulmonary fibrosis, the evaluation of lung lesions induced by bleomycin commonly involves more specialised staining of collagen fibres in the tissue by for example Masson's trichrome (MTC) (192). This staining technique also involves two steps of nuclear and cytoplasmic dye according to a similar principle as the H&E staining, but also contains a third step which stains the collagen fibres resulting in either green or blue staining (193).

To enable more sensitive quantitative measurements of collagen production in the lung, levels of hydroxyproline are often measured. Hydroxyproline is an amino acid which is found in collagen, and the production of this amino acid is therefore indicative of collagen synthesis (194). Hydroxyproline content in the lung can be evaluated through colorimetric assays, generally involving the following steps; firstly, the protein content of the tissue is hydrolysed with acid, and secondly, the free hydroxyproline is converted into the aromatic compound pyrrole, which is able to react with a solution called Ehrlich's solution and produce a colour detected by a spectrophotometer.

Transcriptomic comparison with the human IPF lung indicates that bleomycin-induced pulmonary fibrosis in mouse recapitulates changes seen in patients with rapidly progressive IPF, including dysregulated expression of genes related to cell cycle regulation and signalling pathways such as the platelet-derived growth factor (PDGF) pathway (195). However, the histological changes in the mouse lung induced by bleomycin administration often lack characteristic features seen in the IPF lung, such as fibroblastic foci and hyperplastic alveolar epithelial cells (196). Studies have also reported that single doses of bleomycin induce reversible fibrosis in rodents which is in stark contrast to the irreversibility seen in human IPF (196), although the exact time at which this repair phase starts is variable across the different models. Repetitive approaches of bleomycin dosing have been explored as an optimisation of the model leading to more persistent morphological changes, including more wide-spread collagen deposition and

alveolar epithelial cell hyperplasia characterised by "bronchiolised", honeycombing-like structures, more similar to the morphological changes reported in human IPF (196, 197). Other studies have also shown impact of age and sex of the mice with regards to susceptibility to bleomycin-induced pulmonary fibrosis (198). In the studies described in this thesis, young (around 8 weeks old) female mice are used. Female mice are however reported to be less susceptible to bleomycin-induced pulmonary fibrosis (198). The use of females rather than males is often a practical choice, as this enables housing of several mice in the same cage due to less risk of fighting which is an important consideration when larger studies are performed. Of note however, due to multiple possible routes of administration and varying doses of bleomycin used by the research community, comparison of effects across laboratories is challenging as not all features of disease are equally replicated in every bleomycin study (199). Moreover, regardless of which bleomycin administration strategy is used, the complex *in vivo* models are difficult to interpret with regards to the contribution of each individual cell type to the injury and repair processes. When the target or cell type of interest is known prior to study initiation, transgenic mice with incorporated fluorescent reporters may be used to lineage-trace the expression of specific markers and the cell populations expressing them during the course of injury and repair (200-203).

**Isolation of lung epithelial cells from the mouse lung**

Isolation of cells from tissue is often the next step in evaluation of effects induced *in vivo* in different cell types. In the context of pulmonary fibrosis, epithelial injury and repair in the lung is of particular interest. Numerous studies have been able to base their observations on epithelial cells isolated from the fibrotic mouse lung (34, 129, 143, 145, 202, 203). Mouse tracheal cells are possible to isolate for culture *in vitro* by surgical resection of the trachea from the rest of the lung (204). However, the alveolar epithelial cells are more challenging to isolate as a pure population due to their anatomical location deeply in the lung lobes. The basic steps of alveolar epithelial cell isolation from mouse includes inflation of the lung with enzymatic solutions through the trachea, which is then tied or occluded by for example agarose instillation to prevent backflushing of the enzymatic solution. The incubation with the enzymatic solution causes detachment of the cells from the tissue (205). Following this, epithelial cells are enriched in the isolate by negative selection which separates endothelial cells (positive expression of CD31) and

immune cells (positive for CD45 expression) from the isolate (35). At the end of the protocol, cells can be further purified by positive selection for EpCAM or E-cadherin, which are epithelial surface markers (205). The drawback of this "classic" isolation method is however that this results in a mix of epithelial cell types potentially including cells of both airway and alveolar origin, as they share epithelial surface-associated markers. The impurities caused by the presence of different cell phenotypes in the isolated epithelial cell population may also impact the outcome of the experiments, as cell types might respond differently to various stimuli. Isolation protocols may yield cells of higher purity by cell sorting based on cell-type specific intracellular markers. In such protocols, the cells are fixed and permeabilised before the intracellular staining with fluorescent antibodies specific to the antigens of interest (206). However, such methods are not suitable for isolation of cells intended for continued culture, as the fixative and permeabilising agent kill the cells (35). Alternatively, in animal models, fluorescent reporters might be incorporated into cells which enables sorting of live cells without the need of antibody staining (206). This is however not possible to do in all types of cells. In the case of AEC2 isolation, another possible staining technique involves staining of acidic compartments such as lamellar bodies (207), structures which are highly enriched in this particular cell type (208). However, not all epithelial cell types have organelles which are characteristic enough to enable enrichment based on their presence. Further, some of the used cell type-specific markers are impacted during diseases of the lung, including the AEC2-associated marker SP-C which is often reduced or aberrantly processed in pulmonary fibrosis (108, 209, 210). Thus, while the established protocols for isolating alveolar epithelial cells work well for the healthy lung, they might not work as well in the diseased lung. Moreover, the described protocols do not allow simultaneous isolation of both tracheal and distal lung epithelial cells from the same mouse.

### A novel protocol for lung epithelial cell isolation from the mouse lung

In **Paper II**, the aim was to develop a method enabling isolation of proximal and distal lung epithelial cells from the same mouse. For this purpose, a 3D-printed tool called the 3D-printed lobe divider (3DLD) was developed which physically separates the trachea from the lung lobes of mice (211) (**Figure 11**). This enables isolation of proximal and distal epithelial cells in a way which would also be suitable when cell type-specific markers such as SP-C are affected. The incorporation of this tool into the isolation protocol enables

simultaneous isolation of both proximal epithelial cells and distal epithelial cells from the same mouse. Hence, this optimisation limits the number of mice needed for each type of isolation. This unique approach enables consistent separation of the trachea and main bronchi from the lung lobes, thus eliminating the inconsistency which may be introduced when the trachea is tied off or otherwise blocked during the enzymatic digestion as performed in the classic isolation protocols (205). The location of the knot or the occlusion in the trachea determines how much of the trachea is exposed to the same enzymatic solution as the lung lobes, which determines the proportion of contaminating proximal cell types in the distal lung cell isolate.

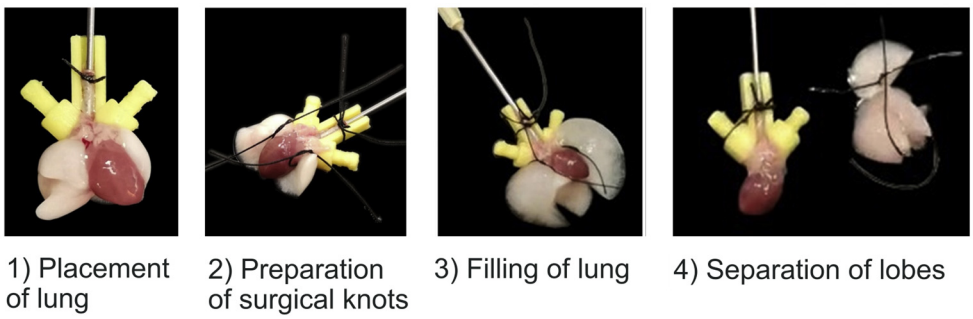



**Figure 11:** Physical separation of the mouse trachea and lung lobes during isolation using the 3DLD tool. Reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 1C published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 6 additional panels.

The proximal and distal cell isolates were evaluated by their expression of cell type-associated markers, such as KRT5 for basal cells and pro-SP-C for the AEC2. Basal cells should have the ability to expand in culture and differentiate into secretory and ciliated cells (17). The differentiation potential of the proximal cell isolates was validated by cell culture at air-liquid interface for 28 days. The proliferative capacities of both the proximal cell isolate and the distal cell isolate was assessed by colony-forming efficiency assays, in which single cells were allowed to form organoids. This assay evaluates the ability of the progenitor cells to proliferate, which is a characteristic feature of progenitor cells (34).

## *Ex vivo* models

*In vivo* models are important assets in the toolbox used for basic research of pulmonary fibrosis and for anti-fibrotic drug development. Nevertheless, *ex vivo* models of the lung offer the possibility to perform studies which would be impossible to conduct in a human being or living animal. This includes experimental setups in which high concentrations of a biological stimulant or drug are used, which would be toxic *in vivo*. Although it could be argued that these are artificial systems because of the fact that such high concentrations do not occur *in vivo*, these experiments give us valuable information about the effects which may normally build up during longer periods of time *in vivo* (and may therefore be missed in the time frame of the *in vivo* experiment). In systems utilising lung tissue, treatment performed *ex vivo* may give valuable information about the structural changes which could occur *in vivo* and therefore, serve as a prior step for prediction of effects or safety issues. Furthermore, recent changes to the legal framework adopted in the United States are now allowing alternatives to animal testing for initial application for a new drug to the FDA (FDA Modernization Act 2.0). Therefore, the development of representable *ex vivo* and *in vitro* models which can be used as such alternatives is becoming increasingly important.

### Fibrosis induction in precision-cut lung slices

*Ex vivo* models based on lung tissue from either human or mouse have been applied extensively in the area of pulmonary fibrosis research, both for disease modelling (212-214) and for drug testing (105, 213, 215-218). Insights into the perturbed reparative mechanisms of the diseased lung can be provided through biopsies from patients with end-stage lung disease. However, as biopsies are taken from patients with already established and often advanced disease stages, this material fails to provide information about the early dysregulation of lung repair. Just prior to the start of this thesis project, recent studies published by my colleagues had described induction of early fibrosis-like changes, such as increased extracellular matrix deposition and tissue structure alterations similar to those observed in the fibrotic lung tissue, in human precision-cut lung slices (PCLS) (212, 213). PCLS are generated by sectioning of agarose-filled lung tissue into slices with a thickness of around 500 µm (219), which can be maintained under cell culturing conditions during several days and used for a variety of applications in lung research (220). One of the most important benefits of using PCLS as

an experimental system is that the native lung tissue structure is preserved in the tissue slice. This enables study of interactions between different cell types and, additionally, easier evaluation of structural changes over time under different culture conditions (212, 221, 222). The latter aspect is more challenging to study in the previously described *in vivo* models. PCLS can be generated from lungs of multiple commonly used species for *in vivo* studies, including the mouse (221) and the rat (222), but also from both healthy and diseased human lung tissue (212, 213, 223). Hence, the PCLS system enables use of human lung tissue in experiments, which is an unachievable concept in studies performed *in vivo*.

Although PCLS can be generated from diseased lung including IPF lungs (224), the viability of the cells might be poor depending on the severity of the disease. Therefore, experimental induction of fibrotic changes in healthy PCLS is an attractive concept. The fibrosis-like changes were induced in the PCLS by stimulation with a fibrosis cocktail containing a mix of the pro-fibrotic mediators TGF-β, PDGF-AB, TNF-α and lysophosphatidic acid (LPA) (212). TGF-β is widely used as a pro-fibrotic stimulus *in vitro* and induces expression of extracellular matrix components and differentiation of fibroblasts into myofibroblasts (225). Moreover, secretion of biologically active TGF-β by alveolar epithelial cells has been linked to fibrotic changes in explant cultures of rat lung (226), thus indicating that it is also an epithelial-associated pro-fibrotic cytokine. Under fibrotic conditions in the lung, TGF-β is secreted by numerous cell types such as epithelial cells, but also by macrophages and mesenchymal cells (227). Similarly to TGF-β, PDGF is an important regulator during later lung development and facilitates the formation of alveolar septae by stimulating alveolar myofibroblast differentiation (228-230). PDGF is secreted by epithelial cells, macrophages and fibroblasts and contributes to the pro-fibrotic conditions as a stimulator of fibroblast proliferation and extracellular matrix production (1, 231). TNF-α can act as an inducer of apoptosis (95) and stimulates secretion of pro-inflammatory and pro-fibrotic cytokines by other cells, including TGF-β (227). Alike TGF-β, TNF-α is also secreted by many cell types in fibrosis, including macrophages, lymphocytes, epithelial cells and endothelial cells (227). LPA is a lipid mediator which forms locally by the active enzyme autotaxin and can trigger fibroblast recruitment and apoptosis of epithelial cells (232). As such, the fibrosis cocktail consisting of TGF-β, PDGF-AB, TNF-α and LPA is a stimulus which is tailored to induce several of the molecular and pathophysiological effects which are also seen in human IPF.

## *In vitro* models

Although the complexity of *ex vivo* models such as PCLS is often considered a benefit, this feature can certainly be a drawback in certain situations. Examples of such situations are studies in which effects on individual cell types are to be evaluated, or when there is a need for studying a specific pathway or stimulus in isolation from the other processes occurring in a multicellular system. For such applications, simpler experimental models are often used. Moreover, using cryopreservable cells in experiments which can be stored until use in assays provides increased control over experiments, as this circumvents the need for fresh tissue. Fibroblast activation is a prominent pathological feature of IPF observed in patients in the clinic and in many *in vivo* models of pulmonary fibrosis. Therefore, the early *in vitro* models of IPF have been heavily focused around fibroblast biology.

### Fibrosis models with primary lung fibroblasts

In **Paper I**, TGF-β-dependent effects were modelled on primary lung fibroblasts isolated from IPF patients. The ability of TGF-β to activate fibroblasts and induce a myofibroblast-like phenotype characterised by increased expression of collagen and α-SMA is well documented in other studies (225) and is a commonly used model of IPF-associated fibroblast activation (233, 234). For such purposes, isolation of fibroblasts from both healthy and IPF lung is often performed (235). The choice of using either healthy or fibrotic lung fibroblasts is highly dependent on the specific question to be addressed. Other studies have explored the transcriptomic differences between lung fibroblasts isolated from healthy lung or the IPF lung, and found enriched expression of certain genes related to ECM receptor interaction, ECM organisation including collagen and wound healing amongst others in the fibrotic lung fibroblasts (236). Therefore, effects related to early fibrosis induction might need to be modelled in a non-diseased source of lung fibroblasts, whereas fibroblast-related effects occurring in the fibrotic lung may be more accurately recapitulated using lung fibroblasts isolated from IPF patients. However, primary lung fibroblasts isolated from subjects diagnosed with IPF may also be more challenging to retrieve than healthy lung fibroblasts, as this requires access to fibrotic lungs specifically. To circumvent the need for access to fresh lung tissue for experiments, commercial sources of healthy or diseased primary lung fibroblasts may instead be used. The drawback of this approach is

however that the commercial lung fibroblasts might not be isolated from the desired location in the lung, which can be of importance for certain types of studies. In **Paper I**, commercially available lung fibroblasts isolated from IPF patients were used for the purpose of exploring the TGF-β-associated effects according to a previously established protocol by my colleagues (233).

### Fibrosis models with primary lung epithelial cells

Along with the establishment of the hypothesis that the alveolar epithelium is the initial site of injury in IPF, the focus intensified on development of *in vitro* systems in which lung epithelial cells are included. Knowledge about the impact of for example mechanical stretch imposed on epithelial cells by the ECM in IPF has led to development of systems such as lung-on-a-chip, in which epithelial and mesenchymal cell types can be incorporated together on materials with different stiffness and structure (237). However, the co-culture of cells in this format is often challenging, and the throughput of lung-on-a-chip systems is still relatively low (238) rendering them difficult for adaptation in for example drug screening.

Historically, *in vitro* fibrosis model development with primary lung epithelial cells has been challenging due to technical difficulties in their isolation and maintenance in culture (239). However, recent advances have significantly improved the possibilities to develop *in vitro* models of fibrosis with lung epithelial cells (240). This includes improved culture techniques such as the 3D-based organoid format, which has enabled extended maintenance of primary lung epithelial cells (240). In such formats, organoids are allowed to spontaneously form from single cells seeded in supporting matrices containing matrix proteins and growth factors, such as Matrigel (241). This culture format has been utilised in studies focusing on the mechanisms driving fibrosis in primary lung epithelial cells isolated from IPF patients (242, 243) and from animal models of pulmonary fibrosis (105, 202, 203, 213). However, in circumstances in which cells are isolated from already diseased lung tissue the cells have likely already undergone certain molecular and phenotypic changes associated with fibrosis. This may be desirable in cases where the aim is to reverse these changes pharmacologically (105, 213), but does not allow for study of the early fibrosis induction phase. Induction of some of the effects seen in IPF, such as upregulation of ECM components and EMT-like phenotypes, have been described in human lung epithelial cells *in vitro* using stimulation with TGF-β (125, 244-246).

However, the poor proliferative capacity of primary lung epithelial cells, particularly applicable to primary AEC2 (239), still restricts these models from applications requiring large numbers of cells including drug screening.

**Derivation of AEC2 from pluripotent stem cells**

To generate proliferative sources of human AEC2 for purposes requiring larger cell numbers, protocols have been developed to derive cells with an AEC2 phenotype from ESC and iPSC (240). In contrast to the embryonic-derived ESC, iPSC are induced by delivery of four transcription factors regulating pluripotency to somatic cells, termed the "OSKM factors"; octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), Krüppel-like factor 4 (KLF4) and cellular-Myelocytomatosis (c-MYC) (163, 164). Around the same time, other studies also reported induction of pluripotency in human somatic cells by delivery of a slightly different combination of transcription factors consisting of OCT4, SOX2, Nanog homeobox (NANOG) and Lin-28 homolog A (LIN28) (247). Interestingly, although NANOG is considered to be a transcription factor which maintains pluripotency in cells, the initial studies reporting the first induction of iPSC found that NANOG was not a necessary component of the OSKM factors for pluripotency induction (163). The OSKM factors can be delivered to cells through various strategies, including integrating methods (such as retroviral and lentiviral transfection), or by non-integrating methods (adenoviral or plasmid transfection), or by completely DNA-free methods (RNA or protein delivery) (248). The OSKM factors initiate a sequence of events in the nucleus of the somatic cell involving methylation of DNA, modification of histones and chromatin remodelling, although the precise binding sites of the OSKM factors and order of the events remain incompletely understood (249). Although it has been shown that c-MYC is not necessarily required for the reprogramming of somatic cells, inclusion of c-MYC facilitates the binding of OCT4, SOX2 and KLF4 to sites with open chromatin to initiate the early steps of reprogramming which rapidly enhances the efficiency of reprogramming (250). Generally however, the reprogramming efficiency of iPSC from somatic cells seldom exceeds 1 % of the initial cell number (248). However, this can be different when the starting somatic cell type is of a progenitor origin, such as haematopoietic cells or myeloid progenitors which may reach a reprogramming efficiency of up to 10-30 % (251). Due to the fact that not all cells are reprogrammed uniformly by the OSKM factors, cell colonies expressing markers of

pluripotency such as OCT4 and stage-specific embryonic antigen-4 (SSEA-4) need to be selected for further expansion and assessment. The general criterion for pluripotency shared by ESC and iPSC is the ability to differentiate into all germ layers present in embryos. This ability can be assessed by different types of assays; the ultimate functional assay includes their ability to form teratomas (tumours comprised of cells from multiple germ layers and lineages) or contribute to embryonic development of an organism through chimera development (often assessed in mice) (248). However, from a practical point of view these assays are time-consuming and expensive. On a more molecular level, these assays are not able to provide information about the purity of cells expressing pluripotency markers in the population. Therefore, the functional assays need to be complemented with molecular readouts such as flow cytometry, which are quicker than the functional assays (248).

The finding that pluripotency can be induced in somatic cells, which have historically been regarded as terminally differentiated cells, necessitated further investigation around whether the iPSC in fact is a suitable alternative to the ESC (252). Studies comparing the transcriptomes of iPSC and ESC lines have shown that iPSC in early passages (p5-9) initially differ transcriptomically from ESC in sets of genes related to processes such as transcription, cell proliferation and organ development (253). These transcriptomic differences become smaller in the comparison of iPSC in late passages (p54-61) with ESC, indicating that the differentially expressed gene set is related to specific suppression of the somatic cell fate and incomplete reprogramming occurring in the early culture phases of iPSC (253). Other studies have however found very few transcriptomic differences in their comparisons of iPSC with ESC lines, and described that iPSC and ESC share key similarities in histone methylation and genes occupied in these regions, associated with pluripotency (254). Notably, none of these studies could conclude that the differences were consistent enough to classify iPSC as a different cell type than ESC (253, 254). Interestingly, other reports have described differences in gene expression patterns between iPSC and ESC which could not be attributed to their different origin, but rather to the laboratory they have been derived in (255). Collectively, the true differences between iPSC and ESC lines are challenging to delineate unless they are differentiated in parallel under the exact same culture conditions and in the same laboratory. A different aspect considered in comparisons of ESC and iPSC is the higher occurrence of genomic instability reported in iPSC reported by several studies (256-258). Such instabilities include

chromosomal abnormalities, aberrant methylation or somatic mutations. These may either already be present in the somatic cell, especially if the iPSC is induced from an elderly individual, during the reprogramming stage or during subsequent culture. Notably however, some of these instabilities do also occur in ESC at a seemingly similar rate to iPSC (259) and the variations occurring are not uniform across cell lines suggesting that there is a lack of common mechanism driving the acquisition of the genomic abnormalities. Therefore, quality control such as karyotyping of the obtained cell banks after derivation are important irrespectively of the source of the pluripotent cells.

From a therapeutic perspective, the use of ESC-derived cell therapies have shown promising effects with respect to the safety and efficacy profile in treatment of macular degeneration and dystrophy (260, 261). In the clinical trials, patients were followed for 1-2 years after transplantation. However, ESC do express major histocompatibility complex (MHC) class I molecules and are hence capable of activating T cells through antigen presenting cells, which may lead to allogeneic rejection of the transplanted cells over time (262). The choice of iPSC as an alternative to ESC in cell therapy is therefore favourable due to the possibility to derive autologous iPSC from the somatic cells of patients which are to receive the cell therapy treatment. However, for the applications of modelling developmental aspects or disease mechanisms, there is no consensus in the scientific community around whether iPSC or ESC are superior over the other. In fact, the key steps in the differentiation protocols towards cells with the AEC2 phenotype have been described using both iPSC and ESC lines (263-267) (**Table 3**). Direct cell reprogramming, a technique in which target cell types are induced from somatic cells by omitting the pluripotency state (268), has been explored for the derivation of epithelial cells with a lung-like phenotype (269) but has not yet been applied to derive alveolar epithelial cells.

**Table 3:** Key advancements leading to the differentiation of cells with the AEC2 phenotype from ESC and iPSC.

| Year | Cell origin (species) | End stage | Key findings | References |
|---|---|---|---|---|
| 1998 | Primary AEC2 (rat) | AEC2 | KGF/FGF7 retains AEC2 phenotype in culture | (270) |
| 2002 | Foetal lung epithelial cells, 13-20 wk (human) | AEC2 | DCI in medium promotes AEC2 phenotype in culture | (271) |
| 2004 | ESC (mouse) | DE | Activin A or serum in culture medium induces DE fate | (272) |
| 2005 | ESC (human) | DE | Activin A together with low serum in culture medium induces DE fate | (273) |
| 2011 | ESC (human)<br>iPSC, derived from dermal fibroblasts (human) | AFE | Dual inhibition of TGF-β and BMP signalling induces AFE fate | (274) |
| 2012 | ESC (mouse)<br>ESC (human) | DE | Enrichment of cells with DE fate by selection of SOX17+ cells | (275) |
| 2012 | ESC (mouse) | Lung and thyroid progenitors | Enrichment of lung and thyroid progenitor cells using a Nkx2-1$^{GFP}$ reporter under serum-free culture conditions | (276) |
| 2012 | ESC (mouse and human)<br>iPSC, derived from dermal fibroblasts (human) | Lung progenitors | BMP4, FGF2 and WNT necessary for induction of NKX2-1+ lung progenitors | (277) |
| 2012 | ESC (human) | Lung progenitors | WNT necessary for maintenance of AEC2 phenotype | (278) |
| 2014 | ESC (human)<br>iPSC, derived from dermal fibroblasts (human) | Mix of airway and alveolar epithelial cells | CHIR99021, FGF10, FGF7, BMP4 and RA promote induction of NKX2-1+ lung progenitors | (279) |
| 2016 | ESC (human) | AFE | RA is a key factor mediating induction of NKX2-1+ lung progenitors in response to BMP and WNT signalling | (280) |
| 2017 | ESC (human)<br>iPSC, derived from dermal skin fibroblasts or PBMC (human) | Lung progenitors | Enrichment of NKX2-1+ lung progenitors based on selection of CD47$^{hi}$/CD26$^{lo}$ cells | (281) |
| 2017 | ESC (human)<br>iPSC, derived from dermal skin fibroblasts or PBMC (human) | Alveolar progenitors | High WNT signalling promotes alveolar epithelial fate | (282) |
| 2017 | ESC (human)<br>iPSC, derived from dermal skin fibroblasts or PBMC (human) | AEC2 | Temporal regulation of WNT signalling promotes AEC2 maturation and proliferation | (265, 266) |

Abbreviations: ESC = embryonic stem cell; AEC2 = alveolar epithelial type 2 cell; KGF = keratinocyte growth factor; FGF = fibroblast growth factor; DCI = dexamethasone, cyclic adenosine monophosphate, 3-isobutyl-1-methyxanthine; DE = definitive endoderm; iPSC = induced pluripotent stem cell; TGF-β = transforming growth factor β; BMP = bone morphogenetic protein; AFE = anterior foregut endoderm; SOX17 = sex determining region Y-box 17; NKX2-1 = NK2 homeobox 1; WNT = wingless-related integration site; RA = retinoic acid; PBMC = peripheral blood mononuclear cells.

The initial step of the AEC2 differentiation protocol involves differentiation of the pluripotent stem cells into definitive endoderm (DE) (272) (**Figure 12**). For induction of the DE, a commercially available kit called the StemDiff DE kit can be used (265), which contents are not fully disclosed. However, other studies have demonstrated that induction of DE in mouse ESC requires either short exposure to serum, or culture in the presence of activin A under serum-free conditions (272). To check for efficiency of DE induction, cells can be stained based on expression of the surface markers c-KIT and CXCR4, which should both be highly expressed at this stage (273, 281). Alternatively, the induction efficiency may be evaluated by intracellular staining of the transcription factor SOX17 which is enriched in the DE population (275), however this requires fixation and permeabilisation of the cells which kills them.

Further, the cells are differentiated into anterior foregut endoderm (AFE), which requires dual inhibition of TGF-β and bone morphogenetic protein (BMP) signalling (274). This is achieved by medium addition of the small molecule SB-431542, which is an inhibitor of the TGF-β1 receptor, and dorsomorphin, which inhibits the BMP signalling (265). Distinguishing the AFE from the posterior foregut endoderm can be made through intracellular staining for the transcription factor SOX2, which should be enriched in the anterior population, and CDX2, which is enriched in the posterior population (283). However, as is the case for SOX17 staining, the intracellular staining of SOX2 and CDX2 requires fixation and permeabilisation of the cells.

From the AFE stage, the cells are differentiated into lung progenitors by the addition of the small molecule CHIR99021, which activates Wnt signalling by inhibiting glycogen synthase kinase 3 (GSK-3), together with BMP4 and retinoic acid (280), which drive the AFE cells into lung fate (263, 276, 281). Successful differentiation of lung epithelial cells from this stage requires expression of the transcription factor NK2 homeobox 1 (NKX2-1) (284), which is induced by the factors in the lung progenitor differentiation medium. However, as this is an intracellular marker, staining for this marker would require fixation and permeabilisation for selection by fluorescence-activated cell sorting (FACS) which kills the cells. Other reports have identified that selection of cells at the lung progenitor stage based on high expression of CD47 and low expression of CD26, which are both surface markers suitable for live cell sorting, enables enrichment of cells with high expression of NKX2-1 (281). Following the enrichment of NKX2-1-expressing lung progenitors, the cells are differentiated towards alveolar fate by temporal

regulation of Wnt signalling through addition and withdrawal of CHIR99021, which enables maturation and proliferation of cells with the AEC2 phenotype (265, 282), fibroblast growth factor 7 (FGF7, also termed KGF) which has been shown to preserve the AEC2 phenotype (270) and stimulate the proliferation of AEC2 (285), and the described media additives dexamethasone, cyclic adenosine monophosphate (cAMP), and 3-isobutyl-1-methyxanthine (IBMX) which enable lung maturation (271). At this stage, the mature iAEC2 can be maintained as organoids in Matrigel (241) and expanded through passaging (265, 266).

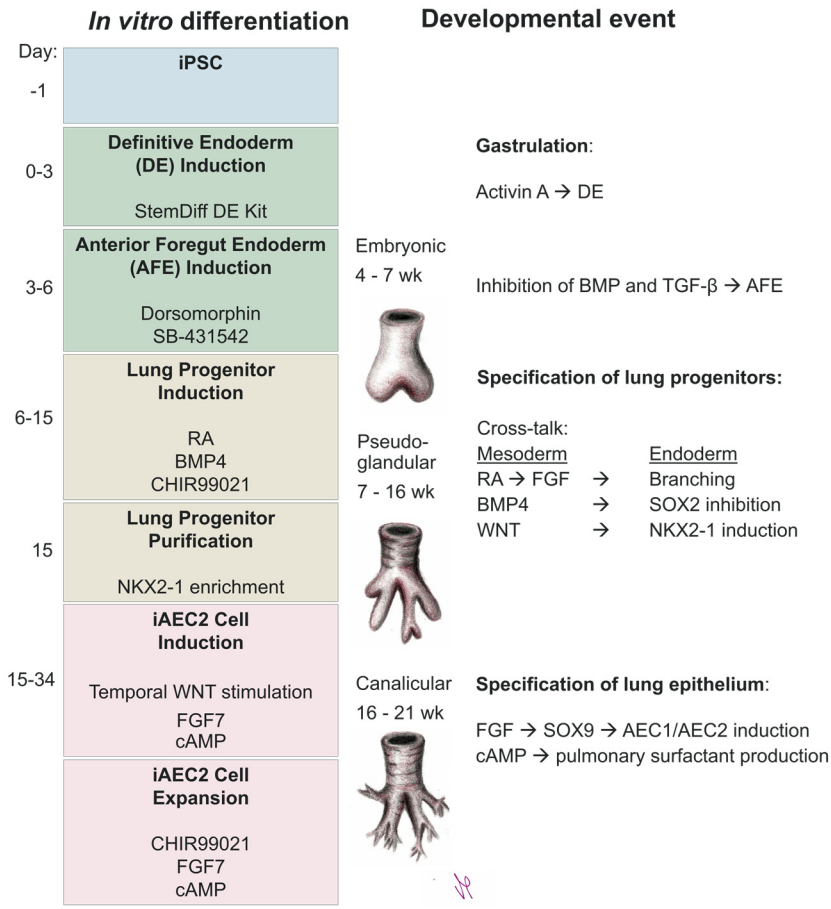



**Figure 12:** Overview of the differentiation protocol from induced pluripotent stem cells (iPSC) to alveolar epithelial type 2 cells (iAEC2) used in Paper III, based on the original protocol as described by Jacob and colleagues (265, 266) and with the corresponding key events occurring during human lung development based on Eenjes and colleagues (286). DE = definitive endoderm; AFE = Anterior foregut endoderm; RA = retinoic acid; BMP4 = bone morphogenetic protein 4; SOX2 = sex determining region Y-box 2; NKX2-1 = NK2 homeobox 1 transcription factor; WNT = Wingless-related integration site; FGF7 = fibroblast growth factor 7; cAMP = cyclic adenosine monophosphate. Illustrations by Victoria Ptasinski.

The iPSC-derived AEC2 (iAEC2) at the end of this differentiation protocol have been described to be transcriptionally similar to primary foetal distal alveolar progenitors around week 21 of human development (265), corresponding to the canalicular developmental stage (1). At this point, the AEC2 phenotype in the derived cells can be validated through a number of readouts (208):

Morphological readouts:

1. Presence of structures which resemble lamellar bodies by for example transmission electron microscopy or staining with LysoTracker, which is a dye staining acidic structures such as lamellar bodies (207).

AEC2-associated marker expression:

1. Alveolar epithelial genes, such as the surfactant protein genes *SFTPC* and *SFTPB* through quantitative real-time polymerase chain reaction (qRT-PCR) or other transcriptomic techniques.
2. Processed, mature forms of surfactant protein B and C by for example Western blotting which is indicative of functional lamellar bodies, or by imaging in which surfactant proteins should be visible in vesicular, intracellular patterns.
3. Other "omic"-based techniques, *e.g.* proteomics and lipidomics, in which the derived cells can be compared to existing datasets of primary AEC2.

Functional readouts:

1. Exocytosis of surfactants upon stimulation with secretagogues, which stimulate the secretion of stored surfactant from intracellular vesicles.
2. Proliferation and self-renewal capacity, for example through organoid culture of the cells which can be evaluated by colony-forming efficiency assays.
3. Transdifferentiation into AEC1-like phenotypes *in vitro*, by for example culturing in 2D conditions (265, 287), and assessment of upregulation of AEC1-associated markers such as for example *AQP5* and *PDPN*. Of note, the upregulation of AEC1-associated markers is not a definite criterion for transdifferentiation into functional AEC1, which are capable of gas exchange. Established assays for gas exchange measurement are however lacking.

4. Engraftment into injured lungs and lineage-tracing of derived cells to assess their potential to contribute to alveolar epithelial repair, consistent with the described function of AEC2 *in vivo*.

### A novel *in vitro* model of fibrotic, transitional alveolar epithelial cells

In **Paper III**, iAEC2 were differentiated using the protocol as previously described by Jacob and colleagues which yields cells of AEC2-like phenotype (265, 266). The differentiated iAEC2 organoids were used to build a miniaturised model in which fibrotic aberrant epithelial phenotypes can be induced (288) by addition of the same components of the fibrosis cocktail as earlier described by Alsafadi and colleagues (212) (**Figure 13**).

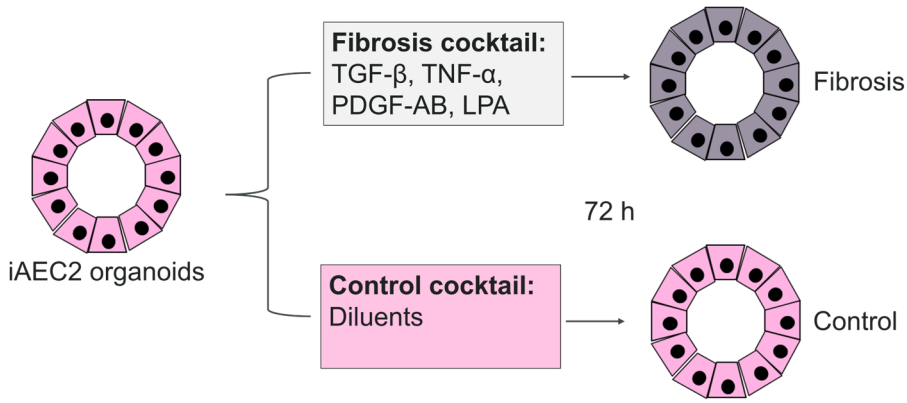



**Figure 13:** Overview of fibrosis cocktail stimulation of iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids. Figure adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figure permitted under the Creative Commons Attribution 4.0 International License. TGF-β = transforming growth factor β; TNF-α = tumour necrosis factor α; PDGF-AB = platelet-derived growth factor subunits A and B; LPA = lysophosphatidic acid. Original figure 1A published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 3 additional panels.

The use of the fibrosis cocktail allows for timely control of induction of the fibrotic changes, which is needed to adapt the model for anti-fibrotic drug screening purposes. In contrast to many other organoid systems in which the alveolar epithelial cells require co-culture with mesenchymal cells (128, 167, 169, 172, 173), our system with iAEC2 organoids does not. As such, this culture system is more accessible to laboratories which do not have access to fresh lung tissue for isolation of mesenchymal cells.

# Visualisation of lung tissue structures

Once a cell type or structure of interest is defined, studying its location in the normal and fibrotic lung is a natural next step. Traditional techniques such as histology or staining of cells with fluorescent antibodies are commonly used for this purpose. However, these techniques require prior embedding of a smaller piece of tissue either in paraffin or in a cryo matrix for freezing of the specimen. This is necessary for human lung specimens due to the large size, but for the smaller mouse lung it is possible to embed an entire lung lobe. However, to be able to stain the lung tissue with stains like H&E or antibodies, it needs to be sectioned in thin slices which are put on glass slides. This step may impact the native structure of the specimen, such as the structure of the alveoli, and make them less representative of what the lung structure looks like *in vivo*.

## Optical clearing

Organs containing water, proteins and lipids are normally opaque and require optical clearing prior to imaging, which is a process of chemical removal of proteins, lipids and bleaching of pigments to avoid refraction and scattering of the light during microscopy (289, 290). A variety of optical clearing protocols have been developed for different imaging purposes, such as visualisation of fluorescent proteins, dyes based on lipids, or fluorescent antibodies used during immunolabelling (290). After these steps are complete, the refractory index of the tissue specimen is matched with that of the solution it is later imaged in (289, 290). This makes the tissue specimen look transparent when immersed in the solution.

## Light-sheet fluorescence microscopy

Light-sheet fluorescence microscopy (LSFM) uses a sheet of laser which illuminates only a thin section of the sample (291, 292). As the illumination source and the detection lens are placed perpendicular to each other, it is possible to image areas localised deeply in the tissue specimen while the extent of photobleaching of the fluorochromes is minimised (292). The benefit of optical tissue clearing in combination with imaging by LSFM as opposed to traditional immunofluorescence imaging is that large pieces of tissue remain intact. Further, the acquisition time using LSFM is considerably shorter than using confocal microscopy.

### A novel protocol for optical clearing and immunolabelling of the mouse lung

In **Paper IV**, the focus was placed on the development of a protocol enabling improved visualisation of whole mouse lungs immunolabeled with fluorescent antibodies. The described clearing strategy is based on hydrophobic clearing (dehydration and removal of lipids from the tissue) with solutions containing organic solvents, alike the previously described clearing protocol termed iDISCO (293). Previously described clearing protocols are commonly based on immersion and incubation of the tissue in the different solutions to allow their diffusion into the tissue (289, 290). In the protocol described in **Paper IV**, the main steps of the clearing and immunolabelling protocol are instead performed by inflation of the lung tissue through the trachea.

## Sequencing techniques

A significant portion of the results described in **Paper I, II** and **III** rely on sequencing techniques and bioinformatic tools to determine the effect on RNA transcripts in various conditions.

### RNA sequencing

In **Paper I-III**, a technique termed RNA sequencing (RNAseq) was utilised to determine the changes of RNA transcripts on bulk level in either mouse lung tissue or cells (**Paper I** and **II**) or in human cells (**Paper III**). The first step involves extraction of RNA from the tissue and assessment of the extent of degradation. RNA is readily degraded by endogenous RNases, and if this occurs the sequencing will be unable to identify transcripts which can later be mapped to genes during the data processing. The next step involves reverse transcription into cDNA and library preparation and the exact protocol used for these purposes varies depending on the type of sequencing. The types range from sequencing of the total RNA from a sample or selected types of RNA, including ribosomal RNA (rRNA) or messenger RNA (mRNA). Once the library is prepared, it is sequenced and the data is bioinformatically processed. The pipelines used for the data processing, which involves alignment of the reads from the sequencing to the genes, estimation of the read counts and normalisation may vary between studies

(294) and they are therefore described in more detail separately in each of the papers included in this thesis. However, the different pipelines in the end generate similar formats of data output being lists of expression values for each of the detected genes and lists of significantly altered (differentially expressed) genes. Therefore, data may be compared across RNAseq datasets even though different analysis pipelines have been used (294).

### Single-cell RNA sequencing

Differences in specific cell populations in the tissue material may be masked when exploring population-averaged measurements with techniques such as bulk RNAseq. During recent years, transcriptomic techniques with single-cell resolution have been developed allowing for study of expression patterns in individual cells and identification of novel cell subpopulations in the lung (38-40, 104). Single-cell RNA sequencing (scRNAseq) requires the isolation of RNA from single cells, the conversion of the RNA into DNA, and library preparation for sequencing. There are now several sequencing techniques available, each of them providing various levels of precision and sensitivity depending on the sample type and size (295). However, compared with bulk RNAseq, many existing scRNAseq methods have low capture efficiency meaning that only a small fraction of each cell's transcriptome is represented in the final sequencing libraries (295). Therefore, many scRNAseq techniques have limited sensitivity and are often unable to detect transcripts present in low abundance, such as for example different isoforms of the same gene or very rare transcripts. Deeper sequencing possible with bulk RNAseq additionally facilitates more accurate pathway analyses which was of importance for the analyses performed in **Paper I**, **Paper II** (211) and in **Paper III** (288). Other challenges associated with scRNAseq are the different methods of cell clustering, for which there is currently no consensus and which impacts the data output significantly (295). It is hence more challenging to compare scRNAseq output across datasets, as the same cell types might be annotated differently in the datasets. Further, scRNAseq is often more costly than bulk RNAseq, which makes scRNAseq a relatively inaccessible analysis technique for many laboratories.

# Bioinformatic techniques

In **Papers I, II** and **III**, bioinformatic analyses were applied to explore biological effects from the obtained sequencing data in the RNAseq experiments. In **Paper I**, those consisted of gene ontology analyses based on the lists of differentially expressed genes and simpler comparisons with other public sequencing datasets from the human lung. In **Paper II** (211) and **Paper III** (288), the findings from the bulk RNAseq datasets indicated that there could be differences in cell phenotypes between the compared samples, which lead to exploration of bioinformatic tools for estimating cell proportions from bulk sequencing data.

### Deconvolution of bulk RNAseq data to estimate cell proportions

Comparing the deeper coverage of bulk RNAseq and the single-cell resolution of scRNAseq, it is undoubtfully clear that the most optimal scenario would be to have both features in a sequencing analysis. For this reason, bioinformatic tools involving "mapping" of bulk RNAseq data to reference scRNAseq datasets called deconvolution techniques have been developed (296). The benefit of this approach is that the algorithm uses all genes detected in the bulk RNAseq dataset to compare with the gene expression signatures in each of the populations in the reference scRNAseq dataset (296). Hence, bias dependent on the selection of individual cell-type associated markers is minimised. For example, the marker *KRT8* which is described to be characteristic for the transitional alveolar epithelial cell population arising in pulmonary fibrosis is actually widely expressed in different clusters of epithelial cells, and is thus not specific to the transitional alveolar epithelial cell population (127). Important factors to consider when applying the deconvolution technique is that the bulk RNAseq dataset ideally needs to be generated from a similar source as the scRNAseq dataset used as the reference. This techniques was shown to be successful in deconvolution of bulk RNAseq data from human PCLS, for which a human lung reference scRNAseq dataset was used (297). Although this was an example in which smaller lung tissue pieces were mapped to whole lung, the deconvolution technique is also applicable when comparing cells to whole lung datasets. In this thesis, this was demonstrated in **Paper II** where the isolated mouse epithelial cells were compared to a reference scRNAseq dataset from whole mouse lung (211), and in **Paper III** in which iAEC2 organoids were compared to a reference scRNAseq dataset from human IPF lungs (288).

# Results and Discussion

## Paper I

The quest for finding molecular mechanisms in IPF which are amenable to pharmaceutical intervention began with a transcriptomic analysis of the bleomycin model of pulmonary fibrosis in mouse. Recent literature indicates that long non-coding RNA transcripts are implicated in fibrotic diseases of the heart, kidney and lung (298-304). They are believed to act through versatile mechanisms regulating availability of other RNA transcripts and the transcription of other genes (305, 306). Hence, they are a potentially attractive group of targets for future therapeutics against pulmonary fibrosis, which is a disease involving interactions between many different molecular mechanisms (1).

In particular, a long non-coding RNA transcript named nuclear paraspeckle assembly transcript 1 (*NEAT1*) has recently been described to have potential roles in pulmonary fibrosis. *NEAT1* is a component of the nuclear paraspeckle structures, an organisational structure in the nuclei around which other regulatory factors reside (307, 308). Loss of *Neat1* in non-haematopoietic cells has been reported to induce apoptosis in fibrotic mice (309), whereas induced *NEAT1* expression in human lung epithelial cells *in vitro* promotes induction of the EMT phenotype and collagen production (300, 303). In **Paper I**, the behaviour of *NEAT1* was first explored in the bleomycin model of pulmonary fibrosis in mouse, followed by step-wise exploration of the associated fibrotic processes common to both mouse pulmonary fibrosis and human IPF, and ending with demonstration that the effects seen in the mouse bleomycin mouse model can be translated to activated primary human lung fibroblasts isolated from patients with IPF (**Figure 14**).

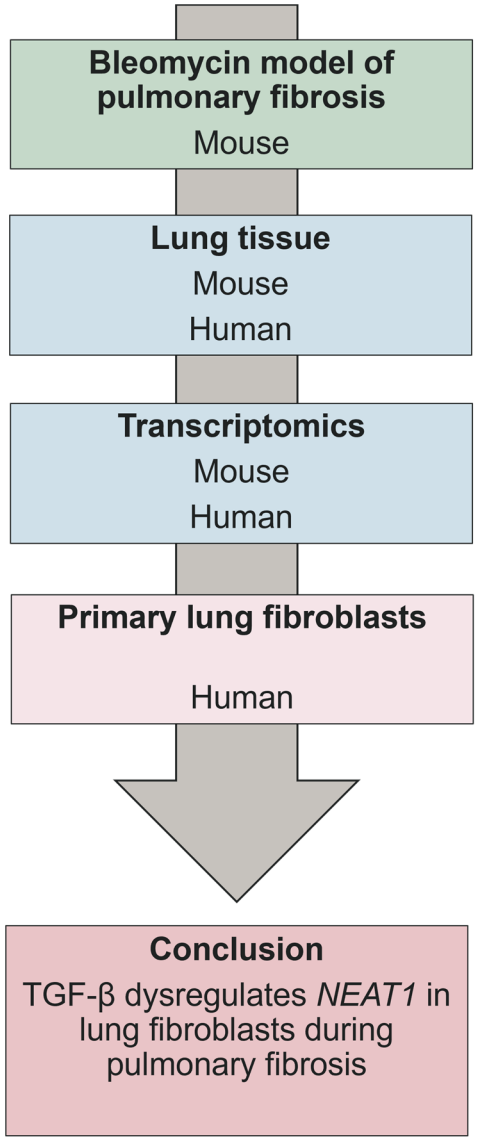



**Figure 14:** Step-wise exploration of the long non-coding RNA transcript *NEAT1* in pulmonary fibrosis, as described in Paper I included in this thesis.

As published studies using human lung epithelial cells indicated that effects on *NEAT1* could be linked to active TGF-β signalling (300, 303), an additional group was included in the bleomycin experiment setup which received treatment with a small molecule inhibiting the activin-receptor like kinase 5 (310) to which TGF-β is able to bind. Bulk RNAseq of mouse lungs

indicated that bleomycin induces loss of *Neat1* expression and that this is prevented by inhibition of TGF-β signalling. Further, the loss of *Neat1* in the mouse lungs was significantly correlated to the extent of dense, fibrotic areas in the mouse lung tissue and increases in deposited collagen in these affected areas.

The main conclusion from the experiment in the bleomycin mouse model is that the lower level of *Neat1* in the fibrotic mouse lung is associated with molecular changes occurring primarily in the dense, fibrotic areas of the lung. This observation was further strengthened by the *in situ* hybridization staining of the *NEAT1* transcripts in both mouse and human fibrotic lung, where significantly less expression of *NEAT1* was observed in the dense areas of the lung tissue. *NEAT1* is expressed as two isoforms, of which the longer isoform *NEAT1_2* is expressed in lower levels in the mouse lung (311). Although there could have been potential differences in expression patterns between these two *NEAT1* isoforms, the results from the analyses in **Paper I** indicate that both isoforms are less expressed in the dense regions of the mouse and human lung.

Areas which are classified as dense in the image analyses, *i.e.* areas with deposited collagen and mesenchymal cells, are also present in the healthy lung around the airways and main blood vessels. Thus, one caveat with this analysis is that the image analysis is not able to accurately distinguish between fibrotic and non-fibrotic dense tissue. Therefore, further analyses were needed to link effects associated with the loss of *NEAT1* in the dense, fibrotic lung tissue. Comparison of the transcriptomic changes in mouse and human pulmonary fibrosis (312) enabled establishment of signatures of genes associated with dysregulated *Neat1* expression. The expression of these gene signatures were projected onto a publicly available scRNAseq dataset of human IPF lung (39). This analysis indicated that decreased *NEAT1* expression correlates with gene signatures related to activated fibroblasts and myofibroblasts in human IPF. Lastly, this hypothesis was further explored by performing *in vitro* experiments with human primary lung fibroblasts from individuals with IPF, in which loss of *NEAT1* expression was dependent on TGF-β signalling and occurred simultaneously with induction of collagen expression, alike in the bleomycin mouse model. Thus, the conclusion from the experiments in **Paper I** is that the bleomycin-induced effects on *Neat1* expression in the mouse translate specifically to TGF-β-dependent effects on *NEAT1* expression in human fibrotic fibroblasts.

## Paper II

The study undertaken in **Paper I** is an example of how potential IPF-associated therapeutic targets are selected and evaluated pre-clinically. Targets are often initially identified as interesting *in vivo* in animal models or in patient samples, and further evaluated by *in vitro* experiments in relevant cell types (90, 117, 233, 309). Thus, the ability to isolate cell types of interest from the lung tissue is an important aspect of drug discovery. However, there has been a historical lack of harmonisation between different cell isolation protocols due to that studies have tended to focus on cell types present either in the trachea and airway regions (204) or in the alveolar region (35, 205), and rarely on both simultaneously.

The application of the 3DLD tool which was developed in **Paper II** enables isolation of cells from the trachea and lung lobes from the same mouse (211). The focus in **Paper II** was on the epithelial cell types. The initial aim was to compare whether the application of the 3DLD tool during the isolation process yielded epithelial cell isolates which perform differently under cell culture conditions and are of different phenotype compared with the classic protocols. When cultured as organoids, the distal lung epithelial cells isolated with the 3DLD tool yielded organoids which were smaller in size than the organoids grown from distal lung epithelial cells isolated with the classic protocol by ligation of the trachea (211) (**Figure 15**).

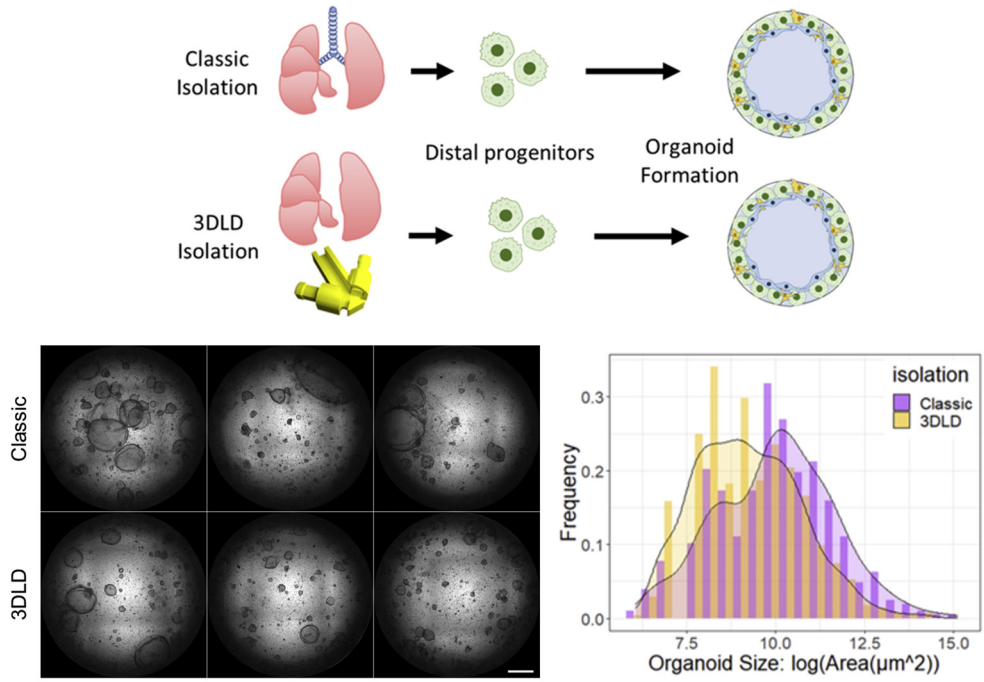



**Figure 15:** Comparison of the classic isolation strategy with isolation using the 3D-printed lobe divider (3DLD) (top panel). Representative images (brightfield microscopy) of organoids formed after 14 days in 3D culture (bottom left panel) and their frequency size distribution (bottom right panel). n = 3 individual mice. Scale bar: 1 mm. Reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 3A, 3B and 3E published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 4 additional panels.

Next, bulk RNAseq was performed of the freshly isolated distal lung epithelial cell pellets and their respective organoids to delineate whether the differences in organoid sizes were due to transcriptomic differences introduced by the two isolation methods. Although the transcriptomic profiles of the organoids were similar irrespectively of the isolation method used, larger differences could be seen in the freshly isolated distal lung epithelial cell pellets (211) (**Figure 16**).

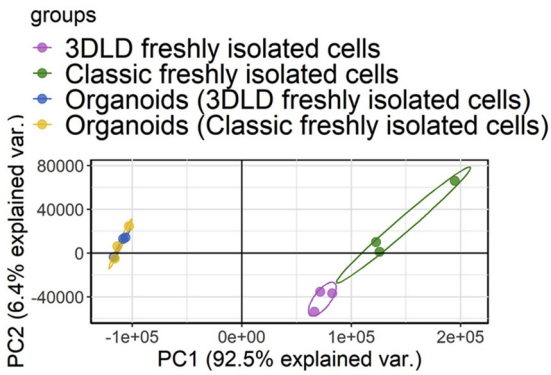



**Figure 16:** Principal component analysis (PCA) of the distal lung epithelial progenitors, sequenced as either fresh cell pellets or after organoid culture and isolated with either the classic method or using the 3DLD. Reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 3F published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 6 additional panels.

The observation that there were larger transcriptomic differences in the freshly isolated cell pellets than in the organoids indicated that this could be due to variations in the initially isolated cells between the classic and 3DLD-based isolation protocols. To explore whether this was the case, the bulk RNAseq data from the freshly isolated distal lung cell pellets was used for deconvolution using a public scRNAseq dataset from mouse lung (129) (**Figure 17**).

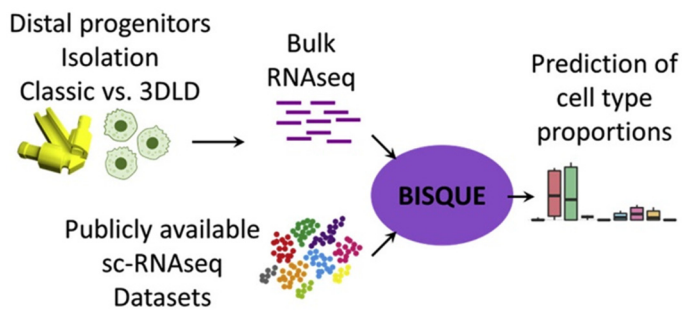



**Figure 17:** Deconvolution of a bulk RNAseq dataset of cell pellets isolated with the classic protocol or by using the 3DLD tool. The cell proportions are estimated based on a reference scRNAseq dataset from mouse lung published by Strunz and colleagues (129). Reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 4A published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 9 additional panels.

Interestingly, the deconvolution predicted a larger proportion of basal cells in the distal lung epithelial cell pellets isolated with the classic protocol compared with the distal lung epithelial cell pellets isolated with the 3DLD protocol (211) (**Figure 18**).

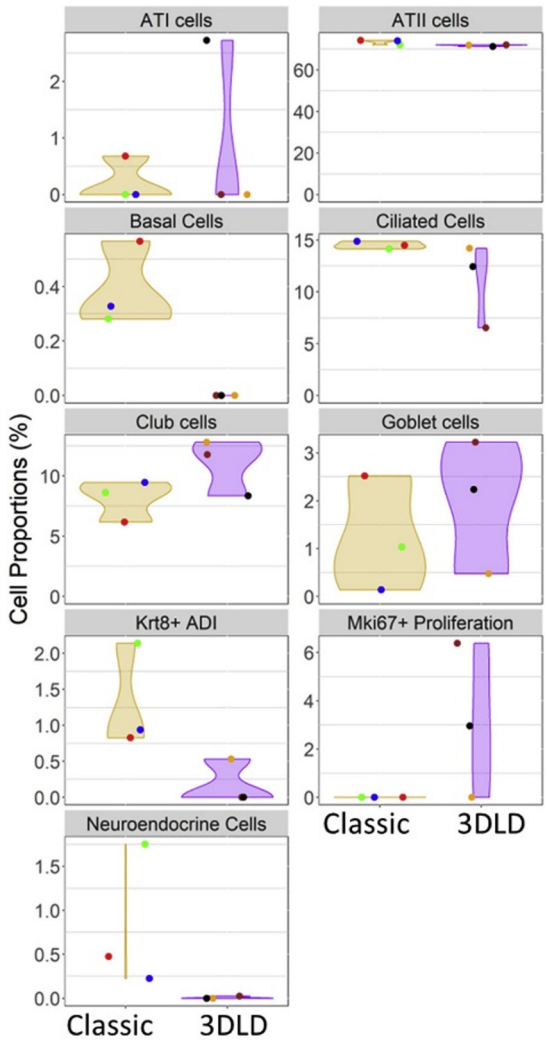



**Figure 18:** Cell proportions estimated by reference-based deconvolution of the bulk RNAseq dataset of cell pellets isolated with the classic protocol or by using the 3DLD tool, based on the reference scRNAseq dataset from mouse lung published by Strunz and colleagues (129). Figure is reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 4D published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 9 additional panels.

To outline whether the predicted differences in cell proportions in the freshly isolated cell pellets isolated with the two different methods impacted the phenotype of the organoids they form, z-scores were calculated for each of the genes in each of the organoid and pellet samples in the dataset (by comparing the expression of a gene in each sample to the mean expression of the same gene based on all samples). To more easily link the gene expression changes to the cell phenotypes analysed in the deconvolution analysis of the cell pellets, genes characteristic of the cell clusters described in the reference scRNAseq dataset (129) were specifically chosen for the heatmap (**Figure 19**). Interestingly, the largest differences in the gene expression z-scores in the chosen genes were due to the organoid culture format, irrespectively of the initial isolation method. What became clear was that there were apparent differences in expression of several genes characteristic for the clusters in the reference scRNAseq dataset (129), rather than changes just in individual cell type-specific markers. Thus, this analysis indicates a shift in cell identity induced by culture of the cells *in vitro* in the organoid format, irrespectively of the initial cell isolation method.

Interestingly, acquisition of proximal lung epithelial markers in cells with an initial distal lung epithelial phenotype *in vitro* has also been described in recent studies using organoids of human iPSC-derived AEC2 (313) and co-cultures of human primary AEC2 with lung mesenchyme (128). Alike the observations made in the organoids of mouse distal lung epithelial cells in **Paper II**, the acquisition of the proximal epithelial phenotypes in the aforementioned studies occurred spontaneously over time in culture. Collectively, these observations point towards a possible limitation of the *in vitro* organoid culture format of both mouse and human distal lung epithelial cells. In future studies, it will be critical to understand the contributing factors to the phenomenon of "proximalisation" in the distal lung epithelial organoids *in vitro* in more detail.

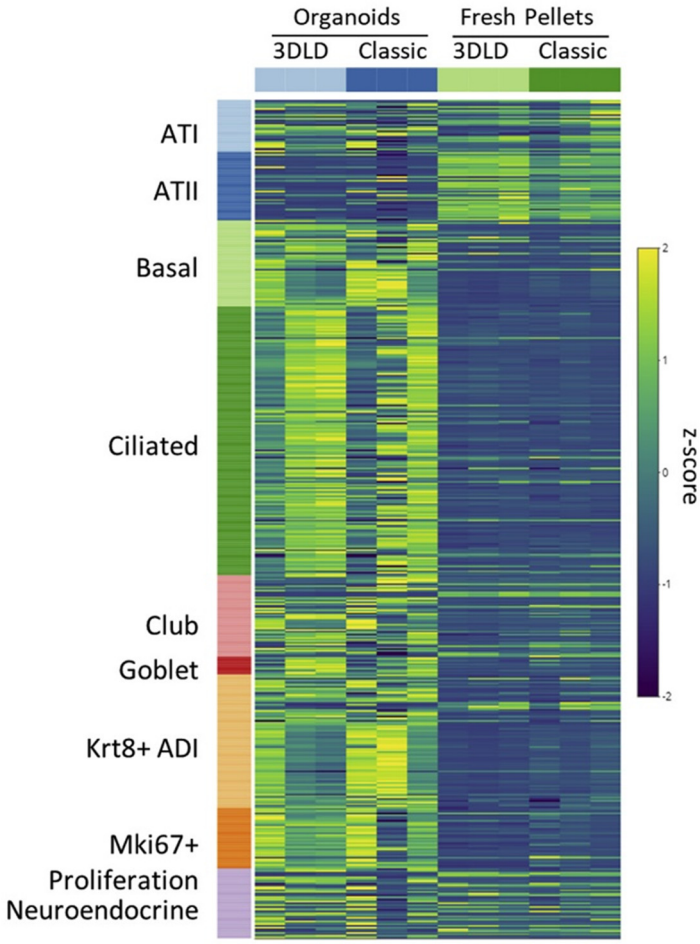



**Figure 19:** Heatmap representing the z-scores of distal progenitors isolated with classic or 3DLD methods, sequenced either as fresh pellets or as organoids. The z-score is calculated as gene[sample] − gene[mean of all samples]/SD. Genes are selected based on the markers of each cluster, as denoted in the reference scRNAseq dataset by Strunz and colleagues (129). Figure is reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 4E published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 9 additional panels.

The observations at that point did however not explain the morphological differences in organoid size between the two cell isolation protocols. One potential explanation could be that the differences in organoid size could have been due to increased presence of proximal lung epithelial cells in the isolate from the classic protocol, as indicated in the deconvolution analysis. To test this hypothesis, distal lung epithelial cells were first isolated using the 3DLD protocol and then proximal lung epithelial cells isolated with the

same protocol from the same animal were added in varying proportions. Interestingly, addition of proximal lung epithelial cells already at the lowest proportion of 1% to the distal lung epithelial cell isolate from the 3DLD protocol yielded organoids larger in size than without any addition of proximal lung epithelial cells (211) (**Figure 20**).

Hence, the conclusion in **Paper II** is that distal lung epithelial cells isolated with classic protocols based on trachea ligation contain larger proportions of contaminating proximal lung epithelial cells, and that isolation using the 3DLD tool minimises the proportion of co-isolated proximal lung epithelial cells. Thus, it was possible to demonstrate that the 3DLD tool enables distal lung epithelial cell isolation from the mouse lung with greater purity. Moreover, the 3DLD tool enables isolation of cells which is independent of cell type-specific surface markers, which enables use of this tool without prior knowledge of such markers. Thus, this isolation method permits isolation of rare cell types which might not share the same, well-established surface markers as the better characterised and more abundant cell types. These aspects are important for all studies utilising mouse lung epithelial cell isolation for downstream analyses, such as sequencing or *in vitro* experiments.

Although **Paper II** focused on the isolation of lung epithelial cells with the 3DLD tool, it may theoretically be applicable for isolation of other cell types located in the trachea and lung lobes of the mouse. This could for example include immune cells and fibroblasts. A potential application of the 3DLD tool may hence be in the context of the study described in **Paper I** in this thesis. For example, the 3DLD tool may potentially be used for isolation of fibroblasts from the mouse trachea and lung lobes, to add another dimension to the described effects on *NEAT1* in pulmonary fibrosis.

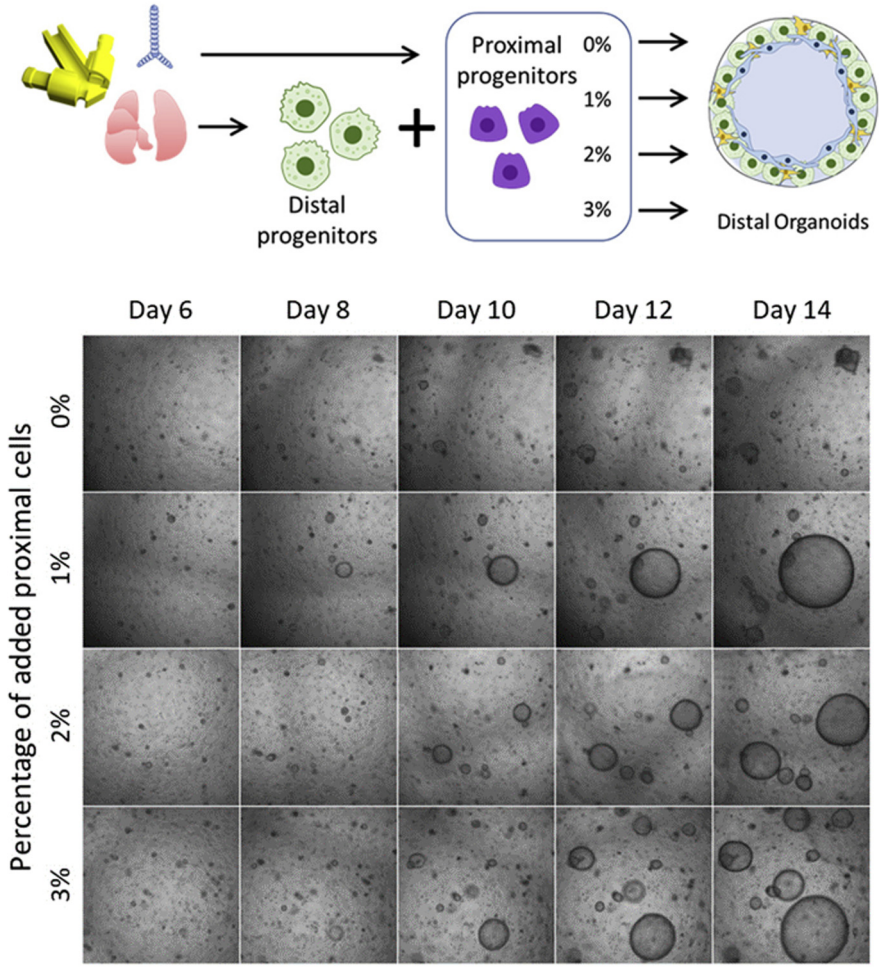



**Figure 20:** Manual addition of proximal epithelial cells to distal cell isolates to study impact on organoid size. Images are representative of organoid sizes over time in 3D culture formed from distal lung epithelial cell isolates with addition of proximal lung epithelial cells in defined percentages (of seeded number of cells). Proximal epithelial cells and distal lung epithelial cells were isolated from the same mouse using the 3D-printed lobe divider (3DLD). n = 1 mouse in 6 experimental replicates. Reused from Paper II included in this thesis by Alsafadi and colleagues (211). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 4H and 4I published in: "Simultaneous isolation of proximal and distal lung progenitor cells from individual mice using a 3D printed guide reduces proximal cell contamination of distal lung epithelial cell isolations". Stem Cell Reports, Vol. 17, Issue 12, 2022, p. 2718-2731. The original figure contains 8 additional panels.

## Paper III

Repetitive injury of the alveolar epithelium leading to aberrant alveolar epithelial behaviour is proposed to be a contributing factor to the development of pulmonary fibrosis (314). Recent scRNAseq datasets have given us insight about previously unknown, disease-specific subpopulations of epithelial cells in the IPF lung (38-40, 104). However, the factors inducing these aberrant epithelial subpopulations and the potential origin of these subpopulations remained unknown due to the lack of experimental systems in which the aberrant epithelial reprogramming could be mimicked. To study the mechanisms contributing to the initiation of pulmonary fibrosis and the associated aberrant epithelial reprogramming in the lung, experimental models allowing for controlled induction of the early IPF-associated changes were needed. However, appropriate models allowing for study of fibrosis induction based on human alveolar epithelial cells were lacking at the start of this thesis project. The development of such models gained speed during the course of this thesis, and a vast majority of these were based on stem-cell derived epithelial cells in co-culture systems with mesenchymal cells. These models were successful in providing valuable information about the genetic factors inducing some of the fibrotic changes (165, 167, 168, 171), or about the early responses to known pro-fibrotic stimuli such as TGF-β (169, 173). However, the effects of these factors specifically on the alveolar epithelial cells (and not through interaction with mesenchymal or immune cell types) were less clear from these studies. Through the strategy to firstly, differentiate iAEC2 from iPSC with no known predisposition of IPF (265, 266) and secondly, stimulate the iAEC2 with the fibrosis cocktail (212) as described in **Paper III**, it was possible to observe effects related to early fibrotic responses induced specifically in the iAEC2 under conditions mimicking those in IPF (288).

The original study describing the fibrosis cocktail and its application to human PCLS demonstrated the ability of this cocktail to induce histological changes in lung tissue structure (212). Interestingly, stimulation of iAEC2 organoids with the fibrosis cocktail induced a dense morphology of the organoids, which was possible to quantify by assessment with light microscopy (288) (**Figure 21**).

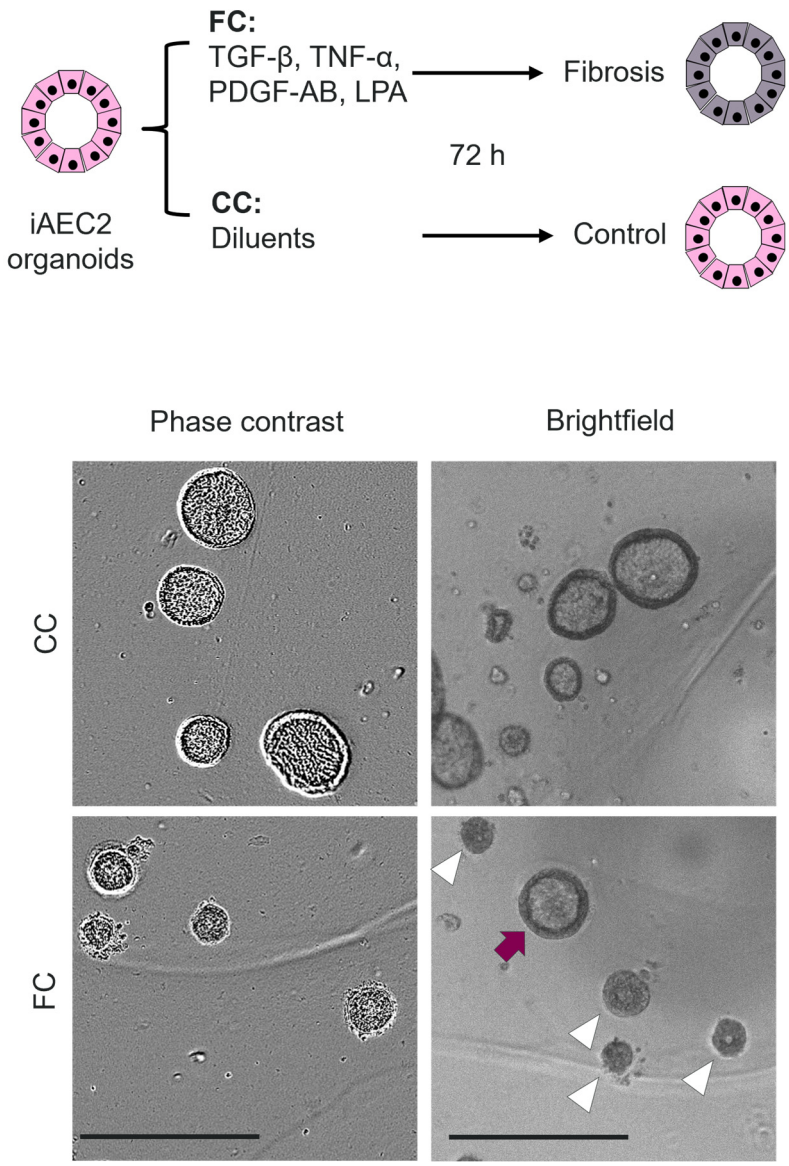



**Figure 21:** Fibrosis cocktail (FC) induces a dense morphology in the iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids. Microscopy images acquired with 4x magnification. Scale bar: 500 µm. Purple arrow shows an organoid with a normal morphology similar to that of control organoids, white arrow heads show organoids with a dense morphology. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 1A and 1B published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains two additional panels.

This morphological change was associated with broad transcriptomic changes elicited by the fibrosis cocktail as evaluated by bulk RNAseq, related to known processes occurring in the IPF lung including senescence and telomere dysfunction (288) (**Figure 22**). Interestingly, senescence has been described to be a process characteristic of the $KRT8^{hi}$ transitional alveolar epithelial cells present in IPF, and is not observed in similar cells in more acute types of lung injuries such as acute respiratory distress syndrome (ARDS) (315). Many of the other pro-fibrotic processes which were induced in our dataset, such as apoptosis and ECM expression, are known to be induced by the components included in the fibrosis cocktail (95, 125, 231, 232). DNA damage and dysfunction in telomere maintenance are reported to be linked to both familial cases of pulmonary fibrosis (120) and adult-onset IPF (121), supporting the hypothesis that these processes can either be induced due to genetic predispositions (120) or through other events occurring later in life, such as smoking (316). However, the mechanism by which the factors of the fibrosis cocktail induce DNA damage and telomere dysfunction needs further investigation.

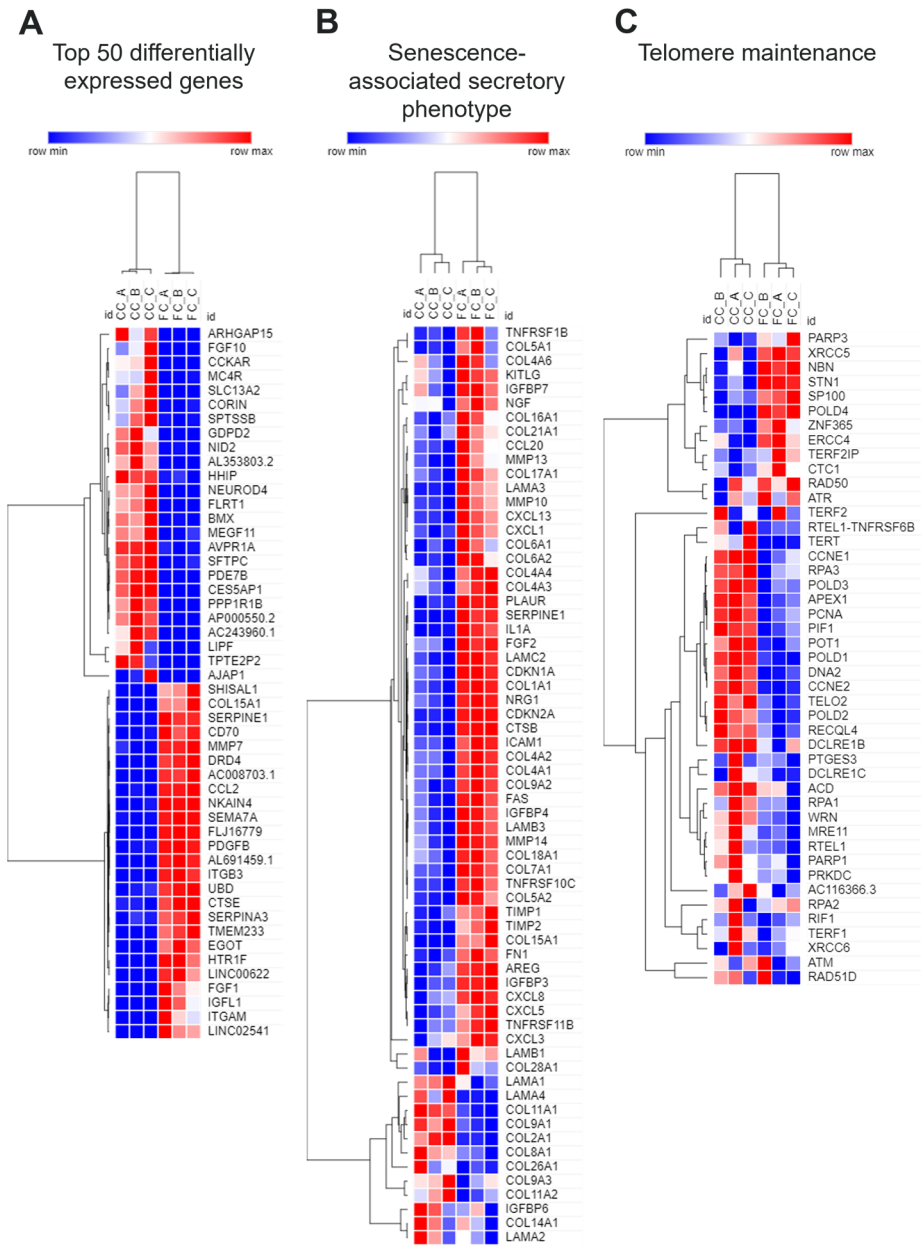



**Figure 22:** Transcriptomic differences in fibrosis cocktail (FC)-stimulated iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids compared with control cocktail (CC)-stimulated organoids. A) Top 50 differentially expressed genes. B) Differentially expressed genes of senescence-associated secretory phenotype factors, based on Table 1 by Coppé and colleagues (102). C) Differentially expressed genes related to GO_BP:0000723: Telomere maintenance. For A-C: Genes are defined by absolute log2 fold change ≥ 0.7 and p(adj) < 0.05. n = 3 batches of iAEC2 organoids. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures S6A, S6C and S6D published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains one additional panel.

The next step included evaluation of whether the iAEC2 model could address the lack of models representative of the aberrant epithelial reprogramming described in IPF. Specifically, focus was placed on changes induced by the fibrosis cocktail related to the aberrant epithelial phenotypes described in the scRNAseq datasets of the IPF lung (38, 39). Therefore, reference-based deconvolution of the bulk RNAseq dataset was performed to estimate the cell proportions based on a publicly available dataset of the human IPF lung, which had identified fibrotic epithelial phenotypes including the *KRT17*$^{+}$*/KRT5*$^{-}$ aberrant basaloid cells (39). Interestingly, the deconvolution estimated an increase in the proportion of cells with the *KRT17*$^{+}$*/KRT5*$^{-}$ aberrant basaloid phenotype along with loss of the AEC2 phenotype upon fibrosis cocktail stimulation (288) (**Figure 23**).

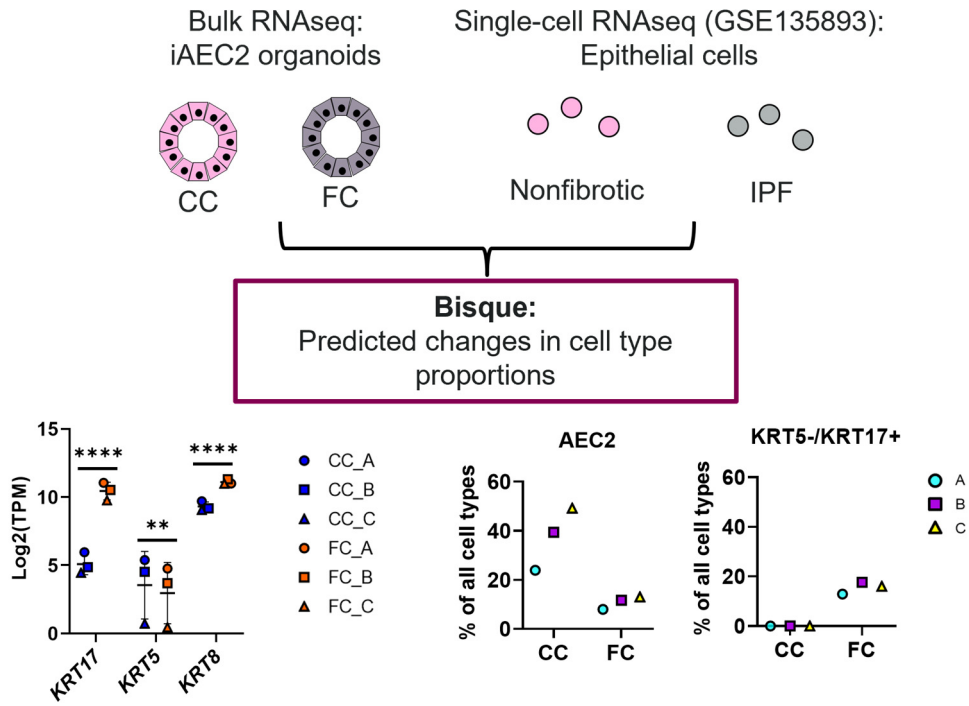



**Figure 23:** Reference-based deconvolution of bulk RNAseq data from fibrosis cocktail (FC)-stimulated iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids based on a public scRNAseq dataset from human IPF lung (39) predicts loss of alveolar epithelial type 2 cell (AEC2) phenotype and acquisition of aberrant basaloid phenotype in iAEC2 organoids upon FC stimulation. n = 3 batches of iAEC2 organoids. Adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 4A, 4B and 4C published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 3 additional panels.

As a scRNAseq dataset defines the cell types based on their transcriptome, the induction of the *KRT17*$^{+}$/*KRT5*$^{-}$ cell phenotype was additionally confirmed in the iAEC2 organoids on protein level by immunofluorescent staining. Intriguingly, the strong KRT17 staining was mostly located to the organoids exhibiting a dense phenotype in the fibrosis cocktail-stimulated cultures (288), indicating that the dense morphology of the fibrotic iAEC2 organoids is linked to the aberrant reprogramming of the cells (**Figure 24**).

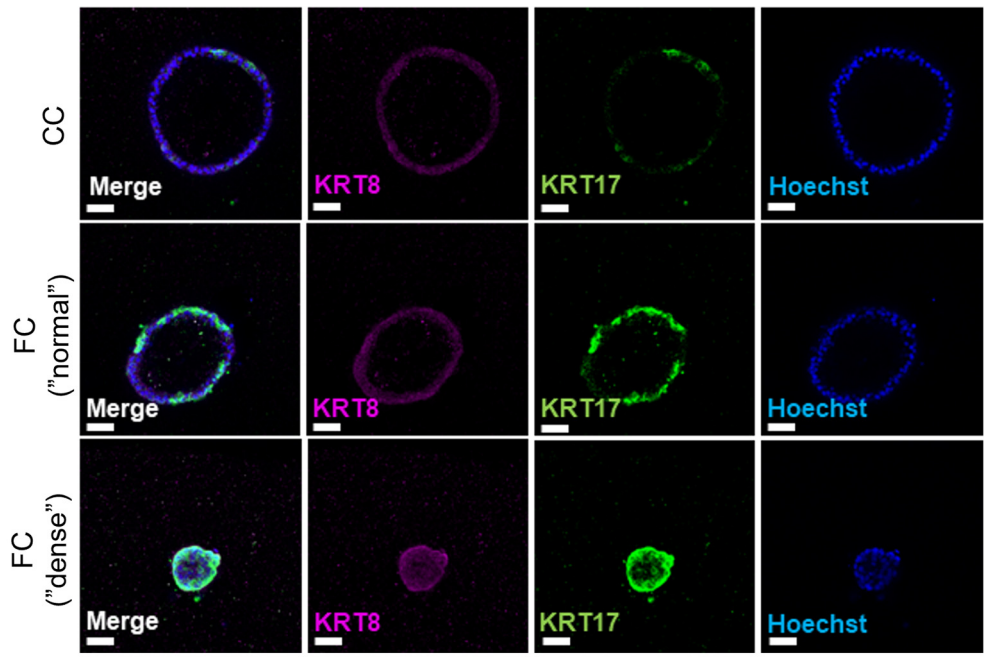



**Figure 24:** FC induces expression of keratin (KRT) 8 and 17 in iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids, associated with aberrant basaloid phenotypes in the IPF lung. Scale bar: 50 µm. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 4D published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 5 additional panels.

During the same period of time as this study was performed, a different report described the induction of the ABI phenotypes in co-cultures of primary healthy hAEC2 with AHLM (128). The ABI populations are proposed to be transitional states characterised by varying expression of KRT8 and KRT17 on the trajectory to the metaplastic $KRT5^{+}$ cells present in human IPF lungs around the honeycombing structures (128). The findings from this report led to exploration of similar phenotypic states in the iAEC2 cultures upon fibrosis cocktail stimulation. Reference-based deconvolution analysis of the bulk RNAseq dataset was performed to estimate the proportions of cells with the same ABI phenotypes as reported in the scRNAseq dataset of primary hAEC2 organoids co-cultured with the AHLM (128). Consistent with the earlier analyses, this deconvolution predicted loss of the AEC2 phenotype and acquisition of the two ABI phenotypes (288) (**Figure 25**). In the original study, the ABI states were acquired after 7 days in culture and without any additional stimuli apart from the presence of AHLM (128). In contrast to this report, the ABI phenotypes are not acquired spontaneously in the iAEC2 system described in **Paper III** (288), which enables control over the timing of this induction. The acquisition of the ABI phenotypes is also more rapid (3 days) in the system described in **Paper III**, which is desirable from a modelling perspective as this will allow use of the model in time-sensitive applications such as for example drug screening.

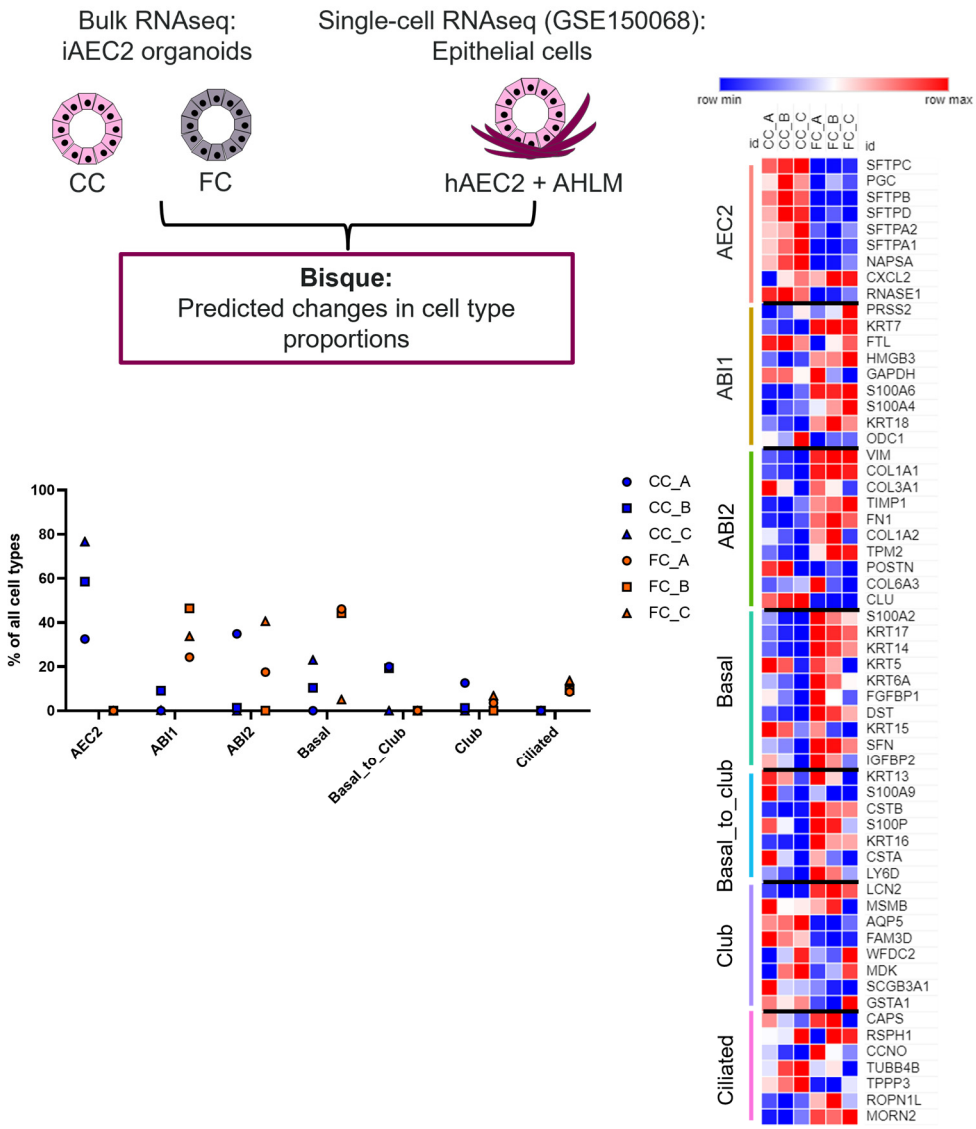



**Figure 25:** Reference-based deconvolution of bulk RNAseq data from fibrosis cocktail (FC)-stimulated iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids based on a public scRNAseq dataset from primary human alveolar epithelial type 2 (hAEC2) organoids co-cultured with adult human lung mesenchyme (AHLM) (128) predicts loss of the alveolar epithelial type 2 cell (AEC2) phenotype and acquisition of alveolar-basal intermediate (ABI) phenotypes in iAEC2 organoids upon FC stimulation. n = 3 batches of iAEC2 organoids. Adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 5A, 5C, and 5E published in: “Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids”. Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 2 additional panels.

Consistent with the findings presented in the study using human PCLS (212), the fibrosis cocktail induced expression of several matrisome-associated components in the iAEC2 organoids (288) including several types of collagens, fibronectin (FN) and tenascin C (TNC) (**Figure 26**).

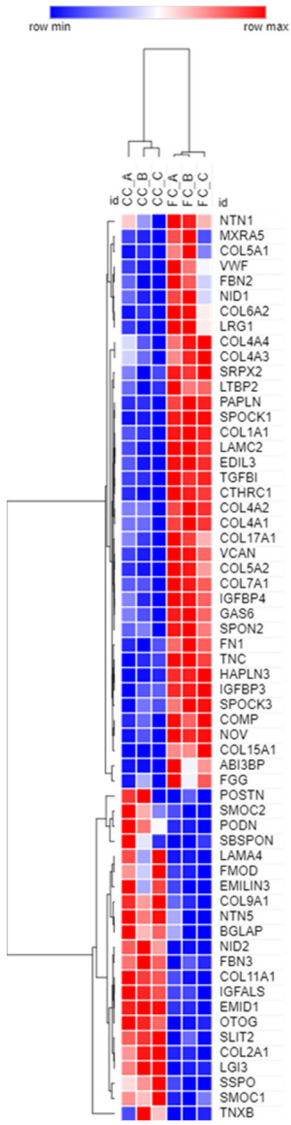



**Figure 26:** Fibrosis cocktail (FC) induces expression of extracellular matrix (ECM) in iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids. Matrisome-associated genes were selected by p(adj) < 0.05. n = 3 batches of iAEC2 organoids. Use of figure permitted under the Creative Commons Attribution 4.0 International License. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Original figure 6A published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 5 additional panels.

Moreover, immunostaining under non-permeabilising conditions showed that the fibrotic iAEC2 organoids are capable of collagen type 1 (COL1) secretion and thus able to contribute to the excessive ECM deposition along with myofibroblasts in IPF (**Figure 27**). This observation is also consistent with the described expression of ECM-associated transcripts by the *KRT17*$^{+}$*/KRT5*$^{-}$ aberrant basaloid cells (38), collectively strengthening the conclusion that cells with this phenotype are induced in the iAEC2 system described in **Paper III**.

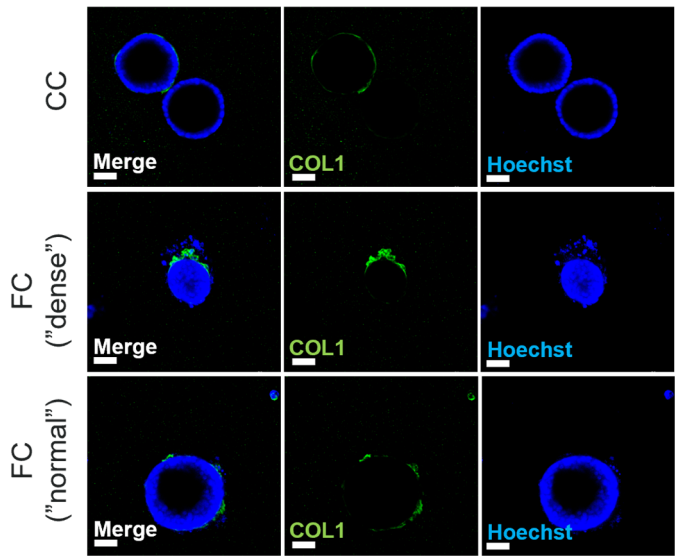



**Figure 27**: Fibrosis cocktail (FC) induces expression of extracellular matrix (ECM) in iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids. Immunofluorescence of collagen type 1 (COL1) was performed under non-permeabilising conditions to visualise secretion. Scale bar: 50 µm. n = 3 batches of iAEC2 organoids. Use of figure permitted under the Creative Commons Attribution 4.0 International License. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Original figure 6F published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 5 additional panels.

After focusing on the induced effects of the fibrosis cocktail related to the aberrant epithelial reprogramming characteristic of IPF, the technical flexibility of the described iAEC2 system was evaluated. A desirable feature of a chronic lung disease model is persistence of the disease-related effects over time. This concept was tested in **Paper III** by performing a stimulation of the iAEC2 organoids with the fibrosis cocktail, followed by withdrawal of the cocktail to allow for potential repair of the induced injury by the iAEC2. Interestingly, withdrawal of the fibrosis cocktail did not lead to reversal of the induced fibrosis-associated effects (288), suggesting that the fibrosis

cocktail induces irreversible effects in the iAEC2 (**Figure 28**). The sustained fibrosis-associated changes over time in this system is an important aspect in the context of modelling pulmonary fibrosis, as spontaneous repair is considered as one of the major drawbacks of other models including the *in vivo* bleomycin model (196, 197). As such, the iAEC2 fibrosis model described in **Paper III** is a less complex alternative to the models in which spontaneous resolution of fibrosis occurs.

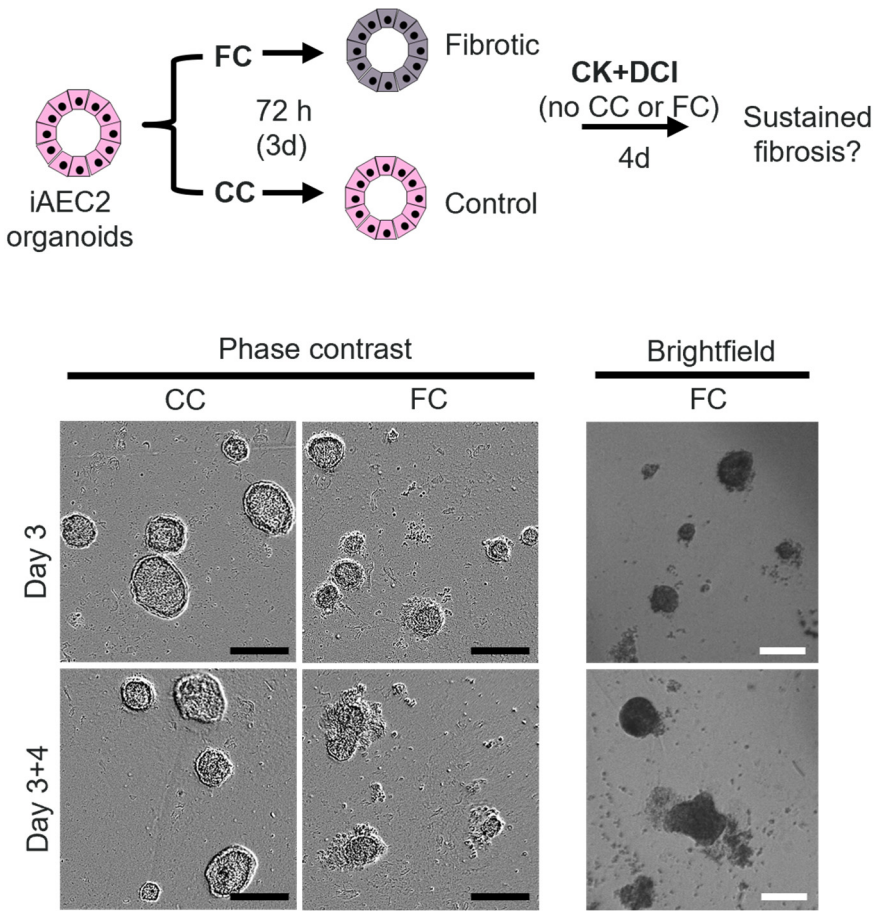



**Figure 28:** Fibrosis cocktail (FC) induces effects in iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids which persist after withdrawal of the FC. Microscopy images acquired with 4x magnification. Scale bar: 250 µm in phase contrast images, 200 µm in brightfield images. Adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 7A and 7B published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 7 additional panels.

The fact that the disease-relevant effects of the fibrosis cocktail do not resolve spontaneously indicate that the iAEC2 fibrosis model may be of potential interest in applications such as evaluation of anti-fibrotic compounds, specifically tailored to repair the alveolar epithelium. Clinically approved compounds with this mode of action which could be tested in this system were however lacking (88). Therefore, a proof-of-concept experiment was performed in which treatment with the clinically approved IPF therapeutics nintedanib and pirfenidone was included simultaneously with the fibrosis cocktail stimulation, in order to see whether fibrotic iAEC2 organoids were responsive to the treatment (**Figure 29**).

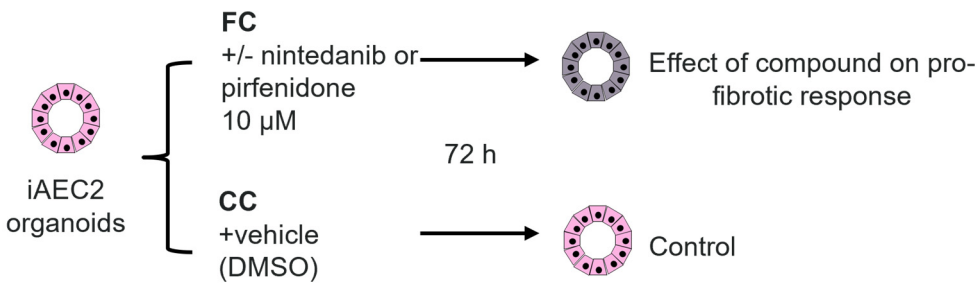



**Figure 29:** Anti-fibrotic treatment of iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids with nintedanib and pirfenidone simultaneously with fibrosis cocktail (FC) or control cocktail (CC) stimulation. Reused from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figure permitted under the Creative Commons Attribution 4.0 International License. Original figure 8A published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 9 additional panels.

Intriguingly, nintedanib prevented the induction of the dense morphology in the iAEC2 organoids, which was associated with reduction of expression of other genes reported to be dysregulated in IPF including *FN* (fibronectin) (317) and *IGFBP3* (insulin growth factor-binding protein 3) (318) (**Figure 30**). Thus, this finding indicates that the change in organoid morphology may serve as a phenotypic readout indicative of effects on the fibrotic phenotype of the iAEC2. Although treatment with nintedanib also reduced the expression of genes related to the EMT process, including *VIM* (vimentin) and *CDH2* (N-cadherin) (126), there were no effects of the treatments on the expression of any of the surfactant genes (288) (**Figure 31**).

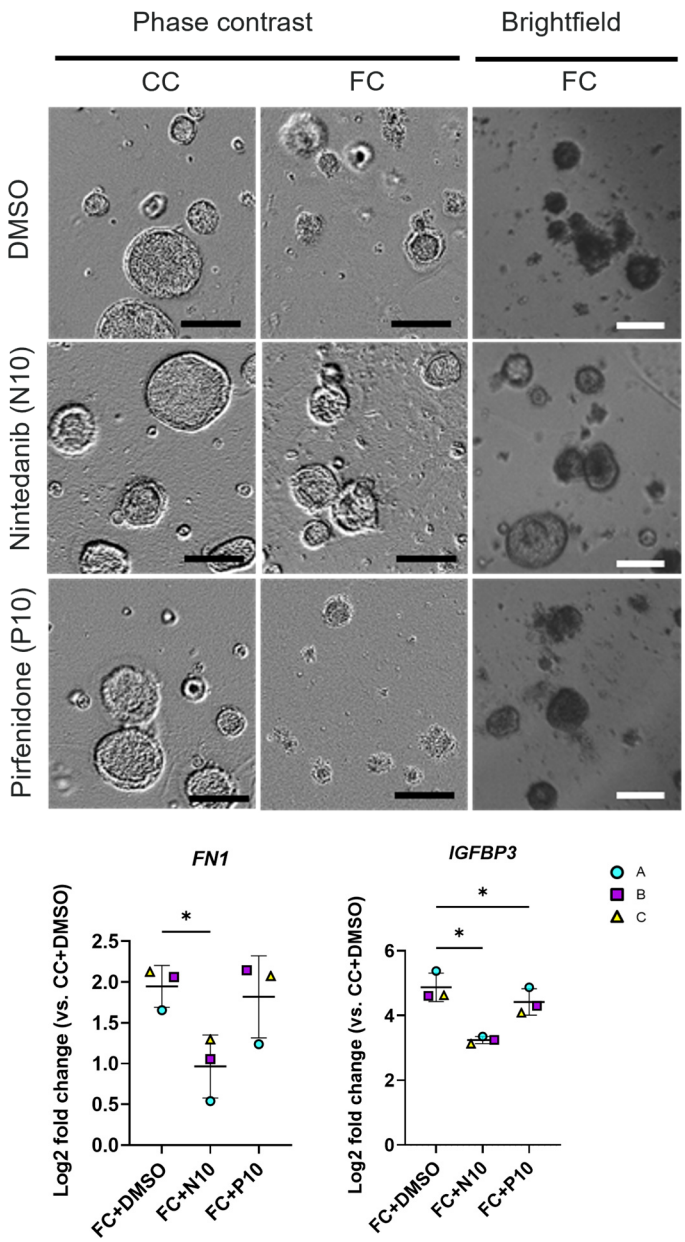



**Figure 30:** Effects on iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids stimulated with fibrosis cocktail (FC) or control cocktail (CC) following anti-fibrotic treatment. Microscopy images acquired with 4x magnification. Scale bar: 250 µm in phase contrast images, 200 µm in brightfield images. Expression of genes measured by qRT–PCR related to extracellular matrix (ECM) production and pro-fibrotic signalling. * = $p < 0.05$ by repeated measures (i.e. paired) one-way ANOVA, if significant with Dunnett's multiple comparisons test (comparisons to FC+DMSO). n = 3 batches of iAEC2 organoids. Adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 8B, 8E and 8G published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 7 additional panels.

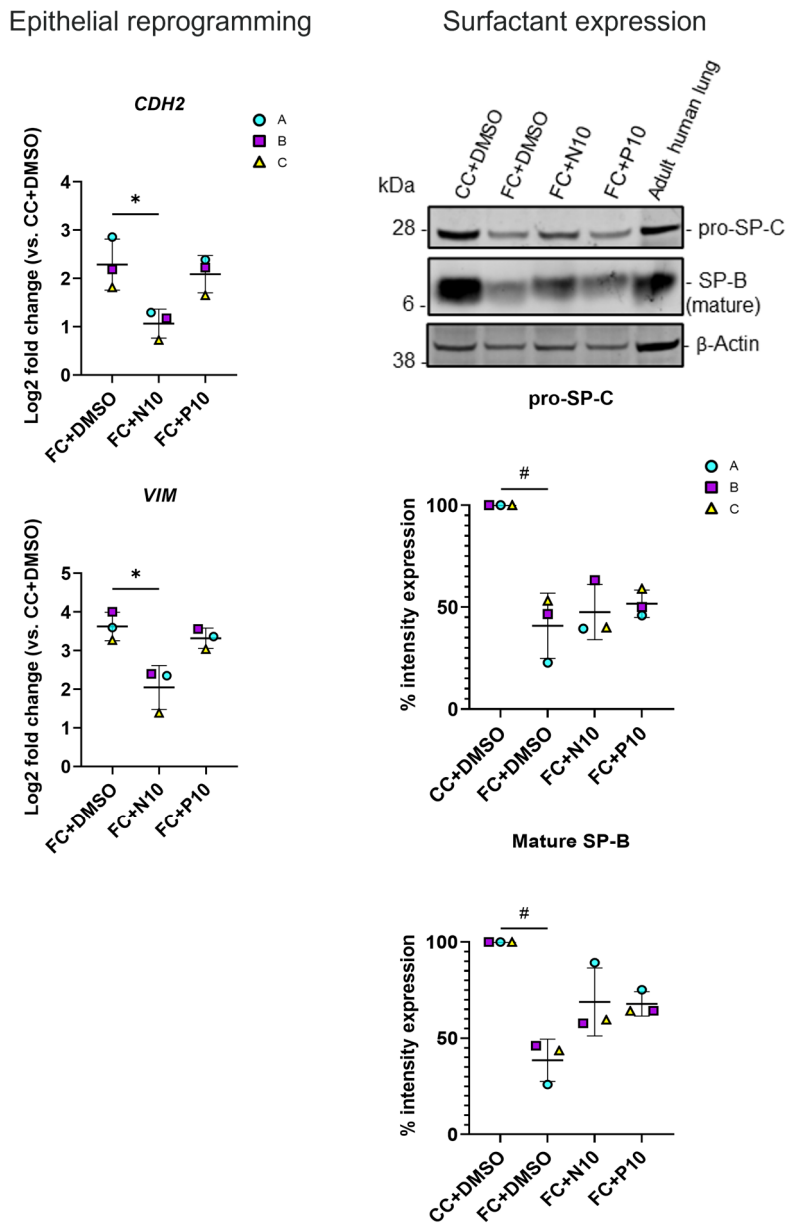



**Figure 31:** Effects on responses associated with epithelial reprogramming in fibrotic iPSC-derived alveolar epithelial type 2 cell (iAEC2) organoids following anti-fibrotic treatment. Expression of genes measured by qRT–PCR related to epithelial reprogramming. * = $p < 0.05$ by repeated measures (i.e. paired) one-way ANOVA, if significant with Dunnett's multiple comparisons test (comparisons to FC+DMSO). Western blot of intracellular protein lysates from iAEC2 organoids. Adult human lung tissue lysate is positive control. # = $p < 0.05$ by one sample t-test on the percentage intensity compared with a hypothetical value of 100 (CC+DMSO set to 100 %). n = 3 batches of iAEC2 organoids. Adapted from Paper III included in this thesis by Ptasinski and colleagues (288). Use of figures permitted under the Creative Commons Attribution 4.0 International License. Original figures 8H and 8J published in: "Modeling fibrotic alveolar transitional cells with pluripotent stem cell-derived alveolar organoids". Life Science Alliance, 2023, Vol. 6, No. 8. The original figure contains 8 additional panels.

Although the fibrotic iAEC2 organoids treated with nintedanib exhibited a less fibrotic phenotype than the fibrotic organoids without anti-fibrotic treatment, the lack of effect on the expression of surfactant proteins indicates that they are still not fully restored. Hence, this experiment demonstrated that the model is of potential interest to be adapted for purposes of drug screening for compounds with effects specifically targeting the alveolar epithelium under fibrotic conditions.

Collectively, the findings in **Paper III** show that the iAEC2 fibrosis system enables controlled induction of the fibrotic, transitional alveolar epithelial phenotypes *in vitro* without the presence of mesenchymal cells in the system. On one hand, this limits our ability to model the cross-talk between the epithelium and mesenchyme under fibrotic conditions. Co-culture experiments including both epithelial and mesenchymal cells enable studies of the mesenchymal niche impacting the induction and maintenance of the aberrant cell types in disease. This concept has been demonstrated in studies of pulmonary fibrosis using primary hAEC2 organoids co-cultured with lung mesenchyme (128) and in many of the stem cell-derived organoid models (166, 168, 171, 172). Further, due to the lack of mesenchymal cells, our system is less suitable for modelling of the mechanical stretch imposed on the alveolar epithelial cells by activated myofibroblasts which is believed to be a possible component in the pathogenesis of IPF. For example, *ex vivo* experiments in which mechanical stretch was applied to fibrotic lung tissue have demonstrated a link to release and activation of latent TGF-β1, an effect which was interestingly not observed in healthy lung tissue (319). On the other hand, our iAEC2 fibrosis model provides a unique opportunity to study the effects of defined fibrotic stimuli on cells with the alveolar epithelial phenotype, which is otherwise challenging in co-culture models. The mesenchyme-free iAEC2 system also reduces the variability which is associated with primary mesenchymal cells or isolated mesenchymal tissue. The concept of creating epithelial-only systems for modelling pulmonary fibrosis is also important from a therapeutic perspective, as aberrantly activated epithelium may secrete factors which stimulate surrounding mesenchymal cells to produce excessive ECM. Thus, therapeutic targeting of the factors secreted by fibrotic epithelial cells may be an attractive approach, and our model would be suitable for use in development of such drugs. Of note, the aberrant epithelial phenotypes induced by the fibrosis cocktail in the iAEC2 system are comparable to the effects seen in other studies in which mesenchymal cells were also present (128, 172). Thus, it is possible that the factors included in the fibrosis cocktail represent some of

the factors which are secreted by the mesenchyme during the induction of the aberrant epithelial phenotypes in pulmonary fibrosis. If so, pharmacological targeting of the factors included in the fibrosis cocktail or the response of the alveolar epithelium to these factors may be potentially effective therapeutic strategies in the treatment of IPF.

**Paper IV**

Structural information is particularly important in the assessment of diseases such as IPF, in which the lung structure is significantly impacted. Therefore, visualisation of structures in the lung tissue is a key readout in studies utilising animal models, lung tissue explants or any other system in which structural information is possible to obtain. Moreover, visualisation strategies which are applicable across different species enable evaluation of how well the structural changes translate from experimental models to human disease.

The lung is an organ with considerable structural complexity constituting of cells, proteins and lipids, which all have varying refractive indexes. These characteristics cause an opaque appearance of the lung tissue, which prevents it from being imaged as a whole organ by traditional fluorescence microscopy techniques due to considerable scattering of the light (290). In **Paper IV**, a clearing strategy was optimised for the mouse lung to overcome this issue. First, the possibility to visualise lung lesions induced by bleomycin in mice was evaluated with a technique that is label-free and which also enables visualisation of the entire lung. An immersion-based clearing workflow based on the iDISCO protocol (293) was applied to optically clear lungs from bleomycin-injured mice by dehydration and removal of lipids. The hydrophobic clearing preserves the structural alterations of proteins in the injured lung tissue (290, 293) and these could be visualised through LSFM capturing autofluorescence at 488 nm. Thus, this technique enables visual inspection of the lung lesions in all possible dimensions inside the tissue. In other, more traditional techniques reliant on sectioning of the lung, the structural information becomes limited to only two dimensions and thus, a significant portion of the structural information is lost. The capability presented in **Paper IV** may therefore serve as a readout for evaluation of the lesions induced by bleomycin and their distribution throughout the entire mouse lung.

In contrast to many other organs, the lungs can be filled by inflation through the trachea. The optimised clearing strategy developed in **Paper IV** (**Figure 32**), termed inFLATION, is utilising this feature to enable increased penetration of the clearing solutions throughout the entire lung lobe. Mouse lungs cleared by the inflation-based workflow displayed increased optical clearance as assessed by visual comparison in parallel with an immersion-based workflow. Thus, this indicates that optical clearing by inflation enables clearing of the mouse lung with increased efficiency compared to clearing by immersion.

The improved workflow for the clearing in the inFLATION protocol can be applied to study the lung lesions in bleomycin-injured mice by autofluorescence in a similar way as was performed using the immersion-based workflow. However, characterisation of the cell types and structural proteins present in those lesions require combination of the improved clearing protocol with immunolabelling. By developing a pump-based system allowing for continuous circulation of the antibody solution through the trachea, improved penetration of the fluorescent antibodies was enabled and it was possible to demonstrate staining of AEC2, airways and the lymphatic system. Comparing with an immersion-based workflow, this strategy yielded more even distribution of the fluorescent signal from the antibody staining as evaluated by imaging using LSFM. This observation demonstrates that the inFLATION protocol enables deeper penetration of the antibodies into the lung tissue during staining, which improves the quality of the staining. On the other hand, while this protocol is suitable for staining of cells and structures in the lung, not all organs can be inflated. Without the possibility of inflation and continuous circulation, being the key steps of the inFLATION protocol, the application of this protocol becomes challenging. Thus, the inFLATION protocol is applicable to organs with structures that can be utilised for connection to the pumping apparatus used during the staining procedure, such as the lung as demonstrated in **Paper IV** but potentially also the heart (320) and the liver (321).

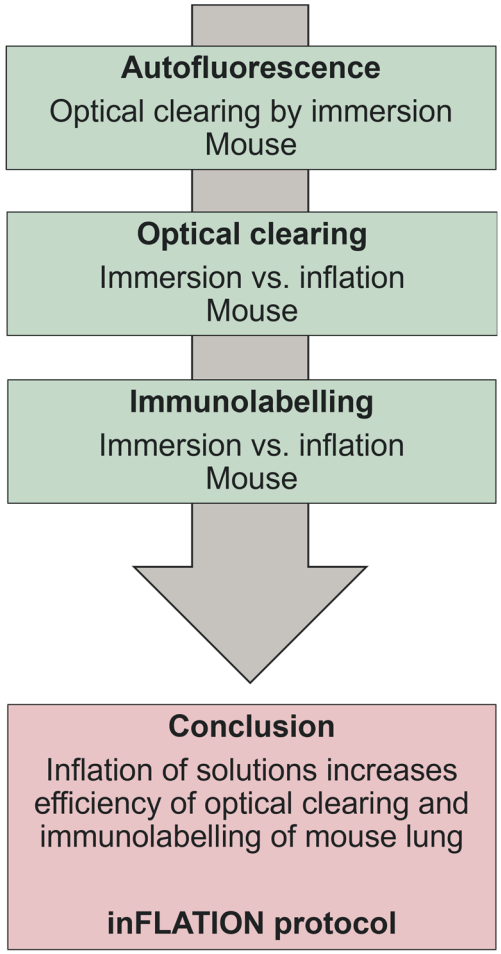



**Figure 32:** Overview of the development of the inFLATION protocol, as described in Paper IV in this thesis.

# Conclusion and future outlook

In this thesis, the aspect of lung injury and repair under fibrotic conditions was studied using several different models and techniques. In **Paper I**, the effects associated with the dysregulation of *NEAT1* was examined using the bleomycin model of pulmonary fibrosis and lung tissue samples from human IPF patients. The results described in **Paper I** open up for further exploration of *NEAT1* as a potential biomarker associated with myofibroblast induction in human IPF. To confirm whether *NEAT1* is suitable as a biomarker, further studies are needed which can link the levels of *NEAT1* in human IPF patients to other disease-related characteristics, such as lung function measurements (322). To increase the feasibility of measuring patient levels of *NEAT1* in the future, the clinical samples would need to be of easily accessible nature such as plasma, in which *NEAT1* present in extracellular vesicles can be detected (323, 324). A different aspect which would be interesting to explore is whether *NEAT1* is predictive of disease severity or progression in IPF patients, alike what has been shown for other chronic lung diseases including asthma (325) and COPD (326). Other aspects which were not explored in detail in **Paper I** is the role of *NEAT1* in the fibrotic myofibroblast activation process. Future studies in which modulation of expression of *NEAT1* is performed by knock-down (300) or overexpression (327) in target cell types will aid in this evaluation. In addition, exploration of *NEAT1* expression in different cell types at different time points in the bleomycin model would provide further information about the stage of pulmonary fibrosis induction during which *NEAT1* is most affected. The 3DLD tool developed in **Paper II** can be utilised for such purposes. The application of the 3DLD tool in the mouse bleomycin model would enable evaluation of *NEAT1* expression in both proximal and distal lung epithelial cells, and potentially also in other cell types, from the same mouse.

Modelling of fibrosis induction in specific cell types of the lung will become increasingly important in enhancing the understanding of the aberrant epithelial cell phenotypes, with respect to their potential origin and their function in IPF. Moreover, such models will be necessary for the

identification and development of novel anti-fibrotic therapeutics. The novel fibrosis model based on iAEC2 organoids developed in **Paper III** will be suitable for such applications (288). Other models in which the fibrosis-associated aberrant epithelial phenotypes have been described rely on complex cell isolations from human lung tissue (128, 130), in contrast to the model described in **Paper III** (288). This model will also enable studies of the effects of defined stimulants to better characterise the signalling pathways inducing the aberrant epithelial reprogramming during pulmonary fibrosis. In addition, it provides a platform which can be adapted for various screening purposes, such as for example phenotypic screening in which the factors inducing the dense morphology and aberrant epithelial phenotype can be explored. Genetic modulation of the cells can be incorporated into the model described in **Paper III** to enable screening for genes inducing or preventing the aberrant epithelial phenotypes during fibrotic conditions. Moreover, the establishment of the fibrosis model described in **Paper III** using iAEC2 derived from iPSC opens up the possibility to include iAEC2 from patient-derived iPSC, with various genetic backgrounds of disease. Thus, this feature enables fibrosis modelling and anti-fibrotic drug testing on cells with complex genetic backgrounds known to be predisposing for the development of IPF, based on available clinical data from the patient the cells are derived from. This is a challenging aspect when using cells of embryonic origin, as it is not possible to know whether their genetic background will in fact lead to the development of IPF in an adult lung. Although ESC allow for genetic editing using for example the CRISPR/Cas9 system, such edits normally target individual genes known beforehand to contribute to the development of disease. In reality, a genetic background of a patient developing IPF is likely more complex and hence less feasible to mimic using gene editing in the laboratory. Lastly, the model can be adapted for drug screening purposes to evaluate compounds with effects on the AEC2 phenotype or induction of the aberrant epithelial phenotypes under fibrotic conditions.

The ability to visualise specific cell types in their native structural setting in the lung tissue will become increasingly important as we acquire more knowledge about the aberrant cell phenotypes arising in IPF. Although the inFLATION protocol which was developed in **Paper IV** was optimised based on lung tissue from healthy mice, it can be applied to study lung lesions in disease models such as the bleomycin model. The improved penetration of the antibodies during immunolabelling with inFLATION may enable visualisation of rare, disease-specific subpopulations of cells, such as the

transitional alveolar epithelial cells characterised by higher expression of KRT8 which have been described in the fibrotic mouse lung (127, 129, 197). Considering that the lung tissue structure is preserved during the entire labelling and imaging process, this capability will increase the understanding of the potential interactions of the disease-specific cells based on their location in the lung tissue.

In conclusion, the findings described in this doctoral thesis contribute to increased understanding of the pathophysiology of IPF. Further, the development of versatile techniques reported in this thesis will be valuable contributions to the broader field of lung research. Finally, the work described in this thesis contributes to the understanding of the processes regulating the induction of the recently described fibrotic epithelial cell types, which are novel and attractive therapeutic targets in IPF.

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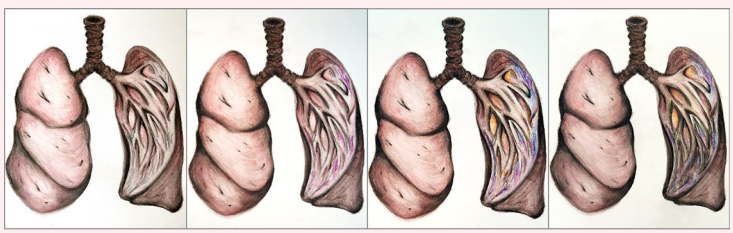
# About the Author

I graduated with a Master's degree of Medical Science with a major in Biomedicine from Lund University. Already as a child, I had an interest in exploring one of the largest mysteries in this world – the human body. My fascination with the lung began in my high school anatomy classroom, where I for the first time witnessed a lung expanding during air filling. Later, I became familiar with the smallest building blocks of the lung, the cells, as a trainee in the pharmaceutical industry. In this dissertation, I am proud to present the scientific advances included in my doctoral thesis, which I hope will also serve as steps on the way to a better future world through healthier lungs for us all.

## About the cover

During our entire life, our lungs defend us bravely against all harmful particles and infections we encounter daily. With resilience, they take on challenges from our environment to keep us breathing and alive.

The cover of this doctoral dissertation was created in a step-wise process. Firstly, detailed anatomical structures representing the healthy, uninjured lung were created by graphite drawing. Secondly, the scars acquired over time by the lung from each of the daily battles against the outer world were painted with watercolours, depicted from left to right. The vivid colours in the injured lung represent my first impression after seeing a histological section of the fibrotic lung – messy and dysfunctional, but at the same time intriguing and beautiful.



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