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Abstract

Lung function is highly dependent on the structure of the lung parenchyma. Remodeling of the alveolar compartment is a central part of the pathology of chronic lung disease such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). Pathological lung remodeling occurs as an interplay between cells and the extracellular matrix (ECM) which in addition to its structural role influence most aspects of cell behavior. This thesis aimed to improve on the available *in vitro* methods for studies of cell-ECM interaction in the alveolar compartment of the human lung.

A technique to produce acellular lung slices for cell culture from clinical lung resection material with preservation of native 3-dimensional structure and ECM composition was established and evaluated. Protein turnover in cell cultures in decellularized lung slices (DLS) was analyzed by mass spectrometry implementing amino acids labeled with stable heavy isotopes to distinguish between cell and DLS derived ECM proteins. The work entailed study of ECM production of primary human lung fibroblasts and primary human alveolar epithelial type II cells (AECII) in DLS from derived from human lungs. In addition, a device for mechanical stretching of lungs slice cultures have been designed and evaluated.

Fibroblasts and AECII cultured in DLS secreted ECM components that were incorporated in structure of the lung slices. Fibroblasts showed to be highly responsive to their extracellular milieu and when cultured in IPF derived ECM, they produced ECM recapitulating pathological alterations of the ECM scaffold. In a comparison with fibroblast culture on standard plastic tissue culture surfaces the deposition of ECM proteins was hampered compared DLS culture. DLS culture of AECII showed that these cells produced a wide array of ECM proteins including interstitial matrix components mostly associated with cells of mesenchymal origin. AECII from explanted healthy and end stage COPD lungs were almost indistinguishable in a proteomic evaluation and with limited transcriptomic differences. Healthy AECII did however display a significant plasticity in ECM production when stimulated with the profibrotic growth factor TGF-ß1, upregulating many markers of IPF remodeling. Finally, the novel stretch device proved to be compatible with lung slice culture and capable to induce a cellular stretch responses.

This thesis provide insight into how lung ECM can dictate cellular function and presents new techniques for the study of interactions of cells and ECM lung parenchyma. The presented model systems have potential to improve on the *in vivo* relevance of preclinical experimental research by better representing normal and pathophysiological conditions. Finally, the description of a broad capacity for ECM production by alveolar epithelial cells raises interesting questions about their importance in alveolar ECM homeostasis pathological remodeling.

Key words: extracellular matrix, decellularization, mass spectrometry, biomechanics, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, alveolar epithelial cell, fibroblast, lung slices

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To my children, Astrid and Didrik

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Introduction

Our lungs are impressive feats of engineering that can withstand being stretched a billion times during a lifetime, with a flow of about 7,500 litres of blood going through them every day, passing through thin capillaries just wide enough for red blood cells to squeeze through. These capillaries are held just a few microns from the inhaled air as part of the thin walls of the alveoli. These delicate structures are usually maintained and repaired to a functional condition despite assaults from inhaled pathogens and air pollutants. However, when the repair mechanism fail, severe disease might develop with loss of functional alveoli and thereby lung function.

To understand what happens in the lung in pathological remodelling, we need to understand what happens to both cells and the extracellular matrix (ECM) and how they interact. Much knowledge about how cells produce ECM and how this is regulated have been gained from studies of cells cultured in monolayers on glass or treated plastic surfaces. These stiff, simple culture surfaces generally support efficient expansion of cells and are easy to handle, but they are also a very artificial milieu affecting all aspects of cellular function. As an alternative, ECM based 3D culture models based on decellularized ECM from the tissue of interest have the potential to significantly improve the clinical translatability of *in vitro* experimental studies.

The work in this thesis aims to improve on available methods to investigate the interaction between cells and ECM, using culture models based on decellularized human lung. The generated data add to the knowledge of how cells in the lung are affected by their ECM environment, and how they in turn can alter their ECM composition. Ultimately, I hope that the described methodology will be used to better our knowledge of how we can help patients with lung diseases breathe more easily.

Original articles

Article I

Rosmark O, Åhrman E, Müller C, Elowsson Rendin L, Eriksson L, Malmström A, Hallgren O, Larsson-Callerfelt A-K, Westergren-Thorsson G, Malmström J. **Quantifying extracellular matrix turnover in human lung scaffold cultures.** Sci Rep. 2018 Dec 3;8(1):5409.

Article II

Rendin LE, Löfdahl A, Åhrman E, Müller C, Notermans T, Michaliková B, Rosmark O, Zhou X-H, Dellgren G, Silverborn M, Bjermer L, Malmström A, Larsson-Callerfelt A-K, Isaksson H, Malmström J, Westergren-Thorsson G. **Matrisome properties of scaffolds direct fibroblasts in idiopathic pulmonary fibrosis.** Int J Mol Sci. 2019;20(16).

Article III

Oskar Rosmark, Måns Kadefors, Johan Malmström, Göran Dellgren, Anders Ericsson, Sandra Lindstedt, Oskar Hallgren, Anna-Karin Larsson Callerfelt, Gunilla Westergren Thorsson. **Alveolar epithelial cells are competent producers of interstitial extracellular matrix with disease relevant plasticity in a human in vitro 3D model.** Manuscript.

Article IV

Oskar Rosmark, Arturo Ibáñez-Fonseca, Johan Thorsson, Göran Dellgren, Oskar Hallgren, Anna-Karin Larsson Callerfelt, Linda Elowsson Rendin, Gunilla Westergren-Thorsson. **A tunable physiomimetic stretch system for precision cut lungs slices and recellularized human lung scaffolds.** Manuscript.

Original articles published during the course of the PhD studies, but not included in the thesis

Bagher M, Larsson-Callerfelt A-K, Rosmark O, Hallgren O, Bjermer L, Westergren-Thorsson G. **Mast cells and mast cell tryptase enhance migration of human lung fibroblasts through protease-activated receptor 2.** Cell Commun Signal. 2018 Dec 15;16(1):59.

Gil-Ramirez A, Al-Hamimi S, Rosmark O, Hallgren O, Larsson-Callerfelt A-K, Rodríguez-Meizoso I. **Efficient methodology for the extraction and analysis of lipids from porcine pulmonary artery by supercritical fluid chromatography coupled to mass spectrometry.** J Chromatogr A. 2019;1592.

Gil-Ramírez A, Rosmark O, Spégel P, Swärd K, Westergren-Thorsson G, Larsson-Callerfelt AK, Rodríguez-Meizoso I. **Pressurized carbon dioxide as a potential tool for decellularization of pulmonary arteries for transplant purposes.** Sci Rep. 2020 Dec 1;10(1):1–12.

Bagher M, Rosmark O, Elowsson Rendin L, Nybom A, Wasserstrom S, Müller C, Zhou X-H, Dellgren G, Hallgren O, Bjermer L, Larsson-Callerfelt A-K, Westergren-Thorsson G. **Crosstalk between Mast Cells and Lung Fibroblasts Is Modified by Alveolar Extracellular Matrix and Influences Epithelial Migration.** Int J Mol Sci. 2021 Jan 6;22(2):506.

Müller C, Rosmark O, Åhrman E, Brunnström H, Wassilew K, Nybom A, Michaliková B, Larsson H, Eriksson LT, Schultz HH, Perch M, Malmström J, Wigén J, Iversen M, Westergren-Thorsson G. **Protein Signatures of Remodeled Airways in Transplanted Lungs with Bronchiolitis Obliterans Syndrome Obtained Using Laser-Capture Microdissection.** Am J Pathol. 2021 Aug 1;191(8):1398–411.

Selected abbreviations

AEC	Alveolar epithelial cells
AECI	Alveolar epithelial type I cells
AECII	Alveolar epithelial type II cells
BM	Basement membrane
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
COPD	Chronic obstructive pulmonary disease
CS	Chondroitin sulphate
DDA	Data dependent acquisition
DIA	Data independent acquisition
DLS	Decellularized lung slice
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HS	Heparan sulphate
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
LC	Liquid chromatography
m/z	Mass-to-charge-ratio
MMP	Matrix metalloprotease
MS	Mass spectrometry
PCLS	Precision cut lung slices
SILAC	Stable isotope labelling by amino acids in cell culture
spA	Surfactant protein A

spB	Surfactant protein B
spC	Surfactant protein C
spD	Surfactant protein D
TGF-	ß1 Transforming growth factor ß1
TIMP-3	Tissue inhibitor of metalloproteinases-3
UIP	Usual interstitial pneumonia
αSMA	α-Smooth muscle actin

Background

In the great oxygenation event about 2.3 billion years ago, the oxygenic photosynthesis of cyanobacteria increased the atmospheric oxygen levels and paved the way for aerobic respiration[1]. The further development of multicellular life required the development of a circulatory system to transport oxygen and nutrients to the inner parts of organisms and to remove waste products, including carbon dioxide[2]. Specialised interfaces for gas exchange between the circulatory system and the environment have since developed, such as gills and lungs, the latter well suited for terrestrial life, limiting evaporation of water while providing efficient exchange of the air interfacing with the blood of the circulatory system[3]. The principle for movement of air in and out of the mammalian lung is similar to that of a pair of bellows, with inspiration driven by active dilation of the thoracic cavity by the diaphragm and muscles of the rib cage, and expiration by in the inherent elasticity of the lungs. This means that the structure of the human lungs has two main functions, to provide surfaces for blood to come in close proximity to inspired air and to facilitate the movements of breathing (Figure 1). Any perturbation of the lung structure, fine-tuned by evolution, may impair lung function and thereby one of the requisites for human life.

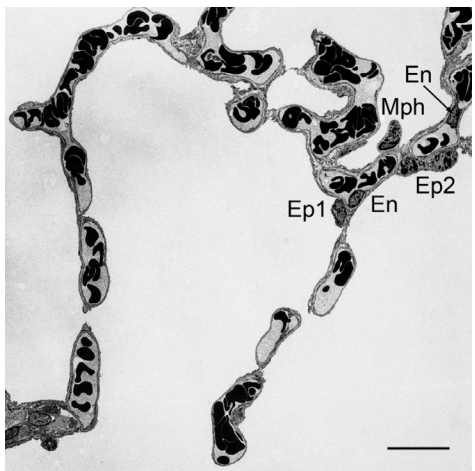

Figure 1. Micrograph of dog lung alveoli. Air fills most of the volume and the alvolar walls are dominated by capillaries filled with dark irregularly shaped red blood cells. The air facing surfaces are lined by alveolar epithelial type I (Ep1) and II (Ep2) cells, capillaries by endothelial cells (En) and alveolar macrophages (Mph) are seen inside the alveolar airspaces. Scale bar 20 µm. Adopted with permission from Weibel 2017[4].

Lung physiology

Gas exchange

Movement of oxygen and CO_2 in the alveolar airspaces occur predominantly by diffusion[5], and the diffusion of gases must pass the alveolar air-blood tissue barrier. Diffusion across this membrane follows Fick's law:

$$\dot{V}gas = \frac{A}{T} * D * (P_1 - P_2)$$

Where the rate of gas flow (V̇) is proportional to the area (A), the diffusion coefficient for the gas (D) and the partial pressure gradient (P_1-P_2), while inversely proportional to the tissue thickness (T). Thus, human lungs have a structure providing large alveolar surface area estimated to 130 m^2, with thin alveolar membranes (Figure 1)[4]. The alveolar air-blood tissue barrier is only 0.2-0.4 µm thick in its simplest form, where it is composed of the alveolar epithelial lining and the capillary endothelium, with an interposed shared basement membrane (BM). However, the average thickness of this barrier is about 2.2 µm as the capillaries run along the connective tissue fibres that provides structural integrity to the alveolar septa, thereby having a thicker diffusion barrier on one side[6]. Uptake of oxygen in a healthy lung is normally not limited by diffusion capacity, it might however become so at high altitudes where the partial pressure of oxygen is lower, and especially during physical exercise where the cardiac output increases and the transit time for blood through the pulmonary capillaries decreases, and thus the time available for diffusion[7]. In pathological condition the thickness of the alveolar wall might increase, which will, apparent from the equation above, limit oxygen diffusion. Oxygen diffusion is driven by a large gradient in partial pressure of around 60 mmHg between the incoming blood and the alveolar air, created by an efficient sequestering of oxygen by the haemoglobin of red blood cells, CO_2 on the other hand, have a much lower pressure gradient of about 5 mmHg driving its diffusion, but its diffusion coefficient is about 20 higher due to higher solubility compared to oxygen. The higher diffusibility of CO_2 means that it is less sensitive to the thickness of the alveolar walls, however the CO_2 exchange is highly sensitive to changes in the partial pressure gradient, and thus to changes in the ratio between ventilation and perfusion.

Mechanics of breathing

The driving forces for breathing at resting conditions can largely be explained by the active contraction of the diaphragm, lowering the pressure in the thoracic cavity and thus sucking air in, and by the passive contraction of the lung as the diaphragm

relaxes, pushing air out[8]. As the diaphragm contracts, it pushes the abdominal contents downwards which also pushes the lower ribs laterally by the increase abdominal pressure, thereby expanding the thoracic cavity. Additional inspiratory muscles help expand the thoracic cavity by moving the ribs, which have been described as having a buckle handle movement. Imagine the ribs as a series of bucket handles attached to the spine at a downwards slanting angle, when they are lifted upwards, resulting in a more perpendicular angel to the spine, the enclosed volume increases. The external intercostal muscles help lift the ribs in inspiration, and with more forceful inspiration, accessory muscles of inspiration are activated , the scalenes and sternocleidomastiodeus of the neck[8]. The most important muscles of expiration are the abdominal muscles, which pull the rib cage downward while increasing the abdominal pressure, thereby displacing the diaphragm upwards, together with the internal intercostal muscles which aid the downward swing of the ribs[8].

The lungs are not fixed to the inner walls of the thoracic cavity, but they nevertheless move together as they are tightly opposed with only a thin layer of fluid between them. This fluid resides in the pleural cavity which is a minimal space between the visceral pleura lining the lungs, and the parietal pleura lining the chest wall. The plural fluid lubricates the plural surfaces and allows them to slide against each other as the shape of plural cavity changes with breathing[9]. The plural cavity has a resting pressure of about -6 cm H_2O[9], caused by the elastic recoil of the lung, which also drive expiration in quiet breathing.

The force behind the elastic recoil of the lung is provided by the deformation of the tissue and the surface tension of the fluid lining the surfaces of the lung[10]. Surface tension is regulated by the secretion of pulmonary surfactant, primarily by alveolar epithelial type II cells (AECII). Of the surfactant proteins, the small strongly hydrophobic surfactant proteins B (spB) and C (spC) are considered most important in reducing surface tension, spC being exclusively produced by AECII[11]. Pulmonary surfactant is composed mainly of phospholipids, but also have a protein content of about 10%[11]. In 1955, Pattle published result from experiments done with lung fluid material suggesting that it could dramatically reduce the surface tension at the air-liquid interface in the lung[12]. Later experiments found that the reduction in surface tension was greater at lower distension and showed higher surface areas during exhalation compared to inspiration at a given pressure (Figure 2)[13]. The effect of the surface tension in the alveoli is often somewhat erroneously explained using Laplace's law, describing the effect of surface tension of curved surfaces[14]. This physical principle states that inward pressure exerted by the surface tension of a model spherical alveolus would be inversely proportional to the radius of this alveolus. This in turn would lead to the collapse of the smaller alveoli into larger alveoli due to the higher inward pressure experienced by alveoli with smaller radii, which would be counteracted by the function of the pulmonary surfactant. However, the shape of the alveoli is not spherical, but rather polyhedral with plane walls which

means that Laplace´s law would only be applicable at the corners formed at the intersections between the walls[4]. Furthermore, as the walls of one alveolus is shared with adjacent alveoli, collapse of any individual alveoli is resisted by the radial traction from the surrounding tissue, which is likely the prevailing mechanism preventing alveolar collapse[14]. This mechanism would not depend on a perfect match between the size distribution of alveoli and the surface tension lowering effect of surfactant. Surfactant is however critical for normal lung function as evident by the successful use of exogenous surfactant in treatment of infant respiratory distress syndrome in premature infants with an insufficient surfactant production[11]. The application of Laplace's law in the lung is likely more relevant for smaller airways not supported with cartilage, which are more prone to collapse at low lung volumes than the alveoli[14].

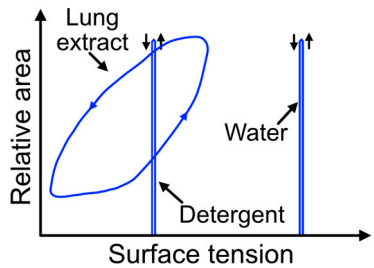

Figure 2. Schematic overview of the surface tension reduction by lung surfactant based on experiments performed by Clements in 1957[13]. The surface tension is lower at low lung volumes preventing the collapse of airways at the end of expiration. A large hysteresis is seen with a higher surface tension at a given area (lung volume) at inspiration compared to expiration.

There is not only an interdependence between adjacent alveoli in maintaining the structure of the tissue, but also an interdependence between the surface tension and tissue forces in regard to lung elasticity[15]. Changes in the surface tension will change the shape of alveoli, and thus the stress put on surrounding tissue elements. At functional residual capacity, the elastic recoil is thought to originate about equally from surface tension and tissue forces, but at higher lung volumes the contribution from the tissue increases[10,16]. The most important structural components contributing to the mechanical properties of the lung are the elastin and collagen fibres in the alveolar tissue[17]. Collagen fibres dominate the alveolar septa, and elastic fibres are concentrated to the intersections between alveolar septa and the entrance rings were alveolar ducts open into alveoli. At lower lung volumes expansion occurs predominantly by unfolding and reorganization of fibres and only at higher lung volumes does stretching of fibres become apparent, which is associated with a marked increase in elastic recoil. Cells in the lung can exert forces

on the underlying ECM, e.g. via focal adhesions and hemidesmosomes, but are not considered to have a large direct impact on over all mechanical properties[16].

In summary, together with surfactant secretion, a correct composition and organization of ECM components, especially in the alveolar compartment, is crucial for the mechanical properties of the lung.

The alveolar epithelium

The alveolar epithelial cells (AEC) are divided into the surfactant producing AECII and the alveolar epithelial type I cells (AECI) which covers about 95% of the surface area of the alveoli (Figure 3). The AECI cells are stretched out across the alveolar surface, fulfilling a barrier function while minimizing the thickness of the diffusion barrier. The AECI is highly effective in covering a large surface, compared to the endothelial cells of the alveolar capillaries, they cover more than three times the surface area per cell[18]. The AECII which cover a relatively small surface area have been found to be about twice as numerous as the AECI cells, accounting for 16 % of cells resident in alveoli[18]. The more numerous AECII cells are regarded as progenitor cells in the alveolar epithelium and can differentiate into AECI cells. The time needed to replace the AEC in mice have been estimated to 4-5 weeks[19], but in response to hyperoxic epithelial damage, the turnover time was reduced to 3 days[20].

As an interface towards the surrounding environment, the alveolar epithelium may be subjected to toxic and infectious substances. Examples of important infectious agents include influenza and corona viruses[21,22], that cause damage to the AEC in severe cases, with a histopathological pattern of diffuse alveolar damage and acute respiratory distress syndrome (ARDS)[23,24]. Even though endothelial damage is part of the lung pathology caused by SARS-CoV-2[24], the damaged epithelial barrier may be of particular importance for the formation of lung oedema as it is the main permeability barrier in alveoli[25]. The role of AECII in infectious diseases stretches beyond being a target of viral infections as they play part in innate immunity by secretion of surfactant protein A (spA) and D (spD). These surfactants are part of the collecting family of proteins, together with mannose binding lectin (MBL), known to activate the lectin pathway of the complement system. The collectins all have central collagenous domains and C-terminal carbohydrate binding domains, and consists of trimeric polypeptide subunits which assemble into even larger octadecamers (spA) and dodecamers (spD)[26]. The carbohydrate binding domains have been shown to opsonize both bacterial and viral pathogens, promoting their uptake by, e.g. alveolar macrophages and neutrophils, both by direct attachment to cell surface receptors and by aggregation of particles[27]. These surfactant proteins also regulate the expression of inflammatory mediators and pathogen recognising receptors from phagocytes[26]. There are also data indicating direct bactericidal[28,29]

and antifungal effects of spA and spD[30,31]. In addition, these surfactant proteins seem to influence the activation of dendritic cells and T-lymphocytes and facilitate clearance of apoptotic cells by alveolar macrophages[32–37]. Besides the spA and spD mediated immune functions of AECII cells, *in vitro* studies have suggested that they may aid the recruitment of dendritic cells by secretion of the chemokine CCL20[38] as well as macrophage resistance to mycobacterial infection via pro-inflammatory cytokines[39]. AECII have also been suggested to induce T-cell tolerance to antigens by presenting antigens on class II MHC molecules without expression of co-stimulatory proteins[40].

It have long been known that epithelial cells from diverse tissues such as cornea, kidney glomeruli and conducting airways produce ECM components[41–43]. Also, AEC have been shown to produce ECM proteins[44], and as for other epithelial cells the described protein products are mainly BM or cell adhesion proteins such as laminin, collagen type IV and fibronectin. Glomerular epithelial cells of the kidney have shown to alter its secretion of ECM proteins in response both mechanical stimuli and transforming growth factor ß1 (TGF-ß1)[45], the latter in a way that differ from mesangial cells of the glomeruli[42]. The full capacity of ECM production of AEC is not well studied.

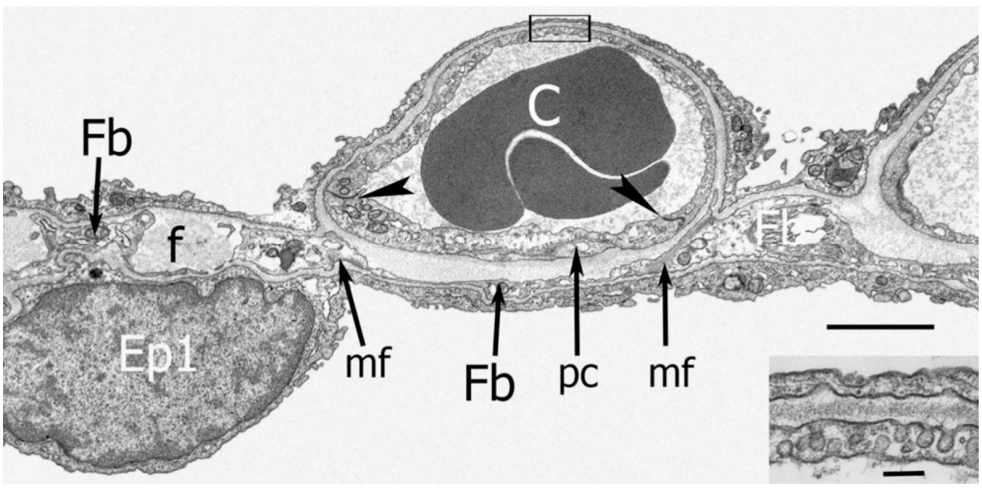

Figure 3. Micrograph of the human alveolar septum showing a capillary (C) with two intercellular junctions of the endothelium (arrowheads)and a pericyte process (pc), a type 1 epithelial cell (Ep1) and fine extensions of a fibroblast (Fb) that is tightly related to fibers (f) in the interstitial matrix and forms myofibrils (mf) that span across the septum. The box outline the minimal barrier for gas dissusion whose structure is shown in the inset: the epithelium and endothelium are separated only by their fused basement membranes. Scale bars 2 µm and 0.1 µm in the inset. Adopted with permission from Weibel 2017[4].

Lung fibroblasts

Fibroblasts are cell of mesodermal origin found in the stroma of most tissues, they have long been recognised as important for maintenance of homeostasis of the ECM. Maintenance of a tissue specific ECM milieu together with production of numerous paracrine factors create niches for other tissue resident cells, e.g. AEC[46]. In addition, they have immune regulating properties and are crucial for wound healing in which their ECM production is accelerated[47]. The fibroblast population is heterogenous with differing phenotypes identified both within and between tissues[48,49]. This heterogeneity is likely facilitated by a significant plasticity which is manifested *in vitro* by the ability to transdifferentiate into adipocytes, chondrocytes and osteoblasts, when provided with appropriate stimuli[50,51]. The fibroblast seem to have dual roles in wound healing, both supplying most of the ECM substrate necessary for healing to occur, and limiting the inflammatory reaction[47,52]. The immunosuppressive capacity showed in fibroblasts have in large been attributed to suppression of lymphocyte proliferation, a capacity increased after INF-γ stimulation which induces HLA-II expression in fibroblasts[53–56].

No unique markers have been identified for fibroblast, however the marker combinations attributed to mesenchymal stem/stromal cells (MSC) by the International Society for Cellular Therapy (ISCT) in 2006, are similarly expressed in fibroblasts, likely reflecting that these cell types belong to the same population of cells with considerable plasticity[51,57,58]. The above described characteristics of fibroblast are part of what characterizes MSC, and described differences between these cell types are of the same magnitude as those seen between fibroblasts in different tissues and culture conditions[48,49,51]. There have been suggested that MSC and fibroblast might be distinguished based on epigenetic profiles[59], however available comparisons often involve cells taken from different tissues which complicates the interpretation of such results[60,61].

Cellular responses to mechanical strain in the lung

In resting conditions, the linear strain, i.e. deformation, of the alveolar walls have been estimated to be between 4 and 10%, which can be increased to up to 20% during exercise[10]. Deep breaths have long been known to stimulate secretion of surfactant in the lung[62–64], and studies of isolated AECII cells have found them to release pre-formed surfactants in response strain[65,66]. One study using a human AECI cell line showed that stretch of AECI can indirectly stimulate surfactant release from AECII cells[67]. This stretch response can be part of the physiology of yawning, where stiffening of the lung and atelectasis can be counteracted by stimulation of surfactant secretion by a deep breath, e.g. a yawn[68]. The same

mechanisms are also relevant at the moment for a new-born's first breath[68]. Stretch of AECII cells can induce differentiation into AEC1, which have been shown in rat AECII cultured with static stretch *in vitro*[69] and in tracheal occlusion models in animals[70], where airway occlusion results in lung distension due to fluid accumulation. Mechanical strain has also been shown to stimulate proliferation of foetal AECII in animal models[71,72], which is in line with the fact that mechanical stimuli is critical for normal lung development[73].

Excessive stretch of AEC is thought to be involved in the pathology of lung fibrosis where stretch induced damage to the epithelium might lead to release of damage-associated molecular patterns (DAMPs)[74,75]. Damage induced by mechanical stress may also induce oxidative damage and ER stress in AEC, which have a role in the pathogenesis of both chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF)[76]. With prolonged damage a dysregulated or insufficient repair response may initiate fibrotic remodeling[77]. In addition, there are evidence for stretch induced release of the pro-fibrotic growth factor TGF-ß1 from AECII as well as the interstitial collagen type I[44]. Signalling from epithelial cells might activate fibroblasts that contribute to ECM remodelling. Fibroblasts are responsive to the stiffness of their surrounding environment and a stiff substrate stimulate both migration and induces and increased ECM production[78]. Regulation of gene and protein expression for both ECM components and ECM modifying enzymes as well as signalling mediators have been documented in response to different modes of both cyclic and static stretch[79,80]. Also endothelial cells and macrophages have been shown to be responsive to mechanical stimuli, which together with fibroblast and AEC constitute the most prevalent resident cell types in alveoli[18]. Fluctuations in shear stress (disturbed blood flow) and substrate stiffness can promote endothelial to mesenchymal transition (Endo-MT)[81,82]. With established sensitivity to mechanical cues from the surrounding, it would not be unreasonable to expect a capacity in endothelial cells to respond also to stretching of the capillary walls of the alveoli. Macrophages exhibit a complex response to stretch with reports of both up- and downregulation of the secretion of pro-inflammatory mediators[83,84], while regenerative responses may be promoted by static and inhibited by cyclic stretch[84].

Lung extracellular matrix

The ECM is an essential component of multicellular life, binding cells together in complex structures making up the tissues and organs of complex organisms. In addition to the structural role of ECM, it takes part in a constant interplay with resident cells whose behaviour is influenced by the ECM and in turn can modify the composition and structure of the ECM. Lung ECM can be divided up into four main categories, interstitial matrix, BMs mainly underlying epithelial and endothelial cells, blood plasma and cartilage, this text will focus on the first two categories.

Basement membranes

Basement membranes (BM) constitute a specialised form of ECM that typically underlie sheets of cells serving as a connection point to the underling stroma. The dominating structural components of BM are laminins, type IV collagen, nidogens and proteoglycans[85]. The composition of BM differs depending on location, the overlying cell type and developmental state, exemplified by the 16 laminin variants found in human BMs[86]. Laminins are considered fundamental to the structure of all BMs and are network forming heterotrimeric proteins composed of α-, β- and γ-chains[86]. The laminin networks are complemented by a collagen type IV reticulum, providing strength and stability to the BMs[85]. Collagen type IV also form trimers based on 6 different α-chains of which three combinations have been found *in vivo*, viz. α1α1α2, α3α4α5 and α5α5α6[87]. The laminin and collagen networks are further stabilized by the glycoproteins nidogen-1 and -2, as well as the proteoglycan perlecan. Perlecan is a large heparan sulphate (HS) and chondroitin sulphate (CS) proteoglycan ubiquitous to BMs that influence a multitude of cellular processes, e.g. adhesion, angiogenesis, autophagy migration, inflammation and wound healing[88,89]. Another HS proteoglycan abundant in alveolar BMs is agrin, its name originating from its role in inducing aggregation of acetylcholine receptors at neuromuscular junctions[90], it is also found in glomerular BM of the kidney, where it is important for molecular filtation[91]. Agrin have been proposed to function as a protease inhibitor in the lung[91].

In addition to the above core constituents of the BM, a multitude of other proteins link it to the interstitial matrix and overlying cells. One important group of such proteins is the integrins, which is a family of 24 cell surface proteins that are important for cell-ECM as well as cell-cell attachment[92]. Several of these receptors bind to laminin and collagens found in the BM. Another cell attachment protein is collagen type XVII which is a transmembrane protein part of hemidesmosomes anchoring epithelial cells to their BM[93]. Other members of the collagen superfamily link the BM to the interstitial matrix, e.g. collagen type VI and type VII[94], the latter is present mainly in bronchial and bronchiolar BMs in the normal lung[95], linking BM collagen type IV and laminin 332 to collagen type I in the interstitial matrix.

Interstitial matrix

The interstitial matrix of the lung is rich in fibrillar collagens such as collagen type I and III, as well as elastic fibres, giving structure and mechanical properties to the tissue[96]. The stability and mechanical properties of the triple helical collagens are not only determined by their intrinsic structure, but also on modifications by proline hydroxylase and lysyl oxidases (LOX) occurring during and post translation, and enabling collagen crosslinking and thereby stable protein structures[97]. Elastic fibres are large structures consisting of highly crosslinked elastin polymers and

microfibrils consisting of numerous protein species, with over 30 proteins described as part of microfibrils or microfibril-elastin interface[98]. The microfibrils are crucial for the assembly of the elastin polymers from the soluble tropoelastin precursors[99]. Elastin fibres are believed to be a very long-lived molecules with a turnover rate estimated to 74 years in human lungs[100]. They exhibits a linear stress strain relationship over the whole range of normal lung movements which contribute to the lungs ability to return to it resting state at exhalation[101]. The microfibrils have large amounts of HS and CS proteoglycans bound to them, and proteoglycans are abundant in the interstitial matrix as a whole[99,102]. Proteoglycans are composed of a core protein with attached glycosaminoglycan (GAG) side chains composed of long unbranched chains of repeating disaccharide units of which one of the two sugar units is an amino sugar[102]. In addition to the proteoglycans, hyaluronic acid (HA) is a prominent GAG-molecule in ECM, that is not associated to any protein core[103]. The polysaccharide chains are rich in negatively charged sulphate and carboxyl groups which generate repulsive electrochemical forces. These repulsive forces stabilize alveolar structures, providing resistance to folding and collapse of alveolar septa[104] and inhibition of proteoglycan synthesis was shown to be enough to initiate emphysematous remodelling in rat lungs[105]. The proteoglycans which are present throughout the ECM have large structural diversity largely due to the variation in the sulphation and acetylation pattern of the GAG-chains. The electrochemical charge and an structural diversity of proteoglycans facilitate the binding of a large array of cytokines, growth factors, enzymes and morphogens, which influence cell and tissue function[106].

Pathological alveolar remodelling

Changes in the lungs structure, often termed remodelling, are part of several disease states and may lead to substantial suffering for the afflicted individuals. Remodelling may occur in the setting of acute lung injury (ALI) with associated fibroproliferative disease[107], but below focus will be on a subset of lung pathologies with a more protracted disease development. Chronic lung diseases include a number of disease entities with pathology typically developing over years to decades, examples include Asthma, COPD and interstitial lung diseases (ILDs). ILD is an umbrella term for are a large group of parenchymal lung diseases that commonly involve fibrotic lung remodeling. It is an heterogenous group of diseases which can develop after a number of different exposures including viral infections, occupational exposures to dusts or toxic chemical, after medical treatments such as radio- and chemotherapy and as part of rheumatic diseases such as scleroderma and rheumatoid arthritis[108]. However, the most common form lacks a clear causative agent, even if tobacco smoke is an important risk factor, this form is called idiopathic pulmonary fibrosis (IPF). About 10% of IPF cases belong to what is

called familial pulmonary fibrosis on the basis on inherited mutations in single genes that increase the risk of disease development, e.g. in genes for mucin 5B, spC or telomerase[109]. The prognosis for IPF patients is grim, with a median survival time of about 3 years after diagnosis[110], even with the best available treatment, however some patients survive for much longer. The work presented in this thesis focuses on the processes behind the turnover and remodelling of the ECM in alveoli. IPF and COPD have been studied which both exhibit pathological alveolar remodelling. The remodelling of the alveolar compartment in these diseases are superficially opposite, with loss of tissue in emphysema in COPD and excessive ECM deposition and scarring in IPF (Figure 4).

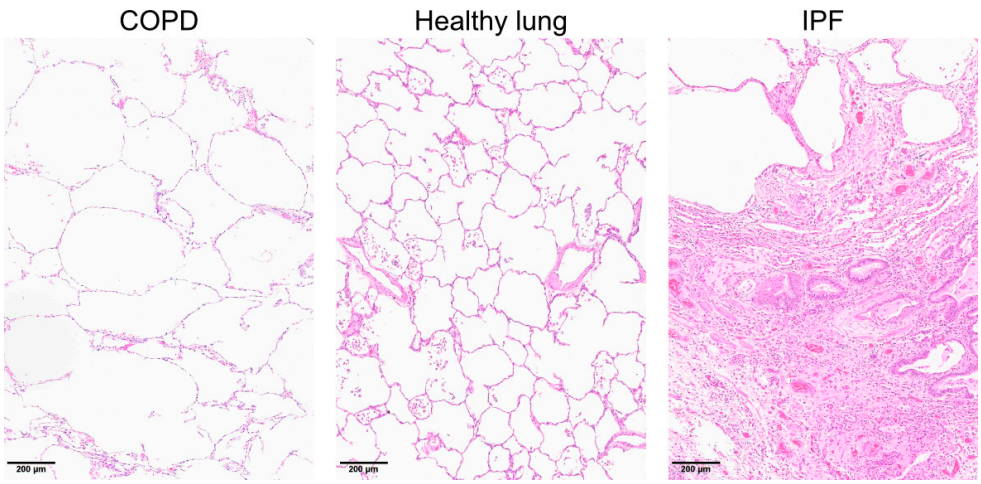

Figure 4. Pathological remodeling in COPD and IPF. The normal alveolar architecture are lost with destruction of alveolar septa in COPD and excessive ECM deposition in IPF. The heterogenus remodellnig seen in IPF is exemplified by emphysematous alveoli in the top part of the IPF section, which can occur alongside alveoli with both fibrotic and a normal apperance.

Chronic obstructive pulmonary disease

In 2019, before the Covid-19 pandemic, COPD was responsible for about 6% of the deaths in the world, with more than 80% of these deaths occurring in low and middle income countries[111]. COPD is a disease characterized by progressive airway obstruction with pathological alterations primarily in the small airways, usually divided up into two major components, chronic bronchitis and emphysema[112]. Chronic bronchitis involves inflammation, mucus hypersecretion and fibrotic remodelling in conductive airways, which leads to airflow obstruction and air trapping in distal lung units. In emphysema, the lung parenchyma is destroyed, i.e., a loss of alveolar walls, this both limits the surface area available for gas exchange and contribute to small airway collapse and airflow obstruction due to the loss of airway tethering.

The process leading to alveolar remodelling in COPD is believed to start with a reaction to inhaled toxic gases and/or particles, where tobacco smoke is the most prominent source, however 25-45% of COPD cases can be attributed to other risk factors[113,114]. Emphysema in human lungs was first described by Laënnec in 1830[115]. The later discoveries of the link between α1-antitrypsin deficiency and pan-lobular emphysema[116] and that emphysema can be experimentally induced by instillation with proteolytic enzymes such as papain[117], lead to the hypothesis of proteolytic overactivity behind development of emphysema[112]. As there is a link between cigarette smoking, inflammation and the number of leukocytes present in lung tissue, proteolytic activity related to inflammation, and especially neutrophil elastase, have been implicated in the development of emphysema[112]. The prevalence and magnitude of emphysema increases with cigarette smoke exposure, however only about 40% of smokers develops emphysema and only 15% of smokers develops COPD[118]. There is currently no clear explanation for the variable sensitivity to cigarette smoke. In addition, disrupted proteoglycan synthesis[105] and apoptosis of alveolar endothelial and epithelial cells[119,120] have been found to induce emphysema in animal models, without or with very limited inflammation, suggesting that inflammation might not be the only pathway of importance.

The idea of a protease-antiprotease imbalance behind emphysema development have experimental support in animal models where intratracheal elastase deposition results in emphysema in mice[121]. The mechanism for this is sometimes described as a selective breakdown of elastin and elastic fibres[122], even of neutrophil elastase also degrade other ECM components such as collagens type I-IV, fibronectin and proteoglycans[123]. However, additional support of the importance of elastin degradation in emphysema comes from studies showing elevated blood and urine levels of elastin specific degradation products in patient with emphysema[124,125], and decreased levels of elastin in emphysematous human lungs[126]. Also, other proteases have been implicated in emphysema development, these include a number of matrix metalloproteases (MMPs), considered to be derived mainly from macrophages, some of the best documented are MMP-10, -12, and -28[127,128]. Also an inhibitor of MMPs, tissue inhibitor of metalloproteinases-3 (TIMP-3) may be of importance in emphysema development, it has affinity for all known MMPs and bind with high affinity to proteoglycans in the ECM[129]. A proteomics study have found TIMP-3 to be upregulated in COPD tissue together with MMP-28[128], and mice lacking TIMP-3 develop spontaneous emphysema[130]. Despite the apparent loss of tissue in emphysema, the pathology has characteristics of an aberrant tissue turnover with increased matrix production as well as breakdown. Serum levels of markers for both production and breakdown of collagens are increased in COPD and correlate with disease severity[122,131]. Increased collagen deposition have been described in centrilobular emphysema seen in COPD[126]. The increased collagen deposition seem to correlate with loss of elastin and this have been interpreted as an ongoing repair in response to inflammation driven injury[126].

ECM turnover disturbances in COPD have also been seen for GAGs with an especially clear link to acute exacerbations of COPD (AECOPD)[122], which may lead to an accelerated decline in lung function[132]. Elevated serum levels of the HA degrading hyaluronidase-1 in COPD patients correlate with lower lung function and increased mortality, and levels of released HA fragments in the blood at a stable state was associated with time to death and also correlated with the severity of an ongoing exacerbation[133]. In addition to HA, both CS and HS are affected in COPD, with increased levels in bronchiolar lavage (BAL) in AECOPD, correlating with MMP levels, indicating release by degradation of the proteoglycan core protein[134]. Levels of heparan sulphate proteoglycans (HSPGs) are reduced in patients with severe emphysema and elastase mediated degradation of core proteins have in animal models resulted in HS release, which in turn may reduce a HS mediated inhibition of proteases[135]. Considering the multitude of functions of GAGs in the lung, e.g. aiding fibrillogenesis of elastin and collagens, inhibition of protease and neutrophil activity, binding of growth factors and cytokines and provision of structural integrity to the alveolar walls[122,135], a disturbance in their turnover may have profound impact on tissue function. Although great progress has been made into mapping the inflammatory component in COPD, much remains to be learned regarding the (dys-)regulation of ECM turnover and the contribution of different cell types in the development of emphysema.

Idiopathic pulmonary fibrosis

The pathological remodelling in IPF primarily involves the alveolar parenchyma and is characterised by an heterogeneous pattern of excessive ECM accumulation[136].The IPF diagnosis rest on multiple criteria and are generally reached after a multidisciplinary conference, but the strongest evidence for IPF comes from histopathological evaluation of a surgical lung biopsy showing a pattern of what is called usual interstitial pneumonia (UIP)[137]. However, a diagnosis can today often be based on the clinical presentation together with a finding of the radiologic correlates of UIP in high resolution computed tomography scans[137]. The distribution of the pathological remodelling have been shown to match the theoretical distribution of mechanical stress in the lung during breathing, indicating a role of mechanical stress in the pathogenesis of IPF[74,75]. Release of the pro-fibrotic mediator TGF-ß1 in response to mechanical strain have been shown from both AECII and mast cells[138,139], which could then propagate fibrotic remodelling, decreasing local lung compliance and thus increasing the mechanical stress on adjoining alveoli, establishing a feed-forward loop.

Repeated damage to the alveolar epithelium is at the centre of the dominating explanatory model for IPF, and downstream senescence of AECII are of special interest[140,141]. IPF is very much a disease of ageing, with a doubling of the incidence for each decade after 50 years of age[142]. Risk factors such as smoking may lead to

increased AECII turnover and several genetic risk factors are related to telomere maintenance[142]. This together with findings of shortened telomers in AEC point toward replicative senescence as a part of IPF pathogenesis[142,143]. Other processes such as mitochondrial dysfunction and ER-stress also results in dysfunctional epithelial cells that secrete mediator that activate and recruit fibroblasts, initiating a remodelling process leading to excessive ECM deposition[77,144,145]. Several mediators for the epithelial to fibroblast signalling have been identified and include TGF-ß1, platelet derived growth factor (PDGF), and members of the Wnt family of proteins[141]. The activated fibroblasts adopt a myofibroblast phenotype which have an increased ECM production with collagen type I being the prototypical protein, they also exhibit an enhanced contractile ability coupled to reorganization and increased synthesis of α-smooth muscle actin (αSMA)[146]. More recent research including studies using next generation sequencing techniques have revealed that the myofibroblast population is highly heterogenous and with different sub phenotypes within a myofibroblasts population[146]. The fibroblasts in IPF afflicted lungs exhibit phenotypic changes that likely contribute to disease progression. Physiological wound healing reactions are limited by apoptosis of fibroblast when the supply of activating stimuli abates, but IPF fibroblast show resistance to both apoptosis and anti-proliferative signals[141]. One characteristic of the histopathological UIP pattern is the presence of fibroblastic foci, were aggregations of fibroblast embedded in ECM lays under a layer apoptotic or hyperplastic AEC in the alveolar parenchyma, this feature has been suggested to be related to an enhanced migratory capacity and ECM invasive fibroblast phenotype[147].

The fibroblastic foci in IPF are considered to be remodelling hot spots which are rich in glycoproteins such as versican, tenascin C, fibronectin and periostin[141,148]. The BM structure is compromised in these areas and genes for BM constituents such as collagen type IV and laminins have been reported to be downregulated in IPF tissue[148,149]. Fibrillar collagens, foremost collagen type I and III are present in the remodelled tissue with a relatively high proportion of collagen type III in early lesion with thickened alveolar walls, but with a gradual switch toward mainly collagen type I in more mature scar tissue. In view of the net accumulation of fibrillar collagens, it is of interest that also collagen degradation is upregulated in IPF patients with upregulation of several MMPs in the lungs and elevated blood levels of collagen degradation[148,150], reflecting an elevated turnover. Among other ECM components, GAGs have been shown to be enriched in IPF tissue with elevated HS and CS levels[151].

Current anti-fibrotic treatment

At this date there are two drugs, nintedanib and pirfenidone, that have been shown to slow down loss of lung function in IPF patients and have thereby been dubbed anti-fibrotic. However, there is no solid evidence that they actually slow done ECM deposition and scar formation *in vivo*[142]. Nintedanib is a tyrosine kinase inhibitor

that inhibit PDGF, fibroblast growth factor and vascular endothelial growth factor receptors, this gives an *in vitro* effect on fibroblasts, limiting their differentiation, proliferation, migration, ECM production[152]. The mechanism behind pirfenidone is less well defined but have an antiproliferative effect on fibroblasts *in vitro* and also inhibit αSMA and pro-collagen-I expression[153].The addition of these two substances to the therapeutic arsenal is an important milestone as they are the first treatments that have been able to slow disease progression and improve survival[110]. However, short of a lung transplantation, there is no cure for IPF and disease halting and reversing treatments are sorely needed.

Decellularized tissue in the study of lung biology

In vivo alternatives

Good experimental models are crucial for progress within medical science. Things that cannot be safely or efficiently evaluated in humans must be tested in some sort of model system trying to recapitulate the human condition. Animal studies have had, and have a crucial role allowing for well controlled experiments while capturing all aspect of a mammalian physiology. However, results from animal models used for testing of new pharmaceuticals and mechanisms behind human disease often fail to translate in subsequent human trials[154,155]. Part of these problems can probably be attributed to physiological differences between humans and common utilized animal species such as rats and mice, but even results from studies in primates have a poor track record[156]. In addition, problems with the relevance of achieved results, there is ethical concerns with experimentation in living animals and such studies are generally costly compared to *in vitro* experimentation. However, animal experimentation will likely remain an important component in toxicology and pharmacology research for the foreseeable future, as available *in vitro* models are far from recapitulating the integrated organ functions of a mammalian physiology. Without abandoning animal studies all together, much can be done to minimize suffering of experimental animals adhering to the 3 R framework of replacement, reduction and refinement[157], where 3D lung slices can be useful tools to achieve these goals.

Precision cut lung slices as ex vivo models

An alternative to the use of living animals for studies of lung biology is to use lung tissue from either animals or human material obtained from a clinical interventions, e.g. part of a lung resected due to lung cancer[158]. One established platform for this kind of studies is called precision cut lung slices (PCLS), were slices of tissue are prepared that are thin enough to allow for gas and nutrient exchange through diffusion while thick enough to preserve the tissue architecture and cellular complexity of the tissue. The use of thin tissue slices with a reproducible thickness

were first established for studies of liver function[159] (the same acronym is used) and later adopted for lung tissue[160]. As lung tissue are heterogenous in their consistency due to the air content, the tissue is commonly filled with low melting point agarose to facilitate sectioning. The agarose solution based on a physiological salt buffer or tissue culture medium are injected as a liquid and form a gel as the tissue are chilled below normal body temperature, which also helps preserve cell viability. The tissue is then sectioned to a defined thickness between 0.1-1 mm, commonly with a slicer equipped with an oscillating blade. Such tissue slices have been utilized for studies of lung metabolism, toxicology, pharmacology, airway and blood vessel contractility and disease mechanism[158,161]. As many tissue slices can be produced from the lungs of one animal or from one resection specimen, the PCLS technique offers good control over biological variability and cost-effective high throughput experimental designs. Even of animal tissue is still used, the number of animals can generally be reduced and moving experiments *ex vivo* spares the animal from any associated suffering. The lung slices can be maintained with preserved tissue functions for up to a week[158], and some recent studies suggest that that time can even be doubled with optimized culturing protocols[162,163]. While the decay of the PCLS might be a limiting factor for some studies, another limitation is linked to the core advantage of this model system. As the cellular complexity of the lung is preserved in the PCLS, it can be difficult to distinguish processes related to specific cell types, something desirable in many mechanistic studies.

Properties of decellularized tissue

Biological scaffolds consisting of ECM can be produced by removing the cellular components of organs or tissues. The ideal result is a culture substrate devoid of cells but with conserved 3D architecture containing structural and signalling components of the extracellular milieu of the native tissue[164]. The produced scaffolds should then supply introduced cells with the structural and compositional signalling cues and cell adhesion sites that are present in the *in vivo* microenvironment[165]. The properties of the ECM in decellularized scaffolds affect fundamental cellular functions such migration, proliferation, and differentiation. Scaffold materials from humans have the potential to capture changes in the tissue related to ageing, which is relevant for many of our most common diseases and often hard to replicate in animal models[165–167]. When the scaffold material is sourced from patients, the effect of pathological remodelling of the ECM on cell function can be studied to investigate mechanism behind the disease. Examples from lung tissue include the induction of a fibrotic phenotype in fibroblasts and pericytes when cultured on scaffolds from IPF pateints[168–170]. Although large advances have been made within the field of bioengineering with artificial biomimetic materials for *in vitro* cell culture, they are far from capturing the full complexity of the *in vivo* ECM environment[171]. Hybrid solution also exist where decellularized tissue are mechanically and/or enzymatically disrupted and used to create homogenous culture materials, often in the form of hydrogels[171]. This methodology has some benefits,

one being that cells can be mixed into material before the gelling occurs, helping to distribute cells throughout the culture substrate, something which can be problematic with dense ECM scaffolds. However, this technique removes the native organization of the ECM structure which can be of importance. In a study using lungs scaffolds, emphysematous scaffolds did not support the growth of endothelial, epithelial and mesenchymal cells as well as scaffolds from healthy tissue, but these differences disappeared when cells were cultured on homogenates of the scaffold materials[167].

In addition to the use of decellularized tissue scaffolds for studies of basic biology and disease mechanisms, there are a considerable interest in the use of such scaffolds for tissue engineering purposes. While not a focus in this thesis, one important aspect of decellularization is key to the use of decellularized tissues for clinical applications, namely that the decellularization process removes most of the immunogenic material in the tissue, making it possible to implant both allogenic and xenogeneic material without invoking an immune rejection[172–174]. Products based on decellularized tissue are already approved for clinical use, e.g. materials made from dermis or demineralized bone, for repair of ligaments and tendons in orthopaedics and bone defects in dentistry[175]. An active research field are trying to create more complex tissues and even whole organs for transplantation, either based on scaffolds from cadaveric human organs or from animals, repopulated either with allogenic or autologous cells[176–178].

Decellularization techniques

The goal in decellularization is generally to remove cells without compromising the ECM structure or composition, this is however not an easy task and the end results of any decellularization procedure of today is a compromise between thorough cell removal and preservation of ECM integrity. Where the optimal compromise lie might depend on the intended application, e.g. complete removal of potentially immunogenic cellular material might be a higher priority for material intended for *in vivo* implantation than for material used to study disease driving properties of the ECM *in vitro,* where preservation of disease related ECM features is crucial[165]. Decellularization agents can be subdivided into physical, chemical or enzymatic and often a combination is employed[164]. Physical disruption and removal of cells might entail mechanically scraping of epithelial cell layers[179] or changes in temperature and pressure to rupture cells, e.g. freeze-thaw cycles where ice crystal disrupts cell membranes[180–182]. Common chemical agents include a variety of different detergents where combinations of ionic and non-ionic detergents are commonly used to efficiently solubilise proteins and nucleic acids as well as lipids[164]. Strong ionic detergents like sodium dodecyl sulphate (SDS) denature proteins and are effective in removal of nucleic acids and proteins, but which unfortunately also reduces the GAG and growth factor content in the ECM and damages collagens[164,183]. Non-denaturing zwitterionic detergents such as 3-((3-cholamidopropyl)

dimethylammonio)-1-propanesulfonate) (CHAPS) and Sulfobetaine-10 (SB-10) have been suggested to be less damaging to ECM, but also less efficient in removing cellular proteins[184–186]. Also, solvents like alcohols and acetone are employed which both lyse cells by dehydration and remove lipids, however they tend to crosslink ECM proteins and lead to protein precipitation[164]. Many decellularization protocols also employ hypo- and/or hypertonic solution for cell lysis and disruption of DNA-protein interaction[181,187,188]. The pH of the solutions used is of critical importance, acids and bases are used to disrupt nucleic acids and solubilize proteins, which of course also affects the remaining ECM. In one study the pH of a hypertonic CHAPS containing decellularization solution was varied between 8 and 12, which led to very different results. The tissue architecture and GAG-content was better preserved at lower pH and despite a higher residual DNA-content the tissue decellularized at pH 8 invoked a lesser immune response when implanted subcutaneously in rats[189]. More targeted decellularization agents include enzymes such as nucleases that helps mobilize DNA and RNA from the tissue[190]. Other enzymes like trypsin and dispase have also been employed with the goal of severing the cells attachments to the ECM, which of course also damage ECM proteins[179,191,192]. Another commonly employed strategy to detach cells is the use of chelating agents such as ethylenediaminetetraacetic acid (EDTA), sequestering metal ions such as Ca^{2+} necessary for cell adhesion via integrins[179,188].

The decellularization agents described above can be applied to the tissue in different ways, and for intact organs and large tissue pieces perfusion through the vasculature is a common method[182,193,194]. The vasculature provides routes for the decellularization solutions to reach every part of the tissue through the capillary beds, however flow might not be equally distributed and to ensure flow to all parts of a tissue the pressure might need to be increased to an extent were the pressure gradient risk damaging the ECM. Lung also offers an alternative route for decellularization through the airways[182,195], which however might be less efficient as this route does not allow for continuous unidirectional flow. When an intact vasculature is not available, which is often the case with smaller patient derived tissue samples, tissue might be divided into thin slices to facilitate decellularization in short time spans, thus limiting the exposure to ECM-damaging agents in the decellularization solutions[196]. Another suggested strategy to efficiently decellularized tissue is the use of supercritical fluids, and in particular supercritical CO_2. A supercritical fluid is a substance at a temperature and pressure above its critical point were there does not exist separate gas and liquid phases. Supercritical fluids diffuse efficiently though materials and for CO_2, the critical state can be reached at physiological temperatures. As an relatively inert substance CO_2 is attractive for decellularization as it should in principle do minimal damage to the ECM and leave no toxic residues. A publication from 2008 by Sawada et al[197]. claimed efficient removal not only of phospholipids but also of DNA from aortic tissue with the addition of ethanol to the supercritical CO_2. Attempts to replicate these findings were done at the early stages of the work for this thesis in pursuit of

an efficient method that could be used for lung decellularization. However, the results of the Sawada paper on DNA removal could not be replicated despite resorting to considerably harsher decellularization conditions[198,199]. Supercritical fluids do not seem to be a silver bullet for decellularization, however in conjunction with other decellularization agents such as nucleases, it still holds potential, and already have an established role in sterilization of decellularized materials[200,201].

Evaluation of decellularization protocols is a difficult task, and different studies often come up with somewhat contradictive results, and which parameters that are emphasised vary between studies. The situation is further complicated by the fact that results may be tissue specific. Most protocols use a combination of different agents, and differences in one factor, such as the pH or the duration of the exposure may a significant impact on the resulting tissue scaffolds, which can explain some of the inconsistencies in the presented results. Hence, protocol optimisation and comparisons need to be done in very well-structured manner. One readout in where there exists some consensus, is that the levels of residual DNA should be as low as possible. Some commonly referenced minimal criteria was suggested by Crapo et al.[164] and state that no nuclear material should be visible in haematoxylin or 4′,6-diamidino-2-phenylindole (DAPI) stained sections, the levels of double stranded DNA should be below 50 ng/mg ECM dry weight, and tissues should be free of DNA fragments >200 base pairs in length.

The decellularization technique used in the work presented in this thesis combines physical, chemical, and enzymatic agents for decellularization[202]. First, a freeze-thaw cycle which includes storage of the frozen samples contribute to cells lysis. Secondly, a decellularization solution previously evaluated for perfusion decellularization[193], is applied to thin lungs slices replicating the physical dimensions used for PCLS. This solution contains a zwitterionic detergent (CHAPS), a chelating agent (EDTA) and a hypertonic sodium chloride concentration in a phosphate buffer at pH 8. Finally, residual nucleic acids are degraded using a broad-spectrum endonuclease.

Mass spectrometry based proteomics

A proteome is the set of proteins expressed in an organism, organ, tissue or cell, and the field of proteomics aims at a comprehensive and quantitative characterization of proteomes. Protein production and degradation, i.e. turnover, is highly dynamic and cellular responses to environmental stimuli and disease related perturbations are hence reflected in the proteome[203]. As main effectors of most biological functions, proteins dictate the phenotype of cells and tissues. As a widely employed high throughput methodology for proteomics studies, the term proteomics is often used to refer specifically to mass spectrometry (MS)[204].

Generation of mass spectra

The dominating MS-proteomics approach is called bottom-up or shotgun proteomics[205], and is what is used in three of the articles in this thesis. With this technique, information about the proteins in a sample are reconstructed from identified peptide fragments. Before injection into the mass spectrometer for analysis, proteins are digested by enzymes, separated, commonly by liquid chromatography (LC), and ionized, e.g. by electrospray ionization (ESI)[205]. These so-called precursor ions generate a first MS1 spectra and can be separated based on their mass-to-charge ratio (m/z). Precursor ions are then led to a collision cell where they are fragmented further, typically via collision with a gas and the generated product ions are then separated according to their m/s and detected to produce a MS2 spectra[205].

The generated mass spectra can then be matched against *in silico* predicted mass spectra in order to identify the peptides in the samples that can subsequently be mapped to specific proteins. The relative abundance of a protein can then also be estimated using based on the signal intensity from precursor or product ions.

Protein quantification accuracy

There exist several different quantification techniques for MS that generate either absolute values for protein abundance, or more commonly relative quantification with varying accuracy[206]. Absolut quantification techniques generally involves adding peptide standards of known concentrations to samples. One example of this is the use of so-called AQUA-peptides, which is synthetic peptides containing stable heavy isotopes that are added to the protein digest, which can serve as a reference for the peptides of interest. Synthesis of these peptides is however expensive, and the technique requires prior knowledge of the target peptides[206].

More commonly used techniques yield relative quantifications that allows for comparison of protein abundance between samples, e.g. treatment and control groups. The data from MS-analysis is sensitive to variation in the upstream sample processing steps, and also in the LC-MS performance[206]. To improve the accuracy of the relative quantifications, samples can be labelled and then processed and injected together and separated again in the data processing steps. Peptides can be given sample specific isobaric labels after protein digestion, allowing multiple samples to be pooled and handled as on in subsequent steps[207,208]. This not only improves the accuracy of the quantifications but simplifies sample processing and often also decrease the time needed for LC-MS-analysis, increasing throughput. Even further reduction in the variation introduced in sample processing can be achieved by introducing the labels already at the cell culture stage or in living animals. This is called metabolic labelling and is done by using isotope labelled amino acids, a technique called stable isotope labelling by amino acids in cell culture (SILAC)[209]. Labelling is usually done with heavy isotope containing arginine and lysine, which is present at the C-terminal end of peptides produced by trypsin which

is commonly used for protein digestion in MS-workflows. The main drawback of this technique is the high cost of the labelled amino acids, especially if you are to include it in the feed of animals[206]. Also, the SILAC approach have previously offered less multiplexing potential than the isobaric tag techniques, but with an increasing set of available labelled amino acids this difference might be diminishing[210].

In label free quantification, samples are not multiplexed and as such are sensitive to variation introduced in sample handling and run-to-run variability in the LC-MS. Meticulous planning and execution of sample processing is important for good quality results. However, label free quantification experiments are easy to perform and cost-effective, as it does require expensive mass labelling reagents and are commonly used and computational procedures like the MaxLFQ[211] have made it possible to compensate for some technical variation between samples.

In this thesis, stable heavy isotope labelled amino acids have been used in cell cultures in ECM scaffolds, analogous to the SILAC technique described above. However, this have been done not to minimize variation between experimental groups, but to separate the proteins of the scaffold from newly synthesised ECM proteins from repopulating cells. Samples from different groups has subsequently not been pooled in the processing and LC-MS analysis steps. This essentially means that metabolic labelling has been used in a label free quantification workflow.

Data acquisition modes

In tandem MS (MS/MS) a selection of precursor ions is often chosen for subsequent fragmentation and generation of a MS2 spectra. This method is called data dependent acquisition (DDA), where the top *n* most abundant precursor ions are selected for fragmentation[205]. The alternative where all precursor ions in a specific MS1 m/z-range are fragmented is called data independent acquisition (DIA). The most common method for generation of DIA data is called SWATH (Sequential Windowed Acquisition of All Theoretical Fragment ions), in which the mass spectrometer cycles through windows 10-25 m/z wide, this generates complex mass spectra which demands more computational power for downstream protein identification[205,212]. Lower computational demands, lower cost and better compatibility with labelling techniques such as SILAC contribute to a widespread use of DDA, the latter being a strong reason for the use of DDA in the studies of this thesis. Two main drawbacks with the use of DDA are the poor coverage of low abundant peptides and the stochasticity that exists in which precursor ions that will be selected for fragmentation[213], resulting in problems with missing values in the resulting data sets. The development of new software tools that lowers the computational demands of working with DIA are however making this an increasingly attractive option[214].

The matrisome

As a fundament of multicellular life, many constituents of the ECM are highly conserved in the evolutionary tree, and based on key protein domains found in ECM proteins complemented with manual curation, an *in silico* definition of ECM and ECM-associated proteins have been developed[215]. This so called matrisome is a practical way to structure data from proteomics studies The matrisome currently includes 274 core matrisome proteins subdivided into three groups, viz. collagens, ECM glycoproteins and proteoglycans, along with 753 ECM-associated proteins also dive din into three subgroups, viz. ECM affiliated proteins, ECM regulators and secreted factors[216]. The matrisome grouping of ECM and ECM-associated proteins are used for the structuring and presentations of data in this thesis.

Present investigation

The overall aim of this thesis was to improve on the available *in vitro* methods to study human parenchymal lung cells in a relevant ECM environment. The intended use of these methods was primarily in studies of disease mechanisms behind chronic lung diseases. Readouts were focused on ECM remodelling and the reciprocal interaction between cells and their ECM milieu and how ECM composition and structure influence cell functions. The objectives of the included studies were:

Article I: Establish a method for *in vitro* culture of lung cells in decellularized ECM from human lungs, together with a mass spectrometry proteomics approach differentiating cell derived proteins from the ECM culture substrate.

Article II: Investigate potential alterations in ECM synthesis from fibroblast invoked by ECM from lungs of patients with IPF as compared to ECM from healthy lungs.

Article III: Assess the ECM synthesis capabilities of AECII cells from healthy and COPD-afflicted lungs together with changes in this capacity in healthy cells induced by the profibrotic growth factor TGFß-1.

Article IV: Development of a platform for studies of cells in a human lung ECM environment subjected to physiologically relevant mechanical strain.

Results and discussion

Article I

Quantifying extracellular matrix turnover in human lung scaffold cultures

Fibroblasts have long been recognised as important ECM producing cells and fibroblasts from different tissues have been studied extensively *in vitro,* mapping their protein production during numerous experimental conditions[49,217,218]. As cell functions are highly dependent on the culture substrate, the very stiff plastic surfaces most commonly used in cell culture might foster cell phenotypes far from those found *in vivo*[219]. However, the character and extent of such differences is poorly characterized. In attempts to increase the relevance of *in vitro* studies cell culture can be performed on more *in vivo* like substrates, here decellularized tissue can supply both compositional and structural similarity to the ECM substrates that cells experience *in vivo*[165]. The decellularized tissue is rich in structural ECM components that might be produced also by the cultured cells. Sensitive proteomics studies on ECM turnover in these culture setting needs to be able to discriminate the proteins from cultured cells from the constituents of the decellularized ECM.

Short summary

- A novel method for cell cultures in decellularized human lung slices (DLS)
- Protein labelling with amino acids with stable heavy isotopes were used to differentiate newly produced proteins from the pre-existing ECM
- The composition of the fibroblasts produced matrisome changes with time in culture
- Fibroblasts culture in DLS led to a greater deposition of ECM and especially of proteoglycans compared to culture on tissue culture plastic

New culture system using decellularized lung slices

Cryosectioned slices of distal human lung were decellularized and used for culture of primary human fibroblast. The decellularized tissue composed mainly of alveolar tissue with some small bronchioles and blood vessels maintained the overall tissue structure. The relatively mild decellularization protocol made possible by the lung slice format allow for good preservation of elastic fibres, and immunohistological staining for collagen type IV and scanning electron microscopy (SEM) showed preserved continuous BMs. The decellularized lung slices (DLSs) was found to consist mainly of core matrisome proteins with collagens being the dominating protein group, they also had low amounts of residual DNA, which is commonly used to evaluate decellularization efficiency[164].

Primary fibroblasts from one human donor were allowed to repopulate the DLS for 25 days and showed an initially high proliferative activity at day 6 of culture, which had abated by day 9. The decrease in proliferation occurred in parallel with a macroscopically visible contraction of the tissue by the cells, likely due to a myofibroblast like phenotype with a high contractile capacity. There were no signs of substantial cell death in the cultures with consistently low levels of apoptotic cells evaluated by a TUNEL assay in PFFE-tissue, speaking against toxicity form residual detergent after decellularization. Overall, the developed DLS based culture system support cell culture for at least 25 days.

ECM remodelling in vitro

Stable isotope labelled amino acids in the culture medium was successfully used to enable separation of cell and DLS derived proteins in subsequent MS- analysis. The repopulating fibroblasts quickly became the dominating source of many of the proteins of the matrisome associated groups, which had been efficiently removed during decellularization. Some of these proteins are intracellular or plasma membrane bound and followed the proliferation profile of the cultures Also some core matrisome proteins were deposited in substantial amount already at day 6, this includes fibronectin, tenascin-C and versican, all known to play important roles in the initial steps of wound healing[220,221]. However, the DLS was the dominating source for most proteins belonging to the core matrisome groups at the initial timepoints. Some of these proteins were accumulating throughout the culture period, and the ratio between newly synthesised and pre-existing protein gradually rose. Prominent among the proteins that increased in abundance at later timepoints were BM proteins such as nidogens and laminins, but also the proteoglycans and several collagen type VI-sub chains followed this trend. The continued accumulation of BM components and proteoglycans such as decorin and biglycan, which are important for collagen fibre assembly and maintenance[222,223], could be interpreted as part of the tissue remodelling seen in the later stages of wound healing *in vivo*.

Different matrisome production compared to monolayers on tissue culture plastic

The fibroblasts cultured on tissue culture plastic had reached confluency at day 6, which should mean that the cell number were at a somewhat steady state at the later timepoints, similar to the scaffold cultures. MS analysis of matrisome proteins showed that the core matrisome proportion was about 3-fold at day 6 in the scaffold cultures compared to the monolayer cultures. The difference core matrisome proportion diminished over time due to increased deposition of ECM glycoproteins and collagens in the monolayer cultures. However, in the scaffold cultures the proportion of proteoglycans increased considerably over time, while it remained low in the monolayer cultures, which also lacked the gradual increase in BM proteins seen in the scaffold culture.

Of note, fibroblasts in monolayer culture were cultured far beyond confluency and a significant ECM accumulation did only appear post confluency. Standard culture procedures generally include passaging the cells before the reach confluency, which mean that they never face a rich ECM milieu. As cells in the scaffold cultures experience a complex ECM milieu, more similar to the *in vivo* situation, a more physiological relevant cell phenotype could be expected.

General conclusions

The culture platform based on decellularized lung ECM supplies a 3-dimensional lung ECM milieu and allows for cell proliferation and maturation. The pre-existing ECM profoundly influences the production of new ECM in by fibroblast, further studies are warranted to evaluate if this reflects a greater in vivo relevance for these studies in this kind of culture setting. Simultaneous MS evaluation of cell and scaffold derived proteins using stable isotope labelled amino acids is a useful tool in this setting and can easily be applied to other experimental systems exploiting decellularized biological scaffold.

Article II

Matrisome properties of scaffolds direct fibroblasts in idiopathic pulmonary fibrosis

Profound changes of the ECM architecture are evident in lungs of IPF patients, but how this affect cellular function is not well established. Both tissue stiffness and composition have the potential to affect cell function. A previous study showed with analysis of polysome-associated RNA that IPF derived lung ECM induced gene translation reinforcing the fibrotic remodelling, proposing a ECM driven positive feedback loop [168]. With the established isotope labelling technique from article I, it was possible to study the effect of IPF derived ECM on the deposition of ECM proteins by repopulating lung fibroblasts. Drawing from experiences from article I where the fibroblast quickly condensed the tissue and obliterated the alveolar architecture, this study implemented a holder that by circumferential attachment prevented collapse of the lung slices.

Short summary

- A patient derived fibroblasts cell line was cultured in DLSs from either IPF or control lungs (n = 4 per group)
- Tissue stiffness and density of the ECM scaffolds were measured, and MS-data was scaled and presented as protein intensity per volume unit
- Fibroblasts in fibrotic ECM reproduced many of the fibrosis related aberration of the scaffolds, supporting the notion of pro-fibrotic positive feedback loop

- Basement membrane components dominated among the ECM regulated proteins

Characteristics of decellularized lung slices

As the remodelling of the lung in IPF is highly heterogenous, with more and less fibrotic areas occurring in close proximity, it is of relevance to have some measure of the degree of remodelling in the decellularized lung tissue. Measurements of lung stiffness based on stain-stress curves confirmed that the IPF derived tissue was in general stiffer, however with large variation in the data. Measurements of tissue density gave however more consistent results with higher densities in IPF. The composition of IPF and control lung showed to be similar on a matrisome group level but with a higher density in IPF for all groups other than the secreted factors. On the level of individual proteins, the number of detected proteins in the different matrisome groups was similar between IPF and control, but there were clear differences especially the content of several BM proteins. Specifically, control scaffolds had more laminin (α3, ß3, γ2) and collagen type IV (α3, α4) chains while nidogen 2 and collagen VI chains were more prominent in IPF tissue. IHC for collagen type IV also showed disrupted BM surfaces in the IPF scaffolds, in agreement with known BM alterations in IPF[224]. In addition to BM proteins, several proteoglycans and collagen chains differed between the tissues. IPF scaffolds had a higher content of asporin, lumican, decorin, mimecan, four collagen type VI chains, and one collagen type I.

Regulation of fibroblasts protein production by fibrotic ECM

Analysis of metabolic activity and histone proteins quantified in the MS data indicated that the cellular content increased over time in both control and IPF tissue without systematic differences in cell number between the two conditions. The produced matrisome of the different groups showed a clear intra-group correlation and on matrisome category level the IPF scaffold promoted an increased production of proteoglycans at day 3 of culture, but the difference abated towards the final timepoint that was 9 days. For individual protein there were a large degree of agreement between the protein profile of the scaffolds and the protein deposited by the repopulating cells, exemplified by the BM proteins described above, where the difference in the scaffold was recapitulated in the production of the fibroblasts. This pattern was however not universal, with a protein like nidogen-2 being enriched in IPF scaffolds but showing a more prominent new synthesis in the control cultures, indication additional layers of regulation. Additional BM proteins which did not show up as significantly different in the statistical analysis of differences between IPF and control scaffolds differed among the newly synthesized proteins. These include laminin α5, nidogen-1 and agrin which were more prominent in control cultures. Fibroblasts in IPF scaffold cultures did also recapitulate other aspects of IPF pathology, e.g. a higher production of tenascin-C which is enriched in fibroblastic foci in IPF[148].

General conclusions

Fibrotic lung ECM have the capacity to direct the activity of resident cells and the data support the possibility of a positive feedback loop aggravating fibrotic remodelling in IPF. The relative importance of tissue stiffness to ECM composition remains an open question as these properties of the ECM cannot be easily separated. However, ECM modifying treatments appears as a promising avenue for future IPF therapies.

Article III

Alveolar epithelial cells are competent producers of interstitial extracellular matrix with disease relevant plasticity in a human *in vitro* 3D model.

Epithelial cells are known to produce BM proteins and some publications have also shown the capacity to produce ECM components considered to belong to the interstitial matrix. As alveolar septa are remodelled in both emphysema and IPF we hypothesised that the ECM production by the epithelial cells lining these septa might play a direct role in dieses development. This is in part motivated by the fact that AECII cells are believed to be key drivers of remodelling in IPF, however mainly as initiators of fibroblast mediated remodelling. AECII cells quickly loose many phenotypical traits in traditional *in vivo* culture, and we therefore use cryopreserved primary human AECII directly seeded into decellularized scaffolds without prior *in vitro* expansion. This should in principle help preserve more of their original phenotype even of the isolation and the freeze-thaw process in itself may affect cellular function to some degree. A drawback of this strategy is of course that the available cell number becomes an important limiting factor.

Short summary

- Primary human AECII from healthy and end stage COPD lungs were cultured in DLS from healthy lungs for 13 days
- A third group was created by adding TGF-ß1 as a profibrotic stimuli to healthy cells at day 7 to evaluate a potential plasticity in ECM-production
- Healthy and COPD cells showed initial proliferation and a stable almost identical phenotype between day 7 and 13 evaluated by RNA-seq and MS
- AECII showed to be competent producers of ECM components belonging to both BM and intestinal matrix
- TGFß-1 stimuli altered ECM production similarly to what have been described in mesenchymal cells without loss of AEC marker expression.

Culture of primary human AECII cells in DLS

The cryopreserved cells showed to be able to proliferate when seeded in human DLS from healthy lung, without any obvious difference between healthy- and COPD derived cells. The study did not include RNA-seq analysis of freshly isolated cells, but in comparison with a publicly available data set from AECII isolated using the same surface marker HT2-280, the profile of AECI and AECII markers is similar both at day 7 and 13 of culture. IHC evaluation at day 13 showed that there were widespread spB expression among the cells and some cells still showed expression of pro-spC. A partial differentiation towards an AECI phenotype would be sensile from a physiological view as AECII is thought to replenish the pool of AECI cells after injury *in vivo*. RNA-seq and MS data did not show signs of a clear change between the timepoints, but as these analyses reflect bulk changes in the samples it is hard to draw conclusions on the individual cell level. Some signs of AECI differentiation could however be seen after TGF-ß1 stimuli with upregulation of the ACEI marker ICAM1 at the protein level, but both up and down regulation of AECI markers were seen in the RNA-data. Stimulation with TGF-ß1 could be expected to induce an epithelial to mesenchymal transition in the cells, and some upregulation of mRNA for EMT markers such as Vimentin, fibronectin and N-cadherin were seen, but with preserved expression of E-cadherin and even upregulation of some AECII markers such as spD. Other effects of the TGF-ß1 addition were a reduction in cell metabolism which could likely be related to a reduction in cell numbers as the proportion of proliferative cells positive for Ki67 was reduced in the TGF-ß1 group at day 13. Immunostaining also showed that all visualized cells retained intracellular cytokeratin at day 13, pointing towards a preserved epithelial phenotype throughout the experiment.

ECM production by alveolar epithelial cells

Looking at the ECM expression by cells in the untreated healthy control group at day 13 and comparing the RNA data to a publicly available RNA-seq dataset from healthy parenchymal lung samples the cell expressed 110 out of 168 core matrisome genes found in intact lung and an additional core matrisome 12 genes. Looking on protein level data, the cells, as expected, produced a rich repertoire of BM genes, including all laminin chains commonly found in adult human lung, i.e. laminin α3-5, ß1-3, γ1 and γ2[225,226]. However, the cells also produce a number of chains belonging to fibrillar collagens, i.e. type I, II, III, and V, which is more associated to the interstitial matrix. Other proteins commonly found in interstitial matrix are the small leucine-rich proteoglycans (SLRPs) which associate to fibrillar collagens and are important for ECM organization. The AEC produced 5 SLRP proteins, i.e. biglycan, decorin, fibromodulin lumican and PRELP, whereof the first four are believed to bind TGF-ß1 and may act to inhibit its biological activity[227].

Upon stimulation with TGF-ß1, the healthy cells upregulated gene for a majority of core matrisome genes with a more complex pattern of up- and downregulation of

matrisome associated proteins, e.g. several ECM degrading enzymes such as MMPs were upregulated while others such as MMP-28 and hyaluronidase-1 were downregulated. Among the upregulated genes were periostin, tenacin-C, fibronectin and versican, all known to be upregulated in fibroblastic foci[141,148], i.e. remodelling hotspots in IPF. Of interest is also that on a gene level both collagen VII and all the three chains of laminin 332 were upregulated in response to TGF-ß1, and the laminins was also upregulated on the protein level. Collagen VII is upregulated in IPF and bind to laminin 332[228], that is present under the aberrant epithelium found in remodelled distal airspaces in IPF[229]. This finding would support the possibility of an AECII origin of these cell as opposed to bronchiolar epithelial cells, which is probably the most likely alternative[230].

COPD vs healthy AECII

The transcriptomic and MS data show very limited differences between healthy and COPD derived cells. The MS data analysis found only one differentially expressed protein, class I major histocompatibility complex (MHC class I), that were upregulated at both day 7 and 13 in COPD. Increased levels of this protein have been found in exhaled breath condensate from COPD patients which also have been shown to have increased expression for this protein in AECII. The conservation of a known pathological alteration in AECII cells after 13 days of culture would speak towards phenotypic conservation in our culture system. The very limited overall differences might reflect limited disease associated alterations in this cell type, or possibly a selection in the isolation process. However, if only a healthier subset of COPD derived cells proliferated in our culture system, the metabolic activity in these samples would likely have been significantly lower which was not seen. A higher expression of the surface marker HT2-280 used for isolation on “healthier” cells from COPD patients and their preferential survival remains a possibility. Another intriguing possibility is that the ECM scaffolds from healthy lung directed both COPD and healthy derived cells towards a similar phenotype.

General conclusions

AEC can be cultured in our DLS platform for at least 13 days with retention of important phenotypic markers. AECII from end stage COPD patients show limited sign of pathological alteration when cultures in healthy lung ECM. The alveolar epithelium has the capacity to produce a wide array of ECM components and likely contribute to the homeostasis of both alveolar intestinal matrix and BMs. AECII also show a significant plasticity in response to pro-fibrotic stimuli in the form of TGF-ß1, showing a potential for active participation in pathological remodelling previously attributed mainly to mesenchymal cells.

Article IV

A tunable physiomimetic stretch system for precision cut lungs slices and recellularized human lung scaffolds.

Lungs are moving with every breath and mechanical stimuli exerted on cells, primarily via interactions with the ECM affect cellular function. The introduction of decellularized ECM as a culture substrate aims to better replicate the *in vivo* condition, and a next step in improving the translatability of *in vitro* results is to simulate the mechanical stimuli experienced by cells in the lung. To this end, a device for culture of lung slices with cyclic mechanical stretching of the tissue have been developed. The device is intended to be used both for studies performed with cells cultured in DLS and for studies using slices of native lung with all cellular components in place, also known as precision cut lung slices (PCLS). In this first proof of concept study the functionality of the device and compatibility with tissue culture methods are demonstrated.

Short summary

- A stretch device has been designed that produces mechanical strain in lung slice cultures
- PCLS and repopulated DLS can be cultured in the device and subjected to cyclic stretch without compromising cellular viability
- Cyclic stretch mimicking physiological conditions induced increased surfactant gene transcription in lung epithelial cells after one day of culture

Properties of the novel stretch device

Each stretch device holds up to 8 individual culture wells that each holds one mounted lung slice. The culture wells can be moved on and off the stretch device and are designed to allow for imaging of tissue in situ. All components that come in direct contact with tissue or culture medium can be sterilized by autoclaving and are non-toxic to cells. Most components are reusable and only a thin membrane of silicone and a small silicone gasket need to be replaced between experiments, making the culture system relatively environmentally friendly. The stretch movement are programmable, ranging from no movement to supraphysiological stretch allowing simulation of pathological states, e.g. in studies related to ventilator induced lung injury. Devices with similar functionality have previously been described, but none are commercially available and either have limited throughput capabilities or compromise part of the tissue samples by using glue for attaching them to an elastic membrane[231,232]. The design of the stretch device will be freely shared with the scientific community.

Culture of the epithelial cell line H441 in DLS with cyclic stretching

The epithelia cell line H441 originate from human adenocarcinoma and have properties of both AECII cells and club cells were used in the culture experiment. These cells showed similar proliferative and metabolic activity with or without cyclic stretching that were performed with a change in 2-dimensional area for the lung slice of ~10% in each cycle and with a frequency of 16 cycles per minute. Samples were evaluated after 24 and 96 hours of culture with or without cyclic stretch. Quantitative PCR evaluation showed that at the first timepoint the stretched samples had an increased expression of the gene of spB with a tendency towards higher expression of the genes for the BM proteins laminin α5 and collagen type IV α3 chain, a pattern that interestingly was reversed at the later 96 h timepoint. No effect was seen on the expression of the gene for the intestinal collagen type III α1 chain, a gene that have been shown to be upregulated in ventilator induce lung injury. Previous studies of H441 cells have shown that mechanical strain induced increased proliferation, an effect that were not seen in our experiments, this could possibly be related to an effect of the ECM included in our set-up. The effect shown was not statistically significant as we had a large variation in the data, this can likely be attributed to the qPCR procedure rather than biological variation as metabolic and IHC data did not show a corresponding variation. Even with limited effect of the applied stretch the data indicate a response and further evaluation using different stretch setting and cell types appear motivated.

PCLS under cyclic stretch

Lung is a complex tissue with an intricate 3-dimensional structure inhabited by at least 40 different types of cells[233], a milieu that is not currently possible to replicate *in vitro*. The use of PCLS allows to capture much of this complexity *in vitro* and is a useful platform for both toxicology and pharmacology studies, as well as basic tissue biology research questions[161]. Adding the possibility to mimic physiologically relevant mechanical strain to PCLS might further improve the translatability of achieved results and enable studies of the physiological effect of mechanical stimuli. In this study we show that rat PCLS can be maintained in our device with mechanical stretching at least to a point 96 hours after generation of the slices, with cyclic mechanical stretch during the last 72 hours. The cultured slices showed no loss in metabolic activity and histological evaluation show a well-preserved morphology. Further evaluation of stretch induced responses in the PCLS is warranted.

General conclusions

This work shows the functionality of our in-house designed device for culture of lungs slices with cyclic starching mimicking in vitro mechanical stimuli from breathing. An effect on the surfactant gene expression in response to mechanical stimuli shown a physiologically relevant effect of mechanical stimuli and confirms

an effect on cellular function. The presented device and methodology should have wide applicability for studies of lung tissue function in relation mechanical stimulation of cells.

Concluding remarks

Chronic lung diseases such COPD and IPF is very much connected to the ageing of the lung, which is an intricate structure that need to be maintained throughout life in the face of challenges such infections, air pollutants and continuous mechanical strain. This thesis has focused on finding ways to better understand the biology of alveolar maintenance and remodeling.

The technique for production of decellularized lung slices from clinical specimens described in **article I** have in addition to the work presented in this thesis been used in one published paper and are currently used for ongoing studies within our research group. Hopefully, the results from the comparison with monolayer cultures on plastic can inspire others to incorporate the ECM in their models, and the presented protocol have the benefit of not using any specialized equipment of reagents. One issue with decellularized tissue that might prove to pile on to the reproducibility crisis in medical science is that its composition will vary with the method of decellularization. At this stage there are very few answers to what methods are the best and the current plethora of methodological variation should probably be view as an asset, as it generates data that might help in finding the right way(s) forward. It is unlikely that there will be a "one fits all method", but there is likely room for considerable improvement of current methods. The ability to source the ECM from human material helps capture features unique to the aged human lung, but at the same time increases the complexity of the generated data. One could imagine that we could find key factors in the ECM find ways to create a simplified bioengineered ECM structures that emulate that of the lung.

The idea of using isotope labeled amino acids to distinguish cell derived proteins in culture systems using biological scaffolds was presented by another group while **article I** was under preparation[234]. Both these papers clearly show the utility of this technique which can easily be applied to similar systems in any tissues and can help with understanding ECM turnover, both production and degradation. The latter poorly explored so far, but by following diminishing amounts of scaffold derived proteins or peptide fragments of enzymatic breakdown of proteins (degradomics)[235], the stability of scaffold and cell derived ECM could be followed. The future will tell how useful the scientific community finds this approach. In addition to **article I**, **II** and **III** in this thesis, we have utilized this technique in three separate studies regarding cholangiocyte and cholangiocarcinoma biology in collaboration with a group led by prof. van der Laan at Erasmus University Medical Center.

Regarding the importance of the ECM in guiding cell phenotypes the data from **article II** adds to previous data in supporting the hypothesis of a positive feed-back loop for ECM deposition in IPF. Also, data on AECII cells from **article III** supports the notion of cell instructive properties of ECM, as COPD derived cells had transcriptomic and proteomic profile very similar to healthy cells. Despite existing data on COPD related gene expression changes in AECII cells, additional study of baseline differences between COPD and healthy derived cells and crossover experiments also utilizing COPD-derived ECM would be of value.

Improved understanding of the role of the epithelial cells in maintaining the ECM of alveoli help our understanding of fundamental lung biology and might inspire new ideas of how to combat alveolar remodeling in emphysema and fibrosis. The transcriptomic and proteomics datasets generated in **article III** from human AECII have the potential to serve as a valuable resource to other researchers as we do not presume to have probed all potential questions of interest. More mechanistic studies relating ECM production to a more detailed phenotypic characterization on a single cell level would be valuable to better understand how the epithelium helps maintain alveolar septa. For instance, AECI cell cover most of the alveolar surface and are as such in an ideal position to repair and maintain the alveoli ECM structure, but their role in ECM maintenance is largely unknown.

As the AEC are known to be responsive to mechanical strain, it would be of great interest for future studies to combine the use of the stretch device developed in **article IV** with the proteomics analysis from **article III**. This could give interesting new insights into the stretch driven AECII pathology suggested to drive fibrotic remodeling in IPF. In addition to the use for cell cultures in DLS, adding physiomimetic stretch to PCLS cultures could potentially help to preserve the native state of these complex tissue samples, increasing the usefulness of this important model system. Not going all the way to PCLS in complexity, another natural step to move closer to the *in vivo* setting would be to do co-cultures of different cell types in DLS. Endothelial cells would be one cell type of primary interest, but to get them in to their correct intravascular niche would likely be challenging without a perfusion system. Alveolar macrophages are probably the easiest cell to get into a correct location in the DLS together with AEC. Fibroblasts and AEC would also be interesting, especially for disease modeling in IPF and other ILDs, but for studies of normal physiology fibroblasts invasion into interstitial tissue compartments would be a relevant goal.

Understanding the alveolus makes for a complex puzzle, and this thesis have hopefully contributed with some new pieces getting us closer to a whole, now even more complicated image. Models with greater physiological relevance holds potential to increase the translatability of preclinical research improving research efficiency. Consequently, the scientific value of this thesis will hopefully not be restricted to the data it contains, but grow with the implementation of the presented methodology.

Populärvetenskaplig sammanfattning

Lungorna är skådeplats för några av de mest utbredda och dödliga sjukdomar som drabbar mänskligheten. Kronisk obstruktiv lungsjukdom (KOL) karaktäriseras av tilltagande andningsbesvär med bakomliggande inflammation i små luftvägar och emfysem, där strukturen för lungans gasutbyte bryts ner. Idag är KOL globalt den tredje vanligaste dödsorsaken med mer än 3 miljoner årliga dödsfall, och trots en stor mängd behandlingsalternativ så förblir sjukdomen just kronisk. Rökning är en viktig riskfaktor för KOL, men ca en tredjedel de som drabbas har aldrig rökt. Luftföroreningar, bland annat från förbränning av ved och andra biobränslen för uppvärmning och matlagning tros vara en viktig riskfaktor i stora delar av världen. Andra betydande sjukdomar i lungan är lungcancer, vilket är den cancerform som tar flest liv i Sverige, och nedre luftvägsinfektioner som är en viktig dödsorsak inte minst i fattigare delar av världen. Få har dock undgått faran med nedre luftvägsinfektioner i samband med Covid-19 pandemin, där merparten av de svårast sjuka har allvarligt skadade lungor. Lungorna från avlidna i Covid-19 har ofta omfattande ärrbildning i lungan och det finns farhågor att överlevare av svår Covid-19 sjukdom kan drabbas av bestående och tilltagande lungfibros. Av ett flertal former av lungfibros är idiopatisk lungfibros (IPF) vanligast och saknar en direkt utlösande faktor, även om manligt kön och ålder är viktiga riskfaktorer. Vid IPF blir lungorna succesivt allt stelare och en ond cirkel uppstår där den bildade ärrvävnaden tycks stimulera till ännu mer ärrbildning. Sjukdomen är mycket mindre vanlig än KOL med grovt uppskattat 1000 nya fall i Sverige per år, dock är medianöverlevnaden bara ca 3 år efter diagnos även med behandling.

För att effektivt kunna överföra syre och koldioxid mellan luft och blod måste avståndet däremellan vara kort. Överföreningen av gaser sker i alveolerna där syre passerar en vägg bestånde av en epitelcell, ett tunt lager bindväv och slutligen en endotelcell innan det når blodet, hela denna barriär har ca en femtiondel av ett hårstrås tjocklek. Alveolernas väggar bär upp små blodkärl med en yta uppskattad till ca 115 kvadratmeter, som dock är så tunna att de tillsammans bara rymmer ca 2 dl blod. Utöver att utgöra mötesplatsen mellan blod och luft så behöver alveolernas väggar vara elastiska och kunna sträckas ut vid varje inandning och dra ihop sig vid varje utandning. Förenklat kan sägas att om väggar försvinner, vilket kan ske vid KOL, blir det svårt att tömma lungorna och om de blir tjocka och stela som vid lungfibros, blir det svårt att fylla dem.

Den huvudsakliga målsättningen för denna avhandling har varit att utveckla nya metoder för att studera hur lungans celler och bindväv interagerar med varandra. Med dessa metoder hoppas vi kunna bidra till att öka kunskapen om sjukdomsmekanismer vid lungsjukdomar och på så sätt bidra till att hitta bättre behandlingsmetoder. Bindväven i kroppen framställts ofta som en relativt statisk byggnadsstomme. Dock har tilltagande förståelse utvecklats för att bindväven spelar en aktiv och föränderlig roll för celler och vävnaders funktion. Hos mottagliga individer kan bindvävsförändringar i lungan som uppstått i samband med en skada på vävnaden initiera cellulära processer som leder till ytterligare patologisk omvandling av bindväven. Förutom i kroniska lungsjukdomar som KOL och IPF är kunskap om interaktionen mellan celler och bindväv även viktig för att förstå andra processer som till exempel spridningen av lungcancer.

Inom ramen för denna avhandling har ny metodik utvecklats för att studera samspelet mellan viktiga celltyper i lungans alveolärvävnad och den bindväv som finns där. I den första studien utvecklades en metod för att från human lungvävnad ta fram vävnadssnitt där bindvävens sammansättning och struktur bevaras, samtidigt som de ursprungliga döende eller döda cellerna tas bort. Fibroblaster, vilka är framstående bindvävsproducerade celler, odlades sedan i dessa snitt och med hjälp av ny metodik för masspektrometri (MS) kunde omsättningen av ca två tusen olika proteiner analyseras i dessa odlingar. Försöken visade utöver metodikens användbarhet att bindväven kraftigt förändrade fibroblasternas egna bindvävsproduktion jämfört med den vanligaste odlingsformen på plastytor. Sannolikt kan denna alternativa cellodlingsmodell generera resultat som är mer relevanta för hur cellerna uppträder inuti människokroppen.

I det andra artikeln användes metodiken etablerad i den första studien för att undersöka hur fibroblaster påverkas av en sjuk bindväv från patienter med IPF. Resultaten visade att en positiv återkopplings-loop skapades där celler odlade i sjuk bindväv producerade mer sjuk bindväv. Utöver detta kunde ett antal sjukdomsrelaterade proteiner identifieras som blivit föremål för vidare studier.

I den tredje studien användes återigen samma metodik, men nu med bindväv endast från friska individer och alveolära epitelceller. De alveolära epitelcellerna tros spela en central roll i utvecklingen av både KOL och IPF där de signalerar till fibroblaster och därigenom initierar remodellering av vävnaden. Resultaten visade att celler från KOL-patienter i stort likande celler från friska individer när de odlades i frisk bindväv, vilket ger hopp för framtida behandlingar inriktade på att påverka bindväven. Vi fann även att friska alveolära epitelceller hade förmåga att producera bindvävskomponenter som tidigare främst förknippats med fibroblaster, samt att när ett pro-fibrotiskt stimuli tillsattes så producerade de bindväv likande den som ses vid lungfibros. Resultaten tyder på att synen på epitelcellernas betydelse för bindvävshomeostas och remodellering vid kroniska lungsjukdomar kan behöva omvärderas.

Att odla lungceller i lungbindväv är ett sätt att skapa modellsystem som efterliknar förhållandena i den mänskliga lungan. Den fjärde studien syftade till att även kunna simulera de stimuli celler får från andningsrörelser. Det huvudsakliga arbetet bestod i att utveckla ny apparatur där vävnadssnitt av lunga på ett kontrollerat vis kan sträckas under cellodlingsförhållanden. Den utvecklade apparaturen tillåter simulering av andningsrörelser av varierande amplitud och frekvens med visad effekt på cellfunktion. Metodiken kan användas både för att studera lungcellers funktion under fysiologiska förhållanden och för att studera mekanismer bakom skador som uppstår vid onormala andningsrörelser som kan uppstå vid respiratorvård.

Denna avhandling har bidragit till kunskaper kring hur celler och bindväv samspelar i lungan och hur detta samspel är rubbat vid kroniska lungsjukdomar. Sannolikt av större betydelse är metodiken som utvecklats, vilken redan används i flera efterföljande studier och som förhoppningsvis kommer bidra till forskningsframsteg inte bara inom lungforskningen.

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