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Mesenchymal heterogeneity in the adult human lung

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Mesenchymal heterogeneity in the adult human lung

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Måns Kadefors

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on Friday 4th of November 2022, at 09.00 in Segerfalksalen, BMC, Lund

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Abstract
The human lung is a complex tissue consisting of a heterogeneous mix of different structures and cells, each which a specific role that together contribute to the primary function of the organ: the gas exchange. Different mesenchymal cells populate the interstitial areas around airways and blood vessels and form the connective tissue together with the extracellular matrix. Fibroblasts constitute one group of mesenchymal cells which are potent producers of extracellular matrix, an important part of the tissue niche which is heavily altered during remodeling in fibrotic lung diseases. In addition to providing structure and support, the extracellular matrix is also a part of the dynamic microenvironment that interacts and communicates with the cells. Yet, the different functions of fibroblasts and other mesenchymal cells in the lung under homeostatic and pathophysiological conditions remain elusive. Furthermore, the extensive heterogeneity displayed by fibroblasts and other mesenchymal cells in terms of phenotype and function, which in part can be related to their spatial localization, is not fully understood. Deciphering this heterogeneity is important to better understand specific functions of specialized mesenchymal cells.

The aim of this thesis was to investigate the heterogeneity of fibroblastic mesenchymal cells that reside in the human lungs and to explore how different cellular phenotypes can contribute to mesenchymal-associated functions such as tissue remodeling. The studies included in the thesis are based on experimental work on primary cells derived from human lung tissue. Mass-spectrometry and RNA-sequencing technologies were used to perform comprehensive analysis of the proteome and transcriptome of targeted cell populations. To study mesenchymal cells in heterogeneous tissue material, we also utilized techniques with single-cell resolution, including flow cytometry, single-cell RNA-sequencing and histology, together with distinct anatomical tissue sampling (proximal vs distal airways).

Based on these approaches we could confirm the existence of heterogeneity within the mesenchymal cell compartment, with a specific phenotypical pattern associated with fibroblasts in the peribronchovascular compartment emerging. In paper I, we characterize the proteome of fibroblast from proximal and distal airways and confirm specific protein expression in the bronchvascular regions. In paper II, we explore the functional attribute of colony formation (reflecting proliferative capacity) that has been connected to a mesenchymal progenitor population, and describe a link between this property and an adventitial localization and an adventitial fibroblasts phenotype. In paper III, we describe the existence of a CD26+ adventitial fibroblast phenotype in human lung and show that it is not associated to an actived profibrotic phenotype in idiopathic pulmonary fibrosis, contrary to what other studies have suggested. Finally, in paper IV, we applied our bioinformatical approach to characterize non-fibroblastic cells, alveolar epithelial cell, with a focus on their contribution to ECM remodeling, and revealed an overlooked complexity in their matrix producing capacity that include interstitial matrix proteins commonly associated with mesenchymal cells.

Taken together, the results presented in the thesis describe a phenotypic heterogeneity among mesenchymal cells that appear to be intricately connected to their local environment, highlighting the importance of the extracellular context for cell function. The generated information could contribute to reduce the inconsistency and confusion regarding different terminology used and provide information regarding potential functions of these elusive cells in lung tissue.

Key words
Lung, extracellular matrix, adventitia, fibroblast, mesenchymal cell, alveolar epithelial cell, fibrosis, idiopathic pulmonary fibrosis, mass spectrometry, RNA-sequencing

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Paper I

Måns Kadefors, Arturo Ibáñez-Fonseca, Göran Dellgren, Sara Rolandsson Enes, Anders Aspberg, Madelene W Dahlgren and Gunilla Westergren-Thorsson. **Spatial heterogeneity of fibroblasts in lung tissue.** *Manuscript in preparation.*

Paper II

Måns Kadefors, Sara Rolandsson Enes, Emma Åhrman, Barbora Michaliková, Anna Löfdahl, Göran Dellgren, Stefan Scheding and Gunilla Westergren-Thorsson. **CD105+CD90+CD13+ identifies a clonogenic subset of adventitial lung fibroblasts.** *Sci Rep.* 2021;11(1):24417.

Paper III

Måns Kadefors, Frida Berlin, Marie Wildt, Göran Dellgren, Sara Rolandsson Enes, Anders Aspberg and Gunilla Westergren-Thorsson. **Dipeptidyl peptidase 4 expression is not associated with an activated fibroblast phenotype in idiopathic pulmonary fibrosis.** *Front Pharmacol.* 2022;13(August):1-12.

Paper IV

Oskar Rosmark, Måns Kadefors, Göran Dellgren, Christofer Karlsson, Anders Ericsson, Sandra Lindstedt, Johan Malmström, Oskar Hallgren, Anna-Karin Larsson Callerfelt and 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 to be submitted.*

Selected abbreviations

α-SMA	α-smooth muscle actin
AEC	Alveolar epithelial cell
AEC1	Alveolar epithelial type 1 cell
AEC2	Alveolar epithelial type 2 cell
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DDA	Data-dependent acquisition
DIA	Data-independent acquisition
DLS	Decellularized lung slice
ECM	Extracellular matrix
FACS	Fluorescence-activated cell sorting
IPF	Idiopathic pulmonary fibrosis
MAGP2	Microfibrillar-associated glycoprotein 2
MSC	Mesenchymal stromal cell
scRNAseq	Single-cell RNA-sequencing
TGF-β	Transforming growth factor-β

Introduction

The respiratory system and the lungs

Every cell in the human body requires oxygen to survive and function. The human respiratory system is a collection of organs and structures that work together with the common core purpose of respiration. Through the process of respiration, oxygen is taken up from inhaled air and carbon dioxide generated in the body is expelled through exhalation. At the center of the respiratory system are the lungs, were the gas exchange take place. The respiratory tract, divided into the upper tract (nasal cavity, pharynx and larynx) and the lower tract (trachea, primary bronchi and lungs), provides the piping that transports gases to and from the functional units in the lung, the alveoli.

Anatomy and structure of the lungs

The lower respiratory tract is made up of the trachea that divides into two main bronchi which lead into the lungs (Figure 1). The two lungs are separated into lobes, two on the left lung and three on the right lung. The lungs are each contained within a pleural sac consisting of two opposing mesothelial membranes, the visceral (inner) and parietal (outer) pleura. Within the lungs, the primary bronchi divide into smaller and smaller airways for each dividing generation, forming the bronchial tree, ending up in the smallest airways, the bronchioles, and finally the air-filled sacs, the alveoli, were the gas exchange take place. The alveoli make up the great majority of the lung volume. To support the critical gas exchange between blood and air, the lungs have an extensive vascular network. Pulmonary blood vessels branch along the bronchial tree ending up in networks of the smallest thin-walled blood vessels, the capillaries, that reside in the alveoli walls. These functional structures of the lung are supported and connected by connective tissue, a complex framework of extracellular matrix (ECM) molecules and cells. In addition to structural support, the components of connective tissue also provide mechanical properties necessary for the stretch associated with breathing, and participates in cellular interactions and signaling. The outermost layers of larger airways and blood vessels is called the tunica adventitia and consist of loosely organized connective tissue infused with smaller vessels and is populated with different stromal cells. The adventitia is part of the peribronchovascular interstitium which together with the interstitium of interlobular

septa and the visceral pleura constitute a continuous interstitium with a similar fibrous connective tissue structure.

As the lung provide a unique interface between the outer environment and the inner environment, host defense is the second essential function of the lungs and respiratory tract. The different defense mechanisms are vital for protection and proper function. The first line of defense are the nonspecific responses, including mechanical defense by mucociliary clearance and coughing as well as innate immune responses such as phagocytosis by macrophages[1]. In addition there are the adaptive immune responses supplied by specialized lymphocytes.

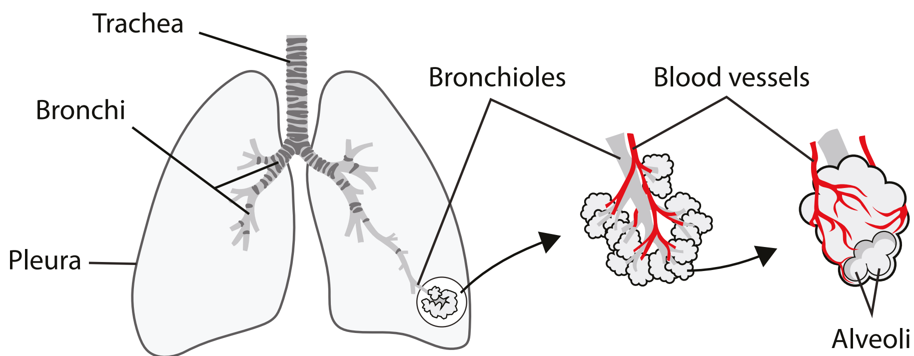

Figure 1. Illustration of the human lungs and the lower respiratory tract with the smallest airways containing the alveoli enlarged.

Lung development

Around four weeks after conception the first resemblance of two lungs start to appear as a primitive trachea and two outpouchings, the lung buds, form from the primitive foregut[2,3]. The lung buds contain endodermal derived progenitors of the airway epithelium which gradually "grow" into the surrounding mesenchyme, a loosely organized connective tissue with unspecialized mesenchymal cells derived from the mesoderm, and form the bronchial tree through the process of branching morphogenesis. This process is regulated through interactions between the mesenchyme and the epithelium, including signaling through fibroblast growth factor and bone morphogenetic protein pathways[3]. In parallel, development of the lung vasculature takes place, originating from the mesenchyme, which is tightly associated with the bronchial tree structure. As the bronchial tree is forming, mesenchymal progenitors differentiate to form smooth muscle cells around airways and blood vessels, pericytes and cartilage surrounding the larger airways, however these processes are not as defined. Alveolar differentiation is initiated after around 16-26 weeks and mature alveoli starts to form at around 36 weeks as fibroblast-like cells from the surrounding mesenchyme invade the immature alveoli and form septa

that divides individual alveoli, contributing to the large surface area. Formation of mature alveoli continues after birth and has been reported to proceed throughout childhood and adolescence[4,5].

Cells and components of the lung

Analysis of the cellular composition of human lungs, utilizing single-cell RNA-sequencing (scRNAseq), has increased our understanding of the cellular complexity and estimates have put the number of different cell types in the lungs as high as 58[6,7]. Yet, the distinction between different cell types and different cell states remains unclear. Among the most common and essential cells in the lungs are airway epithelial cells and alveolar epithelial cells (AEC), endothelial cells of the vascular and lymphatic systems, hematopoietic cells and a variety of mesenchymal cells, e.g. fibroblasts, smooth muscle cells and pericytes[8]. The cells of the lungs are connected to or suspended in the ECM, a three-dimensional molecular network that provide structure, mechanical properties and a plethora of other biological functions to the lungs.

Alveolar epithelial cells

The alveoli constitute the functional units of the lung where the gas exchange take place. These structures are composed of two types of epithelial cells: alveolar epithelial type 1 cells (AEC1) and type 2 cells (AEC2). AEC1 are large, flattened cells that cover a large portion of the alveolar surface with a very thin cell layer that facilitates the diffusion of oxygen and carbon dioxide between the alveolar lumen and the underlying capillaries. Although the number of AEC1 in the lungs is slightly lower than AEC2, the AEC1s cover more than 90% of the alveolar surface[9]. The AEC2 are cuboidal epithelial cells that produce and secrete surfactants that lower the surface tension at the air-liquid interface. In addition, AEC2 help to maintain the integrity and function of the alveoli by serving as alveolar stem cells, with the capacity to self-renew and replenish AEC1 through differentiation[10].

Lung extracellular matrix

The ECM is a network of extracellular proteins and polysaccharide molecules, glycosaminoglycans. Chains of glycosaminoglycans can be attached to core proteins to form a group of heavily glycosylated proteins called proteoglycans.

The lung is a complex organ containing different structures that all have their own distinct ECM environment that support specific structural and cellular functions. The ECM can be divided into basement membranes and interstitial matrix which are intimately connected[11]. Basement membranes are thin, dense sheets of specialized ECM that epithelial and endothelial cells sit on, separating them from the underlying connective tissue[12,13]. The main components of basement membranes are laminin- and collagen type IV-based networks, nidogen and perlecan. The interstitial matrix forms a three-dimensional meshwork enriched in collagen fibers,

containing collagens type I and III, and elastic fibers that support the tissue structure and provide mechanical properties[11]. Cells attach to the ECM through cell adhesion receptors (e.g. integrins) and signal transduction either through individual receptors or clusters of integrins (in focal adhesions) enable cells to respond to the ECM environment and mechanical stimuli (e.g. stiffness)[14,15]. The lung ECM provides multiple binding sites and serves as a reservoir for growth factors and cytokines[16]. Both directly and indirectly, it can affect the local bioavailability of these soluble proteins. The exact composition of basement membranes and interstitial matrix varies between locations and states and provide specific niches that affect cellular behavior.

Mesenchymal cells and fibroblasts

There are many terms that are used to describe fibroblastic mesenchymal-derived cells, which are sometimes used interchangeably and sometimes to denote a more defined cell population. As there is no real consensus on the terminology that should be used to describe these cells, some of the most common terms are defined and discussed here.

Mesenchyme and mesenchymal cells

In developmental biology, the mesenchyme refers to cells and tissue that develop into muscle, cartilage, bone, connective tissue, vascular and lymphatic structures. Mesenchyme is mainly derived from the mesoderm, but can also originate from the ectoderm layer as in the case of neural crest cells. The term mesenchymal cell however has a vaguer meaning and can refer to several different cell types in both developing and adult tissues. The definition of mesenchymal cells is dependent on the context. In theory, it could entail any cell that is derived from the mesenchyme. In adult tissues, the term mesenchymal cells is sometimes used to refer only to connective tissue, cartilage and bone cells and sometimes it is used synonymously with mesenchymal stem cells, a type of adult tissue stem cells.

For the sake of clarity, when the term mesenchymal cell is used in this thesis, it refers to a general population of mesenchyme-derived cells excluding endothelial, epithelial and hematopoietic cells. This population is assumed to be enriched for fibroblastic cells, smooth muscle cells and pericytes.

Fibroblasts

Fibroblasts are mesenchymal connective tissue cells that produce and modulate ECM molecules, providing the scaffolding of all tissues and organs[17]. Although, the

exact composition of the deposited ECM varies between fibroblast populations of different niches and tissues. They are generally described as elongated cells with a spindle-like morphology which can have several processes extending out, although a great level of variability in morphology can be observed in cultures (Figure 2). In contrast to endothelial and epithelial cells which are joined by junctional complexes to form closely connected barriers, fibroblasts are more freely embedded within the ECM framework and they are considered to have a great migratory capacity. Beyond their ECM production, fibroblasts also secrete growth factors, cytokines and other signaling molecules, and are responsive to signals from their environment, contributing to active cell-cell communication[17–19].

Fibroblasts are also the primary effector cells in wound healing. During the initial inflammatory phase after a tissue injury, fibroblast are activated to become myofibroblasts that support the healing process through increased proliferation, contractility and ECM synthesis[20]. Dysregulation of these repair processes can be observed in fibrotic disease and the myofibroblast phenotype is often described as an activated fibrosis-driving cell in these pathological conditions[21].

Currently there are no markers that can be used to specifically identify all fibroblasts. Traditional markers, such as collagen type I, thy-1 membrane glycoprotein (also known as CD90), vimentin and prolyl-4-hydroxylase, are not expressed by all fibroblasts and can also be expressed by non-fibroblastic cells[17,22]. Nevertheless, several markers have been explored to try to delineate functional and spatially distinct fibroblast populations in lung tissue.

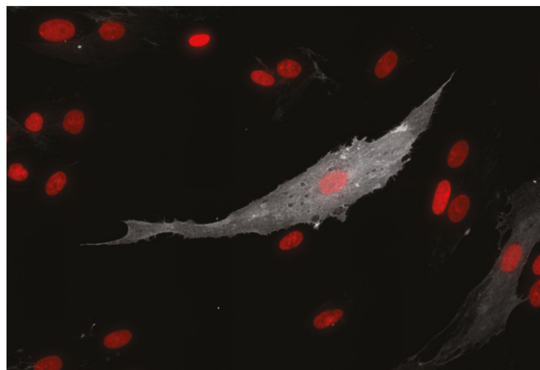

Figure 2. Fibroblast morphology. Immunofluorescence image of a fibroblast in culture. Nuclei are shown in red and the cell body is shown in white.

Stroma

The stroma is the part of the tissue with a structural and support role, in contrast to the parenchyma (i.e. the functional tissue) such as the alveolar epithelium that enable gas exchange. The stroma in lung tissue is not only made up of the connective

tissue, with ECM and mesenchymal cells such as fibroblasts, but can also contain blood and lymphatic vasculature, nerves and interstitial immune cells.

Mesenchymal stromal cells

Multipotent mesenchymal stromal cell (MSC, also referred to as mesenchymal stromal cell and mesenchymal stem cell) is a term used to define a population of fibroblast-like cells with stem/progenitor cell abilities, such as self-renewal capacity and differentiation capacity into mesenchymal lineages. MSC are defined, according to a set of minimal criteria, as (1) plastic adherent cells that can (2) be induced to differentiate into adipogenic, osteogenic and chondrogenic lineages *in vitro*, and (3) express specific surface antigens (they should express CD90, CD73 and endoglin/CD105 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR)[23]. The identification of fibroblast colonies in bone marrow cultures, which could spontaneously form bone when implanted in guinea-pigs, by Friedenstein in 1970, marked the genesis of the MSC field[24]. Although it was not until later, in 1991, that the term "mesenchymal stem cell" was first coined by Caplan, referring to the cells ability to differentiate into different mesenchymal lineages[25]. Since then, a position statement by the International Society for Cellular Therapy, published in 2005, attempted to clarify the distinction between a true mesenchymal stem cell and plastic-adherent cell that display some progenitor characteristics *in vitro*[26], and in 2006, the minimal criteria for defining multipotent MSC were presented[23]. However, confusion and interchangeable use of the terminology and definitions persist to this day. Since their discovery in bone marrow, cells fulfilling the minimal criteria for MSC have been isolated from most organs and tissues[27]. The MSC field has expanded tremendously, with several ongoing clinical trials focusing on MSC as a cell-therapy candidate[28]. However, the therapeutic focus has shifted from their proposed capacity to differentiate and regenerate tissues, to their paracrine actions that can regulate other cells, with a heavy focus on immunoregulatory functions[29].

It remains unclear to which extent cells that fulfil the criteria for MSC in culture represent stem or progenitor cells *in vivo*. The fact that MSC and fibroblasts are seemingly indistinguishable from one another based on their phenotype further complicates the issue[30–32].

Lung mesenchymal cells

Lung fibroblasts

Lung fibroblasts represent a heterogeneous cell group, relating to functional, phenotypical and anatomical diversity[33–37]. Fibroblast cultures established from proximal and distal airways have demonstrated differences in proliferation[34], morphology[35] and gene expression, with increased expression of myofibroblast associated genes in distal cultures[36,37]. Based on the expression of thy-1/CD90, two populations of fibroblast can be separated from rodent lungs, which also display morphological and functional differences in culture, including proliferative ability and response to fibrotic stimuli[33,38–41]. Observations that have shaped our understanding of lung fibroblast biology and heterogeneity are frequently made in *ex vivo* cultures. These cultures may be biased towards a specific phenotype due to the selection of proliferative cells and the plasticity of cells responding to the artificial culture environment. More recently scRNAseq have increased our understanding of the cellular heterogeneity in the native lung by allowing analysis of uncultured cell mixtures in a more unbiased manner. Based on the generated data, multiple novel mesenchymal subsets have been proposed and established cell types have been confirmed or redefined. Some of the reported lung fibroblast populations will be described here, with a focus on the most frequently and well-documented phenotypes. Lung fibroblasts can largely be divided into two groups based on the localization of the interstitium they occupy: fibroblasts in the alveolar interstitium and fibroblasts in the extra-alveolar interstitial spaces, including the peribronchovascular interstitium and interstitium of interlobular septa and visceral pleura (Figure 3).

Fibroblasts in alveolar interstitium

In adult lung, fibroblasts in close proximity to AEC2 are believed to serve as support cells that provide cues that regulate both homeostatic and repair processes[10]. In general, fibroblasts that occupy this space have been referred to as alveolar fibroblasts[6,42], but more specialized alveolar fibroblasts have also been described. Lipid-containing fibroblasts, lipofibroblasts, have been reported in alveoli of rodent lungs and are suggested to support AEC2 surfactant synthesis by transferring neutral lipids, and promote self-renewal and differentiation of AEC2[10,43,44]. However, the existence of a human equivalent has been questioned with limited documentation in human lung tissue[45,46]. Still, the alveolar fibroblast population described by Tsukui et al.[42] and Travaglini et al.[6], express some general lipofibroblast markers, including transcription factor 21, suggesting an overlap between the populations[47]. The mesenchymal alveolar niche cell, identified based on the high expression of platelet-derived growth factor receptor alpha and Axin-2, is another phenotype that has been reported to support alveolar epithelium[48]. Similar to lipofibroblasts, this population

was shown to promote AEC2 self-renewal and differentiation to AEC1 *in vitro*, partly by affecting fibroblast growth factor and bone morphogenetic protein signaling. Interestingly, the gene expression reported by the authors, is also increased in adventitial fibroblasts[6,42], and their spatial distribution was not restricted to the alveoli, suggesting an overlap between this and other fibroblast populations described. Although terminology and reported phenotypes differ, the supportive role of fibroblasts in the alveolar tissue is evident.

The contractile function of myofibroblasts in alveolar septa formation during lung development has been described[49], and the association between a myofibroblast phenotype and pathological remodeling in fibrotic disease is well documented[21]. However, physiological functions of alveolar myofibroblast in adult lung have not been extensively investigated.

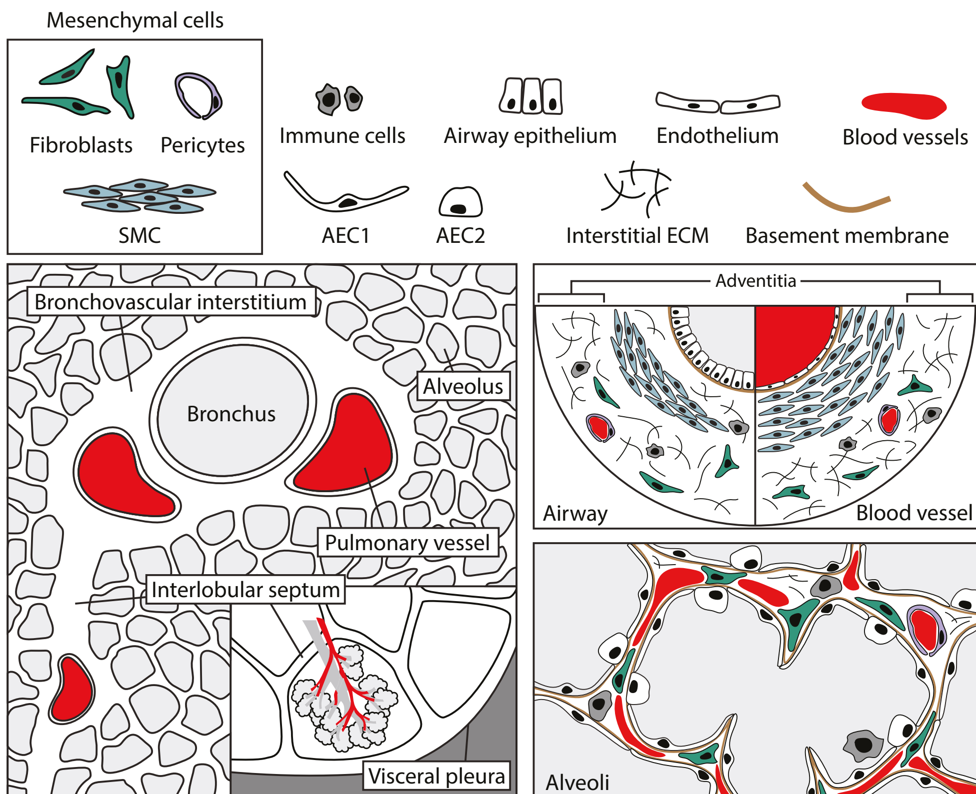

Figure 3. Lung mesenchymal cells populate different structures of the lung. Illustration of different tissue niches in the lung and the cells and structures that occupy them. The peribronchovascular interstitium is continuous with the interstitium of interlobular septa and visceral pleural, forming the extra-alveolar interstitium. The adventitia is the outermost connective tissue layer of larger airways and vessels and is a part of the peribronchovascular interstitium. Mesenchymal cells also populate the alveolar interstitium of the thin alveolar walls. *SMC* smooth muscle cell, *AEC1* alveolar epithelial type 1 cell, *AEC2* alveolar epithelial type 2 cell, *ECM* extracellular matrix.

Fibroblasts in extra-alveolar interstitium

Transcriptomic studies have described a distinct phenotype of fibroblasts that reside within the peribronchovascular interstitium and denoted this population as adventitial fibroblasts based on its localization[6,42]. This is supported by work from Matsushima and coworkers who distinguished a specific fibroblast phenotype associated with collagen fiber-rich connective tissue, as found in tunica adventitia of arteries, interlobular septa and visceral pleura[50].

The fibroblasts in adventitial tissue are reported to display a certain level of heterogeneity with multiple suggested functions, however the extent of mesenchymal heterogeneity is not clear[51]. Within the adventitial tissue their primary function is related to ECM production and mechanical support, however, adventitial fibroblasts have also been described to function as sentinel cells with the ability to sense and respond to various stimuli in their local environment. Interactions between fibroblasts and immune cells are well established and the adventitia is an immunologically active site, with observed immune cell accumulation and the formation of tertiary-lymphoid organs[52]. Furthermore, the peribronchovascular interstitium is enriched in pre-lymphatic and lymphatic structures that drain interstitial fluid from the surrounding alveolar tissue which make this region optimal for immune cells and fibroblasts to respond to a variety of tissue-derived signals[53,54].

Adult lung mesenchymal progenitor cells

The adult lung is believed to harbor mesenchymal progenitors which are proposed to contribute to tissue repair and serve as a reservoirs for different mesenchymal cell types. As with other mesenchymal populations, these have been described by different names and identities in previous literature. Still, MSC persist as a common term and definition of mesenchymal progenitors. Cells that display progenitor properties, including those defined by MSC criteria, have been isolated and studied in lung tissue[55–57]. The prevailing spatial origin documented for lung MSC is the perivascular region, as is the case in most tissues[51,57–61]. So far, the majority of functions ascribed to lung MSC is related to paracrine actions that support other cells and tissues, rather than a direct contribution to regeneration through differentiation. Still, the exact origin and role of these adult mesenchymal progenitors remain poorly defined in native tissue, and it is not known how cells isolated as MSC *in vitro* relate to other mesenchymal cells found in lung.

Lung smooth muscle cells and pericytes

Smooth muscle cells surround airways and blood vessels of different dimensions and represent a set of distinct mesenchymal-derived cells that provide integrity and contractile support to these structures. Airway smooth muscle cells regulate the tone

of the airway, i.e. the dimension of the lumen, and play an important role in airway diseases such as asthma[62]. Similarly, vascular smooth muscle cells contract and relax to control the blood pressure and flow of blood in blood vessels. The smallest blood vessels, the capillaries, are instead wrapped by pericytes that support the endothelial cells through paracrine signals and direct physical contact[63]. With their spindle-shaped morphology, smooth muscle cells can resemble fibroblasts, however they can be distinguished primarily based on their contractile phenotype including increased expression of actin and myosin[64]. The activated myofibroblast on the other hand, that also present a contractile phenotype, remain difficult to discriminate.

Fibroblasts in fibrotic lung disease

Although fibroblast involvement has become increasingly recognized in multiple pathological processes and disease states, fibrosis is the process most commonly associated with fibroblasts. Fibrosis is the pathological result of dysregulated repair processes leading to an excess deposition of ECM components and remodeling of tissue structure.

Activated fibroblasts

In normal wound healing, the activation of a myofibroblast phenotype and associated ECM deposition is transient, ending with apoptosis or de-activation of myofibroblasts and re-establishment of normal functioning tissue[20]. However, in fibrosis, activated fibroblasts persist and continue to proliferate and synthesize ECM leading to an accumulation of deposited ECM[21]. Pathological myofibroblasts are commonly identified based on their increased expression of α-smooth muscle actin (α-SMA) in combination with a high production of ECM, most notably collagen type I. The most prominent drivers of this phenotype *in vitro* and *in vivo* are transforming growth factor-β1 (TGF-β1) and stiffness[65]. However, multiple phenotypes of activated fibroblasts in fibrosis have been reported and the expression of α-SMA appear to be inconsistent[66]. Several potential sources of activated fibroblasts have also been suggested in lung fibrosis, including tissue-resident mesenchymal cells, but also cells of non-mesenchymal origin, such as epithelial cells and bone marrow-derived cells[67]. Among tissue-resident mesenchymal cells, perivascular mesenchymal progenitor cells have been proposed as a source of fibrotic fibroblasts[58,60]. In contrast to these reports, Rock and coworkers described that a heterogeneous population of tissue-resident mesenchymal cells, but not epithelial- and pericyte-derived cells, contributed to myofibroblasts in lung fibrosis[68].

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a fatal lung disease where progressive fibrosis of the lungs destroy the alveolar architecture and stiffens the lung impairing breathing and gas exchange which ultimately leads to respiratory failure[69]. The median survival is only 2-3 years after diagnosis and the median age at the time of diagnosis is 66[70]. It is considered a rare disease, although among the over 200 rare interstitial lung diseases, IPF is the most common, with an incidence between 0.22 and 7.4 cases per 100 000 population annually in Europe[71,72]. The cause of IPF is not known, although there are several identified risk factors including genetic determinants and environmental exposures such as smoking and viral infections[73,74]. The current consensus model of IPF pathobiology suggests that an unspecified repeated injury to the alveolar epithelium leads to dysfunctional repair processes involving epithelial-fibroblast crosstalk that promotes progressive fibrosis. Through the secretion of fibrogenic factors, such as TGF-β1, the malfunctioning alveolar epithelium activates fibroblasts to myofibroblast-like cells that proliferate and synthesize ECM[75]. The changes in tissue composition and mechanical properties further propagate the activation of fibroblasts supporting a positive feedback loop[69]. From a histopathological view, IPF lungs display heterogenous patterns of dense fibrosis with loss of alveolar structures, areas of aberrant re-epithelization including honeycomb cysts, and active fibrotic areas enriched in (myo)fibroblasts referred to as fibroblastic foci. The currently available drugs, nintendanib and pirfenidone, are designed to affect the activity of fibroblasts including their proliferation, myofibroblast differentiation and ECM production[76,77]. In the case of nintendanib, these anti-fibrotic effects are induced through inhibition of the receptor tyrosine kinases platelet-derived growth factor receptor, vascular endothelial growth factor receptor and fibroblast growth factor receptor [77]. However, while these treatments have been shown to slow down decline in lung function, it is not known if they are anti-fibrotic and reduce scarring *in vivo*[78]. Currently, lung transplantation remains the only viable treatment and the closest thing to a cure for advanced stages of IPF. However, lung transplantation has been associated with a poor survival prognosis, although outcomes are improving (median survival of 6.7 years)[79], and together with a shortage of donor lungs, the need for new treatment alternatives is evident.

Aim of thesis

The overall purpose of the thesis was to improve our understanding and characterization of the heterogeneity displayed by mesenchymal cells in the human lung. Specifically, the thesis was centered around the following aims:

Aim I

To investigate the differences between lung fibroblast phenotypes connected to spatial (**paper I**) and functional (**paper II**) heterogeneity

Aim II

To investigate the involvement of distinct fibroblast phenotypes in fibrotic lung disease (**paper III**)

Aim III

To investigate the capacity of cells of non-mesenchymal origin to contribute to extracellular matrix remodeling in lung (**paper IV**)

Methodology

Human lung tissue

In this thesis, studies are performed exclusively on tissues and cells derived from human lungs. The tissue material was mainly derived from explanted lungs of normal donors, in cases when no matching recipient could be identified, and from patients with end-stage chronic lung disease undergoing lung transplantation. As an alternative, animal models represent an important tool to study complex physiological and pathological mechanisms. The dissimilarities between animal and human physiology and the potential inability of animal models to recapitulate human diseases faithfully however, is a problem for the translation to human biology. The use of human tissue therefore constitutes a major strength as the findings are directly translatable. Still, challenges when working with human material include limited availability of tissue and an inability to study biology in a living multi-organ system.

Strategies to isolate fibroblasts

There are different strategies to enrich and isolate fibroblasts for analysis *ex vivo*. The two main methods are the explant method and the tissue dissociation method[80]. Regardless of method, the most common selection criteria used for establishing fibroblast cultures and their identity are plastic adherence and the ability to survive and proliferate in culture. Although specific details of protocols can vary, e.g. depending on tissue type, the general principle for these methods are the same. These will be described below in relation to the protocols used for this thesis.

In the tissue explant method used, tissue material extracted from an organ (e.g. from a biopsy, surgery or autopsy) is dissected into small pieces, which are placed on tissue culture plastic. It is important that the tissue is in contact with the plastic surface so that fibroblasts can migrate out and attach to the plastic. After a few days the first fibroblasts start to emerge, however, the cultures can be maintained for weeks to allow sufficient expansion of fibroblasts and formation of monolayers.

The second common method is the tissue dissociation method that can be used to generate single-cell suspensions from tissue. The tissue dissociation can be

performed enzymatically by adding different enzymes that cleave ECM molecules (e.g. collagenase and hyaluronidase) and adhesion proteins so that cells are detached from the tissue and can be released through the loosened ECM. Prior to enzymatic treatment, the tissue is minced to facilitate penetration of tissue and cell release. Subsequently, these single-cell suspensions can be placed in culture to select for fibroblasts based on plastic adherence and proliferation capacity similarly to what was described for explant cultures. However, another possibility is to perform additional selections of cells based on their immunophenotype, using antibody-based methods including fluorescence-activated cell sorting (FACS), immunomagnetic cell separation and immunodensity cell separation. Based on marker expression, more defined populations can be isolated and the heterogeneity within tissue-derived single-cell suspension can be explored in a more controlled manner.

A benefit with the isolation methods based on selection through plastic adherence and proliferative capacity is the ease of use and minimal hands-on work time. Still, a major drawback is the uncertainty regarding cell heterogeneity, phenotype and origin in the established cultures. This uncertainty is magnified by the influence of culture conditions on cell phenotype and behavior. It is well documented that cells can dramatically change their phenotype when cultured *in vitro*[81–83].

Methods to study heterogeneous cell populations

Flow cytometry and fluorescence-activated cell sorting (FACS)

Flow cytometry is a technique to study optical and fluorescence properties of single cells (or other small particles), based on a combination of fluidics and optics[84]. In multi-parameter flow cytometry, single-cells in a suspension can be immunophenotyped based on their expression of several cell-surface and intracellular antigens. This can be achieved by staining cells with different fluorophore-conjugated antibodies that target antigens of interest and fluorescent dyes (e.g. dyes that bind DNA to enable distinction of dead cells with lost membrane integrity). Stained cells are loaded on a flow cytometer and enter the fluidic system where the sample stream is combined with a sheath fluid stream to form a coaxial flow, with the sample stream as a central core of the stream. Due to the pressure difference between sheath and sample streams, the cells are forced into a single file which enables individual cells to be interrogated in the flow cell. In the flow cell, focused laser beams hit the cells causing light to scatter and fluorescent molecules to be excited. The scattered light reflects different properties of the cells. Forward scatter light is proportional to the cell size and side scatter light is refracted and reflected from the cells, which describe the cells general complexity. The excited

fluorophores and fluorescent dyes emit light at different wavelengths, which pass through a set of mirrors and filter to separate the light from different fluorophores and pass them to detectors.

FACS is a version of flow cytometry where cells can be separated and sorted into different collection tubes or wells in a plate, based on their immunophenotype. The stream leaves the flow cell through a small nozzle that vibrates at a set frequency to generate stable drop formation[85]. The time between interrogation of a cell in the flow cell and droplet formation is carefully determined so that droplets that contain a cell of interest can be charged just before the droplet leaves the stream. The droplet then pass between two deflection plates that generate an electric field that result in an altered trajectory of droplets that are positively or negatively charged, leading them in to different collection vessels. Isolated cell populations can be used for downstream analysis or plated to establish cell cultures.

FACS is the primary method for isolation of cell populations from heterogeneous cell preparations. This is especially true when the target population(s) can only be distinguished based on a combination of several positive and negative markers, as is the case in **paper II**, where the combination of seven markers and dyes are used to isolate mesenchymal subsets. Other antibody-based cell isolation strategies are usually limited to a single parameter, such as immunomagnetic cell separation that is used in **paper IV** to isolate AEC2 using the highly specific marker HT2-280. A limitation with FACS is the increased complexity of the system and analysis, and the time consuming protocol, which can be particularly tedious when rare cell populations are sorted. In addition, some primary cells may be sensitive to the stress from the sorting which may affect their behavior or cause apoptosis. In contrast, immunomagnetic cell separation is a much simpler, cheaper and faster isolation strategy.

Bulk and single-cell RNA-sequencing

RNA-sequencing is based on massively parallel sequencing, which are high-throughput approaches to sequence DNA. In the case of RNA-sequencing, RNA is sequenced indirectly by sequencing the cDNA library of a RNA sample generated by reverse transcription[86]. Prior to cDNA synthesis, ribosomal RNA is commonly excluded through depletion or by enriching for mRNA to achieve an efficient and increased depth of sequencing. For differential expression analysis and in general all analysis of known gene transcripts, short-read RNA-sequencing is the most common method. In **paper II** and **IV**, RNA-sequencing is performed on Illumina systems. In these systems, cDNA is attached to a flow cell and sequenced through the "sequencing by synthesis" approach, where single fluorescently-label nucleotides are incorporated in cycles with imaging occurring at the end of each cycle[87]. After sequencing the computational phase starts. First, the reads are mapped to their corresponding location in an annotated genome (or transcriptome), followed

by assignment of reads to genes/transcripts and finally quantification of their abundance. This is followed by downstream analysis including filtering and normalization, and differential expression analysis. Limitation of RNA-sequencing techniques include sequencing time, cost and data handling. In addition, compared to targeted approaches like qPCR and microarrays, the ability to detect low-expressed transcripts depends on the sequencing depth.

Single-cell RNA-sequencing

The sequencing in scRNAseq experiments, is very similar to standard RNA-sequencing, however, cDNA must first be generated for individual cells. There are different methods to generate single-cell cDNA libraries for sequencing, including limiting cell dilutions and sorting by FACS in multi-well plates and subsequently performing cDNA preparation for each well[88]. A more high-throughput method utilizes microfluidics for droplet-based scRNAseq, which is the method used to generate the datasets analyzed in **paper I** and **II**. In droplet-based scRNAseq, cells are encapsulated with microbeads as an emulsion between a water phase and an oil phase is formed and droplets containing a single cell and a single microbead are generated. The microbeads bear a barcoded oligonucleotide that contain an adapter, a cell barcode sequence, a unique molecular identifier and a primer that initiates reverse transcription of mRNA. The cell is lysed within the droplet and reverse transcription takes place and generates cDNA containing the barcode and unique molecular identifier. The barcode is unique to each cell and will be present in each transcript (cDNA) from that cell. The unique molecular modifier is unique to individual transcripts (cDNA). Due to this barcoding, cDNA from all cells can be combined for sequencing and demultiplexing can be performed at the bioinformatic level to distinguish transcripts corresponding to individual cells. Compared to RNA-sequencing, a challenge with droplet-based scRNAseq is the reduced transcript coverage in single-cells, due to low levels of mRNA capture in the droplets.

Immunohistology and *in situ* hybridization

To study cells *in situ*, in their native tissue, stainings targeting specific antigens and transcripts can be performed by immunohistology and *in situ* hybridization, respectively. These types of stainings allow examination of cells with single-cell and spatial resolution. Dissected tissue material is commonly fixed in formalin and embedded in paraffin to preserve its structure and to enable cutting into thin (e.g. 4 µm) sections that can be stained to visualize tissue features.

In immunohistology, antibodies targeting specific epitopes are used for identification of targets in tissue. To visualize the staining, the primary antibodies are either conjugated to a fluorophore or a secondary fluorophore-conjugated antibody targeting the constant region of the primary antibody is used. Using standard methods, fluorescence-based immunohistology can be used to stain

multiple antigens, but rarely more than 4-5 depending on fluorophores and imaging system.

The targets for *in situ* hybridization are instead nucleic acid sequences, usually RNA transcripts. To visualize a transcript in tissue, a probe that is complementary to the sequence of interest is added to the tissue and hybridizes to the sequence of interest. After the probe has found its counterpart, signal amplification can be performed in different ways and visualized by imaging. In **paper II**, the RNAscope technology is used. The RNAscope probes contain preamplifier binding sites to which an amplifier probe binds, followed by label probes that amplify the signal.

Antibody-based stainings on formalin-fixed tissue can be tricky as the fixation process can change the availability of epitopes. Different pre-treatment alternatives can be used to reveal hidden epitopes, however, finding optimal conditions can be difficult. A benefit of the *in situ* hybridization method, is the reliable chemistry the hybridization is based on, which excludes the need to optimize pre-treatments for each new probe. Another benefit is the possibility to connect expression of extracellular proteins to individual cells by staining for the RNA transcript instead.

CRISPR-Cas9-based gene editing

Gene editing can be used to delete, insert or modify sequences in the DNA of living cells. It represents a useful approach to study the functions of genes in cells and organisms. Gene editing based on the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9) system is currently one of the most powerful and efficient editing tools. The system is composed of a guide RNA and the Cas9 nuclease that together form a ribonucleoprotein complex[89]. The guide RNA contains a sequence that is designed to be complementary to the target of interest and the Cas9 nuclease will then make a double-strand DNA break at the target site. Non-homologous end joining will be performed at the cut through error-prone endogenous DNA repair processes that may result in the addition or deletion of base pairs (indels) that in turn can lead to a frameshift mutation and inactivate the target gene (a strategy used in **paper III**). Through this process knockouts can be generated. With the addition of a DNA template, the system can also be used to change or correct gene sequences or insert new genes.

Mass spectrometry-based proteomics

Proteomics is the study of the proteome, the full set of proteins produced by an organism. In the context of this thesis, the proteome refers to the set of proteins produced by a cell population at a certain timepoint and under the conditions present at that time. Mass spectrometry is an analytical technique that measures the mass-to-charge ration (m/z) of ionized molecules. It can be used to detect the presence and abundance of different types of molecules. In the case of proteomics, the target molecules are peptides and proteins. The method used in this thesis is bottom-up proteomics, in which the existence and quantity of proteins are inferred from peptide fragments[90]. Prior to analysis protein samples are degraded into peptides by enzymes (e.g. trypsin). The peptide samples are first separated by liquid chromatography to allow better coverage in the analysis. After the separation step, the peptides are ionized through electrospray ionization (or other technique), that generates gaseous peptide ions. The m/z of these precursor peptide ions are measured to generate a first mass spectra (MS1). The peptide ions can be further fragmented in a collision cell to generate peptide fragments (product ions) that are analyzed to generate a second mass spectra (MS2). Peptides can be identified by comparing generated spectra with theoretical spectra from *in silico* predictions. The identified peptides are then mapped to the corresponding proteins. There are two methods for the generation of MS2 spectra. In data-dependent acquisition (DDA, used in **paper IV**), the most abundant precursor ions from MS1 spectra are selected for fragmentation. In data-independent acquisition (DIA, used in **paper I** and **II**), all precursor ions within a specified range are selected to generate MS2 spectra[91]. Commonly, multiple windows of overlapping m/z ranges are applied which generates complex mass spectra that requires more sophisticated data analysis. However, the benefit of the DIA approach is the increased depth of the analysis as theoretically, all peptides can be detected and analyzed. In **paper I** and **II**, a label-free quantification is performed. In **paper IV**, stable isotope labeling of amino acids in the cell culture is used to be able to distinguish newly synthesized proteins, labeled with stable isotopes, from proteins derived from the scaffold. The DDA approach is used in this condition as suitable data analysis methods based on DDA are more developed.

Summary of included papers

Paper I

Spatial heterogeneity of fibroblasts in lung tissue

The transcriptomic heterogeneity of lung fibroblasts has been demonstrated in both mice and humans using single-cell transcriptomics[6,42,48,92], and an association between anatomical localization and different phenotypes and functions has been implied. Indeed, previous studies have confirmed that fibroblast cultures derived from proximal and distal airways display different characteristics *in vitro*, including morphological[93], proliferative[34] and gene expression[37] differences. One of the anatomical phenotypes described has been designated adventitial fibroblasts, and represent cells located in the tunica adventitia, the outermost layer of airways and larger blood vessels. This is a immunologically active site and it is reported that specialized adventitial fibroblasts are important regulators of tissue immunity[52]. To better understand the phenotypical differences between anatomically distinct fibroblast subsets, in **paper I** we aimed to investigate the proteome of fibroblasts from proximal airways and distal lung tissue.

Fibroblast cultures were established from dissected bronchi (proximal airway fibroblasts) and parenchymal tissue, taken 2-3 centimeters from the pleura in lower lobes (distal airway fibroblasts), from explanted lungs of normal donors. Using mass spectrometry-based proteomics we could analyze the proteome of fibroblast cultures in an untargeted fashion, identifying and performing relative quantification of thousands of proteins simultaneously. We identified 59 differentially expressed proteins between fibroblast cultures from proximal airways and distal lung, out of which 49 were increased in proximal cultures. Consistent with a connective tissue-producing cell, there was an enrichment of ECM proteins among the proteins increased in proximal cultures, including chains of collagens type I, III , V and VI, as well as ECM glycoproteins fibrillin-1 and fibulin-2. Specifically, several of the identified proteins are associated with collagen- and elastin-based fibrous connective tissue (collagens type I and III, fibrillin-1 and fibulin-2). Non-structural matricellular proteins periostin, osteonectin and thrombospondin-2 were also upregulated in proximal cultures. Adventitial fibroblasts have demonstrated an ability to support tissue-resident lymphocytes by producing factors that promote both survival and chemotaxis in mouse studies. Recently, Gong et al. showed in

mice that adventitial fibroblast promote a immunosuppressive environment through production of prostaglandins that is important for metastasis in the lung[94]. Interestingly, we found that proximal airway fibroblasts had increased expression of enzymes involved in prostaglandin synthesis (prostaglandin G/H synthase 1, prostaglandin I_2 synthase) and an upstream regulator of prostaglandin synthesis (insulin-like growth factor-binding protein 7), suggesting that this may be a conserved feature of adventitial fibroblasts.

To put our *in vitro* proteomic data into a more physiological context we compared the identified proteomes with the transcriptomes of mesenchymal cell populations identified in directly isolated uncultured primary cell suspensions from proximal and distal airways as analyzed by scRNAseq[95]. Analysis of scRNAseq data revealed the distinct difference in distribution of mesenchymal cell phenotypes between the two anatomical regions. While proximal airways were heavily enriched in cells consistent with an adventitial fibroblast phenotype (93% of mesenchymal cells), distal airways displayed a more heterogeneous distribution of mesenchymal cells (30% adventitial fibroblast, 38% alveolar fibroblasts, 12% myofibroblasts and 19% smooth muscle cells and pericytes), which corresponds to the more diverse structural features expected in these samples, including alveoli, vessels and bronchioles.

When we compared the protein expression in proximal cultures with the gene expression in adventitial fibroblast clusters identified in the scRNAseq data, we could observe a large overlap with the gene expression of 22 out of the 49 proximal enriched proteins being upregulated in the adventitial fibroblasts.

One of the proteins upregulated on both RNA (adventitial fibroblast clusters) and protein (proximal airway cultures) level was fibulin-2, an ECM glycoprotein and component of elastic fibers with multiple reported interaction partners, including fibrillin-1 and perlecan[96]. Using immunofluorescence imaging we could confirm the expression of fibulin-2 in adventitial regions with limited expression in surrounding areas. Fibulin-2 could be a useful marker for visualization of adventitial tissue regions and could be used in quantitative image analysis.

The proteomic analysis supported an adventitial origin of the proximal airway cultures and revealed a certain level of conservation of a native phenotype in culture despite subjecting the cells to a stiff plastic substrate. The origin and native phenotype of cultures derived from distal tissue however remain uncertain due to the lack of correlation between upregulated proteins and the gene expression profile in defined fibroblasts populations. Reasons for this could be that the distal culture represents a heterogeneous population of mesenchymal cells, as reflected in the scRNAseq data, or that the *ex vivo* expansion has induced changes to cell phenotype.

The distinction between proximal and distal fibroblast phenotypes could be useful to understand fibrotic diseases that manifest in different regions of the lung. For example, while IPF is associated with the development of peripheral fibrosis, in

asthma and COPD, inflammation and fibrosis around central airways can be observed.

Paper II

CD105$^+$CD90$^+$CD13$^+$ identifies a clonogenic subset of adventitial lung fibroblasts

It is known that the lung possesses a regenerative capacity, a necessity considering the strain and vulnerability posed by the unique interface with the outside environment. While the knowledge of adult progenitors of other cell lineages in lung has increased greatly, we do not know much about the nature of lung mesenchymal progenitors and their involvement in homeostasis and disease.

Fibroblast-like cells that satisfy the MSC criteria and can be isolated as colonies derived from single cells, i.e. clonogenic cells, have been suggested to represent direct *in vitro* progenies of native mesenchymal progenitor populations in multiple tissues[27]. Yet, the native origin and heterogeneity of clonogenic fibroblast-like cells observed in culture remain unclear and the ambiguous MSC phenotype can be demonstrated in most fibroblast-like cultures. Although the clonogenic feature may not determine progenitor status, it implies a high survival and proliferative potential of the origin cell, which are important cellular features in fibroproliferative diseases. Excess fibroblast proliferation is a key component of fibrosis and mesenchymal progenitors that display clonogenic potential have been proposed as a source of fibrosis-mediating fibroblasts in IPF[97]. To fully understand the involvement of lung mesenchymal cells in homeostasis and disease it is crucial to identify and define the mesenchymal populations that are associated with different functional attributes, including high proliferative capacity and survival.

Despite advances in single-cell based technologies and efforts to delineate the cellular landscape in lung, as discussed in **paper I**, a specific subset and identity representing an adult mesenchymal progenitor has not been described. In **paper II,** we aimed to describe the native identity and origin of clonogenic fibroblast-like cells in lung by examining new markers for enrichment of functionally distinct phenotypes.

Originally, we aimed to investigate differences between a proposed lung mesenchymal progenitor cell, defined by MSC characteristics in culture, and the supposedly more differentiated lung fibroblast. As a part of the proteomic investigation performed in **paper I**, we employed the same mass spectrometry-based strategy in **paper II** to compare the proteomes of the two suggested cell types. However, we struggled to pinpoint and describe differences between the two cell

cultures which could sufficiently prove that these were indeed distinct cell populations derived from different cell types in native lung. The cultures were established using two different protocols. The first protocol was a standard protocol commonly used for fibroblast isolation based on tissue explant cultures and a serum containing media. The second protocol was aimed at enriching (or driving) a MSC-like phenotype, using an undisclosed commercial media formulation and selecting for progenies of clonogenic cells after enzymatic degradation of tissue. Both cultures displayed the MSC phenotype, including expression of CD90, CD105 and CD73, and preliminary results (unpublished) also indicated that tri-lineage differentiation could be induced to a similar capacity in both cultures, a notion that has support in the published literature[30]. We also generated data (unpublished) that supported the idea that the observed differences between the described fibroblast and MSC cultures could be driven by culture conditions (media composition). Based on these observations we determined that the *in vitro* cultures were not suitable to study differences between mesenchymal progenitors and other fibroblast-like cells as different origin and identity could not be established. Instead, we aimed to investigate native mesenchymal cell heterogeneity in lung related to a functional trait, the clonogenic capacity. We used the generated proteomic dataset to identify a list of candidate cell-surface markers that could be used to identify and isolate mesenchymal subsets in native uncultured cell suspensions.

In human lung, clonogenic fibroblast-like cells have previously been enriched by isolating a population expressing CD105 and CD90[57]. From the proteomic analysis, candidate markers were selected for which the protein expression was evaluated in native uncultured $CD90^{+}CD105^{+}$ lung mesenchymal cells using flow cytometry. Among the candidate markers aminopeptidase N (also known as CD13) expression distinguished two distinct populations of $CD90^{+}CD105^{+}$ cells. Aminopeptidase N is a metalloprotease with multiple reported functions, including an impact on cell proliferation, but is primarily known for its role as a regulator of peptide activity[98].

To investigate their clonogenic potential, cells from both populations were isolated by FACS and plated at low density. Both $CD13^{-}$ and $CD13^{+}$ derived cells generated colonies, however, there was an increased colony frequency in $CD13^{+}$ derived cultures. In addition, $CD13^{+}$ derived colonies grew larger reflecting an increased proliferative capacity within the population expressing CD13 compared to the population with low or no expression of CD13. This demonstrated a discrepancy between native and *ex vivo* phenotypes as CD13 expression was increased in classical fibroblast cultures compared to the cultures promoting a MSC phenotype. In line with this, we observed that clones derived from $CD13^{-}$ cells upregulated CD13 expression after culture expansion.

To study the populations in greater detail, without exposing them to *in vitro* culture conditions, RNA-sequencing analysis was performed on $CD13^{-}$ and $CD13^{+}$ populations isolated by FACS in combination with analysis of scRNAseq data on mesenchymal enriched lung cells[95]. By comparing the transcriptomes of the isolated

populations with mesenchymal clusters identified in the scRNAseq analysis, we were able to show that clonogenic mesenchymal cells enriched by CD13 were likely to originate from a population of adventitial fibroblasts in human lung. This finding correspond with those of previous studies that have demonstrated an adventitial origin of clonogenic multipotent progenitors in multiple tissues[58,61]. This stands in contrast to reports that have proposed that pericytes are the source of clonogenic cells in multiple tissues[99,100]. While the sources for clonogenic cells in lung is likely to be diverse, and include pericytes, our data suggest that adventitial fibroblasts may be a greater reservoir for highly proliferative clonogenic cells in the lungs.

From the transcriptomic analysis, MFAP5 gene expression was identified as highly specific for the $CD13^+$ population, with no or limited expression in other lung cell populations, including non-mesenchymal cells. The protein product of the MFAP5 gene is microfibrillar-associated glycoprotein 2 (MAGP2), which binds fibrillin-microfibrils, specifically fibrillin-1[101,102]. We could confirm that MFAP5-expressing cells were restricted to loose connective tissue of adventitia and interlobular septa. Similarly in **paper I**, we could show a distinct spatial distribution of fibulin-2, which also binds to fibrillin-1, in adventitial connective tissue. Gene expression of fibulin-2 (FBLN2) and fibrillin-1 (FBN1) were aptly upregulated in the $CD13^+$ population observed in **paper II**. Together, these findings corroborate the adventitial localization of the studied fibroblast population and show that these cells produce a specific ECM profile connected to this tissue region. Of relevance to both regenerative processes and fibrotic disease, both MAGP2 and fibulin-2 can manipulate growth factor signaling by regulating the bioavailability of factors, including TGF-β[103]. MAGP2 can bind directly to TGF-β[103], whereas fibulin-2 competes with TGF-β latent binding proteins for the binding of fibrillin-1[104] which likely contributes to the increased bioavailability TGF-β[105]. Altered matrix production by adventitial fibroblast may affect the local TGF-β signaling and influence the outcome of regenerative and repair process, possibly contributing to the progressive nature of some fibrotic diseases.

These results open the possibility that fibroblasts may functionally be capable of filling the role as mesenchymal progenitors *in vivo* and it may be the adventitial microenvironment that provide the specific cues that support these functions. In line with this theory, Buechler et al. showed that adventitial fibroblasts, sharing a similar phenotype with the $CD13^+$ cells, represent a universal population of fibroblasts that is conserved across multiple tissues and speculated that these adventitial fibroblasts could serve as a source of more specialized fibroblasts[106].

Paper III

Dipeptidyl peptidase 4 expression is not associated with an activated fibroblast phenotype in idiopathic pulmonary fibrosis

There have been several attempts at identifying a cellular source of activated fibroblasts in fibrosis. In some studies, it has been hypothesized that distinct mesenchymal lineages constitute the source of expanded pathological fibroblasts observed in fibrotic tissues[58,60]. Other reports suggest that the cellular origin is not limited to distinct cell lineages, but rather can be derived from multiple mesenchymal and non-mesenchymal sources depending on context[68,92,107,108]. Nevertheless, identification of markers that describe activated fibroblast phenotypes in different fibrotic conditions is important to understand disease heterogeneity and identify targets for anti-fibrotic therapies.

In the screening performed to detect phenotypic heterogeneity using a selection of cell-surface markers in **paper II**, we observed that a small subset of $CD90^+CD105^+$ cells (denoted THY1 and ENG in **paper III**, respectively) expressed the protein dipeptidyl peptidase 4 (also known as CD26, denoted DPP4 in **paper III**). CD26 is a type II transmembrane protein that possess serine protease activity[109,110]. It is well studied for its involvement in glucose regulation and diabetes due to its ability to inactivate incretins, however, it also has other substrates including cytokines and chemokines[111–114]. Non-enzymatic functions of CD26 include its role as an antigen for T-cell activation[115] and its interactions with ECM proteins[116,117].

There have been reports that link the expression of CD26 to a profibrotic fibroblast phenotype in skin[118,119]. In addition, in animal models of lung fibrosis CD26 inhibition has demonstrated an ameliorating effect on fibrosis[118,120]. However, the existence of a CD26-expressing fibroblast phenotype in human lung and in IPF have not previously been investigated. In **paper III**, we aimed to further investigate whether we could identify a CD26-expressing fibroblast phenotype in human lung tissue and explore if such a phenotype is involved and activated in IPF.

Based on flow cytometric and immunohistological analysis of cells and tissue from lungs of normal donors and IPF patients, we identified a population of $CD90^+CD26^+$ lung fibroblasts. In connection to our results from **paper II**, we could identify some $CD90^+CD26^+$ fibroblasts in the adventitia surrounding pulmonary vessels in normal lungs. In fact, we have unpublished data that demonstrate that $CD90^+CD26^+$ lung fibroblasts are $CD13^+$, confirming an overlap between the identified fibroblast populations in **paper II** and **III**.

In contrast to our hypothesis, we observed a decreased relative frequency of $CD90^+CD26^+$ cells in IPF lung tissue compared to normal lung tissue based on flow

cytometric measurements. Through immunohistological stainings we could confirm this quantitatively by the reduced percentage of $CD26^{+}$stained area within $CD31^{-}$ $CD90^{+}$ tissue (excluding $CD31^{+}$ endothelial cells), and qualitatively by the absence of $CD90^{+}CD26^{+}$ cells in histopathological features in IPF tissue, including fibroblastic foci and stroma of remodeled epithelium.

In this study, we specifically investigated the presence of the $CD90^{+}CD26^{+}$ phenotype in end-stage IPF, and thus we are unaware of its presence in earlier stages of the disease. The supposed profibrotic phenotype might be present in earlier stages and associated with fibrogenesis. To understand if there is a mechanistic connection between CD26 expression and lung fibroblast activation, we performed functional *in vitro* studies. Through activation of lung fibroblasts from normal and IPF tissue using the profibrotic factor TGF-β1, we observed a decreased expression of CD26 suggesting a loss of the CD26-expressing phenotype in a profibrotic environment, contrary to what has been reported for skin fibroblasts[118]. Additionally, we inactivated the gene coding for CD26 in lung fibroblast cultures using CRISPR-Cas9 and generated functional CD26 knockout cell lines. Knockout of CD26 did not show any effect on the gene expression of markers associated with fibroblast activation (collagen type I, α-SMA and connective tissue growth factor) in TGF-β1 stimulated fibroblasts. Taken together, the *in vitro* data supported the impression that CD26 expression is not connected to an activated fibroblast phenotype in IPF.

Based on immunofluorescence stainings performed in **paper III**, CD90 expression was absent or limited in fibroblastic foci, consistent with previous reports[121]. Yet, the results from **paper II** suggest that the $CD90^{+}$ population contain fibroblast-like cells with high proliferative potential, a common feature of activated fibroblasts. In fact, we could observe **in paper III** that $CD90^{+}$ cells were abundant in other areas of the remodeled IPF tissue, including in areas directly adjacent to fibroblastic foci Interestingly, these adjacent regions have been described to form an active front of fibrosis with fibroblast-like cells expressing markers of active proliferation[122]. As such, the $CD90^{+}CD26^{-}$ population could remain a promising target in future investigations.

Paper IV

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

Fibroblasts are regarded as the main producers and regulators of interstitial ECM in lung, both during homeostasis and in pathological remodeling. Upon tissue injury,

extensive tissue remodeling can occur in the alveolar tissue (as observed in IPF and demonstrated in **paper III**). Lung epithelial cells, including AEC, are also capable of producing ECM proteins, although studies have mainly demonstrated that they can produce components of their own basement membrane, such as collagen type IV[123,124]. The full extent of their capacity to contribute to lung ECM is not known. We hypothesized that AEC contribute to the production of ECM in alveolar remodeling and that their capacity extends beyond replacing their own basement membrane proteins. Studying AEC *in vitro* remains challenging as AEC2 tend to lose their phenotype upon expansion and AEC1 do not easily expand. To investigate the possibility of AEC to contribute to ECM remodeling in **paper IV**, we cultured primary AEC2 in an *in vitro* three-dimensional model based on decellularized human lung tissue to mimic their native environment and promote maintenance of a native AEC2 phenotype. To achieve a thorough characterization of AEC in this novel model, the AEC proteome and transcriptome was analyzed using mass spectrometry and RNA-sequencing, based on expertise acquired from **paper I-II**.

The *in vitro* model was generated from distal human lung tissue which was cut into slices and treated with a series of detergent solutions to remove cells from the tissue (decellularize) and generate an acellular scaffold, a decellularized lung slice (DLS), with preserved ECM structure[125]. Primary AEC2 were isolated from uncultured single-cell suspensions described in **paper II** and **III** using the AEC2 cell-surface marker HT2-280. To simulate and investigate AEC changes in relation to chronic lung disease, three cell groups were included. One group with cells from normal donors, one group with cells from chronic obstructive pulmonary disease patients and one group with normal donor cells treated with the profibrotic factor TGF-β1 from day 7. The cells were seeded in the DLS and maintained there for up to 13 days, during which the cells appeared active and proliferative as judged by metabolic activity measurements and histological evaluation.

To evaluate the model and understand how the transcriptomic profile is affected in AEC cultures, public datasets containing transcriptomes of native AEC2, AEC organoids and native fibroblasts were analyzed. For the fibroblast transcriptomes, data from the $CD90^{+}CD13^{+}$ population generated in **paper II** was used. The overall transcriptomic patterns, reflected in a principal component analysis, separated AEC cultures from native fibroblasts, native AEC2 and AEC organoids. However, the AEC cultures appeared more similar to the native AEC2 and AEC organoids than the native fibroblasts. Although immunohistology stainings indicated that a majority of the cells had lost HT2-280 and pulmonary surfactant C propeptide expression in culture, mass spectrometry and RNA-sequencing analysis indicated that the cells maintained a stable expression of both AEC2 and AEC1 markers throughout the culture period. As the transcriptomic and proteomic analysis was performed on population-level, the evolution and existence of cellular heterogeneity is difficult to discern from this analysis. However, this could suggest that some of the AEC2 differentiate towards a AEC1-like phenotype resulting in a heterogeneous

population of AEC, which would mimicking a physiological response of the AEC2 in the model.

A main finding in the study was the wide range of ECM proteins that AEC seem able to produce. Compared to the gene expression in lung tissue, AEC cultures expressed 124 out of the 177 so called core matrisome proteins (including collagens, glycoproteins and proteoglycans) expressed in lung. This capacity was confirmed on protein level by mass spectrometry analysis that utilized stable isotope labeling of amino acids in the culture media to enable distinction of newly synthesized proteins produced by the AEC and old proteins derived from the DLS. Out of the 123 ECM proteins (matrisome) detected in DLS scaffold, 108 proteins were produced by the AEC. Among the AEC produced proteins were several collagens, including fibrillar collagens type I and III which are associated with an interstitial matrix. In the culture model, the AEC display an altered transcriptomic profile compared to the native AEC in the reference dataset, which could be attributed to an *in vitro* induced phenotype. As such, the extensive list of ECM proteins produced by AEC in this model does not necessarily correspond to a physiological production pattern, however it suggests a great capacity and level of plasticity which could be activated and contribute to pathological tissue remodeling. This plasticity was further highlighted in the group treated with TGF-β1, in which gene and protein expression was drastically altered, including upregulation of several ECM and ECM-related proteins, many of which have previously been linked to fibrosis and activated fibroblasts. Although the extent of epithelial-mesenchymal transition contribution in lung fibrosis and IPF is uncertain, signs of mesenchymal transition could be observed in the gene signatures of TGF-β1 treated AEC, indicating the potential induction of a mesenchymal phenotype in this model.

There is an obvious focus on fibroblast contribution to fibrotic remodeling in the literature due to the their well-documented capacity to produce and modulate ECM. However, in light of the results presented in **paper IV**, it would be appropriate to consider the potential contribution of AEC to ECM deposition and remodeling when trying to understand the development of fibrosis in future research.

General discussion

This thesis has focused on deciphering the heterogeneity of mesenchymal cells in the human lung to better understand their role in homeostatic and pathophysiological conditions. The papers included in the thesis describe a cell landscape that appears more complex and fluid than what classical cell definitions have inferred.

Techniques with single-cell resolution, including flow cytometry, scRNAseq and histology, together with distinct anatomical tissue sampling (proximal vs distal airways) were applied to study cellular heterogeneity. Through these approaches we could confirm the presence of different mesenchymal phenotypes within the human lung and further expand our knowledge by providing new proteomic characterization related to spatially distinct fibroblast phenotypes (**paper I**) and connect a functional attribute (colony formation/proliferation) to a transcriptomic and spatial identity in native lung tissue (**paper II**). In addition, the connection between a potential fibrosis-associated fibroblast phenotype and IPF was investigated (**paper III**). Finally, we applied our analytical toolkit to characterize non-fibroblastic cells, AEC, with a focus on their contribution to ECM remodeling, and revealed an overlooked complexity in their matrix producing capacity that include interstitial matrix proteins commonly associated with mesenchymal cells.

In **paper I-III** we describe human fibroblastic phenotypes associated with the lung adventitia and peribronchovascular interstitium that show a considerable overlap, most notably based on proteomic and transcriptomic characterizations in **paper I** and **II**. Multiple features with a documented associated to this anatomical region were observed, including an increased expression of several fibrous ECM proteins and an increased prevalence of clonogenic/proliferative cells. Of interest, we could describe several features that may be connected to an overall regulatory fibroblast phenotype and niche in the peribronchovascular interstitium, hinting to potential functions of these elusive cells in lung tissue. Some of the proposed mechanisms by which the peribronchovascular phenotypes could support a regulatory niche include prostaglandin synthesis, peptide-modulating surface enzymes (CD13 and CD26) and the production of a ECM environment that can regulate local signaling.

Markers and traits used to define cell types are not always static as exemplified by the discrepancy between native and *ex vivo* cell characteristics observed in **paper II-IV**, including changes in protein and gene expression. It is tempting to use the

immunophenotype of a cell to predict its origin and fate based on independent observations. However, one should be careful when associating expression of single markers with a specific cell lineage. As observed in **paper II** and **III**, fibroblasts display a large level of plasticity. The fact that *in vitro* culture conditions, including a stiff two-dimensional plastic surface, can have a large effect on cell phenotype and behavior is established, still, the use of cell markers originally defined in *in vitro* cultures, is broadly used. The presented findings highlight the benefit and importance of studying native tissue and uncultured cells, and the necessity of understanding non-physiological effects of *ex vivo* culture systems.

The adventitial fibroblast subset described in **paper II** show a high proliferative capacity and expansion potential *in vitro*. Considering the lack of specific selection in common fibroblast isolation protocols which are mainly based on the capacity of cells to proliferate on plastic, it could be speculated whether cultures established from lung tissue are biased towards a adventitial fibroblast phenotype. However, as described in **paper I**, we were able to observe some preservation of native phenotypes in of fibroblast cultures from distinct anatomical regions, which could speak against this idea.

The term MSC have been associated with several regenerative and stem cell related functions, mainly based on observations in bone marrow. A potential problem in the MSC field are the claims that are formed regarding cellular function *in vivo* in different organs, which are based solely on the fact that a cell *in vitro* can fulfill the seemingly vague criteria of MSC. Measuring colony forming capacity remains a well-used *in vitro* surrogate marker of self-renewal, originally based on a concept derived from the hematopoietic stem cell field. But without further rigorous experimentation, including investigation *in vivo*, care should be taken to infer self-renewal capacity solely based on this read-out. In the context of cell-based therapeutics and tissue engineering, the origin and *in vivo* properties of a cell may be less important, as the cell constitute a product for treatment which can be manipulated in multiple ways as long as a specific function is achieved. However, to understand cellular function in homeostatic and disease process, the inconsistencies between observations made *in vitro* an *in vivo* are crucial to understand.

The lung mesenchymal field is still developing and it will be important to clarify the physiological relevance of cell identities described. Hopefully, the information can contribute to reduce the inconsistency and confusion regarding different terminology and mesenchymal heterogeneity in the lung and take the field one step closer to a consensus of mesenchymal phenotypes and terminology.

Future perspectives

In relation to **paper I** and **II**, it would be interesting to further study functional differences between adventitial and parenchymal fibroblasts. Based on the proteomic and transcriptomic analysis, appropriate markers could be selected to directly isolate fibroblast phenotypes associated with different localization. The generated data analysis could be used as a starting point to understand which functional differences there may be. For instance, increased expression of proteins involved in prostaglandin synthesis could suggest a immunoregulatory niche supported by the adventitial fibroblasts[94]. In relation to this it would be of interest to investigate mechanisms of fibroblast-immune crosstalk which might be relevant for cancer metastasis, asthma and COPD, diseases that partially manifest close to airways and vessels. In this context it would also be warranted to investigate the impact of the spatially distinct ECM produced by fibroblasts on the bioavailability of different signaling molecules.

From the study presented in **paper III**, we concluded that the $CD90^{+}CD26^{+}$ fibroblast phenotype was not increased (and seemingly absent) in end-stage IPF lungs. Studies in animal models of lung fibrosis have shown that CD26 inhibition can have a therapeutic effect[118,120]. It is possible that the observed effect is not directly anti-fibrotic and perhaps due to inhibition of CD26 on other cell types. It is also possible that the observed difference is due to an inability of the models to faithfully imitate IPF progression and pathology. The $CD26^{+}$ fibroblast phenotype could possibly be involved in earlier stages of IPF but disappear in end-stage IPF. It would be of interest to study tissue from earlier stages of the disease, if attainable. It would also be relevant to compare the involvement of $CD26^{+}$ fibroblasts in other fibrotic lung diseases, such as systemic sclerosis-related lung fibrosis, to understand if the reported phenotype is specific to a disease or to an organ.

Taken together, the results presented in this thesis describe a phenotypic heterogeneity among lung cells that appear to be intricately connected to their local environment, highlighting the importance of the extracellular context for cell function.

Populärvetenskaplig sammanfattning

Lungan utgörs av en heterogen blandning celler, var och en med specialiserade funktioner som tillsammans möjliggör det livsnödvändiga gasutbytet mellan blod och luften vi andas. Två huvudsakliga typer av strukturer i lungan är luftvägarna och blodkärlen. Luftvägarna delar sig i ett trädliknande mönster och mynnar ut i miljontals små luftblåsor, alveolerna, där själva gasutbytet sker. Luftträdet och alveolerna är omgivna av ett stort antal blodkärl av olika storlek, där de minsta täcker alveolerna. Dessa strukturer är omgivna och inbäddade in en komplex bindväv som ger både strukturella och icke-strukturella egenskaper. Olika områden i lungan innehåller olika kompositioner av bindvävsceller och bindvävsmolekyler som skapar förutsättningarna för de specifika egenskaperna som associeras med de olika områdena och strukturerna. Celler i bindväven producerar strukturella bindvävsmolekyler men även andra molekyler som deltar i cellsignalering, vilka tillsammans utgör den lokala mikromiljön. Genom denna mikromiljö kan celler kommunicera med varandra och påverka varandras beteende. Vid fibrotisk lungsjukdom bidrar bindvävsceller till att förändra denna miljö och producerar stora mängder bindvävsmolekyler som leder till ärrbildning och förändring av lungstrukturer. Jämfört med andra celler i lungan är bindvävsceller inte lika väl beskrivna, men det verkar finnas en stor heterogenitet med olika cell populationer som tros ha specialiserade funktioner.

Målet med den här avhandlingen är att studera dessa bindvävsceller i lungan för att förstå vilka olika typer som finns och vad de kan ha för funktion i frisk men även sjuk lungvävnad.

Studierna i denna avhandling baseras på experimentella försök gjorda på primära celler från human lungvävnad. För att kunna studera dessa celler på ett omfattande vis användes bland annat masspektrometri för att kunna mäta en stor mängd proteiner (tusentals) i enskilda prover, samt sekvenseringsteknik för att kartlägga genuttryck hos cellerna. Dessa tekniker kombinerades med andra metoder som möjliggör urskiljandet av protein- och genuttryck i individuella celler, för att kunna studera heterogenitet och skillnader mellan olika bindvävsceller.

Analyserna bekräftade att det finns olika bindvävsceller i lungan som uppvisar skillnader i proteinproduktion och genuttryck. En tydlig trend som observerades var att bindvävsceller som ligger kring större luftvägar och kärl uppvisar distinkta uttrycksmönster. Bland annat uttryckte de olika proteiner som kan reglera

funktioner hos celler i deras omgivning och på så sätt forma en specifik mikromiljö. Bindvävsceller i dessa områden visade även en stor förmåga att dela sig och expandera när de sattes i odling på en plastyta, en egenskap som har associerats med självförnyelse och stamcellsegenskaper, men denna koppling är omtvistad.

I dessa områden identifierades en mindre population av bindvävsceller som uttryckte proteinet dipeptidyl peptidas 4, ett protein som har kopplats till patologiska bindvävsceller i fibrotisk sjukdom. Förekomsten av dessa celler undersöktes därför i lungvävnad från patienter med idiopatisk (utan känd orsak) lungfibros. Till skillnad från vad som har rapporterats för andra fibrotiska sjukdomar, hittade vi inga tecken på att bindvävsceller som uttrycker detta protein är delaktiga i idiopatisk lungfibros. Detta tyder på olikheter mellan fibrotiska sjukdomar och att utvecklandet av mer specifika terapeutiska strategier kan behövas.

Slutligen undersökte vi även celler från alveolerna, alveolära epitelceller, som inte är bindvävsceller för att se i vilken utsträckning även dessa cellerna skulle kunna bidra med produktion av bindvävsmolekyler vid sjukdom. Analysen visade att de alveolära epitelcellerna hade en betydligt större kapacitet att bilda dessa bindvävsmolekyler jämfört med vad man tidigare trott, vilket kan tyda på att deras roll i lungsjukdom kan behöva omvärderas.

Studierna som presenteras i denna avhandling visar på en stor diversitet bland lungceller som verkar vara delvis kopplad till deras lokala miljö. Informationen som har genererats skulle kunna bidra till att bättre förstå funktionerna hos bindvävsceller samt hur olika celler bidrar till lungsjukdom och vilka markörer som utmärker patologiska celler.

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