LUND UNIVERSITY

The Human Lung Epithelium in Health and Disease

Wijk, Sofia

2022

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Wijk, S. (2022). *The Human Lung Epithelium in Health and Disease.* [Doctoral Thesis (compilation), Department of Laboratory Medicine]. Lund University, Faculty of Medicine.

Total number of authors:
1

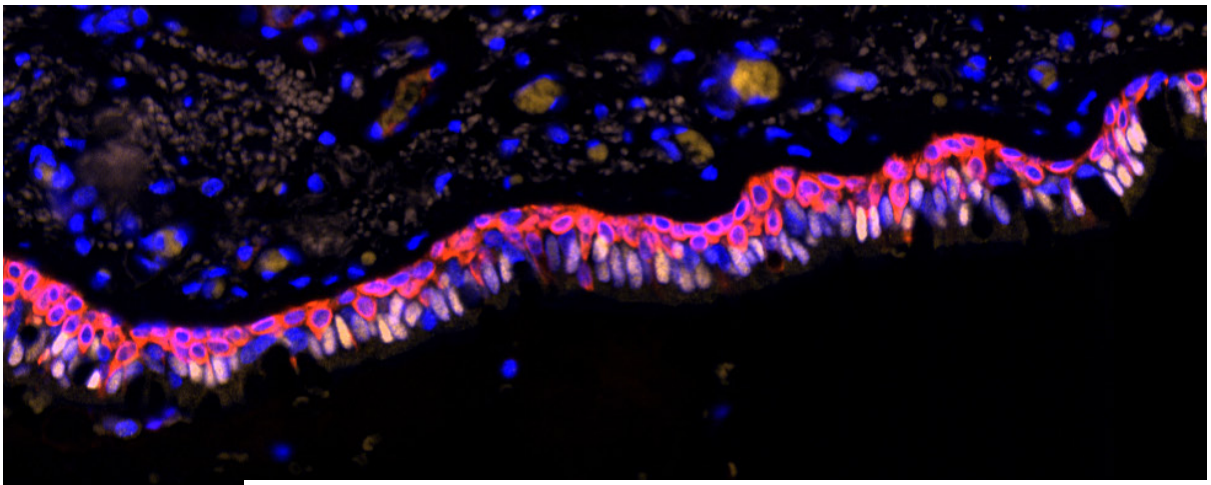
General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

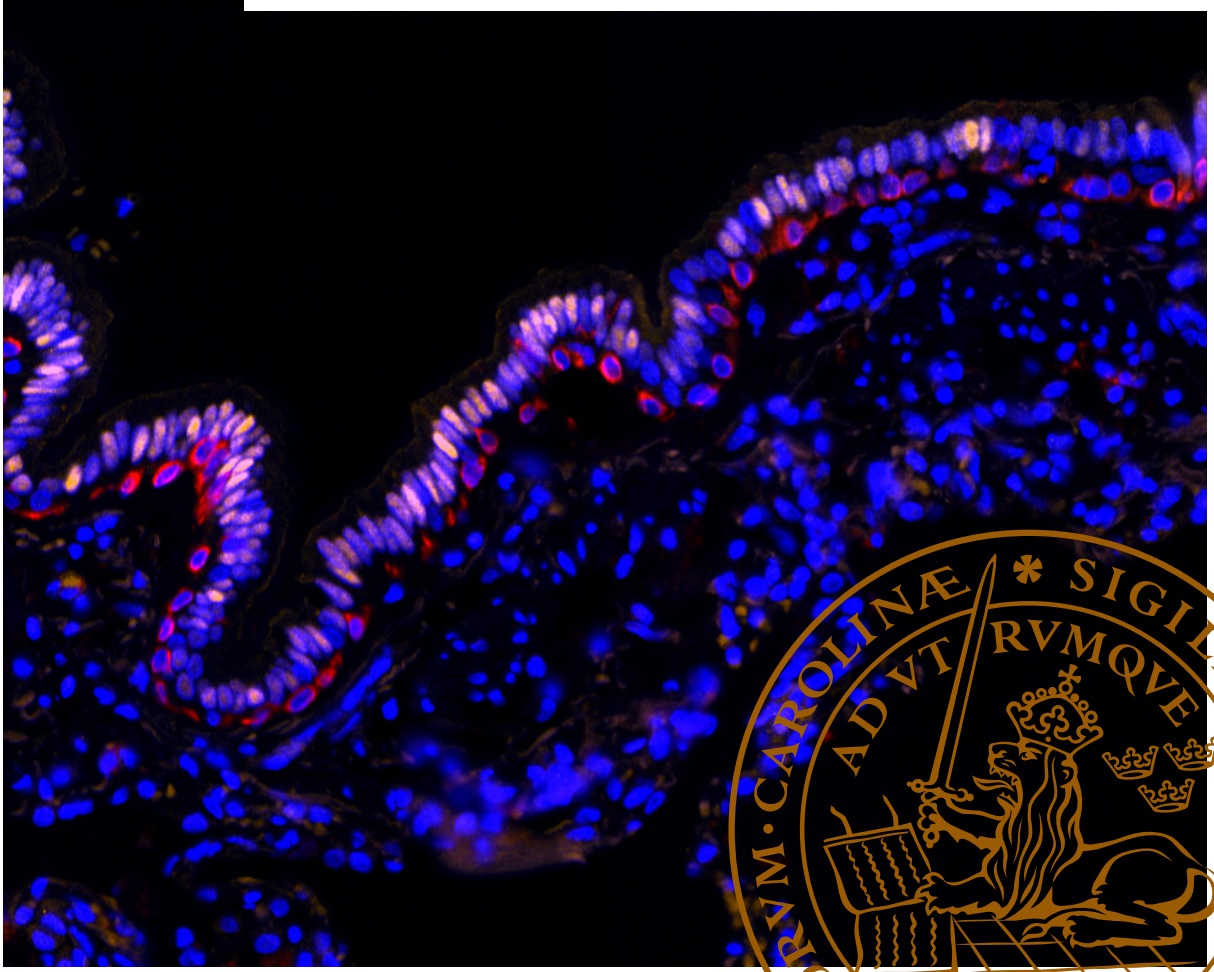
LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



The Human Lung Airway Epithelium in Health and Disease

SOFIA C. WIJK
DEPARTMENT OF LABORATORY MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY



Printed by Media-Tryck, Lund 2022 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Division of Molecular Medicine and Gene Therapy
Department of Laboratory Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2022:106
ISBN 978-91-8021-267-0
ISSN 1652-8220

The Human Lung Airway Epithelium in Health and Disease

The Human Lung Airway Epithelium in Health and Disease

Working toward developing stem cell-based therapy

Sofia C. Wijk

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, BMC A10 in Lund, September 8th at 13:00.

Faculty opponent
Professor Chris Ward
Faculty of Medical Sciences
Newcastle University, UK

Organization	**Document name**
LUND UNIVERSITY | Faculty of Medicine Department of Laboratory Medicine Division of Molecular Medicine and Gene Therapy Lund Stem Cell Center	Doctoral Dissertation
	Date of issue 2022-09-08
Author: Sofia C. Wijk	Sponsoring organization

Title and subtitle The Human Lung Epithelium in Health and Disease – Working toward developing stem cell-based therapy

Abstract

Chronic lung diseases such as COPD and IPF constitute a large burden to society, and currently the only effective treatment for many patients is lung transplantation. Therefore, it is vital to find alternative strategies; the activation of endogenous stem cells or transplantation of new stem cells to repair the damaged tissue offers a novel and promising option. The proximal epithelial layer of the airways is the first tissue to be affected in most diseased lungs, yet the mechanisms behind epithelial remodeling and dysregulated repair of COPD and IPF are not completely known. Elucidating these mechanisms, as well as the signals and cells involved in healthy regeneration, will provide a base for future development of stem cell-based therapy for chronic lung disease.

Basal cells have the capacity to regenerate the epithelium in healthy lungs. Therefore, in **paper I**, we set out to characterize the gene expression of primary human basal cells, and to compare basal cells from healthy and GOLD stage IV COPD patient tissue using single-cell RNA sequencing. We observed a molecular heterogeneity among primary basal cells that was not retained in cultured cells, showing the effects of *in vitro* assays on cellular behavior. Furthermore, we identified upregulated genes and pathways in basal cells from COPD patients that provide future research avenues as potential therapeutic targets. Promisingly, the COPD samples contained a few basal cells that retained a healthy gene expression profile, possibly allowing for induction of endogenous regeneration within the diseased airways as part of future treatment.

In **paper II**, we performed single-cell RNA sequencing on healthy and IPF tissue samples, and identified striking differences in gene expression between their respective ciliated cells. Of note, IPF ciliated cells exhibited downregulation of FTL, a subunit of Ferritin which is responsible for cellular iron metabolism and storage. Since iron accumulation negatively affects IPF pathology, this gene could be a future therapeutic target.

In **paper III,** the aim was to evaluate the effects of aging in healthy lungs, to understand the decline in lung function and regenerative capacity that is observed in older individuals and which processes that may be causing increased risk for developing chronic lung diseases in old age. We therefore performed single-cell RNA sequencing on healthy tissue from 21-40 and 64-75 year-old individuals, and identified several pathways that were upregulated in aged epithelial progenitor and differentiated cells.

Finally, in **paper IV** we report results from single-cell RNA sequencing on squamous cell carcinoma tumor cells. Interestingly, the tumor cells expressed KRT5, and cycling markers such as MKI67. This potentially confirms our hypothesis that dysfunctional basal cells are the origin of tumor formation, and should be targeted as part of improved treatment.

In summary, the vision of finding the right cell types, pathways and genes to target in order to specifically and efficiently treat patients with chronic lung diseases permeates this thesis and the conclusions drawn in each paper provides new insights as well as a basis for further evaluation and functional verification.

Key words: Basal cells, Regeneration, Human, Single-cell, scRNA-seq, COPD, IPF, Stem Cell Therapy

Classification system and/or index terms (if any) N/A

Supplementary bibliographical information N/A		**Language** English
ISSN 1652-8220		**ISBN** 978-91-8021-267-0
Recipient's notes N/A	**Number of pages:** 91	Price N/A
	Security classification N/A	

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature  Date 2022-08-02

The Human Lung Airway Epithelium in Health and Disease

Working toward developing stem cell-based therapy

Sofia C. Wijk

LUND
UNIVERSITY

Cover photo by Sofia C. Wijk

Illustrations by Emil Åberg

Copyright pp 1-91 Sofia C. Wijk

Paper 1 © American Journal of Respiratory Cell and Molecular Biology (2021)

Paper 2 © Cells (2022)

Paper 3 © by the Authors (Manuscript unpublished)

Paper 4 © by the Authors (Manuscript unpublished)

Faculty of Medicine
Department of Laboratory Medicine
Division of Molecular Medicine and Gene Therapy

ISBN 978-91-8021-267-0
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2022

Media-Tryck is a Nordic Swan Ecolabel certified provider of printed material. Read more about our environmental work at www.mediatryck.lu.se

MADE IN SWEDEN

The presence of those seeking the truth is infinitely to be preferred to the presence of those who think they've found it.
from Monstrous Regiment

It's still magic even if you know how it's done.
from A Hat Full of Sky

– Terry Pratchett

Table of Contents

Original articles and manuscripts

Paper I

Human Primary Airway Basal Cells Display a Continuum of Molecular Phases from Health to Disease in Chronic Obstructive Pulmonary Disease.

Wijk SC, Prabhala P, Michaliková B, Sommarin M, Doyle A, Lang S, Kanzenbach K, Tufvesson E, Lindstedt S, Leigh ND, Karlsson G, Bjermer L, Westergren-Thorsson G, and Magnusson M.

Am J Respir Cell Mol Biol 2021 Jul;65(1):103-113. doi: 10.1165/rcmb.2020-0464OC.

Paper II

Ciliated (FOXJ1+) Cells Display Reduced Ferritin Light Chain in the Airways of Idiopathic Pulmonary Fibrosis Patients.

Wijk SC, Prabhala P, Löfdahl A, Nybom A, Lang S, Brunnström H, Bjermer L, Westergren-Thorsson G and Magnuson M.

Cells 2022 Mar 18;11(6):1031. doi: 10.3390/cells11061031

Paper III

A Single-Cell Atlas of the Human Airway Epithelium Suggesting a New Convergence Point in Basal Cell Differentiation and Identifying Transcriptional Changes in the Aging Lung.

Pavan Prabhala, **Sofia C. Wijk**, Stefan Lang, Karina Kanzenbach, Sandra Lindstedt, Shamit Soneji, Leif Bjermer, Gunilla Westergren-Thorsson and Mattias Magnusson (*manuscript)*

Paper IV

Single-Cell Analysis of Primary Human Squamous Lung Carcinoma Shows High Heterogeneity of Tumor-Associated Epithelial Cells.

Pavan Prabhala, **Sofia C. Wijk**, Stefan Lang, Embla Janson , Jesper Andreasson , Kajsa Paulsson, Gunilla Westergren.Thorsson , Hans Brunnström, Sandra Lindstedt and Mattias Magnusson (*manuscript)*

Selected abbreviations

KRT5	Keratin 5
TP63	Tumor protein 63
BC	Basal cell
AECI	Alveolar epithelial cell type I
AECII	Alveolar epithelial cell type II
PNEC	Pulmonary neuroendocrine cell
MSC	Mesenchymal stem cell
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
iPSC	Induced pluripotent stem cell
COPD	Chronic obstructive pulmonary disorder
IPF	Idiopathic pulmonary fibrosis
SCC	Squamous cell carcinoma
GOLD	Global initiative for chronic obstructive lung disease
FEV_1	Forced expiratory volume in 1 second
FEV_1%pred	FEV_1% predicted
FVC	Forced vital capacity
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
NGFR	Nerve growth factor receptor
PDGFR	Platelet-derived growth factor receptor
FGFR	Fibroblast growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
FACS	Fluorescence activated cell sorting
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
PCR	Polymerase chain reaction
RT qPCR	Real-time quantitative PCR
scRNA-seq	Single-cell RNA sequencing
IHC	Immunohistochemistry
IF	Immunofluorescence

Introduction

As with any tissue in the body, over time lung cells become old and die, and are replaced with new cells to maintain a functional organ. This task is performed by stem cells, a population of usually dormant cells, which after activation have the capacity to differentiate into the cell types that need to be replaced. If there is injury to the lung, such as following a respiratory infection or due to inhalation of harmful particles, the stem cells are able to repair the wound and form new tissue by self-replicating and differentiating into all the required cell types – this is referred to as regeneration.

The airway epithelium is a cellular layer consisting of several different cell types, that lines the airways of the lungs and is in constant contact with inhaled air. It is responsive to the outside environment, and therefore represents the lung tissue most susceptible to be injured by pathogens and other harmful particles present in the air. Many chronic lung diseases are characterized by an initial injury to the epithelium that does not get repaired properly. Instead, something in the regenerative process goes awry, activating detrimental pathways that accumulate and start remodeling the cellular structure. In the end, this adversely affects the function of the whole lung.

The incidence of chronic lung diseases is increasing worldwide due to factors like environmental pollution and an aging global population, yet there are no effective treatments for these diseases apart from lung transplantation. Therefore, it is important to study the function and maintenance of the airway epithelium in particular; we need to understand how the epithelium regenerates itself under normal circumstances in order to elucidate the underlying mechanisms behind chronic lung diseases.

The vision for the future is to treat chronic lung diseases with stem cell-based therapy, and the first step is to identify which stem cells have the capacity to produce complete healthy epithelium, as well as which environmental conditions and signals the cells need for effective regeneration. This thesis aims to investigate how the healthy lung is regenerated and explore whether we can harness this knowledge to treat lung diseases that do not yet have a cure.

Background

The human lung airway tree

The structure and function of the human lung can be compared to a tree in some ways. The first is structural; the stem of the tree is the trachea, which divides into progressively smaller airways (bronchi, then bronchioles, then small airways before ending in alveoli) much like the branches of a tree that end in leaves. The second is functional; arguably the most important function of the lung is the gas exchange of oxygen from the environment with carbon dioxide from the body via the thin cellular barrier between airways and blood vessels. Similarly, the leaves of a tree take up carbon dioxide from the air and subsequently release oxygen – a process which occurs in the alveoli, though in the opposite direction (O_2 is exchanged for CO_2).

The lung is a large, complex organ consisting of more than 40 distinct cell types[1], which can be subdivided into epithelial, endothelial, mesenchymal and immune cell groups[2]. The structural framework of the lung is called the extracellular matrix (ECM), a network of cross-linked proteins and other macromolecules that connects all cells, to provide a foundation for structures such as the airways, and enable signaling pathways between cells[3]. Thousands of blood vessels permeate the lung, allowing oxygen uptake for transport by the cardiovascular system. Cartilage and smooth muscle tissue help to maintain the structural properties of the lung by encircling the bronchi and bronchioles/smaller airways respectively, in order to keep them from collapsing[4].

Breathing is mainly controlled by the diaphragm, a muscle horizontally separating the thoracic and abdominal cavity, which when contracting creates a downward force subsequently allowing the lungs to expand and inhalation occurs due to lowered pressure in the thoracic cavity. When the diaphragm relaxes, the thoracic cavity shrinks and the lungs are compressed to exhale[5].

The structure of the human lung airways is illustrated in Figure 1.

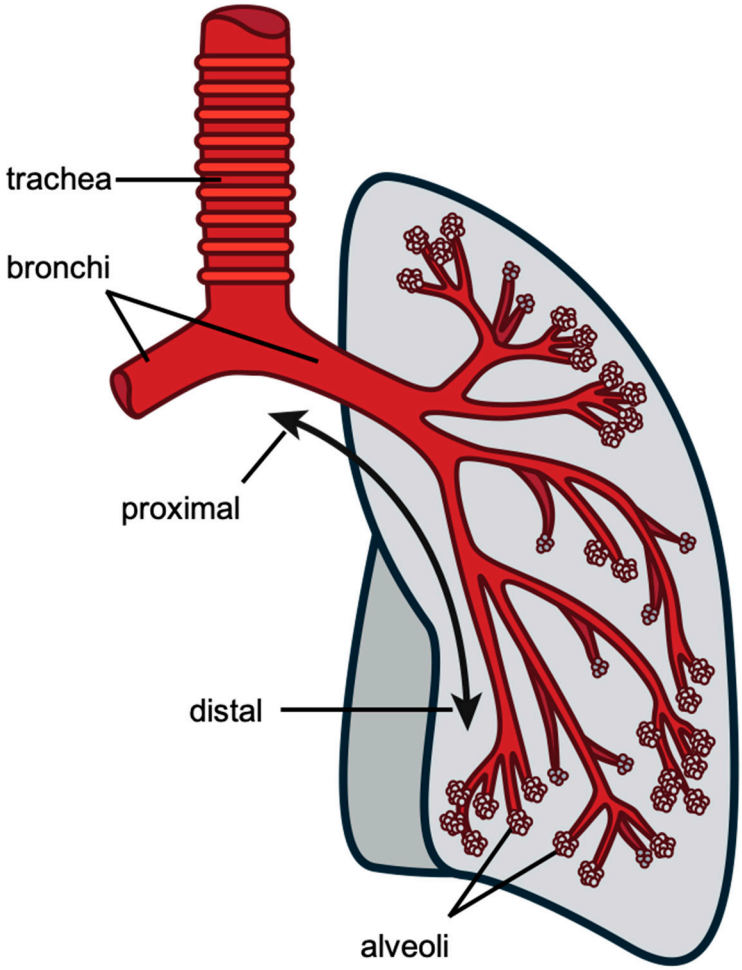


Figure 1. Simplified illustration of the human lung airway structure.
The curved arrow indicates the direction of the proximal-distal axis.

The airway epithelium

The airway epithelium is a cellular layer lining the airways that is in constant contact with inhaled air. It is composed of a large number of different cell types with different functions, all attached to a basement membrane that separates the epithelium from the underlying tissue. The cellular composition, structure and function varies along the "airway tree", thus a directional axis is used to describe the location in relation to the trachea: proximal means close to the trachea and distal means far from the trachea – or close to the alveoli – see Figure 1.

The distal epithelium

The distal epithelium is an important lung compartment for respiratory function, but it is not the main focus of this thesis. Following is therefore a brief overview of the main cellular components.

The distal epithelium resides in the alveoli, where gas exchange occurs. It consists mainly of alveolar cell types I and II (AECI and AECII). AECI cells are long and thin, providing an optimal path for oxygen molecules to diffuse into the blood stream, while AECII cells produce surfactant proteins that regulate the surface tension to avoid collapse of the alveolar space. AECII cells also act as progenitors for AECIs when regeneration in this compartment is required[6]. Figure 2 illustrates the alveolar structure.

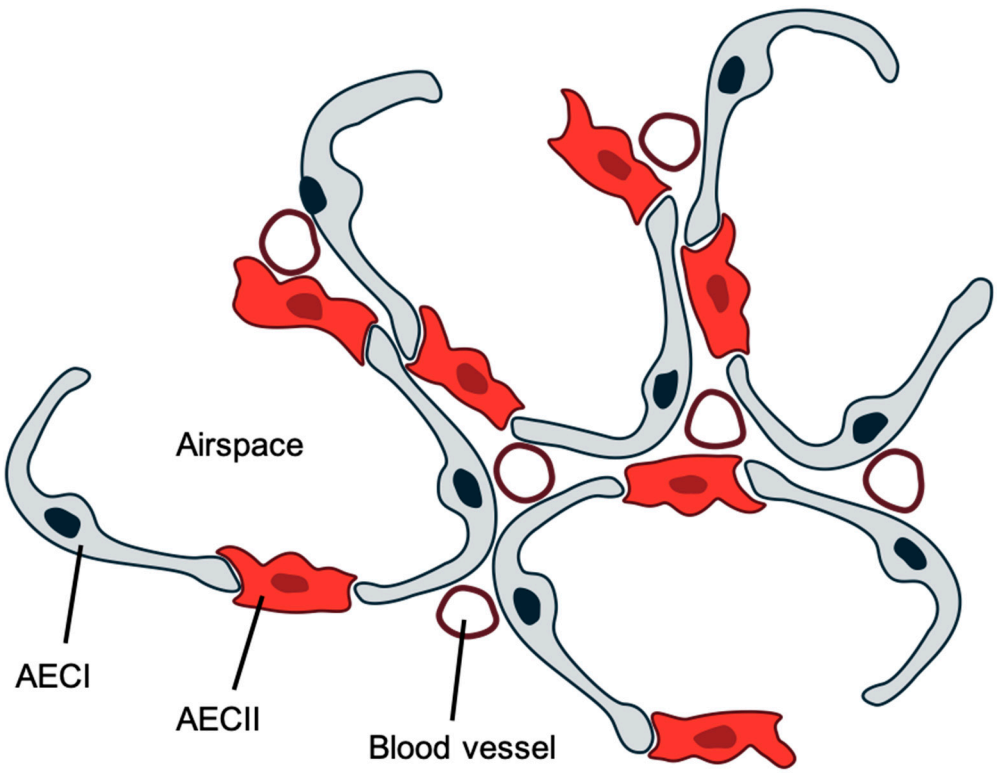


Figure 2. The structure of the alveoli and the main cell types of the distal epithelium.

The proximal epithelium

The exposure to the outside environment requires the epithelium to act as a first line of defense from pathogens and other harmful particles in the inhaled air. The main mechanisms of this defense are carried out by the cells in the proximal epithelium.

The most common cell types in this compartment of the airways are secretory cells, which produce mucus to create a barrier that traps inhaled particles, and ciliated cells, whose cilia generate a constant motion that transports the mucus upwards through the respiratory tract for ejection [7]. Tight junctions between the epithelial cells form an additional protective barrier against outside threats[8], and certain epithelial cells produce anti-microbial peptides and cytokine signaling to attract immune cells if necessary[9].

The most important cell type (for this thesis) in the proximal epithelium is the basal cell (BC), which plays the role of stem cell in this compartment and has the capacity to self-renew and differentiate into the other epithelial cell types for regeneration of new epithelium; both in normal turnover as well as following injury[10]. BCs represent around 6 to 30% of the airway epithelial cells, the number decreasing with the airway size along the proximal-distal axis[11].

A few other rare cell types are dispersed throughout the proximal epithelium; among them intermediate cells, club cells, tuft cells, neuroendocrine cells and ionocytes[12, 13]. Previously, little was known about these rare cell types, and the lack of genetic markers exclusively associated with their identity meant that immunofluorescence or lineage-tracing studies were not able to reliably characterize them. When single-cell RNA sequencing (scRNA-seq) technically improved and became more readily available, these rare cell types could be transcriptionally defined[2].

In this way, ionocytes were recently identified and shown to regulate pH and viscosity of the airway surface liquid (ASL) by regulation of ion transport through CFTR expression[13], though more investigation is needed to determine their role in the airway epithelium. Pulmonary Neuroendocrine Cells (PNECs) sense airway environmental changes such as toxins, allergens, and mechanical stretch, and subsequently release neurotransmitters and neuropeptides. They communicate with other epithelial cells as well as the cerebral[14] and immune[15] systems. Tuft cells (also called brush cells) have a chemosensory function; they respond to the presence of e.g. bacterial peptides and secrete cytokines to activate immune response[16].

The cells of the proximal epithelium form a so-called pseudostratified epithelium; with the ciliated cells, secretory cells and the rare cell types being columnar in shape, reaching from basement membrane to lumen (the airspace within the airways), while the basal cells are cuboidal and not in contact with air. The position of cells within this pseudostratified epithelium can be described as “basal” – closer to the basement membrane – and “apical” – closer to the lumen.

The cell types and structure of the proximal epithelium are illustrated in Figure 3.

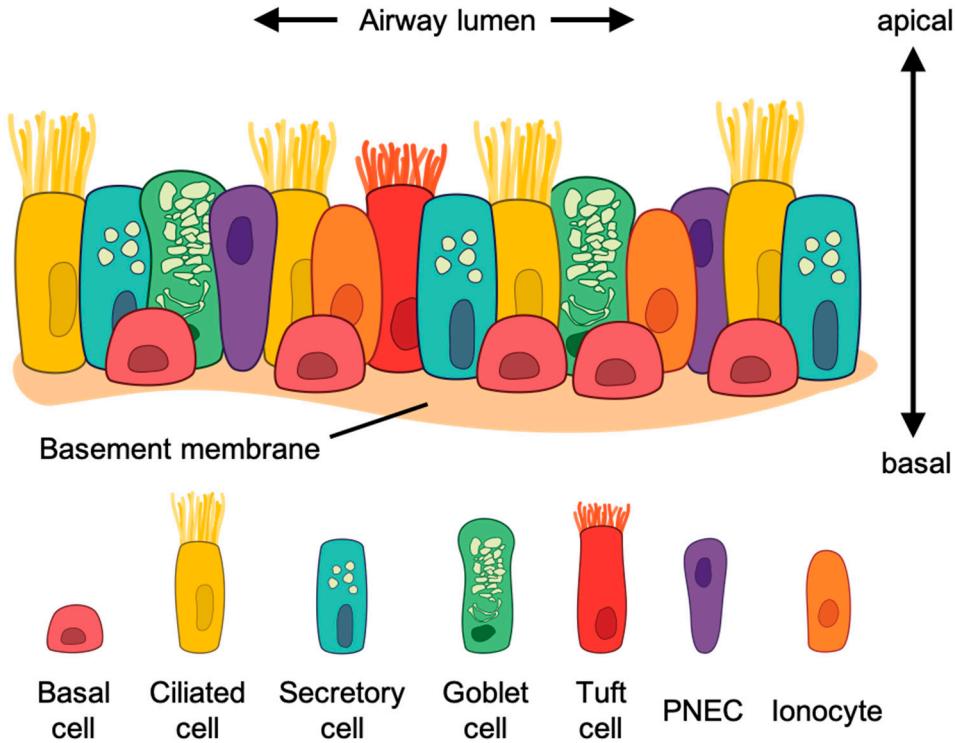


Figure 3. The cell types and structure of the human proximal epithelium.

Basal cells

The basal cell is defined by intracellular TP63 and KRT5 expression, and has long been established as an airway epithelial stem cell, capable of giving rise to differentiated epithelial cells – mainly ciliated and secretory cells both at regular turnover and after injury[17]. Even though many questions still remain on how BCs are regulated, decades of research have identified several important molecular signals determining the fate of BCs. Importantly, FGFR2-mediated SOX2 transcription was shown to maintain BC self-renewal[18], as does WNT signaling through β-catenin which prevents differentiation[19], while LEF-1 maintains BC multipotency[20]. In contrast, NOTCH signaling plays an important role in the differentiation process. Activation of NOTCH2 promotes BC development into secretory cells and inhibits basal-to-ciliated cell differentiation, while inhibition of NOTCH2 and expression of C-MYB gives rise to ciliated cells[21]. In addition, while BCs can promote secretory cell maintenance via NOTCH2 signaling, inhibition of NOTCH2 by the ligands JAGGED1/2 can cause secretory cells to trans-differentiate into ciliated cells[22, 23], while NOTCH3 inhibits secretory cell

differentiation[24]. This illustrates how BCs communicate with their surrounding progenies to maintain epithelial homeostasis, as well as direct wound-healing regeneration; see Figure 4 for a graphical summary.

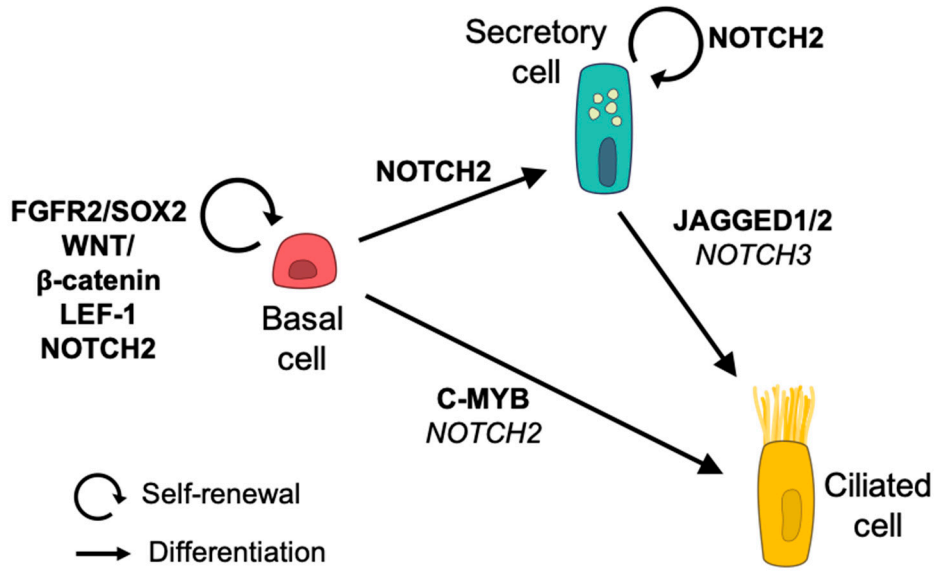


Figure 4. Signaling involved in BC self-renewal and differentiation.
Genes in **bold,** next to arrows, are associated with promoting self-renewal or differentiation processes, while genes in *italics* inhibit these processes.

In recent years, lineage-tracing studies and *in vitro* assays were combined with scRNA-sequencing to show that BCs also give rise to ionocytes and PNECs via a tuft-like intermediary state[13, 25, 26], however further investigation is needed to uncover which signaling mechanisms control the differentiation and maintenance of these cell types.

BCs further interact with their surrounding airway niche cells, such as the mesenchymal compartment of the lung, by activating stromal cells that in turn secrete activation signals to the BCs in a feedback loop. In homeostasis, the epithelium secretes Sonic Hedgehog (SHH) signals that suppress proliferation of adjacent mesenchymal cells, and it has been shown that deletion of SHH in epithelial cells inhibits mesenchymal quiescence which leads to increased proliferative and differentiation activity in epithelial cells[27, 28]. Finally, BCs produce and release IL-33 when stressed, showing that they play a role in recruiting immune cells such as NKT cells and macrophages[29]. Other BC functions include production of laminin for the basal membrane, as well as junctional and adhesive proteins that connect the epithelium to the ECM and protect underlying stromal tissue from inhaled air[10].

The basal cell is proving to be a heterogeneous population, with signs that there may exist subtypes of BCs that have different self-renewal or differentiation capacities. For instance, in areas of active epithelial repair and remodeling, basal cells express KRT14[30], unlike in homeostasis. In addition, Yang et al (2017)[31] detected distinct proximal and distal transcriptomic signatures in BCs taken from proximal and distal airways respectively, indicating that BCs carry out differing functional roles depending on location in order to maintain the specific cellular composition that is required in any specific compartment. Whether this means that distinctly different subtypes of BCs exist, or it merely reflects differing cellular states of the basal cell, is yet unclear.

Moreover, additional scRNA-seq studies have shown instances of varied patterns of gene expression in primary basal cells[24, 32-34]. BCs located more apically in the epithelium, between basement membrane and lumen, were described by Deprez et al (2020)[35] as "suprabasal cells" and characterized by lower expression of TP63 and KRT5 than less mature BCs located basally. Suprabasal cells also expressed the squamous cell marker KRT13, and were shown to be actively cycling leading to a comparison to the mouse $Krt13^{+}$ "hillock BCs" that were identified by Montoro et al(2018) [13].

Subgroups of BCs have been described as "activated BCs, proliferating BCs, secretory primed BCs"[24] and attempts at defining an order of development from inactive through differentiation-primed have been made[34]; yet consensus has not been reached on which newly described basal cell subtypes are valid and how they should be defined. This is largely because of the lack of functional evidence confirming the results from these gene expression studies.

All these epithelial maintenance functions show that BCs are important in maintaining healthy epithelium, yet they are also highly involved in regenerating the epithelium following injury. In disease, BCs are dynamically regulated and pathological BC behavior has been observed as part, and potentially the cause of, several chronic lung diseases. Chronic Obstructive Pulmonary Disease, Idiopathic Pulmonary Fibrosis, Asthma and Squamous Cell Carcinoma all include epithelial remodeling features possibly caused by excessive repair processes. These aberrant processes involve BC and squamous hyperplasia, as well as skewed differentiation towards mucus-producing cells, causing goblet cell hyperplasia[36]. The constant proliferation eventually leads to basal stem cell exhaustion (or metaplasia in cancer), and the subsequent loss in capacity to regenerate the epithelium[37]. Evidence shows that these observations may precede emphysema, leading to the conclusion that accelerated loss of lung function begins with disordered airway BC biology, which thus constitutes a potential target for development of therapies to prevent the progression of disease[38].

In 2009, Rock et al[17] showed BC expression of Nerve Growth Factor Receptor (NGFR), a cell surface marker that can be used in FACS enrichment of basal cells;

which allows for easier purification and selection of basal cells for *in vitro* assays. However, as was explored in **paper I** of this thesis, NGFR does not select exclusively for cells with colony-forming capacity, and investigating the possibility of finding additional markers to purify BCs with stem cell attributes is necessary to facilitate functional study of these cells.

Epithelial regeneration

In homeostasis, *i.e.* the normal, healthy state of lung function, the epithelial cell turnover is very low compared to other epithelial tissues such as gut epithelium[39]. Consequently, epithelial stem and progenitor cells are mostly quiescent until an injury occurs.

However, it is still not completely charted how the human lung epithelium is regenerated and regulated in different conditions; both in homeostasis and in response to acute or chronic injury.

A phenomenon complicating the matter is that the epithelial composition changes gradually along the proximal-distal axis of the human airways[40], with signs that the cell type acting as stem or progenitors may differ accordingly[41]. While the BC has been established as the principal progenitor of secretory and ciliated cells, there have been signs of differentiated cell types also being able to self-renew and even transition into other cell types[39, 42]. However, since many of these assays have been performed under conditions of severe epithelial damage in a mouse lung injury model, or *in vitro* where a small number of progenitors are required to cover a large surface area in epithelium, these instances of epithelial plasticity could also be a process strictly occurring in cases of injury when fast regeneration is needed[43, 44].

Furthermore, the cell number ratio and stem cell function varies between mouse and human lungs[45], making results from experiments on mouse lungs not always applicable to human biology. Most functional studies that have aimed to determine the process of epithelial regeneration have been performed in mouse using *in vivo* lineage tracing; this method is the most appropriate to correctly trace cell origin when studying differentiation, but is not applicable in humans except in *in vitro* models.

Since many chronic lung diseases affect the airway epithelium, it is important to understand how the epithelium regenerates under normal circumstances, as well as to unravel what factors influence the initial spark and development of lung disease over time. In this case, the cells that are responsible for regeneration ostensibly become aberrant and produce dysfunctional tissue. Other disease-promoting factors exhibited by epithelial cells include dysregulation of immune cell recruitment, leading immune cells to over-react and give rise to chronic inflammation which further hinders wound healing[46].

Lung function and aging

Aging is a process that involves changing physiological and molecular properties in the body, slowly leading to decline in organ function. In general, molecular hallmarks of aging include shortening of telomeres, cellular senescence, mitochondrial dysfunction and stem cell exhaustion[47, 48]. In the lung, this increases sensitivity to oxidative stress and other environmental exposures that lead to DNA damage and decreased regeneration, and age is thus one of the largest risk factor in developing chronic lung disease[49]. Since the response to injury is dependent on retained regenerative capacity, insufficient or aberrant wound repair responses such as those occurring in many chronic lung diseases are highly correlated with the cellular and molecular environment in the aging lung.

Stem cell exhaustion has been described in aging lungs, and is believed to be caused by accumulating environmental stress factors as well as epigenetic changes, telomere shortening and mitochondrial dysfunction[49, 50]. In line, it was recently shown that aging results in a reduced number of airways, pointing towards a reduction in regenerative capacity over time[51]. Additional factors that influence stem cell exhaustion are changes in the niche, including ECM interaction as well as signaling from other cells such as fibroblasts and resident immune cells[52]. In the airways, the lack of stem cells – in terms of sheer numbers or progenitor capacity – leads to impaired regeneration and causes both decline in mucociliary clearance and increased epithelial permeability, which in turn increases susceptibility to infection.

Cellular senescence is induced by aging factors such as DNA damage, oxidative stress and mitochondrial dysfunction[53]. A senescent cell exhibits permanent cell-cycle arrest and anti-apoptotic signaling, yet maintains metabolic function with secretion of growth and pro-inflammatory signals. Age-associated deterioration of the immune system leads to impaired clearance of senescent cells in the lung, and accumulation of senescent cells has been strongly linked to chronic lung disease[54]. In addition, age-impaired immune systems are often less responsive to antigens, leading to higher vulnerability to infections such as influenza or COVID-19[55, 56]. In already existing epithelial remodeling, for example in COPD patients, these infections can lead to exacerbations in disease progression. Furthermore, infections may cause the initial injury that induces repeated wound-healing responses which can develop into IPF.

Together, these age-related symptoms of reduced cellular metabolism, accumulation of senescent cells and stem cell exhaustion, form a negative spiral leading to decline in lung function and increased susceptibility to both acute and chronic injury. Unfortunately, as many symptoms of chronic lung disease overlap with regular aging lung physiology[52], errors and delays in diagnosis may postpone crucial treatment. This highlights the importance of studying “normal aging” in the lung, to better differentiate normal progression from processes that

lead to specific disease-related symptoms and pathologies. On a positive note, the existing connections between aging and disease means that efforts to treat and slow down aging may automatically lead to a decline in chronic lung disease development, and vice versa – it is possible that finding new treatments that alleviate or reverse pathologies common in lung disease may also translate to an overall slowing down of lung aging[57].

Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death worldwide, with the majority of deaths occurring in low- and middle-income countries[58]. It has long been thought that cigarette smoking is the primary cause of the disease[59], yet it has since been shown that 25-45% of patients have never smoked. In fact, inhalation of COPD-causing toxic particles are more likely to occur through air pollution and occupational exposure than through smoking, and since almost half the world's population experience these conditions in day-to-day life[60], this may explain the disease occurrence. Importantly, aging is also a large contributing factor for developing COPD[52], greatly increasing incidence even in higher income countries where pollution is not as prevalent but advances in medicine enables the population distribution to grow older[61].

Symptoms and treatment

COPD is defined as a combination of emphysema, airway obstruction and chronic inflammation. It is diagnosed in the clinic through spirometry measurements. The ratio between the volume of air exhaled during the first second (Forced Expiratory Volume during one second – FEV_1) and the total volume of air exhaled (Forced Vital Capacity – FVC) is determined as the patient forcibly exhales as hard as they can manage. If the FEV_1/FVC ratio is lower than 0.70, a COPD diagnosis can be established. The degree of severity is judged based on the FEV_1% predicted value; this is obtained by comparing the patient's FEV_1 value to the mean value from a control group of the same age, weight, height and sex.

GOLD stages I-IV, i.e. mild, moderate, severe and very severe, are classified according to defined cutoff values of FEV_1 % predicted[62], as shown in Table 1.

Table 1. GOLD stage classification and FEV_1% predicted values.

GOLD stage	Degree of severity	FEV_1% predicted
GOLD I	Mild	x ≥80
GOLD II	Moderate	50≤ x <80
GOLD III	Severe	30≤ x <50
GOLD IV	Very Severe	x <30

Treatment typically includes a combination of inhaled bronchodilators, which relax smooth muscle tissue and improves air flow, and corticosteroids, which reduce inflammation[63]. In addition to smoking cessation and reduction of other environmental influences, these treatments can improve quality of life and reduce symptom exacerbations, but will not halt the progression of the disease[64, 65]. In addition, symptoms vary greatly from patient to patient, resulting in treatment response and optimal combination of pharmaceutical administration being difficult to predict[66].

In severe cases, lung transplantation becomes the only option, which may prolong survival, but the procedure is associated with surgery-related risks and complications[67]. The limited access to compatible donor lungs for transplants is an additional obstacle.

Pathology

Several changes in cell behavior and tissue remodeling occur as a consequence of exposure to toxic particles, affecting the various lung compartments differently; all combining to develop into a very heterogeneous disease.

In the alveolar compartment, inflammatory cells respond to inhaled toxins by releasing proteolytic enzymes, breaking down elastins and collagens in the ECM resulting in reduced structural integrity[68]. Furthermore, oxidative stress induces apoptotic signaling causing alveolar cell death through VEGF receptor blockade, adding to alveolar breakdown and loss of function[69].

However, the narrowing and loss of small airways, as a consequence of aberrant and excessive tissue repair, has been shown to precede emphysematous breakdown of the alveoli[70, 71]. This gives an indication that the proximal epithelium is the first compartment to become affected by COPD-initiating dysregulations, and this cell layer should therefore be a priority to investigate in the search for disease-preventing therapeutic targets.

Constant exposure to toxins and stress causes chronic remodeling of both epithelium and smooth muscle; causing the airway walls to thicken. Changes in gene expression

of stem and progenitor populations cause dysregulation of normal cell turnover and differentiation; basal cell hyperplasia is commonly seen in COPD airways. This leads to imbalance of epithelial cell types; goblet cell hyperplasia is common and leads to excess mucus production, and loss of ciliated cells leads to reduced airway clearance and further obstruction[72-74]. Additional effects are squamous metaplasia and loss of tight junctions which weakens the epithelial barrier further. This, in combination with production of pro-inflammatory mediators, causes infiltration of immune cells which adds to airway obstruction[75-78].

In COPD caused by smoking, which has been most extensively studied, there are signs that the bronchial epithelial remodeling is first caused by changes in basal cell behavior[36]. BCs can communicate with the other cells in their environment, as well as the ECM, through production of various growth factors and by expressing receptors on their cell surface[79]. Epithelial-to-Mesenchymal transition (EMT), a feature associated with epithelial wound healing, has been implicated as a COPD-driving process active in smokers, possibly by BCs through TGF-β1/pSMAD pathway[80, 81]. Basal cell hyperplasia is commonly found in COPD epithelial tissue, and it has been shown that smoking causes differential gene expression in BCs[82]. Smoking-related changes include increased immune cell activation through IL-33 and formation of squamous metaplasia EGFR-EGF signaling[83]. Yang et al (2017)[31] found that BCs from small airways in smokers acquired transcriptomic profiles more similar to proximal BCs; losing distally associated genes such as SCGB3A2 and SFTBB an increasingly manifesting EGFR-EGF signaling pathway. This indicates that targeting specific gene pathways in BCs may be key to reverse remodeling of small airways and prevent obstruction.

Risk factors and significance of patient history

As explored, risk factors and causes for COPD are numerous, all contributing in varying ways to the unique remodeling features that each patient can exhibit. Because of this, studies on the mechanisms behind COPD development can be biased by the specific patient cohort that is being studied. In our study (**paper I**), tissue samples were all from individuals living in Sweden, with an environment that differs from, for example, rural India or metropolitan London, UK. Not only do factors such as ambient pollution and open-fire cooking methods play a role[84], but cultural and national differences can influence lung development from a young age such as the amount of time spent outdoors and access to sufficient nutrition[85]; not to mention genetics[86-88]. In addition, individual pre-natal and early life exposure to cigarette smoke, as well as respiratory illness or asthma constitutes risk for sub-normal lung development and a decline in FEV_1 that may persist into adulthood and increase susceptibility to chronic lung disease later in life[89].

This contributes to the complexity of determining what pathological features and mechanistic signals are common to all COPD patients, and what, if any, subgroups

of disease features are exhibited by different patient groups. The fact that COPD diagnosis still only involves spirometry, and the only measured parameter is airflow limitation, is an issue, since this symptom can be caused by a variety of pathogenic features that are not considered. In addition, the rate of lung function decline has been shown to be lower in individuals that had a lower FEV_1 than normal prior to their COPD-related decline[90]. Developing additional tools for diagnosis and phenotyping patients could improve care by allowing for treatment tailored to the specific remodeling mechanisms present in each type of disease pathology[91, 92].

Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is the most common interstitial lung disease, affecting about 50 in 100 000 people[93]. Though much less common than COPD, the disease has a much faster progression of the severity of symptoms resulting in a median survival time of only 2-3 years after diagnosis[94]. As with most other chronic diseases, the highest risk factor for developing IPF is age, with a doubling of incidence every decade after 50 years of age[93]. Other risk factors that have been identified include cigarette smoking and inhalation of other toxins, as well as genetic predisposition[95].

Symptoms and treatment

IPF is characterized by formation of fibrotic scar tissue, due to progressive injury and wound repair in the airways, and excessive deposition of ECM. This results in the lungs becoming stiff and dysfunctional, with patients exhibiting shortness of breath and persistent coughing.

As with COPD, there is no cure available, and patient care focuses on slowing disease progression and preventing exacerbations, as well as improving quality of life through counseling and exercise[96]. There has been some advancement in clinically available pharmaceuticals that reduce fibrosis and inflammation, such as Pirfenidone and Nintedanib. Pirfenidone acts by suppressing TGF-β1 production, which reduces fibroblast proliferation and collagen deposition. In addition, Pirfenidone reduces production of the inflammatory mediator TNF-α[97]. Nintedanib inhibits fibroblast proliferation and migration by blocking platelet-derived, fibroblast and vascular endothelial growth factor receptors PDGFR, FGFR and VEGFR[98]. These medications have been shown to reduce disease progression by up to 50%; however without significantly reducing overall IPF mortality rate[99-101], and unfortunately causing various adverse effects[102].

Lung transplantation remains the option with the best chance of survival, yet this is only possible for a few patients due to the strict selection criteria of both recipient

and lung donor[103]. In addition to the usual risks and complications associated with all transplantation procedures, lung transplantation in IPF patients is especially fraught since patients are generally older and often present with comorbidities[104].

Pathology

As the term idiopathic implies, the mechanisms of disease origin and progression are not fully known. The current agreed upon theory is that genetic predisposition, in combination with a series of micro-injuries to the epithelium, leads to a series of defective alveolar repair processes, epithelial-to-mesenchymal transition, accumulation of fibrotic tissue and deposition of ECM[105]. Histologically, the IPF lung exhibits instances of dense fibrotic tissue such as fibroblastic foci[106], yet also shows bronchiolization and "honeycombing"; cystic spaces with muco-ciliated epithelium[107].

Various genes and cell types have been implicated in this complex process of disease progression. Mutations in an AECII gene, surfactant protein C, have been shown to cause spontaneous fibrosis in mouse lungs[108], and alveolar cell senescence has also been shown to lead to dysfunctional alveolar regeneration and fibrosis[109]. Less distally, the thickening of the small airways and loss of terminal bronchioles has been reported in early disease stages[110]. In addition, IPF patients show overexpression of MUC5B in the bronchioalveolar epithelium, which may lead to excessive mucus production and reduced mucociliary clearance as well as impairment of normal wound healing[111].

The case has also been made that ciliated cells may play a larger role in IPF disease progression than previously thought[112]. The beat movement of cilia becomes important not only for clearance of excess mucus, but also for the possibility of sensing the environment and cell-cell signaling. Cilia have been shown to aid fibroblast migration and promote myofibroblast differentiation through the hedgehog pathway[113].

Recently, several scRNA-seq studies have been performed on lung tissue from IPF patients, which have identified various forms of aberrant BC cell types apparently prevalent in the disease; almost universally found in distal airways and at typical IPF pathological sites with honeycombing, bronchiolization or fibroblastic foci. Xu et al (2016)[114] discovered epithelial cells expressing BC genes like TP63, KRT5 and KRT14, but also a group of "indeterminate" epithelial cells that seemed to express both distal and proximal epithelial cell markers, as well as markers of EMT; suggesting a disease-specific depart from normal epithelial cell identity as well as active pathological change in behavior. Basal-like cells with similar co-expression of epithelial and mesenchymal genes were identified as "basaloid" and "$KRT5^{-}$ /$KRT17^{+}$" respectively[115, 116]. Carraro et al (2020)[24] also described cells they termed "secretory-primed basal (SPB)" in distal IPF lungs, adding to the number of

abnormally behaving epithelial progenitors that have been transcriptionally described; yet the exact contribution and involvement of these epithelial cells in the disease-progressing wound repair process is still undetermined.

A proposed theory for the function of these basaloid cells found in IPF is the capacity to rapidly proliferate, an ability they acquire through initiation of EMT giving them mesenchymal stem cell characteristics. Additionally, they express laminin for ECM deposition through SOX9 activation, which facilitates wound healing and alveolar regeneration[117, 118]. However, basaloid cells from IPF tissue also express senescence genes, possibly indicating an attempt to return to homeostasis[115]. When not properly controlled or disposed of by e.g. macrophages, these senescent BCs accumulate and block wound healing[119].

Taken together, IPF is a disease of airway remodeling that heavily involves airway epithelial cells and BCs in particular. More investigation is needed to understand the interactions between the different disease-promoting processes, and to determine whether BCs can be a therapeutic target in IPF.

The mouse in lung research

The mouse has been studied extensively in biology research, used as a model to understand organs and systems that parallel human biology. Laboratory animal research is useful since it enables the study of developmental biology, and breeding mouse strains with different genetic properties allows for gene knockout studies, lineage tracing, xenogeneic transplants etc.

Both COPD and IPF have been simulated in different mouse models; though due to differences in species biology, this approach may not always generate relevant data.

Differences between mouse and human lungs

The mouse, which is the animal model most often used to study lung development and disease, has proportionately small lungs to its body – the total lung capacity is 6000 times smaller compared to humans[120]. Consequently, many factors vary between the two species; both structurally and in terms of cellular composition. The airways of the mouse have fewer total branches, and cartilage is only present around the upper trachea rather than extending to the bronchi as in humans[121].

The mouse trachea and the first part of the bronchi are quite similar to the human proximal airways; alveolar structure is quite conserved as well. The largest difference resides at the transition between airways and alveoli. In mouse, the distal airways do not contain BCs and instead possess a higher proportion of club cells in the epithelium. The border between mouse distal airways and alveoli is termed

Bronchioalveolar Duct Junction (BADJ), and contains Bronchioalveolar Stem Cells (BASCs) that express secretory cell and AECII markers[122], and were found to give rise to both club cells and AECII cells following injury[123, 124].

In contrast, human distal airways gradually transition into alveoli via "respiratory bronchioles", exhibiting a more cuboidal epithelium where BCs are present, yet in lower numbers than in intermediate airways. Human distal airways do not feature a BADJ, and no cell type identical to the mouse BASC has been identified so far[121]. These differences in airway structure makes studying human distal epithelial regeneration difficult, since mouse models will not behave similarly when replenishing damaged epithelium in these regions. More focus should be put on human-derived *in vitro* models such as organoids and iPSC models of lung development.

Mouse models for lung disease

Mouse and other rodent models of COPD and asthma have been used since the 1990's[125]; yet there are still hurdles to overcome in developing a model that encompasses all the complex, different pathological aspects of the disease[126]. The mouse is the most commonly used model for COPD, mainly because of practicality of handling and availability of reagents such as antibodies[127]. Exposure to cigarette smoke induces COPD-like lesions and emphysema in mice, though limitations of this model include the prolonged exposure needed to induce symptoms and the lack of disease progression after exposure cessation[128]. Other methods to model COPD-like symptoms include administration of proteases such as elastase, in order to break down elastane and induce alveolar breakdown[129], and inducing respiratory viral infections to replicate remodeling due to inflammation[130]. The biological differences between mouse and human make it challenging to model the more severe stages of COPD since mice do not live long enough to develop critical lung function decline[131].

Mouse models for IPF and other fibrotic disorders exist as well; the most common method to induce fibrosis is intranasal/tracheal administration of bleomycin. This causes accumulation of reactive oxygen species, which induces epithelial cell death, inflammation and lastly fibroblast and ECM-mediated fibrosis. Since it is reproducible and in use globally, many comparable studies have been performed in this model[132-134]; however similarly to cigarette smoke-induced COPD, after bleomycin administration mice can recuperate[135]. In addition, the tissue damage occurs locally where the bleomycin landed at injection, often not reaching the distal regions more known to exhibit fibrosis in humans[126].

Other animal models and ethical aspects

Larger animal species have been used to study lung disease due to their closer similarity to humans, with advantages and disadvantages associated with each species following their respective biology. Dogs have larger lungs than mice, as well as a larger mouth which facilitates intratracheal administration, and their natural coughing reflex is relevant when studying COPD. However, species-specific reagents are limited[136]. Pigs have a similar organ-to-body weight ratio to humans as well as a closely resembling lung structure, but have a narrow mouth opening that can hamper experimental procedures[137]. Finally, the lungs of non-human primates like the rhesus macaque are exceptionally similar to humans, down to cell type ratio in the airway epithelium. Being so genetically close to humans this model also allows for the use of human reagents[138]. However, common drawbacks with these larger animal models are the increased costs due to requirements for expertise in handling housing facilities and specialized equipment[131].

There are also ethical implications that need to be considered when using any animal model. The "three R principle" of Replacement, Reduction and Refinement[139] dictates that, whenever possible, alternate methods should be used instead of animal studies. As few animals as possible should be involved, and stress and discomfort due to methodology and procedures should be minimized. In addition, it is vital to make sure the output is maximized in terms of accurate and applicable information gained. Therefore, due to the limitations of modelling and gathering relevant experimental data from these complex diseases in animal models, and to reduce animal suffering, alternate methods should be implemented.

3D organoids and re-cellularized ECM scaffolds derived from patients or iPSCs are a promising possibility to further our understanding of lung disease and test new therapeutic options. Paradoxically however, more knowledge about disease mechanisms and progression is needed to reproduce the appropriate pathologies *in vitro* that can generate relevant conclusions.

Stem cell therapy

Stem cells, due to their ability to self-renew and differentiate into mature cell types with different functions, have long been of interest to medical science as a promising future way of treating various chronic and life-threatening diseases. The most established stem cell therapy currently approved for use in clinics is hematopoietic stem cell therapy (or bone marrow transplantation), which has been used to cure several forms of leukemia and other blood disorders with increasing success rates since the 1970s[140]. In recent years, as stem cells in other tissues have been discovered and their function and characteristics have been determined, the hope to develop cures for other diseases has flourished. Research and clinical trials are

ongoing for neural stem cell therapies targeting neurodegenerative diseases such as Parkinson's and Alzheimer's[141], and using mesenchymal stem cells (MSCs) for wound and bone repair is also being explored[142, 143].

The road from lab to clinic is not without its hurdles. Stem cells, despite their regenerative properties, also carry with them the risk of malfunction such as uncontrolled proliferation and tumor formation, as well as defective differentiation[144]. To develop a clinic-approved stem cell treatment, reliable quality control and robust manufacturing must be in place before even clinical trials can commence, and the requirements for proceeding to the next step is understandably high[145].

However, advancements are reported continuously and the field remains promising. In addition, injection of pluripotent stem cells is only one approach to stem cell-based therapy. Other possibilities include cultivating and directing the differentiation of stem cells *in vitro* to produce complete tissue for application *in vivo*, as well as targeting the patient's own stem cells with known activation/differentiation factors or gene therapy in order to stimulate endogenous regeneration[146].

Stem cell therapy in lung disease

Given the limited effectiveness of current treatments for chronic lung diseases, and the lack of access to lung transplantation for most patients, the need to develop long-term therapies or curative solutions for lung disorders is clear. Once stem/progenitor cells of the epithelium such as BCs and AECII cells were identified, investigations started on whether the regenerative properties of these cells could be harnessed[147].

In mice, transplantation of epithelial progenitor cells has been shown to repopulate damaged epithelium[148, 149]. In various human studies, induced pluripotent stem cells (iPSCs) have been directed to become epithelial progenitors that differentiate into mature epithelial cells *in vitro* [150-152]. These results suggest the possibility of transplanting healthy progenitor cells into patients with acquired diseases such as COPD and IPF, but also allows for possible gene therapy to correct mutation-based disorders such as Cystic Fibrosis (CF). Importantly however, these avenues still need to be thoroughly tested for efficacy and safety *in vitro* and in animal models, before any human trials can be performed[153].

Of note, arguably the most studied, and clinically advanced, stem cell-based approach to treat lung disease is the use of mesenchymal stem/stromal cells (MSCs). These do not differentiate into epithelial or other cell types, but rather act as tissue regeneration support through communication with other cells in their environment; both epithelial and immune cells. In 2013, Weiss et al[154] performed a placebo-controlled, randomized clinical trial on COPD patients who got intravenous

infusions of MSCs, and detected no serious adverse effects or worsening of disease compared to the control group; although no significant improvements were detected either. Likewise, Tzouvelekis (2013)[155] and Chambers (2014)[156] performed phase 1b trials with endobronchial and intravenous MSC injections respectively in patients with mild to moderate IPF, both similarly establishing basic safety of the treatment, though without discernible improvement of symptoms.

More recently, in 2020 Averyanov[157] showed an increase in lung function of IPF patients who got high doses of intravenously injected MSCs, and Acute Respiratory Distress Syndrome (ARDS) seems to be even more responsive to ameliorative treatment with MSCs[158, 159]. Among the more pertinent clinical studies are attempts to use MSCs to treat COVID-19 patients with acute respiratory infections; MSCs derived from umbilical cord[160], menstrual blood[161] and adipose tissue[162] have all been used and proven safe and show slight improvement of symptoms. However, due to the acute nature of the ongoing pandemic, these studies need to be followed up on and improved by including a larger subject group and using more standardized methods[159].

As of July 2022, there are 168 clinical trials registered relating to MSCs and pulmonary disease[163]. However, there are a lot of unknown factors yet to elucidate in order to develop a safe, predictable treatment for lung disorders that can be approved for clinical administration[147]. MSCs have different properties depending on their source of isolation and they react differently in different niche environments[164, 165]; since lung disease pathologies are extremely heterogeneous this makes it difficult to predict and control their effect once administered *in vivo*. In addition, the ECM affects behavior of repopulating cells, as pointed out by Elowson Rendin et al (2021)[166], making the regulation of transplanted MSCs extremely complicated.

Basal cells and stem cell therapy

Basal cells are multipotent progenitors of airway epithelium and hold the promise of stem cell therapy. Various initial studies have been performed in mouse models, to test and optimize BC regeneration. In 2018, Farrow et al[167] tested the effect of epithelial disruption to mouse airways before transplantation of human BCs and found that denudement of the epithelium by polidocanol increased engraftment of the human cells and showed apparent normal epithelium at the end point of the study. This has potential for treating cystic fibrosis in which replacement of CFTR-deficient epithelial cells with gene-edited BCs could vastly improve symptoms.

Ma et al [149] injected *in vitro* expanded human $SOX9^{+}$ ($TP63^{+}$ $KRT5^{+}$) BCs into bleomycin-injured mouse tracheas in 2018. They observed engraftment and some epithelial cell differentiation as well as improved pulmonary function and halting of fibrosis. They followed up on these results by treating two patients with

bronchiectasis with lobar infusion of autologous, *in vitro* expanded $SOX9^+$ BCs, who both reported improvement of symptoms and quality of life. The biggest disadvantage to the study is that unlabeled cells cannot be tracked for confirmation of engraftment and contribution to tissue repair, though the irreversible nature of bronchiectasis led the authors to believe the amelioration of pulmonary function must be due to the transplanted cells. Obviously more studies are needed to confirm safety and reproducibility, as well as long-term follow-up on these two patients.

Ethical considerations

I would be remiss not to bring up the scandal surrounding Paolo Macchiarini and the unethical and unsafe procedures that were performed on several patients with various lung/tracheal disorders. From 2011 to 2014, he performed or was involved in transplants of synthetic tracheas seeded with stem cells from autologous bone marrow in nine patients[168, 169], supposedly due to life-threatening conditions. Though follow-up reports promised good engraftment results and improved quality of life, later complications were revealed such as the synthetic transplants not being coated in functional epithelium and coming loose due to failure to fuse with surrounding tissue. Seven of these patients died soon after procedures, but due to insufficient reporting it is difficult to establish to what extent this was caused by the synthetic tracheas, surgical complications, or other underlying health issues[170]. Several of his papers have since been retracted, either forcibly or by the authors themselves after being called into question. Macchiarini and colleagues at the Karolinska Institute were accused of scientific misconduct, and Macchiarini is as of July 2022 being prosecuted for causing bodily harm[171].

Tracheal reconstruction using different types of bioengineered scaffolds are still being investigated and show promise[172], but the Macchiarini case has highlighted the importance of biocompatibility and pre-clinical safety studies on the materials used, as well as crucial ethical considerations in study design. Furthermore, the scientific community as a whole has been made aware of the value of respecting ethical guidelines and transparent reporting[173].

Other uses for airway stem/progenitor cells

Until stem cell therapy becomes available in the clinic, airway stem/progenitor cells offer useful applications other than direct therapeutic injection. Culturing them *in vitro* together with differentiation-promoting factors enables the formation of 2D/3D airway models and lung organoids, which in turn can be used for pharmaceutical screening, toxicology assays, and studies comparing epithelial tissue derived from healthy versus diseased lungs[174, 175]. An example of *in vitro* airway model use is Air Liquid Interface culture (ALI), as applied by Ji (2018)[176],

who evaluated cellular crosstalk between epithelial and immune cells following exposure to diesel exhaust.

Even more structurally complex models can be explored by seeding progenitor cells on decellularized lung tissue pieces, in order to evaluate the influence of ECM factors on cellular regeneration. This has been done using human bronchial epithelial cells (HBECs) on decellularized scaffolds from COPD donors and healthy controls, which showed an influence of COPD ECM on the ability of COPD HBECs to differentiate[177].

To avoid limitations such as obtaining lung tissue for decellularization, and to increase simplicity and reproducibility, other avenues are being investigated. An example is to use 3D-printing to produce scaffolds made of different combinations of manufactured or ECM-derived gels. These complex structures can later be repopulated with progenitor cells; alternatively cells can be directly printed into the appropriate tissue compartments (referred to as bioprinting)[178]. Methods such as these can be used to investigate cell-ECM interactions and cellular migration during lung development or wound healing, but are also promising targets for *ex vivo* production of transplantable tissue[179].

In brief

There are still many unknowns regarding the function of BCs, their stem cell capacity and interactions with the airway environment, as well as their role in chronic lung disease. The aim of this thesis was therefore to further characterize the primary human airway basal cell in order to further fill in these knowledge gaps, and to examine the possibility of utilizing the BC in stem cell therapy.

Our vision was to approach the airway stem cell field with the same strategy and methods as other research fields involving stem cells and cell-based therapy, setting out to characterize the epithelial stem cell hierarchy by examining the different cell populations at a single cell level.

Aims of thesis

The overall aim of this thesis was to elucidate basal cell identity and behavior in health and disease, to more fully map the normal airway regeneration as well as pin-point the cell(s) of origin in disease-related aberrant repair processes. In connection with this, an additional aim was to identify possible therapeutic targets for chronic lung disease and cancer.

Paper I

- Develop a FACS sorting method to further purify human primary basal cells with colony-forming capacity
- Compare gene expression heterogeneity and colony-forming capacity of primary human basal cells versus cultured basal cells
- Compare gene expression of human basal cells from healthy and COPD tissue

Paper II

- Compare gene expression of airway epithelial cells from healthy and IPF tissue

Paper III

- Create a gene expression atlas of airway epithelial cells from healthy donors
- Investigate possible changes in differentiation pathways utilized by stem/progenitor cells from young and old healthy lung tissue

Paper IV

- Use single-cell RNA sequencing to compare healthy lung epithelial cells to cells from squamous cell carcinoma tumors
- Determine the cell of origin and its possible cancer-driving genes

Methodology

Sample sourcing

Biopsy collection

Human lung tissue samples were obtained from two major sources. As part of a joint project with Lund University Hospital together with other research groups at Lund University, volunteering lung tissue donors were invited to get spirometry measurements and subsequent bronchoscopies, during which proximal biopsies were collected. Both healthy individuals and patients diagnosed with COPD participated.

Bronchoscopy was performed under local anesthetics in accordance with clinical routines, and biopsies were taken from airway branching points at generation 4-6; several biopsies were pooled for each sample to maximize cell yield. Tissue was collected in DMEM/F12 media with 10% FBS and 1% penicillin/streptomycin, and stored on ice for transport to the lab.

Tissue resections from transplants

The second source for human lung tissue used in this thesis was from lung operations performed at Lund University hospital or Sahlgrenska University Hospital. Samples included lung tissue from healthy, diseased donors where the organ was not matched to a transplant recipient, diseased lung tissue from patients receiving a transplant, or tissue from lung tumor removal surgery. Lung tissue was processed immediately upon arrival at the lab following the process described below.

Tissue processing

Lung tissue pieces larger than 5 mm^3 were cut into smaller pieces, before adding to an enzymatic dissociation media made from cell culture media (PneumaCult-Ex) with dispase, collagenase and DNase. Antibiotics were added throughout all

processing steps to minimize contamination. Dissociation was performed on a shaking table at 37ºC for 90 min or at 4ºC overnight, after which the cell suspension was strained using a 100 µm filter. Cells were centrifuged to remove enzymatic solution, and were either frozen for storage in liquid nitrogen in DMEM/F12 with 10% FBS and DMSO, or further analyzed directly.

Tumor tissue was dissociated using a Miltenyi Biotec gentleMACS^{TM} dissociator in combination with a human tumor dissociation kit.

Cell culture

Cells were cultured in PneumaCult-Ex medium with antibiotics, to select for airway epithelial cells with colony-forming ability – i.e. basal cells. Either total cell suspension from enzymatic dissociation of whole lung tissue, or FACS sorted purified cell populations, were added to tissue culture plates coated with bovine collagen I at approximately 10^4 cells per cm^2, and media was changed after 24 h to remove non-adherent cells. Throughout expansion culture, media was changed every 2-3 days.

For passaging or analysis, cells were dislodged using trypsin at 0.05% concentration and incubated for 5 min, and cell detachment was aided by pipetting up and down. Media was added to dilute trypsin concentration, and cells were centrifuged to remove supernatant.

Colony frequency assays

Colony frequency assays were performed by FACS sorting various cell populations and plating in varying cell numbers. When plating whole lung cell suspension, cells were plated at 10^3, 10^4 and 10^5 total cells per cm^2 to balance ratio of BCs to other cell types in each sample. When plating purified BCs, cell number was also titrated and both single cells as well as up to 10^3 cells per cm^2 were assayed.

Media was changed as described above, and forming colonies were observed through a microscope and counted at approximately 7 days after plating. Basal cell colonies were judged by their cobblestone morphology; sometimes when plating whole lung cell suspension, fibroblast colonies were observed but not counted toward the basal cell colony-forming capacity.

Colony frequency was defined as the number of observed colonies divided by the number of originally plated cells.

FACS

Fluorescence Activated Cell Sorting (FACS) is a method that uses epitopes on the cell surface to distinguish between, quantify and sort cell types in a sample. This method can be used to characterize the cellular composition of a sample, or to verify expression of certain genes in gene edited samples, and the sorting function is valuable e.g. when culturing specific cell populations separately.

The FACS method starts by incubating cells with fluorophore-conjugated antibodies that specifically target known cell surface markers. The sample is then aspirated into a stream of buffer pumped through the FACS machinery, and the cells are led through a narrowing tube that lines them up in single file for individual analysis. Next, each cell passes through several lasers at different wavelengths, which excite the fluorescent molecules attached to the antibodies bound on each cell surface.

The resulting emission wavelength is detected and quantified, generating data on which antibodies, and therefore which markers, are present on each cell (and in what numbers). The cells can then be assigned to groups based on the combination of markers they express. Finally, the cells pass through a chamber with an applied electric current, adding charge to specified cells and a following magnetic field is used to divert the cells to different sample collection tubes.

Method

Cells were stained for FACS using directly conjugated antibodies at 1/50 dilution in PBS with 2%FBS, and 7AAD was added at 1/100 dilution directly before analysis for dead cell removal. Flow cytometry analysis and cell sorting was performed on a BD FACS CantoII, a BD Aria IIu or BD Aria III, using BD FACSDiva software for sorting and FlowJo software for analysis and image presentation.

Immunohistochemistry/fluorescence

Staining

Human lung tissue was fixed in formaldehyde and embedded in paraffin directly upon arrival in the lab, and cut into 4 μm thick slices for staining. Tissue sections were rehydrated and stained with hematoxylin and eosin, or treated for antigen retrieval using a PT Tissue link system (Dako, Agilent Technologies), at either high or low pH, before staining with antibodies and fluorophores in sequential incubation to avoid unspecific staining of secondary antibodies. Stained sections were mounted with fluorescent mounting medium.

Visualization and quantification

Images were obtained with a VS120 virtual microscopy slide scanning system (Olympus). Representative images were acquired using the OlyVIA software (Olympus).

Quantification of FTL-expressing cells (**paper II**) was performed using the open software tool Qupath[180].

Single-cell RNA sequencing

Single-cell real-time qPCR

The method used in **paper I** for scRTqPCR was Fluidigm, a platform in which pre-amplified cDNA and Taqman probes are applied in separate wells, which are combined using microfluidics to an array on a microchip where RTqPCR reactions take place.

Method

After antibody staining, basal cells were single-cell FACS sorted to exclude doublets; one cell into each well of a 96-well plate containing cell lysis buffer. The cell lysate was immediately frozen and stored at -80ºC. Pre-amplification was performed using Taqman probes for 48 selected genes, using 27 cycles, and cDNA was stored at -20ºC. RTqPCR was performed on a BiomarkHD and 48x48 Dynamic Array Chip (Fluidigm). The data was normalized using the housekeeping gene GAPDH, and relative intensity was calculated for expression level comparison ($RI=2^{Ct(gene)-Ct(rps18)}$).

Single-cell RNA Sequencing

Single-cell RNA sequencing is performed by generating cDNA libraries from single cells in a sample that are sequenced to obtain a transcriptome. In this thesis, droplet-based scRNA-seq was used; in which cells and microbeads are combined in an oil-water emulsion where water droplets each contain a single cell plus one microbead. Each bead has sequences that cellular RNA attaches to, and during reverse transcription, which occurs within each droplet, cDNA strands with barcoded ends are generated. The barcodes contain a sample-identifying sequence (index), a barcode sequence that is associated with every gene expressed by a specific cell, and a unique molecular identifier (UMI). The UMI permits differentiation between individual RNA molecules, allowing for elimination of multiple transcripts of the

same strand. Thus, the cDNA from all cells can be pooled for the sequencing step while remaining traceable to their cell and sample of origin.

After sequencing, data processing, normalization and analysis is performed using bioinformatics, and lists of differentially expressed genes by specified cell populations can be generated. Gene ontology can be used to identify pathways and processes active in each cell. In addition, various analyses such as unsupervised clustering and differentiation trajectories can be used, to analyze similarities and relationships between cells on a transcriptome level.

Method

The single-cell RNA sequencing libraries were attained using the 10x Chromium system and libraries were sequenced on Nextseq500 or Novaseq600. Cell Ranger pipeline was used to analyze raw data; for exclusion parameters and details on data analyses, see the individual papers.

Results and Discussion

Paper I

Human Primary Airway Basal Cells Display a Continuum of Molecular Phases from Health to Disease in Chronic Obstructive Pulmonary Disorder

COPD is a chronic lung disease that affected 319.9 million people worldwide in 2019[181], and its incidence is steadily increasing. Apart from a severe decrease in life quality for patients, the consequences are also high in terms of the economic burden on society[182]. In addition, the highest incidence is reported in low- to middle-income countries[58], where access to medication and treatment is scarce.

Today there is no cure for COPD apart from lung transplantation, which is not available to most patients[65]. Instead, patients are treated to alleviate symptoms, usually using bronchodilators and corticosteroids. Therefore, we aimed at identifying the cells responsible for disease progression, in order to work toward a possible alternative cell-based treatment for COPD.

The airway epithelium is the first barrier of defense against any inhaled toxins, and thus the epithelial cells are at highest risk of dysregulation due to environmental factors. Specifically the basal cells, the progenitors of the bronchiolar epithelium, have been shown to be the first cell type to acquire aberrant behavior due to smoking inhalation, possibly losing their capacity to repair the airways and thus becoming the initiating spark in COPD[36]. However, at the time when we started work on this project, little was known about the true identity, frequency and location of primary functional human epithelial basal stem cells, and no isolation protocol existed. In fact, most literature was based on precultured primary cells, which leads to an *in vitro* selection bias and other artifacts.

Therefore, our aim with this study was to delineate the basal cell compartment in healthy lungs and in COPD in order to identify any disease-related changes that could hint at potential targets for therapy.

Results

$NGFR^+$ Exclusively Marks Human Basal Cells with Colony-Forming Capacity

We first attempted to purify the primary basal cell population by optimizing a FACS sorting protocol. Healthy human airway tissue biopsies were dissociated into single-cell suspension, stained with flow cytometry antibodies and sorted in different populations. To evaluate our gating strategy, we performed colony-forming experiments in order to verify a basal cell population that retained self-renewal capacity. Comparing $NGFR^+$ FSC^{high} and $NGFR^+$ FSC^{low} cells, the colony-forming capacity was found exclusively in the FSC^{high} population, reinforcing this strategy as a means of further purifying basal cells with progenitor properties. To further validate this result, we index sorted cells from the $NGFR^+$ FSC^{high} and compared the FACS profiles of colony-forming and non-colony-forming cells. The cells were equally distributed throughout the gates, showing that no further sub-division could be done using these markers to select for colony-forming capacity.

Single-Cell RNA Analysis on Primary Human Basal Cells Reveals High Heterogeneity

Our next line of questioning was related to the exhibited heterogeneity of the sorted basal cell population as only 10% of the sorted human basal cells showed self-renewal capacity. We performed real-time qPCR on $NGFR^+$ FSC^{high} single sorted cells. We selected 48 genes to test for based on their association with basal cells, stem cell and differentiation capacity, proliferation, and mature differentiated epithelial cells. We were able to use clustering to identify four subgroups of basal cells, possibly corresponding to different stages of quiescence, activation and differentiation; confirming our hypothesis of primary basal cell heterogeneity and possibly explaining the lack of stem cell properties of some subgroups *in vitro*. We also showed differential expression of several cell surface markers, that can be tested for use additional markers for purifying basal cell sub-populations.

Cultured Human Basal Cells Possess Higher Colony Frequency Potential and Altered Gene Expression Compared with Primary Basal Cells

Subsequently, we tackled the question of whether primary human basal cells change or acquire different characteristics when cultured *in vitro*. Since our experiments show a sevenfold increase in colony-forming capacity between the first and second culture passage, and a change and homogenization in NGFR and EPCAM expression occurs, we hypothesized that culture enriches for colony-forming cells with similar gene expression profile, which could affect the conclusions from any assays performed on pre-cultured cells as opposed to primary cells. We single-cell sorted cultured basal cells and performed RT qPCR using the same 48 genes as previously. The results showed a more homogeneous gene expression within the cultured basal cell group compared with the primary BCs, with KRT14 and KI67

being expressed exclusively in the cultured cell group while only primary BCs expressed HLF; suggesting that cultured basal cells are in a more active proliferating state, similar to that of injury conditions *in vivo*.

Single-Cell RNA Sequencing Identifies a Molecular Continuum of Pathological Changes between Basal Cells from Healthy Airways and Patients with COPD

Finally, we aimed to characterize the global gene expression profiles of primary human basal cells from healthy versus COPD airways at the single cell level. We first analyzed the epithelial cell populations by FACS and IHC, and found a significant increase in BC frequency in the COPD samples. This is supported by other studies where BC hyperplasia is a common find in COPD airways[183]. Next, we performed single-cell RNA sequencing on four healthy and four GOLD stage IV COPD samples. We defined all KRT5 expressing cells as BCs, and unsupervised hierarchical clustering identified 4 clusters of cells with varying numbers of BCs from healthy and diseased patients. Interestingly, cluster 1 contained mostly healthy cells but a few from COPD, while cluster 4 contained mostly COPD cells but some from healthy samples. This suggests that some BCs in end-stage COPD airways still retain a healthy gene expression profile – a promising target for future induction of endogenous regeneration.

Using gene ontology analysis on the significantly upregulated genes in cluster 4, we found that BCs from COPD were expressing genes connected to proliferation, stress and inflammation, suggesting an ongoing damage repair process in response to high levels of stress. BCs in cluster 1, however, expressed healthy functional pathways such as WNT signaling, cell-cell adhesion and mitochondrial activity. Looking at clusters 2 and 3, we interpreted them to represent intermediate stages of a hypothetical disease development process from healthy to end-stage COPD. In order to further investigate this notion of a continuum of BC states, we created a pseudotime vector that illustrated the changes in gene expression in BCs from cluster 1 through cluster 4. Here, we could see genes associated with stress and hypoxia response increase in expression across the vector, indicating a progression from healthy state through a pathological development of COPD symptoms.

Finally, we were interested in GADD45B, a gene that was upregulated in BCs from COPD tissue that is associated with stress response[184]. We performed immunofluorescent staining on fixed and embedded airway tissue sections from healthy and COPD donors in order to test for GADD45B at the protein level. We found high GADD45B expression in $TP63^+$ BCs in COPD tissue, but no expression in healthy BCs, aligning with our sequencing results.

Discussion

Basal cells and their homeostatic properties remain elusive, since they are difficult to purify and study *in vitro*. The lack of surface markers to sort primary BCs has been addressed in this study, and we successfully used FSC^{high} as an additional gate criterion to enrich for BCs with proliferating properties. This is a step forward in our ability to extract BCs from donated tissue, for use in culture assays on primary BCs, and will enable further therapeutic studies creating organoids for pharmaceutic or genetic marker screening. The gating strategy essentially constitutes a selection of larger cells among the $NGFR^{+}$ population. We know that BCs are largely quiescent in non-injured airways[185], so a majority of primary BCs should not be actively cycling, thus being larger. The reason for the seeming correlation between size and colony-forming capacity still remains unclear.

While this gating strategy is certainly a step forward in our ability to purify said population with expansion ability, it remains to be discovered how to study other intermediate basal cell states or cell types. In our single-cell RT qPCR assay, we brought to light the possible presence of BCs that were primed for differentiation, which may not retain self-renewal ability but nevertheless could reconstitute a limited area of epithelial injury. Finding new surface markers that allow us to separate these cell types or states would enable us to further clarify the BC regenerative mechanisms. This is an important area of future study that we initiated by including CD markers in our assay, and should be further pursued given sufficient resources and access to more primary samples.

Numerous other ideas for future study were sparked by this project; for example exploration of CD55 and HLF which both stood out in the qPCR assay as expressed by subgroups of BCs. CD55 expression has been linked to therapeutic resistance in endometrioid and breast cancer tumors by providing protection from induced cell lysis[186, 187], suggesting that CD55-expressing BCs may exhibit a higher proliferation or survival *in vitro*. In contrast, HLF is responsible for maintaining quiescence and stemness in hematopoietic stem cells[188], and it would be interesting to test whether HLF-expressing BCs are similarly regulated. Gene knockout of these genes of interest could be performed to test for functional effects *in vitro* with proliferation/differentiation assays.

A significant point we made is the radical change in gene expression and variance in BCs after culture, which shows how *in vitro* assays based on passaged cells or BC-derived cell lines do not reflect the conditions existing *in vivo*. Since functional assays are crucial to verify results from sequencing assays and to investigate the role of specific genes in cell function, it is important to use primary cells as much as possible. Culture simulates a state of epithelial ablation or injury, where BCs with high proliferation tendencies succeed over quiescent or differentiation-primed cells. this makes the assay replicate conditions of injury-response more than homeostasis.

The upregulation of several genes associated with disease and stress response in BCs from COPD tissue promoted further avenues of study to determine early disease detection markers or therapeutic targets. We demonstrated an increased expression of GADD45B in COPD BCs; GADD45B is a gene demonstrated to respond to stress signals and apoptosis by activation of p38 and the MAPK cascade, as well as to activate p53[184]. Moreover, it has been detected in various cancer tumors and linked to tumor cell survival, for which it has been proposed as a therapeutic target[189]. Interestingly, GADD45B was recently identified as a biomarker and potential therapeutic target in IPF, but not in COPD[190]. The microarray datasets used from that study, however, only contained samples from COPD GOLD stage II patients; the GADD45B levels may have been low and thus disregarded. Taken together, GADD45B remains a gene of interest for further study, and it remains to be determined whether it contributes to COPD pathology and if it could become a target for COPD treatment.

As Shaykhiev (2021) comments in the editorial of our work in the same journal issue[191], limiting our study is the low sample number; both in terms of overall donor numbers (and correspondingly cell numbers), but also in terms of representation of disease stages. COPD is a heterogeneous disease, and analyzing cells from more patients would be of great benefit to separate variation in gene expression due to differing cell types versus patient-to-patient variation. Another valuable addition would be to similarly characterize BCs from lung tissue with less severe disease development, such as patients with stage I-III COPD according to GOLD standard. Nevertheless, Shaykiev highlighted our study as a new and innovative approach to understand disease progression in COPD.

In conclusion, this paper revealed the presence of BCs with healthy gene signatures in end-stage COPD airways. This proposes the exciting avenue of targeting endogenous repair in patients, provided we uncover what makes these cells resistant to the disease changes or how to activate them for regeneration that outcompetes cells with aberrant behavior. In addition, we showed that BC gene expression profiles could be ordered in a continuum of gradual upregulation of genes associated with COPD pathology, such as stress and hypoxia response, cell proliferation and wound healing signals. Studying this virtual progression of disease-related changes could give an indication of pathways to target in order to stop or reverse disease exacerbations, as well as providing new biomarkers to facilitate earlier diagnosis.

Paper II

Ciliated (FOXJ1^{+}) Cells Display Reduced Ferritin Light Chain in the Airways of Idiopathic Pulmonary Fibrosis Patients

IPF is a devastating disease with the short overall median survival of 2-3 years [94] and 5 year survival rate of 48% in Sweden[192]. Aging, and the resulting epithelial cell senescence, is among the highest risk factors for developing the disease[193]. With the worldwide population getting older[61], new therapies need to be developed to more effectively treat IPF patients.

The disease mechanisms are complicated and not yet fully understood; however a currently emerging theory is that injury to the airway epithelium, followed by repeated aberrant repair, is the initial spark of the continuous dysfunctional remodeling of lung tissue in IPF[194]. Previously thought to be a disease of alveolar injury and dysrepair, the mucociliary epithelium, and ciliated cells in particular, have now received interest as important players in disease progression[112, 195-197].

In this study, we wanted to more closely characterize mucociliary epithelial cells from IPF patients, and to compare with healthy counterparts in order to find disease-specific changes. The aim was to identify cell types with aberrant gene expression that may contribute to disease pathology, as well as possible therapeutic targets.

Results

Immunohistochemistry and Immunofluorescence Show Typical Structures Associated with Disease in Lung Tissue from IPF Patients

We obtained lung tissue from healthy donors and IPF patients, and first stained sections containing airways with H&E as well as with KRT5 and FOXJ1 in order to visualize the epithelial structures. As expected, we found regular airways lined with epithelium in the healthy tissue, containing KRT5^{+} BCs and FOXJ1^{+} ciliated cells. In tissue from IPF patients, we also found typical IPF-associated pathological features such as honeycomb cysts; indicating that our subsequent gene expression analyses should give information on disease-related cellular identities.

Single-Cell RNA Sequencing Identifies Molecular Differences between Ciliated Cells from IPF Patients and Healthy Control Cells

Next, we performed single-cell RNA sequencing on cells from healthy and IPF lung tissue; we enriched for epithelial cells by FACS sorting 7AAD^{-} CD45^{-} CD31^{-} cells. We confirmed the presence of epithelial cells using known markers (basal, mucus,

ciliated and alveolar cells). Initial analysis showed that ciliated cells separated into two disease-specific clusters; indicating that ciliated cells exhibited the highest gene expression differences between healthy and IPF epithelial cell types. We identified several genes of interest that were among the top up- and downregulated genes in IPF ciliated cells versus healthy ciliated cells: SYT8, FTO, NEAT1 and FTL, GSTA2, FOS respectively. FTL encodes Ferritin light chain, part of a complex that regulates iron metabolism and intra-cellular storage[198]. Interestingly, excess iron accumulation has been previously reported in IPF lung tissue and is thought to contribute to pathogenesis[199].

Ciliated Cells in IPF Lung Epithelium Exhibit Increased Expression of Ciliated Pathways

Using gene ontology analysis, we identified pathways and biological processes that were upregulated in healthy and IPF ciliated cells (for a detailed list, see **paper II**). As expected, healthy ciliated cells exhibited epithelial cell activity such as epithelial differentiation, cell-cell adhesion and WNT signaling/planar cell polarity, as well as general biological processes such as RNA processing, translation and protein folding, and mitochondrial electron transport. However, IPF ciliated cells extensively upregulated pathways associated with cilium morphogenesis and function. This has been observed in other studies[200], reinforcing our results and suggesting a part in IPF pathogenesis played by ciliated cells.

Ciliated Cells in IPF Patients Display Reduced FTL Protein Levels

Finally, we wished to further study the expression of FTL in ciliated cells, starting by verifying its presence at the protein level. We obtained formalin-fixed, paraffin embedded lung tissue sections from healthy and IPF patients, which we stained using IHC antibodies for FOXJ1 and FTL. Looking at healthy airway epithelial structures, we identified FTL expression in ciliated cells ($FOXJ1^+$); while the expression of FTL in IPF samples was more sporadic and entirely absent in many sections of ciliated epithelium. Further evaluating these findings, we used Qupath[180] to quantify FTL expression in $FOXJ1^+$ cells and found a difference in mean percentage of FTL expression of 21% between healthy and IPF samples.

Discussion

As mentioned, ciliated cells have emerged as a new area of interest in IPF pathogenesis, due to their multi-faceted roles in airway epithelial function. Excess mucin production has been established as a usual IPF symptom, which may lead to airway obstruction, as well as impaired wound repair due to disruption of signaling and cell-cell interaction[111]. It stands to reason that ciliated cells will be affected by this increased need for mucus clearance. Moreover, the ability of cilia to sense the cellular microenvironment contributes to immune signaling through cytokine

antimicrobial production, which may impact mechanisms of disease-associated cellular dysregulation[201]. In addition, cilia coordinate various wound-repair sequences by promoting fibroblast migration[113]. Finally, it has long been known that a regular histological feature of IPF lungs is the presence of honeycomb cysts and areas of bronchiolization, where apparent mucociliary epithelium appears. Taken together, we were interested in characterizing ciliated cells and their role in IPF.

In this study, we identified genes and pathways that were differentially expressed between ciliated cells from healthy and IPF tissue. Pathways connected to cilium formation and maintenance were overrepresented, an interesting observation that leads to further questioning how the activity of ciliated cells may affect disease progression. In a mouse fibrosis model, Kim et al (2022)[197] recently showed a relationship between cilium-associated genes and fibrosis development; particularly interesting was their conditional deletion of a cilium gene (lft88) in $Krt5^+$ cells resulting in decreased formation of $Krt5^+$ cystic pods and delayed ciliogenesis. Although $Krt5^+$ pods have not been observed in humans, they are similar to honeycombing found in IPF patients and are undoubtedly of interest since $Krt5^+$/$KRT5^+$ cells are progenitors of ciliated cells in both mouse and human.

In particular, a few genes of interest were identified from the top differentially expressed genes in ciliated cells. Upregulation of FTO and NEAT1 in IPF cells suggest a link between IPF and cancer pathology, since these genes have both been associated with progression of lung cancer[202-204], though not previously identified in IPF tissue. SYT8, which was also upregulated in IPF ciliated cells, is involved with exocytosis. Active formation of extracellular vehicles contributes to signaling between cells and influences the microenvironment, and has been implicated both in contributing to pathogenesis in lung disease and considered as a therapeutic delivery vehicle[205].

FTL was downregulated in IPF ciliated cells compared to healthy, which we also confirmed as a decreased FTL protein expression in IPF ciliated airways. FTL encodes a subunit of Ferritin, a protein complex responsible for various forms of iron metabolism and safe storage[198, 206]. Iron is an important component of cellular metabolism, but is also prone to free radical formation[207] which is toxic in high amounts. In addition, iron availability to pathogens increases their proliferation and survival, which is why iron levels are regulated by immune cells such as macrophages[208]. In IPF, excess iron accumulation has been reported[209, 210] and has been shown to contribute to lung deterioration in mice[199, 211]. Interestingly, Ali et al (2020)[199] showed that treatment with iron chelators reduced the decline in lung function in a bleomycin-induced mouse model, suggesting the possibility of ameliorating IPF patient symptoms by targeting iron metabolism[212].

Our study found a reduced level of FTL in IPF ciliated cells, and it is possible that impaired iron regulation by the epithelium leads to formation of free radicals leading to oxidative stress, contributing to pathogenesis. Since iron chelator treatment was needed daily for the fibrosis mouse model to improve by reducing free pulmonary iron, an alternate longer-term solution could be to target cellular ability to more effectively store iron in less toxic forms, e.g. by increasing FTL expression. Therefore, it is important to further determine why ciliated cells in IPF exhibited lower FTL levels, such as whether it comes from BCs that give rise to ciliated cells, due to a mutation or other inhibition of gene expression, or whether it is a consequence of other factors in the pathological environment. Should a stem cell therapy treatment be considered for induced regeneration of healthy epithelium in IPF patients, the microenvironment and free iron levels may be necessary to take into account, in case injected BCs or subsequently differentiating ciliated cells lose their ferritin expression due to external factors.

An advantage of our study is the use of human tissue samples since bleomycin-induced rodent fibrosis models do not completely capture the same disease state affecting human patients[135]; though a limiting factor is the availability of patient samples. Furthermore, other aspects need to be further studied to reinforce our conclusions and determine whether FTL expression in ciliated cells is affecting IPF pathogenesis, such as testing for overall lung iron levels in IFP airway tissue. In addition, other genes involved in the ferritin-iron storage function should be tested for, such as ferritin heavy chain (FTH1) and divalent metal transporter 1 (DMT1).

Finally, airway-resident macrophages are greatly involved in iron storage to combat pathogens by limiting accessible iron[213, 214], and have exhibited impaired iron sequestration ability in IPF lungs with excessive iron levels[215]. We detected high FTL expression in macrophages in some airways of our IPF tissue sections, and it remains to be investigated whether these macrophages contribute to iron neutralization.

Paper III

A Single-Cell Atlas of the Human Airway Epithelium Suggesting a New Convergence Point in Basal Cell Differentiation and Identifying Transcriptional Changes in the Aging Lung

In order to develop more effective treatment, such as stem cell therapy, for chronic lung disease, the mechanisms regulating human lungs during regeneration and in steady state need to be described in detail. The epithelium is an important part of lung function, and learning the pathways its stem/progenitor cells go through during repair is crucial. The basal cell, progenitor of the airway, is proving to be heterogeneous in its gene expression and differentiation capacity, with important regulatory mechanisms still unknown.

Furthermore, most chronic lung diseases such as COPD, IPF and lung cancer are negatively associated with aging[216], which argues the importance of delineating the changes that occur within the "healthy" aged lung in order to understand and provide a context for the development of lung disease. Lung function has been shown to be heavily affected by the aging process; both at the organ level, with difficulties breathing (reduced muscle capacity and airway elasticity) and at the cellular level (shorter telomeres, increased expression of cellular senescence markers, increased DNA damage, oxidative stress, and apoptosis)[217]. The aging process may be accelerated by the decline in stem cell function due to stem cell exhaustion similar to what has been described in the hematopoietic system[218]. In addition, older individuals show a significantly reduced ability to repair damage and regenerate a healthy lung compared to younger patients[219]. It has been hypothesized that the inability of aged lung tissue to re-initiate growth signaling may contribute to its slow, restricted, and even failed response to injury. Hence, it is critical to understand how aging impairs the mechanisms of regeneration of the human lung.

To this end, we set out to create a single-cell transcriptomic atlas of healthy human airway epithelial cells with the aim of comparing the gene expression between the old and young cells.

Results

Generating a single cell atlas of the human airways

To generate a comprehensive cell atlas of the human lung airways, we collected human lung bronchoscopies from 8 healthy donors that had never smoked and had normal lung physiology; 5 were categorized as young (23-40 years old) and 3 as aged (64-75). The tissue was processed immediately and sent for sequencing within a maximum time of 2-3 hours after collection. High quality single-cell

transcriptomes were obtained from >42 000 airway epithelial cells using the 10x platform. Using highly variable genes as input, we initially identified cellular clusters corresponding to known epithelial cell populations as well as resident immune cells based on expression of canonical genes. Taken together, we have successfully generated a unique reference library for studying human lung biology during normal physiology and aging.

Transcriptome on human lung airway epithelium

Since one of the main interests of this study was to further delineate the hierarchy within the human lung airway epithelium, we initially selected all $EPCAM^+$ cells. However, we and others have found that EPCAM is not a ubiquitous marker of epithelial cells so we also included $EPCAM^-$ cells that clustered together with the $EPCAM^+$ cells for further analysis. We grouped the clusters based on the canonical markers known for the different cell types in the lung airway epithelium: BCs ($KRT5^+$), suprabasal cells ($KRT4^+$), goblet cells ($MUC5AC^+$), mucociliary cells ($SCGB1A1^+$ $FOXJ1^+$), ciliated cells ($FOXJ1^+$) and ionocytes ($ASCL3^+$). The distribution of these epithelial cells is in line with previous publications.

Pseudotime analysis shows differential gene expression in young and aged epithelial cells

Next, in order to compare the epithelial cell differentiation process between young and old patients, we performed pseudotime analyses on these two age groups separately. Pseudotime is the name given to an artificial time vector (timeline) introduced to plot against gene expression allowing a visualization of the continuous change in gene expression. We generated a pseudotime vector going from BCs to differentiated cells and as expected, while genes associated with BCs decreased, genes connected to differentiated cell types such as goblet cells and ciliated cells increased over “time”. Interestingly, we discovered a range of genes that differed along this virtual time axis between young and old epithelial cells. Several genes associated with protein-folding homeostasis and anti-apoptosis were overexpressed in the aged group, especially in differentiated cells; suggesting that these cells may be experiencing more stress due to age factors. Of note, genes associated with antigen presentation were overall lower expressed in the aged cells, possibly contributing to a higher susceptibility to infections and other airway pathologies in older patients.

RNA velocity identifies origin of basal cell activation and differentiation

The $KRT5^+$ $TP63^+$ BCs are known to have self-renewal capacity and the ability to differentiate into the effector cell types of the human lung airway epithelium. In order to investigate the possibility of changed regeneration capacity in older epithelium, we compared BCs from young and old donors. Interestingly, we identified a number of genes that were differentially expressed between young and

old BCs, suggesting the presence of different cellular states or functions that exist among the BC population. This is consistent with recently published single-cell data.

In order to identify the cluster populated with basal cells primed to differentiate, and thus the starting point of differentiation, we performed RNA velocity analysis on the $KRT5^{+}$ cells. This calculates the ratio of spliced to unspliced RNA in each cell at the time of sequencing, and predicts in which direction the cells are likely to move in terms of cellular maturity[220]. Notably, the velocity analysis showed young and old BCs starting from different points but converging before moving toward further differentiation. We speculate that once the basal cells are stimulated by the environment to differentiate, they go from the quiescent/naïve state through the divergence point identified by velocity. From there the cells may undergo asymmetrical cell division where one cell can differentiate and one cell return to its original quiescent state.

Single cell sequencing identifies transcriptional changes in the aging differentiated cell populations

Finally, we were interested in the effects of aging on the differentiated cell populations, and performed gene ontology analysis on the upregulated genes in aged goblet cells, mucociliary cells and ciliated cells as well as their young counterparts. There were some genes and pathways that were differentially expressed by cells from the two age groups within each cell type. Interestingly, aged goblet cells exhibited upregulation of lung fibrosis and DNA damage response pathways, and mucociliated cells upregulated genes known to be oncogenic such as FOS, JUN and ATF3; indicating a stressful environment existing in the aged epithelium and suggesting that aged epithelial cells may be more prone to cell transformation and cancer development. In contrast, young goblet and mucociliated cells exhibited upregulation of genes associated with the innate immune system, antigen processing and presentation and MHC class II protein binding; suggesting that aging differentiated epithelial cells are losing some of their immune system function and may be more susceptible to disease and infections.

Discussion

Aging is a complicated process with a large number of factors contributing to changes in health and function at the molecular, cellular and organ level. scRNA-seq provides a powerful opportunity to examine the transcriptome in specific cell types, and we have uncovered interesting differences between both progenitors and differentiated cell types in young and aged samples. Due to the complex nature of all the interacting processes occurring during aging, it is difficult to determine which changes in gene expression is due to age in particular, and which are due to other

environmental factors. To strengthen the data, more donors in both age ranges would be beneficial to study, to reduce inter-patient variability bias.

In addition, the differentially expressing genes and transcription factors should be verified in functional assays, in order to determine their possible influence on epithelial function and regeneration. Various mouse models of aging exist, such as telomerase knockout mice, that simulate accelerated aging, and naturally aged wild type mice. These have to some extent been used to determine regeneration capacity after lung injury[221, 222], and could be used to study the effect of overexpressing transcription factors found to be upregulated in aged epithelial cells. However, mouse models of aging mostly exhibit changes in the distal compartment of the lungs, with emphysema-like breakdown of alveoli and loss of AECII cells, while the effect of aging in the proximal epithelium is less well characterized. In addition, it is difficult to recapitulate all effects of aging in a knockout model, since so many different cellular and molecular changes accumulate and drive aging processes forward[223]. Furthermore, the shorter lifespan of mice and the structural differences between mouse and human lungs makes it difficult to translate results from aging mouse models to human biology. To alternatively simulate aging lung epithelium, *in vitro* models using human cells could be used, such as organoids and 3D printed tissue derived from aged donor samples.

In this study, we described the differential gene expression in basal cells from aged and young samples, and aged BCs were found to have a higher level of DNA damage and oncogene expression, as well as a lower level of cell-cell adhesion compared to younger cells. This suggests that age affects the ability of BCs to act as stem cells and repair the epithelium following injury, and falls in line with previous literature showing similar defects in aged tissues[218].

Using RNA velocity and pseudotime analysis, we observed that aged and young BCs reach a point of converging gene expression, while the differentiating cells from the two age groups once again differ in transcriptome. This suggests that the "primed for differentiation" BC state is retained in older individuals, while quiescent BCs, as well as differentiated cell types, differentially express pathways such as cell-cell interactions and signaling during aging. This would also be of interest to study functionally; an option could be to use ALI differentiation culture of young and aged BCs separately and sequence the cells at various time points to confirm these results of converging gene expression profiles that again diverge during differentiation[34]. In connection with this, the effects of certain genes of interest could be tested using inhibitors or gene knockout to see the effects on epithelial regeneration, similar to the method used by Goldfarbmuren et al (2020)[25], who investigated ciliated cell differentiation over time in ALI culture by scRNA-sequencing at several time points and subsequently knocking out FOXN4 in BCs to observe a block in ciliogenesis.

Taken together, we have generated a single cell atlas of the human airway epithelium during healthy aging that provides a good basis for further unraveling the epithelial cell hierarchy in the healthy lung, and have shown interesting age-related differences between lung progenitors that should be studied further.

Paper IV

Single Cell Analysis of Primary Human Squamous Lung Carcinoma Shows High Heterogeneity of Tumor-Associated Epithelial Cells

Every year 1.8 million patients die of lung cancer[224], which represents more deaths than of colon-, breast-, and prostate cancers combined[225]. One of the biggest challenges today is that most patients are diagnosed too late and cannot be operated on, which has a great adverse effect on survival. Thus, patients are often treated with chemotherapy that non-specifically targets all proliferative cells leading to severe side effects and a high relapse rate. Relapse can partly be explained by the heterogeneous cell populations in the tumor, accounting for the different tumor characteristics[226], along with the existence of cancer initiating stem cells (CIC), which are believed to initiate and drive the disease, and to be resistant to chemotherapy[227]. Therefore, more specific treatments are needed that target the CIC in order to prevent or inhibit tumor formation. Although some therapies have been approved that target more common mutations in lung cancer such as ERG, ALK, MET and HER2, due to the CIC and complex tumor heterogeneity most patients develop resistance to their therapy irrespective of treatment[228].

Approximately 40% of all lung cancers are diagnosed as squamous cell carcinoma (SCC)[229], and are believed to be originating from the transformation of the squamous cells lining the airways. Still, how and from what cell SCC arises is not clear; but research has suggested that SCC is caused by initial changes in the airway BCs[230, 231]. In SCC, chronic stress and injury induced by toxins like smoking and pollution can result in BC proliferation (hyperplasia), possibly due to an increased need of repair. The constant proliferation may eventually lead to BC metaplasia and finally cancer[36, 232]. Here, using scRNA-seq on primary tumor tissue, our aim was to characterize the different cell types contained in human lung SCC, to investigate the dynamics within the tumors and potential mechanisms regulating tumor progression, as well as search for a cell of origin to potentially target in future therapy.

Results

Successful single-cell sequencing on human lung squamous cell carcinoma

We performed scRNA-seq on primary SCC cells isolated from fresh tumor tissue. The analysis was then pooled with our healthy single cell atlas as a reference (**Paper III**). In total we analyzed 75 000 cells, 33 500 of which were SCC tumor cells. Unsupervised clustering had cells from the two tumors clustering separately from each other as well as from the healthy cells; which was expected due to the

heterogeneous nature of SCC. However, some cells clustered together with the healthy cells, indicating that the separate clustering was not merely due to individual donor variation.

Interestingly, the majority of tumor cells expressed TP63 and KRT5, BC cell markers that are also used in SCC diagnosis[233]. These cells also expressed MKI67 and TOP2, showing high proliferation compared to epithelial cells in steady state. Combined with the lack of overlap of these cells and SCGB1A1^{+} or FOXJ1^{+} cells, this indicates that the tumors may originate from hyperproliferative BCs that lack the ability to differentiate into secretory or ciliated cells. This is in line with previous findings that place BCs as the cell of origin in SCC tumors[234, 235].

Single-cell RNA sequencing analysis reveals ongoing epithelial-to-mesenchymal transformation and identifies a potential cancer-driving population

We next focused the analysis on the cluster containing cells from tumor 1, due to its high cell number and frequency of tumor cells. Unsupervised clustering divided the tumor in multiple differentially expressing cell populations, showing heterogeneity in gene expression and supporting the theory of clonal heterogeneity within the tumor. Interestingly, EPCAM expression divided the KRT5^{+} cells in two distinct clusters, with the EPCAM$^{low/-}$ cells expressing known mesenchymal genes such as VIMENTIN, COL6A, ACTA2, DECORIN and COL1A2. This indicates a subset of KRT5^{+} cells that are going through EMT, possibly at a different stage of tumor progression.

In order to identify the most naïve cancer cell state, or cell type of origin, we used the RNA velocity algorithm. We were able to identify a cluster of KRT5^{+} EPCAM^{+} cells from which all the RNA velocity arrows pointed outward; indicating that this cluster represented the earliest state of the cancer originating cells in terms of developing into differentially expressing tumor cell types.

Transketolase is highly upregulated in SCC

Comparing the genes expressed by cells in tumor 1 and the healthy cells, we identified two genes that were highly upregulated by the tumor cells; Keratin 6A (KRT6A) and transketolase (TKT). KRTA6 is known to be hyper-expressed in multiple cancer forms and was recently shown to promote proliferation and metastasis via epithelial-to-mesenchymal transition, and to affect cancer stem cell transformation in lung adenocarcinoma[236]. TKT is an enzyme regulating the pentose-phosphate pathway and is important for generating NADPH and nucleotides. Interestingly, TKT has been shown to be overexpressed in multiple cancer forms, counteracting oxidative stress[237] as well as promoting EMT[238]. We used immunofluorescence to verify TKT expression at the protein level, staining paraffin-fixed tissue sections from the sequenced tumors with TKT and KRT5. SCC tumor cells showed clear co-localization of KRT5 and TKT, supporting our gene expression findings.

Discussion

We showed for the first time an in-depth single-cell characterization of primary human lung SCC, illustrating the capacity and importance of scRNA-seq to identify multiple tumor cell stages and several possible mechanisms regulating tumorigenesis. Since CICs are often resistant to chemotherapy and other conventional cancer treatments, it is crucial to find the cell responsible for tumor formation for specific therapeutic targeting. The basal cell has been linked to squamous metaplasia leading to tumor expansion in several studies, and the high expression of TP63 and KRT5 throughout both of our tumor samples reinforces the theory that BCs are the cell of origin of squamous cell carcinoma development. Our scRNA-seq data provides a base for determining the genes and mechanisms responsible for the dysregulation of aberrant BC behavior, which can enable us to find therapeutic targets for halting tumor propagation and possibly preventing relapse.

TKT was upregulated in $KRT5^+$ cells from tumor 1 versus healthy cells, both transcriptionally and at the protein level as confirmed by immunofluorescence. Of note, the expression of TKT was mainly found in the nucleus which is in line with recent findings suggesting that TKT may have an additional non-metabolic role in tumorigenesis[239] apart from promoting EMT and resistance to stress. This finding supports our gene expression analysis and suggest TKT as a potential target for cancer therapy. Tseng et al (2018)[240] showed that oxythiamine, a known inhibitor of TKT, significantly reduced the viability of breast cancer cells in cell culture, as well as inhibited metastasis in an orthotopically injected mouse model. Taken together, this shows promise for TKT inhibitors as possible treatment in lung cancer; though similar assays should be performed on SCC cells to ascertain whether these are similarly affected by the metabolic pathways associated with TKT.

Relapse and death is often caused by metastasis, which has been shown to occur earlier in the disease stage than previously thought[241, 242]. In addition, cells undergoing EMT acquire invasive properties and are able to cross the basal membrane into the bloodstream[243]. Given that we observed EMT-related genes upregulated in a portion of $KRT5^+$ tumor cells, it would be valuable to pursue further studies to assess the potential motility and re-seeding potential of these cells, as well as to investigate the possibility that blocking the EMT process could prevent metastasis.

Pagano et al (2017)[231] identified a subset of cells within an immortalized HBEC cell line exhibiting high motility *in vitro*, and showed that when injected intravenously in mice, these cells were capable of migration and colonization of the lung, suggesting metastatic behavior. It remains to be determined whether this highly motile subset of epithelial cells exists *in vivo*, and whether this migration

behavior can be exhibited by primary SCC tumor cells. However, the link between this motility exhibited by airway epithelial cells with differentiation capacity and our finding that BCs are the origin of SCC tumor formation is worth considering, since the key to prevent metastasis and relapse could be to target these highly motile cells.

We sequenced cells from two tumors obtained from two different patients. Analysis of the cells from tumor 2 will show whether the differences in gene expression between tumor 1 and healthy cells are patient-specific or shared by SCC tumors. Any common markers we could find between tumors from different lung cancer patients would be of high interest to use as future biomarkers for easier diagnosis and tumor characterization; however sequencing of additional tumors is needed to verify this due to the high patient-patient variability.

General discussion

Challenges and considerations

A large portion of the work involved in any scientific study is never acknowledged or published. For this thesis, a lot of initial work went into optimization of the process of sample handling and preparation, to ensure the cells studied maintained as much of their primary characteristics as possible. The success of *in vitro* cell expansion in particular was highly affected by logistical factors such as the time taken from biopsy sampling to culture plating of the cells; we were lucky to have the hospital within a short walking distance, but still had to adjust dissociation and incubation times to keep them at a minimum while extracting as many cells as possible. Another factor that influenced the survival and quality of our cells was cryopreservation; as far as possible, we tried to use fresh cells for culture assays. Similarly, FACS sorting of the cells to purify the population of interest was also detrimental to the colony-forming capacity.

On the topic of sample preparation, when using primary cells in particular, any process they are exposed to can have effects on assay readout, such as the enzymes used to dislodge the cells from the basement membrane. It has been reported that the use of collagenase in tissue dissociation can cause the cells to respond by expressing heat shock proteins and oncogenes like FOS and JUN, which can introduce bias in transcriptomic analyses[244]. In addition, cell death or stress can increase mitochondrial reads in scRNA-seq which has to be taken into account during data processing, and the possibility of certain cell populations being more sensitive to hypoxia than others may lead to a skewed cell ratio in the analysis[245].

A sometimes frustrating aspect of attempting to culture primary cells from lung epithelial tissue is the high risk of contamination, not as a result of failing to use antiseptic laboratory practice but coming from the samples themselves. As described in the introduction, the airway epithelium is in contact with the outside environment and consequently lung tissue samples may contain bacterial or fungal contaminations that can disrupt *in vitro* assays. We used antibiotics and antimycotics in all tissue processing steps as well as culture assays, however there is an unfortunate possibility of gene expression changing in the presence of antibiotics that can be difficult to take into account during data analysis[246].

Single-cell RNA sequencing has emerged in recent years as a powerful tool that not only allows to chart the genes that are being expressed by a certain cell type to an

extensive depth, but also to reveal differences in gene expression between individual cells. This is opening the doors to distinguish hitherto undetectable subtle differences between rare cells with specific roles and functions, and even to detect certain intermediate states that a cell may pass through in a process of differentiation or other morphologic changes.

However, the method also demands consideration of multiple aspects when processing and analyzing data[247]. The first steps include quality control such as exclusion of doublet cells, low quality reads and cells with too high mitochondrial gene counts (which often signifies they were dead/dying at the time of sequencing). The respective limits imposed in this early data processing affect the interpretation of the results later on, for example a certain cell type may be more highly sensitive to stress and may therefore be dismissed from the analysis entirely if the mitochondrial gene limit is set too low. Next, the number of highly variable genes needs to be defined in order to perform dimensionality reduction, which similarly can influence the outcome of the analysis; using too few genes here can reduce the apparent heterogeneity, while too many genes can introduce false variation between similar cells. Once pre-processing of the data is finished, analyses such as visualization, clustering, pseudotime, and differential gene expression can be performed. The interpretation of these results are also dependent on how the analyses were carried out; while there are good practice guides available for how to decide parameters and limits, there is no universally defined standard when it comes to analyzing and presenting results from scRNA-seq assays[248]. Therefore, it is important to understand how these factors affect the biological interpretation of a study, both when drawing conclusions from one's own data as well as when reading others' work.

In addition, it is important and highly valuable to functionally verify scRNA-seq results in order to dismiss artefacts or to gauge the biological significance of transcriptomic differences.

What is a functional assay?

RNA sequencing data determines which genes are being transcribed in a cell at a given time. This gives useful insight into the characteristics and probable identity of that cell; however not all mRNA will be translated into protein and not all proteins will take part in biological processes that significantly affect the function of the cell in a larger context. Consequently, identifying a gene that is being transcribed by a certain cell type can give an indication that that gene is important, but it is still necessary to determine how and what this gene expression contributes to the behavior of the cell. To this end, functional assays can be used.

In vitro assays with gene knockdown or deletion, where the expression of a gene is reduced or deleted, can be used to evaluate outcomes such as changes in cell

survival, proliferation, ability to differentiate etc., to further evaluate the processes the specific gene is involved in or influenced by. ALI culture is used to test for lung epithelial cell differentiation, where BCs are grown on a membrane with culture medium below but exposed to air on top to simulate airway conditions. This culture method has been used to show that loss of TP63 expression causes BCs to lose their ability to differentiate into goblet cells[249]. Organoids, re-cellularized ECM scaffolds and *in vivo* mouse models can also be used for gene knockdown assays, involving increasing complexity since the cell type to be examined will then also be able to interact with environmental structures and signals both from ECM and neighboring cells.

Primary vs immortalized cells

Functional assays using cell culture are very dependent on the ability of the cells in question to survive *in vitro*. *In vivo,* lung epithelial cells are attached to a basement membrane and used to interacting with the complex structure of the ECM and cooperating with neighboring cells, and therefore primary cells do not tend to thrive in culture assays that constitute a simpler environment. In addition, the process of dissociation from whole lung tissue pieces using mechanical and enzymatic forces to dislodge the cells, and the use of cryopreserving steps in between if necessary, are stressful to the cells and contribute to them not immediately succeeding to proliferate.

It took a long optimization process and many samples to establish culture conditions that even managed to produce the colony formation capacity results from primary BCs described in **paper I**. There we obtained an average of 7.4% of BCs able to form colonies *in vitro*, but the variance was high and although we were able to enrich this population with an additional FACS gate criterion, the original proliferative capacity of the cells in their intact organ environment may have been higher than after the cells were subjected to dissociation and FACS sorting.

An additional hurdle to overcome is the access to fresh samples; especially healthy lung tissue since good quality lungs from deceased organ donors are prioritized for lung transplantation, and voluntary biopsy donors can only provide small tissue pieces that contain a lower cell number than desired.

The heterogeneous nature of primary cells, and patient-patient variability, can also give assay readouts that are complex to interpret, since reaction to different conditions to be tested can be highly varied, and large number of repeated assays are required to be able to filter out this variation in signal.

An alternate solution to the difficult nature of functionally assessing primary cells is to use immortalized cell lines. These are cells derived from a single donor, and given the capacity to indefinitely proliferate and survive in culture through induced mutations that disrupt the normal cell cycle, or by increasing telomerase expression

to prevent senescence. Immortalized cells are available in large numbers and easily cultured, making them practical to use in larger scale assays such as toxicology studies or screening for therapeutic targets using molecular libraries. In contrast to primary cells, being genetically identical, they are also homogeneous in their response to such stimuli, which is simpler to interpret but does not give completely accurate readouts when compared to diverse, primary cell populations; not to mention the lack of patient-patient variability that does not get taken into account. In addition, many cell lines do not retain the full range of capabilities that cells exhibit *in vivo*, such as differentiation capacity or signaling molecule production, that are necessary to fully reflect the relevant cellular environment[250, 251].

A third possibility is to use *in vitro* expanded primary cells, i.e. performing several culture and passaging iterations to increase cell number and select for cells that grow well in culture. This means the cells remain genetically unaltered from their primary state, and expanded cells from various donors and disease profiles can be compared. However, these pre-culturing steps have been shown to alter cellular phenotype due to various factors inherent to *in vitro* culture, such as the composition of growth media inducing epigenetic changes occurring in the cells. Ruiz Garcia et al (2019)[34] reported that ALI culture of BCs in two different epithelial cell media solutions showed different differentiation patterns; one condition led to formation of ciliated cells and goblet cells, while the other skewed BCs toward club cell differentiation and no ciliated cells were detected. Another important note about pre-culture steps is the homogenization and increased cell cycling of BCs after passaging, as described in **paper I**. Gene expression may also become skewed compared to primary cells because of the nature of expansion culture, which more closely resembles a wound-healing situation than homeostasis.

The choice between which cells to use becomes necessary to consider on a case-by-case basis. Sometimes practical and logistical factors dictate what studies are possible to perform; nevertheless it is important to take all these aspects into account when designing an assay and interpreting the readout. With the field of organoids and bioengineered lung tissue making great advances, in the future hopefully the *in vivo* conditions of the lung airways will become more realistically simulated in the lab. This means the cells used in these assays will be less affected by an unnatural environment and thus provide a more relevant basis for studying epithelial homeostasis and regeneration.

Conclusions and future perspective

Paper I

- The primary basal cell population with colony-forming capacity can be further purified using $NGFR^+$ FSC^{high} FACS gates.
- Primary human BCs are heterogeneous and quiescent; intermediate and differentiation-primed cell states exist within the population. *In vitro* culture conditions increase BC homogeneity and changes gene expression.
- BCs from healthy and severe COPD patients exhibit a continuum of changing gene expression profiles. Healthy BC profiles were found in COPD tissue, promising future targets for endogenous regeneration.

Paper II

- Ciliated cells in IPF highly express pathways involved in ciliogenesis, as well as genes associated with cancer cell survival.
- FTL is downregulated in IPF ciliated cells, suggesting a connection between iron metabolism and IPF pathology.

Paper III

- Aging lung epithelial cells show signs of DNA damage, oncogene expression, and impaired immune cell signaling.
- Young and aged BCs differ in gene expression, but move toward a convergence point in expression profile when primed for differentiation.

Paper IV

- The BC is the cell of origin in squamous cell carcinoma tumors, and possibly acts as the cancer-initiating cell responsible for resistance to therapy and relapse.
- TKT is expressed by $KRT5^+$ SCC cells and should be investigated as a therapeutic target.

There are several promising research questions to pursue to make further use of the scRNA-seq data we have generated; I have mentioned some ideas for future studies in the discussion of the respective papers. In general, to help the epithelial regeneration field of research, a valuable yet perhaps underestimated avenue of study is to identify more cell surface markers to target with FACS antibodies. The ability to sort and purify each epithelial cell population from primary cell samples would be of great benefit in determining the role and capacity of different cell types. Especially for the rarer cell types, it is essential to be able to purify and enrich this population to have enough numbers to avoid their gene expression profiles getting lost in scRNA-seq data of multiple more common cell types. In addition, BCs with differing gene expression profiles should be tested for self-renewal and differentiation capacity separately, and this would be made convenient if surface FACS markers were identified for each BC subtype.

scRNA-seq can indicate signaling molecules that cells are expressing to communicate with their surroundings, but more information is needed about where the signaling cells are located and which cells they are communicating with. To gain better understanding of cell-cell interactions, recently developed spatial transcriptomic assays would be interesting to employ; this makes it possible to track the gene expression of single cells while still fixed in tissue, showing the location of each mRNA transcript in the cellular niche. Thus we could illuminate the influence of structural parameters such as basal/apical location of epithelial cells, and the presence and proximity to resident immune cells and other cells known to interact with epithelial cells such as MSCs and fibroblasts. This knowledge is necessary to gain an understanding of the niche as a whole and will help us better design both *in vitro* models and cell-based therapy.

In order to work toward stem cell-based therapy for lung disease, more knowledge is needed to correctly combine all necessary factors. We need to employ the necessary progenitor cells, and provide the right signals and niche environment to stimulate regeneration of functional tissue with all required cell types. For stem cell transplantation, we need to find a method to deliver the cells to the right location for engraftment, and finally we need to consider whether the diseased cells must be removed before the delivery of healthy stem cells in order to provide a "clean slate" to avoid relapse of diseased remodeling.

In conclusion, the aim outlined in this thesis was to study the proximal human lung epithelium, to better understand progenitor roles and mechanisms for regeneration. This was done in healthy tissue, to further our knowledge of the normal life cycle of the lung epithelium, and in various lung diseases, to characterize the differences in cell phenotype and behavior leading to pathological progression and epithelial remodeling. The vision of finding the right cell types, pathways and genes to target in order to specifically and efficiently treat patients with these diseases permeates this thesis and the conclusions drawn in each paper provides new insights as well as a basis for further evaluation and functional verification.

Populärvetenskaplig sammanfattning

Kronisk obstruktiv lungsjukdom (KOL) är den tredje största dödsorsaken i världen, enbart föregången av hjärt- och kärlsjukdom och stroke. Varför är denna sjukdom så vanlig, och vad ligger bakom den höga dödligheten? ”Rökning dödar” är välkänt, men 25-45% av de som får KOL har aldrig rökt tobak, och endast 15% av de som rökt hela livet får diagnosen. Kroniska lungsjukdomar som KOL och IPF (idiopatisk lungfibros) tros uppstå till följd av små skador i luftvägarna som inte läks på rätt sätt. Istället för att repareras med frisk vävnad, återbyggs luftvägarna felaktigt vilket leder till nedsatt lungfunktion. I KOL bryts cellväggarna ner och syreupptagning försämras, samtidigt som luftvägarna blir inflammerade och blockeras av slem. I IPF bildas istället ärrvävnad, vilket gör lungorna styva och man förmår inte längre att andas in och ut. Orsaken till dessa patologiska förändringar är inte alltid klar, men riskfaktorer som tobaksrökning, luftföroreningar och hög ålder tycks öka sannolikheten för att drabbas.

Vad är det som gör kroniska lungsjukdomar så dödliga? Idag finns inget botemedel för dessa sjukdomar, man kan bara behandla symptomen och i bästa fall sakta ner sjukdomsförloppet. För nuvarande är därför den enda långsiktiga lösningen att göra en lungtransplantation. Dock är detta alternativ bara möjligt för ett fåtal patienter, och ett stort kirurgiskt ingrepp innebär många risker, särskilt då de flesta av transplantationsmottagarna är äldre med underliggande sjukdomar och läkemedelsbehandlingar som komplicerar operationen. Därför finns ett stort behov av att utveckla mer effektiva behandlingar.

Kontaktytan mellan vår omgivning och kropp, där luften möter lungvävnaden, kallas epitel och består av ett flertal celltyper – alla med sin specifika funktion och roll. Epitelet är kroppens första försvar mot farliga partiklar i inandningsluften, därför är det den mest utsatta vävnaden i lungan och ofta först att ta skada. En frisk lunga har dock förmågan att läka sig själv när små skador sker i luftvägarna. Denna läkningsprocess, så kallad regenerering, sker genom att specifika lungstamceller börjar dela sig och sedan bilda de olika celltyper som normalt utgör frisk vävnad. Därmed repareras skadorna i epitelcellslagret. Denna regenerationsförmåga skulle kunna nyttjas för att utveckla nya behandlingsmetoder för kroniska lungsjukdomar – stamcellsterapi utgör här en lovande möjlighet. Men för att veta hur man bäst skall använda stamcellerna krävs djupare kunskap om lungans biologi och de mekanismer som styr sjukdomsförlopp.

För att förstå hur kroniska lungsjukdomar som KOL och IPF uppstår, krävs det en ökad insikt i vad som händer i epitelets celltyper under sjukdomsförloppet. Cellernas beteende är kopplat till vilka gener de uttrycker, s.k. genuttryck. En frisk epitelcell kan ha en genuttrycksprofil och dess sjuka motsvarighet en annan. Med högteknologiska instrument kan man studera hur genuttrycket i enskilda celler förändras, och skiljer sig åt, mellan frisk och sjuk vävnad. Denna nya kunskap kan ge underlag för utveckling av stamcellsterapeutiska behandlingar.

Att utveckla stamcellsterapi är komplicerat, det är nödvändigt att ge stamcellerna rätt förutsättningar och signaler för att de skall kunna regenerera frisk vävnad. Att kartlägga dessa signaler har varit min forskningsinriktning under doktorandutbildningen, med målet att förstå i detalj hur lungans egna självläkning regleras. Dessutom har jag fokuserat på skillnaderna i genuttryck mellan friska och sjuka epitelceller för att utreda var sårläkningen går fel och ger upphov till sjukdomar kopplade till förändringar i epitelet.

I **studie I** kartlade jag genuttrycket i celler från friska och KOL-sjuka luftvägar. Jag undersökte specifikt basalceller, som är stamcellerna vars roll är att bilda de andra celltyperna som epitelet består av. Detta för att felreglering av basalceller misstänks ligga till grund för förändringarna som orsakar sjukdomen.

Genom att sortera basalcellerna baserat på genuttryck bildades ett spektrum. På ena sidan av spektrumet återfanns mest friska celler medan andra sidan utgjordes av mest sjuka celler. På detta sätt tror vi att vi kan följa utvecklingen av sjukdomen i basalcellerna på gennivå, till exempel ökade uttrycket av gener kopplade till stress och syrebrist gradvis genom spektrumet.

Genom att identifiera och reglera de gener som styr basalceller till att utveckla felaktig epitelsammansättning, skulle vi teoretiskt kunna motverka sjukdomsförloppet. Ett intressant fenomen jag kunde observera var att de sjuka vävnadsproven faktiskt även innehöll basalceller vars genuttryck var snarlikt det i basalceller från frisk vävnad. Detta innebär att KOL-patienter fortfarande har en del egna friska stamceller kvar, en potential som skulle kunna nyttjas för att stimulera regeneration av epitelet utan att behöva transplantera stamceller från donatorer.

I **studie II** gjorde jag en liknande jämförelse mellan genuttryck i epitelceller från friska och IPF-patienters luftvägar. Det är en mer ovanlig sjukdom än KOL, men symptomen är mycket allvarliga och patienter som får diagnosen IPF lever i genomsnitt bara 2-5 år. Här fanns den största skillnaden i genuttryck bland cilierade celler, de celler i epitelet med rörliga flimmerhår på ytan vars huvuduppgift är att transportera ut slem ur lungan. Cilierade celler kan dessutom skicka signaler till cellerna i sin omgivning vilket medverkar till frisk sårläkning, därför är deras roll i IPF intressant att undersöka.

Jag upptäckte att cilierade celler från IPF-proverna uttryckte mycket lägre mängd av ferritin-protein (FTL) jämfört med friska celler. Det är intressant därför att FTL

reglerar mängden fritt järn i och utanför kroppens celler, och ansamling av för mycket järn har tidigare visats vanligt förekommande i IPF-lungor. För höga nivåer av järn i lungan ökar risken för bildning av fria radikaler, vilket skadar epitelcellerna och kan bidra till sjukdomen. Därför är det av intresse att undersöka kopplingen mellan IPF och FTL i epitelcellerna, för att se om det är en möjlig angreppspunkt för att bota eller förhindra sjukdomsutvecklingen.

I **studie III** gjorde jag en jämförelse mellan genuttryck i epitelceller från friska personer uppdelade i två åldersgrupper (23-40 år och 64-75 år). Detta då ålder är en stor riskfaktor för såväl kronisk lungsjukdom som lungcancer. Målet var att kartlägga förändringar i epitelet som uppstår med ålder, för att försöka förstå på cellnivå vad som bidrar till att risken för lungsjukdom ökar.

Resultaten visade att äldre epitelceller, både basalceller och cellsorterna de utvecklas till, uttryckte i högre grad gener med koppling till skador i DNA:t, men även cancer-relaterade gener. Detta innebär att åldringsprocessen förändrar epitelcellernas genuttrycksprofil, vilket troligen påverkar deras förmåga att effektivt läka sår. Detta utgör sannolikt kopplingen till åldersrelaterad kronisk lungsjukdom som ofta uppstår genom en felaktig läkningsprocess. Därför är det intressant att vidare utvärdera effekten av dessa ålders-beroende gener på epitelcellernas funktion.

I **studie IV** fick jag möjligheten att studera genuttryck i lungcancertumörer. Cancerpatienter utvecklar ofta en resistens till kemoterapi och kan få återfall efter behandling. Det är sannolikt att de nya tumörerna utvecklas från en celltyp som går kan gå in i ett tillstånd av dvala. Det skyddar cellerna mot cellgiftsbehandlingen, och efteråt blir de aktiva och återbildar tumören.

Eftersom basalceller har uppvisat överdriven celldelning till följd av stress från rökning och luftförorening, misstänks de vara ursprunget till tumörer i luftvägarna. När jag jämförde genuttrycket i tumörceller med friska epitelceller, hade tumörcellerna mest gemensamt med basalceller. Detta bekräftade vår teori att tumörerna utvecklats från felreglerade basalceller. Genom vidare studier på basalcellerna i tumören skulle vi i framtiden kunna utveckla metoder att påverka dem för att specifikt stoppa tumöråterbildningen och på så sätt effektivt kunna behandla lungcancerpatienter.

Sammanfattningsvis handlar denna avhandling om lungepitelets förmåga till självläkning, och hur omgivningsfaktorer kan få regenerationsmekanismer att slå slint. Mina fyra studier har tillsammans genererat en stor mängd data som utgör en bas för fortsatt forskning, med intressanta indikationer om hur basalcellen kan komma att nyttjas i framtiden som en viktig komponent i ett stamcellsbaserat botemedel för kronisk lungsjukdom.

Acknowledgements

I would first like to thank my supervisor **Mattias**, who gave me the exciting, and challenging, opportunity to be the first PhD student to take on his newly started lung stem cell project. We encountered many challenges during these years, but somehow we worked out a plan in the end and my publications and thesis suddenly came together. Thank you for making it possible for me to complete my PhD studies, despite the time it took and the concessions we had to make along the way.

Next, thank you **Pavan** for joining our little lung group and injecting some sorely needed optimistic outlook and expertise! I could not have published what I did without you, and I am grateful that you stepped in and helped with my work, sometimes putting your own postdoc project on the back burner. Thank you as well for all the pleasant coffee chats, and for your encouragement and advice on my journey to dissertation.

Fellow group member **Simon**, we worked in completely different fields and did not often discuss our projects, but thanks for all the interesting talks on philosophy, psychology and everything else. Thank you to past group member **Alex D**, my first supervisor as a master student, who introduced me to research and taught me to work independently in the lab. Thanks as well to other past group members, **Aurélie** and **Karolina.**

To some of my fellow PhD students and colleagues **Svetlana**, **Ludwig**, **Alex M**, **Sarah**, **Roman**, **Kris**, **Alexandra**, **Anna**, **Leal**, **Els**, **Melissa**, thank you for being such nice people and making me feel less alone in my PhD challenges. Thank you **Christine**, for your work in the research school and for supporting me together with all other students.

The environment at A12 & B12, and Lund Stem Cell Center as a whole, is a dynamic and collaborative workplace, full of scientific resources. I would therefore like to thank all PIs connected to these departments together with the SCC administrative team for maintaining said workplace, as well as organizing social events such as Christmas dinners, retreats and seminars.

I would like to thank my co-supervisor **Gunilla**, for providing collaboration and scientific exchange with other lung researchers. I did not make as much use as I should have of your expertise and supervision, but thank you for always being helpful and encouraging during our meetings and discussions.

Speaking of the lung biology group: thank you **Barbora** and **Anna**, for helping me with immunofluorescence analysis for my first and second paper respectively. A special thanks to **Lena** and **Annika**, who collected lung tissue samples with and for me, and always helped to find samples and fixed tissue slides for my staining experiments.

I am sending big hugs and thanks to my close friends from LTH: **Stina**, **Ida**, **Alisa**, **Emma** and **Kajsa**. Thank you for the company at study sessions during exam periods, without which I probably would have failed some of the courses. Thank you as well for all the crazy fun times we had together inside and outside the classroom. I am so happy for all your accomplishments since we graduated together! You have all been supportive and commiserative during my PhD journey and it's always wonderful to see you, even though it's not as often anymore. Some of you should never have left Skåne!

Finally, I would like to thank my family. **Susanna and Karsten**, thank you for being such supporting and loving parents. You have taught me so much, and I am especially glad that you contributed to my passion for exercise, which helps me to mentally and physically enjoy life. My thanks to you, and to my brother **Stefan**, for believing I can do anything even when I don't.

Thank you to my grandfather **Lars**, for listening on the phone with interest to mundane life anecdotes as well as detailed research explanations. I would also like to show appreciation to my grandparents **Catarina** and **Bernhard**. And thank you **Sanne** and **Lasse**, my wonderful parents-in-law, for welcoming me into their family and enriching my inner circle.

I cannot express how grateful I am to my husband **Emil**. You have been part of my life since before I started my PhD and your presence makes every day better. Thank you for your support, comfort, and help through big and small problems, PhD related and otherwise. Thank you for making my spare time so great that work setbacks don't matter!

References

1. Raslan, A.A. and J.K. Yoon, *WNT Signaling in Lung Repair and Regeneration.* Mol Cells, 2020. **43**(9): p. 774-783.
2. Sun, X., et al., *A census of the lung: CellCards from LungMAP.* Developmental Cell, 2022. **57**(1): p. 112-145.e2.
3. Mouw, J.K., G. Ou, and V.M. Weaver, *Extracellular matrix assembly: a multiscale deconstruction.* Nat Rev Mol Cell Biol, 2014. **15**(12): p. 771-85.
4. Murray, J.F., *The structure and function of the lung.* Int J Tuberc Lung Dis, 2010. **14**(4): p. 391-6.
5. Ratnovsky, A. and D. Elad, *Anatomical model of the human trunk for analysis of respiratory muscles mechanics.* Respiratory Physiology & Neurobiology, 2005. **148**(3): p. 245-262.
6. Crapo, J.D., et al., *Cell number and cell characteristics of the normal human lung.* Am Rev Respir Dis, 1982. **126**(2): p. 332-7.
7. Jeffery, P.K., *Morphologic features of airway surface epithelial cells and glands.* Am Rev Respir Dis, 1983. **128**(2 Pt 2): p. S14-20.
8. Carlier, F.M., C. de Fays, and C. Pilette, *Epithelial Barrier Dysfunction in Chronic Respiratory Diseases.* Front Physiol, 2021. **12**: p. 691227.
9. Mori, K., et al., *Synergistic Proinflammatory Responses by IL-17A and Toll-Like Receptor 3 in Human Airway Epithelial Cells.* PLoS One, 2015. **10**(9): p. e0139491.
10. Rock, J.R., S.H. Randell, and B.L. Hogan, *Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling.* Dis Model Mech, 2010. **3**(9-10): p. 545-56.
11. Boers, J.E., A.W. Ambergen, and F.B. Thunnissen, *Number and proliferation of basal and parabasal cells in normal human airway epithelium.* Am J Respir Crit Care Med, 1998. **157**(6 Pt 1): p. 2000-6.
12. Plasschaert, L.W., et al., *A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte.* Nature, 2018. **560**(7718): p. 377-381.
13. Montoro, D.T., et al., *A revised airway epithelial hierarchy includes CFTR-expressing ionocytes.* Nature, 2018. **560**(7718): p. 319-324.
14. Ouadah, Y., et al., *Rare Pulmonary Neuroendocrine Cells Are Stem Cells Regulated by Rb, p53, and Notch.* Cell, 2019. **179**(2): p. 403-416.e23.
15. Branchfield, K., et al., *Pulmonary neuroendocrine cells function as airway sensors to control lung immune response.* Science, 2016. **351**(6274): p. 707-10.

16. Bankova, L.G., et al., *The cysteinyl leukotriene 3 receptor regulates expansion of IL-25–producing airway brush cells leading to type 2 inflammation.* Science Immunology, 2018. **3**(28): p. eaat9453.
17. Rock, J.R., et al., *Basal cells as stem cells of the mouse trachea and human airway epithelium.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12771-5.
18. Balasooriya, G.I., et al., *FGFR2 is required for airway basal cell self-renewal and terminal differentiation.* Development, 2017. **144**(9): p. 1600-1606.
19. Haas, M., et al., *ΔN-Tp63 Mediates Wnt/β-Catenin-Induced Inhibition of Differentiation in Basal Stem Cells of Mucociliary Epithelia.* Cell Rep, 2019. **28**(13): p. 3338-3352.e6.
20. Jensen-Cody, C.W., et al., *Lef-1 controls cell cycle progression in airway basal cells to regulate proliferation and differentiation.* Stem Cells, 2021. **39**(9): p. 1221-1235.
21. Tsao, P.N., et al., *Notch signaling controls the balance of ciliated and secretory cell fates in developing airways.* Development, 2009. **136**(13): p. 2297-307.
22. Pardo-Saganta, A., et al., *Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations.* Cell Stem Cell, 2015. **16**(2): p. 184-97.
23. Lafkas, D., et al., *Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung.* Nature, 2015. **528**(7580): p. 127-31.
24. Carraro, G., et al., *Single-Cell Reconstruction of Human Basal Cell Diversity in Normal and Idiopathic Pulmonary Fibrosis Lungs.* Am J Respir Crit Care Med, 2020. **202**(11): p. 1540-1550.
25. Goldfarbmuren, K.C., et al., *Dissecting the cellular specificity of smoking effects and reconstructing lineages in the human airway epithelium.* Nat Commun, 2020. **11**(1): p. 2485.
26. Zuo, W.L., et al., *Ontogeny and Biology of Human Small Airway Epithelial Club Cells.* Am J Respir Crit Care Med, 2018. **198**(11): p. 1375-1388.
27. Ruiz, E.J., F. Oeztuerk-Winder, and J.J. Ventura, *A paracrine network regulates the cross-talk between human lung stem cells and the stroma.* Nat Commun, 2014. **5**: p. 3175.
28. Peng, T., et al., *Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration.* Nature, 2015. **526**(7574): p. 578-82.
29. Byers, D.E., et al., *Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease.* J Clin Invest, 2013. **123**(9): p. 3967-82.
30. Ghosh, M., et al., *Human tracheobronchial basal cells. Normal versus remodeling/repairing phenotypes in vivo and in vitro.* Am J Respir Cell Mol Biol, 2013. **49**(6): p. 1127-34.
31. Yang, J., et al., *Smoking-Dependent Distal-to-Proximal Repatterning of the Adult Human Small Airway Epithelium.* Am J Respir Crit Care Med, 2017. **196**(3): p. 340-352.
32. Travaglini, K.J., et al., *A molecular cell atlas of the human lung from single-cell RNA sequencing.* Nature, 2020. **587**(7835): p. 619-625.

33. Vieira Braga, F.A., et al., *A cellular census of human lungs identifies novel cell states in health and in asthma.* Nat Med, 2019. **25**(7): p. 1153-1163.
34. Ruiz García, S., et al., *Novel dynamics of human mucociliary differentiation revealed by single-cell RNA sequencing of nasal epithelial cultures.* Development, 2019. **146**(20).
35. Deprez, M., et al., *A Single-Cell Atlas of the Human Healthy Airways.* American Journal of Respiratory and Critical Care Medicine, 2020. **202**(12): p. 1636-1645.
36. Crystal, R.G., *Airway basal cells. The "smoking gun" of chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2014. **190**(12): p. 1355-62.
37. Ghosh, M., et al., *Exhaustion of Airway Basal Progenitor Cells in Early and Established Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med, 2018. **197**(7): p. 885-896.
38. Wu, M., et al., *Roles of airway basal stem cells in lung homeostasis and regenerative medicine.* Respiratory Research, 2022. **23**(1): p. 122.
39. Rawlins, E.L. and B.L. Hogan, *Ciliated epithelial cell lifespan in the mouse trachea and lung.* Am J Physiol Lung Cell Mol Physiol, 2008. **295**(1): p. L231-4.
40. KNIGHT, D.A. and S.T. HOLGATE, *The airway epithelium: Structural and functional properties in health and disease.* Respirology, 2003. **8**(4): p. 432-446.
41. Parekh, K.R., et al., *Stem cells and lung regeneration.* Am J Physiol Cell Physiol, 2020. **319**(4): p. C675-c693.
42. Tata, P.R., et al., *Dedifferentiation of committed epithelial cells into stem cells in vivo.* Nature, 2013. **503**(7475): p. 218-23.
43. Rawlins, E.L., et al., *The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium.* Cell Stem Cell, 2009. **4**(6): p. 525-34.
44. Rostami, M.R., et al., *Smoking shifts human small airway epithelium club cells toward a lesser differentiated population.* NPJ Genom Med, 2021. **6**(1): p. 73.
45. Alysandratos, K.D., M.J. Herriges, and D.N. Kotton, *Epithelial Stem and Progenitor Cells in Lung Repair and Regeneration.* Annu Rev Physiol, 2021. **83**: p. 529-550.
46. Decramer, M., W. Janssens, and M. Miravitlles, *Chronic obstructive pulmonary disease.* Lancet, 2012. **379**(9823): p. 1341-51.
47. Cho, S.J. and H.W. Stout-Delgado, *Aging and Lung Disease.* Annu Rev Physiol, 2020. **82**: p. 433-459.
48. López-Otín, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
49. Meiners, S., O. Eickelberg, and M. Königshoff, *Hallmarks of the ageing lung.* European Respiratory Journal, 2015. **45**(3): p. 807-827.
50. Issa, J.-P., *Aging and epigenetic drift: a vicious cycle.* The Journal of Clinical Investigation, 2014. **124**(1): p. 24-29.

51. Mercado, N., K. Ito, and P.J. Barnes, *Accelerated ageing of the lung in COPD: new concepts.* Thorax, 2015. **70**(5): p. 482-9.
52. Budinger, G.R.S., et al., *The Intersection of Aging Biology and the Pathobiology of Lung Diseases: A Joint NHLBI/NIA Workshop.* J Gerontol A Biol Sci Med Sci, 2017. **72**(11): p. 1492-1500.
53. Shmulevich, R. and V. Krizhanovsky, *Cell Senescence, DNA Damage, and Metabolism.* Antioxid Redox Signal, 2021. **34**(4): p. 324-334.
54. Aghali, A., et al., *Cellular Senescence in Aging Lungs and Diseases.* Cells, 2022. **11**(11).
55. Goodwin, K., C. Viboud, and L. Simonsen, *Antibody response to influenza vaccination in the elderly: A quantitative review.* Vaccine, 2006. **24**(8): p. 1159-1169.
56. Chen, Y., et al., *Aging in COVID-19: Vulnerability, immunity and intervention.* Ageing Res Rev, 2021. **65**: p. 101205.
57. Schneider, J.L., et al., *The aging lung: Physiology, disease, and immunity.* Cell, 2021. **184**(8): p. 1990-2019.
58. World Health Organization. *Chronic obstructive pulmonary disease (COPD)*. 2022 2022, June 20]; Available from: https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd).
59. Fletcher, C. and R. Peto, *The natural history of chronic airflow obstruction.* British Medical Journal, 1977. **1**(6077): p. 1645-1648.
60. Salvi, S.S. and P.J. Barnes, *Chronic obstructive pulmonary disease in non-smokers.* The Lancet, 2009. **374**(9691): p. 733-743.
61. Raftery, A.E., et al., *Bayesian probabilistic population projections for all countries.* Proc Natl Acad Sci U S A, 2012. **109**(35): p. 13915-21.
62. Vogelmeier, C.F., et al., *Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report. GOLD Executive Summary.* American Journal of Respiratory and Critical Care Medicine, 2017. **195**(5): p. 557-582.
63. Riley, C.M. and F.C. Sciurba, *Diagnosis and Outpatient Management of Chronic Obstructive Pulmonary Disease: A Review.* Jama, 2019. **321**(8): p. 786-797.
64. Todd, J.L. and S.M. Palmer, *Lung transplantation in advanced COPD: is it worth it?* Semin Respir Crit Care Med, 2010. **31**(3): p. 365-72.
65. Singh, D., et al., *Current Controversies in the Pharmacological Treatment of Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med, 2016. **194**(5): p. 541-9.
66. Viniol, C. and C.F. Vogelmeier, *Exacerbations of COPD.* European Respiratory Review, 2018. **27**(147): p. 170103.
67. Inci, I., *Lung transplantation for emphysema.* Ann Transl Med, 2020. **8**(21): p. 1473.
68. Kulkarni, T., et al., *Matrix Remodeling in Pulmonary Fibrosis and Emphysema.* Am J Respir Cell Mol Biol, 2016. **54**(6): p. 751-60.

69. Tuder, R.M., et al., *Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade.* Am J Respir Cell Mol Biol, 2003. **29**(1): p. 88-97.
70. Hogg, J.C., et al., *The Nature of Small-Airway Obstruction in Chronic Obstructive Pulmonary Disease.* New England Journal of Medicine, 2004. **350**(26): p. 2645-2653.
71. McDonough, J.E., et al., *Small-Airway Obstruction and Emphysema in Chronic Obstructive Pulmonary Disease.* New England Journal of Medicine, 2011. **365**(17): p. 1567-1575.
72. Innes, A.L., et al., *Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction.* Chest, 2006. **130**(4): p. 1102-8.
73. Shaykhiev, R., *Emerging biology of persistent mucous cell hyperplasia in COPD.* Thorax, 2019. **74**(1): p. 4-6.
74. Sprott, R.F., et al., *Flagellin shifts 3D bronchospheres towards mucus hyperproduction.* Respir Res, 2020. **21**(1): p. 222.
75. Yaghi, A., et al., *Ciliary beating is depressed in nasal cilia from chronic obstructive pulmonary disease subjects.* Respir Med, 2012. **106**(8): p. 1139-47.
76. Rigden, H.M., et al., *Squamous Metaplasia Is Increased in the Bronchial Epithelium of Smokers with Chronic Obstructive Pulmonary Disease.* PLoS One, 2016. **11**(5): p. e0156009.
77. Shaykhiev, R., et al., *Cigarette smoking reprograms apical junctional complex molecular architecture in the human airway epithelium in vivo.* Cell Mol Life Sci, 2011. **68**(5): p. 877-92.
78. Barnes, P.J., *Inflammatory mechanisms in patients with chronic obstructive pulmonary disease.* Journal of Allergy and Clinical Immunology, 2016. **138**(1): p. 16-27.
79. Evans, M.J., et al., *Cellular and molecular characteristics of basal cells in airway epithelium.* Exp Lung Res, 2001. **27**(5): p. 401-15.
80. Sohal, S.S., et al., *Reticular basement membrane fragmentation and potential epithelial mesenchymal transition is exaggerated in the airways of smokers with chronic obstructive pulmonary disease.* Respirology, 2010. **15**(6): p. 930-8.
81. Mahmood, M.Q., et al., *Transforming growth factor (TGF) β1 and Smad signalling pathways: A likely key to EMT-associated COPD pathogenesis.* Respirology, 2017. **22**(1): p. 133-140.
82. Ryan, D.M., et al., *Smoking dysregulates the human airway basal cell transcriptome at COPD risk locus 19q13.2.* PLoS One, 2014. **9**(2): p. e88051.
83. Shaykhiev, R., et al., *EGF shifts human airway basal cell fate toward a smoking-associated airway epithelial phenotype.* Proc Natl Acad Sci U S A, 2013. **110**(29): p. 12102-7.
84. Gordon, S.B., et al., *Respiratory risks from household air pollution in low and middle income countries.* Lancet Respir Med, 2014. **2**(10): p. 823-60.

85. Agustí, A. and J.C. Hogg, *Update on the Pathogenesis of Chronic Obstructive Pulmonary Disease.* New England Journal of Medicine, 2019. **381**(13): p. 1248-1256.
86. Raghavan, D., A. Varkey, and T. Bartter, *Chronic obstructive pulmonary disease: the impact of gender.* Curr Opin Pulm Med, 2017. **23**(2): p. 117-123.
87. Gilkes, A., et al., *Ethnic differences in smoking intensity and COPD risk: an observational study in primary care.* NPJ Prim Care Respir Med, 2017. **27**(1): p. 50.
88. Hansel, N.N., et al., *Racial differences in CT phenotypes in COPD.* Copd, 2013. **10**(1): p. 20-7.
89. Martinez, F.D., *Early-Life Origins of Chronic Obstructive Pulmonary Disease.* New England Journal of Medicine, 2016. **375**(9): p. 871-878.
90. Lange, P., et al., *Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease.* New England Journal of Medicine, 2015. **373**(2): p. 111-122.
91. Sheikh, K., H.O. Coxson, and G. Parraga, *This is what COPD looks like.* Respirology, 2016. **21**(2): p. 224-36.
92. Hoffman, E.A., et al., *Pulmonary CT and MRI phenotypes that help explain chronic pulmonary obstruction disease pathophysiology and outcomes.* J Magn Reson Imaging, 2016. **43**(3): p. 544-57.
93. Raghu, G., et al., *Incidence and prevalence of idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 2006. **174**(7): p. 810-6.
94. Ley, B., H.R. Collard, and T.E. King, Jr., *Clinical course and prediction of survival in idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 2011. **183**(4): p. 431-40.
95. Wolters, P.J., et al., *Time for a change: is idiopathic pulmonary fibrosis still idiopathic and only fibrotic?* The Lancet Respiratory Medicine, 2018. **6**(2): p. 154-160.
96. Lee, J.S., S. McLaughlin, and H.R. Collard, *Comprehensive care of the patient with idiopathic pulmonary fibrosis.* Curr Opin Pulm Med, 2011. **17**(5): p. 348-54.
97. Ruwanpura, S.M., B.J. Thomas, and P.G. Bardin, *Pirfenidone: Molecular Mechanisms and Potential Clinical Applications in Lung Disease.* Am J Respir Cell Mol Biol, 2020. **62**(4): p. 413-422.
98. Wollin, L., et al., *Potential of nintedanib in treatment of progressive fibrosing interstitial lung diseases.* Eur Respir J, 2019. **54**(3).
99. Sgalla, G., et al., *Idiopathic pulmonary fibrosis: pathogenesis and management.* Respir Res, 2018. **19**(1): p. 32.
100. King, T.E., Jr., et al., *A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis.* N Engl J Med, 2014. **370**(22): p. 2083-92.
101. Richeldi, L., et al., *Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis.* N Engl J Med, 2014. **370**(22): p. 2071-82.

102. Galli, J.A., et al., *Pirfenidone and nintedanib for pulmonary fibrosis in clinical practice: Tolerability and adverse drug reactions.* Respirology, 2017. **22**(6): p. 1171-1178.
103. Weill, D., et al., *A consensus document for the selection of lung transplant candidates: 2014—An update from the Pulmonary Transplantation Council of the International Society for Heart and Lung Transplantation.* The Journal of Heart and Lung Transplantation, 2015. **34**(1): p. 1-15.
104. Laporta Hernandez, R., et al., *Lung Transplantation in Idiopathic Pulmonary Fibrosis.* Med Sci (Basel), 2018. **6**(3).
105. Spagnolo, P., et al., *Idiopathic pulmonary fibrosis: Disease mechanisms and drug development.* Pharmacol Ther, 2021. **222**: p. 107798.
106. Jones, M.G., et al., *Three-dimensional characterization of fibroblast foci in idiopathic pulmonary fibrosis.* JCI Insight, 2016. **1**(5).
107. Seibold, M.A., et al., *The Idiopathic Pulmonary Fibrosis Honeycomb Cyst Contains A Mucocilary Pseudostratified Epithelium.* PLOS ONE, 2013. **8**(3): p. e58658.
108. Nureki, S.I., et al., *Expression of mutant Sftpc in murine alveolar epithelia drives spontaneous lung fibrosis.* J Clin Invest, 2018. **128**(9): p. 4008-4024.
109. Yao, C., et al., *Senescence of Alveolar Type 2 Cells Drives Progressive Pulmonary Fibrosis.* Am J Respir Crit Care Med, 2021. **203**(6): p. 707-717.
110. Verleden, S.E., et al., *Small airways pathology in idiopathic pulmonary fibrosis: a retrospective cohort study.* Lancet Respir Med, 2020. **8**(6): p. 573-584.
111. Seibold, M.A., et al., *A common MUC5B promoter polymorphism and pulmonary fibrosis.* N Engl J Med, 2011. **364**(16): p. 1503-12.
112. Wiscombe, S., I.A. Forrest, and A.J. Simpson, *IPF: time for the (ciliary) beat generation?* Thorax, 2013. **68**(12): p. 1088-9.
113. Cigna, N., et al., *The Hedgehog System Machinery Controls Transforming Growth Factor-β–Dependent Myofibroblastic Differentiation in Humans: Involvement in Idiopathic Pulmonary Fibrosis.* The American Journal of Pathology, 2012. **181**(6): p. 2126-2137.
114. Xu, Y., et al., *Single-cell RNA sequencing identifies diverse roles of epithelial cells in idiopathic pulmonary fibrosis.* JCI Insight, 2016. **1**(20): p. e90558.
115. Adams, T.S., et al., *Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis.* Science Advances, 2020. **6**(28): p. eaba1983.
116. Habermann, A.C., et al., *Single-cell RNA sequencing reveals profibrotic roles of distinct epithelial and mesenchymal lineages in pulmonary fibrosis.* Science Advances, 2020. **6**(28): p. eaba1972.
117. Goodwin, K., et al., *Basal Cell-Extracellular Matrix Adhesion Regulates Force Transmission during Tissue Morphogenesis.* Developmental Cell, 2016. **39**(5): p. 611-625.

118. Bilodeau, C., et al., *TP63 basal cells are indispensable during endoderm differentiation into proximal airway cells on acellular lung scaffolds.* NPJ Regen Med, 2021. **6**(1): p. 12.
119. Xie, T., et al., *Abnormal respiratory progenitors in fibrotic lung injury.* Stem Cell Res Ther, 2022. **13**(1): p. 64.
120. Irvin, C.G. and J.H. Bates, *Measuring the lung function in the mouse: the challenge of size.* Respir Res, 2003. **4**(1): p. 4.
121. Basil, M.C., et al., *The Cellular and Physiological Basis for Lung Repair and Regeneration: Past, Present, and Future.* Cell Stem Cell, 2020. **26**(4): p. 482-502.
122. Kim, C.F., et al., *Identification of bronchioalveolar stem cells in normal lung and lung cancer.* Cell, 2005. **121**(6): p. 823-35.
123. Liu, Q., et al., *Lung regeneration by multipotent stem cells residing at the bronchioalveolar-duct junction.* Nat Genet, 2019. **51**(4): p. 728-738.
124. Salwig, I., et al., *Bronchioalveolar stem cells are a main source for regeneration of distal lung epithelia in vivo.* Embo j, 2019. **38**(12).
125. Groneberg, D.A. and K.F. Chung, *Models of chronic obstructive pulmonary disease.* Respir Res, 2004. **5**(1): p. 18.
126. Rydell-Törmänen, K. and J.R. Johnson, *The Applicability of Mouse Models to the Study of Human Disease.* Methods Mol Biol, 2019. **1940**: p. 3-22.
127. Bartalesi, B., et al., *Different lung responses to cigarette smoke in two strains of mice sensitive to oxidants.* European Respiratory Journal, 2005. **25**(1): p. 15-22.
128. Williams, K. and J. Roman, *Studying human respiratory disease in animals – role of induced and naturally occurring models.* The Journal of Pathology, 2016. **238**(2): p. 220-232.
129. Janoff, A., et al., *Lung injury induced by leukocytic proteases.* Am J Pathol, 1979. **97**(1): p. 111-36.
130. Mizgerd, J.P. and S.J. Skerrett, *Animal models of human pneumonia.* American Journal of Physiology-Lung Cellular and Molecular Physiology, 2008. **294**(3): p. L387-L398.
131. Tanner, L. and A.B. Single, *Animal Models Reflecting Chronic Obstructive Pulmonary Disease and Related Respiratory Disorders: Translating Pre-Clinical Data into Clinical Relevance.* Journal of Innate Immunity, 2020. **12**(3): p. 203-225.
132. Ng, B., et al., *Interleukin-11 is a therapeutic target in idiopathic pulmonary fibrosis.* Sci Transl Med, 2019. **11**(511).
133. Schafer, M.J., et al., *Cellular senescence mediates fibrotic pulmonary disease.* Nat Commun, 2017. **8**: p. 14532.
134. Rangarajan, S., et al., *Metformin reverses established lung fibrosis in a bleomycin model.* Nat Med, 2018. **24**(8): p. 1121-1127.
135. Tashiro, J., et al., *Exploring Animal Models That Resemble Idiopathic Pulmonary Fibrosis.* Front Med (Lausanne), 2017. **4**: p. 118.
136. Chapman, R.W., *Canine models of asthma and COPD.* Pulm Pharmacol Ther, 2008. **21**(5): p. 731-42.

137. Theisen, M.M., et al., *Ventral recumbency is crucial for fast and safe orotracheal intubation in laboratory swine.* Lab Anim, 2009. **43**(1): p. 96-101.
138. Asgharian, B., et al., *Development of a rhesus monkey lung geometry model and application to particle deposition in comparison to humans.* Inhal Toxicol, 2012. **24**(13): p. 869-99.
139. Lewis, D.I., *Animal experimentation: implementation and application of the 3Rs.* Emerg Top Life Sci, 2019. **3**(6): p. 675-679.
140. Müller, A.M., S. Huppertz, and R. Henschler, *Hematopoietic Stem Cells in Regenerative Medicine: Astray or on the Path?* Transfus Med Hemother, 2016. **43**(4): p. 247-254.
141. Sivandzade, F. and L. Cucullo, *Regenerative Stem Cell Therapy for Neurodegenerative Diseases: An Overview.* Int J Mol Sci, 2021. **22**(4).
142. Nourian Dehkordi, A., et al., *Skin tissue engineering: wound healing based on stem-cell-based therapeutic strategies.* Stem Cell Res Ther, 2019. **10**(1): p. 111.
143. Mousaei Ghasroldasht, M., et al., *Application of mesenchymal stem cells to enhance non-union bone fracture healing.* J Biomed Mater Res A, 2019. **107**(2): p. 301-311.
144. Zakrzewski, W., et al., *Stem cells: past, present, and future.* Stem Cell Res Ther, 2019. **10**(1): p. 68.
145. Lovell-Badge, R., et al., *ISSCR Guidelines for Stem Cell Research and Clinical Translation: The 2021 update.* Stem Cell Reports, 2021. **16**(6): p. 1398-1408.
146. Li, X., et al., *Administration of signalling molecules dictates stem cell homing for in situ regeneration.* J Cell Mol Med, 2017. **21**(12): p. 3162-3177.
147. Ikonomou, L., et al., *Translating Basic Research into Safe and Effective Cell-based Treatments for Respiratory Diseases.* Annals of the American Thoracic Society, 2019. **16**(6): p. 657-668.
148. Ghosh, M., et al., *Transplantation of Airway Epithelial Stem/Progenitor Cells: A Future for Cell-Based Therapy.* Am J Respir Cell Mol Biol, 2017. **56**(1): p. 1-10.
149. Ma, Q., et al., *Regeneration of functional alveoli by adult human SOX9(+) airway basal cell transplantation.* Protein Cell, 2018. **9**(3): p. 267-282.
150. McCauley, K.B., et al., *Single-Cell Transcriptomic Profiling of Pluripotent Stem Cell-Derived SCGB3A2+ Airway Epithelium.* Stem Cell Reports, 2018. **10**(5): p. 1579-1595.
151. Jacob, A., et al., *Differentiation of Human Pluripotent Stem Cells into Functional Lung Alveolar Epithelial Cells.* Cell Stem Cell, 2017. **21**(4): p. 472-488.e10.
152. McCauley, K.B., et al., *Efficient Derivation of Functional Human Airway Epithelium from Pluripotent Stem Cells via Temporal Regulation of Wnt Signaling.* Cell Stem Cell, 2017. **20**(6): p. 844-857.e6.

153. Wagner, D.E., et al., *Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Disease 2019.* ERJ Open Res, 2020. **6**(4).
154. Weiss, D.J., et al., *A placebo-controlled, randomized trial of mesenchymal stem cells in COPD.* Chest, 2013. **143**(6): p. 1590-1598.
155. Tzouvelekis, A., et al., *A prospective, non-randomized, no placebo-controlled, phase Ib clinical trial to study the safety of the adipose derived stromal cells-stromal vascular fraction in idiopathic pulmonary fibrosis.* J Transl Med, 2013. **11**: p. 171.
156. Chambers, D.C., et al., *A phase 1b study of placenta-derived mesenchymal stromal cells in patients with idiopathic pulmonary fibrosis.* Respirology, 2014. **19**(7): p. 1013-8.
157. Averyanov, A., et al., *First-in-human high-cumulative-dose stem cell therapy in idiopathic pulmonary fibrosis with rapid lung function decline.* Stem Cells Transl Med, 2020. **9**(1): p. 6-16.
158. Wilson, J.G., et al., *Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial.* Lancet Respir Med, 2015. **3**(1): p. 24-32.
159. Chen, L., et al., *Mesenchymal stem cell-based treatments for COVID-19: status and future perspectives for clinical applications.* Cell Mol Life Sci, 2022. **79**(3): p. 142.
160. Lanzoni, G., et al., *Umbilical cord mesenchymal stem cells for COVID-19 acute respiratory distress syndrome: A double-blind, phase 1/2a, randomized controlled trial.* Stem Cells Transl Med, 2021. **10**(5): p. 660-673.
161. Xu, X., et al., *Evaluation of the safety and efficacy of using human menstrual blood-derived mesenchymal stromal cells in treating severe and critically ill COVID-19 patients: An exploratory clinical trial.* Clin Transl Med, 2021. **11**(2): p. e297.
162. Sánchez-Guijo, F., et al., *Adipose-derived mesenchymal stromal cells for the treatment of patients with severe SARS-CoV-2 pneumonia requiring mechanical ventilation. A proof of concept study.* EClinicalMedicine, 2020. **25**: p. 100454.
163. Clinicaltrials.gov. 2022 2022-07-05]; Available from: https://clinicaltrials.gov/ct2/home.
164. Islam, D., et al., *Identification and Modulation of Microenvironment Is Crucial for Effective Mesenchymal Stromal Cell Therapy in Acute Lung Injury.* American Journal of Respiratory and Critical Care Medicine, 2019. **199**(10): p. 1214-1224.
165. Silva, L.H.A., et al., *Strategies to improve the therapeutic effects of mesenchymal stromal cells in respiratory diseases.* Stem Cell Res Ther, 2018. **9**(1): p. 45.
166. Elowsson Rendin, L., et al., *Harnessing the ECM Microenvironment to Ameliorate Mesenchymal Stromal Cell-Based Therapy in Chronic Lung Diseases.* Front Pharmacol, 2021. **12**: p. 645558.
167. Farrow, N., et al., *Epithelial disruption: a new paradigm enabling human airway stem cell transplantation.* Stem Cell Res Ther, 2018. **9**(1): p. 153.

168. Jungebluth, P., et al., *RETRACTED: Tracheobronchial transplantation with a stem-cell-seeded bioartificial nanocomposite: a proof-of-concept study.* The Lancet, 2011. **378**(9808): p. 1997-2004.
169. Jungebluth, P. and P. Macchiarini, *RETRACTED: Airway Transplantation.* Thoracic Surgery Clinics, 2014. **24**(1): p. 97-106.
170. Kremer, W. *Paolo Macchiarini: A surgeon's downfall*. 2022-07-06]; Available from: https://www.bbc.com/news/magazine-37311038.
171. Karolinska Institutet (Stockholm). *The Macchiarini case timeline*. 2022-07-06]; Available from: https://news.ki.se/the-macchiarini-case-timeline.
172. Damiano, G., et al., *Current Strategies for Tracheal Replacement: A Review.* Life (Basel), 2021. **11**(7).
173. Teixeira da Silva, J.A., *Ethical perspectives and ramifications of the Paolo Macchiarini case.* Indian J Med Ethics, 2017. **2**(4): p. 270-275.
174. Chu, H.W., et al., *CRISPR–Cas9-mediated gene knockout in primary human airway epithelial cells reveals a proinflammatory role for MUC18.* Gene Therapy, 2015. **22**(10): p. 822-829.
175. Brewington, J.J., et al., *Detection of CFTR function and modulation in primary human nasal cell spheroids.* Journal of Cystic Fibrosis, 2018. **17**(1): p. 26-33.
176. Ji, J., et al., *Multi-cellular human bronchial models exposed to diesel exhaust particles: assessment of inflammation, oxidative stress and macrophage polarization.* Part Fibre Toxicol, 2018. **15**(1): p. 19.
177. Hedström, U., et al., *Impaired Differentiation of Chronic Obstructive Pulmonary Disease Bronchial Epithelial Cells Grown on Bronchial Scaffolds.* Am J Respir Cell Mol Biol, 2021. **65**(2): p. 201-213.
178. De Santis, M.M., et al., *Extracellular-Matrix-Reinforced Bioinks for 3D Bioprinting Human Tissue.* Adv Mater, 2021. **33**(3): p. e2005476.
179. De Santis, M.M., et al., *How to build a lung: latest advances and emerging themes in lung bioengineering.* European Respiratory Journal, 2018. **52**(1): p. 1601355.
180. Bankhead, P., et al., *QuPath: Open source software for digital pathology image analysis.* Sci Rep, 2017. **7**(1): p. 16878.
181. Adeloye, D., et al., *Global, regional, and national prevalence of, and risk factors for, chronic obstructive pulmonary disease (COPD) in 2019: a systematic review and modelling analysis.* Lancet Respir Med, 2022. **10**(5): p. 447-458.
182. Gutiérrez Villegas, C., et al., *Cost analysis of chronic obstructive pulmonary disease (COPD): a systematic review.* Health Economics Review, 2021. **11**(1): p. 31.
183. Auerbach, O., et al., *Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer.* N Engl J Med, 1961. **265**: p. 253-67.
184. Salvador, J.M., J.D. Brown-Clay, and A.J. Fornace, Jr., *Gadd45 in stress signaling, cell cycle control, and apoptosis.* Adv Exp Med Biol, 2013. **793**: p. 1-19.

185. Wansleeben, C., et al., *Stem cells of the adult lung: their development and role in homeostasis, regeneration, and disease.* Wiley Interdiscip Rev Dev Biol, 2013. **2**(1): p. 131-48.
186. Saygin, C., et al., *CD55 regulates self-renewal and cisplatin resistance in endometrioid tumors.* J Exp Med, 2017. **214**(9): p. 2715-2732.
187. Wang, Y., et al., *CD55 and CD59 expression protects HER2-overexpressing breast cancer cells from trastuzumab-induced complement-dependent cytotoxicity.* Oncol Lett, 2017. **14**(3): p. 2961-2969.
188. Komorowska, K., et al., *Hepatic Leukemia Factor Maintains Quiescence of Hematopoietic Stem Cells and Protects the Stem Cell Pool during Regeneration.* Cell Rep, 2017. **21**(12): p. 3514-3523.
189. Cretu, A., et al., *Stress sensor Gadd45 genes as therapeutic targets in cancer.* Cancer Ther, 2009. **7**(A): p. 268-276.
190. Maghsoudloo, M., et al., *Identification of biomarkers in common chronic lung diseases by co-expression networks and drug-target interactions analysis.* Mol Med, 2020. **26**(1): p. 9.
191. Shaykhiev, R., *Airway Basal Cells in Chronic Obstructive Pulmonary Disease: A Continuum or a Dead End?* American Journal of Respiratory Cell and Molecular Biology, 2021. **65**(1): p. 10-12.
192. Gao, J., et al., *Baseline characteristics and survival of patients of idiopathic pulmonary fibrosis: a longitudinal analysis of the Swedish IPF Registry.* Respiratory Research, 2021. **22**(1): p. 40.
193. Barnes, P.J., J. Baker, and L.E. Donnelly, *Cellular Senescence as a Mechanism and Target in Chronic Lung Diseases.* American Journal of Respiratory and Critical Care Medicine, 2019. **200**(5): p. 556-564.
194. Chakraborty, A., et al., *Emerging Roles of Airway Epithelial Cells in Idiopathic Pulmonary Fibrosis.* Cells, 2022. **11**(6).
195. Walentek, P., *Signaling Control of Mucociliary Epithelia: Stem Cells, Cell Fates, and the Plasticity of Cell Identity in Development and Disease.* Cells Tissues Organs, 2021: p. 1-18.
196. Stancil, I.T., J.E. Michalski, and D.A. Schwartz, *An Airway-Centric View of Idiopathic Pulmonary Fibrosis.* Am J Respir Crit Care Med, 2022.
197. Kim, E., et al., *Aberrant Multiciliogenesis in Idiopathic Pulmonary Fibrosis.* Am J Respir Cell Mol Biol, 2022.
198. Vidal, R., et al., *Expression of a mutant form of the ferritin light chain gene induces neurodegeneration and iron overload in transgenic mice.* J Neurosci, 2008. **28**(1): p. 60-7.
199. Ali, M.K., et al., *Critical role for iron accumulation in the pathogenesis of fibrotic lung disease.* J Pathol, 2020. **251**(1): p. 49-62.
200. Yang, I.V., et al., *Expression of cilium-associated genes defines novel molecular subtypes of idiopathic pulmonary fibrosis.* Thorax, 2013. **68**(12): p. 1114-21.
201. Kuek, L.E. and R.J. Lee, *First contact: the role of respiratory cilia in host-pathogen interactions in the airways.* Am J Physiol Lung Cell Mol Physiol, 2020. **319**(4): p. L603-l619.

202. Li, J., et al., *The m6A demethylase FTO promotes the growth of lung cancer cells by regulating the m6A level of USP7 mRNA.* Biochem Biophys Res Commun, 2019. **512**(3): p. 479-485.
203. Ding, Y., et al., *FTO Facilitates Lung Adenocarcinoma Cell Progression by Activating Cell Migration Through mRNA Demethylation.* Onco Targets Ther, 2020. **13**: p. 1461-1470.
204. Yu, X., et al., *NEAT1: A novel cancer-related long non-coding RNA.* Cell Prolif, 2017. **50**(2).
205. Kubo, H., *Extracellular Vesicles in Lung Disease.* Chest, 2018. **153**(1): p. 210-216.
206. Cozzi, A., et al., *Iron detoxifying activity of ferritin.* FEBS Letters, 1990. **277**(1-2): p. 119-122.
207. Beaufay, F., et al., *Polyphosphate Functions In Vivo as an Iron Chelator and Fenton Reaction Inhibitor.* mBio, 2020. **11**(4).
208. Cassat, J.E. and E.P. Skaar, *Iron in infection and immunity.* Cell Host Microbe, 2013. **13**(5): p. 509-519.
209. Puxeddu, E., et al., *Iron laden macrophages in idiopathic pulmonary fibrosis: The telltale of occult alveolar hemorrhage?* Pulmonary Pharmacology & Therapeutics, 2014. **28**(1): p. 35-40.
210. Ali, M.K., et al., *Role of iron in the pathogenesis of respiratory disease.* Int J Biochem Cell Biol, 2017. **88**: p. 181-195.
211. Neves, J., et al., *Disruption of the Hepcidin/Ferroportin Regulatory System Causes Pulmonary Iron Overload and Restrictive Lung Disease.* EBioMedicine, 2017. **20**: p. 230-239.
212. Ogger, P.P. and A.J. Byrne, *Lung fibrosis enters the iron age(†).* J Pathol, 2020. **252**(1): p. 1-3.
213. Ganz, T., *Macrophages and systemic iron homeostasis.* J Innate Immun, 2012. **4**(5-6): p. 446-53.
214. Wang, W., et al., *Ferritin H is a novel marker of early erythroid precursors and macrophages.* Histopathology, 2013. **62**(6): p. 931-40.
215. Allden, S.J., et al., *The Transferrin Receptor CD71 Delineates Functionally Distinct Airway Macrophage Subsets during Idiopathic Pulmonary Fibrosis.* Am J Respir Crit Care Med, 2019. **200**(2): p. 209-219.
216. Cho, W.K., C.G. Lee, and L.K. Kim, *COPD as a Disease of Immunosenescence.* Yonsei Med J, 2019. **60**(5): p. 407-413.
217. Lowery, E.M., et al., *The aging lung.* Clin Interv Aging, 2013. **8**: p. 1489-96.
218. de Haan, G. and S.S. Lazare, *Aging of hematopoietic stem cells.* Blood, 2018. **131**(5): p. 479-487.
219. Navarro, S. and B. Driscoll, *Regeneration of the Aging Lung: A Mini-Review.* Gerontology, 2017. **63**(3): p. 270-280.
220. La Manno, G., et al., *RNA velocity of single cells.* Nature, 2018. **560**(7719): p. 494-498.
221. Jackson, S.R., et al., *Partial pneumonectomy of telomerase null mice carrying shortened telomeres initiates cell growth arrest resulting in a*

limited compensatory growth response. Am J Physiol Lung Cell Mol Physiol, 2011. **300**(6): p. L898-909.
222. Paxson, J.A., et al., *Age-dependent decline in mouse lung regeneration with loss of lung fibroblast clonogenicity and increased myofibroblastic differentiation.* PLoS One, 2011. **6**(8): p. e23232.
223. Melo-Narváez, M.C., et al., *Lung regeneration: implications of the diseased niche and ageing.* Eur Respir Rev, 2020. **29**(157).
224. World Cancer Research Fund International. *Lung cancer statistics*. 2022 2022-07-13]; Available from: https://www.wcrf.org/cancer-trends/lung-cancer-statistics/.
225. American Cancer Society. *Key Statistics for Lung Cancer*. 2022 2022-07-13]; Available from: https://www.cancer.org/cancer/lung-cancer/about/key-statistics.html.
226. Sosa Iglesias, V., et al., *Drug Resistance in Non-Small Cell Lung Cancer: A Potential for NOTCH Targeting?* Front Oncol, 2018. **8**: p. 267.
227. Reya, T., et al., *Stem cells, cancer, and cancer stem cells.* Nature, 2001. **414**(6859): p. 105-11.
228. Hirsch, F.R., et al., *Lung cancer: current therapies and new targeted treatments.* Lancet, 2017. **389**(10066): p. 299-311.
229. Li, Y., et al., *Transcriptomic and functional network features of lung squamous cell carcinoma through integrative analysis of GEO and TCGA data.* Sci Rep, 2018. **8**(1): p. 15834.
230. Hynds, R.E. and S.M. Janes, *Airway Basal Cell Heterogeneity and Lung Squamous Cell Carcinoma.* Cancer Prev Res (Phila), 2017. **10**(9): p. 491-493.
231. Pagano, P.C., et al., *Identification of a Human Airway Epithelial Cell Subpopulation with Altered Biophysical, Molecular, and Metastatic Properties.* Cancer Prev Res (Phila), 2017. **10**(9): p. 514-524.
232. Sarode, P., et al., *Epithelial cell plasticity defines heterogeneity in lung cancer.* Cellular Signalling, 2020. **65**: p. 109463.
233. Cao, B., et al., *Use of four genes in exosomes as biomarkers for the identification of lung adenocarcinoma and lung squamous cell carcinoma.* Oncol Lett, 2021. **21**(4): p. 249.
234. Zakaria, N., et al., *Targeting Lung Cancer Stem Cells: Research and Clinical Impacts.* Front Oncol, 2017. **7**: p. 80.
235. Succony, L., et al., *Lrig1 expression identifies airway basal cells with high proliferative capacity and restricts lung squamous cell carcinoma growth.* Eur Respir J, 2022. **59**(3).
236. Yang, B., et al., *KRT6A Promotes EMT and Cancer Stem Cell Transformation in Lung Adenocarcinoma.* Technol Cancer Res Treat, 2020. **19**: p. 1533033820921248.
237. Xu, I.M., et al., *Transketolase counteracts oxidative stress to drive cancer development.* Proc Natl Acad Sci U S A, 2016. **113**(6): p. E725-34.
238. Li, M., et al., *Transketolase promotes colorectal cancer metastasis through regulating AKT phosphorylation.* Cell Death Dis, 2022. **13**(2): p. 99.

239. Qin, Z., et al., *Transketolase (TKT) activity and nuclear localization promote hepatocellular carcinoma in a metabolic and a non-metabolic manner.* J Exp Clin Cancer Res, 2019. **38**(1): p. 154.
240. Tseng, C.-W., et al., *Transketolase Regulates the Metabolic Switch to Control Breast Cancer Cell Metastasis via the α-Ketoglutarate Signaling Pathway.* Cancer Research, 2018. **78**(11): p. 2799-2812.
241. Tanaka, F., et al., *Circulating tumor cell as a diagnostic marker in primary lung cancer.* Clin Cancer Res, 2009. **15**(22): p. 6980-6.
242. Okumura, Y., et al., *Circulating tumor cells in pulmonary venous blood of primary lung cancer patients.* Ann Thorac Surg, 2009. **87**(6): p. 1669-75.
243. Polyak, K. and R.A. Weinberg, *Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits.* Nat Rev Cancer, 2009. **9**(4): p. 265-73.
244. van den Brink, S.C., et al., *Single-cell sequencing reveals dissociation-induced gene expression in tissue subpopulations.* Nat Methods, 2017. **14**(10): p. 935-936.
245. Hewitt, R.J. and C.M. Lloyd, *Regulation of immune responses by the airway epithelial cell landscape.* Nature Reviews Immunology, 2021. **21**(6): p. 347-362.
246. Ryu, A.H., et al., *Use antibiotics in cell culture with caution: genome-wide identification of antibiotic-induced changes in gene expression and regulation.* Scientific Reports, 2017. **7**(1): p. 7533.
247. Luecken, M.D. and F.J. Theis, *Current best practices in single-cell RNA-seq analysis: a tutorial.* Mol Syst Biol, 2019. **15**(6): p. e8746.
248. Andrews, T.S., et al., *Tutorial: guidelines for the computational analysis of single-cell RNA sequencing data.* Nature Protocols, 2021. **16**(1): p. 1-9.
249. Arason, A.J., et al., *deltaNp63 has a role in maintaining epithelial integrity in airway epithelium.* PLoS One, 2014. **9**(2): p. e88683.
250. Ferreira Lopes, S., et al., *Primary and Immortalized Human Respiratory Cells Display Different Patterns of Cytotoxicity and Cytokine Release upon Exposure to Deoxynivalenol, Nivalenol and Fusarenon-X.* Toxins (Basel), 2017. **9**(11).
251. Hiemstra, P.S., et al., *Human lung epithelial cell cultures for analysis of inhaled toxicants: Lessons learned and future directions.* Toxicology in Vitro, 2018. **47**: p. 137-146.