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Epithelial Cell Reprogramming in Idiopathic Pulmonary Fibrosis

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List of abbreviations

ADI	Alveolar differentiation intermediate
AT1	Alveolar type 1
AT2	Alveolar type 2
Ccna1	Cyclin A1
Ccnd2	Cyclin D2
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CT	Computed tomographic
CTNNB1	Catenin β-1
DNAH6	Dynein axonemal heavy chain 6
DNAH7	Dynein axonemal heavy chain 7
DNAI1	Dynein axonemal intermediate chain 1
EMT	Epithelial cell adhesion molecule
EpCAM	Epithelial cell adhesion molecule
FDA	Food and Drug Administration
FZD	Frizzled receptors
GPCRs	G protein-coupled receptors
GPR87	G-protein coupled receptor 87
HBEC	Human bronchial epithelial cells
Hopx	Homeobox only protein x
IPF	Idiopathic pulmonary fibrosis
KEGG	Kyoto Encyclopedia of Genes and Genomes
Krt	Keratin
LAMB3	Laminin subunit β-3
LAMC2	Laminin subunit γ-2
LEF1	Lymphoid enhancer-binding factor 1
MUC5B	Mucin-5B
Nkd1	Protein naked cuticle homolog 1
PCLS	Precision-cut lung slices
RAS	Respiratory airway secretory
ROS	Reactive oxygen species
SA-β-GAL	Senescence-associated β-galactosidase
SASP	senescence-associated secretory phenotype
Sftpc	Surfactant Protein C
TGF-β	Transforming growth factor beta
Tle1	Transducin-like enhancer protein 1
TP63	Tumor protein 63
UIP	Usual interstitial pneumonia
WISP1	WNT1-inducible signaling protein-1
WNT	Wingless-INT

List of publications

M. Lehmann, **Q. Hu**, Y. Hu, K. Hafner, R. Costa, A. van den Berg, and M. Königshoff. "Chronic Wnt/B-Catenin Signaling Induces Cellular Senescence in Lung Epithelial Cells." *Cell Signal* 70 (Jun 2020): 109588. https://doi.org/10.1016/j.cellsig.2020.109588.

Q. Hu*, K. Heinzelmann*, Y. Hu, E. Dobrinskikh, M. Ansari, M. C. Melo-Narváez, H. M. Ulke*, et al.* "Single Cell Rna Sequencing Identifies G-Protein Coupled Receptor 87 as a Basal Cell Marker Expressed in Distal Honeycomb Cysts in Idiopathic Pulmonary Fibrosis." *Eur Respir J* (Jun 16 2022). https://doi.org/10.1183/13993003.02373-2021.
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Your contribution to the publications

1.1 Contribution to paper I

M. Lehmann, **Q. Hu**, Y. Hu, K. Hafner, R. Costa, A. van den Berg, and M. Königshoff. "Chronic Wnt/B-Catenin Signaling Induces Cellular Senescence in Lung Epithelial Cells." *Cell Signal* 70 (Jun 2020): 109588. https://doi.org/10.1016/j.cellsig.2020.109588.

My contribution in this presenting work included:
Experimental design, *in vitro* models, *ex vivo* models, senescence detection, imaging analysis, figures preparation and assisting with manuscript writing.

In particular:

a) Setting up the in vitro cell line model (MLE12 cells) of WNT/β-Catenin induced senescence (Figure 3).
b) Preparation of WNT-conditioned medium.
c) Detecting the activation of WNT/β-Catenin pathway after CHIR 99021 stimulation (Figure 3A) in MLE12 cells; measuring senescence cells via SA-β-GAL (Figure 3B) and FACS-based staining (Figure 3C) after acute and chronic WNT/β-Catenin pathway activation in MLE12 cells then doing statistics.
d) Detecting senescence gene expression on transcription level and protein level after acute and chronic WNT/β-Catenin pathway activation (Figure 3D).
e) Quantifying senescence cells via FACS-based SA-β-GAL staining (Figure 4F) after acute and chronic WNT/β-Catenin pathway activation (WNT conditioned medium) in mouse primary AT2 cells then doing statistics.
f) Generation and treatment of mouse Precision-Cut Lung Slices (PCLS).
g) Setting up the ex vivo model (PCLS) of WNT/β-Catenin induced senescence (Figure 5).
h) Detecting WNT pathway genes and senescence genes after acute and chronic WNT/β-Catenin pathway activation (CHIR99021 and WNT conditioned medium stimulation) in PCLS (figure 5A and Figure 5B).
i) Immunofluorescence staining and co-staining of p21 with E-Cadherin in PCLS to detect senescence cells and cell types.
j) writing the manuscript and discussion among authors.

1.2 Contribution to paper II

Q. Hu*, K. Heinzelmann,*, Y. Hu, E. Dobrinskikh, M. Ansari, M. C. Melo-Narváez, H. M. Ulke*, et al.* "Single Cell Rna Sequencing Identifies G-Protein Coupled Receptor 87 as a Basal Cell Marker Expressed in Distal Honeycomb Cysts in Idiopathic Pulmonary Fibrosis." *Eur Respir J* (Jun 16 2022). https://doi.org/10.1183/13993003.02373-2021.
* Authors contributed equally.

My contribution in this presented work includes:
Experimental design, pre-processing of single cell RNA-sequencing data, cell type annotation of single cell RNA-sequencing data, differentially expressed genes analysis and GPR87 gene identification, manuscript writing and figures preparation.

In particular:

a) Performing the pre-processes of single cell RNA-sequencing data, including quality control, removing ambient RNA contamination, data normalization, data batch correction, highly variable genes selection, cell cycle regression, data visualization.
b) Annotating cell types in the single cell RNA-sequencing data, including cell clustering, marker gene identification, clustering annotation.
c) Uniform manifold approximation and projection (UMAP) visualization of cell clustering and heatmaps visualization of cell marker genes of each cluster (Panel/Figure1 A).
d) Showing cell distribution via UMAP visualization of healthy donor and IPF groups (Panel/Figure1 B).
e) Analyzing differentially expressed gens of IPF EpCAM+ epithelial cells compared with donor samples and visualizing the top-15-fold change of genes and top-15-fold change genes mapping to Transmembrane Signaling Receptor Activity genes (Panel/Figure1 C); identifying the KRT5, KRT17 and GPR87 within the top-regulated genes.
f) Checking the transmembrane signaling receptor genes and identifying the GPR87 as of the robustly regulated genes in the (supra)basal cell population across all samples (Panel/Figure1 D).
g) Checking GPR87 gene expression in the built-up single cell dataset as well as two others public IPF datasets and finding out GPR87 was highly expressed in (supra)basal cell population and aberrant basaloid cells in IPF (Panel/Figure1 E and Panel/Figure1 F).
h) Identifying KRT17 as one of the highly positive correlated genes via correlation analysis of GPR87 genes; Several airway remodeling categories were identified via functional annotation enrichment analysis of those positive correlated genes (Panel/Figure1 L).
i) Preparing figure (Panel/Figure1 K and Panel/Figure1 M).
j) writing the manuscript and discussion among authors.

1.3 Contribution to paper III (Apendix)

Y. Hu, C. Ciminieri, **Q. Hu**, M. Lehmann, M. Königshoff, and R. Gosens. "Wnt Signalling in Lung Physiology and Pathology." Handb Exp Pharmacol 269 (2021): 305-36. https://doi.org/10.1007/164_2021_521

My contribution in this presenting work included:
Structuring the manuscript and manuscript writing.

In particular:

a) Co-writing the session of “Cell type-specific expression patterns of Wnt ligands/Fzd receptors.
b) Co-writing the session of “Normal lung aging”.
c) Co-writing the session of “Lung cancer”.
d) Co-writing the session of “Pulmonary vascular diseases”.

2. Introductory summary

2.1 Background

2.1.1 The lung and lung structure

The lung is the primary organ of the respiratory system in humans. It has the function of gas exchange by inhaling oxygen from the air and simultaneously releasing carbon dioxide into the atmosphere. Over 40 cell types have been identified in the lung, while more and more novel cell types were reported in recent years due to the advancement of technology.

Based on structure and function, the lung can be categorized into two parts, the conducting region, and the respiratory region [1]. The conducting region comprises the trachea, the bronchi, and bronchioles, while the respiratory region consists of the terminal bronchioles and the alveoli. The major cell types of the epithelium in the conducting airway include club, basal, goblet and ciliated cells. The ciliated cell is the dominant population of airway epithelium, the proportion of ciliated cells increases with bronchi generation. Ciliated cells have a columnar shape resting on the basement membrane and possess apical cilia or microvilli which distinguishes them from other cell types. The epithelium in conducting airways is key for secreting mucus which, together with the airway epithelium, forms the first line preventing from inhaled environmental pathogens. The various cell types and proportions vary in the different regions of the bronchial tree.

The most distal part of the lung is comprised of alveoli, the primary units for gas exchange. There are an average of 480 million alveoli in the adult human lung [2]. Alveoli consist of two epithelial cells: alveolar type 2 (AT2) cells and alveolar type 1 (AT1) cells. AT1 cells show larger and flat shape and are wrapped by capillary networks mediating gas exchange. AT2 cells are responsible for producing and secreting surfactant protein and are considered as progenitor cells of AT1 cells [3]. Upon injury, AT2 cells have the ability of proliferation and differentiation into AT1 cells to repair the alveolus [4, 5].

2.1.2 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is an age-related chronic and lethal lung disease with a progressive and irreversible loss of lung function. The IPF lung is characterized by usual interstitial pneumonia (UIP) pattern, a distinct histological lesion and a definitive diagnosis pattern generally detected by high-resolution thin section computed tomographic (CT) scans or surgical lung biopsy [6]. Honeycomb cysts, aberrant bronchioles filled with mucus, are a typical feature of pulmonary fibrosis and a key criterium in the diagnosis of UIP [7]. IPF has a prevalence of 10-60 cases per 100,000 people with an incidence of 2-30 cases per 100,000 person-years [8]. In aging population (65 years old or elder), the estimated prevalence of IPF reaches up to 400 cases per 100,000 people [9]. Two drugs, Pirfenidone (Esbriet®) and Nintedanib (Ofev®), have been approved by U.S. Food and Drug Administration (FDA) for the treatment of IPF. However, both drugs can only slow down but not stop disease progression [10]. Therefore, understanding IPF pathogenesis and developing new therapeutic strategies has significant clinical implications.

2.1.3 Cellular reprogramming in IPF

Progenitor cells in adult tissue have the ability of self-renew as well as the ability to differentiate into multiple cell types. Physiological maintenance of tissue homeostasis relies on the fine regulation of progenitor cell activity. In the lung tissue, several populations of progenitor cells are resident in distinct regions and form specific niches with surrounding cells to maintain tissue homeostasis. For example, AT2 cells serve as progenitor cells in the distal lung and are responsible to replace AT1 cells, whereas basal cells and club cells are progenitors for airway epithelial cells. Upon injury, progenitor cells can activate multipotency and reprogram to change cell identity either directly or via intermediate states. Regeneration of the alveolus requires temporal and spatial control of epithelial cell behaviors. Aberrant reprogramming of epithelial cells may lead to impaired regeneration culminating in chronic lung diseases. Cellular senescence and aberrant activation of intracellular developmental pathways are commonly observed in fibrotic epithelial cells in the lung, likely leading to reprogramming. These findings pose the question whether developmental pathways directly or indirectly impact cellular senescence upon injury as part of the cellular reprogramming observed in the IPF lung.

2.1.3.1 Cellular senescence is a hallmark of aging lung

Aging is one of the most significant risk factors for chronic lung diseases, which are the third leading cause of mortality worldwide. The lung continues to develop after birth. Lung function generally peaks at around 25 years of age and declines after 35 years of age [11]. The aging lung features a progressive loss of physiological integrity, impaired respiratory function, and increased vulnerability to disease [12]. Hallmarks of the aging lung include cellular senescence, stem cell exhaustion and mitochondrial dysfunction [13]. Cellular senescence is a process of permanent cell cycle arrest in response to various stressors. It has emerged as a potentially important contributor to aging and age-related disease, therefore gaining attention as an attractive target for therapies targeting age-associated diseases [13, 14]. Cellular senescence, in young organisms, is considered as a tumor suppressive mechanism that prevents the growth of damaged cells, consequently maintaining tissue homeostasis and protecting from cancers. However, the accumulation of senescent cells was widely observed in different aged organisms. Senescent cells in aging individuals showed an increase in their rate of generation coupled with a decline in the rate of clearance. This abnormal accumulation of senescent cells has various harmful effects on tissue homeostasis [15]. Furthermore, young mice transplanted with small numbers of senescent cells developed persistent physical dysfunction [16]. Cellular senescence is characterized by a distinct secretory phenotype, senescence-associated secretory phenotype (SASP), which produces high levels of inflammatory cytokines, growth factors, proteases. SASP has powerful autocrine and paracrine activities, contributing to aging and aging-related disease [17].

2.1.3.2 Cellular senescence contributes to IPF

The current paradigm considers IPF as the result of impaired repair/regenerative responses and aberrant tissue remodeling [18, 19]. Alveolar epithelial cells are central players in the process of lung repair/regeneration, and the reduction of their regenerative potential is causal to the pathogenesis of IPF [20]. The cellular senescence reprogramming of alveolar epithelial cells can distinctly alter the alveolar regenerative capacity during lung injury. For instance, exposure to bleomycin induced cellular senescence

in rat primary AT2 cells and impaired alveolar re-epithelialization [21]. Additionally, targeting senescent epithelial cells attenuated experimental lung fibrosis [22, 23]. Therefore, studying cellular phenotypes in subtypes of epithelial cells may reveal mechanisms of pro/anti-regenerative reprogramming that causes why alveolar epithelial cells lose regenerative potential.

The age-dependency of IPF, and the fact that cellular senescence is a central hallmark of cellular aging, raise the possibility that cellular senescence contributes to the development of IPF. Indeed, accumulating evidence demonstrates that cellular senescence is one of the prominent features of IPF [22-24]. The senescence of different cell types such as alveolar epithelial cells and airway epithelial cells has been observed in lungs of patients with IPF [25, 26], even though it remains unclear whether senescence of different cell types results in distinct contributions to disease mechanisms in IPF. The SASP of senescent fibroblasts is pro-fibrotic [22] and the secretion of SASP in primary mouse AT2 cells was increased in experimental lung fibrosis [23]. In our previous study, we detected increased senescence staining for primary AT2 cells in fibrotic mouse model as well as in the lung tissue of IPF patients [23], which, together with recently published reports [22, 27, 28], strongly indicates that cellular senescence is a major phenotype of the reprogrammed lung epithelium in IPF. However, the mechanisms driving cellular senescence in specific cell types and how this might predispose to pulmonary fibrosis development remain unknown.

2.1.4 Developmental Pathways in IPF

Many developmental signaling pathways including WNT, Hedgehog, TGF-β, and Notch signaling pathway show low activity in the adult lung, while getting reactivated during injury repair. Increased activity of these developmental pathways is linked to the pathogenesis of IPF [26, 29-31]. Given the common spatial and temporal regulation in physiological process, aberrant activation of developmental pathway is likely to result in severe consequences [32, 33]. WNT pathway and TGF-β pathway are within vital signaling pathways highly involved in both lung homeostasis and progression of IPF [34, 35].

2.1.4.1 WNT pathway in normal lung aging and IPF

WNT signaling compromising the canonical and non-canonical WNT pathways regulates the different stages of lung development. Unbiased genetic screening methods and confirmatory studies indicate aberrant WNT pathway activities in adult lung following injury, contributing to pathogenesis of pulmonary fibrosis [26, 36-39]. For example, the expression of WNT ligands (such as WNT-1, WNT-7B, WNT-10B) and WNT receptors (such as CTNNB1, LEF1, FZD2, and FZD3) was upregulated in human lung tissue with IPF if compared to donor tissue [40]. Further studies identified epithelial cell sub-population with higher WNT/β-catenin pathway activities in both human lung fibrosis and fibrosis animal models [41-43]. The increase in WNT/β-catenin pathway activities seem to be an early event in both experimental and idiopathic pulmonary fibrosis [26, 37, 39, 44]. Evidence showed WNT1-inducible signaling protein-1 (WISP1) was upregulated in human IPF tissue and mediated the pathogenesis of bleomycin-induced lung fibrosis in mice [36]. The siRNA-mediated WISP1 knockdown blunted TGFβ1 induced IL-6 over-expression [45]. Therefore, targeting the WNT pathway and its downstream mediators might provide a promising way for anti-fibrotic therapeutics. Indeed, pharmacological inhibition of WNT/β-catenin pathway showed attenuation of bleomycin-induced lung fibrosis [46-48].

An aging-associated dysregulation of several WNT target genes was detected. Specifically, WNT target genes including Ccna1 (Cyclin A1), Ccnd2 (Cyclin D2) [49], Nkd1, Lef1, Tle1 [50] were identified reduced during mouse lung aging, whereas Wisp-1 [51] and c-Myc [52] were considered over-expressed. The altered activity of WNT pathway during lung aging are spatially and temporally regulated. For example, lower expression of WNT-2 was detected in lung mesenchymal stromal cells while the expression was up-regulated in airway epithelial cells in aging mice [53, 54]. However, more studies are required to decipher the function of WNT pathway in region-specific niche during aging and development of IPF.

2.1.4.2 TGF-β signaling pathway in IPF

Transforming growth factor (TGF)-β signaling pathway is an evolutionarily conserved developmental signaling pathway. It regulates multiple cellular processes not only in the developing embryo but also in adult organism including cell proliferation, differentiation, migration, and cellular senescence. TGF-β signaling pathway has pleiotropic effects on various cell types in the lung. For example, TGF-β promotes the growth of fibroblasts [55], while suppressing the proliferation of epithelial cells in the lung. Aberrant activation of TGF-β has been indicated in both the animal models of pulmonary fibrosis and lung tissue from patients with IPF [56, 57]. Upon repeated injury, TGF-β activity promotes the activation of fibroblasts into myofibroblasts and enhances secretion of extracellular matrix such as collagens, leading to the collapse of lung structure. These effects are features of IPF. Furthermore, TGF-β mediates the epithelial–mesenchymal interactions during acute lung injury and the development of pulmonary fibrosis. TGF-β activation on epithelial cells drives adjacent fibroblasts cells into collagen-producing cells [58]. Notably, TGF-β can induce epithelial–mesenchymal transition (EMT), which has consequences for disease progression in both lung cancer and lung fibrosis [59-61]. The TGF-β pathway can be activated by several mechanisms, including reactive oxygen species (ROS), PH, proteolysis, thrombospondin and integrins [35]. Therefore, TGF-β effects are context-dependent and TGF-β acting on various niches induces distinct cellular responses. For example, TGF-β can not only maintain the self-renewal of stem cell, but also controls differentiation of multiple cells lineages [62]. In the airway region, TGF-β is involved in epithelial reprogramming, subepithelial fibrosis, leading to airway remodeling [63]. Moreover, TGF-β was linked to cellular senescence. IPF fibroblast in niche enriched with TGF-β was observed with a profibrotic SASP [64]. In vitro studies demonstrated TGF-β was able to induce cellular senescence in primary human bronchial epithelial cells (HBECs) [25]. However, the molecular mechanism on how TGF-β induces cellular senescence and airway remodeling remain unclear.

2.2 Hypotheses and Objectives

IPF is a progressive and irreversible lung disease of unknown cause. It has a high prevalence in the aged population. Cellular features in the IPF lung include extensive reprograming of epithelial cells, impaired cellular crosstalk with immune and mesenchymal cells, and subsequent accumulation of extracellular matrix. The two approved drugs available for treatment can both only slow down but not stop the fibrosis process. Therefore, investigation on IPF pathogenesis leading to the development of novel therapeutics are of clinical importance. Currently, it is thought that repetitive injury of epithelial cells leading to cellular reprogramming triggers the development of pulmonary fibrosis by abnormal re-epithelization and remodeling of the lung. Cellular senescence and aberrant activation of intracellular developmental pathway are commonly observed in fibrotic epithelial cells in the lung, likely leading to reprogramming [22, 26]. These findings pose the question whether developmental pathways directly or indirectly impact cellular senescence as part of the cellular reprogramming observed in the IPF lung. Furthermore, increased cellular senescence and developmental pathway activities were detected in both airway epithelial cells [25, 65] and alveolar epithelial cells [23, 26] in IPF. Considering region-specific niches in the lung, it is necessary to specifically define cellular reprogramming in airway and alveolar regions.

We hypothesized that the senescent reprogramming of both airway epithelial cells and alveolar epithelial cells contributes to the development and progression of IPF and that this process is mediated by developmental signaling pathway, such as WNT or TGF-β signaling, both of which are known to be main profibrotic mediators.

The aims of this study were to A) characterize the cell reprogramming, particular cellular senescence in both alveolar epithelial and airway epithelial cells; B) evaluate the contribution of WNT signaling pathway in alveolar epithelial cells and pathogenesis of pulmonary fibrosis; C) study the effect of TGF-β signaling pathway on senescence of airway epithelial cells and airway remodeling.

2.3 Summary

IPF is a chronic lung disease with progressive scarring and continuous decline of lung function, usually affecting the aged population. Its incidence and prevalence increase with age and the median overall survival time of IPF patients was 4.5 years [66]. Even though there are two approved drugs (Pirfenidone vs. Nintedanib), both of them can only slow down the progression but not completely stop the scarring [10]. Therefore, it is of high clinical relevance to develop novel therapeutics. However, the mechanism of pulmonary fibrosis is incompletely understood, which limits therapeutic options. Here we thought to investigate the hypothesis that cellular reprogramming in epithelial cells drives pathogenesis of IPF. To do so, we evaluated the effect of developmental pathway and cellular senescence in both alveolar and airway epithelial cells.

The WNT signaling pathway is a key signaling pathway during lung development. It further serves as a regulator to maintain tissue homeostasis in the adult lung. Recent studies demonstrated activities of WNT pathway in lung injury repair and lung disease. We reviewed the WNT pathway in lung under both physiological and pathological condition (Apendix A) [34]. The WNT pathway is one of the targets for lung regeneration. Based on our experience and previous work, we summarized regulatory roles of the WNT pathway on lung function under both physiological conditions, aging and pathogenesis. We further discussed potential options for targeting WNT pathway for treatment of chronic lung disease.

WNT pathways, including canonical and non-canonical pathway, have been indicated in the development of pulmonary fibrosis [26, 36-39], however, the underlying mechanisms remain unclear. In the first publication [67], our data showed that chronic stimulation of WNT/β-catenin pathway by either CHIR or WNT3A ligands induced cellular senescence in mouse primary AT2 cells. Notably, chronic WNT3A treatment on precision-cut lung slices (PCLS) also resulted in cellular senescence and most senescent cells are E-cadherin+ epithelial cells. Additionally, mouse primary AT2 cells stimulated with chronic WNT/β-catenin activity showed increased expression of fibrotic/transient epithelial cell marker, Keratin (Krt) 8, indicating a fibrotic phenotype [68].

Upon injury, cellular reprograming is a common physiological process to maintain tissue homeostasis. Aberrant cellular reprogramming may contribute to chronic lung diseases [43, 68-70]. IPF is an aging associated lung disease, however, how aging impairs cellular reprogramming and further contributes to the development of IPF remain unclear. In the first publication [67], we compared the cell population in young (3 months) and old mice (16–24 months), identifying declined population of epithelial cells (EpCAM+) in old mice. We further demonstrated increased activity of β-catenin and senescence-associated β-galactosidase (SA-β-GAL) in primary AT2 cells in aging mice. AT2 cell in old mice gradually lost their regenerative potential, showing declined gene expression of Surfactant Protein C (*Sftpc*) and increased transcript level of *Hopx*. The reduced progenitor cell capacity of old AT2 cells were further confirmed by organoid formation assay. In the second publication [71], we aimed to further characterize epithelial cell subpopulations in the IPF lung. The single cell RNA sequencing dataset we generated on EPCAM+ enriched cells revealed an enlarged population of airway epithelial cells and sharply declined population of AT2 cells in IPF patients, similar to our findings in the aged mouse

lung [67]. Moreover, analysis of differentially expressed genes indicated cytokeratins including KRT5, KRT17, KRT15 and KRT6A are among the most upregulated genes in IPF, which indicates the airway and alveolar epithelial reprogramming and remodeling.

Considering the region-specific niches and the alteration of cell type proportion, the cell reprogramming within alveolar region and airway regions may be distinct. In the second publication [71], we identified G-protein coupled receptor 87 (GPR87) as a novel marker of airway basal cells within honeycombs cysts in IPF. Notably, stimulation of developmental signaling pathway, the TGF-β pathway, on primary human HBECs led to increased expression of GPR87. Overexpression of GPR87 in vitro impaired the differentiation of KRT5+ basal cells, which indicated GPR87 may play important role on bronchiolization and honeycomb cyst formation. Importantly, gene functional enrichment analysis indicated GPR87 related genes are enriched in TGF-β pathway signaling and cellular senescence.

Overall, in this project, we focused on phenotyping lung epithelial cells and exploring molecular mechanisms of how cellular reprogramming contributed to pulmonary fibrosis. We identified cellular senescence as a common type of reprogramming in both alveolar and airway epithelial cells. Then we investigated the role of developmental pathway within both alveolar and airway regions and demonstrated the activation of developmental signaling pathway contributing to epithelial cell senescence. Our work highlighted the significance and connection of developmental signaling pathway and cellular senescence during the development of IPF.

2.4 Discussion

2.4.1 Epithelial cell damage and its contribution to IPF

Lung epithelial cells provide a complex barrier as the first defense against environmental risk factors, such as toxins, cigarette smoke, antigens. They are vital not only for tissue homeostasis but also for rapid repair after acute or chronic lung injury. Repair of acute lung injury is usually initialized with an inflammatory response, recruitment of immune cells, and epithelial cell proliferation and differentiation. Depending on the injury location and the severity of the injury, various progenitor cells are responsible for restoring the damaged epithelium. In the respiratory zone, AT2 cells serve as the progenitor cells and replace damaged alveolar epithelial cells. Whereas, in the conducting zone, both club and basal cells are considered as multipotent progenitor cells capable of regenerating the bronchial epithelium [72, 73]. Notably, club cells can serve as progenitor cells differentiating into either ciliated cells or alveolar epithelial cells [73, 74].

Dysregulated repair processes can result in chronic lung disease. IPF results from aberrant wound healing responses, featuring multiple cycles of epithelial cell injury and activation [75]. Repeated injury may lead to exhaustion of the alveolar epithelial progenitor cells pool. During pathogenesis, epithelial cells can undergo EMT, a trans-differentiation process during which epithelial cells lose their repair capacity and acquire mesenchymal features [76]. In our single cell dataset generated in this project, we demonstrated that the proportion of AT2 cells was significantly declined in IPF lung tissue, while the proportion of airway epithelial cells was increased. This alteration of cell type proportions between donors and IPF is consistent with previous reports [43, 70]. Furthermore, we identified expression of senescence-associated genes in IPF epithelial cells, including AT2 cells, basal cells. Similarly, we detected an enlarged population of senescent AT2 cells in aging mice. IPF has been widely considered as an aging-associated lung disease. The cellular senescence of epithelial progenitor cells is likely to be a driver of IPF.

The WNT signaling pathway plays critical roles during lung development and lung regeneration after injury. The cell niche dominated by WNT signaling molecules is critical for alveolar cell fate. For example, the niches supported by neighboring fibroblast expressing Wnt5a helps AT2 cells maintaining stemness during homeostasis, while upon injury, AT2 cells with autocrine Wnts signaling rapidly proliferate and regenerate lost alveolar cells (Nabhan et al. 2018). WNT activities, including canonical and non-canonical WNT, were detected in areas of lung injury [77-80]. Previous work identified WNT activity in both alveolar and bronchial epithelium in IPF lung tissue [26, 36]. In this project, we demonstrated an enlarged proportion of epithelial cells showing active WNT/β-catenin pathway in the aged mice lung [67]. Notably, Paxson and colleagues demonstrated reduced WNT/β-catenin activity in mesenchymal stromal cells in aging mouse lung tissue [53]. This indicates the WNT signaling pathway is spatiotemporally controlled during aging.

Recent studies indicated the existence of an intermediate cell state of AT2-AT1 cells, showing features of both AT2 cells and AT1 cells [68, 81-84]. Notably, aberrant accumulation of these intermediate cell state may mediate disease pathogenesis. For example, a study led by Duke University identified a bipotent progenitor, AT0 state, which origi-

nated from AT2 cell, differentiating into AT1 cells in physiological lung repair while differentiating into respiratory bronchioles secretory cells (TRB-SCs) in IPF [84]. Therefore, identifying transient epithelial cells and cell reprogramming may enhance our understanding of the origin and progression of chronic lung diseases, which have a high medical need, and may lead to novel therapies impacting the lives of patients worldwide.

2.4.2 Alveolar epithelial cell reprogramming in IPF

Alveolar epithelial cells are key players of gas-exchange in the lung. The adult lung has around 480 million alveoli and each single alveolus has the mean size of 4.2×10^6 μm^3. AT1 cells are flat squamous cells and the main component of air-blood barrier. AT2 cells have a cuboidal shape and are responsible for producing and secreting surfactant into the alveolus. Upon injury, AT2 cells can maintain the integrity of alveolar epithelial cells via proliferation and differentiation into AT1 cells. Yao et al. demonstrated that adult mouse AT2 cells with lower expression of Sin3a became senescence, and Sin3a loss of function in AT2 cells is likely to contribute to lung fibrosis via p53 pathway activation [69]. Furthermore, they indicated senescence but not loss of AT2 cells as a driver of progressive lung fibrosis [69]. These findings highlighted the significance of epithelial cell reprogramming, in particular cellular senescence, in the development of pulmonary fibrosis.

In this project, we identified cellular senescence of AT2 cells and the exhaustion of alveolar progenitor cells in the lung in aging mouse, which may contribute to the development of fibrotic pathogenesis. Aging is one of the risk factors for lung fibrosis, which is linked to the accumulation of cellular senescence during injury in both fibrotic mouse and human lung tissue. The accumulated senescent cells can result in exhaustion of stem cells and altered intercellular communication, further impairing the injury repair and regenerative processes [15]. Recent progress in high-throughput technologies helped to identify several transient cell states during either physiological lung repair or the pathogenesis of pulmonary fibrosis. Strunz and colleagues identified Krt8+ alveolar differentiation intermediate (ADI) during lung injury, showing expression of senescence-associated genes, oxidative phosphorylation pathways, as well as EMT, p53, MYC, and NFkB [68]. All these pathways have been linked to fibrogenesis [22, 85-89]. Further studies indicated Krt8+ ADI are the transitional stem cell state preceding AT1 cells and inhibition of WNT/β-catenin activity reduced the formation of Krt8+ ADI and AT1 cell states in vitro [68]. Notably, we identified a cluster of AT2 cells showing high activity of WNT/β-catenin signaling in aging mice in this project. Upon single cell RNA sequencing analysis, Yao and colleagues showed AT2 cells from IPF patients were enriched with cellular senescence and WNT/β-catenin activity [69]. Therefore, investigating WNT/β-catenin pathway and cellular senescence on alveolar epithelial cells are likely to provide new insights on mechanism of IPF pathogenesis.

2.4.3 Chronic WNT/β-catenin activity induces cellular senescence of Alveolar epithelial

Genetic mice with deficient klotho, a negative WNT/β-catenin regulator, display an accelerated aging phenotype with enlarged senescent cell population and reduced stem cell numbers in skin and intestine [90]. Even though aberrant WNT/β-catenin activity

has been showed in distal lung epithelial cell dysfunction [44, 91], its role during lung aging and pulmonary fibrosis is unclear.

In this project, our data revealed that the activation of WNT/β-catenin pathway drives cellular senescence, further inducing a fibrotic phenotype in mouse primary AT2 cells. Aberrant WNT/β-catenin activity was reported in IPF [26, 92], indicating a key role of WNT/β-catenin pathway in the development of IPF. Notably, induction of AT2 cell senescence was detected under chronic but not acute WNT/β-catenin stimulation. Moreover, mouse primary AT2 cells stimulated with non-canonical WNT pathway (WNT5A) did not show senescence. Co-stimulation with non-canonical WNT pathway (WNT5A), it showed declined capacity of WNT/β-catenin to induce senescence. These findings highlighted that the functional outcomes of WNT/β-catenin activity highly relied on the timing, concentration, and duration of stimulation. During aging and pathogenesis of pulmonary fibrosis, continuous injuries and cellular reprogramming of alveolar epithelium increased and likely maintained the chronic WNT/β-catenin activities. It is well known that WNT/β-catenin pathway is a developmental pathway and essential for stem cell function during homeostatic conditions. Therefore, this project indicated the significance of fine-tuning developmental pathways like the WNT/β-catenin signaling pathway to prevent the accumulation of cellular senescence and pathogenesis of pulmonary fibrosis.

2.4.4 Airway epithelial cell reprogramming in IPF

Airway epithelial cells are among the first line of defense preventing from antigens, toxins, and viruses. Growing evidence supports the hypothesis that inappropriate repair of airway epithelial cells drives airway remodeling and chronic lung disease [1, 93]. Accumulation of basal cells within the alveolar area were reported in IPF lung tissue [94-96]. These alveolar basal cells may either originate from p63+ progenitors cells and migrate into injured alveolar area [97, 98] or originate directly from resident AT2 cells [95, 99]. Investigators from Yale University identified aberrant basaloid cells in IPF lung tissue, expressing general basal cell markers KRT17, TP63, LAMB3 and LAMC2 but without expression of KRT15 and KRT5. These aberrant basaloid cells display profibrotic and senescent features [43]. Those cells are enriched in IPF but not detected in healthy lung, which was further confirmed by research group from Vanderbilt University [70]. Notably, these aberrant basaloid cells show similarities in transcriptomic to the Krt8+ ADI cells which are an intermediate cell state upon mouse lung injury [43, 68, 70]. Additionally, both the aberrant basaloid cells and Krt8+ADI cells showed features of cellular senescence and epithelial-mesenchymal transition (EMT). However, the exact function and origin of these cells remain unclear. Basil et al. demonstrated respiratory airway secretory (RAS) cells as progenitors of alveolar type 2 cells, displaying roles in regenerating alveolar niche [100]. Therefore, transient epithelial cells are critical for maintaining alveolar hemostasis and the process of pulmonary fibrosis.

Honeycomb cysts are derived from distal airway containing a mucociliary pseudostratified epithelium dominated by basal and ciliated cells [101]. However, the mechanisms of bronchiolization and terminal bronchiole remodeling during the pathogenesis of IPF remains unclear. Ciliated cells were detected in the honeycomb areas and most of these areas were uniformly ciliated [101]. Basal cells are considered as progenitor cell for both secretory and ciliated cells in the healthy adult human lung. Basal cells are characterized by gene expression of KRT5, KRT15, KRT17 and TP63. Jonsdottir et al. demonstrated a

fibroblastic foci in IPF contains a layer of TP63+ basal cells lacking ciliated and goblet cells. These fibroblastic foci showed both E-Cadherin and N-Cadherin positive cell, indicating cadherin switch and epithelial-to-mesenchymal transition (EMT) under this area. Further studies suggested that TP63+ basal cells can lead to phenotypic changes and act as progenitor cells of EMT in IPF pathogenesis [102].

Transcriptomic studies indicated that patients with more microscopic honeycombing formation showed high expression of cilium-associated genes (DNAI1, DNAH6, DNAH7 and RPGRIP1L). Furthermore, the higher expression of cilium -associated genes is linked to the expression of airway mucin gene MUC5B and basal marker gene KRT5 [103].

KRT5 is a well-known marker of suprabasal and basal cells. Increasing evidence suggested KRT5+ progenitor cells are ectopically present in fibrotic areas, especially in honeycomb cysts, in IPF tissue. Similar to IPF, Krt5-expressing basal cells were detected in honeycomb-like cyst in mouse models [98, 104], and the Krt5+ basal cells promoted cyst formation [104]. Further study showed dysregulation of cilium genes on Krt5+ progenitor cells leads to defective ciliogenesis, contributing to the pathogenesis of bleomycin induced pulmonary fibrosis [105]. In this project, we performed single cell RNA sequencing on EPCAM+ enriched cells and demonstrated that cytokeratin genes including KRT5, KRT17, KRT15, KRT6A are most upregulated gene in IPF, which is consistent with other studies [103, 105]. The dysregulated cytokeratin gene signature is suggestive of airway remodeling during the IPF pathogenesis. Therefore, identifying Krt5+ basal cell subpopulation and dissecting their characterization can enhance our understanding of IPF pathogenesis. We further identified G-protein coupled receptor (GPR) 87 as a novel surface marker of distal bronchioles and KRT5+ basal progenitor cells in IPF. GPR87 belongs to the largest family of membrane receptors which translate extracellular stimulation into intracellular signals. Additionally, G protein-coupled receptors (GPCRs) are frequently implicated in disease process and show popularity as therapeutic target for drug discovery. Therefore, investigation on GPR87+ basal cells can enhance our understanding of IPF progression and may lead to novel therapies preventing IPF development.

2.4.5 TGF-β pathway drives cell reprogramming of bronchial epithelial cells and airway remodeling

The TGF-β pathway is a developmental pathway, regulating cellular processes in both lung health and disease. The TGF-β activity has been demonstrated in both IPF and airway remodeling [63, 106]. Upon injury, epithelial cells showed increased TGF-β activity. For example, $\alpha_v\beta_{6,}$ an integrin that mediating the activation of TGF-β pathway, is low expressed in normal lung epithelial cells but highly expressed in injured lung epithelial cells [107]. Evidence demonstrated injury signal transmitted to TGF-β pathway rely on G protein–coupled receptors (GPR) [108, 109]. G protein-coupled receptors (GPR), also known as seven-transmembrane domain receptors, are attractive pharmacological targets. In this project, we identified GPR87 as one of the highly regulated transcripts in our single cell dataset from IPF patients. Furthermore, basal cells showed robustly upregulated GPR87 in IPF tissue in our dataset as well as in other public datasets [43, 70]. Even though we cannot detect aberrant basaloid cells in our dataset likely due to cell isolation methodology, GPR87 was enriched in aberrant basaloid cells (KRT5−/KRT17+ cells) of public dataset [43, 70]. Overexpression of GPR87 has been reported in various

of cancers, where it promotes EMT [110-114]. Notebly, GPR87 was linked to dysregulated cell cycle control [115-117], which is a feature of epithelial cell reprogramming and the driver of homeycome cysts development in IPF [101, 104, 118]. Moreover, in situ studies indicated GPR87 expression in KRT5+ cells localized to distal bronchioles and honeycomb cysts in IPF, which highlight the role of GPR87 in cell reprogramming and airway remodeling during the development of IPF. Interestingly, overexpression of GPR87 was detected in lung cancer and is likely to promote tumor cell invasion [119, 120]. Furthermore, IPF is known as a high-risk factor for lung cancer [121]. These findings suggests that GPR87 might contribute to both diseases and highlight the importance of shared mechanisms of lung cancer and fibrosis [122].

Functional annotation enrichment analysis of our single cell dataset showed that genes correlating to GPR87 were enriched in TGF-β production, keratinocyte differentiation and extracellular matrix remodeling, all pathways described in IPF pathogenesis. Notably, stimulation with TGF-β promoted the expression of GPR87 in human bronchial epithelial cells (HBECs). Furthermore, modulation of GPR87 in primary HBECs cultures resulted in impaired airway differentiation and ciliogenesis. Moreover, GPR87 gene expression was positively correlated with the expression of senescence gene CDKN2A (Spearman's rank correlation, rs= 0.84). KEGG pathway analysis demonstrated GPR87 correlated genes were enriched in P53 signaling pathway further corroborating a link to cellular senescence. These discoveries further support our hypothesis that developmental pathway, such as TGF-β pathway, drive cellular reprogramming of airway epithelial cells and airway remodeling in IPF.

2.4.6 Limitation and further directions

Overall, in this project, we investigated cell reprogramming of both alveolar and airway epithelial cells during the pathogenesis of IPF. We identified an enlarged proportion of alveolar epithelial cells showing active WNT/β-catenin pathway in the aged mouse lung and demonstrated that distal lung alveolar epithelial cells can developed senescence after chronic WNT/β-catenin stimulation. On the one hand, WNT/β-catenin signaling pathway orchestrates lung repair and regeneration upon injury but induces cellular senescence on the other hand, which may drive chronic lung disease. These conflicting roles of WNT/β-catenin activity indicate that WNT/β-catenin signaling is under fine regulation during lung regeneration to ensure proper injury repair. Indeed, the spatiotemporally dynamic of WNT pathway has been indicated in lung regeneration and lung diseases [54, 123]. In this project, even though we demonstrated chronic WNT/β-catenin activity induced cellular senescence, the mechanisms how WNT activity switches the function from regeneration into cellular senescence, further contributing to disease development, remain unclear. Investigation on precise control of WNT activities during distinct phases of IPF pathogenesis is limited due to the access to lung tissue from different stages of disease. In this project, we didn’t observe the cellular senescence of lung epithelial cells induced by non-canonical ligand WNT5A as did by canonical ligand WNT3A. However, both canonical WNT acitivity and non-canonical WNT activity have been demonstrated in IPF development [26, 124]. Therefore, the function of non-canonical WNT activity during lung repair and IPF progression requires further investigation.

Cellular senescence is a hallmark of the aging lung and is considered as a risk factor for IPF. In this project, we linked developmental pathway activity to cellular senescence in

IPF. However, our understanding of senescence in IPF onset versus progression remains extremely limited. Heterogeneity in senescence include different cell types but further different potential triggers. Therefore, characterization of the initiating trigger for senescence and the subsequent senescent cell phenotype during different phases of IPF are in high demand and require further work.

We identified GPR87 as a novel surface marker and potentially druggable target of KRT5+ basal progenitor cells. Those GPR87+ basal cells are senescent and likely contribute to bronchiole remodeling and honeycomb cyst development in IPF. Notably, investigators identified Krt8+ ADI cells and basaloid cells in IPF tissues. GPR87+ basal cell detected in this project showed similarity of transcriptomics to both Krt8+ ADI cells and basaloid cells with expression of cytokeratin genes, senescent features and EMT features. All these three novel cell types are considered as intermediated cell state which may be involved in the IPF progression. However, the function and origin of these novel cell types as well as their similarity and distinction remain poorly understood. Thus, further functional investigations on these novel cell types are required. Spatial transcriptomics and lineage trajectory analysis would shed light on the origin of these cell types and their fate. These investigations will further clarify the underlying mechanism of how cell reprogramming drive pulmonary fibrosis and may lead to novel therapies.

3. Paper I

M. Lehmann, **Q. Hu**, Y. Hu, K. Hafner, R. Costa, A. van den Berg, and M. Königshoff. "Chronic Wnt/B-Catenin Signaling Induces Cellular Senescence in Lung Epithelial Cells." *Cell Signal* 70 (Jun 2020): 109588. https://doi.org/10.1016/j.cellsig.2020.109588.

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Chronic WNT/β-catenin signaling induces cellular senescence in lung epithelial cells

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ABSTRACT

The rapid expansion of the elderly population has led to the recent epidemic of age-related diseases, including increased incidence and mortality of chronic lung diseases, such as Idiopathic Pulmonary Fibrosis (IPF). Cellular senescence is a major hallmark of aging and has a higher occurrence in IPF. The lung epithelium represents a major site of tissue injury, cellular senescence and aberrant activity of developmental pathways such as the WNT/β-catenin pathway in IPF. The potential impact of WNT/β-catenin signaling on alveolar epithelial senescence in general as well as in IPF, however, remains elusive. Here, we characterized alveolar epithelial cells of aged mice and assessed the contribution of chronic WNT/β-catenin signaling on alveolar epithelial type (AT) II cell senescence. Whole lungs from old (16–24 months) versus young (3 months) mice had relatively less epithelial ($EpCAM^+$) but more inflammatory ($CD45^+$) cells, as assessed by flow cytometry. Compared to young ATII cells, old ATII cells showed decreased expression of the ATII cell marker *Surfactant Protein C* along with increased expression of the ATI cell marker *Hopx,* accompanied by increased WNT/β-catenin activity. Notably, when placed in an organoid assay, old ATII cells exhibited decreased progenitor cell potential. Chronic canonical WNT/β-catenin activation for up to 7 days in primary ATII cells as well as alveolar epithelial cell lines induced a robust cellular senescence, whereas the non-canonical ligand WNT5A was not able to induce cellular senescence. Moreover, chronic WNT3A treatment of precision-cut lung slices (PCLS) further confirmed ATII cell senescence. Simultaneously, chronic but not acute WNT/β-catenin activation induced a profibrotic state with increased expression of the impaired ATII cell marker Keratin 8. These results suggest that chronic WNT/β-catenin activity in the IPF lung contributes to increased ATII cell senescence and reprogramming. In the fibrotic environment, WNT/β-catenin signaling thus might lead to further progenitor cell dysfunction and impaired lung repair.

1. Introduction

Physiological lung aging contributes to changes in lung function and susceptibility to a wide range of chronic lung diseases (CLD), such as chronic obstructive pulmonary diseases (COPD) or idiopathic pulmonary fibrosis (IPF) [1,2]. Several aging hallmarks are observed in CLDs, however, our current knowledge of the main similarities and/or differences between normal lung aging and CLD pathogenesis is limited and needs to be extended to further identify potential therapeutic options in CLDs that target aging-associated mechanisms.

The distal lung epithelium consists of different airway and alveolar epithelial cells, which are essential for homeostasis and proper function of the alveolus. Notably, alveolar epithelial type (AT) II cells secrete surfactant proteins such as Surfactant Protein-C (*Sftpc*/ SP-C) and serve as progenitor cells for ATI cells, which are indispensable for gas exchange [3,4]. Injury and loss of distal lung epithelial cells are major hallmarks of many CLDs, including IPF. IPF is thought to result from aberrant and continuous activation of injured distal lung epithelial cells leading to alterations in the cellular phenotype that contributes to a failure in repair and regeneration (also referred to as “reprogramming”) [4,5]. More recently, several aging mechanisms have been implicated in ATII cell reprogramming [2,6], however, the underlying molecular

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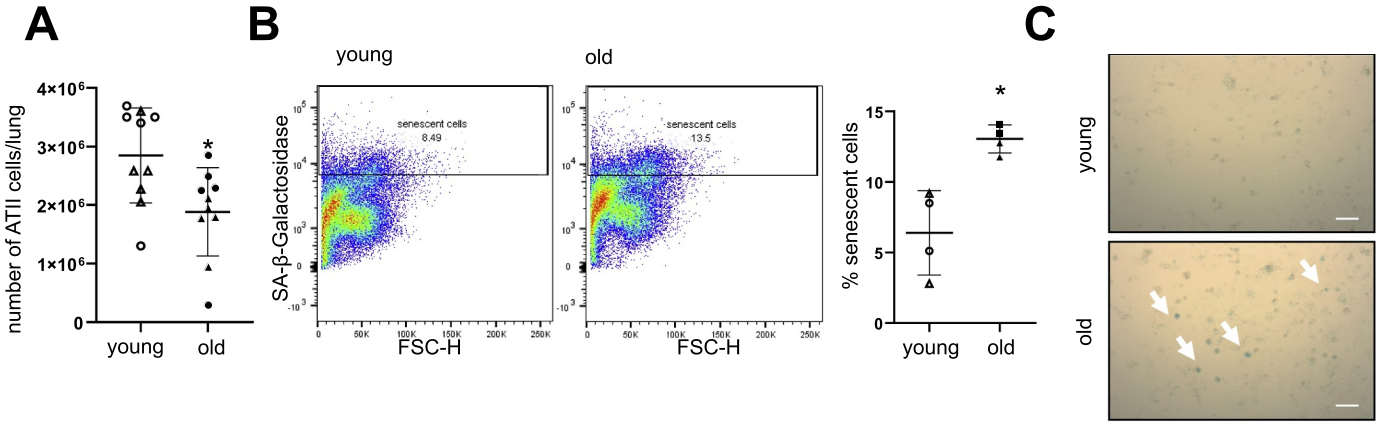

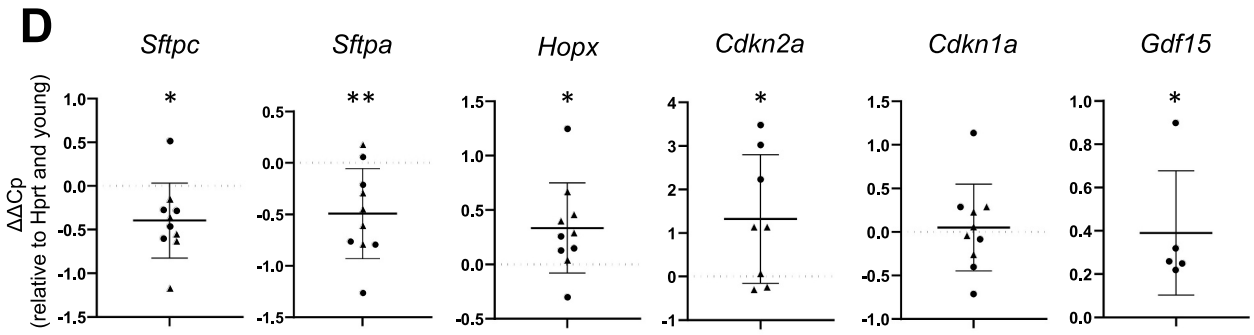

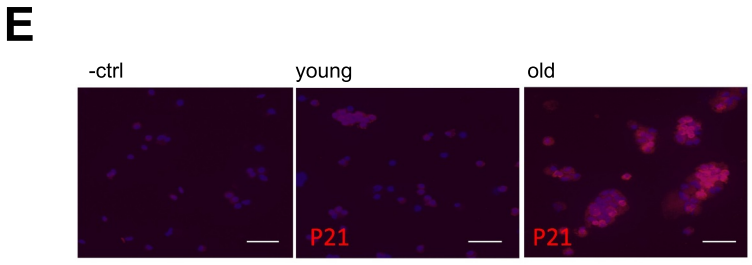

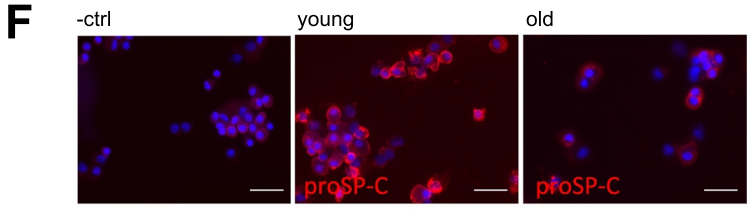

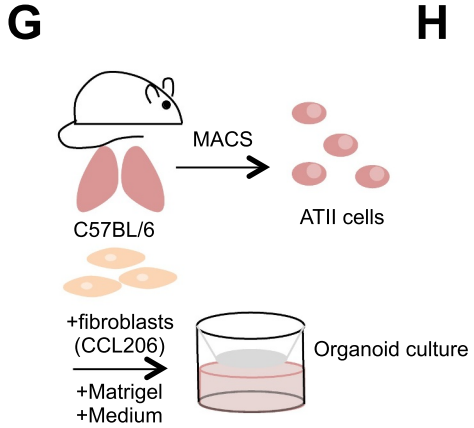

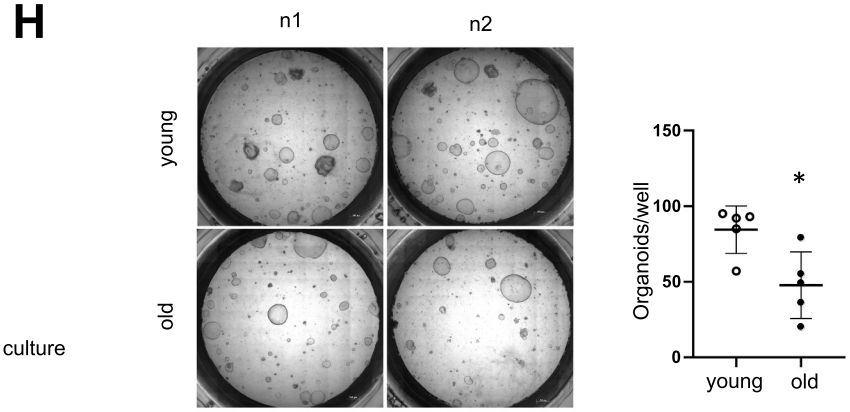

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Fig. 1. Phenotype of ATII cells in aged mice. **(A-H)** Lungs from young (6–21 weeks) or old (16–24 months) mice were harvested and ATII cells were isolated. **(A)** Total number of isolated ATII cells per mouse, $n = 10$. **(B)** Freshly isolated ATII cells were analyzed for SA-ß-galactosidase activity by FACS $n = 4$ or **(C)** by conventional SA-ß-galactosidase staining 2 days after plating, image representative of n = 4, arrows depict positive cells, Size bar represents 50 μm. **(D)** Freshly isolated ATII cells were analyzed for alveolar epithelial cell and senescence markers by qPCR. Values were normalized to *Hprt* and corresponding young controls. $n = 5–10$. **(*E*-F)** Cytospins of freshly isolated ATII cells were stained for **(E)** P21 or **(F)** proSP-C protein. Image representative of a $n = 3$, Size bar represents 50 μm. **(G-H)** Freshly isolated ATII cells were combined with Matrigel and CCL206 fibroblasts and used for an organoid assay as outlined in **(G)** and representative pictures are shown at day 14 of organoid differentiation in **(H)** as well as quantification of numbers of organoids per well, $n = 5$. Data are presented as mean ± s.d. Circles represent C57BL6/J mice, triangles represent C57BL6/N mice. Significance was assessed with one sample *t*-test compared to a hypothetical value of 0 (D) or Mann Whitney-test (A, B, H) Significance: $*P < .05$, $**P < .01$.

mechanisms contributing to the aging phenotype in IPF, remains largely unexplored.

Ten hallmarks of the aging lung were described and in particular stem cell exhaustion, cellular senescence, and extracellular matrix dysregulation have been shown to contribute to the aging phenotype [2]. Cellular senescence is characterized by irreversible cell cycle arrest due to augmented levels of cell cycle inhibitors p16INK4a and p21CIP1 [7,8], high activity of senescence-associated β-galactosidase (SA-β-gal) as well as secretion of senescence-associated secretory phenotype (SASP), by which senescent cells significantly impact the (micro-)environmental niche [9]. While cellular senescence is a physiological process, required for the regulation of embryogenesis [10,11] and prevention of tumor cell proliferation [8], aberrant accumulation of senescent cells has further been demonstrated to exhibit deleterious effects on tissue homeostasis [8,12], for example by contributing to stem/progenitor cell exhaustion [13].

Increased senescent epithelial cells and their associated SASP have been linked to IPF [14,15]. Different stressors can induce cellular senescence [8,12]. Senescence is triggered by a persistent DNA damage response that is initiated by extrinsic (UV damage, chemotherapeutic drugs, γ-irradiation) or intrinsic (telomere attrition, oxidative stress, hyperproliferation) insults. In addition, oncogene-induced senescence (OIS) is a specific type of premature senescence, which is classically triggered by hyperactivation of oncogenes such as Ras or BRAF [16] but also activation of WNT/β-catenin signaling can result in OIS [17,18].

The WNT signaling pathway regulates a number of cellular processes, including cellular migration, proliferation and differentiation. WNT proteins are secreted, cysteine-rich glycosylated proteins that can activate the β-catenin-dependent (canonical) WNT pathway (such as WNT3A) or the β-catenin-independent (non-canonical) WNT (such as WNT5A) pathway, by binding to various transmembrane receptors (Frizzled 1–10). In both developing and adult lung, WNT/β-catenin signaling controls progenitor cell function and regulates tissue homeostasis [19–23]. Importantly, aberrant WNT/β-catenin signal activity has been demonstrated in human and experimental lung fibrosis [19,24–26] and linked to distal lung epithelial cell dysfunction [27–29]. Moreover, increased WNT/β-catenin activity has been demonstrated to lead to accelerated aging [18,30]. Its role in the aging lung, however, is still under-investigated [31]. Here, we aimed to elucidate the role of WNT/β-catenin signaling in the process of normal lung aging and its contribution to cellular senescence and reprogramming of ATII cells.

2. Materials and methods

2.1. Animals

Young or old pathogen-free C57BL/6 N or *J* mice were obtained from Charles River or Jackson Laboratory and housed in rooms with constant humidity and temperature with 12 h light cycles and free access to water and rodent chow. Mice were sacrificed and lungs were used for collection of whole lung tissue, ATII cells or PCLS. For all experiments in Fig. 3–6 C57BL6/N mice were used, for experiments in Fig. 1 C57BL6/N or *J* mice were used as indicated. Specific ages of mice were as follows: Fig. 1A 16–24 months 6–20 weeks, Fig. S1A 16–21 months 6–20 weeks, Fig. 1B 16–24 months, 6–12 weeks, Fig. 1C 16–24 months, 6–12 weeks, Fig. 1D 16–24 months, 6–21 weeks, Fig. 1 E/F 14–24 months, 21–24 weeks, Fig. 1H 20–24 months 10–20 weeks, Fig. 2 14–18 months, 8–16 weeks, Figs. 3-6: 6–12 weeks.

TCF/Lef:H2B/GFP (TCF-GFP, The Jackson Laboratory, 013752) mice of 56–80 weeks were used for aging analysis. Young adult TCF-GFP mice of 8–16 weeks were used as control. All animal experiments were performed according to the institutional and regulatory guidelines of University of Colorado Institutional Animal Care and Use Committee.

2.2. Isolation of primary murine alveolar epithelial cell type II (pmATII) cells

The pmATII cells were isolated as previously described [28,32] with slight modifications. In brief, lungs were filled with dispase (Corning, New York, NY, USA) and low gelling temperature agarose (Sigma Aldrich, Saint Louis, MO, USA) before tissue was minced and the cell suspension was filtered through 100-, 20-, and 10-μm nylon meshes (Sefar, Heiden, Switzerland). Negative selection of fibroblasts was performed by adherence on non-coated plastic plates. Macrophages and white blood cells were depleted with CD45 and endothelial cells were depleted with CD31 specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cell purity was assessed routinely by analysis of endothelial (CD31), mesenchymal (α-SMA, CD90), epithelial (EpCAM, panCK and proSP-C), and hematopoietic cell (CD45) markers by immunofluorescence or flow cytometry.

For the analysis of WNT-GFP epithelial cells and for the organoid experiments, isolation was performed as described above. No depletion of fibroblasts was performed, the CD45 and CD31 depleted single cell suspension was further enriched for epithelial cells by positive selection using EpCAM (CD326) Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.3. Flow cytometry

A single cell suspension was generated by dispase treatment, mincing and serial filtering as described above. Cells were washed once in FACS buffer, stained with anti-mouse CD326 (Ep-CAM), APC (Biolegend 118,214), anti-mouse CD45, PE (Biolegend 103,106) or respective IgG controls (Biolegend 400,512, 400,608) for 20min at 4 °C in FACS buffer, washed once and analyzed. FACS-based detection of SA-β-galactosidase was performed as previously described [14,33]. Briefly, pmATII or MLE12 cells were incubated with Bafilomycin A1 (100 nM, Enzo Life Sciences, Farmingdale, NY; USA) and C_{12}FDG (33 μM, Life technologies, Carlsbad, CA; USA) for 1 and 2 h, respectively, directly after isolation or at day 2 of culture. Cells were trypsinized and washed. Stained cells were analyzed with a FACS LSRII (BD Bioscience, San Jose, CA; USA). Positive populations were quantified by FlowJo software (Tomy Degital Biology Co., Ltd., Tokyo, Japan).

Cells from old or young adult WNT-GFP mice were stained by anti-mouse EpCAM conjugated with APC (Biolegend, 118,214) or APC rat IgG2a isotype control for EpCAM (BioLegend 400,511) in dark for 15 min at room temperature, followed by PBS washing and centrifuge at 300 *g*, 15 °C for 5 min. Then the cells were resuspended in PBS with 1% FBS and 25 mM HEPES. DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, final concentration 2 μg/ml) was added to the cell

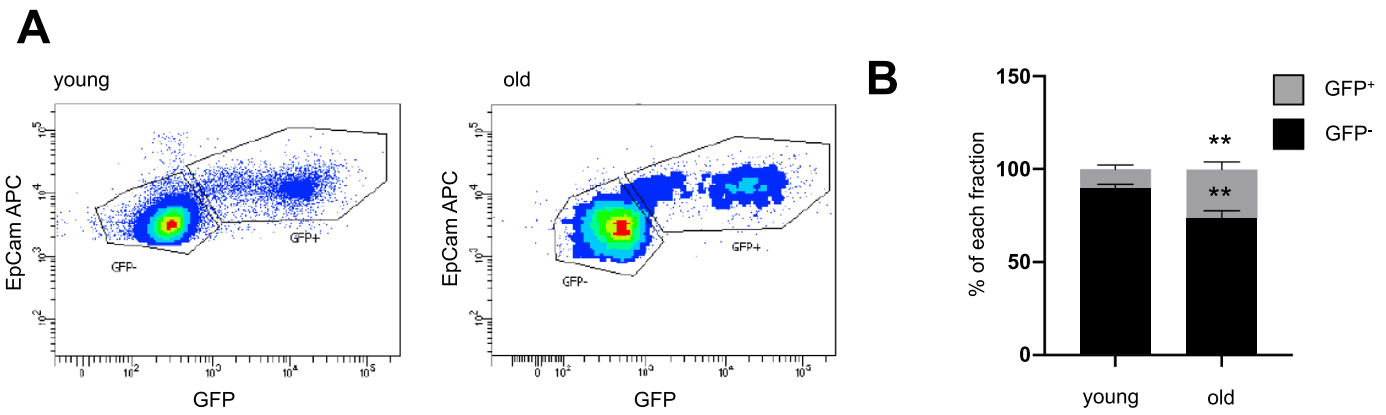

Fig. 2. WNT activity is increased in ATII cells from aged mice. **(A-B)** Lungs from young (3 months) or old (18 months) WNT GFP mice were harvested and ATII cells were isolated and analyzed for WNT activity (GFP^+) by FACS. Data are presented as mean ± s.d. $n = 6$ old, $n = 14$ young. Significance was assessed with a two-way Anova followed by Sidak's multiple comparison test. Significance: **P < .01.

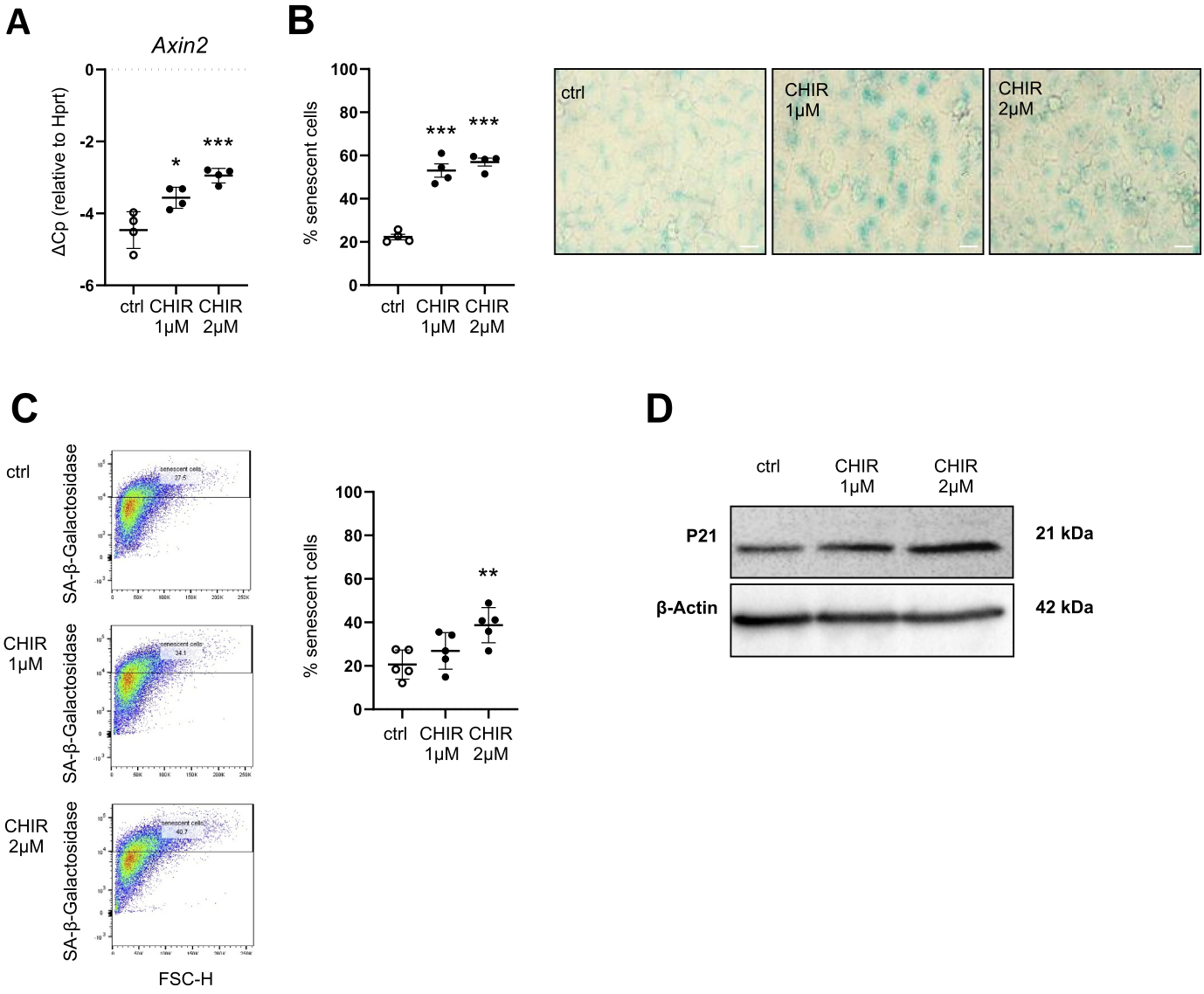

Fig. 3. Chronic WNT stimulation induces cellular senescence in MLE12 cells.
(A-D) MLE12 lung epithelial cells were treated with 1 μM CHIR or 2 μM CHIR for 7d **(A)** qPCR analysis for WNT target gene *Axin2* normalized to *Hprt* levels was perfomed. $n = 4$. **(B)** SA-β-galactosidase activity was measured by conventional staining after 7d, representative of n = 4. Size bar represents 50 μm. **(C)** SA-β-galactosidase activity was measured by FACS-based staining after 7d, n = 5. **(D)** Western Blot for P21 was performed, β-actin was used as a loading control. Blot is representative of n = 4. Data are presented as mean ± s.d. Significance was assessed with a one-way Anova followed by Tukey's multiple comparison test. Significance: *P < .05, **P < .01, ***P < .001.

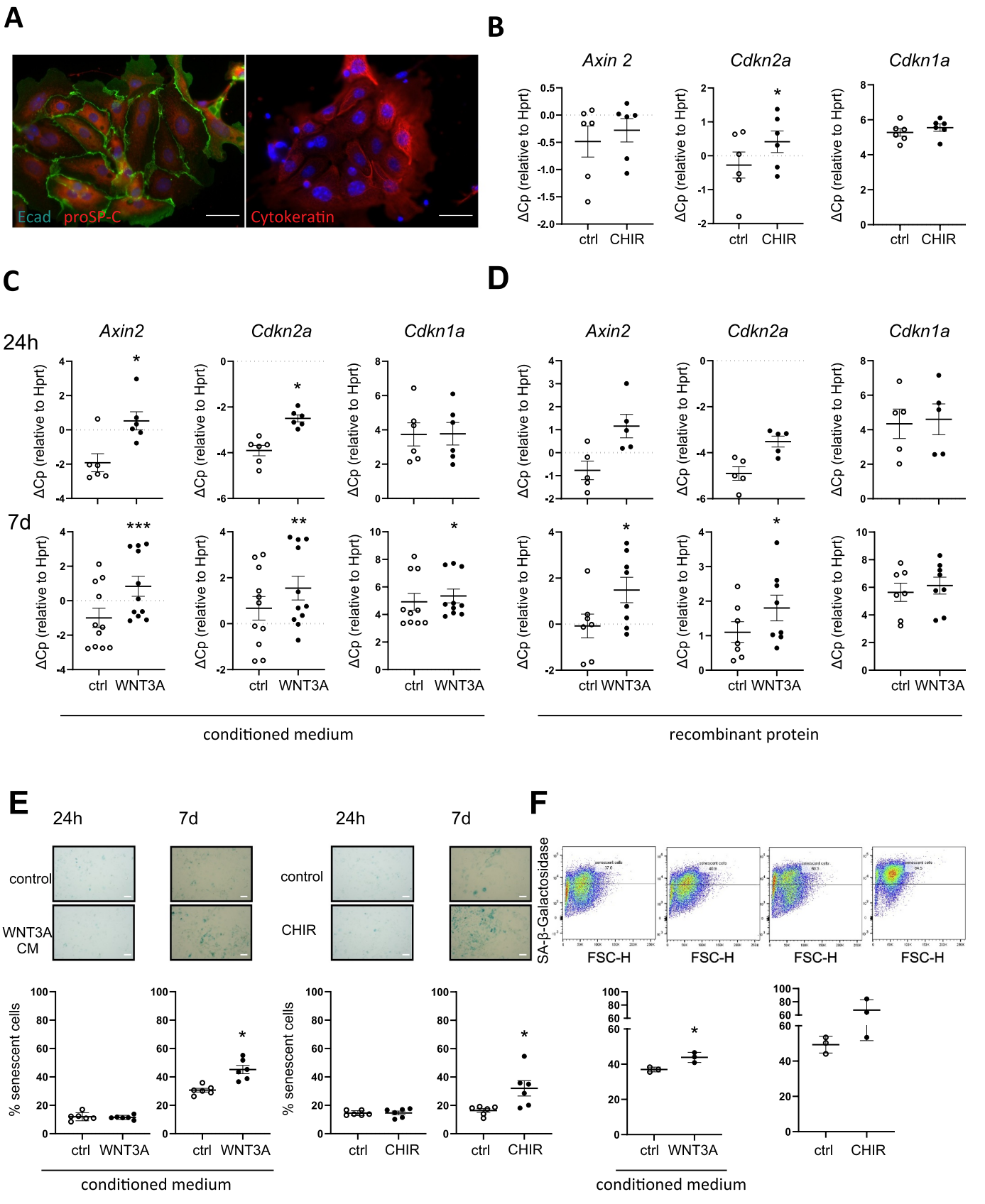

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Fig. 4. Chronic WNT stimulation induces cellular senescence in pmATII cells.
(A) ATII cells were stained by Immunofluorescence for pro Surfactant Protein-C (proSP-C), E-Cadherin (Ecad) or Cytokeratin. **(B)** ATII cells were treated with 1 μM CHIR for 7d, n = 6**, (C)** conditioned medium from WNT3A-overexpressing L-cells (WNT3A CM; 1:1) for 24 h (n = 6) or 7 days (n = 10) **(D)** recombinant mWNT3A for 24 h (n = 5) and 7d (*n* = 8). **(B-D)** qPCR analysis for WNT target gene *Axin2* and senescence markers *Cdkn1a* and *Cdkn2a* normalized to *Hprt* levels was performed. **(E-F)** ATII cells were treated with 1 μM CHIR or WNT3A CM and SA-β-galactosidase activity was measured by **(E)** conventional staining after 24 h and 7d, n = 6 or by **(F)** FACS-based staining after 7d, *n* = 3. Significance was assessed by Wilcoxon matched-pair signed rank test (B-D) and Student's t-test (E-F). Significance: **p* < .05, **P < .01.

suspension before analysis or sorting. GFP reporter activity in the $EpCAM^+$ population was assessed based on fluorescence intensity using FACSDiva software (BD Bioscience). The analysis was performed by FACS Fortessa cell analyzer (BD Bioscience).

2.4. Senescence-associated (SA)-β -galactosidase staining

pmATII cells or precision-cut lung slices (PCLS) were prepared from C57BL6/N wildtype (WT) mice as previously described [25] and cultured in multi-well plates. Cytochemical staining for SA-β-galactosidase was performed using a staining Kit (Cell Signaling Technology, Danvers, MA), according to the manufacturer's instructions. Images were acquired using a Zeiss Axiovert40C microscope. The percentage of senescent cells was determined by counting of total and SA-β-galactosidase-positive cells in 3 random microscopic fields per condition (100× magnification).

2.5. Preparation of WNT-conditioned medium (CM)

Mouse fibroblasts-like L-cells stably expressing WNT-3A or WNT-5A were used to obtain WNT-CM according to a standardized protocol [34]. Parental L-cells (control: ATCC CRL-2648), L-WNT-3A cells (ATCC CRL-2647) L-WNT-5A cells (ATCC CRL-2814), were cultured in DMEM/F12 medium supplemented with 10% FCS, 100 mg/l streptomycin, and 100 U/ml penicillin. WNT CM or control CM was prepared according to the ATCC guidelines and as previously published [34]. In short, confluent L-cell cultures were split 1:10 and cultured for 4 d in supplemented DMEM/F12 medium in 10-cm culture dishes. After 4 d, the medium was collected and the cells were cultured for another 3 d in fresh DMEM/F12 medium with supplements. The second batch of medium was collected after 3 d and mixed with medium of day 4 (ratio 1:1). The combined medium, which is referred to as CM, was filtered and stored at −20 °C till further use.

2.6. Cell culture

In experiments using pmATII cells, cells were seeded, cultured for 48 h in ATII cell medium (DMEM (Sigma Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (both Life Technologies, Carlsbad, CA), 3.6 mg/ml glucose (Applichem GmbH, Darmstadt, Germany) and 10 mM HEPES (PAA Laboratories) containing 10% FCS (PAA Laboratories, Pasching, Austria). Then the ATII cells were treated with ATII cell medium supplemented with 5% FCS and containing DMSO, 1 μM CHIR 99021 (CHIR) or 100 ng/ml recombinant mouse WNT3A (RnD Systems, 1324-WN, Minneapolis, MN, USA dissolved in 0.1% BSA in PBS) or treated with WNT3A conditioned medium mixed with ATII cell medium (1:1; final FCS concentration 5%). MLE12 cells were purchased from ATCC (CRL-2110) and maintained in DMEM/F12 (Gibco®, USA) medium containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded at 25 000 cells per well in a 6-well plate and allowed to adhere for 24 h. Cells were then treated every 48 h with DMSO, 1 μM CHIR 99021 (CHIR) and 2 μM CHIR in DMEM/F12 supplemented with 5% FCS or treated with WNT3A conditioned medium mixed with DMEM/F12 (1:1; final FCS concentration 5%).

2.7. Organoid culture

Organoids were cultured as previously described [3,35]. Briefly, MLg (ATCC CCL-206) mouse lung fibroblasts were proliferation-inactivated with 10 μg/ml mitomycin C (Merck, Darmstadt, Germany) for 2 h. 20.000 primary mouse ATII cells were resuspended in 50 μl media and diluted 1:1 with 20.000 MLg cells in 50 μl growth factor reduced Matrigel (Corning, New York, USA). Cell mixture was seeded into 24-well plate 0,4 μm trans-well inserts (Corning, New York, USA). Cultures were treated at day 0 and every 2nd or 3rd day in DMEM/F12 containing 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-alanyl-L-glutamine, Amphotericin B (Gibco), insulin-transferrin-selenium (Gibco), 0.025 μg/ml recombinant human EGF (Sigma Aldrich, St Louis, USA), 0.1 μg/ml Cholera toxin (Sigma Aldrich, St Louis, USA), 30 μg/ml bovine pituitary extract (Sigma Aldrich, St Louis, USA), and 0.01 μM freshly added all-trans retinoic acid (Sigma Aldrich, St Louis, USA). 10 μM Y-27632 (Tocris) was added for the first 48 h of culture. Microscopy for organoid quantification at day 14 was performed using a LSM710 system (Zeiss) containing an inverted AxioObserver.Z1 stand.

2.8. Generation and treatment of PCLS

Precision-Cut Lung Slices (PCLS) were generated as previously described [14,20]. Briefly, lungs were flushed through the heart with sterile sodium chloride solution and filled with low gelling temperature agarose (2%, A9414; Sigma) in precision-cut lung slices (PCLS) medium (DMEM/Ham's F12 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Sigma Aldrich)). Next, lobes were cut with a vibratome (Hyrax V55; Zeiss, Jena, Germany) to a thickness of 300 μm (speed 10–12 μm·s − 1, frequency 80 Hz, amplitude of 1 mm). PCLS were treated with WNT3A or control conditioned medium mixed with PCLS medium (1:1; final FCS concentration 5%). RNA was isolated and gene expression was measured by qRT-PCR.

2.9. Immunofluorescence staining

PCLS were fixed with 4% paraformaldehyde (PFA) for 20 min, then blocked with 5% normal goat serum (Abcam) for 1 h. After incubation with primary antibody (p21 1:200 (ab188224, Abcam, Cambridge, UK)); (at 4 °C overnight and secondary antibody at room temperature for 1 h, staining was evaluated via confocal microscopy (LSM 710; Zeiss, Oberkochen, Germany). For immunofluorescence staining experiments, ATII cells were seeded on poly-l-lysin treated coverslips. Cells were stopped at day 2 and fixed with ice-cold acetone-methanol (1:1) for 10 min and washed 3 times with 0.1% BSA in PBS. Next, cells were permeabilized with 0.1% Triton X-100 solution in PBS for 20 min, blocked with 5% BSA in PBS for 30 min at room temperature and incubated with primary antibodies (proSP-C 1:100 (AB3786, Millipore, Darmstadt, Germany), *E*-Cadherin 1:200 (610181, BD, Franklin Lakes, NJ, USA), Cytokeratin 1:500 (Dako, Glostrup, Denmark), followed by secondary antibodies, 1 h each. DAPI (Roche, Basel, Switzerland) staining for 10 min was used to visualize cell nuclei. Next, coverslips were fixed with 4% PFA for 10 min, mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and visualized with an Axio Imager microscope (Zeiss, Oberkochen, Germany). Cyto Spins were obtained by centrifugation of freshly isolated pmATII cells (10 min 300 g, 100.000cells/spin). Cells were fixed with 4% PFA, and blocked

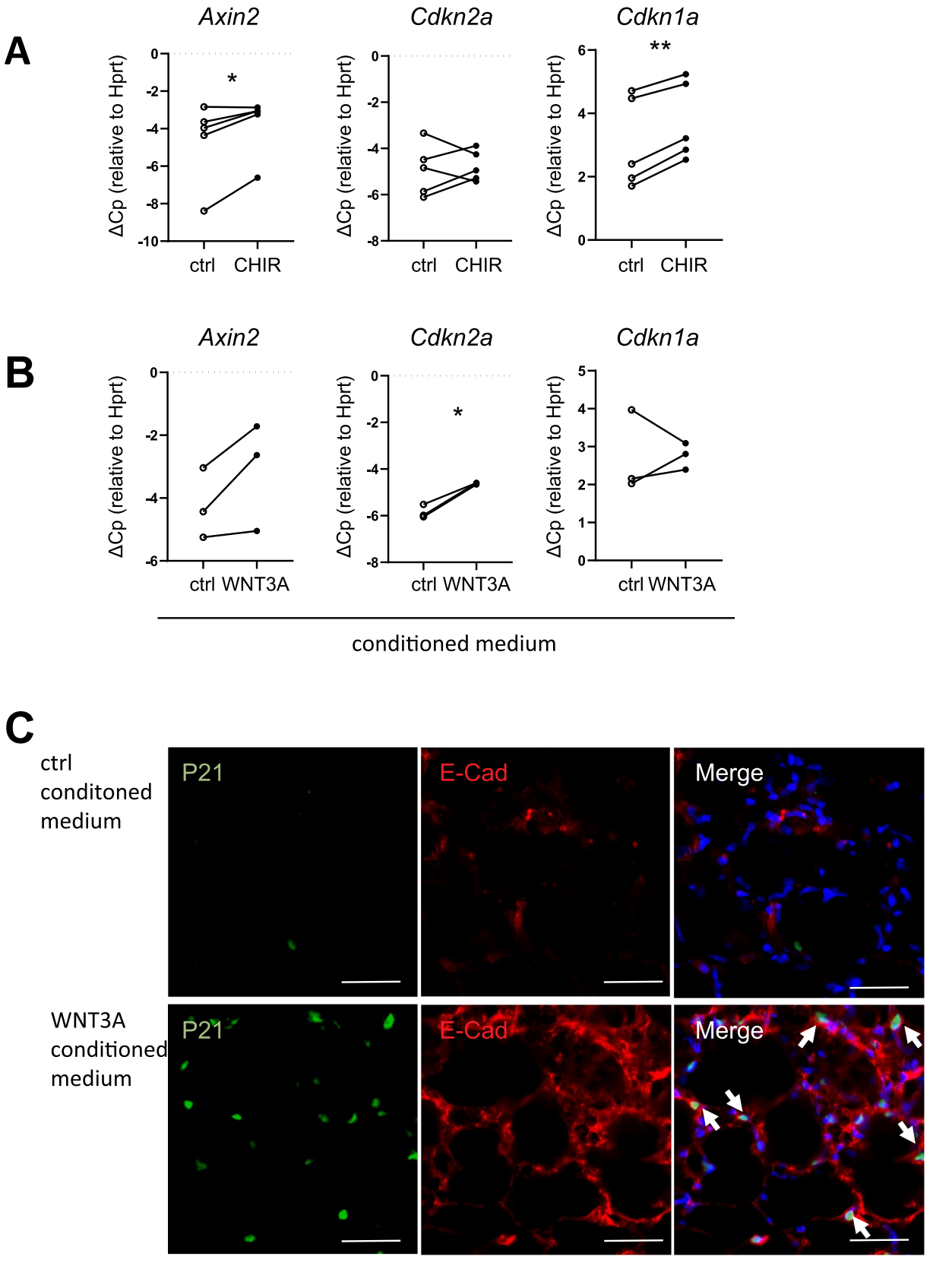
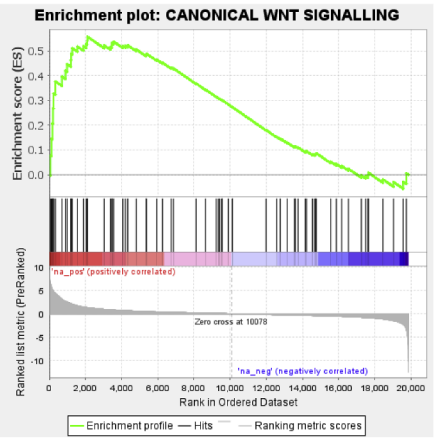


Fig. 5. Chronic WNT stimulation induces and cellular senescence in epithelial cells.
(A-C) Preciscion cut lung slices (PCLS) were prepared from young mice and treated with **(A)** 2 μM CHIR for 7 days, n = 5, or **(B)** conditioned medium from WNT3A-overexpressing L-cells (WNT3A CM; 1:1) for 7 days. n = 3. **(A-B)** qPCR analysis for WNT target gene *Axin2* and senescence markers *Cdkn2a* and *Cdkn1a* was performed and normalized to *Hprt* levels. **(C)** Representative images of immunofluorescence staining for P21 and CDH1 (E-CAD) in PCLS prepared from young mice and treated with WNT3A CM for 7 days. Fluorescent images represent a 400× magnification. The scale bar represents 50 μm. Representative of n = 3. Significance was assessed by paired Student's t-test (A-B). Significance: *p < .05, **P < .01.

A



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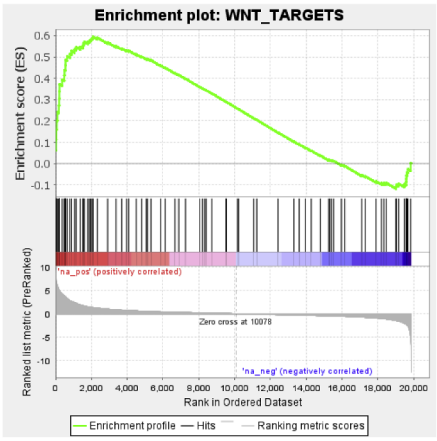
Normalized Enrichment Score: 1.574
Nominal p-value: 0.004



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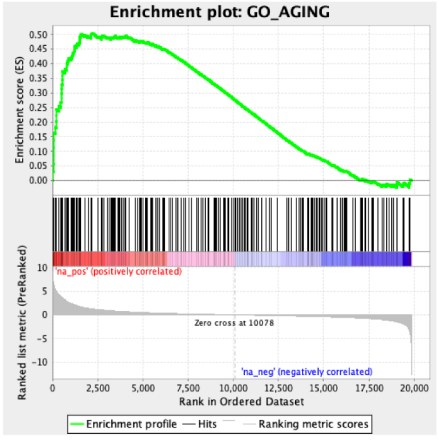

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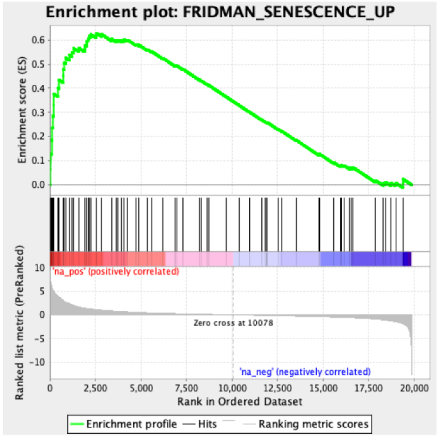

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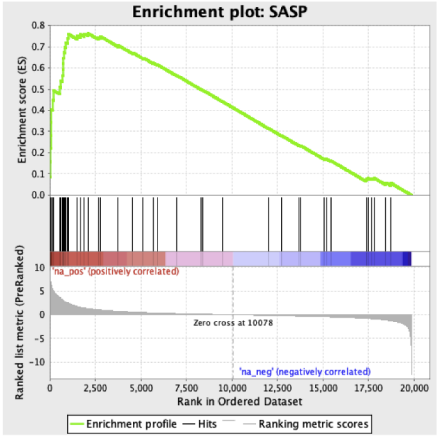

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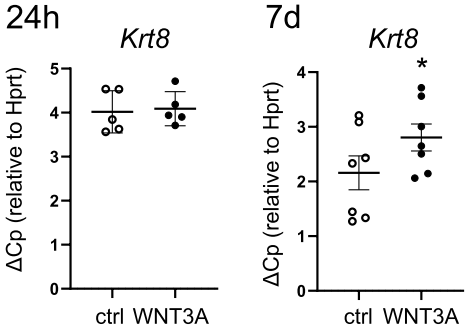
C



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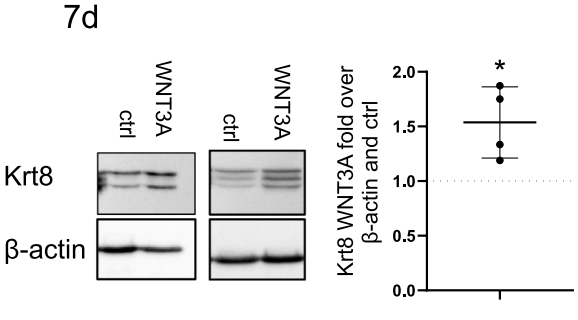

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Fig. 6. Chronic WNT stimulation induces Keratin 8 (Krt8) and cellular senescence in epithelial cells.
(A-B) Gene set enrichment analysis was performed on a published RNA sequencing dataset from hATII cells from Donor/IPF patients GSE94555 (Xu et al., 2016, JCI Insight). The dataset was tested for the enrichment of **(A)** WNT/β-catenin (GO: 0060070 and https://web. stanford. edu/group/nusselab/cgi - bin/wnt / target_genes) or **(B)** for aging (GO:0007568), senescence (Fridman et al., Oncogene, 2008 [40]) or senescence associated secretory phenotype (SASP; Coppé, Annu Rev. Pathol) [9]) lists. **(C-D)** pmATII cells were treated with WNT3A CM for 24 h (n = 5) or 7 days (*n* = 7). **(D)** qPCR analysis of the fibrotic epithelial marker *Krt8* normalized to *Hprt***(D)** Two representative western blots of Krt8. Quantification of Krt8 signal over β-actin normalized to Ctrl CM is shown on the right. n = 4. Data are presented as mean ± s.d. Significance was assessed by Wilcoxon matched-pair signed rank test (C) and one sample t-test compared to a hypothetical value of 1 (D). Significance: *p < .05, **P < .01.

with 5% goat Serum (Abcam, ab7481) for 30 min. Cells were subsequently incubated with the respective primary antibody at RT for 2 h in PBS containing 0.1% BSA, (proSP-C (Merck Millipore, AB3786, Darmstadt, Germany), p21 (Abcam, ab188224) followed by incubation with the fluorescently labeled secondary antibody (goat anti-rabbit Alexa Fluor 555, Life Technologies). DAPI staining (Life Technologies, 62248) was used to visualize cell nuclei and cytospins were analyzed using an Axio Imager microscope (Zeiss, Oberkochen, Germany).

2.10. Cytotoxicity assay

Cytotoxicity of CHIR99021 (4423-Tocris) was evaluated using an LDH-cytotoxicity detection kit (Roche 11644793001) according to manufacturer's instructions. MLE12 cells were cultured in 24-well plates in 10% DMEM for 7 days and were treated with CHIR99021 every second day (2 μM). After day 6 the medium was changed to DMEM containing 0.1% FCS containing CHIR and supernatant was collected at day 7 and incubated with reaction mixture. TritonX-100 was used as a positive control and DMEM as a negative control. Cytotoxicity was calculated as % of the positive control.

2.11. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR, qPCR)

Total RNA was extracted using the PEQLAB Total RNA extraction Kit (PEQLAB, Erlangen, Germany) according to the manufacturer's instructions. For PCLS, RNA was extracted as previously described [14,20,36], with minor variations. Briefly, 3 pieces of PCLS each sample were snap frozen in liquid nitrogen and kept at −80 °C until isolation was done. Frozen PCLS was homogenized using Tissue Lyser II (QIAGEN, Hilden, Germany) and then incubated with triazol reagent (Sigma, St Louis, USA) on ice for 30 min. Cell debris were removed by centrifuging samples at 1000 xg for 5 min and the supernatant were cleaned by PerfectBind RNA Columns (peqGOLD Total RNA Kit, Erlangen, Germany) and DNase I (Applichem, Darmstadt, Germany). Cleaned RNA was eluted from column using RNase-free Water and stored at −80 °C.

cDNAs were generated by reverse transcription using SuperScriptTM II (Invitrogen, Carlsbad, CA, USA). Quantitative (q)RT-PCR was performed using Light Cycler 480 detection system and SYBR Green (Roche Diagnostics, Mannheim, Germany). Hypoxanthine phosphoribosyltransferase (HPRT) was used as a reference gene.

Relative gene expression is presented as ΔCt value (ΔCt = [Ct Hprt]-[Ct gene of interest]). Relative change in transcript level upon treatment is expressed as ΔΔCt value (ΔΔCt = ΔCt of treated sample-ΔCt of control).

Primers:

Gene	Forward primer	Reverse primer
mCdkn2a	CGGGGACATCAAGACATCGT	GCCGGATTTAGCTCTGCTCT
mCdkn1a	ACATCTCAGGGCCGAAAACG	AAGACACACAGAGTGAGGGC
mAxin2	AGCAGAGGGACAGGAACCA	CACTTGCCAGTTTCTTTGGCT
mKrt8	ACATCGAGATCACCACCTACC	GGATGAACTCAGTCCTCCTGA
mHprt	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
mGdf15	TCGCTTCCAGGACCTGCTGA	TGGGACCCCAATCTCACCTCT

2.12. Western blotting

Cold RIPA buffer plus protease and phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany) was added to the cells which were washed twice by PBS. The plate with cells was kept on ice for 30 min, swirled occasionally for uniform spreading. Then, cells were scraped and the lysate was collected to a microcentrifuge tube. The tube was centrifuged at ~14,000 *g* for 15 min to pellet the cell debris. The supernatant was transferred to a new tube and the protein concentration was quantified using Pierce™ BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific). Equal amounts of protein were loaded with 4× Laemmli loading buffer (150 mM Tris HCl [pH 6.8], 275 mM SDS, 400 nM dithiothreitol, 3.5% (*w/v*) glycerol, 0.02% bromophenol blue) and subjected to electrophoresis in 17% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat dried milk solution in TRIS-buffered saline containing 0.01% (*v/v*) Tween (TBS-T) (Applichem, Darmstadt, Germany) for 1 h and incubated with primary antibodies (anti p21, MAB88058, Merck Millipore (Billerica, MA, USA); anti β-actin, A3854; anti-Krt8/TROMA-I; DSHB-Developmental Studies Hybridome Bank at the University of Iowa) at 4 °C overnight. Next, blots were incubated for 1 h at RT with secondary, HRP-conjugated, antibodies (GE-Healthcare) prior to visualization of the bands using chemiluminescence reagents (Pierce ECL, Thermo Scientific, Ulm, Germany), recording with ChemiDocTMXRS + system and analysis using Image Lab 5.0 software (Biorad, Munich, Germany).

2.13. Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) was performed using the GSEA software [37,38] on a previously published single-cell RNA sequencing dataset from IPF and control human isolated ATII cells (GSE94555) [39]. A pre-ranked gene list was generated from normalized data and based on log2 fold change. Enrichment of a gene set in one distinct phenotype was considered significant with a false discovery rate (FDR) q-value < 0.05 and a nominal *p*-value < .05. Five different gene set lists were used: Wnt target genes (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes, The Wnt Homepage – Wnt target genes, last accessed 23 September 2019), Canonical Wnt signaling (gene ontology ID GO: 0060070), Aging (gene ontology ID GO:0007568), Senescence [40] and SASP [9].

2.14. Statistical analysis

All data is presented as mean ± s.d. and was generated using GraphPad Prism 8. The number of biological replicates is indicated in each experiment. Statistical significance was evaluated with either Wilcoxon signed-rank test, Mann-Whitney *U* test or repeated-measures one-way ANOVA followed by Newmann-Keuls multiple comparison test, with one-sample *t*-tests in comparison to a hypothetical value of 0 or 100 or two-way ANOVA followed by Sidak's multiple comparison test where appropriated. Differences were considered to be statistically significant when $P < .05$.

3. Results

3.1. Old lung epithelial cells are senescent and exhibit impaired progenitor cell function

We aimed to investigate lung epithelial cells in lungs of young (3 months) compared to old (16–24 months) mice. Analysis of a single cell suspension of the whole lung revealed a relative decrease of the epithelial ($EpCAM^+$) cell population, while the percentage of $CD45^+$ cells was significantly increased in the old mice (Fig. S1A), which is consistent with recent reports demonstrating lung "inflammaging" [6]. We used well-established protocols to isolate ATII cells from the single cell suspension and observed less cell numbers in old animals compared to young ones (Fig. 1A). This finding was irrespective of size and bodyweight of the animals (Suppl. Fig. 1B). Old ATII cells exhibited increased activity of the senescence marker senescence-associated β-galactosidase (SA-β-gal) as assessed by flow-cytometry (Fig. 1B, 5.69 ± 2.64% senescent cells in young mice; 12.90 ± 0.94% senescent cells in old mice; $p < .05$) or conventional light microscopy, with SA-β-gal high cells stain in blue (Fig. 1C). Furthermore, we observed significantly increased *Cdkn2a* and *Gdf15* gene expression levels, indicative of increased cellular senescence in old ATII cells (Fig. 1D). In contrast, we observed reduced gene expression of *Surfactant Protein C* (*Sftpc)* and *Surfactant Protein A (Sftpa)* in old ATII cells compared to young ones (Fig. 1D).The transcript level of *Hopx*, a protein implicated in bipotent ATII/ATI progenitors, was increased in old ATII cells (Fig. 1D). The upregulation of P21 protein expression as well as the downregulation of proSP-C protein expression was confirmed by immunofluorescence (Fig. 1E; F respectively). These data support the idea that ATII cells are exhausted in old lungs. To further determine the progenitor cell potential of these cells, we placed primary ATII cells in an organoid assay (Fig. 1G) [3,35,41]. Notably, old primary ATII cells formed significantly fewer organoids as compared to cells isolated from young animals (Fig. 1H). Altogether, these data indicate that the aged lung contains ATII cells with increased cellular senescence and reduced progenitor cell potential.

3.2. Old ATII cells display increased WNT/β-catenin activity

WNT/β-catenin signaling has been implicated in lung epithelial cell progenitor function [27–29,42] and aberrant ATII cell reprogramming in IPF [18,30,31]. Thus, we wondered if WNT/β-catenin signaling contributes to lung aging and potentially cellular senescence. In order to assess WNT/β-catenin activity in ATII cells from young or old mice, we used a reporter mouse line that expresses GFP under the control of multimerized TCF/Lef DNA binding sites, thus faithfully recapitulating WNT/β-catenin-signaling activity (WNT-GFP mice) [43]. We observed increased WNT/β-catenin activity in old ATII cells as compared to the young mice (Fig. 2A and B; 10.50 ± 8.30% GFP^+ cells in young mice versus 26.3 ± 9.23% GFP^+ cells in old mice; $p < .01$).

3.3. Chronic WNT/β-catenin signaling induces cellular senescence in ATII cells

We next asked whether increased WNT/β-catenin-activity results in ATII cell senescence. To this end, we activated WNT/β-catenin-signaling chronically with CHIR99021 (CHIR), a GSK3-β inhibitor that leads to direct β-catenin-accumulation, a key feature of WNT/β-catenin pathway activation [20]. Prolonged CHIR treatment for 7 days in a murine ATII cell line (MLE12 cells) induced a strong, dose-dependent induction of WNT/β-catenin signaling, as measured by the gene expression of the bona fide WNT target gene *Axin2* (Fig. 3A). No cytotoxicity of CHIR was observed (Fig. S2A, C). At the same time, chronic WNT/β-catenin activation led to increased SA-β-gal activity as assessed by flow cytometry as well as conventional light microscopy (Fig. 3B, C). In addition, we observed increased *Cdkn1a (p21)* transcript (Suppl Fig. S2B) as well as P21 protein levels (Fig. 3D). Similarly, we treated primary ATII cells, which expressed high levels of proSP-C, E-cadherin and Cytokeratin (Fig. 4A), with CHIR and found increased *Axin2* and *Cdkn2a (p16)* expression (Fig. 4B). In order to investigate whether specific WNT ligands exhibit similar effects, we treated primary mouse ATII cells with WNT3A, a WNT ligand, which is increased in IPF [24,27]. We used either conditioned medium from L-cells overexpressing WNT3A [44] (Fig. 4C, E, F) or recombinant WNT3A (Fig. 4D). Consistently, WNT3A induced the transcript level of senescence markers *Cdkn2a* and *Cdkn1a* accompanied by *Axin2* (Fig. 4C, D). Both WNT3A and CHIR led to increased SA-β-gal activity after 7 days of treatment (Fig. 4E, F). Gene expression of *Cdkn2a* was induced rapidly after 24 h of WNT3A treatment, whereas *Cdkn1a* expression as well as SA-β-gal was induced only by chronic stimulation after 7 days. Notably, stimulation of ATII cells with conditioned medium containing a non-canonical WNT ligand, WNT5A, did not induce signs of cellular senescence (Fig. S3A,B). Co-treatment of cells with both WNT ligands revealed that the non-canonical ligand WNT5A reduced the ability of WNT3A to induce senescence. Gene expression of *Axin2*, *Cdkn2a* and *Cdkn1a* (Fig. S3C) as well as SA-β-gal activity (Fig. S3D) was significantly reduced, thus further confirming that canonical WNT/β-catenin signaling induces ATII cell senescence. Finally, we aimed to investigate whether WNT3A is able to induce cellular senescence in a 3D lung environment and subjected precision-cut lung slices (PCLS) from young mice to chronic WNT stimulation. CHIR or WNT3A treatment led to increased gene expression of senescence markers *Cdkn2a* and *Cdkn1a* (Fig. 5A, B). Importantly, prolonged WNT3A treatment also resulted in P21 protein expression, primarily in E-$cadherin^+$ epithelial cells, as monitored by immunofluorescence staining (Fig. 5C).

3.4. Chronic WNT/β-catenin stimulation induces a fibrotic phenotype in ATII cells

WNT/β-catenin activity has been linked to a fibrotic epithelial cell phenotype by several studies [27–29,42] and our data further indicate that WNT/β-catenin contributes to cellular senescence. Thus, we next aimed to investigate the potential overlap of WNT/β-catenin signaling and cellular senescence in the fibrotic epithelium of IPF. In a published dataset from primary human ATII cells isolated from Donor and IPF patients (GSE94555 [39]), we found a concomitant and significant gene set enrichment of both, WNT signaling (gene list from WNT/β-catenin GO: 0060070 and https://web. stanford. edu/group/nusselab/cgi - bin/wnt / target_genes, Fig. 6A) as well as aging, cellular senescence and senescence associated secretory phenotype (SASP) (gene lists from: aging (GO:0007568), Senescence [40], or senescence associated secretory phenotype (SASP) [9]), in fibrotic ATII cells (Fig. 6B). In line with these findings, we found a recently described marker of fibrotic epithelial cells, Keratin 8 [32,45], to be induced by chronic, but not acute WNT3A stimulation in pmATII cells (Fig. 6C, D, corresponding senescence and *Axin2* expression in Fig. 4C).

4. Discussion

Aging is a major risk factor for the development of IPF, however, which aging mechanisms contribute to IPF development remains underinvestigated and how these are regulated, is largely unknown [2]. Here, we provide evidence of cellular senescence and ATII progenitor cell exhaustion in the aging mouse lung, which might predispose to CLD development. There is increasing evidence suggesting that senescent cells accumulate in aging tissues and organs, thereby impairing physiological repair and regenerative processes, thus leading to organismal aging [8,12]. ATII cell reprogramming, including cellular injury and hyperplasia is a central phenotype observed in IPF [4,5,46]. Aged mice exhibit a higher susceptibility to lung fibrosis development, which correlated with the burden of senescent cells upon injury [15,47]. In accordance, ATII cells have been shown to exhibit cellular senescence

and SASP secretion in IPF and further show signs of telomere attrition and oxidative stress [14,46]. It remains unclear, however, which signaling pathways drive the aging phenotype in (impaired) ATII cells.

In this study we observed that WNT/β-catenin signaling is increased in aged lungs, which is consistent with findings in other organs [18,30]. Moreover, we demonstrate that active WNT/β-catenin signaling contributes to ATII cell senescence, potentially leading to progenitor cell exhaustion. Similarly, increased WNT/β-catenin has also been linked to cellular senescence in other organs and conditions, including normal embryogenesis [10,11]. Aberrant activity of WNT/β-catenin is well described in IPF [19,24–26] and plays a critical role for ATII progenitor cell function [21,23]. Notably, we observed that chronic WNT/β-catenin stimulation robustly induced cellular senescence, whereas shorter stimulation did not result in the same phenotype. *Cdkn2a* (encoding for p16) has been described as a WNT/β-catenin target gene, consistent with the induction we observed after 24 h in primary ATII cells [48], in contrast to this, *Cdkn1a* is not upregulated at 24 h but only at a later timepoint. Induction of *Cdkn2a* is not sufficient to establish a full senescence phenotype as shown by the fact that SA-ß-galactosidase is increased only after 7d but not after 24 h of WNT stimulation. These findings underline that timing, concentration and duration of WNT/β-catenin can lead to different cellular and functional outcomes. While WNT/β-catenin signaling increases with aging; in IPF, this is enhanced by continuous injuries and reprogramming of the lung epithelium, likely further promoting prolonged and chronic WNT/β-catenin activity. Accordingly, inhibition of β-catenin signaling attenuates bleomycin-induced lung fibrosis [26,49]. Whether these treatments decrease the burden of senescent cells has not been investigated yet.

Notably, only canonical, but not non-canonical WNT signaling was able to induce cellular senescence. In addition, the co-activation of both WNT pathways prevented canonical WNT-driven senescence induction, further underlining the importance of proper crosstalk between these pathways. Dysregulation of both, canonical and non-canonical WNT signaling likely contributes to cellular senescence and aging. Studies exploring the expression of WNT ligands in the aging lung are sparse, however, the non-canonical ligand WNT5A was found increased in the aging lung in several reports [5,34,50,51]. Whether WNT5A is upregulated as a feedback mechanism in response to increased senescence or potentially contributes to other aging mechanisms needs to be further investigated.

Under homeostatic conditions, WNT/β-catenin signaling is essential for stem cell function, including the progenitor cell potential of ATII cells [35,41]. We have recently demonstrated that modulation of WNT/β-catenin signaling alters ATII cell-based organoid growth and numbers and further pro-fibrotic activation of the supporting mesenchyme skewed WNT/β-catenin signaling and led to impaired organoid formation [41,52]. Here, however, we observed that aged ATII cells were characterized by increased WNT/β-catenin signaling but displayed a reduced capacity to form organoids, suggesting a defective progenitor cell function. Similarly, fibrotic ATII cells or ATII cells with shortened telomeres have a decreased organoid forming capacity, further suggesting alveolar progenitor cell dysfunction as a contributor to IPF [53,54]. Senescence of progenitor cells can lead to cell exhaustion and further senescent niche cells might affect neighboring cells in a cell-autonomous manner by secretion of SASP components that negatively affect progenitor cell function [13]. Along these lines, recent reports describe senescent cells in a latent, stem-like condition and highlight WNT/β-catenin as a major signaling factor in the establishment of this stemness associated senescence (SAS). Altogether, these findings highlight the intricate and overlapping role of WNT signaling as a simultaneous stem-cell factor and senescence inducer [55,56].

Our analysis of a single cell suspension of the whole lung revealed a relative increase in inflammatory cells accompanied by a relative decrease of epithelial cells, consistent with a recent report analyzing single cell sequencing from the aged lung [6]. Analyzing ATII cells by flow cytometry, however, is limited in determining total cell numbers, resulting often in an underestimation of cells, probably due to cell loss during tissue processing [57,58]. The gold standard to determine total cells numbers in situ remains stereology. Indeed, a recent study found no change in total ATII cell numbers between young and old mice [59]. As such, it is important to note, that while we observe reduced relative numbers of ATII cells by FACS analysis we can not exclude that this finding is in part due to a relative increase of inflammatory cells.

The following considerations have to be taken into account given our experimental setup: Throughout the paper, we used primary ATII cells isolated using a well-documented and established isolation protocol [35]. These cells are characterized by high enrichment of $EpCAM^+$ and $proSP\text{-}C^+$ cells [58]. While it is well-known that this leads to a high enrichment of ATII cells, we cannot fully exclude that other rare cell populations are present. Moreover, we used different strains (C57BL6/J or /N) as well as the broad range of ages, which might lead to increased variability, however, in our study we observed consistent changes across different strains. We further found that the impairment of progenitor cell function is already apparent in mice aged 16–18 months and not significantly different from even older animals.

While senescent cells most likely accumulate in everybody's lungs, not everybody develops a disease, such as IPF. Thus, other environmental as well as autonomous factors are likely required to develop disease. Notably, telomere attrition is a driving force in IPF and mutations in telomerase genes have been found in familial and sporadic cases of IPF [60]. Telomere attrition is well known to induce cellular senescence [8,12]. Interestingly, telomere dysfunction in ATII cells, but not mesenchymal cells, led to increased cellular senescence, stem cell failure and lung fibrosis [53,61]. Moreover, other hallmarks of aging such as mitochondrial dysfunction contribute to IPF [2,62] and thus convey increased disease susceptibility in addition to increased cellular senescence.

Targeting senescence as a potential therapeutic target is of high interest. Recent advances in the field have led to the development and testing of drugs that target cellular senescence, including senolytics. Senolytics have been effectively shown to attenuate disease in mouse models of various diseases, including pulmonary fibrosis [7,8,14,15] with first in human studies currently being performed [63]. However, current senolytics are rather broad and concerns with regards to their suitability have been raised. While the senolytics target predominantly senescent cells, not every cell type and every type of senescence seems to be affected by these drugs [63]. In IPF, several cell types have been reported to become senescent, including fibroblasts [15,47] and epithelial cells. It is still a matter of ongoing research, how cell type-specific senescence contributes to IPF pathogenesis. A recent study demonstrated ATII cell-specific induction of cellular senescence was able to drive the development of pulmonary fibrosis in mice [64]. Interestingly, myofibroblast senescence restricts fibrosis in organs other than the lung [8,12] and recent studies suggest a similar mechanism in the lung [61]. It is intriguing to envision the potential of future therapeutics to address the induction and cell-type specificity of cellular senescence that determines not only the susceptibility to chronic lung disease but also the potential that interference with this process could be developed for novel treatment options for IPF.

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Author contributions

ML, MK: conception and design of research. ML, QH, YH, RC planned experiments. ML, QH, YH, RC, KH, AvdB, performed experiments and analyzed the data. ML, QH, MK wrote the manuscript. All authors approved the final version of the manuscript.

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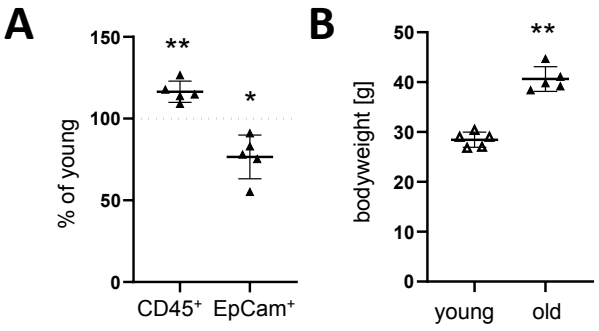
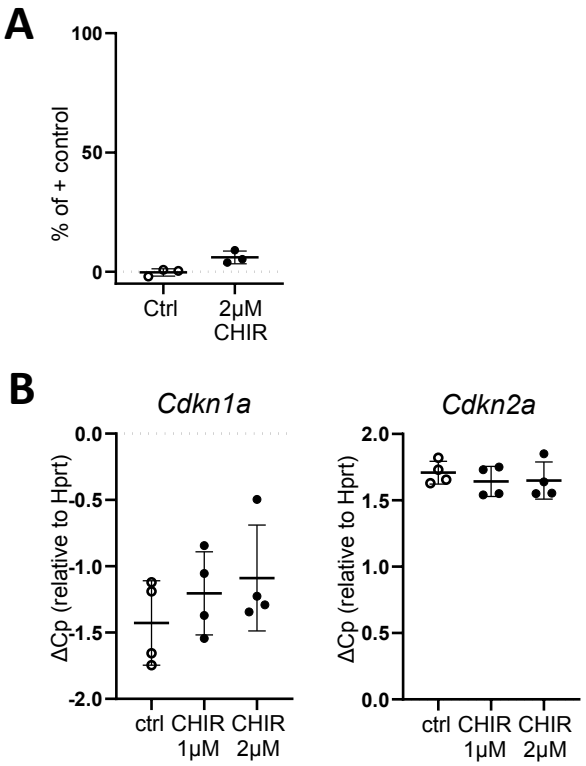
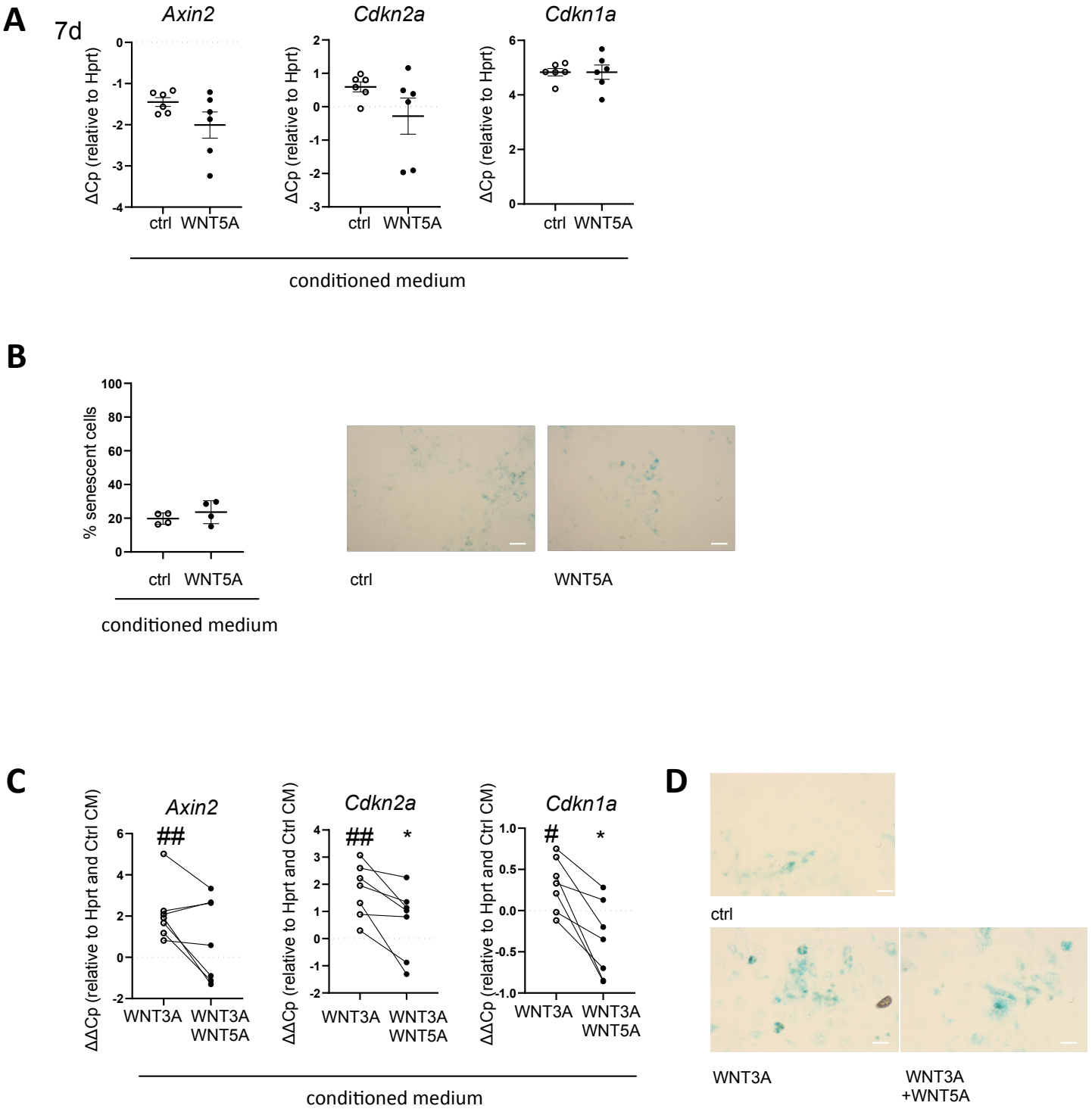
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



4. Paper II

Q. Hu*, K. Heinzelmann,*, Y. Hu, E. Dobrinskikh, M. Ansari, M. C. Melo-Narváez, H. M. Ulke*, et al.* "Single Cell Rna Sequencing Identifies G-Protein Coupled Receptor 87 as a Basal Cell Marker Expressed in Distal Honeycomb Cysts in Idiopathic Pulmonary Fibrosis." *Eur Respir J* (Jun 16 2022). https://doi.org/10.1183/13993003.02373-2021.
* Authors contributed equally.

Single-cell RNA sequencing identifies G-protein coupled receptor 87 as a basal cell marker expressed in distal honeycomb cysts in idiopathic pulmonary fibrosis

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To the Editor:

Idiopathic pulmonary fibrosis (IPF) is a devastating and life-threatening lung disease characterised by epithelial reprogramming and increased extracellular matrix deposition leading to loss of lung function. Prominent histopathological structures in the distal IPF lung include honeycomb cysts in the alveolar space [1]. These are heterogeneous bronchiolised areas that feature clusters of simple epithelium with keratin (KRT)5^{+} basal-like cells interspersed with pseudostratified epithelium containing differentiated, hyperplastic epithelial cells, as well as aberrant ciliated cells [2–5]. Recent single-cell RNA sequencing studies of whole lungs from IPF and donor tissue revealed cellular subtypes unique to IPF, including basaloid KRT5^{-}/KRT17^{+} cells present in the distal lung [6–10]. However, IPF distal bronchiole KRT5^{+} basal cell subtypes still remain poorly characterised and their disease contribution remains under-investigated. Here, we report *G-protein coupled receptor (GPR) 87* as a marker of distal bronchioles and KRT5^{+} basal-like cells in IPF. We generated single cell transcriptomes from EpCAM^{+} cells isolated from parenchymal lung tissue from three IPF patients and three age-matched healthy donors. In short, fresh non-fixed human lung tissue from de-identified healthy donors and explants from IPF patients with end-stage disease was received from National Jewish Hospital/UC Health University of Colorado Hospital (Denver, CO, USA) (COMIRB 11–1664). Right lower or middle lobes of healthy donor (n=3, two males both aged 66 years, and a 68-year-old female) and IPF patient tissue (n=3, two males aged 45 and 65 years, and a 68-year-old female), respectively, were used. All tissues were obtained from non-smokers. Human lung tissue was homogenised and tissue was digested by dispase/collagenase (collagenase: 0.1 U·mL^{-1}; dispase: 0.8 U·mL^{-1}; Roche). Samples were successively filtered through nylon filters (100 µm and 20 µm) followed by a percoll gradient and CD45 MACS sorting (Miltenyi Biotec). After FACS, EpCAM^{+}/DAPI^{-} live single epithelial cell suspensions were used for single-cell RNA sequencing (scRNAseq). Detailed single cell methodology and data processing and analysis is reported in the GitHub repository (https://github.com/KonigshoffLab/GPR87_IPF_2022). The raw data have been deposited in NCBI's Gene Expression Omnibus with accession number GSE190889. Using the 10x Genomics platform, we generated a dataset of 46 199 cells and found nine distinct cell clusters, including main progenitor cell types of the alveolar region and distal airways as well as rare cell types, such as suprabasal cells, recently reported in the healthy lung (figure 1a) [11]. Cells from both conditions were found in all clusters with differentially distributed clusters between healthy and IPF (figure 1b). In line with previous single cell data [6–8], ciliated cells were predominantly found in IPF while ATII cells were largely present in non-diseased lungs, further suggesting a loss of ATII cells and distal bronchiolisation in IPF. Honeycomb cysts are an important histopathological criteria for the diagnosis of IPF; however, mechanistic insight in the process of bronchiolisation and remodelling of the terminal bronchiole in IPF remains scarce. To shed light into cell populations potentially contributing to honeycomb cysts, we analysed differentially expressed genes in all epithelial clusters and found cytokeratins such as *KRT6A*, *KRT5*, *KRT17*, and *KRT15* among the most upregulated genes in IPF (figure 1c). KRT5 is a well-characterised marker of basal and suprabasal cells, and KRT5^{+} cells strongly accumulate in distal IPF lung tissues, mostly in areas of honeycombing [3, 4, 12]. To further identify cellular surface markers and potential pharmacological targets that might be expressed in KRT5^{+} cells, we analysed transmembrane signalling receptors (GO:0004888) in all epithelial cells and found *GPR87*, a G-protein coupled receptor with unknown function in IPF, to be

Shareable abstract (@ERSpublications)
Bronchiolisation and honeycombing are features of IPF. ScRNA sequencing identified GPR87 as a novel marker of basal cells in IPF, enriched in honeycomb cysts. GPR87 overexpression resulted in aberrant airway cell differentiation. https://bit.ly/3i4dXeT

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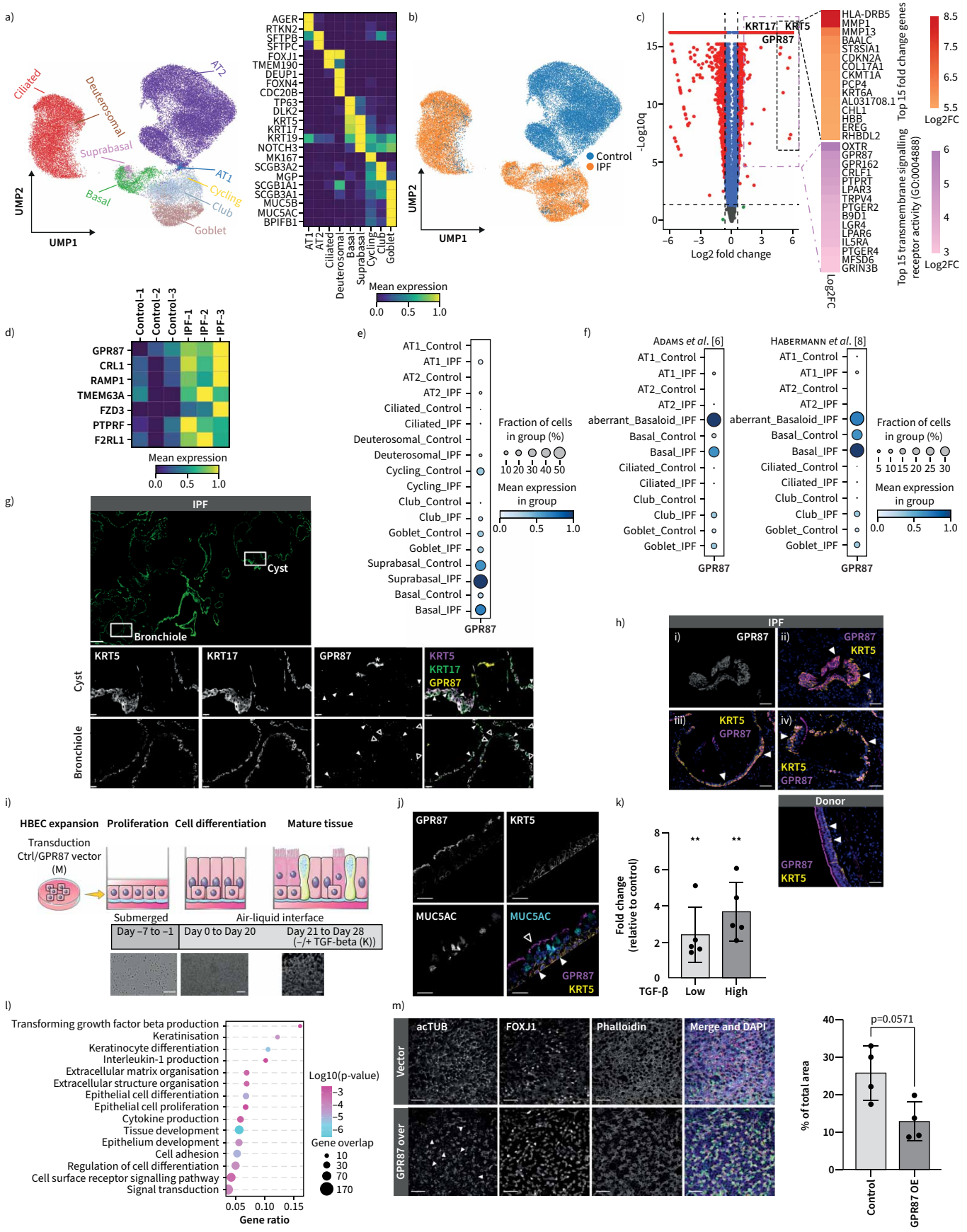
a)
Ciliated
Deuterosomal
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Basal
AT2
AT1
Cycling
Club
Goblet
UMP2
UMP1
AGER
RTKN2
SFTPB
SFTPC
FOXJ1
TMEM190
DEUP1
FOXN4
CDC20B
TP63
DLK2
KRT5
KRT17
KRT19
NOTCH3
MK167
SCGB3A2
MGP
SCGB1A1
SCGB3A1
MUC5B
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Mean expression
0.0 0.5 1.0
b)
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Log2 fold change
HLA-DRB5
MMP1
MMP13
BAALC
ST8SIA1
CDKN2A
COL17A1
CKMT1A
PCP4
KRT6A
AL031708.1
CHL1
HBB
EREG
RHBDL2
OXTR
GPR87
GPR162
CRLF1
PTPRT
LPAR3
TRPV4
PTGER2
B9D1
LGR4
LPAR6
IL5RA
PTGER4
MFSD6
GRIN3B
Log2FC
Top 15 fold change genes
8.5 7.5 6.5 5.5
Log2FC
Top 15 transmembrane signalling receptor activity (GO:0004888)
6 5 4 3
Log2FC
d)
Control–1
Control–2
Control–3
IPF–1
IPF–2
IPF–3
GPR87
CRL1
RAMP1
TMEM63A
FZD3
PTPRF
F2RL1
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AT1_Control
AT1_IPF
AT2_Control
AT2_IPF
Ciliated_Control
Ciliated_IPF
Deuterosomal_Control
Deuterosomal_IPF
Cycling_Control
Cycling_IPF
Club_Control
Club_IPF
Goblet_Control
Goblet_IPF
Suprabasal_Control
Suprabasal_IPF
Basal_Control
Basal_IPF
GPR87
Fraction of cells in group (%)
10 20 30 40 50
Mean expression in group
0.0 0.5 1.0
f)
Adams et al. [6]
Habermann et al. [8]
AT1_Control
AT1_IPF
AT2_Control
AT2_IPF
aberrant_Basaloid_IPF
Basal_Control
Basal_IPF
Ciliated_Control
Ciliated_IPF
Club_Control
Club_IPF
Goblet_Control
Goblet_IPF
GPR87
Fraction of cells in group (%)
5 10 15 20 25 30
Mean expression in group
0.0 0.5 1.0
g)
IPF
Cyst
Bronchiole
KRT5
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GPR87
KRT5 KRT17 GPR87
h)
IPF
i) GPR87
ii) GPR87 KRT5
iii) KRT5 GPR87
iv) KRT5 GPR87
i)
HBEC expansion
Proliferation
Cell differentiation
Mature tissue
Transduction Ctrl/GPR87 vector (M)
Submerged
Air-liquid interface
Day –7 to –1
Day 0 to Day 20
Day 21 to Day 28 (–/+ TGF-beta (K))
j)
GPR87
KRT5
MUC5AC
MUC5AC
GPR87 KRT5
k)
Donor
GPR87 KRT5
Fold change (relative to control)
TGF-β
Low
High
**
**
l)
Transforming growth factor beta production
Keratinisation
Keratinocyte differentiation
Interleukin-1 production
Extracellular matrix organisation
Extracellular structure organisation
Epithelial cell differentiation
Epithelial cell proliferation
Cytokine production
Tissue development
Epithelium development
Cell adhesion
Regulation of cell differentiation
Cell surface receptor signalling pathway
Signal transduction
0.05 0.10 0.15
Gene ratio
Log10(p-value)
–3 –4 –5 –6
Gene overlap
10 30 70 170
m)
acTUB
FOXJ1
Phalloidin
Merge and DAPI
Vector
GPR87 over
p=0.0571
% of total area
Control
GPR87 OE

FIGURE 1 Expression of GPR87 in accumulated basal progenitor cells within the idiopathic pulmonary fibrosis (IPF) lung. a) Uniform manifold approximation and projection (UMAP) visualisation shows unsupervised transcriptome clustering, revealing nine distinct cell clusters. Heatmap shows the highest expressed marker genes of each cluster. b) UMAP visualisation showing distribution of healthy donor and IPF cells to different clusters. c) Volcano plot of differentially expressed genes (red, log2 fold change (FC) >0.6, q<0.05) in IPF EpCAM$^+$ epithelial cells compared with donor samples, zooming in gene sets with top-15 fold change and top-15 fold change genes related to transmembrane signalling receptor activity (GO:0004888). d) Heatmap of transmembrane signalling receptor genes robustly regulated in the (supra)basal cell population across all individual tissue samples. Dotplots show GPR87 expression in our (e) and another two publicly available datasets [6, 8] (f), respectively. g) GPR87 mRNA was detected by RNAscope (Advanced Cell Diagnostics, number 471861) and combined with fluorescent immunolabelling of basal cell markers KRT5 and KRT17 in IPF lung sections (n=6) (KRT5: Biolegend 905901; KRT17: Abcam ab51056) Top row: lower magnification of a distal lung area with several remodelled airways (scale bar: 200 µm). Higher magnification of a representative cyst and a bronchiole are presented below in higher magnification (scale bar: 20 µm). KRT5$^+$/KRT17$^+$/*GPR87*$^+$ cells are indicated by arrowheads. KRT5$^-$/KRT17$^+$/*GPR87*$^+$ cells are indicated by open triangles. Non-cellular staining is marked by an asterisk. h) Lung tissue sections of IPF (n=3) and healthy donor (n=2) were co-immunolabelled for GPR87 (Novus Biologicals NBP2-16728) and KRT5. Nuclei are visualised by DAPI staining. Protein expression of GPR87 alone is shown in subpanel (i) and co-immunolabeled with KRT5 in (ii). Two more representative areas of remodelled airways with merged protein expression are shown in (iii) and (iv). Representative double positive cells for respective markers are indicated by arrowheads. Scale bar: 50 µm. i) Scheme of primary human bronchial epithelial cell (HBEC) isolation and air–liquid interface (ALI) culture (reproduced from Servier Medical Art (smart.servier.com) with permission). HBECs were isolated from healthy donors (n=3) and cultured on rat-tail collagen type I under submerged conditions, either transduced with lentivirus (empty vector (Origene, PS100092) or human GPR87 ORF (Origene, RC218486L3)) (m), and/or directly transferred and cultured on collagen type IV membranes, airlifted (day 0) and differentiated to a mature epithelium within 21 days. Transforming growth factor (TGF)-β treatment (R&D, 240-B-002, 2 or 4 ng·mL^{-1}) was performed at day 21 and every other day till day 28 (four times in total) (k). Shown are phase contrast images for dish cultured cells and early ALI (left, middle; scale bars: 250 µm, 100 µm), and a confocal image of acetylated tubulin (acTub) (Abcam ab24610) to visualise late ALI (mature epithelium, right; scale bar: 25 µm). j) Vertical membrane sections of mature ALI cultured HBECs were immunolabeled for GPR87, basal cell marker KRT5 and secretory cell marker MUC5AC (Abcam ab3649) (n=2). Representative double positive cells for respective markers are indicated by arrowheads. Scale bar: 25 µm. (We also observed cilia staining, as indicated by open triangles, and based on our single cell dataset ciliated cells might also express GPR87.) k) Airlifted donor HBECs were stimulated with low (2 ng·mL^{-1}) and high (4 ng·mL^{-1}) concentrations of TGF-β, as described in (i). GPR87 gene expression was assessed by qPCR in five independent donor cell lines. GAPDH was used as an housekeeper gene control (huGPR87-fw (ACCTATGCTGAACCCACGC), -re (CCGTGCAGCTCGTTATTTGG); huGAPDH-fw (ACTAGGCGCTCACTGTTCTC), -re (AATACGACCAAATCCGTTGACTC)). Two-tailed Mann–Whitney test was performed to determine statistical significance. **: p<0.01. n=5. l) Functional annotation enrichment analysis of GPR87 positive correlated genes reveals several categories of airway remodelling. m) HBECs were transduced with lentivirus containing the full ORF of GPR87 to generate a stable overexpression of GPR87 (GPR87-over). Empty backbone-vector alone was used as a control (vector). Cells were cultured on ALI till day 21 and co-immunolabelled for acTub and FOXJ1 (Invitrogen 14-9965-82). DAPI and phalloidin stainings were performed to visualise nuclei and cellular integrity. Cells with no/shortened cilia are indicated by arrowheads. Representative images of n=4 are shown. Scale bar: 25 µm. Areas covered by cilia were quantified with ImageJ [17]. Two-tailed Mann–Whitney test was performed to determine statistical significance.

one of the highest regulated transcripts (figure 1c). Importantly, when we analysed transmembrane signalling receptors specifically in the (supra) basal cell population across individual tissue samples, we observed a strong and robust increase of *GPR87* (figure 1d). A limitation of our scRNASeq dataset is the small sample size used for scRNASeq (n=3 each); thus, we further confirmed upregulation of *GPR87* in (supra) basal cells in comparison to other epithelial cells not only in our own (figure 1e) but in two additional independently published datasets (figure 1f) [6, 8]. Notably, GPR87 showed further enrichment in basaloid KRT5$^-$/KRT17$^+$ cells, a cell type which we did not detect in our dataset (figure 1f).

We focused on GPR87 for our subsequent studies for several reasons: First, it belongs to the class of G-protein coupled receptors, which are intensively studied drug targets with attractive pharmacological accessibility. Second, although classified as an orphan receptor, profibrotic ligands have been discussed, such as lysophosphatidic acid [13]. Third, GPR87 has been linked to aberrant cell cycle control [14, 15], which is a feature of epithelial reprogramming and bronchiolisation/honeycomb cyst development in IPF [1]. Thus, we aimed to investigate GPR87 expression within the distal IPF lung and its potential contribution to airway cell differentiation and bronchiolisation in IPF.

We confirmed GPR87 epithelial cell expression and distribution within the IPF lung *in situ* using fluorescent immunolabelling and RNAscope of human tissue section as previously described [4, 16]. RNAscope detected *GPR87* RNA in KRT5$^+$ cells in areas of bronchiolisation and honeycomb cysts in distal IPF tissue sections, respectively (figure 1g; arrowheads). The *GPR87* RNA was also found in KRT5$^-$/KRT17$^+$ cells (figure 1g; open triangles). In addition, GPR87 protein was observed in clusters of KRT5$^+$ basal cells in IPF lungs as well as in some KRT5$^+$ cells in non-diseased lungs (figure 1h; arrowheads). GPR87 function was further investigated in an air–liquid interface (ALI) cell culture model of primary human bronchial epithelial cells (HBECs), mimicking *in vivo*-like differentiation of basal cells to

more mature cell types, including ciliated and secretory cells (figure 1i) [4, 16]. GPR87 was expressed in $KRT5^+$ basal cells of our human ALI culture (figure 1j). Transforming growth factor (TGF)-β treatment, inducing fibrotic epithelial reprogramming, led to increased *GPR87* expression in mature ALI cultures (figure 1k). This was consistent with the functional annotation enrichment analysis of our scRNAseq data, which revealed tissue development, keratinocyte differentiation and extracellular matrix remodelling, as well as TGF-β production; all indicative of altered epithelial airway differentiation and integrity, to be correlated with GPR87 (figure 1l). Moreover, GPR87 overexpressing HBECs cultured at ALI displayed impaired differentiation of $KRT5^+$ cells into mature airway cells evidenced by altered epithelial structure and a decrease in cilia coverage (mean±SD 27.65±6.21% for the control, compared to 12.90±4.47% for the GPR87 overexpression) (figure 1m).

Our data suggest that overexpression of GPR87 leads to impaired airway cell differentiation of $KRT5^+$ basal cells, and thus support the hypothesis that GPR87 might contribute to bronchiolisation and honeycomb cyst formation. It will be important to further study the functional consequences of GPR87 expression in basal cells *in vivo* and to analyse whether inhibition of GPR87 would be able to revert impaired airway cell differentiation and prevent TGF-β induced fibrotic reprogramming, thus serving as a potential therapeutic target.

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Apendix A: Paper III

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WNT Signalling in Lung Physiology and Pathology

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Abstract

The main physiological function of the lung is gas exchange, mediated at the interface between the alveoli and the pulmonary microcapillary network and facilitated by conducting airway structures that regulate the transport of these gases from and to the alveoli. Exposure to microbial and environmental factors such as allergens, viruses, air pollution, and smoke contributes to the development of chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and lung cancer. Respiratory diseases as a cluster are the commonest cause of chronic disease and of hospitalization in children and are among the three most common causes of morbidity and mortality in the adult population worldwide. Many of these chronic respiratory diseases are associated with inflammation and structural remodelling of the airways and/or alveolar tissues. They can often only be treated symptomatically with no disease-modifying therapies that normalize the pathological tissue destruction driven by inflammation and remodelling. In search for novel therapeutic strategies for these diseases, several lines of evidence revealed the WNT pathway as an emerging target for regenerative strategies

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in the lung. WNT proteins, their receptors, and signalling effectors have central regulatory roles under (patho)physiological conditions underpinning lung function and (chronic) lung diseases and we summarize these roles and discuss how pharmacological targeting of the WNT pathway may be utilized for the treatment of chronic lung diseases.

Keywords

Ageing; Asthma; COPD; Fibrosis; Lung; Lung cancer; Regeneration; WNT

1 Introduction

The main physiological function of the lung is gas exchange of O_2 and CO_2, mediated at the interface between the alveoli and the pulmonary microcapillary network and facilitated by conducting airway structures that regulate the transport of these gases from and to the alveoli. Being in direct contact with the outside world, the lung needs to be resilient against microbial and environmental exposures such as bacteria, viruses, fungi, air pollution, and many other factors. Unfortunately, microbial exposures not seldomly trigger respiratory infections, whereas exposure to environmental factors such as allergens, air pollution, and smoke contributes to asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and lung cancer. Respiratory diseases as a cluster are the commonest cause of chronic disease and of hospitalization in children and are among the three most common causes of morbidity and mortality in the adult population. Many of these chronic respiratory diseases are associated with inflammation and structural remodelling of the airways and/or alveolar tissues and can often only be treated symptomatically with no disease-modifying therapies that are able to stop or even reverse the pathological inflammation and remodelling of lung tissue.

In search for novel therapeutic strategies for these diseases, the WNT pathway is increasingly in the focus as a major contributor to physiological repair but further to pathophysiological lung inflammation and remodelling (Skronska-Wasek et al. 2018; Vladar and Konigshoff 2020; Burgy and Konigshoff 2018). WNT signalling is explained in more detail elsewhere in this handbook. Briefly, the WNT family of secreted glycoproteins consists of 19 members, some of which with (partially) overlapping binding and signalling characteristics involving both the FZD family of G protein coupled receptors and non-FZD receptors such as the receptor tyrosine kinases ROR2, RYK, and PTK7 (Burgy and Konigshoff 2018). The WNT pathway is classically subdivided in a branch dependent on WNT/FZD-mediated stabilization of the intracellular signalling effector β-catenin through mechanisms involving the co-receptors LRP5/6 and inactivation of the β-catenin destruction complex composed of Axin2, APC, GSK-3, and CK1. β-catenin is a cofactor of several transcription factors, leading to the activation of transcriptional programs in cell-type specific manners. In addition, β-catenin-independent signalling functions exist, involving activation of Ca^{2+} signalling, ROCK signalling, JNK signalling among other pathways associated with cytoskeletal rearrangements and gene transcription (Baarsma et al. 2013a). These two branches are often referred to as β-catenin-dependent and β-catenin-independent.

Here we summarize the central regulatory roles that WNT proteins, their receptors, and signalling effectors have in the physiological and pathophysiological processes underpinning lung function and (chronic) lung disease (Skronska-Wasek et al. 2018; Baarsma and Konigshoff 2017; Lehmann et al. 2016) and discuss how pharmacological targeting of the WNT pathway may be utilized for the treatment of these diseases. In view of the detailed descriptions of the biochemistry and intracellular signalling of these WNT/FZD interactions elsewhere in this handbook, our focus will be on the (patho)physiological roles of WNT signalling in the lung across all stages of life, and in the major respiratory diseases including asthma, COPD, IPF, lung cancer, as well as pulmonary vascular diseases.

2 Physiology of the Lung: Role of WNT Signalling

2.1 Anatomy of the Lung

The human respiratory system is a complex ensemble which guarantees intake of oxygenated air through inhalation and expulsion of CO_2-enriched air through exhalation. It can be generally divided into upper and lower respiratory tract. The upper respiratory tract serves as point of entry, conduction and later exit for the air through the nose, pharynx, larynx, and trachea.

The lungs are part of the lower respiratory tract. The human left lung is comprised of two lobes – superior and inferior, while the right lung includes a middle lobe and therefore comprises three lobes. The lungs start from a bifurcation of the trachea into two primary bronchi. These extend into bronchial trees, branching into progressively smaller ramifications defined as secondary and tertiary bronchi, and bronchioles which lead through the alveolar ducts to the prime site for gas exchange, the alveoli (Fig. 1a).

Starting from the upper conductive airways into the bronchi and the bronchioles, we observe a pseudostratified epithelium with distinct spatial composition and function. Together with neuroendocrine and basal cells, the airways comprise of multi-ciliated and secretory cells that have the important role of maintaining mucociliary clearance. Among the secretory cells, goblet cells are predominantly found in the upper larger airways, while club cells are mainly found in the smaller airways. Separated from the epithelium by a basal membrane, the mesenchyme contains submucosal glands, fibroblasts, smooth muscle cells, nerves, and blood and lymphatic vessel (Wansleeben et al. 2013; Hogan et al. 2014; Nikolic et al. 2018; Tirouvanziam et al. 2009). The terminal bronchioles are defined as the respiratory airway, a region with a monolayer of cuboidal epithelium that leads into the alveoli (Hogan et al. 2014; Basil et al. 2020).

The alveoli represent the most distal part of the lung and are characterized by a specific lobular structure surrounded by a network of capillaries that maximizes the surface available for gas exchange. Here we find a thin epithelium comprised of at least two key cell types: alveolar type I (ATI) and alveolar type II (ATII) cells. ATI are flat and elongated cells specialized in gas exchange; they share the basal lamina with the underlying pulmonary capillary endothelial cells (PCEC) to optimize the efficiency of gas exchange. ATII cells are cuboidal cells which produce and secrete pulmonary surfactant, thereby reducing the surface tension of the alveolar surface area to prevent the lungs from collapsing while breathing.

Further, ATII cells serve as progenitor cells for ATI cells (Nikolic et al. 2018; Tirouvanziam et al. 2009; Basil et al. 2020; Ruaro et al. 2021).

There are several types of stromal cells in the interstitial region, including mesenchymal cells, pericytes, endothelial cells, and immune cells. These cells constitute the alveolar niche, a supporting microenvironment for epithelial progenitor cells which plays a major role during both homeostasis and injury (Ruaro et al. 2021; Hogan 2020). The mesenchyme is comprised of different cell types involved in a cross-talk with the epithelium through secretion of growth factors and other key signalling pathways, including WNT. Several types of fibroblasts have been described mainly in mouse, including myofibroblasts, matrix fibroblasts, and lipofibroblasts-like alveolar niche fibroblasts (MANCs); these niche cells produce WNT proteins, which act mainly through WNTβ-catenin signalling to replenish the AXIN2+ epithelial progenitor pool during development and repair (Frank et al. 2016; Lee et al. 2017; Zepp et al. 2017; Nabhan et al. 2018; Ushakumary et al. 2021). Our knowledge of the cell types that populate the various sections of the human respiratory system is continuously evolving, as new subtypes of cells are discovered through single-cell RNA-sequencing of the lung in steady state and disease.

2.2 Cell Type: Specific Expression Patterns of WNTs/FZDs

WNT signalling is a key mediator of cellular cross-talk in the lung. Specific interactions between epithelial, mesenchymal, immune, and endothelial cells have been described in development, homeostasis, and disease, and in the recent years, the advent of single-cell RNA-sequencing has allowed us to identify cell-specific expression of WNT proteins and receptors (Adams et al. 2020; Habermann et al. 2020; Reyfman et al. 2019; Travaglini et al. 2020).

While some WNT proteins and receptors are widely expressed in all cell types, it is important to highlight the ones that seem rather tissue- or cell-specific or have been described to be involved in signalling in specific cellular compartments: Expression of β-catenin-dependent WNT proteins such as WNT-7B or WNT-3A is mostly restricted to the epithelium, with WNT-3A highly enriched in ATI cells (Adams et al. 2020; Habermann et al. 2020; Reyfman et al. 2019; Travaglini et al. 2020). Recently, it was described that ATI cells in the early lung express WNT-3A, WNT-7A, and WNT-7B, which interact with FZD1 and LRP6 on transient secondary crest myofibroblast (Zepp et al. 2021).

In the mouse alveoli, a specific WNT niche has been proposed comprising of a single fibroblast constitutively expressing WNT-5A (and potentially other WNT proteins) to provide juxtacrine signals to the neighboring ATII cell, thereby maintaining stem cell homeostasis and cell fate (Nabhan et al. 2018). In human, within the ATII population, a WNT-responsive lineage has recently been identified to be positive for the WNT target gene Axin2. These cells, named alveolar epithelial progenitors (AEP), comprised 29% of the human ATII population and contributed to functional alveolar epithelial regeneration after injury (Zacharias et al. 2018).

WNT-5A and WNT-5B expression has been primarily linked to fibroblasts, although recent human single-cell data suggest further expression in other cell types in healthy lungs as well,

e.g. some epithelial cells (Adams et al. 2020; Habermann et al. 2020); for example, a subset of ATII cells expressing, among others, WNT-5A, LRP5, TCF4, was identified in the adult human lung (Travaglini et al. 2020). WNT-5A and WNT-2 were further identified in subsets of mesenchymal cells at postnatal day 1 in mouse, including smooth muscle and fibroblasts (Guo et al. 2019).

Notably, it has been observed that secretion of WNT proteins and activation of WNT target genes can be mutually exclusive in some cells. For example, a recent study showed through single-cell RNA-seq and in situ RNA hybridization that in both human and mouse, expression of the *WNT7B* gene is abundant in small airway epithelial cells, such as club cells, but expression of the WNT target gene *AXIN2* was not found in these cells, while it was detected only in the alveolar region (Reyfman et al. 2019). In human, among FZDs, FZD_1 appears to be mostly expressed in the mesenchyme, FZD_4 is highly expressed in the vascular compartment, but also, similarly to FZD_5 and FZD_6, enriched in ATI and ATII cells (Habermann et al. 2020; Travaglini et al. 2020). Additionally, co-receptors LRP5 and LRP6 are widely expressed in the human lung epithelium, mesenchyme, and endothelium; ROR1 and ROR2 are co-expressed in several subtypes of human lung fibroblasts, while ROR1 is also enriched in ATI cells (Habermann et al. 2020; Reyfman et al. 2019; Travaglini et al. 2020).

LGR5 and LGR6 have been identified as markers of mesenchymal cells in the adult murine lung. LGR6+ cells, including a sub-population of smooth muscle cells in the airway epithelium, promote airway differentiation of epithelial progenitors via WNT-FGF10 cooperation. In the alveoli LGR5+ cells are found, which produce WNT-3A and WNT-5A, promoting alveolar differentiation of both club and ATII cells (Lee et al. 2017; Raslan and Yoon 2020).

2.3 Lung Development

While our understanding of the homeostatic role of WNT signalling in the adult lung is limited, our knowledge about its involvement in orchestrating lung development in mouse has been extensively documented. Numerous WNT genes, including *Wnt2, Wnt2b, Wnt5a, Wnt7b*, and *Wnt11* are expressed at various stages in the developing lung (Harris-Johnson et al. 2009; Lako et al. 1998; Li et al. 2002; Rajagopal et al. 2008). WNT signalling interplays with other key developmental signalling pathways, such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and sonic hedgehog (SHH), in a spatial-temporal manner that allows lung specification and full formation of the respiratory system (Rock and Hogan 2011).

Lung development is historically divided into five stages which are similar in human and rodents, although they have been more extensively defined in mouse. Today, these stages are condensed into two main ones: a branching stage that corresponds to the pseudoglandular stage, which in mouse starts at embryonic day (E) 9.5 and ends around E16.5, during which distal epithelial progenitors give rise to the conducting airway epithelium, and an alveolar differentiation stage that starts around E16.5 until several weeks after birth in mouse, and 2–3 years in human, during which distal epithelial progenitors give rise to bipotential alveolar

epithelial progenitors that will differentiate directly into alveolar type I (ATI) and ATII cells (Volckaert and De Langhe 2015).

Lung specification begins before the embryonic stage, around E9 in mouse and gestation week 5 in human, from a respiratory primordium which is comprised of endodermal cells that start expressing the respiratory lineage factor Nkx2.1 (Chen 2015; Herriges and Morrisey 2014). These cells are localized in the ventral wall of the anterior foregut endoderm, and by E9.5 in mouse and around 28 days in human, they evaginate and form the two primary lung buds, leading into the embryonic stage (Herriges and Morrisey 2014; Morrisey and Hogan 2010). These buds are comprised of three cell layers: in addition to the inner endoderm-derived epithelial layer, there is a thin outer mesothelium, and a mesoderm layer which will give rise to the mesenchyme and is in continuous cross-talk with the endoderm to direct branching and cell differentiation (Volckaert and De Langhe 2015; Herriges and Morrisey 2014; Hines and Sun 2014; Rawlins 2011).

At this initial stage, β-catenin-dependent WNT signalling is required to define the respiratory fate in the anterior foregut: in fact, mutant mice with combined loss of the *Wnt2* and *Wnt2b* genes lose the Nkx2.1+ lung endoderm progenitors population which leads to impaired branching of the lung buds and trachea formation; at the same time, specification of other foregut derived tissues is not affected, indicating that WNT-2 and WNT-2B regulation is specific to lung (Goss et al. 2009). A similar result is achieved with conditional inactivation of *Ctnnb1* (β-catenin) in mouse foregut endoderm (Harris-Johnson et al. 2009).

During the pseudoglandular stage, the lung buds undergo a process defined as “branching morphogenesis,” to create a tree-like structure. At this stage there’s a differential expression of specific markers which will control the proximal-distal axis and cell fate; WNT/β-catenin signalling is also involved in this axis determination, as inactivation of *Ctnnb1* in the mouse pseudoglandular stage causes enhanced formation of the conducting airways while restricting formation and differentiation of the peripheral lung (Mucenski et al. 2003). Furthermore, WNT signalling is required for bud formation by inducing apical constriction, while loss of WNT signalling is necessary for air sac formation in the canalicular-saccular stages (Fumoto et al. 2017). Additionally, WNT/β-catenin signalling is involved in the upstream regulation of other essential pathways including N-myc, BMP4, and FGF signalling, acting as a regulator of a molecular hierarchy that promotes distal while repressing proximal airway development (Shu et al. 2005). Interestingly, specific combinations of WNT proteins, such as the interaction between mesenchymal WNT-2 and epithelial WNT-7B, play an essential role in branching morphogenesis, proximal-distal patterning, and development of distal lung progenitors (McCulley et al. 2015; Miller et al. 2012). WNT-7B is also involved in regulating mesenchymal proliferation, late epithelial maturation, and pulmonary vascular smooth muscle differentiation and/or survival (Shu et al. 2005).

During the branching morphogenesis phase, the β-catenin-independent WNT planar cell polarity (PCP) pathway is also involved, as mice mutant for PCP proteins such as Vangl2

(Van Gogh homolog) and Celsr1 (Flamingo homolog) form hypoplastic distal lungs with reduced airway branching (Vladar and Konigshoff 2020; Yates et al. 2010).

Recently, an active role for ATI cells in lung development has been described, with several WNT proteins, such as WNT-7A and WNT-3A, produced primarily by ATI cells that are required for myofibroblasts and alveolar formation (Zepp et al. 2021).

Other WNT proteins which signal through the β-catenin-independent pathway cause aberrant lung development in mutant mice: for example, in *Wnt5a*(−/−) mice there's an increased proliferation of both epithelial and mesenchymal cell compartments, causing overexpansion of the distal airways and thickened intersaccular interstitium that leads to delayed lung maturation and causes alterations in the FGF-10, BMP4, and SHH signalling, once again proving the interplay between different epithelial-mesenchymal signalling pathways (Skronska-Wasek et al. 2018; Li et al. 2002; Li et al. 2005; Yin et al. 2008). Furthermore, WNT-5A signalling through ROR1/ROR2 is essential for the later stage of alveologenesis, as postnatal WNT-5A inactivation in mice results in hypoalveolarization of the lungs, due to myofibroblast defects (Li et al. 2020).

In conclusion, a vast array of WNT proteins and receptors are expressed in specific cells and phases of lung development and promote epithelial-mesenchymal cross-talk with other signalling pathways, initiating key steps in the developmental process; our knowledge of these interactions provides a precious insight into the understanding of how these same processes may be involved in regeneration of the adult human lung.

2.4 Epithelial Cell Specification/Regeneration in Adulthood

Unlike organs with high epithelial turnover, such as skin and intestine, the lung is relatively quiescent with slow turnover at homeostasis, however, it exhibits a potential rapid repair upon acute injuries (Rawlins and Hogan 2006; Leeman et al. 2014). Mounting evidence showed that multiple regional specific lung epithelial progenitor cells can differentiate to other epithelial cells to maintain homeostasis or repair in response to injuries (Rawlins and Hogan 2006; Leeman et al. 2014). Remarkably, collective data demonstrated that both WNT/β-catenin and β-catenin-independent WNT signalling pathways are crucial for the functions of nearly all of these progenitor cell function, including self-renewal and differentiation, at homeostasis and during regeneration (Raslan and Yoon 2020) (Fig. 1).

Basal cells, marked by expressions of transcription factor TRP63, cytokeratin 5 (KRT5), and the nerve growth factor receptor (NGFR), are found in human airways proximal to the respiratory airways, and in murine airways proximal to main bronchi (Basil et al. 2020). They maintain airway epithelial structure by self-renewing and differentiating to goblet cells, club cells, or ciliated cells (Fig. 1b). Previous studies demonstrated that activation of WNT/β-catenin signalling was required for basal cell proliferation and promoted their differentiation to ciliated cells, instead of secretory cells (goblet and club cells) (Brechbuhl et al. 2011; Giangreco et al. 2012). In the submucosal gland, a population of basal like progenitor cells called myoepithelial cells were identified to regenerate basal cells and subsequently differentiate to other epithelial cell types upon activation of transcription factor Lef1/TCF7 of WNT/β-catenin signalling pathway (Lynch et al. 2018). Therefore, WNT/β-

catenin signalling controls the stemness and differentiation of the proximal airway epithelial progenitor cells. The role of β-catenin-independent WNT signalling in basal cells, however, remains unknown.

In the distal airways, club cells are dome-shaped airway epithelial progenitor cells, which represent ~20% of the bronchiolar epithelium in humans and 50–70% in mice (Okuda et al. 2019; Ito et al. 2000; Boers et al. 1999). Club cells can be identified by expression of a secreted protein called CCSP (also known as CC10, secretoglobin 1A1, or uteroglobin). They possess host defense, barrier maintenance, and progenitor functions (Zuo et al. 2018). In homeostasis, club cells maintain airway epithelial structure by self-renewing followed by differentiation to daughter club cells and ciliated cells (Rawlins et al. 2009). In addition, several studies demonstrated that the club cells formed alveolar organoids expressing ATII cell marker Surfactant Protein C (SPC) in vitro, suggesting the potential of murine club cells to differentiate to ATII cells at homeostasis.

Furthermore, the functional heterogeneity of murine proximal and distal airway club cells has been confirmed in several disease models (Chen et al. 2012; Kim et al. 2005; Liu et al. 2019). Club cells in the trachea are able to differentiate to ciliated cells upon tracheal injury (Rawlins et al. 2009). Cells at the bronchioalveolar-duct junction that express CCSP and SPC were identified as bipotential progenitor cells (Kim et al. 2005). These $CCSP^{pos}/SPC^{pos}$ bronchioalveolar stem cells (BASCs) contribute to airway epithelial repair by replenishing club cells after naphthalene treatment, and they also contribute to alveolar repair by replacing ATII cells after bleomycin challenge (Kim et al. 2005; Liu et al. 2019). In addition, a distinct sub-population of Uroplakin3a+ club cells are resistant to naphthalene and regenerate both airway and alveolar epithelia after bleomycin-induced injury (Guha et al. 2017). Hence, there is strong support for critical club cell functions in lung repair.

The role of WNT signalling in the progenitor function of club cells remains largely unknown. With deficiency of a WNT/β-catenin co-receptor RYK, the club cells were shown to contribute to goblet cell hyperplasia following airway injury, suggesting a potential role of WNT/RYK signalling in the airway repair by club cells (Kim et al. 2019). However, another study showed that deletion of β-catenin did not affect airway regeneration by club cells, arguing whether RYK controls club cell fate through β-catenin pathway or other unrelated signalling pathways. Importantly, using organoid culture, two studies showed that activating WNT/β-catenin signalling with WNT-3A and the GSK3β inhibitor CHIR99021 promoted alveolar but not bronchiolar differentiation of club cells (Lee et al. 2017; Hu et al. 2020). Intriguingly, a study showed that overexpression of an active form of β-catenin leads to abnormal ATII cell differentiation in the conducting airways (Mucenski et al. 2005). These data suggest the critical role of WNT/β-catenin signalling in fate decision of club cells (Lee et al. 2017; Hu et al. 2020). Future studies are need to dissect the potential heterogeneous roles of WNT signalling pathways in different subsets of club cells in homeostasis and diseases.

In the alveoli, ATII cells function as progenitor cells to proliferate and differentiate into ATI cells in vivo, however, infrequently at steady state (Barkauskas et al. 2013; Desai et al. 2014). Importantly, most of ATII cells do not show active WNT signalling at homeostasis.

Upon alveolar injury, several distinct ATII sub-populations were reported to be activated to repair the damaged alveolar tissue, including the aforementioned BASC cells, a sub-population of Axin2-positive ATII cells, and an integrin α6β4-positive SPC-negative cell (Nabhan et al. 2018; Zacharias et al. 2018; Kim et al. 2005; Liu et al. 2019; Chapman et al. 2011). Among these progenitor sub-populations, the Axin2-positive ATII cells require activation of WNT/β-catenin signalling for proliferation but not for differentiation into ATI cells (Nabhan et al. 2018). Further, a study showed that ATII progenitor cells required WNT/β-catenin signalling for cell survival and migration post bleomycin-induced injury (Flozak et al. 2010; Tanjore et al. 2013). For ATII-ATI trans-differentiation, both WNT/β-catenin and β-catenin-independent WNT signalling were demonstrated to be critical. The β-catenin-independent WNT-5A/protein kinase C (PKC) signalling enhances β-catenin/p300 interactions to promote ATII cell differentiation (Rieger et al. 2016). Furthermore, both WNT-5A and WNT-5B were recently shown to inhibit WNT/β-catenin signalling and repress proliferation of ATII cells. Unlike WNT-5A, WNT-5B also inhibits ATII-ATI trans-differentiation (Wu et al. 2019). These studies suggested that a fine-tuned temporal control of WNT/β-catenin and β-catenin-independent WNT signalling balance is required during alveolar progenitor proliferation and differentiation.

2.5 Mesenchymal Cell Specification/Differentiation in Adulthood

Airway and alveolar mesenchymal cells such as smooth muscle cells and fibroblasts express high levels of particularly WNT-5A and WNT-5B, which appear to contribute to the maintenance of mesenchymal features such as contractility and ECM production (Koopmans et al. 2016a). These WNT proteins cooperatively interact with TGF-β in the regulation of these functions, in part because TGF-β augments the expression of both WNT-5A and WNT-5B in airway smooth muscle and in lung fibroblasts (Kumawat et al. 2013). The effects of TGF-β on WNT-5A expression are mediated by TGF-β-activated kinase 1 (TAK1), which regulates recruitment of the transcription factor SP1 to the WNT-5A promoter to induce gene expression (Kumawat et al. 2013).

WNT-5A/B and TGF-β subsequently regulate expression of mesenchymal marker proteins such as sm-α-actin, by cooperating at the level of actin polymerization in a RhoA/ROCK controlled manner (Koopmans et al. 2016a). In turn, actin polymerization drives the expression of contractile apparatus genes via myocardin-related transcription factor A (MRTFA) (Wang et al. 2002). In addition to WNT-5A, WNT-11 has similar functions (Kumawat et al. 2016).

Although these effects of WNT-5A/5B/11 are not mediated by classical β-catenin-dependent pathways (Kumawat et al. 2013), this does not mean that β-catenin signalling does not play a role in mesenchymal cells. In fact, β-catenin expression is inducible in response to TGF-β and plays a role in ECM production by TGF-β (Baarsma et al. 2011; Lam et al. 2011). This is not mediated by FZD/AXIN2-dependent β-catenin stabilization, but rather by GSK-3 phosphorylation-dependent activation of β-catenin, redirecting β-catenin to SMAD and CBP dependent gene expression (Gottardi and Konigshoff 2013; Koopmans et al. 2016b) and in that respect, distinct from “classical” WNT/β-catenin signalling. A

similar role for β-catenin-dependent, yet WNT/FZD independent signalling was described for PDGF-induced cell proliferation of airway smooth muscle cells (Nunes et al. 2008).

Mesenchymal cells exert important secretory functions with important roles in physiology and disease. The proinflammatory cytokines IL-6 and IL-8 are secreted from lung fibroblasts in a WNT-5B dependent manner through interactions with the FZD_2 and FZD_8 (van Dijk et al. 2016), whereas smooth muscle derived WNT-5A contributes to Th2 cytokine production and allergic inflammation (Koopmans et al. 2020). Moreover, mesenchymal cells contribute secreted growth factors such as WNTs and FGFs, which support epithelial plasticity and repair. Crucially, there appears to be a distinct segregation of mesenchymal cell functions based on WNT-responsiveness and -activity. In line with what has been outlined above, mesenchymal cells that do not express high levels of "classical" WNT/β-catenin signalling markers are TGF-β responsive and typically enriched in smooth muscle markers such as sm-α-actin and ECM-markers such as collagen 1 (Zepp et al. 2017). In contrast, there appears to be a sub-population co-expressing the WNT/β-catenin marker AXIN2 at high levels along with PDGFR-α with supportive roles in epithelial cell plasticity and repair (Zepp et al. 2017). A distinct segregation of mesenchymal cells supportive of repair can also be made on the basis of expression of LGR subtypes with LGR5+ cells preferentially supporting alveolar epithelial repair and LGR6+ cells preferentially supporting airway repair (Lee et al. 2017). In fact, singlecell sequencing of mouse lung mesenchymal cells identified at least five sub-populations of cells each with distinct WNT and LGR expression profiles (Lee et al. 2017). We lack a good understanding at present of the distinct features of each of these mesenchymal lineages, their functions and dependence on WNT, which will need to be addressed in future studies.

3 Pathology: Role of WNT Signalling

3.1 Normal Lung Ageing

Ageing is characterized by cellular changes that are summarized by nine hallmarks (López-Otín et al. 2013), many of which are influenced by the WNT pathway. Several studies suggest that WNT/β-catenin signalling actively contributes to ageing: Mice deficient for the negative WNT regulator klotho demonstrated increased cellular senescence in skin, intestine, and testis accompanied by reduced stem cell numbers leading to an accelerated ageing phenotype (Liu et al. 2007). Similarly, Brack et al. detected active WNT/β-catenin signalling in aged myogenic progenitors and showed a critical role for WNT signalling in lineage conversion from a myogenic to a fibrogenic lineage (Brack et al. 2007).

While WNT signalling has been investigated in the context of chronic lung disease (see later paragraphs), detailed studies on WNT signalling during normal *human* lung ageing are scarce. There are a couple of reports describing a change of WNT expression in the aged lung, many of which focused on mouse tissue. The expression of WNT-3A decreased along with increased WNT inhibitor, FRZB in ageing mouse lung (Hofmann et al. 2014). Moreover, WNT-2 exhibited reduced expression in lung mesenchymal stromal cells isolated from older mice (12 months old) when compared to young mice (3 months old) (Paxson et al. 2013). In contrast to this, airway epithelial cell isolated from aged mice showed higher expression of WNT-2 and lower expression of WNT-7A (Aros et al. 2020). Wu et al.

describe an upregulation of the β-catenin-independent WNT-5A and WNT-5B in aged whole mouse lungs (Wu et al. 2019). In contrast to the initial studies discussed above that linked active β-catenin signalling to ageing, these latter studies suggest a (potentially cell-type specific) shift from β-catenin-dependent to β-catenin-independent WNT proteins in the aged lung. In addition to the shift of WNT proteins in lung ageing, there is also evidence showing ageing-associated dysregulation of WNT target genes. Several WNT target genes including *Tle1, Lef1, Nkd1* (Hofmann et al. 2014), *Ccnd2* (Cyclin D2), *Ccna1* (Cyclin A1) (Watson et al. 2020) were found to be reduced in the ageing mouse lung, while *c-Myc* (Zhang et al. 2012a) and *Wisp-1* (Chanda et al. 2021) show increased expression. Another study exploring differential expression of WNT target genes in the ageing mouse lung (Hofmann et al. 2014) also observed a decrease in WNT/β-catenin signalling with age on a whole lung level. However, these studies focus on whole lung tissue, and thus cell-specific changes might be diluted. Along these lines, Lehmann et al. described an increased population of epithelial cells displaying high β-catenin activity in the ageing lung (Lehmann et al. 2020a). In contrast, Paxson et al. reported declined β-catenin gene expression in mesenchymal stromal cells from murine aged lungs (Paxson et al. 2013). Data on normal human lung ageing is even more scarce but some reports suggest a regulation similar to the mouse (Kovacs et al. 2014; Zhou et al. 2018). Interestingly, Baarsma et al. demonstrated enhanced expression of β-catenin-independent WNT-5A in senescent primary human lung fibroblasts linked to impaired endogenous lung repair in COPD (Baarsma et al. 2017). These current data indicate a spatiotemporally controlled shift in WNT signalling during ageing, similar to what has been described in other ageing processes, such as in hematopoietic stem cells (Florian et al. 2013).

Ageing-related alterations in cells and cell niches trigger stem cell exhaustion, one hallmark of lung ageing (Meiners et al. 2015), leading to impaired regenerative capacity of the lung. As mentioned above, WNT signalling has been implicated as a contributor to cellular senescence: WNT-5A is found in senescent lung fibroblasts (Baarsma et al. 2017), while WNT/β-catenin is able to induce lung epithelial senescence (Lehmann et al. 2020a). Additionally, Kovacs et al. reported WNT-5A and WNT-11 expression to be reduced in epithelial (EpCAM1+) and increased in nonepithelial (EpCAM1−) cells during senescence, further highlighting a potential WNT signal shift and cellular senescence (Kovacs et al. 2014).

Mitochondrial dysfunction and metabolic distress along with increased ROS production is another hallmark of ageing implicated in lung diseases (Bueno et al. 2015; Braidy et al. 2011). Active WNT/β-catenin signalling during ageing has been linked to increased production of reactive oxygen species (ROS) in mesenchymal stem cells (Zhang et al. 2013; Zhang et al. 2011). Inhibition of the WNT signalling pathway via overexpression of the secreted frizzled-related protein 2 (SFRP2) in A549 (a human lung epithelial cell line) led to mitochondrial dysfunction (Li et al. 2019). The cofactor nicotinamide adenine dinucleotide (NAD+) and its reduced form NADH are key regulator of metabolism, and an age-dependent decline in NAD+ levels and NAD+/NADH ratio was detected in the lung (Braidy et al. 2011; Massudi et al. 2012).

Telomere shortening, believed to drive ageing phenotypes, is associated with lung function decline (Birch et al. 2018) and lung alveolar integrity depends on proper telomere maintenance (Lee et al. 2009). Even though it is not clear how WNT signalling in the lung contributes to telomere length, a regulatory loop connecting WNT signalling and telomerase activity has been suggested in other organs (Zhang et al. 2012b; Park et al. 2009). On the one hand, transient activation of the WNT/β-catenin pathway might induce telomerase reverse transcriptase expression and elevates telomerase activity in different cell lines (Zhang et al. 2012b). On the other hand, telomerase, by serving as a cofactor, might directly modulate WNT/β-catenin in mouse embryonic stem cells (Park et al. 2009).

In summary, although reports of WNT signalling activity in the ageing (human) lung are still limited, there is an emerging concept of a cell-type specific regulation and shift in WNT signalling probably contributing to multiple hallmarks of lung ageing. These findings further warrant cell-type specific studies of β-catenin-dependent and independent WNT signalling in the aged human lung and the modulation of WNT signalling as a potential therapeutic option to target/reverse cellular ageing processes.

3.2 Asthma

Asthma is an obstructive airways disease that affects around 300 million people worldwide. Characteristic clinical features of asthma include airway hyperresponsiveness to specific (e.g., histamine, methacholine) and non-specific (e.g., allergens, cold air) stimuli, and associated with airway inflammation and remodelling. Asthma is a highly heterogeneous disease. Whereas most asthma patients experience symptoms resulting from Th2 dependent eosinophilic inflammation associated with allergies, additional inflammatory phenotypes of asthma exist, often resulting in severe disease. A detailed literature review on the role of WNT signalling in asthma was published previously (Koopmans and Gosens 2018). Here, we will focus on key findings and on findings contributed since this review was published.

Genetic association studies have often been used to predict the functional involvement of genes or pathways of genes in the etiology of asthma. Genomewide association studies have identified two single nucleotide polymorphisms in the *WNT11* gene region (El-Husseini et al. 2020). Candidate gene approach studies identified additional roles for genetic variations in the *FAM13A* gene in asthma (Kerkhof et al. 2014), which was originally identified to associate with COPD (Cho et al. 2010). Additional genetic associations were found for WNT-1-inducible-signalling pathway protein-1 (*WISP-1*) and WNT inhibitory factor-1 (*WIF-1*) in children diagnosed with mild to moderate persistent asthma, though both of these were identified on the basis of candidate gene approaches (Sharma et al. 2010). The nature and pathophysiological roles of these WNT pathway genes in asthma are not fully elucidated. WNT-11 is expressed in airway smooth muscle and regulates contractile genes (Kumawat et al. 2016), whereas FAM13A is a protein that supports the degradation of β-catenin (Jiang et al. 2016). WISP1 and WIF1 are both secreted proteins capable of modulating WNT signalling directly. The exact roles of these genes could well reach beyond direct functional effects on airway inflammation or remodelling, however, as these genetic associations may also be explained on the basis of changes in lung development with subsequent impact on asthma susceptibility (Sharma et al. 2010).

Nonetheless, evidence does point to a direct role for WNT signalling in asthmatic inflammation and remodelling. In line with what has been outlined above in the section on mesenchymal cells, WNT-5A appears to play a role in remodelling of the airway smooth muscle as smooth muscle-specific overexpression of WNT-5A aggravates sm-α-actin expression in combination with allergen exposure. Smooth muscle-specific WNT-5A overexpression augments the expression of Th2 cytokines as well, and in fact direct application of WNT-5A to Th2 cells obtained from asthma patients revealed regulation of the Th2 cytokine IL-31 by this WNT protein (Koopmans et al. 2020). Moreover, eosinophils isolated from asthma patients activate *WNT5A* gene expression in airway smooth muscle more than eosinophils obtained from healthy subjects (Januskevicius et al. 2016). In contrast, whole lung application of recombinant WNT-5A does not impact on allergic inflammation, whereas application of recombinant WNT-1 represses Th2 dependent inflammation in a mouse model of asthma (Beckert et al. 2018), possibly indicating distinct roles for WNT/β-catenin-dependent (protective) and WNT/β-catenin-independent (detrimental) signalling in the development of asthma, or for cell-specific roles of WNT signalling in individual features of the disease. The latter possibility seems most likely as WNT/β-catenin-dependent signalling is not protective towards all features of asthma and induces both β-catenin stabilization and the secretion of proinflammatory cytokines such as IL-8 and CCL8 from human mast cells.

3.3 COPD

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of mortality worldwide (Barnes et al. 2015; Anne et al. 2015). Although preventable and treatable, COPD remains incurable in part because of an incomplete understanding of its cellular mechanisms driving the disease. COPD comprises two major clinical conditions: chronic bronchitis (CB) and emphysema. CB is characterized by chronic inflammation, airway epithelial remodelling, and mucus obstruction. It is typically thought of as condition affecting the central airways even though small airways with internal diameters of <2 mm serve as the main site of airflow limitation (Hogg et al. 1968). With more recent advances in high resolution computed tomography, small airway disease (SAD) is now recognized as an event in early COPD, with 40–56% of terminal airways in early GOLD I and II stage patients (Capron et al. 2019; Higham et al. 2019; Burgel et al. 2011). On the other hand, emphysema is characterized by irreversible destruction of parenchymal lung tissue and airspace enlargement associated with abnormal inflammation and subsequent protease/antiprotease imbalance. COPD is caused by several stressors, such as tobacco smoke and air pollution (Barnes et al. 2015; Tuder and Petrache 2012). In both CB and emphysema, the WNT/β-catenin signalling is generally found to be down-regulated, whereas the β-catenin-independent WNT signalling is upregulated (Qu et al. 2019). These data suggest a similar WNT signal shift as observed in the ageing lung, which is in line with an emphysema phenotype developing in the normal human lung upon ageing.

In CB, β-catenin was shown to be reduced in the small airway epithelial cells (Wang et al. 2011), while several components of the β-catenin-independent WNT signalling pathway were found to be increased in CB patients, including WNT-4, WNT-5B, and Frizzled-8 (FZD_8) (Qu et al. 2019; Wang et al. 2011; Durham et al. 2013; Spanjer et al. 2016a; Heijink

et al. 2016). In addition, a WNT inhibitor, secreted frizzled-related protein 2 (SFRP2), was found to be increased in both healthy smokers and COPD patients (Qu et al. 2019; Wang et al. 2011). Similarly, previous studies have demonstrated that WNT-3A and FZD_4 were down-regulated in the alveolar epithelium in murine and human emphysema, associated with elevated β-catenin-independent WNT protein WNT-5A expressed by lung fibroblasts (Baarsma and Konigshoff 2017; Desai et al. 2014; Qu et al. 2019; Kneidinger et al. 2011; Skronska-Wasek et al. 2017; Beers and Morrisey 2011; Stabler and Morrisey 2017).

Notably, ectopic activation of WNT/β-catenin signalling using GSK3β inhibitors induced intrinsic alveolar repair in mouse models of emphysema and human precision cut lung slices derived from COPD patients (Kneidinger et al. 2011; Uhl et al. 2015). These studies demonstrated that tissue regeneration can be initiated in adult human emphysematous lungs and that WNT/β-catenin signalling pathway serves as a potential therapeutic target to initiate tissue regeneration in emphysema. However, the identity of the progenitor cells that respond to WNT/β-catenin activation upon chronic injury to regenerate alveoli, and the role of WNT/β-catenin signalling during their transition from quiescence to activation in homeostasis and disease remains largely unknown. Studies using transgenic mouse models with WNT activity reporters have identified a sub-population of ATII cells labeled by WNT/β-catenin target gene *Axin2* that can be activated to regenerate alveolar tissue after acute injuries (Nabhan et al. 2018; Zacharias et al. 2018). Whether these cells can be activated in emphysema to repair lung tissue is unknown. Furthermore, another study has identified a distal lung epithelial progenitor population containing club cells and a small population of ATII cells, which respond to ectopic WNT activation to form alveolar organoids in mouse emphysema model induced by porcine pancreatic elastase, suggesting a potential role of both club and ATII cells in WNT/β-catenin induced tissue repair in COPD (Hu et al. 2020). In a more recent study, using a cigarette smoke induced mouse COPD model, Conlon et al. identified lymphotoxin β-receptor (LTβR) on epithelial cells interacts with TNF superfamily members expressed by the immune cells of a tertiary lymphoid structure in COPD known as inducible bronchus-associated lymphoid tissue (iBALT). LTβR signalling functions as an inhibitor of WNT/β-catenin signalling by activating non-canonical NF-κB signalling. Strikingly, blockade of LTβR activated WNT/β-catenin signalling in the alveolar epithelial progenitor cells and promoted tissue repair (Conlon et al. 2020). Although these studies support the notion that activating WNT/β-catenin signalling is beneficial for tissue regeneration in COPD, it is important to point out that WNT/β-catenin signalling was found upregulated in idiopathic lung fibrosis and lung cancer, as discussed below. Therefore, further studies are needed to identify disease-specific targeting WNT/β-catenin driven tissue repair. The recent development of “WNT agonist surrogates” that allows the functional study of WNT signalling and the delineation of FZD subtype specific effects can serve as an important tool in our understanding of cell-type specific WNT signalling outcomes in the lung (Janda et al. 2017).

3.4 Lung Cancer

Lung cancer is the second most commonly diagnosed cancer and the leading cause of mortality among all cancer types, which accounts for 18% of the total cancer death worldwide (Sung et al. 2021). The 5-year survival rate of lung cancer patients post

diagnosis is only about 10–20% (Sung et al. 2021). Lung cancer is classified into two major types: small cell lung cancer (SCLC, 15% of all lung cancers) and non-small cell lung cancer (NSCLC, 85%) (Leeman et al. 2014; Herbst et al. 2008; Travis et al. 2013). Regardless of its smaller proportion, SCLC is one of the most aggressive and lethal forms of malignant diseases, which is characterized by aberrant neuroendocrine marker expression, genomic instability, rapid tumor growth, and high metastatic potential (Schwendenwein et al. 2021). Unfortunately, most SCLC patients already showed metastatic spread at the time of diagnosis, due to lack of early detection strategy (Schwendenwein et al. 2021). On the other hand, the most common lung cancers, NSCLCs, are further classified into three distinct histological subtypes: adenocarcinoma (ADC; 50% of NSCLC), squamous cell carcinoma (SCC; 40% of NSCLC), and large cell carcinoma (10% of NSCLC) (Leeman et al. 2014; Chen et al. 2014). ADCs are identified by glandular histology and expression of thyroid transcription factor 1 (TTF1; also known as NKX2–1) and keratin 7 (KRT7), which are markers found in the distal lung (Leeman et al. 2014; Travis et al. 2013; Chen et al. 2014; Imielinski et al. 2012). However, SCCs are characterized by expression of markers of pseudostratified epithelial cells in the proximal airways, including cytokeratin 5 (CK5), cytokeratin 6 (CK6), transcription factors SRY-box 2 (SOX2), and p63. Lastly, large cell carcinoma does not express either markers of ADC or SCC and remains largely unstudied (Leeman et al. 2014; Travis et al. 2013; Chen et al. 2014; Imielinski et al. 2012).

Studies using single-cell RNA-sequencing and numerous genetically engineered mouse models of specific lung cancer subtypes have suggested that distinct lung epithelial progenitor cells may undergo malignant transformation in lung cancer subtypes. For instance, neuroendocrine cells were shown to form SCLC tumor with *Tp53* and *Rb* knockout (Leeman et al. 2014; Song et al. 2012; Noguchi et al. 2020). Further, both BASC and ATII cells were demonstrated to contribute to Kras-activated ADC (Leeman et al. 2014; Kim et al. 2005; Xu et al. 2012). For SCC, basal cells were proposed to be the cellular origin (Giangreco et al. 2012; Lu et al. 2010). Genetic mutations in the progenitor cells were believed to contribute to cancer initiation. High-throughput NGS studies have identified multiple oncogenic mutations in NSCLC, including *KRAS* and epidermal growth factor receptor (*EGFR*) in ADCs, discoidin domain-containing receptor 2 (*DDR2*), *FGFR1, FGFR2, FGFR3* and genes in the PI3K pathway for SCC (Chen et al. 2014; Cancer Genome Atlas Research Network 2014). Many clinical inhibitors targeting these oncogenes have been used and significantly improved the treatment of NSCLC in the past decade, however, many patients developed resistance to these inhibitors within 9–12 months of treatments (Chen et al. 2014).

Intriguingly, mutations in genes of WNT/β-catenin signalling are predominant in many cancer types but rarely found in lung cancers (Skronska-Wasek et al. 2018; Stewart 2014). However, overexpression of β-catenin, WNT proteins, and receptors, as well as inactivation of β-catenin destruction complex components, such as APC and AXIN2, was commonly identified in resected NSCLC samples (Stewart 2014; Garcia Campelo et al. 2011). Nguyen et al. suggested that activation of WNT/β-catenin/TCF pathway was a determinant of metastasis to brain and bone during lung adenocarcinoma progression (Nguyen et al. 2009). Moreover, accumulating evidence demonstrated that abnormal activation of WNT signalling was associated with increased resistance factors, and that inhibiting WNT signalling can

overcome the resistance to certain drugs against NSCLC (Song et al. 2019). Single-cell RNA-sequencing analysis revealed that samples from patients exhibiting drug resistance under therapy showed relative low expression of WNT/β-catenin-associated pathway genes *SUSD2* and *CAV1* (Travis et al. 2013). Altogether, these data indicate that aberrant activation of WNT/β-catenin signalling plays a role in both, lung tumorigenesis and drug resistance under anti-cancer therapies. However, whether inhibiting β-catenin is beneficial to lung cancer treatment remains controversial. Some studies indicated that activation of β-catenin may inhibit metastasis through regulating E-cadherin and increase cell–cell contact (Skronska-Wasek et al. 2018; Schwendenwein et al. 2021). In addition to WNT/β-catenin signalling, overexpression of β-catenin-independent WNT protein WNT-5A was found to elevate cell proliferation predominantly in SCC, which is highly associated with cigarette smoke (Skronska-Wasek et al. 2018; Janda et al. 2017; Chen et al. 2014). Importantly, WNT-5A was shown to activate β-catenin signalling in the stromal cells and to induce vascularization, and therefore enhance the tumor-stromal interaction (Chen et al. 2014). In contrast, another WNT protein, WNT-7A functions as an tumor suppressor, and was found down-regulated in NSCLC (Janda et al. 2017). Therefore, dysregulation of β-catenin-dependent and independent WNT signalling plays a pivotal role in NSCLC. The precise mechanisms regulating the functions of specific WNT proteins and their interactions with other oncogenes in each subtype of NSCLC remain largely unknown and require more studies.

3.5 IPF

Idiopathic Pulmonary Fibrosis (IPF) is a chronic, age-associated lung disease with poor prognosis and limited therapeutic options (Lederer and Martinez 2018). To date, lung transplantation remains as the only curative treatment and novel therapies, targeting pathogenic pathways are urgently needed. IPF is characterized by the excessive accumulation of extracellular matrix which leads to the destruction of functional lung tissue. Accumulating evidence suggests a role of epithelial cell reprogramming in disease initiation by inhibiting endogenous repair capacities of stem cells (Selman and Pardo 2020). Unbiased screening approaches and several follow-up studies unequivocally revealed an aberrant WNT signature in IPF and experimental lung fibrosis (Konigshoff et al. 2008; Konigshoff and Eickelberg 2010; Konigshoff et al. 2009; Chilosi et al. 2003; Yang et al. 2007; Lam et al. 2014; Martin-Medina et al. 2018; Vukmirovic et al. 2017). In human as well as experimental disease, an increase in active WNT/β-catenin signalling was described (Konigshoff et al. 2008; Konigshoff and Eickelberg 2010; Konigshoff et al. 2009; Chilosi et al. 2003; Yang et al. 2007; Lam et al. 2014) (Sucre et al. 2018), likely as an early event during pathogenesis with changes observed already in rather normallooking parenchyma of the human diseased lung (Rydell-Tormanen et al. 2016). Importantly, low-density lipoprotein receptor-related protein 5 was discovered as a genetic marker of IPF and furthermore proved to have a prognostic value (Lam et al. 2014). Recent single-cell sequencing approaches led to the transcriptional characterization of lung cells in unprecedented detail. These unbiased approaches further led to the identification of epithelial cells population enriched in β-catenin-dependent WNT signalling in both experimental and human lung fibrosis (Adams et al. 2020; Reyfman et al. 2019; Strunz et al. 2020; Xu et al. 2016). In experimental lung fibrosis, the appearance of transitional stem cell states, such as transitional Krt8+ ATII

(ADI) cells, has been identified, which exhibit an active β-catenin signature. Further studies also suggest distinct WNT-expressing versus WNT-responsive epithelial cells in the IPF lung, suggesting the control of the WNT pathway by distinct niches in the lung (Nabhan et al. 2018).

Mechanistically, aberrant WNT/β-catenin signalling led to impaired epithelial cell function such as increased cellular senescence, expression of fibrotic markers, induction of Krt8, and increased proliferation (Selman and Pardo 2020; Konigshoff and Eickelberg 2010; Chilosi et al. 2003; Lehmann et al. 2020b; Chilosi et al. 2013). Importantly, also WNT-5A is upregulated in IPF and experimental lung fibrosis and contributes to increased proliferative capacity of primary human lung fibroblasts (Martin-Medina et al. 2018). This represents an important difference to other disease scenarios in the lung, such as COPD or the ageing lung, where a shift of β-catenin-dependent and independent WNT signalling has been observed (Baarsma et al. 2017; Kneidinger et al. 2011; Skronska-Wasek et al. 2017). Importantly, WNT-5A was found on extracellular vesicles in the BALF of IPF patients as well as secreted by fibrotic primary human lung fibroblasts, suggesting that extracellular vesicles in the IPF lung function as carriers for WNT proteins thereby extending their ability for intercellular communication (Parimon et al. 2019).

Targeting WNT/β-catenin pathway has also therapeutic potential in IPF. Inhibition of β-catenin signalling by pharmacological agents was shown to attenuate bleomycin-induced lung fibrosis (Henderson Jr. et al. 2010; Ulsamer et al. 2012; Wang et al. 2014). Similarly, administration of an siRNA directed against β-catenin resulted in reduced bleomycin-induced fibrosis load (Kim et al. 2011). In addition, the WNT/β-catenin target gene WNT inducible signalling protein (WISP) 1 has been identified as a profibrotic protein and inhibition of WISP1 attenuated the development of experimental lung fibrosis in vivo (Konigshoff et al. 2009; Klee et al. 2016).

As illustrated by these in vivo studies, targeting the WNT pathway and its downstream mediators might be a promising therapeutic approach. It will be important to develop treatments that do not interfere with normal wound healing or regeneration, illustrated by the worsening of lung fibrosis observed after epithelial-specific removal of β-catenin in murine fibrosis (Tanjore et al. 2013). Additionally, ATII cells display active WNT signalling needed for regeneration (Nabhan et al. 2018). This underlines that it is crucial to target disease-specific WNT signalling which is characterized by the involvement of additional cofactors and interactions with other profibrotic signalling pathways such as TGFβ/SMAD (Gottardi and Konigshoff 2013; Ulsamer et al. 2012; Zhou et al. 2012).

In summary, (re-) shifting the disease- and cell-specific parts of the WNT/β-catenin pathway thereby restoring the “right amount and the right flavor” of WNT signalling is an important although challenging therapeutic strategy for IPF (Gottardi and Konigshoff 2013).

3.6 Pulmonary Vascular Diseases

Pulmonary vascular disease (PVD) is a medical term for a large and diverse group of pathologies that affects the blood vessels within the lungs. PVD is characterized

by remodelling of microvessels and abnormal angiogenesis. WNT signalling has been implicated in several PVDs.

There are two main types of PVDs: pulmonary embolism (PE) and pulmonary hypertension (PH). PE is a blockage of an artery in the lungs and causes lung injury due to obstructed blood flow, decreased oxygen levels in the blood. The majority of PE initials from other parts of the body and travels through the bloodstream to the lung. Thereby, we will only discuss the PH here. PH is caused by high blood pressure in the pulmonary arteries. It can be progressive, even turning to fatal disease if untreated early.

PH is characterized by vascular narrowing and increased pulmonary vascular resistance, due to pulmonary injury and abnormal pulmonary vascular remodelling (Humbert et al. 2019). Given the role of WNT signalling in angiogenesis (Olsen et al. 2017) and accumulating evidence showing WNT signalling involved in PH pathologies, the development of WNT-based therapeutics becomes amenable. The defection of WNTless (*Wls*), a gene that encodes for a transmembrane protein that mediates WNT protein secretion, impaired pulmonary vascular differentiation and peripheral lung morphogenesis (Cornett et al. 2013), whereas embryos and newborn mice with *Wnt7a* knockout exhibit severe defects in mesenchymal proliferation and vascular development in lung (Shu et al. 2002).

Pulmonary arterial hypertension (PAH), characterized by high blood pressure in the arteries of the pulmonary artery, is a type of PVD. A study investigating genetic signatures across multiple cell types in pulmonary arterial hypertension showed the activation of the WNT/β-catenin pathway in both heritable PAH (HPAH) and idiopathic PAH (IPAH) (West et al. 2014), indicating the role of WNT pathway in maintaining pulmonary vascular homeostasis. The activation of β-catenin-independent WNT/PCP pathway was demonstrated in IPAH, in particular in the endothelial layer, with significant upregulation of WNT-11, DAAM1, DSV, and RHO-kinase (Laumanns et al. 2009). WNT-5A, a known WNT/PCP pathway activating protein, was further identified as a key mediator for the recruitment of pericyte and for the establishment of epithelium–pericyte interactions to promote pulmonary angiogenesis (Yuan et al. 2019). Furthermore, de Jesus Perez et al. demonstrated both β-catenin-dependent and independent (WNT/RHOA/RAC1) WNT pathways are required in BMP-2–mediated protection of pulmonary artery endothelial cells (de Jesus Perez et al. 2009). However, inhibition of WNT/β-catenin signalling by LRP5/6 inhibitors attenuates hyperoxia-induced PH and decreased pulmonary vascular smooth muscle cell proliferation in neonatal rats (Alapati et al. 2013). Further study conducted by Sklepkiewicz et al. suggested that glycogen synthase kinase 3 beta (GSK3β), a member of β-catenin destruction complex, promoted vascular remodelling processes with proliferation of arterial smooth muscle cells in pulmonary hypertension (Sklepkiewicz et al. 2011). In conclusion, the regulation of WNT signalling pathway in vascular remodelling in PA pathologies varies from ligand-receptor combination, as well as cell types.

Apart from vascular remodelling via endothelial cells and smooth muscle cells, activation of WNT/β-catenin signalling has been implicated in the suppression of mesenchymal progenitor cells (MPC) differentiation into functional pericytes, thereby driving a phenotype of persistent microvascular dysfunction and abnormal angiogenesis (Gaskill et al. 2017).

Similarly, Summers et al. suggested activation of WNT/β-catenin signalling in MPC induced emphysema-like distal lung remodelling and exacerbated vascular injury (Summers et al. 2020).

Moreover, proinflammatory activation is another major mechanism involved in PA, which has been linked to WNT signalling. Various perivascular inflammatory cells including monocytes, macrophages, T lymphocytes were identified in plexi-form lesion (Tuder et al. 1994), while several of cytokines including interleukin (IL)-1, IL-1β, IL-6, connexin 36, fractalkine (FKN) were implicated on the lesion of PAH (Tuder et al. 1994; Itoh et al. 2006; Simonneau et al. 2013). WNT signalling pathways were suggested in both proinflammatory and anti-inflammatory process. In general, WNT-5A pathway is a crucial trigger of inflammatory activation in macrophages, with upregulated expression of the proinflammatory genes *IL6, IL1B, CXCL8*, and *CCL4* (Pereira et al. 2009). In contrast, WNT-3A/β-catenin signalling mediates anti-inflammatory effects (Schaale et al. 2011). There are rare reports linking WNT pathway with inflammation in lung, in particular in PA pathologies. It is reported that β-catenin-independent WNT-5B signalling induces IL-6 and CXCL8 secretion in human lung fibroblasts (van Dijk et al. 2016). Li et al. suggest that WNT/β-catenin signalling acts as a negative feedback loop, attenuated cell injury from excessive inflammatory reactions by suppressing inflammation in alveolar epithelial cells (Li et al. 2014). However, further in-depth investigations on how WNT pathway contributes to inflammatory-medicated PA pathologies are needed.

4 Conclusions and Perspectives

The studies summarized above support major roles for WNT signalling in lung physiology and pathophysiology and highlight a number of emerging key concepts. Probably the most compelling concept is the WNT signalling switch from WNT/β-catenin signalling to β-catenin-independent signalling in ageing, in chronic lung disease and in response to TGF-β. In light of the supportive roles of WNT/β-catenin signalling in epithelial repair and regeneration, such studies call for approaches to inhibit WNT/β-catenin-independent signalling or activate WNT/β-catenin-dependent signalling as regenerative pharmacological approaches towards restoration of lung architecture in these chronic diseases. Such approaches have been considered, mostly using GSK-3 inhibitors, which successfully prevent and restore changes in lung remodelling in animal models of LPS-induced lung injury (Baarsma et al. 2013b), in animal models of elastase-induced emphysema (Kneidinger et al. 2011), and in lung slices of COPD patients (Uhl et al. 2015). Additional data indicate that targeted agonism of FZD_4 (Skronska-Wasek et al. 2017) or antagonism of FZD_8 (Spanjer et al. 2016a; Spanjer et al. 2016b) is beneficial in restoration of lung injury. Such subtype selective FZD agonists and antagonists are not yet widely available but are on the horizon in view of the recent development of surrogate WNT proteins that function as FZD-LRP5/LRP6 heterodimers to activate WNT/β-catenin signalling in mammalian cells (Janda et al. 2017). Furthermore, WNT agonists may be used in concert with in vitro expanded stem cells for transplantation purposes (Fig. 2). Though not yet demonstrated for lung injury, organoid transplantation of salivary gland epithelial cells is enhanced by WNT proteins to functionally restore salivary gland function in response to irradiation injury (Maimets et al. 2016) thus serving as a promising proof-of-concept study. Finally, it is likely that to

successfully intervene in these complex diseases, it will be necessary to move away from the approach to target a singular signalling pathway, but further take into account additional pathways that drive distinct disease processes. These approaches remain challenging within the translational pipeline and for potential clinical trials. Thus, integration of these concepts of early multi-pathway targeting will be essential (Fig. 2).

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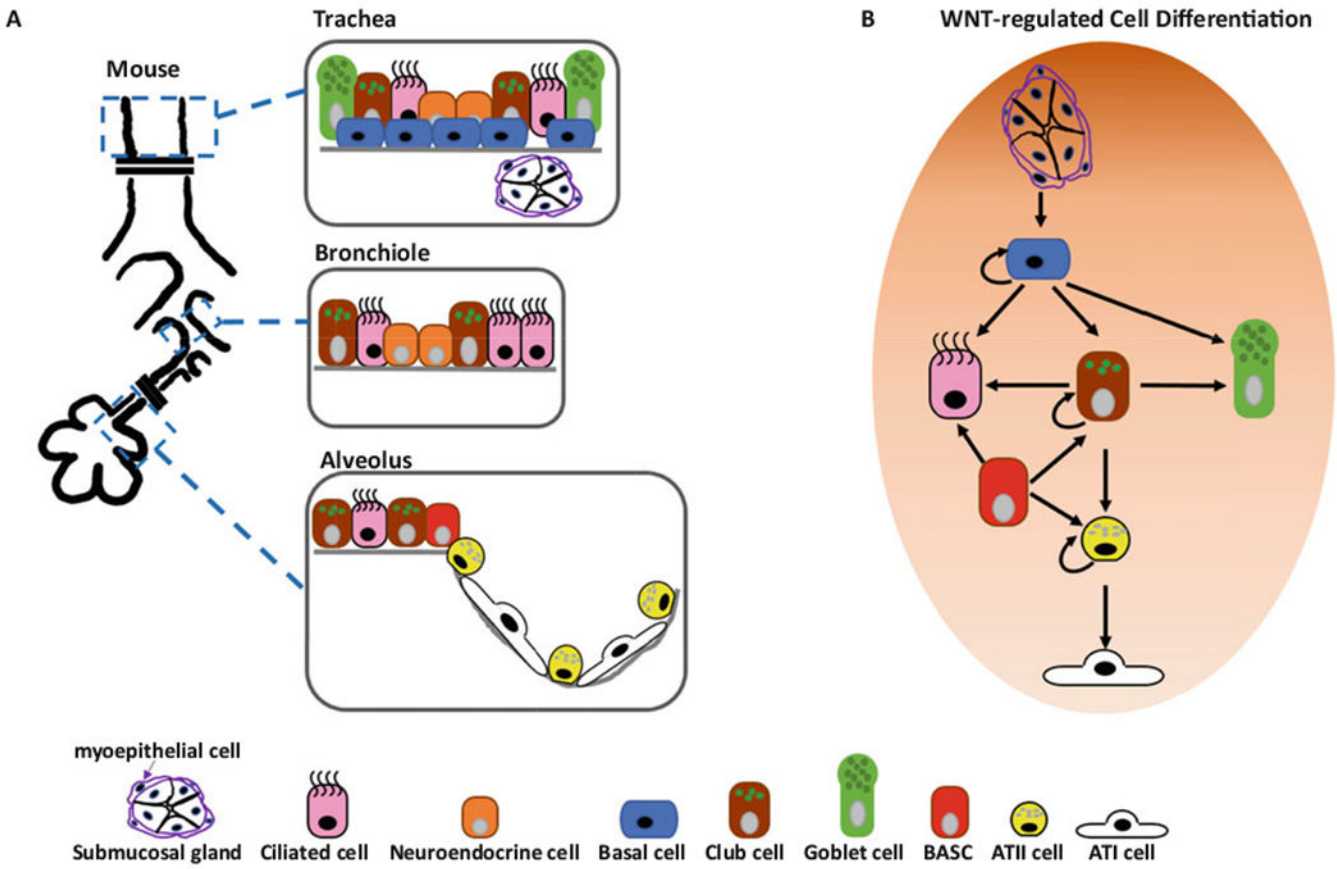

Fig. 1.

WNT signalling controls lung epithelial cell differentiation. (**a**) Mouse lung epithelial structure and cell types in trachea, bronchiole, and alveolus. (**b**) Lung epithelial cell differentiation processes under control of WNT signalling pathways

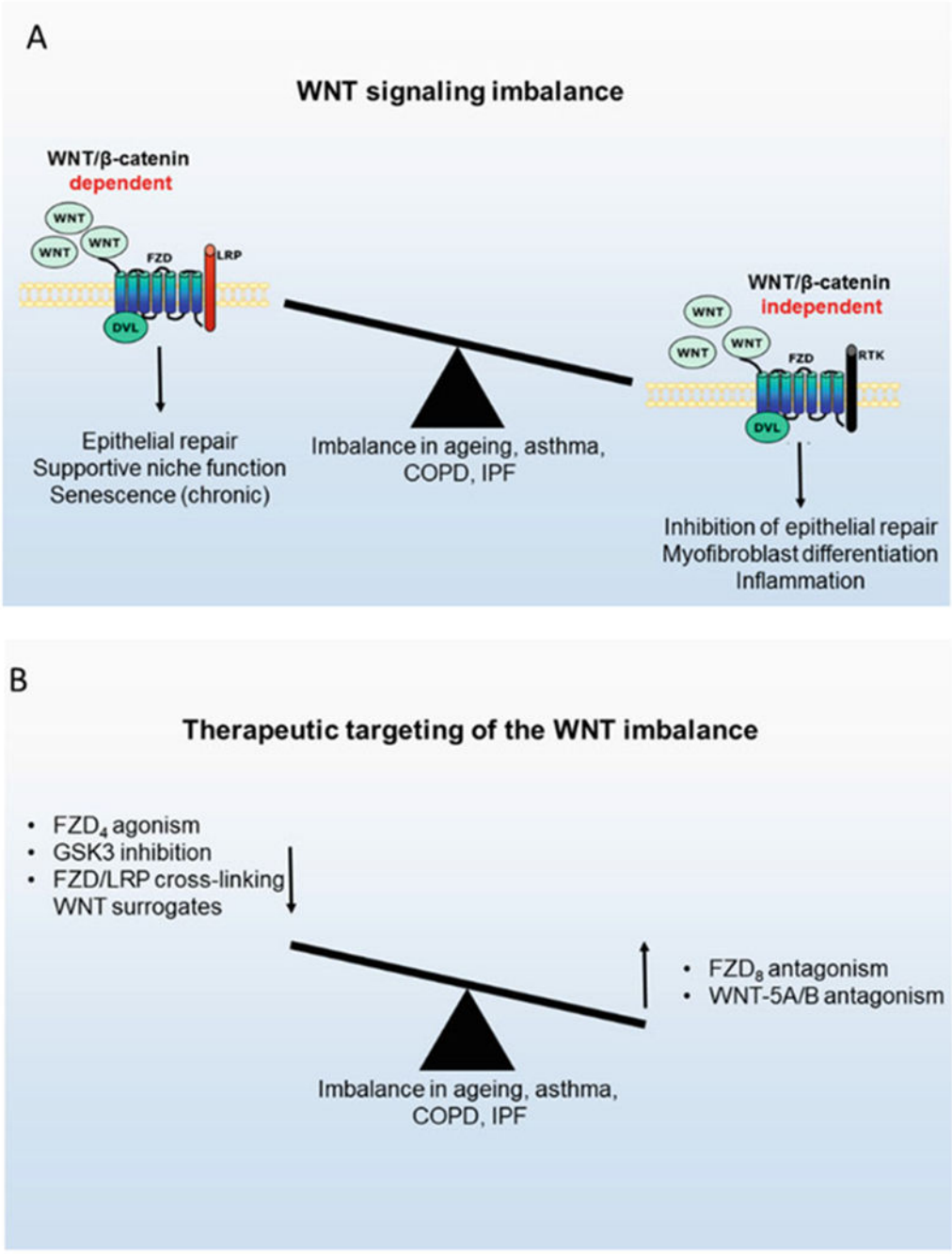

Fig. 2.

The nature and therapeutic targeting of the WNT signalling imbalance in chronic lung diseases. (**a**) Lung ageing and chronic lung diseases such as asthma, COPD, and IPF are characterized by reduced WNT/β-catenin-dependent signalling and increased WNT/β-catenin-inde-pendent signalling, which contributes to impaired epithelial repair, myofibroblast differentiation, and inflammation. (**b**) Restoration of this imbalance may

hold therapeutic promise, either by amplifying WNT/β-catenin-dependent signalling or by reducing WNT/β-catenin-independent signalling

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