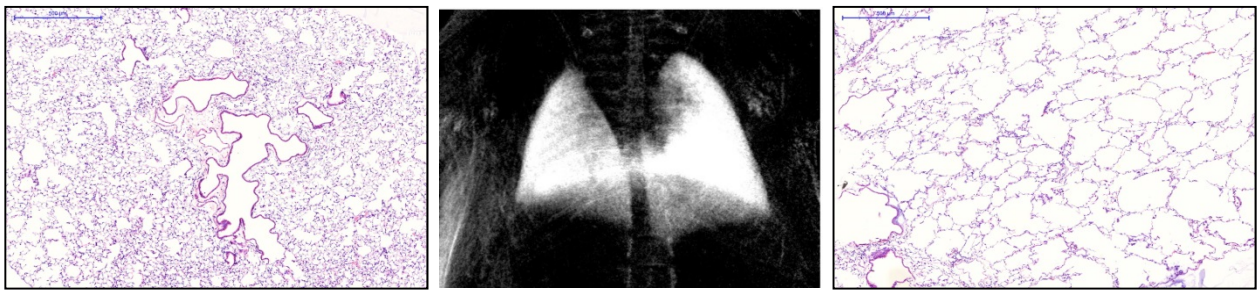
WNT/Frizzled Signaling in COPD and Emphysema



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PhD Thesis

From Lung Repair and Regeneration (LRR)

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WNT/Frizzled signaling in COPD and emphysema

Thesis for the attainment of the degree

Doctor of Philosophy (Ph.D.)

at the Faculty of Medicine

at the Ludwig-Maximilians-Universität München

by

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From Częstochowa, Poland

Munich, 2017

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Date of oral defence:	13.12.2017

Table of Contents

List of abbreviations

A1AT	alpha-1 antitrypsin
ABC	non-phosphorylated (active) β-catenin
APC	adenomatous polyposis coli
ATI	alveolar epithelial type I cell
ATII	alveolar epithelial type II cell
AXIN	axis inhibition protein
BALF	bronchioalveolar lavage fluid
BASC	bronchioalveolar stem cell
BMP	bone morphogenic proteins
CAMKII	calmodulin-dependent protein kinase II
CBR2	carbonyl reductase 2
CKI	casein kinase I
COPD	chronic obstructive pulmonary disease
CRD	cysteine-rich domain
CS	cigarette smoke
CSE	cigarette smoke extract
CYP1A1	cytochrome P450, family 1, member A1
DKK	dickkopf
ECM	extracellular matrix
EV	empty vector
FA	filtered air
FCS	fetal calf serum
FGF	fibroblast growth factor
FVC	forced vital capacity
FZD	frizzled
GPCR	G protein-coupled receptor
GSK3-β	glycogen synthase kinase 3-β
HPRT	hypoxanthine guanine phosphoribosyl transferase
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor 1

IL-6	interleukin-6
IP3	inositol 1,4,5-triphosophate
JNK	c-Jun-N-terminal kinase
LGR5	leucine-rich repeat-containing G protein-coupled receptor 5
LiCl	lithium chloride
LOX	lysyl oxidase enzyme
LRP5/6	low density lipoprotein receptor-related protein 5/6
MMP12	metalloproteinase 12
NFAT	nuclear factor of activated T cells
OE	overexpressing vector
PAN-CK	pan-cytokeratin
PBS	phosphate-buffered saline
PCLS	precision-cut lung slices
PCP	planar cell polarity
PFA	paraformaldehyde
PGF	placental growth factor
phATII	primary human alveolar epithelial type II cells
pLRP6	phosphorylated low-density lipoprotein receptor-related protein 6
PLC	phospholipase C
pmATII	primary murine alveolar epithelial type II cells
Pro-SPC	pro-surfactant protein C
qPCR	quantitative reverse transcription polymerase chain reaction
RIPA	radioimmunoprecipitation assay buffer
sFRP	secreted frizzled-related protein
SHH	sonic hedgehog
SPARC	secreted protein acidic cysteine-rich, osteonectin, basement-membrane protein 40
T1α	podoplanin, lung type I cell membrane associated glycoprotein
TCF/LEF	T-cell specific transcription factor/lymphoid enhancer-binding factor
TGF	transforming growth factor
VA	valproic acid

VEGF	vascular endothelial growth factor
WNT	wingless/int1

1. Introductory summary

1.1. COPD

More than 300 million people worldwide suffer from chronic obstructive pulmonary disease (COPD) and COPD is predicted to become the third leading cause of death by the year 2020 (Decramer *et al.* 2012; Vos *et al.* 2012). The disease is associated with poor prognosis and has a high socioeconomic burden (Sullivan *et al.* 2000). COPD can affect all compartments of the lungs and due to its heterogeneity is not a single disease but rather an umbrella term describing airflow limitations. The two major features of COPD are small airway disease (SAD) and emphysema. SAD involves airway inflammation, mucus hyperproduction, airway wall remodeling and peribronchial fibrosis leading to progressive narrowing of the conducting airways resulting in air trapping (Chung 2001; Hogg *et al.* 2004; Sturton *et al.* 2008), whereas emphysema describes destruction of the alveolar wall leading to loss of surface area required for gas exchange (Rabe *et al.* 2007; Minai *et al.* 2008) [Normal and COPD lung depicted in Fig. 1]. The severity of COPD is mainly estimated based on the degree of airflow limitation assessed by spirometry and classified according to the Global Initiative for Chronic Obstructive Pulmonary Disease (GOLD) (stage I-IV). It is calculated by the ratio of forced expiratory volume in one second (FEV_1) (volume of air that after full inspiration can forcibly be blown out in one second) to the forced vital capacity (FVC) (volume of air that after full inspiration can forcibly be blown out) (Rabe *et al.* 2007).

The cause of COPD and emphysema remains unknown, but several risk factors such as cigarette smoking, indoor and outdoor pollution and genetic associations (eg. alpha 1-antitrypsin (A1AT) deficiency) have been identified. Regardless of the cause of emphysema, there are several common pathological mechanisms, which appear to be conserved across the disease spectrum. Emphysema has been linked to increased apoptosis of both alveolar epithelial and endothelial cells (Rangasamy *et al.* 2004; Petrache *et al.* 2005). Airspace enlargement is a defining feature of emphysema, but the location varies depending on the cause. Airspace enlargement mostly occurs in the upper lobe (centrilobular emphysema) in smokers and lower lobes (panlobular emphysema) in patients with A1AT deficiency (Hogg *et al.* 2009). However, in all instances, the lung fails to repair following injury.

Recent studies suggest that impaired endogenous lung tissue maintenance and, in particular, damaged alveolar epithelial cell repair processes further contribute to emphysema development and progression, resulting in irreversible changes and loss of surface area for gas exchange (Sharafkhaneh *et al.* 2008; Taraseviciene-Stewart *et al.* 2008; Tuder *et al.* 2012; Boucherat *et al.* 2016). Despite our increasing knowledge about disease pathomechanisms, currently there is no causal therapy available for COPD. Pharmacological intervention with bronchodilators or corticosteroids only decelerates the disease progression and lung transplantation thus remains the only option in the end stage disease (Barnes 2010). Identification of novel pathomechanisms is critical for developing new potential therapies for these patients.

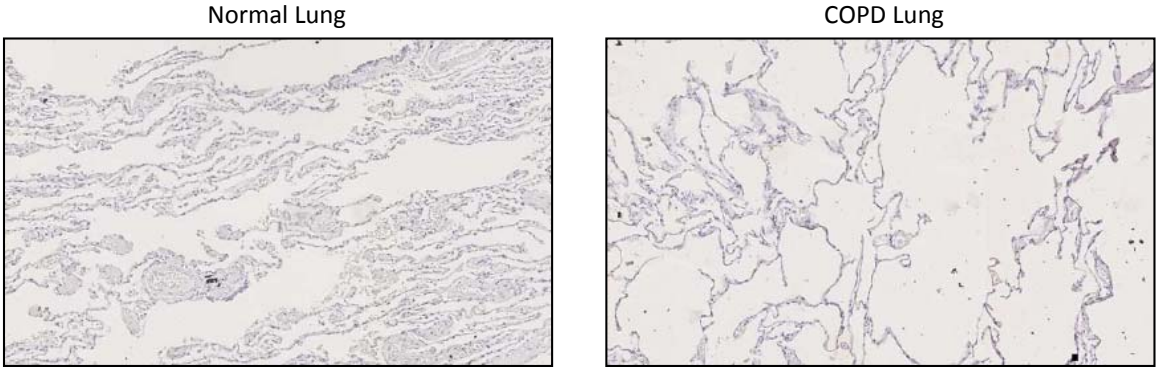

Fig. 1. Structure of the tissue obtained from normal and COPD lung (Miller *et al.* 2016)

1.1.1. COPD pathogenesis

1.1.1.1. Cigarette smoke

Several proposed hypotheses exist regarding COPD pathogenesis. Air pollution and biomass fuel exposure, especially in low-income countries, are increasing and are known to be a major contributor to the increase in disease prevalence. However, the main known risk factor for the development of the disease is tobacco smoking (Mannino *et al.* 2007; Salvi *et al.* 2009) and up to 50% of heavy smokers develop COPD (Rennard *et al.* 2006; Vos *et al.* 2012), indicating an interplay between environmental exposure and genetic predisposition.

Cigarette smoke (CS) contains more than 4700 chemical compounds, among them toxins, oxidants and carcinogens, and is a major source of oxidative stress (Smith *et al.* 2000) inducing DNA adducts, peroxidation of lipids and protein modifications (Church *et al.* 1985; Cai *et al.* 2009; Liu *et al.* 2010). Interestingly, despite the role of cigarette smoke in initiating COPD pathogenesis, quitting smoking does not reverse the disease, however, smoking cessation has been shown to slow down lung function decline (Tashkin *et al.* 2009; Decramer *et al.* 2012). This irreversibility has been postulated to be due to epigenetic changes induced by cigarette smoke (Stampfli *et al.* 2009; Besingi *et al.* 2014; Schamberger *et al.* 2014), persistence of oxidative stress (Louhelainen *et al.* 2009), altered microbiome colonization (Marsland *et al.* 2014), or sustained activation of adaptive immune responses, leading to irreversible tissue destruction (Rutgers *et al.* 2000; Morissette *et al.* 2014).

1.1.1.2. Genetic susceptibility and protease/antiprotease imbalance

In addition to cigarette smoke and environmental exposures, genetic susceptibility is also known to play a major role in COPD development. In 1963 Laurell and Eriksson published a report about emphysema patients exhibiting A1AT deficiency. A1AT is an inhibitor of neutrophil elastase, and thus prevents tissue destruction. This report suggested for the first time the contribution of protease/antiprotease imbalance to the emphysema development (Laurell *et al.* 1965). Subsequently, researchers utilized lung instillation of rodents with elastase (mimicking elastase released by neutrophils) and showed that it caused emphysematous changes (Kuhn *et al.* 1976; Janoff *et al.* 1977; Snider *et al.* 1984) confirming contribution of protease/antiprotease imbalance to disease development.

Macrophages and neutrophils are the primary inflammatory cells, which have been shown to release elastolytic enzymes. Macrophages play a central role in recruiting other cells, such as neutrophils, especially upon cigarette smoke, and release several proteases, including matrix metalloprotease (MMP)12, also known as macrophage metalloelastase (Hautamaki *et al.* 1997). Mice deficient for neutrophil elastase (Shapiro *et al.* 2003) or MMP12 (Hautamaki *et al.* 1997) were protected against cigarette smoke-induced emphysema, thus indicating that the presence of these proteases is necessary for disease development. Interestingly, cigarette smoke has also been shown to decrease A1AT activity in rats (Janoff

et al. 1979). Thus, it appears that cigarette smoke can directly modify the protease/antiprotease balance.

1.1.1.3. Deranged ECM and repair mechanisms in COPD

One prominent feature of emphysema is the failure of the lung to rebuild damaged tissue. Changes in the extracellular matrix (ECM) surrounding the conducting airways, alveolar cells and vasculature have been shown to contribute to COPD development (Bidan *et al.* 2015). The ECM is comprised of fibrous proteins (collagens and elastin) and structural/adhesive proteins (fibronectin, laminin) (Pelosi *et al.* 2007) and exhibits a variety of functions, including maintenance of structural integrity, role in cellular adhesion, migration, osmotic activity, as well as serves as a source of multiple growth factors and cytokines (Bonnans *et al.* 2014). Thus, changes in the ECM can have a detrimental effect on a number of cell functions.

Elastic fibers, due to their mechanical properties, provide great elasticity to the alveolar tissue (Mercer *et al.* 1990) and elastosis (septal inflammation and fiber fragmentation) leads to disorganization of the alveolar wall (Negri *et al.* 2000; Rocco *et al.* 2003). It has been shown that distal lung parenchyma of COPD patients exhibits reduced expression of elastin (Wright 1961; Chrzanowski *et al.* 1980) and the content of elastic fibers has been demonstrated to be reduced in the disease in both the airway wall as well as the alveoli (Black *et al.* 2008; Anstey *et al.* 2014). The amount of elastic fibers has been shown to correlate with FEV_1, indicating an influence of reduced elastin on reduced lung function in COPD patients. Moreover, it is well known that the administration of elastase enzyme, which depletes elastin in the lung, results in the development of emphysema (Goldstein *et al.* 1978; Kononov *et al.* 2001; Inoue *et al.* 2003; Ishizawa *et al.* 2004; Ito *et al.* 2005; Kawakami *et al.* 2008), underlining the importance of proper shaped elastin fibers in the lung physiological function. Restoration of functional elastin fibers or inhibition of further destruction of elastic fibers is therefore an appealing target for repair or regeneration strategies.

1.1.1.4. Aging and senescence

Aging of the lung has also been hypothesized to contribute to lung function decline and several hallmarks of aging, such as cellular senescence, are shared with the pathology of COPD (Meiners *et al.* 2015). The decline in lung function has been associated with emphysema-like changes in the aging lung (Janssens *et al.* 1999), however, destruction of the wall is not observed in such "senile" emphysema, where only enlargement of alveoli occurs (Janssens *et al.* 1999). Accelerated aging has been proposed to be driven by oxidative stress, telomere shortening and failure in the repair of damaged DNA (Ito *et al.* 2009). Interestingly, murine models of premature aging have been shown to exhibit increased susceptibility to cigarette smoke-induced lung tissue damage (Teramoto *et al.* 1994; Sato *et al.* 2007; Fukuchi 2009), which is in agreement with the notion that age predisposes to COPD development.

1.1.1.5. Deranged developmental signaling pathways

Recently, there has been increasing evidence that deranged developmental pathways may be implicated in COPD disease onset and progression. Several of these pathways have been targeted pharmacologically in various *in vivo* and *ex vivo* models and show promise. In particular, the Wingless/int1 (WNT) signaling pathway has emerged as playing a major role in COPD pathogenesis and several other chronic lung diseases (Kneidinger *et al.* 2011; Uhl *et al.* 2015; Boucherat *et al.* 2016). However, the exact mechanisms by which this pathway is altered in COPD remains incompletely understood, making pharmacological targeting challenging.

1.2. WNT signaling pathway

The WNT pathway is a developmental pathway, which is highly conserved among species. It has been shown to be important not only during embryogenesis but also postnatally in tissue repair (Konigshoff *et al.* 2010; Whyte *et al.* 2012; Baarsma *et al.* 2013). WNT signaling has been classically divided into canonical (β-catenin-dependent) and several non-canonical (β-catenin-independent) pathways [Fig. 2]. WNT signaling consists of at least 19 WNT ligands

that signal through 10 seven-transmembrane Frizzled (FZD) receptors, low-density lipoprotein receptor related proteins (LRP) 5 and 6 and many various co-receptors (e.g. ROR2, RYK), and extracellular modulators (e.g. Dickkopf (DKK) and secreted Frizzled-related proteins (sFRP)). WNT ligands have been classified historically based on their amino acid sequence rather than their functional properties and they have been suggested to be able to compensate for each other. However, it is now well established that some of the ligands have a higher capacity to facilitate specific and distinct WNT pathways. For instance, WNT3A is the most studied ligand known to induce canonical WNT signaling, but WNT1, WNT2, WNT8 and WNT10B have also been shown to facilitate this WNT signaling. On the other hand, WNT4, WNT11 and the broadly investigated WNT5A ligand mediate non-canonical WNT signaling (Niehrs 2012; Al Alam *et al.* 2013; Baarsma *et al.* 2013).

With regard to receptors for the WNT pathways, the FZD family is a part of atypical G protein-coupled receptors (GPCRs) (Foord *et al.* 2005). FZDs share common structural characteristics and contain the following elements: (i) an extracellular N-terminal signal sequence, followed by (ii) a cysteine-rich domain (CRD), implicated in WNT binding, (iii) the transmembrane and intracellular domains, and (iv) an intracellular C-terminus (Schulte 2010). Distinct WNT ligands have been shown to bind with different affinities to different FZD receptors, required for transducing both canonical and non-canonical WNT signaling (Grumolato *et al.* 2010; Dijksterhuis *et al.* 2015). However, to date, little is known about specific and distinct WNT-FZD interactions. As binding of WNT ligands to FZD receptors is an initial step into regulating pathway activity, manipulation of the pathway at this level is an attractive pharmacological target.

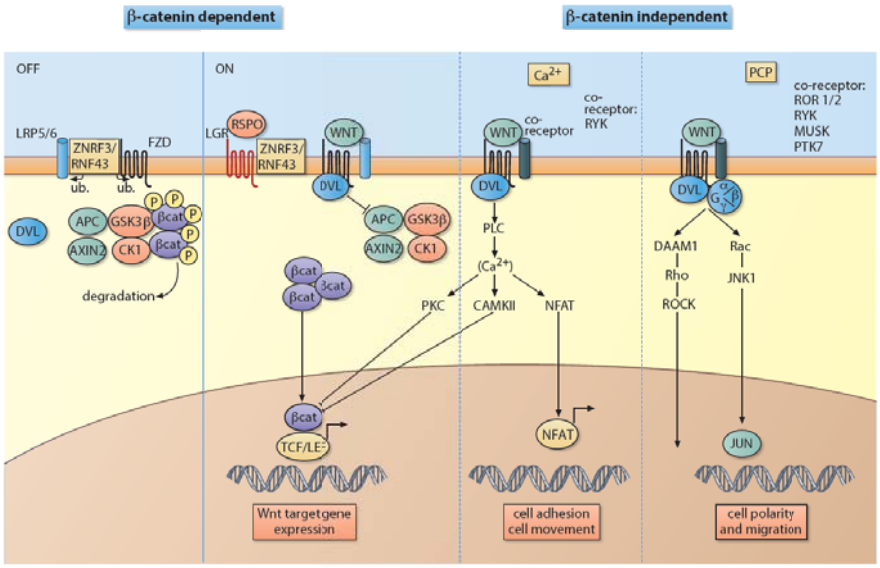

Fig. 2. Schematic representation of canonical WNT/β-catenin and non-canonical: PCP and WNT-Ca^{2+} signaling pathways (Skronska-Wasek *et al*., in preparation)

1.2.1. Canonical WNT (/β-catenin) signaling

Canonical WNT (/β-catenin) signaling is the most well-characterized WNT pathway. In the absence of canonical WNT ligands, the active destruction complex, consisting of axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3-β (GSK3-β) and cysteine kinase I (CKI), phosphorylates β-catenin at its N-terminal residues. Phosphorylated β-catenin is subsequently ubiquitinated and degraded by β-transducin repeat containing protein (Logan *et al.* 2004; Clevers 2006; Angers *et al.* 2009; MacDonald *et al.* 2009). On the other hand, when canonical WNT ligands bind to the membranous receptors (FZD and LRP5/6), the destruction complex is recruited to the membrane and GSK3-β and CKI phosphorylate the LRP5/6 receptor. This results in the inactivation of the destruction complex and leads to the inhibition of phosphorylation of β-catenin. Unphosphorylated β-catenin cumulates in the cytoplasm and can be translocated to the nucleus where it binds to the members of the T-cell specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family, which leads to the transcription of canonical WNT target genes. Canonical WNT target genes seem to be cellular- and tissue-context dependent and our knowledge in this matter is steadily increasing, however, for a majority of cells, the most well established target genes are

canonical WNT pathway components Axin2 and leucin-rich repeat-containing G protein-coupled receptor (Lgr5). Functionally, WNT/β-catenin signaling has been described to be involved in cellular proliferation and differentiation (Logan *et al.* 2004; Clevers 2006; Angers *et al.* 2009; MacDonald *et al.* 2009; Konigshoff *et al.* 2010; Niehrs 2012; Nusse 2012; Baarsma *et al.* 2013), processes known to be impaired in COPD.

1.2.2. Non-canonical WNT signaling

To date, several distinct non-canonical (β-catenin-independent) WNT signaling pathways have been described. The most studied are the planar cell polarity (PCP) pathway acting via c-Jun-N-terminal kinase (JNK) and the WNT-Ca^{2+}, Ca^{2+}-dependent pathway (Niehrs 2012; Nusse 2012). In β-catenin-independent pathways, WNT ligands bind with FZD receptors, but LRP5/6 activation is not required. Instead, several co-receptors, which are present in the membrane (e.g. ROR2, RYK, PTK7), are involved in transducing the signal into the distinct cellular pathways.

In the PCP pathway, signaling is transduced via small GTPases and activates Rac1, RhoA and JUN-N-terminal kinase (JNK). This signaling pathway has been implicated in tissue morphogenesis, cytoskeletal rearrangements and epithelial cell polarity (Niehrs 2012). The PCP and WNT/β-catenin signaling can antagonize each other. For instance, WNT5A-dependent non-canonical WNT signaling has been shown to inhibit WNT3A-induced WNT/β-catenin signaling (Bryja *et al.* 2007; Niehrs 2012).

In the WNT-Ca^{2+} pathway, binding of WNT ligands is associated with activation of phospholipase C (PLC), which leads to the formation of inositol 1,4,5-triphosophate (IP3) and 1,2 diacylglycerol (DAG), resulting in intracellular increase of Ca^{2+}. This subsequently activates calmodulin-dependent protein kinase II (CAMKII), protein kinase C (PKC) and the nuclear factor of activated T cells (NFAT) transcription factor. Similarly to the PCP signaling, the WNT-Ca^{2+} pathway is known to antagonize WNT/β-catenin signaling, and to be involved in developmental processes, but also has been linked to cancer and inflammation (De 2011; Niehrs 2012; Baarsma *et al.* 2013).

1.2.3. WNT signaling in lung development

As described above, both canonical WNT/β-catenin and non-canonical WNT signaling pathways play a role during lung morphogenesis (Pongracz *et al.* 2006; De Langhe *et al.* 2008).

During development, the lung arises from the anterior foregut endoderm at the laryngotracheal groove (Morrisey *et al.* 2010). Branching morphogenesis starts around embryonic day 10.5 (E10.5) in the mouse, when the lung-bud epithelium invades the surrounding mesenchyme (Warburton *et al.* 2005; Cardoso *et al.* 2006; Rockich *et al.* 2013) and precise coordination between the mesenchyme and epithelium is required for organogenesis (Shannon *et al.* 2004; Hines *et al.* 2014). This process is known to be controlled by several different factors and signaling pathways, such as WNT pathways, transforming growth factor (TGF), fibroblast growth factor (FGF), sonic hedgehog (SHH) and bone morphogenic proteins (BMPs) (Hogan *et al.* 1998; Cardoso *et al.* 2006). It has been shown that distinct WNT ligands and receptors are temporally and spatially expressed during morphogenesis (Ota *et al.* 2016). For instance, FZD4, FZD7 and LRP5/6 have been localized in the developing human lung (Zhang *et al.* 2012) and shown to exhibit highly cell specific expression patterns in the murine lung during development (FZD4 and FZD7 in mesenchyme and LRP5/6 in epithelium) (Wang *et al.* 2005). Several WNT ligands have been identified in the developing lung: WNT2 in the distal mesenchyme (Levay-Young *et al.* 1992) and WNT7B in the epithelium (Shu *et al.* 2002), whereas WNT5A has been found in both cell entities (Li *et al.* 2002). Moreover, transgenic deletion of WNT7B (Shu *et al.* 2002) led to impaired mesenchymal growth and vascular development and lack of WNT2A/2B activity resulted in complete lung agenesis (Goss *et al.* 2009). On the other hand, transgenic mice overexpressing WNT5A exhibited reduced epithelial branching morphogenesis and distal airspace enlargement, accompanied by decreased Shh signaling (Li *et al.* 2005), suggesting that WNT pathways interact with different other signaling pathways during lung development.

Collectively, WNT signaling is crucial for proper lung development and alterations in WNT pathway components lead to lung defects or even agenesis. Moreover, these developmental pathways have been shown to play an important role in postnatal tissue maintenance and

repair (Whyte *et al.* 2012), making them interesting candidates for pharmaceutical targeting.

1.2.4. WNT signaling in COPD and tissue repair

Adult lung tissue exhibits self-renewal capacity, albeit to a low extent. In a landmark case study report, Butler *et al.* describe lung regeneration in a 15-year follow up study after partial pneumonectomy in a 33-year-old woman as a treatment for adenocarcinoma. They observed significant improvement of lung function that they attributed to growth of new alveoli (Butler *et al.* 2012). This case report supports the concept that the adult lung may possess more capacity to repair than previously thought. To date many different endogenous stem/progenitor cell populations have been identified in the lung (Rock *et al.* 2012) and their capacity to promote regeneration of the damaged tissue has been shown to be dependent on the WNT signaling activity (Beers *et al.* 2011). WNT signaling has been identified as playing a critical role in regulating specification and differentiation of pluripotent cells to airway epithelial lineages. WNT signaling is needed for mouse embryonic stem cells and for the differentiation of human induced pluripotent cells (hiPSC) to differentiate to airway progenitors (Mou *et al.* 2012; McCauley *et al.* 2017).

It has been demonstrated that the loss of the transcription factor GATA6 induced WNT signaling which in turn led to concurrent expansion of bronchioalveolar stem cells (BASCs), an endogenous progenitor cell population shown to give rise to club and alveolar epithelial type II (ATII) cells in the murine lung (Kim *et al.* 2005; Zhang *et al.* 2008). Also ATII cells are known to have a progenitor potential. Upon injury they can clonally expand *in vivo* and give rise to ATI cells, as well as they have a capacity to form 3D organoid structures when cultured *ex vivo* in Matrigel with stromal cells (Barkauskas *et al.* 2013; Desai *et al.* 2014). Interestingly, inhibition of the WNT/β-catenin pathway in ATII cells interfered with ATII-to-ATI cell trans-differentiation (Flozak *et al.* 2010; Mutze *et al.* 2015), underlining the importance of active WNT signaling to exert their progenitor potential.

WNT/β-catenin signaling is known to play a role in cell proliferation and renewal (Chilosi *et al.* 2012), especially following injury. For instance, in mice lacking β-catenin in alveolar epithelium, instillation of bleomycin led to more severe fibrosis and accelerated epithelial cell death (Tanjore *et al.* 2013). Additionally, knock down of β-catenin delayed repair of

alveolar epithelium (Zemans *et al.* 2011). Thus, it appears that β-catenin in the epithelium is critical for tissue repair. Interestingly, activation of the WNT/β-catenin pathway with lithium chloride (LiCl) via GSK3-β inhibition attenuated cigarette smoke- and elastase-induced emphysema in mice *in vivo* (Kneidinger *et al.* 2011). Furthermore, it has been recently shown that re-activation of WNT/β-catenin signaling in *ex vivo* COPD patient-derived precision cut lung slices (PCLS) induced expression of epithelial markers (surfactant protein C (SPC) and Podoplanin) (Uhl *et al.* 2015), indicating a role of WNT/β-catenin signaling in maintaining alveolar epithelial function (Sharafkhaneh *et al.* 2008; Flozak *et al.* 2010).

Dysregulation of the WNT signaling might thus result in impaired repair processes and lead to the development of several lung diseases, such as COPD. Emphysema, the major pathological feature of COPD, has been shown to be associated with reduced WNT/β-catenin signaling (Kneidinger *et al.* 2011; Wang *et al.* 2011; Uhl *et al.* 2015; Jiang *et al.* 2016), particularly in ATII cells [Fig. 3] (Kneidinger *et al.* 2011). Moreover, decreased β-catenin has been observed in the airway epithelium of smokers (Guo *et al.* 2015) and reduced WNT pathway components have been detected in the lungs of mice exposed to cigarette smoke (Kneidinger *et al.* 2011). However, the cause of deranged WNT/β-catenin signaling and the involved components remain unknown.

Together, identification of aberrant components of the WNT pathway in COPD patients could help to develop novel treatments inducing lung tissue repair and restoration of tissue function.

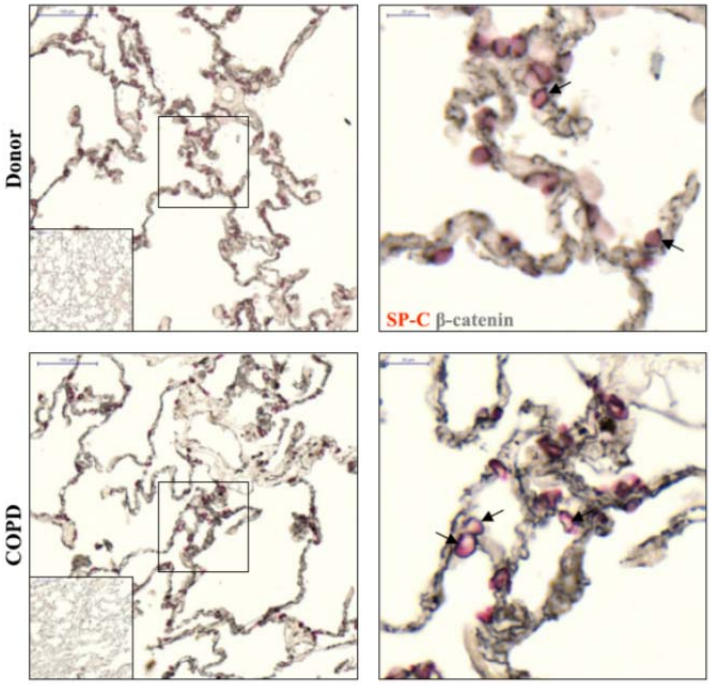

Fig. 3. IHC staining of SPC (red) and β-catenin (grey) in alveolar region of the lung obtained from Donor and COPD patient (Kneidiger *et al*., AJRCCM, 2011)

1.3. Hypothesis and Objectives

It is known that WNT/β-catenin signaling activity is decreased in COPD and emphysema, as well as that induction of this pathway holds promise for repair of the lung tissue. What remains unknown is if there are any WNT pathway components, FZD receptors or WNT ligands, deranged in the disease, that could potentially be used as pharmacological targets.

We hypothesized that targeting of WNT ligands or FZD receptors altered in COPD/emphysema can lead to increased WNT/β-catenin signaling and induce distal lung tissue repair.

The aims of this thesis were to: A) comprehensively analyze the expression of FZD receptors in human COPD as well as different mouse models of emphysema, B) investigate the potential influence of identified deranged receptors on WNT signaling and repair of epithelial cells, C) characterize the role of the non-canonical WNT5A ligand in the development of COPD/emphysema and its contribution to impaired tissue repair, D) investigate the therapeutic potential of targeting of the WNT5A ligand.

1.4. Publications included in this thesis

Reduced Frizzled Receptor 4 Expression Prevents WNT/β-catenin-driven Alveolar Lung Repair in COPD

Skronska-Wasek W, Mutze K, Baarsma HA, Bracke KR, Alsafadi HN, Lehmann M, Costa R, Stornaiuolo M, Novellino E, Brusselle GG, Wagner DE, Yildirim AÖ, Königshoff M. Am J Respir Crit Care Med. 2017 Jul 15;196(2):172-185.

Noncanonical WNT-5A Signaling Impairs Endogenous Lung Repair in COPD

Baarsma HA, **Skronska-Wasek W**, Mutze K, Ciolek F, Wagner DE, John-Schuster G, Heinzelmann K, Günther A, Bracke KR, Dagouassat M, Boczkowski J, Brusselle GG, Smits R, Eickelberg O, Yildirim AÖ, Königshoff M. J Exp Med. 2017 Jan; 214(1):143-163.

1.5. Summary

COPD is a progressive and fatal disease with no causal therapies available. The disease is described by irreversible airflow limitations due to small airway disease, destruction of the alveolar walls leading to distal airway enlargement (emphysema) and impaired tissue repair. Mechanisms underlying the development and progression of the disease are not yet fully understood. It is known that canonical WNT signaling activity is decreased in COPD and that the induction of the pathway attenuates experimental emphysema. However, which deranged specific receptor or ligand contributes to the observed impaired WNT signaling remains unknown.

In the first publication (Skronska-Wasek *et al.* 2017), screening for deranged FZD receptors in human COPD and elastase-induced mouse model of emphysema revealed decreased level of FZD4. Further investigation showed downregulation of FZD4 receptor in the lung tissue of smokers and mouse model of cigarette smoke-induced emphysema and in ATII cells obtained from COPD patients. Moreover, FZD4 expression was demonstrated to regulate WNT signal activity and further epithelial cell proliferation, ATII-to-ATI cell trans-differentiation and wound closure. Additionally, FZD4 positively regulated expression of elastic components indicating a role in elastogenesis.

The second publication (Baarsma *et al.* 2017) revealed up-regulation of non-canonical WNT5A ligand in the lung of COPD patients and in two experimental models of emphysema. Moreover, we demonstrate increased expression of WNT5A in fibroblasts from COPD patients and upon COPD-related stimuli like cigarette smoke and cellular senescence. Functionally, WNT5A attenuated canonical WNT signaling and interfered with epithelial wound closure and ATII-to-ATI cell trans-differentiation. *In vivo*, WNT5A overexpression led to airspace enlargement in elastase-induced emphysema and blocking of WNT5A attenuated tissue destruction and improved lung function parameters in elastase- and cigarette smoke-induced emphysema.

Taken together, we show that the decrease of WNT/β-catenin signaling observed in COPD/emphysema might be caused by deranged expression of a critical WNT/β-catenin signaling pathway component, the FZD4 receptor, or the overexpression of the WNT5A ligand. We also demonstrate that targeting of the receptor or the ligand can lead to the

positive regulation of WNT signaling inducing repair of lung tissue which could be beneficial in the treatment of COPD/emphysema.

1.6. Contribution

First publication (Skronska-Wasek *et al.* 2017)

In vivo experiments with exposure to cigarette smoke (CS) (Supplementary Fig. E3D, E8A); isolation of pmATII cells (Fig. 2C/E, 5A, 6C/D/E/F, Supplementary Fig. E3C/D, E6A, E8A); *in vitro* experiments with CSE (Fig. 2D/E, 6C/E/G, Supplementary Fig. E3D); transfection of the cells (Fig. 3A/B/3D, 4C/E/G, E5A/B); *in vitro* treatments with FzM1/WNT3a/VA (Fig. 3A/B/C/D, 4A/B/C/F, 5A, 6B/D, Supplementary Fig. E9A/B); WST-1 assay (Fig. 4A, 4C); qPCR (Fig. 1A, 2D, 3A, 6A/B/C/D/I, Supplementary Fig. E1A, E3C, E5A, E6A, E9A/B); immunofluorescence staining (Fig. 1B, 2C/E, 4D/E, Supplementary Fig. E1B, E3B, E5B); Western blot (Fig. 2B, 3C, 5A, Supplementary Fig. E3D, E7A); scratch assay (Fig. 4F/G); ELISA (Fig. 6E/F/K); organoid formation assay (Supplementary Fig. E6B); design of the experiments, preparation and editing of the figures and manuscript.

Second publication (Baarsma *et al.* 2017)

In vivo experiments with SFTPC rtTA TetO WNT5A mice and elastase (Fig 1E/F/G/H); *in vivo* experiments with WNT5A blocking antibodies and elastase (Fig. 7A-F, Fig. 8); *in vivo* experiments with BOX5 and cigarette smoke (Fig. 7G-J); editing of the manuscript.

2. Publication I

3. Publication II

4. Discussion

WNT signaling in COPD and emphysema

One of the hallmarks of COPD is impaired tissue repair. The role of WNT/β-catenin signaling in repair processes is well known in several different organs and blocking of this pathway impairs the regenerative capacity of several different tissues (Cho *et al.* 2006; Kawakami *et al.* 2006; Stephens *et al.* 2010; Ramachandran *et al.* 2011). Activation of the WNT/β-catenin pathway observed during bleomycin-induced lung injury has been shown to promote alveolar epithelial surivival and migration (Flozak *et al.* 2010). Moreover, inhibition of WNT signaling decreased the potential of the cells to proliferate (Qyang *et al.* 2007; Stoick-Cooper *et al.* 2007). Interestingly, WNT/β-signaling has been previously described to be inhibited in COPD patients and smokers, as assessed with decreased β-catenin gene expression in lung tissue (Guo *et al.* 2015; Jiang *et al.* 2016), as well as decreased β-catenin nuclear localization in ATII cells of COPD patients (Kneidinger *et al.* 2011). Moreover, we have shown attenuated active-β-catenin expression in murine models of elastase- and cigarette smoke-induced emphysema (Kneidinger *et al.* 2011; Uhl *et al.* 2015; Baarsma *et al.* 2017; Skronska-Wasek *et al.* 2017). However, the reason for the decrease in WNT signaling remains incompletely understood.

To date, several components of the WNT pathway have been shown to be altered in the disease. We found FZD4 receptor (Skronska-Wasek *et al.* 2017) and WNT5A ligand (Baarsma *et al.* 2017) to be deranged in human COPD and murine models of emphysema. WNT5A, the classical non-canonical pathway ligand, was found to be increased in COPD patients but also in both used mouse models of emphysema. Interestingly, WNT5A-induced non-canonical WNT signaling has been widely shown to antagonize canonical WNT signaling (Mikels *et al.* 2006; Nemeth *et al.* 2007; Baarsma *et al.* 2017), although the exact mechanism of how this occurs remains unknown. With regard to receptor expression, Kneidinger and colleagues have shown decreased FZD1 and FZD2 expression in lungs from the murine model of elastase-induced emphysema (Kneidinger *et al.* 2011). In line with this, FZD1, but also FZD8 have been shown to be decreased in the small airway epithelium of smokers and COPD patients (Wang *et al.* 2011). We identified FZD4 to be downregulated in ATII cells from the lungs of COPD patients (Skronska-Wasek *et al.* 2017). FZD4 has been reported to transduce both canonical WNT/β-catenin (Tickenbrock *et al.* 2008) and non-canonical WNT signaling

(Robitaille *et al.* 2002), indicating that this capacity is likely tissue-/organ-/cell type- and injury-dependent. We demonstrated that in the alveolar epithelial cells, FZD4 facilitates canonical WNT/β-catenin signaling (Skronska-Wasek *et al.* 2017). Thus, we identified a new component of the canonical WNT pathway that is deranged in COPD and a novel inhibition mechanism of the canonical WNT pathway through altered WNT5A levels and activity.

Potential of WNT signaling in lung tissue repair

The activation of the WNT pathway has been shown to positively regulate tissue repair in several organs (Ito *et al.* 2007). WNT signaling has been shown to be elevated at early stages upon injury (Chen *et al.* 2007; Kim *et al.* 2007; Leucht *et al.* 2008; Petersen *et al.* 2009) and specifically in the lung (Beers *et al.* 2011; Villar *et al.* 2011), which could be interpreted as an attempt of the tissue to regenerate. Interestingly in the lung, activation of the WNT pathway via inhibition of GSK3-β with LiCl attenuated elastase- and cigarette smoke-induced emphysema (Kneidinger *et al.* 2011). Additionally, administration of LiCl in *ex vivo* cultured COPD-derived PCLS led to the increase in expression of epithelial markers pro-SPC and T1α as compared to time-matched controls, indicating the potential contribution of WNT signaling in epithelial repair (Uhl *et al.* 2015). However, while the activation of WNT signaling is now known to be beneficial for the lung tissue repair, how to best accomplish this remains unknown. Thus, we focused on identifying deranged components of the WNT pathway (e.g. receptors) that could be targeted more specifically to induce WNT signaling. Activation of WNT/β-catenin signaling transduced by FZD4 receptor not only positively influenced proliferation of epithelial cells, but also accelerated wound closure (Skronska-Wasek *et al.* 2017). In line with the concept that activation of the canonical WNT pathway is beneficial for alveolar repair, we found that activation of the WNT/β-catenin pathway, via inhibition of the non-canonical WNT5A ligand, in murine models of cigarette smoke- and elastase-induced emphysema *in vivo*, led to attenuation of lung pathogenesis, as measured with improved lung function parameters and histology (Baarsma *et al.* 2017). On the other hand, blocking of WNT/β-catenin signaling *in vitro* with inhibition of FZD4 led to the opposite effect and decreased proliferation and delayed wound closure (Skronska-Wasek *et al.* 2017).

Additionally, in our recent studies, we found that FZD4 and WNT5A not only modulate WNT/β-catenin signaling in alveolar epithelial cells, but further influence differentiation of

the alveolar epithelium. Blocking of the FZD4 receptor or downregulation of the canonical WNT/β-catenin pathway with WNT5A inhibited ATII-to-ATI-cell trans-differentiation as assessed by attenuated upregulation of T1α protein expression over time of culture (Baarsma *et al.* 2017; Skronska-Wasek *et al.* 2017). Moreover, loss of FZD4 function decreased organoid formation capacity by ATII cells (Skronska-Wasek *et al.* 2017). Similarily, *in vivo* inhibition of the WNT/β-catenin pathway via WNT5A overexpression exacerbated airspace enlargement in elastase-induced emphysema in mice (Baarsma *et al.* 2017). These studies further support the role of WNT/β-catenin signaling in alveolar epithelial cell repair.

Contribution of cigarette smoke to the lung pathology observed in COPD and emphysema

Cigarette smoke is the main risk factor for chronic lung diseases and is thought to contribute both to the destruction of the lung tissue and to impaired repair capacity. Markers of oxidative stress are increased in COPD indicating that oxidative stress might play a role in the disease development (MacNee 2001; Rahman *et al.* 2002). Oxidants present in cigarette smoke are known to induce inflammatory responses in the lung, resulting in the accumulation of macrophages and the recruitment of neutrophils (Rennard *et al.* 2006). Both inflammatory cell types are sources of elastolytic enyzmes, such as neutrophil elastase and MMP12, respectively, which according to the "protease-antiprotease" hypothesis contribute to the injury of the alveoli and the development of emphysema (Hiemstra *et al.* 1998; Rennard *et al.* 2006).

We and others have shown that cigarette smoke inhibits WNT/β-catenin signaling and deregulates expression of WNT pathway components (Kneidinger *et al.* 2011; Wang *et al.* 2011; Guo *et al.* 2015). We have demonstrated that WNT5A is increased in COPD patients as well as in cigarette smoke-induced emphysema and *in vitro* in cigarette smoke-exposed lung fibroblasts (Baarsma *et al.* 2017). Interestingly, WNT4, another non-canonical WNT ligand, has been previously shown to be increased in COPD patients, especially in bronchial epithelial cells, and to potentiate cigarette smoke-induced inflammation (Heijink *et al.* 2013), another hallmark of COPD. Both WNT4- and WNT5A-induced non-canonical WNT signaling have been widely shown to antagonize the canonical WNT pathway and in turn attenuate repair processes as shown with delayed wound closure (Baarsma *et al.* 2017). On

the other hand, WNT3A, the classical inducer of canonical WNT signaling, has been found to be decreased in cigarette smoke-exposed bronchial epithelial cells (Guo *et al.* 2015).

In addition to dysregulation at the WNT ligand level, FZD receptors have been shown to be deranged upon cigarette smoke exposure. We have found that FZD4 expression was not only decreased in smokers, but even further downregulated in COPD patients and correlated with the severity of the disease, indicating that there are additional factors, other than cigarette smoke, contributing to FZD4 loss (Skronska-Wasek *et al.* 2017). Moreover, direct exposure of bronchial (Guo *et al.* 2015) and alveolar epithelial cells (Skronska-Wasek *et al.* 2017) led to decreased FZD4 expression. Additionally, in previous studies *in vivo* cigarette smoke exposure decreased FZD1 expression in the mouse lung (Kneidinger *et al.* 2011) and downregulated FZD1 and FZD8 in smokers (Wang *et al.* 2011). *In vitro* exposure to cigarette smoke decreased expression of FZD1 and FZD2 in bronchial epithelial cells (Heijink *et al.* 2013; Guo *et al.* 2015) and FZD2 in alveolar epithelium (Heijink *et al.* 2013).

While cigarette smoke exposure correlates with tissue destruction, cessation of smoking does not reverse the progression of alveolar destruction. This indicates an impaired ability of the lung to repair after removal of injurious insults. This impaired repair might be partially explained by the onset of a persistent inflammation present in the lung, but additional mechanisms are most likely involved. Changes upon cigarette smoke, such as the loss of FZD4 receptor shown by us, might be irreversible, leading to a cascade of events including attenuation of canonical WNT signaling and further disturbed tissue repair.

Apoptosis and its contribution to COPD/emphysema development

Apoptosis represents another pathological feature observed in emphysema that has been linked to cigarette smoke exposure. Apoptosis is a normal process during early development and branching morphogenesis, however, its occurrence in healthy adult lung is uncommon (Scavo *et al.* 1998; De Paepe *et al.* 1999). Several reports exist bridging emphysema and cell apoptosis in both human (Segura-Valdez *et al.* 2000) and rodents (Tuder *et al.* 2000). Noteworthy, cigarette smoke exposure has been linked with increased apoptosis *in vitro* (Nakamura *et al.* 1995; Aoshiba *et al.* 1997; Aoshiba *et al.* 2000) and *in vivo* in the murine lung (Obot *et al.* 2004; Rangasamy *et al.* 2004; Bartalesi *et al.* 2005). Interestingly, we found that blockade of FZD4 with FzM1 led to increased cleaved caspase 3 staining (data not

shown), indicating a potential role of this receptor in apoptosis processes, which might lead to impaired proliferation and repair. In addition, several factors responsible for the tissue loss observed in emphysema have been proposed, such as placental growth factor (PGF) (Tsao *et al.* 2004), vascular endothelial growth factor (VEGF) (Kasahara *et al.* 2000; Tang *et al.* 2004), TNF-α and oxidative stress (Takabatake *et al.* 2000). Interestingly, administration of superoxide dismutase prevented alveolar cell apoptosis, and inhibition of apoptosis prevented oxidative stress and emphysema, showing that both processes - oxidative stress and apoptosis - interact in alveolar wall destruction (Tuder *et al.* 2003).

Collectively, apoptosis plays a major role in the development of emphysema and inhibition of this process, for instance via increasing FZD4 receptor abundance, might be beneficial.

Cellular senescence and its contribution to COPD/emphysema

In addition to environmental insults, aging and especially cellular senescence have been ascribed a role in COPD development and impaired tissue repair. Aging is described as a failure of the organ to maintain and repair, which resembles a similar state observed in emphysema. During normal aging, the lung exhibits a progressive airspace enlargement and a hallmark of aging is present in emphysematous lung and COPD (Faner *et al.* 2012). Alveolar epithelial and endothelial cells from emphysematous lungs have been shown to exhibit enhanced expression of senescent markers p16 and p21 and shortened telomeres (Tsuji *et al.* 2006). Additionally, prematurely aged mice exhibit enhanced airspace enlargement (Teramoto *et al.* 1996). Senescent cells, present in the diseased lung, are thought to release inflammatory cytokines and matrix proteases, which could contribute to impaired repair potential, alveolar inflammation and altered extracellular matrix observed in emphyematous and aged lungs (Campisi 2005; Sokocevic *et al.* 2013; Wagner *et al.* 2014). Importantly, cigarette smoke has been shown to induce aging and senescence in alveolar epithelial cells, linking oxidative stress with cellular senescence (Tsuji *et al.* 2004; Nyunoya *et al.* 2006). Additionally, it has been reported that COPD lungs possess shortened telomeres (compared to non-smokers), another hallmark of aged organ (Campisi 2005), and that oxidative stress contributes to the observed effect (Lombard *et al.* 2005).

WNT signaling has also been linked to aging. Canonical WNT/β-catenin signaling has been shown to be decreased (Kneidinger *et al.* 2011), whereas non-canonical WNT signaling increased (Kovacs *et al.* 2014) in aged lungs. Additionally, we found that the level of *FZD4* expression negatively correlated with age in human lung tissue and we observed that expression of the FZD4 receptor was decreased in old mice (>12 months) in comparison to young animals (<3months) (Skronska-Wasek *et al.* 2017). Further, we have observed that oxidative stress (induced with H_20_2 exposure) led to decreased FZD4 expression in primary mouse ATII cells. Interestingly, we also found increased WNT5A expression in senescent primary human lung fibroblasts (phLF) of smokers, as well as from individuals with COPD compared with respective nonsenescent fibroblasts (Baarsma *et al.* 2017). Further it has been shown that non-canonical WNT5A is able to negatively affect stem cell aging and indicates a potential contribution of age-associated shift to non-canonical WNT signaling to COPD pathogenesis (Florian *et al.* 2013). Therefore, the reduced repair potential observed in COPD/emphysema may be partially explained by a dysregulation of WNT signaling with aging and its contribution to senescent cell activity present in these lungs. Thus, reactivation of the pathway could prevent cellular senescence or clear senescent cells and in turn positively influence repair of the lung tissue.

Deranged elastogenesis in COPD/emphysema

Ultimately, irreversible structural changes in the alveoli and loss of surface area available for gas exchange is what causes a loss in tissue function and lowers the quality of life for patients. Elastic fibers are important components of the ECM, providing the elasticity of the alveoli (Mercer *et al.* 1990). Elastic fibers consist of an elastin core and a mantle of fibrillin-rich microfibrils (Kielty *et al.* 2002). Maturation of functional fibers requires cross-linking of elastin monomers, which depends on the activity of the enzyme lysyl oxidase (Lox and Loxl 1-4) (Rosenbloom *et al.* 1993). Alveolar fiber content has been shown to positively correlate with lung function parameters (Cardoso *et al.* 1993) and breakdown of elastic fibers in the lung is one of the main features of COPD (Cardoso *et al.* 1993). Elastin content has been shown to be decreased in the alveolar region as well as in small airway walls of COPD patients (Black *et al.* 2008). Interestingly, animals heterozygous for elastin are known to be more susceptible to develop cigarette smoke-induced emphysema than wild type animals

(Papaioannou *et al.* 2010). Moreover, cigarette smoke has been shown to decrease elastin re-synthesis in hamsters after elastase instillation (Osman *et al.* 1985) and has been shown to decrease protein elastin content *in vivo* (Seimetz *et al.* 2011).

In addition to changes in elastin synthesis levels, cigarette smoke has been linked to increased elastolytic activity of macrophages (White *et al.* 1979; Hautamaki *et al.* 1997). Further, inflammatory mediators such as MMP12 are known to both modulate elastin gene expression and lead to decreased accumulation of elastic fibers in the tissues (Taraseviciene-Stewart *et al.* 2008; Tuder *et al.* 2012). Moreover, Lox enzymes have been shown to be inhibited by smoke (Laurent *et al.* 1983), preventing the assembly of mature elastic fibers In support of the concept that an imbalance between elastin synthesis, deposition and protease activity exists in COPD. We have shown that expression of elastin and elastogenic components, including Lox and Loxl are decreased in ATII cells not only upon cigarette smoke exposure, but, interestingly, inhibition of FZD4 was sufficient for reducing elastin, elastogeneic components and Lox enzymes (Skronska-Wasek *et al.* 2017). Importantly, overexpression of FZD4 prevented cigarette smoke-induced downregulation of elastin expression in epithelial cells (Skronska-Wasek *et al.* 2017).

In order to explore potential mechanistic links of how FZD4 regulates elastogenesis, we explored secreted factors which participate in this process. Elastin has been shown to be induced by insulin-like growth factor (IGF-1) (Srisuma *et al.* 2010), an epithelilal cell-derived cytokine, and demonstrated to positively regulate WNT signaling (Desbois-Mouthon *et al.* 2001; Jin *et al.* 2008). Interestingly, IGF-1 has also been shown to be reduced in COPD (Kythreotis *et al.* 2009; Papaioannou *et al.* 2010; Ye *et al.* 2012). We found that both cigarette smoke exposure and inhibition of FZD4 led to decreased secretion of IGF-1 by alveolar epithelial cells and cigarette smoke treatment inhibited expression of *Igf-1* in *ex vivo* cultured PCLS (Skronska-Wasek *et al.* 2017). Importantly, treatment with valproic acid (VA), leading to induction of FZD4 expression (Smirnova *et al.* 2014), led to concurrent increases in IGF-1 secretion in PCLS (Skronska-Wasek *et al.* 2017). This indicates that treatment inducing FZD4 receptor expression may also have the potential to positively influence elastogenic cytokines such as IGF-1, which could in turn lead to improved elastogenesis and structural repair in diseased lung, in parallel to WNT/β-catenin-driven alveolar epithelial repair.

Collectively, we show that the decrease of WNT/β-catenin signaling observed in COPD/emphysema might be caused by deranged expression of a critical WNT/β-catenin signaling pathway components – the FZD4 receptor, or the overexpression of the WNT5A ligand. We also demonstrate that targeting of the receptor or the ligand can lead to the positive regulation of WNT signaling, inducing repair of lung tissue. Therefore, pharmacological treatment boosting expression of FZD4 receptor or stabilizing the receptor at the membrane and/or attenuation of WNT5A ligand availability either with specific blocking antibody or treatment reducing secretion of the ligand could be beneficial in the treatment of COPD/emphysema.

5. Limitations of the studies and future directions

We have shown deranged expression of WNT pathway components – FZD4 receptor and WNT5A ligand – in COPD and suggest that these deregulations lead to attenuated WNT signaling and impaired tissue repair observed in the disease. However, the presented studies have some limitations.

In the first study, we focused on impaired tissue repair observed in COPD and attributed it to the decrease in FZD4 expression, driven in part by cigarette smoke. However, cigarette smoke is not the only factor contributing to the downregulation of the FZD4, as shown by a further decrease of receptor expression between smokers and COPD patients. Whether these COPD patients had lower FZD4 before disease onset remains unknown. Identification of additional, presumably genetic factors, or factors upstream of FZD4 regulation would be of interest to better understand the background of the disease, enabling development of targeted drugs.

It is noteworthy that in addition to epithelial cells, other cell types like endothelial and mesenchymal cells are crucial for the maintenance and repair of the tissue and we focused in this study only on one cell type. Endothelium, especially in the terminal alveoli, is essential together with epithelial cells for proper gas exchange and mesenchymal cells and the extracellular matrix provide necessary niche for the proper function of epithelial cells. It would be necessary to further investigate how FZD4 modulation affects the function of other cell types, important for the lung repair.

In the second study, we used the SFTPC rtTA TetO WNT5A mice, where WNT5A is

overexpressed in SPC^+ ATII cells. However, as we showed in our study, fibroblasts are known to be the main source of WNT5A. As WNT5A is known to be secreted on extracellular vesicles, which are known to carry cell-specific cargo, it is unknown if the source of WNT5A plays a significant role in exerting its effects. Thus, it would be of interest to further study the contribution of fibroblast-derived WNT5A to COPD/emphysema development. Moreover, for our *in vivo* experiments we used two approaches: a) WNT5A blocking antibody and b) BOX5, known to bind to multiple FZD receptors. As the WNT ligands share high homology with one another, our blocking antibody approach may have additionally blocked other WNT ligands. Therefore, we cannot exclude that the observed effect is only WNT5A-dependent and additional experiments would be needed to further decipher specificity for WNT5A. In this regard, an *in vivo* experiment with blocking AB/BOX5 in mice overexpressing WNT5A would confirm our findings. Furthermore, in the cigarette smoke-induced emphysema, we only used a 10 day exposure for our study, which is an acute model. Longer exposure (e.g. 6 months) would allow us to study not only the inflammatory response but to furter analyze tissue remodeling.

The work presented here has undoubtly added important pieces to our understanding of the mechanisms underlying the development of COPD and it has opened new exciting avenues.
We have shown a contribution of cigarette smoke to decreased FZD4 expression. However, the exact mechanism leading to this attenuation remains to be elucidated and identification of upstream players modulating the receptor expression (e.g. kinases), that could be pharmacologically targeted seems to be of great interest. Moreover, how restoration of the FZD4 receptor (especially in ATII cells) would influence the repair of diseased lung remains a key question. Interestingly, some mice strains (depending on their genetic background) are more prone to develop emphysema than the others. Analysis of the FZD4 receptor abundance in these mouse strains and its correlation with the severity of the disease would be of interest.
One of the most exciting directions seems to be further investigation of pharmacologically activating FZD4 using valproic acid, an FDA-approved compound. While the exact mechanism of how FZD4 is induced by valproic acid treatment remains unknown, it would be of interest to further explore its potential in the repair of the lung tissue in *in vivo* murine

models of emphysema. Additionally, it would be interesting to explore if there is any correlation between valproic acid usage and lung function in a clinical cohort.

In our second study, we have identified 3 different forms of the WNT5A ligands in human COPD (45kDA, 49kDA and 230kDA). However, what they exactly represent, if they are mono- and multimers or different splice variants remains to be investigated. Further analysis of their physiological properties would be of great interest. Moreover, we have shown that blocking of WNT5A with the antibody or using BOX5 exert a beneficial effect in mouse models of emphysema, however, confirmation of this influence in human tissue, by utilizing human PCLS for example, would further underline the potential of targeting of WNT5A ligand for lung tissue repair.

Targeting of WNT signaling either via modulation of FZD4 receptor or WNT5A ligand seems to be very promising. Identification or synthesis of the compounds and drugs inducing expression of FZD4 receptor or blocking production, secretion or binding of WNT5A leading to the activation of repair mechanisms in the lung tissue is an important next step.

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Acknowledgements

I would like to thank the whole, fantastic MK lab (Aina, Ana, Andrea, Astrid, Babsi, Carlo, Carola, Cedric, Chiharu, Darcy, Florian, Franzi, Hani, Henrik, Hoeke, John-Poul, Julia, Kathrin, Lara, Mareike, Maria, Marlene, Martina, Melanie, Nadine, Rabea, Rita, Sarah, Simone, Stephan, Verena) for their support and many, many discussions. I would like to thank especially Melanie, Kathrin and Darcy for being incredible mentors, for all their patience and constant help. I am very grateful to all the members of the CPC for their kindness, openness and generosity, especially to Deniz, Korbinian and Christina (Meiners Lab), Jie and Önder (Yildirim Lab), Daniela (Eickelberg Lab), Chanze (Stöger Lab). I would also like to thank Diana and Katharina (Dietrich Lab) for their help. I am very grateful to all my peers from the Research School for all the seminars and journal clubs that we had to go through, that gave me opportunity to learn a lot. I would like to thank the Research School coordinators (Doreen and Camille) for organizing seminars and events and for all the opportunities that came along with the participation in the Research School. I am grateful to my Thesis Committee, Christina Scheel, Melanie Königshoff and Oliver Eickelberg, for stimulating discussions and great suggestions. Lastly, I would like to greatly thank my friends (especially Magda and Bartek) and my family (my parents, Kasia, Łukasz) for all the support and for making it possible.

Affidavit

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I hereby declare, that the submitted thesis entitled:

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is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others have been quoted or reproduced, the source is always given.

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