The WNT receptor Frizzled-8 in pulmonary remodelling and inflammation
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Chapter 1

General introduction
Chapter 1

Preface
The objective of this thesis is to establish the functional role of the Frizzled-8 receptor and its cognate WNT ligands in lung diseases, with focus on its role in chronic obstructive pulmonary disease (COPD). Lung diseases as COPD, asthma and pulmonary fibrosis are characterised by aberrant inflammatory and repair processes that show overlap in expression of growth factors and inflammatory cytokines. The pathophysiology of these diseases is not yet fully understood and current treatment options are not curative. Recent studies have indicated an important role for WNT signalling in the development of these lung diseases. We previously found the expression of the WNT receptor Frizzled-8 (FZD8) to be increased in human lung fibroblasts of COPD patients. This thesis will investigate the functional role of the FZD8 receptor, and its cognate WNT ligands.

The WNT signalling pathway
The wingless/intergrase-1 (WNT) signalling pathway is essential to cell polarity and cell fate determination during embryonic development [1]. In adult tissue, WNT signalling contributes to tissue homeostasis via the maintenance of stem cell pluripotency, inflammation, repair processes and self-renewal [2, 3]. The WNT signalling pathway is made up of WNT ligands that can bind cell surface Frizzled (FZD) receptors to activate downstream signalling cascades [4]. Signalling that occurs via β-catenin is called canonical signalling, while all other routes are indicated as noncanonical signalling.

WNT ligands
WNT ligands are cysteine-rich, glycosylated, lipid-modified secreted proteins that function as growth factors throughout the body. There are currently 19 known mammalian WNT ligands that have been defined by amino-acid structure instead of functional properties [5, 6]. All WNT ligands share a signal sequence for secretion, several highly charged amino acid residues and potential glycosylation sites [7], while the number of glycosylation sites differs per WNT molecule. The cysteine residues in WNT ligands are important for the binding properties of the molecule. Of the 23 cysteine residues found in WNT-1, 22 are conserved in the other WNT ligands [7, 8]. This suggests a strong overlap in binding site interactions. WNT ligands can bind to the N-terminal cysteine-rich domain (CRD) of the FZD receptors in a highly specific manner [9]. The crystal structure of Xenopus WNT8 with mouse FZD8 has been identified (figure 1) and shows four main regions of concentrated amino acid conservation, one of them being opposite of the WNT-binding patch for the FZD receptor. This has been proposed to be a binding site for co-receptors [9].

Secreted WNT ligands are strongly hydrophobic because of palmitoylation, which is why they preferably bind to the extracellular matrix (ECM) or associate with the cell membrane [10]. Their hydrophobic nature also contributes to their short distance signalling range, however mechanisms have been described for long distance WNT signalling as well. It has been suggested that both short and long distance transportation of WNT ligands is possible via transcytosis/retromer function in endosomal trafficking vesicles [11, 12]. Another suggested manner of extracellular transport of WNT ligands over short distances involves argosomes in the ECM. Argosomes are lipoprotein particles that can bind the palmitoylated WNT ligands [11]. Additionally, heparan sulfate glycosaminoglycans (GAG)s facilitate the extracellular movement by stabilizing WNT ligands [10, 13].
Posttranslational modifications, including palmitoylation and glycosylation appear to be necessary for WNT transport and function, although their precise roles are not yet fully elucidated. Palmitoylation by the acyl-transferase porcupine facilitates N-glycosylation [14] and is necessary for WNT secretion [12, 15, 16] as well as binding to the FZD receptor [9, 17, 18]. The mechanism of WNT secretion into the extracellular space is not fully understood, but is facilitated by the protein complex Wntless/Evenness interrupted (Wls/Evi) [16, 19-21]. Glycosylation by an oligosaccharyl transferase complex (OST) [14] increases WNT interactions with heparin sulphate proteoglycans on the cell surface of WNT responding cells [11]. Specific interactions with GAG-modified proteins facilitate the extracellular movements of WNT ligands [10], but it has also been proposed that palmitoylation targets WNT ligands to specific extracellular domains [11], thereby influencing movement of WNT ligands.

At the time that WNT ligands were discovered, it was not clear how these growth factors activated downstream signalling. While the class of FZD receptors was already known, it took several years before it was clear that they were the cognate receptors in the WNT signalling pathway [4]. Now, a variety of WNT receptors is known. Apart from FZD receptors, WNT ligands can bind the transmembrane tyrosine kinase-related receptor RYK that has a WNT binding domain [22] and ROR2 that has a CRD motif similar to FZD receptors [23]. RYK and ROR2 can function as co-receptors, but they are not indispensable for activation of WNT signalling as FZD receptor dimerization upon binding can be sufficient to activate the downstream signalling cascade [24]. In addition to RYK and ROR2, also protein tyrosine kinase 7 (PTK7) and lipoprotein receptor-related protein (LRP)5/6 are known to function as co-receptors of the WNT signalling pathway. While co-receptors are not essential for WNT signalling, it is believed that they are important in determining downstream signalling. As receptor or as co-receptor RYK can function...
in both the canonical and noncanonical WNT signalling cascade [22], while activation of ROR2 [25] and PTK7 [26] (alone and together [27]) have been shown to inhibit canonical signalling, and activate noncanonical signalling pathways. LRPS/6 is as co-receptor essential for canonical WNT signalling [28].

**Frizzled receptors**
In mammalian tissue, 10 FZD subtypes are known. FZD receptors are characterized by a large extracellular domain that is made up of an assumed signal sequence at the N-terminus followed by a sequence of 120 amino acid (primarily α-helical proteins [9]) that contain 10 highly conserved cysteine residues called the CRD. The CRD appears to be the ligand binding site [8, 9] and is followed by a differing region of 40 – 100 amino acids that form a flexible, seven transmembrane segments and a cytoplasmic C-terminal tail [29, 30]. The transmembrane segments are separated by short extracellular and cytoplasmic loops. FZD receptors share several features with G protein coupled receptor (GPCR)s [31, 32] and there are multiple indications that both canonical and noncanonical WNT signalling can involve G proteins [32]. However, the specific molecular mechanisms for FZD activation are still unknown.

WNT/FZD signalling can be downregulated by zinc and ring finger (ZNRF)3 and ring finger protein (RNF)43, which have E3 ubiquitin ligase activity that leads to turnover of activated FZD receptors [33, 34]. When a WNT ligand is bound, ZNRF3 expression is increased and as a negative feedback loop, a mechanism that acts to inhibit WNT signalling by internalizing the activated WNT/FZD/LRP complex. In this process of WNT receptor turn-over, the phosphoprotein Dishevelled (DVL) functions as an adapter protein by binding ZNRF3 and RNF43 to FZD [35]. Next to WNT ligands, several FZD receptors including FZD8 can bind R-spondins [36-39]. R-spondins enhance WNT signalling because of their binding properties to LGR receptors, which act to neutralize the activity of ZNRF3 and RNF3 [40]. In contrast, specifically for the FZD8 receptor, connective tissue growth factor [41] and insulin-like growth factor-binding protein-4 [42] can antagonize FZD8 by direct binding.

As stated before, lipid modification by palmitoylation of WNT ligands is necessary for FZD binding [9, 17, 18]. Palmitoylated WNT ligands can interact with the CRD of FZD receptors [9]. Because of the homology in FZD structure, it is speculated that all FZD CRDs will bind WNT ligands [43]. It is not clear how the WNT ligands or the FZD receptors determine downstream signalling specificity. There are indications that FZDs are the main players herein as substitution of amino acids in FZD-CRD impacts on preferences of binding of WNT subtypes. For instance, Met14 at the centre of the part where the WNT C-terminal CRD binds to the FZD CRD is conserved in FZD5 and FZD10, but substituted by Val, Glu or Asp in FZD1, FZD2, FZD3, FZD6 and FZD7 [9]. However, because of the highly conserved binding sites in the FZD CRD, it is unlikely that there is a large difference in affinity of individual WNT ligands for individual FZD receptors [9]. Indeed, any WNT ligand can activate several downstream signalling cascades, depending on the receptor that is expressed [25]. More recently, it was proposed that most WNT ligands can bind to most FZD receptors and that the downstream signalling that will be activated by a specific WNT/FZD combination is dependent on the combination of WNT ligands and FZD receptors present in a cell system [44]. In any case, binding of a WNT ligand to a FZD receptor, induces FZD interaction with DVL at the cytoplasmic site [18, 45-47], followed
by downstream signalling cascades. As mentioned earlier, DVL also facilitates the turn-over of activated FZD receptors [35] and in this way functions both as an activator and an inhibitor of WNT signalling.

Canonical WNT signalling
β-Catenin has a pivotal role in the canonical WNT signalling pathway. In the absence of an activated FZD receptor, β-catenin is phosphorylated and degraded by a destruction complex that is made up of Axin, glycogen synthase kinase (GSK)3, casein kinase (CK)1 and adenomatosis polyposis coli (APC). If WNT and FZD form a ternary complex with co-receptor LRP5/6, intracellular DVL is activated and recruits Axin and GSK3 to the ternary complex. This leads to the disassembly of the β-catenin destruction complex and a subsequent accumulation of β-catenin. β-Catenin will then translocate to the nucleus, where it activates among others the T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors, resulting in the transcription of WNT responsive genes [48], such as Axin2 [49] and cyclin D1 [50].

Noncanonical WNT signalling
WNT ligands can also induce multiple β-catenin independent signalling pathways [32] which are called noncanonical signalling pathways. New noncanonical signalling pathways are still discovered, for instance activation of p38 mitogen activated protein kinase by WNT ligands has recently been shown [51-53]. Established noncanonical WNT signalling routes include WNT/RAC, WNT/RHO, WNT/RAP, WNT/ROR, WNT/planar cell polarity (PCP), WNT/ Ca2+ and WNT/cGMP signalling [54, 55]. Three noncanonical signalling pathways that are known to involve FZD activation, namely the WNT/PCP pathway that signals via RhoA/c-Jun N-terminal kinase (JNK), the WNT/Ca2+ pathway that signals via calcium and the WNT/cGMP pathway that signals via cyclic guanosine 3′5′-monophosphate (cGMP).

WNT/PCP pathway
Activation of the WNT/PCP pathway can occur via a ternary WNT/FZD complex with co-receptor RYK [56], ROR2 [57], PTK7 [26] or ROR2 and PTK7 combined [27]. WNT ligand binding results in the recruitment of intracellular DVL and the subsequent activation of small Rho GTPases to regulate actin cytoskeleton remodelling and the activation of JNK, which can have three effects. Rho/JNK signalling can regulate actin cytoskeleton remodelling, increase the intracellular calcium concentration and activate transcription factors thereby regulating gene expression [58].

WNT/Ca2+/cGMP pathway
Binding of a WNT ligand to its FZD receptor supposedly activates a heterotrimeric G protein [32, 59], thereby regulating intracellular calcium [31] and cGMP levels [60]. In the WNT/Ca2+ pathway, the activated G protein induces the activation of phospholipase C (PLC), which stimulates the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to diacylglycerol (DAG) and inositol triphosphate (IP3) via hydrolysis. IP3 can initiate a rise in intracellular calcium via its receptors on the endoplasmic reticulum. DAG will activate protein kinase C (PKC). Activation of PKC leads to actin cytoskeleton remodelling. Calcium can also activate calmodulin to induce contraction in smooth muscle cells and calmodulin can activate calcineurin. Calcineurin dephosphorylates nuclear factor of activated T cells
(NFAT) transcription factors, which then translocate to the nucleus and activate gene transcription [61]. In addition, next to intracellular calcium levels, FZD2 has been shown to regulate intracellular cGMP levels via G protein activation [62, 63]. Phosphodiesterase (PDE)6 is the proposed effector for this signalling route [63, 64].

**WNT signalling in chronic lung diseases**

WNT-1 (at that time int1) was discovered as a proto oncogene in a mouse model for breast cancer [65]. Since then, aberrant WNT signalling has been implicated in multiple types of cancer, including lung cancer [66]. WNT signalling is also found to play an important role in (embryonic) lung development [67-69] as well as inflammatory processes [70] and tissue repair and remodelling in the adult lung [71-74]. These pathophysiologic processes have their implications for chronic lung diseases such as COPD, asthma and pulmonary fibrosis.

**COPD**

COPD is a complex, progressive lung disease with a high prevalence especially in the elderly that is the fourth cause of death worldwide with a predicted increased burden in the future. In the Western world, the main risk factor of COPD is cigarette smoking. Other important risk factors include outdoor air pollution, indoor air pollution for instance caused by biomass cooking, heating in inadequately ventilated rooms or occupational dust and chemicals [75]. The vast majority (80-90%) of COPD patients are (ex-)smokers, although only 10-15% of the smoking population develops COPD. Over the last decades, it has become apparent that genetic susceptibility is also underlying COPD development [76]. In addition, impairment in lung growth and lung development (by any cause) is also associated with a higher risk for COPD development later in life [77, 78].

COPD is defined by four grades (1-4) of severity in the global initiative for chronic obstructive lung disease (GOLD) guidelines. These grades reflect the degree of airflow obstruction that is expressed as the forced expiratory volume in 1 second (FEV₁) after full inspiration, divided by the total volume of exhaled air upon forced expiration (FVC). COPD is diagnosed by a ratio of FEV₁/FVC < 0.7. A lower ratio reflects more severe airflow obstruction. The development of airflow obstruction in COPD is progressive and not fully reversible. This obstruction is caused by a combination and interplay of increased airway resistance and decreased elastic recoil. Increased airway resistance is caused by bronchoconstriction, chronic inflammation, mucus hypersecretion and remodelling of the small airways i.e. increased airway smooth muscle mass, myofibroblast differentiation and abundant ECM production. Decreased elastic recoil is caused by parenchymal destruction leading to a loss of alveolar wall attachments called emphysema, contributing to changes in airway resistance [75].

COPD is characterised by a predominant neutrophilic inflammation [79]. Inhaled noxious particles or gases first encounter epithelial cells and macrophages, causing oxidative stress, damage and activation of pro-inflammatory pathways [80]. Activated epithelial cells and macrophages produce pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α and the chemokine ligands CXCL1, CXCL8, CXCL9, CXCL10 and CXCL11 [81, 82]. IL-1β, TNF-α and IL-6 amplify inflammatory processes and are increased in COPD [81]. CXCL1 and CXCL8 are also increased in COPD and attract neutrophils and monocytes via their receptor chemokine receptor CXCR2. Monocytes
differentiate to macrophages in the lung [79], to help in the first line of defence against foreign invaders. CXCL9, CXCL10 and CXCL11 attract T helper (Th1) lymphocytes and type 1 cytotoxic T cells (Tc1) via CXCR3. However, T lymphocytes in COPD are predominantly CD8+ cytotoxic cells. Also TH2-type cytokines, such as IL-4, are elevated in COPD [82], especially during exacerbations [83]. Epithelial damage can also lead to the production of growth factors such as transforming growth factor (TGF)-β and epidermal growth factor (EGF) [82] and both growth factors are increased in COPD [82]. EGF regulates mucus secretion from goblet cells in the epithelium via its receptor EGFR [84, 85] and TGF-β is a key factor in fibroblast activation.

The fibroblast plays a key role in repair and remodelling processes in COPD. TGF-β activates fibroblasts to produce ECM proteins and promotes their differentiation into myofibroblast [86-88]. Myofibroblasts have a more contractile profile than fibroblasts, which is characterized by an increased expression of contractile markers such as α-smooth muscle(sm)-actin [87, 89]. In turn, the composition of the ECM regulates the structural and functional properties of the lung and the cells embedded herein [89-91]. ECM protein can be broken down by proteases such as matrix metallopeptidase/protease (MMP)-9. An imbalance in the breakdown and production of ECM proteins can lead to the development of emphysema [92]. In contrast, fibroblasts in the small airways are activated by growth factors to produce ECM proteins and contribute to the repair process. A shift in the balance to this side contributes to the development of fibrotic processes leading to airway wall thickening [93].

COPD is an umbrella-term for small airways disease, chronic bronchitis and parenchymal destruction. Within the COPD patient population, there are different phenotypes. For instance, not all COPD patients have additional chronic bronchitis. Chronic bronchitis is defined by chronic cough and idiopathic sputum production for at least three months per year for two consecutive years [75]. Enhanced EGFR signalling upon epithelial damage can cause mucus cell metaplasia [94]. Mucus cell metaplasia is characterized by more mucus in the airways both by an increased production of mucus and by a decreased mucociliary transport from the airways [95], and manifests itself as chronic mucus hypersecretion (CMH). CMH is a risk factor for COPD [96] and is associated with bronchial inflammation and accelerated lung function decline [97].

COPD is considered as a treatable disease [75], however current treatment options are not curative. The most effective treatment option is smoking cessation, as this results in an almost normal pattern of lung function decline, albeit with a lower starting point [98].
Figure 2: The natural history of chronic airflow obstruction, adapted from Fletcher and Peto [98]. FEV₁ declines gradually and continuously with increasing age. Smoking causes a steeper decline in lung function in susceptible smokers, but not in non-susceptible smokers. Smoking cessation at any age almost fully restores the natural pattern of lung function decline albeit at a lower starting point [98].

Anti-inflammatory treatment in COPD consists of inhaled corticosteroids and/or oral PDE4 inhibitors [75]. Inhaled corticosteroids dampen the inflammation in some of the COPD patients, in particular when there is an additional eosinophilic inflammation, but there is no hard evidence that they can improve lung function decline. This minimal effect is mainly due to corticosteroid resistance [99]. PDE4 inhibitors are added to corticosteroid or bronchodilator treatment in severe COPD with chronic bronchitis and a high risk of exacerbations [75]. Bronchodilators (adrenergic β-receptor agonists) can improve lung function [100, 101], and there are indications that they also affect the underlying inflammation [102, 103] and remodelling [103], but there is no evidence yet that they can stop or reverse the long-term decline in lung function, indicating that new pharmacological approaches are urgently awaited.

Asthma
Asthma is a heterogeneous obstructive airway disease that affects people of all ages and has an increasing incidence worldwide. The mechanisms responsible for asthma are multifactorial, involving both genetic and environmental factors. Allergy is amongst the strongest predispositions, but it cannot explain the prevalence of asthma alone and non-atopic factors exist as well [104, 105]. In addition to exposure to indoor and outdoor allergens (allergic asthma), for instance also exercise or cold air, irritants and viral infections (non-allergic asthma) can trigger an asthmatic reaction, leading to airflow obstruction. Airflow obstruction in asthma is at least in part reversible and assessed with a bronchodilator reversibility test. Asthma is further characterised by bronchial hyperresponsiveness that is often associated with airway inflammation and remodelling and is an important determinant of the severity of the disease [105-107]. Asthma
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severity is defined according to the composite asthma severity index (CASI) that is based on combined information on impairment, risk and the amount of medication needed to maintain control [108].

Mast cells play an important role in inflammation in asthma and can be activated directly by allergenic factors via immunoglobulin E or indirectly through non-allergenic factors and epithelial derived cytokines. Upon activation, mast cells release their contractile mediators, such as histamine and leukotrienes that lead to bronchoconstriction and attract inflammatory cells. In contrast to COPD, airway inflammation in asthma is mainly eosinophilic in nature and characterized by a Th2-type immune response, involving cytokines such as IL-4, IL-5, IL-9 and IL-13 [81, 109-112]. IL-4 and IL-13 stimulate B-cells to synthesize immunoglobulin E (IgE), IL-5 promotes eosinophil inflammation, IL-9 stimulates mast cell proliferation and IL-13 is a main regulator of mucus production in the airways. Similar to COPD, IL-1β, TNF-α and IL-6 also play a pro-inflammatory role in asthma [113, 114].

Airway remodelling in asthma is induced by TGF-β, EGF and vascular endothelial growth factor (VEGF) and includes structural changes such as airway smooth muscle hypertrophy and hyperplasia, goblet cell metaplasia, bronchial vascular remodelling as well as basement membrane thickening and subepithelial fibrosis [115-118]. Structural changes in the airways contribute to the persistent component of airway hyperresponsiveness [119], lung function decline and persistent airflow obstruction [120]. Parenchymal destruction is a process that by definition does not occur in asthma, but can be seen in asthma patients with additional COPD [121, 122].

Airflow obstruction is in most asthmatic patients spontaneously reversible or reversible by treatment with bronchodilators or corticosteroids. Nevertheless, some patients remain symptomatic despite treatment and treatment does not cure the underlying inflammation and remodelling [123, 124]. For instance, neutrophilic inflammation in severe asthma patients does not respond to the standard corticosteroid treatment [125].

Pulmonary fibrosis
Pulmonary fibrosis is characterised by the abundant formation of scar tissue in the parenchymal lung tissue and is presumed to be caused by repetitive micro injuries to the lung as a result of cigarette smoke, environmental exposure to larger dust particles such as metal dust, silica or coal dust, viral infections or a connective tissue disorder [126]. Pulmonary fibrosis is determined by recognition of a distinct pattern of interstitial fibrosis/scar tissue through high resolution computed tomography (HRCT) and is not defined by different stages of disease. Ultimately, chronic oxygen deficiency in the blood due to impaired diffusion capacity leads to organ failure and eventually death [126]. Pulmonary fibrosis is often a secondary effect of other diseases, but in the case of idiopathic pulmonary fibrosis (IPF), the exact cause is unknown. IPF is a chronic progressive fibrotic interstitial pneumonia that upon diagnosis has an estimated median survival rate of two to five years [127].

While it was previously thought that the fibrosis is a consequence of chronic inflammation, recent studies indicate that repeated epithelial damage in genetically predisposed subjects may be of more importance [126, 127]. Repeated injury to alveolar epithelial cells combined with defective repair mechanisms lead to so called fibroblast
foci that consist of myofibroblasts and scar tissue. Fibroblasts at the damaged site differentiate under influence of TGF-β into myofibroblasts. In addition, fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and VEGF contribute to the (vascular) remodelling seen in pulmonary fibrosis [126]. While remodelling in asthma and COPD occurs mainly in the (small) airways, in pulmonary fibrosis, the parenchymal lung tissue is mostly affected [128, 129].

Recently, new anti-fibrotic therapies are being used in IPF patients, or have entered Phase I-III studies, according to the ATS/ERS/JRS/ALAT statement on IPF [126]. Still, according to this official document, the current medicinal treatment options for IPF are without definitive, confirmed advantages [126]. In general, there is a need for new and better treatment options for the above mentioned lung diseases and for this, we need to get a better understanding of the underlying mechanisms that contribute to the development of these diseases.

WNT signalling in COPD, asthma and pulmonary fibrosis
While COPD, asthma and pulmonary fibrosis are distinctly different diseases, there are some important aspects that overlap, most evidently the changes in fibroblast function. Next to their structural role, fibroblasts exert cellular responses associated with airway remodelling and inflammation, including proliferation, ECM protein production and the secretion of inflammatory cytokines. One of the main players in airway remodelling is TGF-β [86]. TGF-β can be secreted from the epithelium upon damage and subsequently activate fibroblasts, which leads to dysregulated ECM production and differentiation of fibroblasts into the more contractile myofibroblast [87]. Damaged epithelium can also secrete IL-1α, IL-1β, TNF-α and EGF [81] which can stimulate fibroblasts to secrete inflammatory cytokines [130]. In this way, fibroblasts also play a role in airway inflammation by regulating inflammatory cytokine secretion.

There are multiple indications that the WNT signalling pathway is involved in airway remodelling and inflammation in various lung diseases. WNT/β-catenin signalling was increased in ovalbumin-challenged mice and allergen-induced airway inflammation and remodelling in these animals were attenuated by treatment with β-catenin small interfering (si)RNA [131]. In a mouse model of allergic airway disease, pharmacological inhibition of GSK-3β reduced the expression of MUC5AC [132]. As GSK-3 normally inhibits canonical WNT signalling, these data suggest that canonical WNT signalling negatively regulates allergen-induced mucus secretion in the airways. However, it should be realized that GSK-3 has additional functions in the cell [133]. Nuclear β-catenin was found to be upregulated in IPF patients [134], but whether this is a cause or a consequence of the disease is not clear [135]. In addition, several studies found activation of WNT/β-catenin signalling in IPF [71, 136], or showed that inhibition of WNT/β-catenin signalling attenuates (experimental) IPF [137-139]. In COPD, decreased canonical WNT signalling was found in emphysematous lesions [140, 141].

Furthermore, TGF-β has recently been shown to interact with the WNT signalling pathway [74, 142-144]. Recent studies from our group showed that β-catenin is required for TGF-β-induced extracellular matrix production by airway smooth muscle cells [73] and that TGF-β-induced WNT-5B and FZD8 mRNA expression is increased in pulmonary fibroblasts of COPD patients [74]. It was also reported that high FZD8 gene expression in lung tissue of IPF patients correlates with more rapid disease progression [145].
Furthermore, nicotine induced epithelial-mesenchymal transition via TGF-β1 and WNT-3A/β-catenin signalling in human bronchial epithelial cells [146]. In addition, inhibiting DVL suppressed TGF-β-induced signalling in murine fibroblasts and reduced bleomycin-induced pulmonary fibrosis in a mouse model [147].

TGF-β is able to induce WNT-1-inducible signalling pathway protein (WISP)-1 in human bronchial smooth muscle cells and both TGF-β and WISP-1 induced bovine smooth muscle proliferation and hypertrophy [148]. Ovalbumin challenge induced WISP-1 and a WISP-1 antibody partly suppressed ovalbumin-induced airway smooth muscle hypertrophy in a rat animal model [148]. Additionally, a single nucleotide polymorphism (SNP) in the WISP-1 gene is associated with lower FEV$_1$ in COPD [149], further strengthening a possible important role for WISP-1 in obstructive lung diseases. WISP-1 as well as WNT-7B transcript levels were specifically upregulated in mice with bleomycin-induced acute lung injury and mice with bleomycin-induced pulmonary fibrosis [150].

Next to WISP-1, stimulation with TGF-β increased WNT-5A in normal human lung fibroblasts and in human airway smooth muscle cells [151]. WNT-5A mRNA and protein expression was also found to be increased in fibroblasts from lung tissue of patients with interstitial pneumonia compared with normal lung fibroblasts [152]. In addition, our group previously showed WNT-5A to be increased in asthmatic airway smooth muscle cells [144] and in whole lung tissue of COPD patients [53]. In line with this, we showed that TGF-β induced WNT-5A in human airway smooth muscle cells [144] and that this occurs via TGF-β-activated kinase (TAK)-1 [153]. WNT-5A in turn regulated TGF-β-induced ECM production in human airway smooth muscle cells [144] and human lung fibroblasts [152], and increased fibroblast proliferation [152]. Furthermore, WNT-5A increased resistance to H2O2-induced apoptosis and WNT-5A knockdown by specific siRNA induced the apoptosis marker caspase-3 [152], indicating that WNT-5A is a proliferative ligand that regulates remodelling. Additionally, WNT-5A [154, 155] and FZD5 [154] have recently been shown to function as pro-inflammatory in the lung, whereas WNT/β-catenin signalling inhibits neutrophil attraction by alveolar epithelial cells [156].

There are several other WNT ligands that have been shown to play a role in the pathophysiology of lung diseases. WNT-1 and WNT-2 have been shown to regulate proliferation of lung fibroblasts induced by conditioned medium of mesenchymal stem cells [157]. WNT-4 expression was shown to be increased in epithelial cells of COPD patients, but downregulated after stimulation with cigarette smoke extract [52, 158]. WNT-7A mRNA expression is increased in sputum of asthma patients compared to sputum of healthy controls [131]. WNT-10B has also been shown to act anti-inflammatory by modifying the T cell response to allergen-challenge in wild-type (WT) and WNT-10B-deficient mice [159].

Next to WNT ligands, WNT antagonists play a role in lung diseases. Tobacco smoke causes an upregulation of secreted Frizzled-related protein (SFRP)-2, an inhibitor of WNT signalling, in epithelial cells [160], suggesting inhibition of WNT signalling by smoking may lead to the development of emphysema. Interestingly, SFRP-1 was found to be upregulated in the lungs of emphysema patients, while its expression is also necessary for normal alveolar development [161], indicating dual roles for WNT signalling in developmental and disease mechanisms. In another study, SFRP-1 was shown to counteract TGF-β-induced signalling in pulmonary fibroblasts and alveolar epithelial cells, however it had no effect on bleomycin-induced fibrosis in a mouse model [162], indicating that the effects of SFRP-1 are location dependent.
Chapter 1

Scope of this thesis
As described above, WNT ligands and FZD receptors appear to play a role in inflammatory and remodelling processes that contribute to the development of various lung diseases. Changes in fibroblast function contribute to chronic lung diseases such as COPD, asthma and pulmonary fibrosis. We previously found WNT-5A, WNT-5B and FZD8 expression to be increased in lung fibroblasts of patients with COPD. Therefore, we hypothesized that WNT-5A/WNT-5B and FZD8 signalling functionally contribute to the development of inflammation and remodelling in the lungs and the overall aim of this thesis is to establish the functional roles of WNT-5A/WNT-5B and FZD8 in various lung diseases, with focus on its role in COPD.

In chapter 2, we investigated the role of FZD8 signalling in vitro in TGF-β-induced fibroblasts activation and explored the ligands of FZD8 in these effects. Furthermore, we studied a possible role for FZD8 in bleomycin-induced TGF-β signalling in vivo, using FZD8 deficient mice.

TGF-β induces ECM proteins like fibronectin and collagen Iα1, however it reduces decorin expression and even more so in COPD patients [163]. TGF-β and decorin are known to operate in a negative feedback loop were TGF-β reduces decorin [163], but decorin is able to bind TGF-β thereby reducing TGF-β-induced signalling [164]. Decorin has recently been shown to be able to bind WISP-1 [165]. Based on their structures, we hypothesized that decorin is also able to bind WNT-5B. Therefore we investigated in chapter 3 whether decorin was able to reduce functional WNT-5B signalling, by studying the effect of recombinant decorin on WNT-5B-induced fibroblast activation.

In chapter 4, we studied whether there is a specific role for FZD8 in the chronic bronchitis phenotype. Acute cigarette smoke-induced airway inflammation was studied in vivo in wild-type and FZD8 deficient mice. We performed genetic association studies and lung expression quantitative trait loci (eQTL) analyses for FZD8 to evaluate polymorphisms in FZD8 and their relationship to tissue expression in chronic bronchitis. Next, we investigated whether FZD8 was differentially expressed in fibroblasts of GOLD grade 4 COPD patients with CMH and fibroblasts of patients without CMH. To investigate the functional effects of this potential difference, we studied inflammatory cytokine secretion from these fibroblasts and their effect on mucus secretion in vitro using air-liquid-interface (ALI) cultured primary airway epithelial cells.

Airways are in direct contact with the outside world and epithelial cells function as the first defence barrier. Upon epithelial damage, signalling from the epithelium to the underlying fibroblasts is altered. In COPD, asthma and pulmonary fibrosis, TGF-β is an important player in epithelial-fibroblast communication and this is an important aspect in disease development. TGF-β has recently been shown to interact with the WNT signalling pathway [74, 142-144], but if WNT signalling is involved, and if so in what way is not known. WNT ligands and FZD receptors are differentially expressed in epithelial and mesenchymal cells and since WNT ligands are known to function in an autocrine/paracrine manner over short distances, it is likely that they play a role in the communication between epithelial and mesenchymal cells. There are some indications that WNT signalling is indeed involved in epithelial-mesenchymal communication or at least on mesenchymal function. For instance, epithelial WNT-7B was found to be necessary for smooth muscle development through regulation of mesenchymal differentiation [166]. To elaborate further on the apparently important role of WNT signalling in the communication between epithelial
cells and fibroblasts, we investigated in chapter 5 which WNT ligands are involved herein. To study this, we used co-cultures of either primary airway fibroblasts of COPD grade 4 patients or MRC-5 human lung fibroblasts with ALI differentiated primary airway epithelial cells of healthy donors.

Others have shown that WNT-5A is upregulated in asthma patients and functions as a ligand for FZD8 [144]. In chapter 6, we investigated whether FZD8 has a functional role in allergen-induced inflammation and remodelling in vivo. Wild-type and FZD8 deficient mice were sensitized to ovalbumin and subsequently challenged with ovalbumin or saline (control group).

In chapter 7, all the results presented in this thesis are discussed within the broader perspective of the development of inflammatory and remodelling mechanisms in the different lung diseases, emphasizing possible future therapeutic strategies.
Chapter 1

References


Chapter 1


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General introduction


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