The WNT receptor Frizzled-8 in pulmonary remodelling and inflammation
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Chapter 7

General discussion and main conclusions
Preface
The objective of this thesis was to establish the functional role of the WNT receptor FZD8 and its ligands in various chronic lung diseases, including COPD, asthma and IPF, with particular focus on COPD. The research in this thesis shows that FZD8 contributes to processes underlying tissue remodelling and inflammation in these diseases. We show that FZD8 is involved in TGF-β-induced fibroblast activation in vitro and in bleomycin-induced fibrosis in vivo. In addition, we report that WNT-5B is a FZD8 ligand in fibroblast activation and that the effects of WNT-5B on fibroblast activation can be inhibited by the proteoglycan decorin. Furthermore, we show that FZD8 is associated with chronic bronchitis, and is involved in cytokine secretion in vitro in human lung fibroblasts and in acute cigarette-smoke-induced airway inflammation in a mouse model. We demonstrate that FZD8 expression in fibroblasts regulates mucin mRNA expression in differentiated airway epithelial cells via secretion of IL-6 and CXCL8, suggesting an important role for fibroblasts in mucus secretion in the lung via this receptor. We did not find a major role for FZD8 in allergen-induced airway inflammation, indicating a context-specific role for FZD8. Furthermore, we studied WNT signalling in epithelial-fibroblast cross-talk in a co-culture of differentiated airway epithelial cells and lung fibroblasts. Here, we found a regulatory role for WNT-4 in IL-6 secretion that is induced upon co-culturing epithelial cells and fibroblasts. Collectively, our data suggest that the FZD8 receptor significantly contributes to remodelling and inflammatory processes that underlie chronic lung diseases. In addition, we find that WNT/FZD signalling is important in the bidirectional communication between epithelial cells and fibroblasts.

**FZD8 in pulmonary remodelling**
TGF-β is an important regulator of remodelling in various lung diseases as it drives myofibroblast differentiation and ECM protein production [1, 2]. We previously found that TGF-β-induced FZD8 expression is higher in pulmonary fibroblasts of GOLD grade 2 and 4 COPD patients compared to non-COPD controls [3]. These high expression levels of FZD8 suggest a role in COPD where remodelling via abnormal fibroblast activity contributes to the disease development. In line with our previous findings, we show in chapter 2 that TGF-β induces FZD8 expression in normal primary lung fibroblasts and that TGF-β-induced fibroblast activation is in part regulated by the FZD8 receptor. Furthermore, bleomycin-induced collagen deposition and fibronectin expression, a process that is regulated by TGF, was reduced in whole lung homogenates of FZD8−/− mice compared to WT mice. Our data might indicate that inhibiting FZD8 activation reduces TGF-β-induced remodelling not only in bleomycin-induced lung fibrosis, but also in other fibrotic lung diseases.

We found that WNT-5A and WNT-5B may contribute to FZD8 signalling in remodelling processes in the lung. Our group previously showed that TGF-β induces WNT-5A expression in airway smooth muscle cells and that WNT-5A regulates TGF-β-induced ECM protein production via FZD8 in these cells [4]. In addition, TGF-β-induced WNT-5B expression is increased in primary lung fibroblasts of COPD grade 2 and grade 4 patients compared to those of non-COPD controls [3]. In chapter 2, we show that TGF-β also induces WNT-5A in human lung fibroblasts. Interestingly, WNT-5B but not WNT-5A mimics TGF-β-induced fibroblast activation and TGF-β induced WNT-5B that signals via FZD8. These findings suggest that in lung fibroblasts WNT-5B is the primary ligand for FZD8.
In human airway smooth muscle cells, TGF-β induced primarily WNT-5A and FZD8 and both WNT-5A and FZD8 regulated TGF-β-induced ECM protein expression [4], suggesting that WNT-5A functions as a ligand of the FZD8 receptor in TGF-β-induced airway smooth muscle remodelling. In combination with our data, these data in airway smooth muscle cells indicate that multiple ligands can bind to the same FZD receptor and that WNT/FZD couples activated downstream signalling can be cell-type specific. The effects of WNT-5A on ECM production seem to be specific for airway smooth muscle cells [4, chapter 2], while WNT-5B has its effect specifically on fibroblasts [3, chapter 2].

TGF-β-induced effects can be reduced by binding to the core protein of the ECM glycoprotein decorin [5]. We show in chapter 3 that decorin is also able to reduce WNT-5B-induced fibroblast activation and that decorin is able to bind WNT-5B. This could mean that reduced WNT-5B signalling can be achieved by antagonizing WNT-5B via direct binding and in that way reduces WNT-5B-induced signalling. The finding that decorin can inhibit WNT-5B-induced fibroblast activation is important for unravelling the physiological mechanisms that regulate WNT-5/FZD8 signalling.

**FZD8 in pulmonary inflammation**

In this thesis, we also show that FZD8 signalling is pro-inflammatory. We found an association between a SNP in the FZD8 region and chronic bronchitis [chapter 4], indicating a possible role for FZD8 in inflammation. Acute cigarette smoke exposure induced less neutrophils and lymphocytes in FZD8−/− mice compared to WT mice [chapter 4]. We did not see effects of FZD8 knockdown in bleomycin- [chapter 2] and allergen-induced inflammation [chapter 6]. This could be due to the obvious fact that cigarette smoke, bleomycin and ovalbumin cause a different kind of inflammation, although TGF-β, IL-1β and EGF are common important signalling molecules involved in the pathophysiology of all these disease models and FZD8 is important in all these signalling routes. Therefore, a role for FZD8 in all three models might be envisioned. Nevertheless, cigarette smoke exposure causes a mainly neutrophilic inflammation, while allergen challenge causes an eosinophilic inflammation [6, 7]. Bleomycin-induced inflammation is initially neutrophil-driven, but later on comprises a mix of inflammatory cells [8]. Our data indicate that FZD8 has a selective role in neutrophilic inflammation.

We show that FZD8 contributes to chronic mucus hypersecretion as seen in chronic bronchitis [chapter 4], but not in goblet cell hyperplasia in a mouse model of allergic asthma [chapter 6]. Airway goblet cell hyperplasia and mucus hypersecretion are seen in both asthma and the chronic bronchitis phenotype in COPD, however the underlying mechanisms differ [9, 10]. The type 2 cytokines IL-4 and IL-13 are important cytokines in chronic mucus hypersecretion and IL-13 also in chronic bronchitis, [11]. Both IL-13 and IL-4 are increased in asthma [12]. In neutrophilic inflammation, it has been shown that mucus secretion is regulated via neutrophil elastase [13] and we found in chapter 4 that IL-6 and CXCL8 from fibroblasts induce MUC5AC mRNA expression in differentiated epithelial cells, and that CXCL8 but not IL-6 induces goblet cell hyperplasia. We found that FZD8 contributes to IL-6 and CXCL8 [chapter 4] but not IL-13 protein secretion [chapter 6]. Since allergen challenge induces IL-13, but not so much IL-6 or CXCL8, these findings can explain why we found in chapter 6 that FZD8 does not contribute to an in vivo model of allergen-induced goblet cell hyperplasia [chapter 6]. Neutrophil elastase, IL-6 and CXCL8 are hallmarks of neutrophilic inflammation. Therefore these findings strengthen
the contention that the role for FZD8 in inflammation is specific for CXCL8 mediated neutrophilic inflammation.

**FZD8 in lung development**
In embryonic development, the primary FZD receptor expressed at the gastrulation stage is the FZD8 receptor [14], indicating that the FZD8 receptor is essential for embryonic development. We and others show that FZD8 might be important for but not indispensable as FZD8\(^{-/-}\) mice were born according to normal Mendelian ratio and survived after birth with no differences in survival compared to WT mice [15, chapter 2, chapter 4 and chapter 6]. Macroscopic baseline lung structure showed no gross differences between FZD8\(^{-/-}\) mice and WT mice. We show in chapter 6 that airway collagen expression and IL-4 protein levels in whole lung tissue are lower in FZD8\(^{-/-}\) mice compared to WT mice, while we find no difference between FZD8\(^{-/-}\) and WT mice for collagen deposition and IL-4 protein levels after allergen challenge. This indicates that FZD8 plays a differential role in developmental and allergic mechanisms.

**Intracellular FZD8 signalling**
FZD8 is a receptor of the WNT signalling pathway and is characterised as a 694 amino-acid polypeptide with the characteristic frizzled-like cysteine-rich domain, seven transmembrane domains, and a C-terminal tail [16] with DVL binding capacity [17].

It has been suggested that FZD8-regulated proliferation in lung cancer cell lines occurs through activation of the β-catenin/TCF/LEF canonical WNT pathway [16]. FZD8 was classified as a typical canonical WNT receptor [18-20]. However, there are several indications for downstream signalling mechanisms that are not involved in canonical WNT signalling. R-spondin has been shown to activate canonical WNT signalling by binding to both the canonical co-receptor LRP6 and FZD8, however without forming a ternary complex [21]. Since LRP6 is activates canonical signalling, and does not have to couple to FZD8 for doing this, this finding suggests that LRP6 activates canonical WNT signalling through a different mechanism than via FZD8. In addition, the CRD of FZD8 was able to antagonise WNT-3A-induced β-catenin accumulation [22] and while FZD8 has been shown to be involved in WNT/β-catenin signalling in human mesenchymal stem cells, FZD8 was shown to be negatively correlated with WNT/β-catenin activation status [23]. Moreover, FZD8 was shown to be to mediate noncanonical WNT-5A signalling [4] and micro-RNA-375 inhibits WNT/β-catenin signalling while it increases FZD8 expression [24]. Here, we show that FZD8-mediated human lung fibroblast activation is not established via canonical signalling, but rather via noncanonical signalling [chapter 2].

Activation of canonical or noncanonical WNT signalling was originally believed to be solely dependent on the co-receptor that was activated by WNT-FZD coupling. It has become clear that the downstream signalling is also dependent on the concentration and selectivity of the WNT ligands for different receptors. For most FZD receptors, both canonical and noncanonical signalling routes have been described, while WNT ligands generally are selective either toward canonical or noncanonical signalling. For instance, WNT-3A has until now only been shown to signal via canonical WNT signalling and WNT-5B via noncanonical WNT signalling. On the other hand the mainly noncanonical WNT-5A has been shown to activate β-catenin in very high concentrations [25], indicating that WNT-induced downstream signalling effects are concentration-dependent. If one considers
the WNT ligands and FZD receptors that are present in a cell system, it is tempting to speculate that the combination determines downstream signalling.

FZD receptors are primarily activated by WNT ligands, but since multiple WNT ligands can bind to a specific FZD receptor, ligand affinity and the concentration of the ligand are important. WNT-5A expression is high both in airway smooth muscle cells and fibroblasts; in fibroblasts WNT-5B expression is higher compared to WNT-5B expression in airway smooth muscle cells [3, 4]. We show in that TGF-β induced WNT-5A, WNT-5B and FZD8 expression in lung fibroblasts and airway smooth muscle cells. Remarkably, for TGF-β-related remodelling in human lung fibroblasts WNT-5B/FZD8 signalling is important [chapter 2], while in airway smooth muscle cells WNT-5A and FZD8 play major roles. Next to remodelling, recombinant WNT-5A and WNT-5B can induce the expression of inflammatory cytokines IL-6 and CXCL8 in human lung fibroblasts [26] and we show that FZD8 signalling is important for IL-1β-induced IL-6 and CXCL8 secretion. However IL-1β does not affect WNT-5A expression and reduces WNT-5B expression in primary lung fibroblasts of COPD grade 4 patients [chapter 4], which may imply that IL-1β-related inflammation via FZD8 involves another (WNT) ligand.

WNT signalling in epithelial-mesenchymal communication
Both epithelial cells and mesenchymal cells can secrete various WNT ligands and express various FZD receptors. These secreted proteins can have autocrine and paracrine effects. We show in chapter 5 that epithelial and mesenchymal mRNA expression of WNT ligands and FZD receptors differ; WNT-4 and WNT-7B are higher expressed in epithelial cells compared to fibroblasts, while for WNT-5A and WNT-5B it is the other way around. This implies that also the spectrum of WNT ligands secreted by the cells and FZD receptors that are present on the cells differ. When differentiated epithelial cells are co-cultured with fibroblasts, epithelial WNT-4 and WNT-7B mRNA expression as well as IL-6 and CXCL8 secretion by fibroblasts are increased [chapter 5]. This increase is additive for IL-6, while for CXCL8 it is synergistic. We suggest in chapter 5 that this difference is due to the inhibitory role of WNT-4 on IL-6 secretion. WNT-4 has been previously shown to act pro-inflammatory on epithelial cells, as it can induce epithelial CXCL8 secretion [27, 28]. In our co-culture model, we studied the effect of WNT-4 on IL-6 and CXCL8 secretion from fibroblasts and found an anti-inflammatory role for WNT-4. These findings emphasize again the different effects that the same WNT ligand can have on different cells.

It is important to study WNT signalling in models with increasing complexity to appreciate the effects on different levels of organization in the living organism. Earlier studies from our group showed that in a co-culture of human bronchial epithelial cells with human lung fibroblasts, epithelium-derived IL-1α induces IL-6 and CXCL8 secretion from fibroblasts [29]. IL-1α and IL-1β are known to function via the same receptor and in chapter 4 we showed that IL-1β-induced IL-6 and CXCL8 secretion by fibroblasts is regulated by FZD8. WNT-5A [4] and WNT-5B [chapter 2] are ligands of FZD8 and both WNT-5A and WNT-5B are able to induce IL-6 and CXCL8 secretion in human lung fibroblasts [26]. Therefore, we hypothesized that WNT ligands contribute to the secretion of IL-6 and CXCL8 from fibroblasts that is induced upon co-culturing epithelial cells and fibroblasts. In chapter 5, we studied the functional role of WNT ligands in epithelial-mesenchymal communication in a co-culture system of differentiated human airway epithelial cells with human lung fibroblasts. In our co-culture model, we inhibited all WNT secretion,
using the porcupine inhibitor IWP-2 to see whether WNT signalling was involved in IL-6 and CXCL8 secretion by fibroblasts. After treatment with IWP-2, we found a further increase in IL-6 secretion by fibroblasts and then we specifically inhibited single WNT ligands using neutralizing antibodies to determine which WNT ligand was responsible for the increase in IL-6 secretion by fibroblasts in our co-culture model. In this system, we found an anti-inflammatory effect for epithelial WNT-4 on the secretion of IL-6 but not CXCL8 by fibroblasts. Intriguingly, no role for WNT-5A nor WNT-5B was found in IL-6 nor CXCL8 secretion by fibroblasts co-cultured with differentiated airway epithelial cells. This was unexpected, because previous studies showed that WNT-5B and to a lesser extent WNT-5A induce IL-6 and CXCL8 secretion from human lung fibroblasts [26] This could be explained by the fact that there are more pro-inflammatory proteins that contribute to CXCL8 and IL-6 secretion and that in co-culture, their effect eclipsed the contribution of WNT-5A and WNT-5B. Second, it could be that when WNT-5A is inhibited, WNT-5B takes over its function and vice versa, and that therefore an effect on cytokine secretion could be seen when both WNT-5A and WNT-5B will be inhibited. To see whether specific WNT ligands can compensate each other’s absence, multiple WNT ligands should be inhibited at the same time in our model. Either way, the fact that our results concerning the effect of WNT-5A and WNT-5B on IL-6 or CXCL8 secretion by fibroblasts in this co-culture system are in contrast with previous findings in separately cultured fibroblasts, shows the importance of multicellular models when investigating WNT signalling and stresses the need for ex or in vivo models to study the role of WNT signalling in inflammation.

**Therapeutic prospects**
The importance of increased FZD8 signalling in both remodelling and inflammatory processes in the lung suggests that antagonising this receptor may have therapeutic benefits in various chronic lung diseases. On the other hand, the protective and destructive effects of altered WNT signalling make it difficult to see clear possibilities for interference in this pathway.

There have been indications that antagonising FZD8 can have protective effects on inflammation and remodelling [30]. Antagonizing FZD8 by using FZD8 siRNA made cells more sensitive towards chemotherapy (with taxotere) [20, 31]. Another strategy for antagonising ligand binding to FZD8 is by using the fusion protein OMP-54F28. The immunoglobulin Fc domain and CRD of FZD8 compete with the FZD8 receptor for its ligands, and was able to reduce tumour growth [32].

An important aspect of developing WNT/FZD medication is targeting this medicine to the right cells, as WNT/FZD signalling is highly context dependant. WNT signalling in the lung is a complex system that can lead to canonical and noncanonical signalling and is also used for communication between different cell types [chapter 5]. It is therefore important that we take into account not only cell type specific signalling when we study WNT-FZD signalling to develop new drug targets, but as much as possible the whole lung, including all cell types and their surroundings.

A definite pre for using a FZD8 antagonist in treating pulmonary remodelling and inflammation is that although we found cell specific effects in chapter 2 and chapter 5, in a FZD8 knockout mouse we found that bleomycin-induced collagen [chapter 2] and cigarette-smoke-induced neutrophilic inflammation [chapter 4] are specifically inhibited in the lungs without notably affecting other vital functions. A difficult aspect herein is
that the induced pathophysiological effects are only partially inhibited, indicating that therapeutic strategies should target more upstream signalling proteins or additional signalling routes next to FZD8.

In **chapter 3**, we suggest that restoring decorin levels could antagonise WNT-5B-induced fibroblast activation. While this is a promising suggestion, it should be taken into account that other proteins, such as collagens in the ECM have binding properties for WNTs and FZDs as well [33]. For therapeutic interference in the WNT signalling pathway, it would be easier to target the receptor to block downstream signalling effects instead of targeting the ligand. One reason for this is that multiple (WNT) ligands can bind to one receptor, therefore targeting the receptor will block all downstream signalling. Another reason is that when fibroblasts are stimulated with pro-fibrotic TGF-β or pro-inflammatory IL-1β, of the FZD receptors only FZD8 expression is up-regulated whereas the expression of multiple WNT ligands is changed upon stimulation [3, 4, **chapter 2**]. Therefore, a FZD8 receptor antagonist would be more useful than a WNT-5 antagonist. Capturing WNT ligands by the addition of decorin could still be a useful target when it appears to have its inhibiting effect via receptor internalisation as in that way the receptor is also blocked. To explore the therapeutic possibility of blocking FZD8, we need to further investigate the use of specific FZD8 siRNA and the FZD8 specific fusion protein. In addition, the possibilities of using a FZD8 antagonists should be explored. For all future studies that further investigate FZD8 as a possible drug target, it would be useful to properly visualize FZD8 using specific antibodies. To be able to do this, these antibodies first need to be developed. This will be the next challenge.

**Main conclusions:**

- FZD8 regulates in part TGF-β-induced pro-fibrotic signalling.
- WNT-5B, but not WNT-5A, functions as a ligand for FZD8 in fibroblast activation.
- TGF-β-induced activation of β-catenin happens independent of the canonical WNT signalling pathway.
- The proteoglycan decorin functionally antagonizes WNT-5B-induced effects on fibroblast activation.
- FZD8 is associated with chronic mucus hypersecretion as a clinical outcome of chronic bronchitis.
- Fibroblasts play a regulatory role in mucus secretion by the epithelium via FZD8.
- Acute cigarette smoke-induced neutrophilic inflammation in a mouse model is regulated by FZD8.
- FZD8 is not involved in allergen-induced inflammation and remodelling in a mouse model.
- FZD8 regulates basal collagen deposition and basal IL-4 protein levels **in vivo**.
- WNT-4 negatively regulates IL-6 secretion from fibroblasts in a co-culture of differentiated epithelial cells with fibroblasts.
- FZD8 is a possible drug target for chronic bronchitis and lung fibrosis.
References


General discussion and main conclusions


