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Preface

The objective of this thesis was to establish the role of noncanonical WNT signalling in chronic obstructive pulmonary disease (COPD). Specifically, we studied the role of WNT-5A and WNT-5B in inflammatory processes, and the effect of oxidative stress on WNT-5A and WNT-5B-mediated signalling. In addition, the role of parenchymal tissue disruption in enhanced airway narrowing was investigated. Finally, we studied the interaction between noncanonical WNT signalling, ageing and tissue damage to the lung, with a focus on WNT-5B and FZD8. The research in this thesis shows that WNT-5B, and to a lesser extent WNT-5A, contributes to processes underlying COPD pathogenesis.

WNT signalling imbalance

WNT signalling, both canonical and noncanonical, is tightly regulated in both the developing and adult lung and is involved in stem and progenitor cell function and tissue homeostasis (1-4). As this pathway is crucial for normal development and homeostasis it is not surprising that recent studies indicate that an imbalance in WNT signalling contributes to chronic lung diseases such as COPD. Increased activation of canonical signalling is associated with tissue fibrosis (5, 6). In contrast, decreased canonical signalling as is the case in COPD is associated with emphysema (7, 8). Importantly, emphysema can be attenuated by LiCl-mediated WNT/β-catenin activation in both experimental models of emphysema and in patient-derived ex vivo lung tissue cultures (7, 9). In addition, it has recently been demonstrated that the COPD susceptibility gene FAM13A contributes to β-catenin degradation (10). Most studies investigating the role of WNT signalling in lung disease pathogenesis focused on the canonical pathway. However, recent studies demonstrate a crucial role for noncanonical WNT signalling in lung diseases as well. Dysregulated expression of noncanonical WNT-4, WNT-5A and WNT-5B has been observed in fibroblasts and airway epithelium of COPD patients as compared to controls (11-14). WNT-5B protein expression is significantly higher in the airway epithelium from smokers with COPD than non-smokers as well as control smokers (13). WNT-5B protein expression levels did not differ between non-smokers and control smokers, which indicates that the higher WNT-5B
expression in COPD is not smoking, but disease-related (13). Our group previously showed that TGF-β-regulated fibroblast activation is mediated via the WNT-5B receptor FZD8 (15), and that FZD8 has a pro-inflammatory role in chronic bronchitis (16). In line with these findings, we show in chapter 2 that WNT-5B induces FZD2 and TAK1-mediated inflammatory processes in pulmonary fibroblasts, and in chapter 3 that WNT-5B mediates fibroblast activation. In chapter 2 we show that WNT-5A and FZD2 gene expression levels are increased in whole lung tissue homogenates from COPD patients. Notably, we demonstrate that WNT-5B-induced IL-6 and CXCL8 secretion is higher in airway fibroblasts from COPD patients as compared to controls (14). Recent studies show that pulmonary fibroblasts regulate repair and inflammatory processes differently depending on their site of origin (bronchial vs. parenchymal) (17-19). Dessalle and colleagues demonstrated that basal IL-6 secretion is lower, whereas basal CXCL8 secretion is higher in human bronchial fibroblasts as compared to parenchymal fibroblasts (19). It could therefore be possible that WNT-5B-mediated cytokine secretion is different in parenchymal than in bronchial effects. However, in chapter 2 we demonstrate that WNT-5B increases both IL-6 and CXCL8 secretion in MRC-5 lung fibroblasts significantly as well, indicating that WNT-5B-mediated inflammatory processes are likely similar in both parenchymal and bronchial fibroblasts. Together, these results indicate enhanced activation of noncanonical WNT-5B signalling in COPD, favoring a pro-fibrotic and inflammatory environment.

**Oxidation of WNT-5B enhances inflammatory response even further**

Oxidative stress is recognized as an important driver of COPD pathogenesis, contributing to inflammation and airway remodeling (20). Recent studies indicate that oxidative stress can influence WNT signalling. For example, it has been demonstrated that ROS cause dissociation of nucleoredoxin (NRX) from DVL, which enables DVL to activate the downstream signalling pathway (21). In addition, Tiki, a protease required for head formation in Xenopus embryos, antagonizes WNT signalling by inducing Wnt3a oxidation. Tiki cleavage of Wnt3a results in oxidation and oligomerization through inter-Wnt disulfide bond formation. This oxidation and oligomerization results in inactivation of Wnt3a-mediated signalling, as Wnt3a oligomers fail to bind to their receptors (22). Although these studies show an effect of oxidative stress or oxidation on
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WNT signalling, these studies do not show a direct effect of oxidative stress on WNT-FZD interaction and signalling. Therefore, in chapter 3, we aimed to assess the effect of oxidative stress on WNT-5A and WNT-5B ligand-receptor interactions and the downstream signalling. We demonstrate that exposure of WNT-5B to H\textsubscript{2}O\textsubscript{2} results in oxidation of cysteine residues, and that treatment of MRC-5 and HBE cells with oxidized WNT-5B results in higher cellular activation. Interestingly, we show that oxidized WNT-5B induces an even higher inflammatory response in MRC-5 fibroblasts, as reflected by further increased IL-6 and CXCL8 protein levels. Together, these results indicate that oxidation of WNT-5B potentiates the noncanonical signalling pathway. This suggests that the high levels of oxidative stress present in COPD may contribute to the observed WNT signalling imbalance by enhancing noncanonical signalling, although this remains to be elucidated as the oxidation capacity in lung tissue of COPD patients may be different from the H\textsubscript{2}O\textsubscript{2}-driven oxidation capacity in our PCLS model. Whether oxidative stress could alter canonical WNT signalling via a similar mechanism is currently unknown. As all WNT ligands contain cysteine residues at evolutionary conserved sites, it is likely that oxidative stress could also influence canonical signalling by directly altering WNT-FZD interaction. However, canonical WNT signalling is decreased in COPD as a result of defective β-catenin transmission (7, 8). Therefore, it may be that direct oxidation of canonical WNTs will affect COPD pathogenesis to a lesser extent than oxidation of noncanonical WNTs. This idea also raises the question whether the canonical/noncanonical imbalance in COPD could be a consequence of increased levels of oxidative stress, or whether a pre-existing imbalance renders an individual prone for developing COPD and more susceptible to the effect of oxidation on noncanonical signalling. It has been demonstrated that lung-specific WNT-5A overexpression increases airspace enlargement in elastase-induced emphysema in vivo (23). Baarsma et al. showed that inhibition of WNT-5A in vivo attenuated emphysema, and restored expression of β-catenin-driven target genes (23). These results support findings from earlier studies that demonstrate that noncanonical signalling inhibits canonical WNT signalling, resulting in decreased β-catenin stabilization and/or downstream signalling (24, 25). The study by Baarsma et al. supports the hypothesis that an imbalance in WNT signalling, consisting of increased noncanonical and decreased canonical
signalling leads to increased susceptibility for developing COPD. Whether this imbalance also leads to an increased susceptibility for increased noncanonical WNT signalling due to WNT ligand oxidation remains to be elucidated.

**Ageing enhances noncanonical WNT signalling**

In chapter 5 our aim was to study the interaction between noncanonical WNT signalling, ageing and tissue damage to the lung. Recent studies indicate that noncanonical WNT signalling is increased during ageing in the lung (23, 26-28), while canonical signalling is decreased (29, 30). In chapter 5 we show that gene expression levels of p16 (a senescence marker), Nkd1 (a negative regulator of canonical WNT signalling), and of Wnt-5b were increased in aged WT as compared to young WT mice. Gene expression levels of Nkd1, Dkk2 (another negative regulator of canonical WNT signalling), Wnt-5a, and Wnt-5b correlated with p16, confirming that the noncanonical WNT signalling is ageing-related. Notably, treatment of PCLS from young WT mice with recombinant WNT-5B decreases gene expression levels of alveolar Type I and II makers to a similar extent as elastase treatment. This finding implies that WNT-5B, similar to elastase, induces COPD-like pathophysiology in young mice, as alveolar type I and II marker gene expression is reduced in COPD (31, 32). These data fit with studies showing that progenitor cells in the lung, such as airway basal cells or alveolar type I cells, demonstrate reduced regenerative capacity in COPD (33, 34). Interestingly, WNT-5A expression in induced sputum of COPD patients is dependent on disease severity, and correlates with the age of the patient independently of the disease severity (23). These data, together with the data from chapter 5, indicate that an age-dependent increase in noncanonical WNT signalling might contribute to increased COPD susceptibility. A limitation to chapter 5 is that the effect of ageing on canonical signalling was not assessed directly. Although we observed increased noncanonical WNT signalling, we did not assess whether canonical signalling was decreased in our model. Therefore, we can only hypothesize that a switch from canonical to noncanonical signalling occurred in our set-up. This remains to be elucidated, although the increased expression of Nkd1 and Dkk2 in aged mice support the hypothesis that canonical WNT signalling is reduced in this group. Studies in other organs than the lung support the theory of a canonical to noncanonical signalling switch during ageing as well. Recently it was
demonstrated that WNT-5A induces aging of young hematopoietic stem cells (HSCs), and that aged HSCs have increased expression levels of WNT-5A while active nuclear β-catenin levels are reduced. (27). These findings are supported by studies showing that WNT-5A antagonizes canonical signalling in HSCs (25), and that reduced canonical WNT activity in aged HSC correlates with impaired T-cell differentiation (35).

It remains to be elucidated why a switch from canonical to noncanonical signalling takes place during ageing. According to the developmental drift theory of ageing, later-in-life acting mutations or genes are under far weaker selection than early-in-life acting genes, as in the wild most individuals die of extrinsic causes such as predation before late-acting mutations or genes can take effect. As extrinsic mortality is generally high in nature, the extent of natural selection decreases rapidly with age (36). Therefore, genotypes that have beneficial effects early in life may become detrimental later in life. This trade-off of early benefits and late costs are known as antagonistic pleiotropy (36). The WNT signalling pathway is a tightly regulated pathway which is beneficial during development, while it has both positive and negative effects on ageing (as reviewed by Gruber et al) (37). Hence, the WNT pathway is a likely candidate for the concept of antagonistic pleiotropy. Indeed, it has been shown in C. elegans that the WNT pathway is tightly regulated and beneficial during development, whereas it becomes dysregulated during ageing, affecting lifespan (38). These results indicate that the WNT pathway may indeed undergo developmental drift, which might explain the complicated and often seemingly contrasting effects of this pathway during ageing. Although this concept is not a mechanistic explanation and needs further elucidation, the results from chapter 5 appear to be supportive of the concept of antagonistic pleiotropy. In chapter 5 we demonstrate that noncanonical WNT signalling increases during ageing, indicating that noncanonical WNT pathway starts to drift with age. Furthermore, it may be speculated that developmental drift of noncanonical WNTs enhances ageing, as knockdown of the WNT-5B receptor FZD8 has an inhibiting effect on the expression of p16.

Increased levels of oxidative stress present during ageing (39, 40) might also influence the shift from canonical to noncanonical WNT signalling. As mentioned above, the WNT pathway is susceptible to both indirect and direct
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effects of oxidative stress. In chapter 3 we showed that oxidation of WNT-5B enhanced cellular activation and downstream signalling, increasing inflammatory processes. As the level of oxidative stress increases during ageing, it may be that noncanonical signalling is enhanced, resulting in an imbalance in WNT signalling. In summary, there is evidence for increased noncanonical WNT signalling during ageing, and in COPD. This switch from canonical to noncanonical signalling does not appear to be exclusive for the lungs, but may be a general mechanism involved in ageing.

Fiber structural changes in emphysema

The role of structural alterations in the parenchyma of COPD patients in enhanced airway narrowing is increasingly recognized (41). Elastolytic enzymes and oxidative stress present in COPD alter the parenchyma and ECM, resulting in a loss of elastic recoil end emphysema (41, 42). This loss of elastic recoil is likely to affect airway mechanics and enhance bronchoconstriction in COPD. To investigate the hypothesis that parenchymal disruption enhances airway narrowing, we developed a comprehensive ex vivo PCLS model. In chapter 4 we demonstrate that ex vivo elastase, but not H2O2, treatment disrupts the parenchymal compartment and enhances MCh-induced airway narrowing in PCLS. Notably, we demonstrate that elastase treatment increases the Lmi and disrupts the structural organization of both elastin and collagen fibers. In addition, elastase treatment decreases gene expression of both alveolar Type I and II markers (Aqp5, Rage and Sftpc). Our PCLS model mimics important pathophysiological characteristics of COPD, as COPD is characterized by an altered ECM, alveolar Type I and II injury, and enhanced airway narrowing.

Altered fiber organization in COPD

An important structural change in COPD is the decreased expression and disorganization of elastin fibers (41-43). Recent studies demonstrate that expression of genes associated with elastogenesis is altered in COPD (44, 45). Among the most upregulated genes were fibulin-5 (FBLN5), elastin (ELN), latent transforming growth factor β binding protein 2 (LTBP2) and microfibrillar associated protein 4 (MFAP4), which are all implicated in elastogenesis. Alterations in elastin expression are already present in mild to
moderate COPD, and seen in both airways and alveoli of COPD patients (46). Recent studies demonstrated that exacerbations of COPD resulted in elevated levels of circulating fragments of structural proteins (47, 48). Notably, specific fragments are found to be associated with specific COPD phenotypes (49). These findings suggest that there may be a link between the rate of ECM turnover during COPD exacerbations and disease progression. Furthermore, alveolar wall elastin fiber structure is altered in patients with severe COPD. Compared to healthy subjects, elastin fibers from COPD patients are significantly less densely packed, unraveled and loose (50). This indicates that, even though elastin expression is similar in both mild to moderate and severe COPD, the disruption of the structural organization of elastin might contribute to the continuous decline of elastic recoil observed in small airways and parenchyma of patients with COPD (41). The same appears to be the case for collagen in COPD. Studies on the total expression of collagen in COPD are inconsistent, but it has been observed that collagen fibers are more disorganized in severe COPD as compared to mild to moderate COPD (51). Importantly, it has been shown that long-term inhaled corticosteroids treatment partially changes the composition of the ECM in moderate-severe COPD, which is associated with an increased long function (52). Together, these studies underline the importance of ECM and its structural organization in COPD pathogenesis.

In chapter 4 we demonstrate that elastase treatment disrupts the structural organization of both elastin and collagen fibers, similar to what is observed in COPD. Interestingly, the effect of elastase treatment on elastin and collagen fiber organization was very profound whereas the increase in Lmi was moderate. The relatively mild elastase treatment already enhanced airway narrowing. These findings stress the importance of the structural organization of elastin and collagen fibers with respect to tissue mechanics. These findings may also explain why McDonough and colleagues observed that small airways already narrowed before the onset of obvious emphysematous destruction in COPD patients (53). McDonough et al. assessed emphysema by measuring the Lmi, but they did not assess possible elastin and collagen fiber changes in the tissue cores. It could therefore be possible that, although Lmi was not increased yet, pulmonary elastic recoil was already reduced due to a disrupted fiber
organization, resulting in small airway narrowing. Whether it is the case that fiber changes occur prior to the development of emphysema (as defined by an increase in Lmi) needs to be further investigated. However, the results from chapter 4 support this hypothesis, as with even a mild increase in Lmi, the elastin and collagen fiber organization was already altered drastically. In addition to its structural role, the ECM is now also increasingly recognized for its bioactive role in regulating cellular responses, and as such it may be a driving factor for respiratory disease pathology (54). Therefore, future studies should focus on the role of fiber organization and ECM-mediated cellular responses in the development of emphysema. It would be interesting to investigate whether fiber organization could be used as a marker to predict the development of emphysema and airway narrowing.

**Noncanonical signalling and elastin**

An upregulation of elastin gene expression is observed in *in vivo* elastase-induced emphysema models (23), which supports the findings of studies demonstrating that elastin is among the highest up-regulated genes in COPD (44, 45). Recent findings show that noncanonical WNT signalling also contributes to an impaired elastogenesis. *In vitro* WNT-5A treatment upregulates elastin gene expression in primary mouse alveolar Type II cells, while *in vivo* WNT-5A overexpression downregulates tropoelastin protein expression (23). In addition to this direct negative effect, noncanonical signalling might also have an indirect effect on elastogenesis. It has been shown that activation of canonical WNT signalling reduces degradation of elastin and improves the linear deposition of elastin in alveolar walls in COPD patient-derived three-dimensional *ex vivo* tissue cultures (9). Noncanonical signalling can inhibit canonical signalling, and therefore might have an indirect negative effect on elastogenesis as well. These findings are supported by our results in chapter 5. In chapter 5 we demonstrate that aged WT mice are less sensitive to the effects of elastase treatment as compared to young WT mice, as Lmi and MCh-induced airway narrowing do not increase significantly. As discussed in chapter 5, this is most likely the effect of an already lower basal expression and/or altered elastin organization in aged WT mice. We also demonstrate that noncanonical signalling is ageing-related. Furthermore, our preliminary findings demonstrate that aged FZD8KO-/- mice have lower p16 gene
expression levels, are partially protected from ageing-related alveolar damage, and are more sensitive to the effects of elastase on Lmi than aged WT mice. Although it needs further investigation, it may be possible that aged FZD8KO/-mice have higher basal elastin expression and/or better elastin fiber organization than aged WT mice due to the reduced noncanonical signalling. Higher basal levels of elastin could explain why elastase treatment is able to enhance Lmi in the aged FZD8KO/- mice, but not in aged WT mice. Future research should determine whether and how noncanonical WNT signalling contributes to the disturbed elastogenesis observed in COPD.

The observation that elastase failed to increase MCh-induced airway narrowing or Lmi in aged WT mice reflects a limitation to our PCLS model in mimicking COPD pathophysiology. Loss of elastic recoil due to reduced expression or disorganization of elastin fibers is associated with normal lung ageing (55-57). In COPD however, these changes in elastin fibers are even more enhanced as compared to age-matched controls, likely due to increased elastase expression among others (46, 58). As elastase failed to increase Lmi or airway narrowing in aged WT mice PCLS, this may indicate that elastin fiber organization in the aged mouse lung is regulated differently as compared to the aged human lung. It could also mean that other factors than elastase alone are involved in elastin fiber regulation, or that the 16 hour elastase stimulation is too short to induce an effect in aged WT mice PCLS. As PCLS are only viable for a limited amount of time, long-term stimulation with elastase in this model is not possible. Taken together, PCLS from aged WT mice are less suitable as a model for elastase-related pathophysiological aspects of COPD. To model elastase-induced biomechanical changes as observed in COPD it is therefore preferable to use PCLS from young WT mice.

**Therapeutic prospects**

Noncanonical WNT signalling via WNT-5A, WNT-5B, FZD2 and FZD8 is clearly involved in COPD pathology. As demonstrated in this thesis and elsewhere it is involved in inflammatory processes, fibrotic responses, emphysema, and in ageing of the lung. Antagonizing noncanonical signalling by interfering with the above-mentioned WNTs and FZDs may therefore have beneficial therapeutic effects in the treatment of COPD. However, targeting
noncanonical signalling is challenging because of several reasons. First, the WNT signalling pathway is a very complex system. WNT ligands may activate both the canonical and noncanonical pathways, and can have beneficial or adverse effects depending on the cellular context and FZD receptors. Hence, targeting potential WNT/FZD medication to the right cells is crucial. WNT signalling is not only cell context, but also tissue and organ dependent. For this reason, the whole lung needs to be taken into account as much as possible when novel WNT-FZD drug targets are developed. The comprehensive PCLS model set up in chapter 4 could therefore serve as a good tool for the screening of early effects of novel drug targets.

In chapter 2 we demonstrate that in vitro knock-down of FZD2 inhibits WNT-5B-mediated CXCL8 secretion, with a potential role for FZD8. In addition, in chapter 5 our preliminary data suggest that in vivo knock-down of FZD8 partially protects against ageing-related alveolar damage. As WNT-5B and WNT-5A are upregulated in COPD and mediate inflammatory processes, these ligands may be therapeutic targets. However, a challenge in targeting WNT signalling at the ligand level is that multiple WNTs can bind to one FZD, and that the same WNT can have beneficial or adverse effects depending on the context. For therapeutic interference it is therefore more efficient to target the receptor to block the downstream signalling effects, ideally in a cell or tissue specific manner. Antagonizing FZD2 and FZD8 therefore represent promising therapeutic targets. Earlier work by our group demonstrates that FZD8KO mice are not affected basally in their vital functions (16), which is advocative for the use of a FZD8 antagonist in the treatment of inflammatory processes and alveolar damage.

When developing new WNT/FZD drug targets for the treatment of COPD it is important to take into account the role of oxidative stress. In chapter 3 we show that oxidation of the WNT-5B alters WNT-FZD interaction by altering the WNT structure. As COPD is characterized by high levels of oxidative stress is it possible that both WNTs and FZDs are structurally modified in COPD patients. To target WNT signalling effectively in COPD, it is important to assess whether WNTs and FZDs are structurally altered in COPD patients by using for example mass spectrometry. Structural alterations can then be taken into account when developing FZD antagonists or WNT-binding proteins to ensure
optimal binding capacity. Targeting the oxidative stress itself in COPD could be another strategy. As typical radical scavenger treatments such as vitamin E and other dietary antioxidants show minimal improvement in COPD, other strategies should be followed. Preventing the generation of ROS by a pharmacological approach which inhibits oxidant species is therefore required (59).

Taken together, interfering with noncanonical WNT signalling represents a promising therapeutic target in the treatment of COPD. However, in order to do so, FZD-specific antibodies or small molecule inhibitors need to be developed. As WNT signalling is crucial in not only COPD but in many other diseases as well, development of these antibodies could mean the next breakthrough in the search of novel therapeutics.
Main conclusions

- WNT-5B, and to a lesser extent WNT-5A, mediate inflammatory responses in pulmonary fibroblasts via FZD2, with a potential role for FZD8.
- WNT-5B-mediated inflammatory responses are increased in pulmonary fibroblasts from COPD patients as compared to non-COPD controls.
- Oxidation of WNT-5B and WNT-5A alters cysteine residues within the ligands and alters ligand-receptor interaction.
- Oxidation of WNT-5B enhances downstream signalling, increasing inflammatory processes.
- Elastase treated PCLS are a useful model to mimic the effects of parenchymal ECM disruption on airway narrowing and repair in COPD.
- WNT-5A, WNT-5B, Dkk2 and Nkd1 are positively correlated with ageing
- WNT-5B, FZD2 and FZD8 are possible drug targets for COPD and ageing-related lung tissue damage
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