WNT pathway activation in COPD: a two way street between signalling and pathology
van Dijk, Eline Margaretha

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 23-12-2018
Chapter Five

Noncanonical WNT signalling increases during ageing and induces alveolar damage

E.M. van Dijk*1,2, S. Culha1,2, R.Gosens1,2

1 Department of Molecular Pharmacology, University of Groningen, the Netherlands.
2 Groningen Research Institute for Asthma and COPD (GRIAC), University Medical Center Groningen (UMCG), University of Groningen, Groningen, the Netherlands.
Abstract

Background: COPD is a chronic lung disease characterized by airflow obstruction, impaired alveolar repair, and accelerated lung ageing. Several recent studies indicate dysregulated expression of noncanonical WNTs and FZDs in COPD. This dysregulation is associated with impaired repair and with ageing of the lung. In the present work, our aim was to study the effect of ageing on the interaction between lung structure and airway function, and to determine the role of noncanonical WNT signalling, in ageing-related lung tissue damage.

Methods: Lungs of young (14-40 weeks old) and aged (48-52 weeks old) C57Bl/6J mice were sliced and treated ex vivo with elastase (2.5 μg/ml, 16 hours) or WNT-5A or 5B (500 ng/ml, 24 hours). Following treatment, parenchymal structure, airway narrowing, and gene expression levels of alveolar Type I and II cell repair were assessed.

Results: Compared to young mice, PCLS from aged mice show increased gene expression of p16, altered expression of alveolar markers, and disrupted elastin and collagen fiber organization at baseline. Elastase alters parenchymal structure, alveolar marker gene expression, and airway narrowing in young animals only, inducing a phenotype with airway contractility resembling the contractility seen in PCLS of aged mice. In addition we show that noncanonical WNT signalling is correlated with ageing, and decreases alveolar marker expression in young, but not in old, mice.

Conclusion: We demonstrate that airway constriction is increased in aged mice, likely due to elastin fiber disorganization. In addition, we show that noncanonical WNT signalling increases during ageing, and induces alveolar damage, similar to elastase-induced damage. Taken together, our findings support the hypothesis that noncanonical WNT signalling is altered in ageing, likely contributing to pulmonary tissue damage.
Introduction

Chronic obstructive pulmonary disease (COPD) is a debilitating and life-threatening disease. The major risk factor for developing COPD is long-term tobacco smoke exposure (1, 2). COPD is characterized by an abnormal inflammatory response of the lungs to noxious particles or gases, and this abnormal response is associated with progressive airflow limitation. Important pathophysiological features that contribute to the continuous decline of lung function and increased airway obstruction are inflammation, bronchoconstriction, and an abnormal increase in air spaces (emphysema) among others (3-5). COPD has recently been characterized as a disease of accelerated lung ageing (4, 6). It is well-established that lung function declines with increasing age (7). Decline in lung function, as measured by reduced FEV1, is a slow process spanning several decades. For this reason, the prevalence of COPD rises sharply from the age of 40, hence most patients with COPD are of late middle or old age (8, 9). Currently COPD is the fourth leading cause of death worldwide and it is expected to be the third leading cause of death by 2020 (10). Available treatments can delay disease progression to some extent, but recovery or normalization of loss of lung function is not possible. Therefore, better insights into the mechanisms underlying COPD pathology are needed to develop novel and more effective therapies.

Recent studies demonstrate that noncanonical WNT signalling is altered in COPD at the expense of canonical WNT/β-catenin signalling. Autocrine or paracrine secreted WNT ligands are cysteine-rich proteins which bind to Frizzled (FZD) receptors via disulfide bonds and subsequently induce various downstream signalling pathways in the cell (11, 12). In canonical WNT/β-catenin signalling, WNT ligands bind to FZD and lipoprotein receptor-related protein (LRP) cell surface receptors. After WNT-FZD binding, cytosolic β-catenin is stabilized and translocated to the nucleus. Following nuclear translocation, WNT target genes involved in tissue repair and remodelling are transcribed (13). Noncanonical WNT signalling is mediated via intracellular calcium and c-Jun N-terminal kinase (JNK). These pathways regulate cell motility and gene transcription (14, 15) and antagonize canonical, β-catenin dependent gene transcription.
Dysregulated expression of noncanonical WNT-4, WNT-5A and WNT-5B has been observed in COPD and is associated with increased inflammatory processes and impaired repair (16-20). Furthermore, single nucleotide polymorphisms in the WNT-5A and WNT-5B receptor Frizzled-8 (FZD8) associate with chronic bronchitis, and FZD8 regulates airway inflammation, and TFGβ-induced profibrotic signalling (21, 22). Recently, it was shown that WNT4 and WNT-5A expression is increased in the senile lung and contribute to myofibroblast-like differentiation (23). In addition, ageing of mouse hematopoietic stem cells (HSCs) was associated with a shift from canonical to noncanonical WNT signalling due to elevated expression of WNT-5A (24). Although several studies indicate a role of noncanonical WNT signalling in ageing and COPD, the precise role and the interaction between noncanonical WNTs, ageing and COPD remains poorly understood.

Previous work by our lab demonstrates that elastin fiber degradation by \textit{ex vivo} elastase treatment of precision cut lung slices (PCLS) mimics structural and biomechanical aspects of COPD pathophysiology, reflected by decreased gene expression of alveolar markers, emphysema and enhanced airway narrowing (\textit{chapter 4}) (25). In the present study, we aimed to investigate the interaction between noncanonical WNT signalling, ageing and tissue damage to the lung. To study this interaction, we had two aims. First, we used the above-mentioned \textit{ex vivo} PCLS model to investigate the effect of ageing on lung structure, senescence and alveolar markers, and airway function using PCLS of young and aged mice. Second, we studied the expression levels of WNT-5A and WNT-5B in young and aged mice and investigated the effect of WNT-5A and WNT-5B on alveolar makers in young and aged mice. The benefit of our \textit{ex vivo} PCLS model is that it enables all the experimental conditions to be performed within the same animal. We hypothesized that noncanonical WNT signalling would increase during ageing, inducing tissue damage similar to damage seen in COPD.
Materials and Methods

Antibodies and reagents: Recombinant human/mouse WNT-5A and human WNT-5B were purchased from R&D systems (Abingdon, UK). Methacholine (MCh) was obtained from ICN Biomedicals (Zoetermeer, the Netherlands). Alexa Fluor® 488 Phalloidin was purchased from Life technologies. Mouse anti-E-cadherin was obtained from BD Biosciences (Bedford, MA, USA), and Cy3-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove PA, USA). Elastase from porcine pancreas Type IV and chloroquine were received from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Animals: C57bl/6J male and female mice were obtained from Innoser (Lelystad, The Netherlands). Animals were divided in two age groups: young (age 14-40 weeks, body weight 23-41 g, n=20), and aged (age 48-52 weeks, body weight 30-54 g, n=13). Animals were maintained on mouse chow and tap water ad libitum in a humidity- and temperature-controlled room at 24 °C with a 12 h light/dark cycle. All experiments were performed according to the national guidelines and upon approval of the experimental procedures by the local Animal Care and Use committee of Groningen University, DEC number 6815A.

Precision-cut lung slices: Precision-cut lung slices were prepared as described previously (26). Animals were euthanized by subcutaneous injection with ketamine (40 mg/kg, Alfasan, Woerden, The Netherlands) and dexdomitor (0.5 mg/kg, Orion Pharma, Mechelen, Belgium). Subsequently, the trachea was cannulated, and the animal was ex-sanguinated via the aorta abdominalis. Lungs were filled through the cannula with a low melting-point agarose solution (1.5% final concentration (Gerbu Biotechnik GmbH, Wieblingen, Germany) in CaCl₂ (0.9 mM), MgSO₄ (0.4 mM), KCl (2.7 mM), NaCl (58.2 mM), NaH₂PO₄ (0.6 mM), glucose (8.4 mM), NaHCO₃ (13 mM), HEPES (12.6 mM), sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100), pH = 7.2). Subsequently, lungs were placed on ice for 15 min, in order to solidify the agarose for slicing. The lobes were separated and tissue cores were prepared of the individual lobes, after which the cores were sliced at a thickness of 250 μm. Slicing was performed in medium composed of CaCl₂ (1.8 mM), MgSO₄ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH₂PO₄ (1.2 mM), glucose (16.7 mM), NaHCO₃ (26.1 mM), HEPES...
(25.2 mM), pH = 7.2, using a tissue slicer (CompresstomeTM VF-300 microtome, Precisionary Instruments, San Jose CA, USA). Tissue slices were incubated at 37°C in a humid atmosphere under 5% CO₂/95% air. Slices were washed every 30 min (four times in total). PCLS were incubated in DMEM supplemented with sodium pyruvate (1 mM), MEM non-essential amino acid mixture (1:100; Gibco® by Life Technologies), gentamycin (45 μg/ml; Gibco® by Life Technologies), penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (1.5 μg/ml; Gibco® by Life Technologies). Slices were cultured at 37°C in a humidified atmosphere under 5% CO₂/95% air in 12-well tissue culture plates, using 3-4 slices per well. Slices were incubated DMEM or DMEM with elastase (2.5 μg/ml, Sigma Aldrich) for 16 h. Following treatment, slices were washed twice with medium and incubated for another 24 hours with DMEM or DMEM with recombinant WNT-5A (500 ng/ml) or WNT-5B (500 ng/ml) after which slices were collected. Previous work from our lab (unpublished) demonstrated that mouse lung slice viability is preserved after 72 hours of culturing, as mitochondrial activity did not change. This indicates that the lung slice is viable for at least three days. Our experiments were all performed within 56 h after sacrifice.

**mRNA isolation and real-time PCR:** Total RNA was extracted from PCLS by automated purification using the Maxwell 16 instrument and the corresponding Maxwell 16 LEV simply RNA tissue kit (Promega, Madison, USA) as per the manufacturer’s instructions. Equal amounts of total RNA (1 μg) were then reverse transcribed using the Reverse Transcription System (Promega, Madison, USA). 1 μl of 1:3 diluted cDNA was subjected to real-time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands) using FastStart Universal SYBR Green Master (Rox) from Roche Applied Science (Mannheim, Germany). Real-time PCR was performed with denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 5 minutes at 72°C. Analysis of RT-PCR data was performed using LinRegPCR analysis software (27, 28). Primer sets used to analyse gene expression are shown in Table 1.
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Table 1. Primers used for RT-PCR analysis.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse B2m</td>
<td>Fwd 5′-ACCGTCTACTGGGATCGAGA-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-TGCTATTTCTTTCTGCGTGCAT-3′</td>
</tr>
<tr>
<td>Mouse Rpl13a</td>
<td>Fwd 5′-AGAAGCAGATCTTGGAGTTACGG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GTTCACACCAGGAGTCCGT-3′</td>
</tr>
<tr>
<td>Mouse Aqp5</td>
<td>Fwd 5′-CTTGTTGGGATCTACTTACCG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-AAATAGAGGATTGCAAGCCAGG-3′</td>
</tr>
<tr>
<td>Mouse T1α</td>
<td>Fwd 5′-TCACCCCAATAGAGATGGCTTG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GGGCCAAGTGGGAACGTCTCCT-3′</td>
</tr>
<tr>
<td>Mouse Rage</td>
<td>Fwd 5′-CAGGGCTCTGTGGGATGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-TTACAGCTCTGCACGTTCTC-3′</td>
</tr>
<tr>
<td>Mouse Con43</td>
<td>Fwd 5′-TCTTCTTCTCAGGACTTCGCTC-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-TCTGAAAATGAGGAGACCGACACA-3′</td>
</tr>
<tr>
<td>Mouse Sftpc</td>
<td>Fwd 5′-GGAGGAGCGGAGAACTCAGAA-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GGAGGAGGCTTGAGTACATA-3′</td>
</tr>
<tr>
<td>Mouse p16</td>
<td>Fwd 5′-CGGGGACATCAAGACATCGT-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GGGGGATTTAGCTCTGCT-3′</td>
</tr>
<tr>
<td>Mouse p21</td>
<td>Fwd 5′-CTTGTGCTTGCTTGCACT-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-TGGGCACTTGAGGTTTCT-3′</td>
</tr>
<tr>
<td>Mouse Trp53</td>
<td>Fwd 5′-GAGAGTATTTCCACCTCAAGATCGG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GCAGCTTGGGCTTTCCT-3′</td>
</tr>
<tr>
<td>Mouse WNT-5A</td>
<td>Fwd 5′-CTGGGGAGAAACATCGACT-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-TGAGGAGGCCGAGCACCTCA-3′</td>
</tr>
<tr>
<td>Mouse WNT-5B</td>
<td>Fwd 5′-AGTTTGAGTTCCCGAGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-CAGGGCACTTGAGGACCC-3′</td>
</tr>
<tr>
<td>Mouse Nkd1</td>
<td>Fwd 5′-TAGACCTTGCGGGGAGTAGAG-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-GTCAAGGAGGGTGAAGAGGC-3′</td>
</tr>
<tr>
<td>Mouse Dkk2</td>
<td>Fwd 5′-ACCGCTGCAAATAATGGGAA-3′</td>
</tr>
<tr>
<td></td>
<td>Rev 5′-CGTACCGATGGGCTCTTCCTC-3′</td>
</tr>
</tbody>
</table>

Tissue staining and confocal laser scanning microscopy to visualize parenchymal cells: To visualize the parenchyma of the PCLS, slices were stained for F-actin and E-cadherin. PCLS were fixed for 15 min at 4°C in cytoskeletal buffer (CB) (10 mM Tris base, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂ and 5 mM glucose at pH 6.1) containing 3% paraformaldehyde (PFA). PCLS were then permeabilized by incubation for 5 min at 4°C in CB containing 3% PFA and 0.3% Triton X-100. Subsequently, PCLS were washed twice with 4°C CB. For immunofluorescence microscopy, fixed PCLS were first blocked for
1 hour at room temperature in Cyto-TBS buffer (200 mM Tris base, 154 mM NaCl, 20 mM EGTA and 20 mM MgCl₂ at pH 7.2) containing 1% bovine serum albumin and 2% normal donkey serum. PCLS were incubated with primary antibody (E-Cadherin, 1:100, BD biosciences) overnight at 4°C in Cyto-TBS containing 0.1% Tween 20 (Cyto-TBST). The next day, PCLS were incubated with Alexa Fluor® 488 Phalloidin (1:100, Life technologies) and Cy3-conjugated secondary antibody (1:50, Jackson ImmunoResearch) for 2 h at room temperature in Cyto-TBST containing 1% BSA. Between incubation steps slices were washed with Cyto-TBST. Following staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen). Fluorescence was determined with a confocal laser scanning microscope (CLSM) equipped with true confocal scanner (TCS; SP8 Leica, Heidelberg, Germany), using a 200x lens. To avoid bleed through, sequential scans were performed. AlexaFluor488 was excited using the 488 nm blue laser line, and Cy™3 was excited using the 552 nm green laser line. All images were recorded in the linear range, at an image resolution of 1024x1024 pixels and with a pinhole size of 1 Airy unit, while avoiding local saturation. The images presented here show a single z-scan. ImageJ 1.48d was used to further process images (29).

2-photon imaging and autofluorescence to visualize the ECM: 2-Photon and multiphoton excitation fluorescence (MPEF) imaging were used to visualize organized and intact collagen, and elastin polymers, respectively, as described previously (30). Following stimulation with elastase, PCLS were washed twice with PBS and directly mounted on coverslips. Under excitation at 820 nm, the collagen bundles naturally emitted a second harmonic generation signal collected around 410 nm. Elastin was visualized by using its endogenous fluorescence. Images from elastin were generated by using infrared laser (excitation wavelength 880 nm). The measured broadband emission spectrum ranged from 455 to 650 nm with a peak at ~500 nm.

Mean linear intercept (Lmi): To assess emphysema in the PCLS, the mean linear intercept (Lmi) was determined as a measure of mean distance of free airspace, as described previously (31). Following staining with Alexa Fluor® 488 Phalloidin (1:100, Life technologies) the alveolar structure was visualized by confocal microscopy (magnification 200x). Two fields per animal were used to determine Lmi. As the lungs were filled with agarose under a varying pressure,
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Lmi differs between animals due to the experimental protocol. However, in this experimental set-up animals served as their own control as all experimental conditions were performed within the same animal. Hence, the treatment effect on Lmi was normalized (percent basal) within the animal, and these normalized values were compared between animals.

Airway narrowing studies: Airway narrowing studies were performed on untreated slices and on slices treated with elastase (2.5 μg/ml). Dose response curves for MCh (10⁻⁹M – 10⁻³M) were recorded, after which the airways were dilated using the bitter taste receptor agonist chloroquine (10⁻³M, Sigma-Aldrich). A nylon mesh and metal washer were used to fixate the lung slice, as described previously (32). Lung slice images were captured in time-lapse (1 frame per 2 seconds) using a microscope (Eclipse, TS100; Nikon). To quantify airway luminal area, image acquisition software (NIS-elements; Nikon) was used. Luminal area is expressed as percent basal.

Data analysis (statistics): Values reported for all data are represented as mean ± SEM. The statistical significance of differences between means was determined on log transformed data by Student’s t-test, repeated measures ANOVA, or by 2-way ANOVA, followed by a Bonferroni correction where appropriate. Differences were considered to be statistically significant when p<0.05.
Chapter Five

Results

Gene expression levels of senescence and alveolar repair markers in aged mice

Our first aim was to describe differences between young and aged mice. Therefore, we assessed basal gene expression levels of markers of senescence and alveolar epithelial repair. First, we investigated basal gene expression levels of p16, p21, and Trp53 as markers of cellular senescence (33). Aged animals showed increased p16 gene expression levels, whereas p21 and Trp53 expression levels did not differ significantly as compared to young mice (Figure 1 A-C). Next, markers of alveolar epithelial repair were assessed. Alveolar epithelial cells are classified as alveolar Type I and alveolar Type II cells. Type I cells maintain the alveolar structure and are responsible for gas diffusion toward the alveolar capillaries. Type II cells are the primary source of surfactant proteins, next to being the progenitor cells of Type I cells. Both cell types are crucial for maintaining alveolar tissue integrity, and the loss of these cells directly impacts the whole parenchyma (34). COPD is characterized by a loss of type I and II cells and hence alveolar structure integrity (35). To study the basal alveolar epithelial repair marker expression, several markers were chosen: T1α and Aqp5 (specific for alveolar Type I cells), Con43 and Rage (both alveolar Type I cell associated), and Sftpc (specific for alveolar Type II cells) (36, 37). Some of these markers such as Aqp5 and Sftpc are decreased in COPD, and this decrease is correlated with a lower lung function (38, 39). Compared to young mice, PCLS from aged mice show increased basal gene expression levels of Rage and T1α, and decreased levels of Con43 (Figure 1 D-F). In addition, Aqp5 expression levels were reduced whereas Sftpc levels were increased, although not significantly (Figure G-H).
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Figure 1. Basal mRNA expression of senescence and alveolar markers is altered in aged mice. PCLS from young (n=5-8) and aged (n=4-11) were incubated in DMEM for 40 hours. (A-C) Basal gene expression levels of p16 were increased in aged mice, whereas p21 and trp53 were similar in aged and young mice. (E-H) Basal gene expression levels of Rage and T1 were increased in aged mice whereas Con43 was decreased, and Aqp5 and Sftpc were not altered significantly. The statistical significance of differences between means was determined by Student’s t-test. *p<0.05 compared to young mice.
Elastase alters Lmi, alveolar Type I/II gene expression levels, and airway narrowing in young animals.

Next, we investigated the effect of elastase treatment on lung structure and airway function. Previous work from our lab (chapter 4) (25) demonstrated that elastase treatment of PCLS from young mice enhances airway narrowing as a consequence of parenchymal disruption. As basal gene expression levels of alveolar repair markers were altered in aged mice, we were interested if elastase would have a different effect in this group. To assess emphysema in the PCLS, the Lmi was determined as a measure of mean distance of free airspace. Following elastase treatment, the mean free distance in air spaces was increased in young, but not in aged mice, as compared to basal (set at 100%) as reflected by an enhanced Lmi (young mice 120.6 % (p=0.04), aged mice 108.8 % (p=0.14)) (Figure 2). In addition, 2-Photon and MPEF imaging of elastase-treated slices of young mice showed a disrupted elastin and collagen fiber organization. Under basal conditions, both elastin and collagen fibers were clearly present (Figure 3A). The elastin fibers had a straight appearance whereas the collagen fibers were coiled (Figure 3B). After elastase treatment the elastin fibers were absent whereas the collagen fibers became more stretched (Figure 3C). 2-Photon and MPEF imaging of aged animals showed a far weaker signal for collagen fibers as compared to young mice, indicating disorganization of collagen fibers (Figure 3D). In addition, the collagen fibers were stretched instead of coiled (Figure 3E). Elastin fibers were present, although not as obvious as in young mice (Figure 3E). Following elastase treatment, elastin fibers were absent in aged mice (Figure 3F). Furthermore, elastase treatment decreased gene expression of Rage, Aqp5 and Sftpc in young animals (Figure 4A-C) whereas only Sftpc was decreased in aged mice (Figure 4C). In addition, elastase treatment decreased Sftpc further in aged than in young mice (Figure 4C). Elastase did not affect Con43 or T1α gene expression in either young or aged mice (Figure 4D,E). Finally, we studied the airway functionality following elastase treatment by investigating the effect of elastase on MCh-induced airway narrowing. Elastase enhanced MCh-induced airway narrowing in young mice as shown by a significant increase in pEC50 (5.87 vs 6.50, p=0.007), and showed a trend for increased Emax (47.96 vs 67.30 % contraction, p=0.06) (Figure 5A). Maximal airway contraction in aged mice under basal conditions was the same as maximal airway contraction in young mice under elastase
Noncanonical WNT signalling increases during ageing and induces alveolar damage

conditions. In addition, elastase treatment did not increase pEC50 (6.00 vs 5.80, p=0.54) or Emax (64.65 vs 69.00 % contraction, p=0.85) significantly in aged mice (Figure 5B). Interestingly, when values for all conditions were combined for the young animals, we found pEC50 correlated with Lmi, whereas Emax did not (Figure 6A,B). In aged animals, both pEC50 and Emax did not correlate with Lmi (Figure 6A,B). Taken together, these findings indicate that elastase alters Lmi, aveolar marker gene expression levels and airway narrowing in young animals only. In addition, elastase induces an aged phenotype in PCLS from young mice, with airway contractility resembling the contractility seen in basal aged PCLS.

**WNT-5B is correlated with ageing and increases tissue disruption**

Our second aim was to study the role of noncanonical WNT signalling in ageing and tissue damage. Therefore, we first assessed basal gene expression of the noncanonical ligands WNT-5A and WNT-5A, and of the negative regulators of canonical WNT signalling Nkd1 and Dkk2. Gene expression of Nkd1 was slightly increased in aged mice, although not significantly (Figure 7C). WNT-5A expression showed a trend for increasing in aged mice (Figure 7A). In addition, gene expression of WNT-5B and Dkk2 was significantly higher in aged mice (Figure 7B,D). Interestingly, when values for young and aged were combined, we found expression of Nkd1, Dkk2, WNT-5A, and WNT-5B correlated with p16 (Figure 8A-D). Next, we investigated the effect of exogenously added recombinant WNT-5A or WNT-5B on gene expression of alveolar markers. Stimulation of PCLS with WNT-5A did not alter any of the alveolar markers as compared to basal in aged mice (data not shown). Interestingly, in young mice, WNT-5B decreased gene expression of Aqp5, Rage, and Sftpc, similar to the decreased gene expression seen after elastase treatment (Figure 9A). In contrast, WNT-5B increased gene expression of Con43 in aged animals, whereas the other alveolar markers were not affected (Figure 9B). Taken together, noncanonical WNT signalling is correlated with ageing, and decreases alveolar marker expression in young, but not in old, mice.
Chapter Five

Figure 2. Elastase increases Lmi in young mice only. PCLS of young (n=7) and aged (n=13) mice were exposed to elastase (2.5 μg/ml) for 16 hours. After stimulation, slices were washed twice with medium and incubated for 24 hours in medium. Following incubation, slices were stained for F-actin filaments (green) and E-cadherin (red) and Lmi was assessed as % basal. Elastase increased Lmi in young mice only. The statistical significance of differences between means was determined on log transformed data by Student’s t-test (A). Data represent mean±SEM, *p<0.05 compared to basal control.
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Figure 3. Elastase disrupts the structural organization of elastin and collagen in young mice only. PCLS of young (n=2) and aged (n=2) mice were exposed to elastase for 16 hours. After stimulation, slices were washed twice with medium and incubated for 24 hours in medium. 2-Photon and multiphoton excitation fluorescence (MPEF) imaging were used to visualize collagen and elastin polymers. The elastin and collagen fiber organization was less obvious in aged mice as compared to young mice, with stretched instead of coiled collagen fibers. Elastase treatment disrupted elastin fiber organization in both young and aged mice, while collagen fibers were stretched in young mice only.
Figure 4. Elastase alters mRNA expression levels of Type I and II cell markers in young mice only. PCLS of young (n=6-8) and aged (n=10) were exposed to elastase (2.5 μg/ml, n=9) 16 hours. After stimulation, slices were washed twice with medium and incubated for 24 hours in medium. The statistical significance of differences between means was determined on log transformed data by Student’s t-test followed by a Bonferroni correction. Data represent mean±SEM, *p<0.05 compared to basal control, #p<0.05 compared to young mice.
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Figure 5. Elastase enhances MCh-induced airway narrowing in young mice only. PCLS from young (n=10) and aged (n=10) mice were exposed to elastase (2.5 μg/ml) 16 hours. After stimulation, slices were washed twice with medium and incubated for 24 hours in medium. Following incubation, MCh-induced airway narrowing was assessed. Lung slice images were captured in time-lapse (1 frame per 2 seconds) using an inverted phase contrast microscope (Eclipse, TS100; Nikon). Airway luminal area was quantified using image acquisition software (NIS-elements; Nikon), and expressed as percent basal. (A) MCh-induced airway narrowing following elastase treatment in young mice. (B) MCh-induced airway narrowing following elastase treatment in aged mice. The statistical significance of differences between means was determined on log transformed data by one-way ANOVA followed by Bonferroni testing. Data represent mean±SEM, *p<0.05 compared to basal control.
Figure 6. An increased pEC50 value correlates with an increased Lmi value in young animals only. (A) Lmi (μm) and pEC50 values for all conditions were combined. It was found that an increased pEC50 value correlates with an increased Lmi (R²=0.2838, p<0.05) in young, but not in aged animals. (B) Emax values did not correlate with Lmi in either young or aged animals.
Figure 7. Basal mRNA expression of noncanonical WNT signalling markers is increased in aged mice. PCLS from young (n=5-6) and aged (n=5-6) were incubated in DMEM for 40 hours. (A-B) Basal gene expression levels of Wnt-5a and Nkd1 were increased in aged mice, albeit not significantly. (C-D) Basal gene expression levels of Wnt-5b and Dkk2 were significantly increased in aged mice. The statistical significance of differences between means was determined by Student’s t-test. *p<0.05 compared to young mice.
Figure 8. Increased expression of noncanonical markers correlates with increased p16 expression. (A-D) Expression levels of Wnt-5a, Nkd1, Wnt-5b, Dkk2 and p16 for young and aged mice were combined. It was found that increased expression levels of Wnt-5a, Nkd1, Wnt-5b, and Dkk2 correlate with increased expression levels of p16 ($R^2=0.6807$, 0.6229, 0.4582, and 0.6667, respectively, $p<0.05$).

Figure 9. WNT-5B alters mRNA expression levels of Type I and II cell markers in young mice only. PCLS of young (n=5) and aged (n=7-11) were exposed to WNT-5B (500 ng/ml) for 24 hours. The statistical significance of differences between means was determined on log transformed data by Student’s t-test followed by a Bonferroni correction. Data represent mean±SEM, *p<0.05 compared to basal control.
Noncanonical WNT signalling increases during ageing and induces alveolar damage

**Figure S1.** Basal mRNA expression of p16 is decreased in aged FZD8-/- mice. PCLS from aged (n=4) wildtype mice and aged (n=6) FZD8-/- mice were incubated in DMEM for 40 hours. The statistical significance of differences between means was determined by Student’s t-test. *p<0.05 compared to aged WT wildtype mice.

**Figure S2.** Elastase increases Lmi aged FZD8-/- mice. PCLS of aged wildtype mice (n=13) and aged FZD8-/- (n=6) mice were exposed to elastase (2.5 μg/ml) for 16 hours. After stimulation, slices were washed twice with medium and incubated for 24 hours in medium. Following incubation, slices were stained for F-actin filaments (green) and E-cadherin (red) and Lmi was assessed as % basal. Elastase increased Lmi in aged FZD8-/- mice only. The statistical significance of differences between means was determined on log transformed data by Student’s t-test (A). Data represent mean±SEM, *p<0.05 compared to basal control.
Discussion

COPD is a life-threatening and incurable disease characterized by a continuous decline of lung function, and is described as a disease of accelerated lung ageing. It is increasingly recognized that noncanonical WNT signalling is altered in COPD, enhancing inflammatory processes and pro fibrotic signalling. Previous work by our lab demonstrates that elastase treatment of PCLS mimics structural and biomechanical aspects of COPD pathophysiology. In the present study, we used our PCLS model to investigate the interaction between noncanonical WNT signalling, ageing and tissue damage to the lung. We show that gene expression of markers of senescence and alveolar repair is altered in aged compared to young mice. Furthermore, our findings indicate that elastase alters Lmi, alveolar marker gene expression and airway narrowing in young animals only. In addition, elastase induced an aged phenotype in PCLS from young mice, with airway contractility resembling the contractility seen in basal aged PCLS. Noncanonical WNT signalling was correlated with ageing, and WNT-5B treatment decreased alveolar marker expression in young, but not in old, mice.

Our first aim was to compare the effect of elastase treatment in young and aged mice. Our previous study demonstrated a clear relationship between lung structure and lung function in young animals following ex vivo elastase treatment ([chapter 4]) (25). Elastase treatment decreased alveolar Type I and II marker gene expression levels, and increased Lmi, which was correlated to enhanced MCh-induced airway narrowing. These structural and functional changes are similar to those seen in COPD pathophysiology, suggesting an aged phenotype in elastase-treated PCLS. Interestingly, elastase treatment did not induce these changes in PCLS of aged mice. Whereas in PCLS of young mice elastase treatment decreases gene expression levels of Rage, Aqp5, and Sftpc, elastase only decreased Sftpc in aged mice. Even though basal expression levels of Sftpc were higher in aged mice than in young mice, the effect of elastase on Sftpc expression was more pronounced in aged mice. This suggests that aged mice are more susceptible to elastase-induced alveolar Type II cell damage than young mice. These findings are in line with studies demonstrating that aged mice have impaired regeneration of Type II cells following influenza infection and that Type II cells are susceptible to telomere dysfunction (40, 41).
In summary, elastase treatment decreases alveolar Type I and II marker gene expressions in young mice, whereas only Sftpc is decreased in aged mice.

The observation that elastase does not have the same effect on the Lmi of PCLS of young and aged mice could be explained via several mechanisms. First, the aged animals might have had stiffer lung tissue as a result of ageing-related ECM changes, rendering the tissue more resistant to the effects of elastase. However, this seems unlikely as gene expression levels of matrix markers such as collagen and fibronectin did not differ between young and old mice (data not shown). In addition, aged animals showed more disorganization of collagen fibers than young mice. These results are in line with a study showing that ageing-related increase of collagen in C57BL/6 mice is delayed until 26 months of age (42). Secondly, it could be that the aged mice had less elastin fibers present in the parenchyma as a reduction and disorganization of elastin protein is associated with ageing (42-44). This is a more likely explanation, as elastin fibers were less obviously present in aged mice under basal conditions. The observed disorganization and stretch of collagen fibers in aged mice was clearly evident and supports the hypothesis of reduced elastin fibers in the aged mice. Earlier studies concerning pulmonary elastin and collagen fiber organization indicate that at low levels of strain, elastin fibers bear most of the strain while the adjacent collagen fibers are in a wavelike configuration. At higher levels of strain the collagen fibers become stretched, preventing further distension of the alveolar ducts (45). Ageing-related elastin degradation may therefore explain the observed stretched collagen fibers in aged mice under basal conditions. Taken together, elastase treatment only enhances Lmi in young animals. The lack of effect of elastase on Lmi in aged animals is most likely explained by ageing-related reductions in elastin.

The difference in elastin fiber organization between young and aged mice could also explain why exogenously added elastase failed to enhance airway narrowing in aged mice. A disrupted elastin fiber organization leads to an impaired elastic recoil of the parenchyma, enhancing airway narrowing (chapter 4) (25). As the basal elastin fiber organization was already disorganized in aged mice, exogenously added elastase would have little extra effect. Indeed, MCh-induced airway narrowing in untreated PCLS of aged mice was the same as MCh-induced airway narrowing in elastase-treated PCLS of
young mice. This finding confirms the role of the elastin fiber organization in airway narrowing and likely explains why elastase treatment did not have any further effect. Taken together, our findings demonstrate that elastase treatment induces an aged phenotype in PCLS from young mice, mimicking structural and functional aspects of COPD pathophysiology. In addition, aged mice are less suitable to study the relation between lung structure, lung function, and lung repair processes in an \textit{ex vivo} PCLS elastase model, as elastin fiber organization is already altered in aged animals.

Our second aim was to study the interaction between noncanonical WNT signalling, ageing and tissue damage to the lung. Gene expression levels of the senescence marker p16, the negative regulator of canonical WNT signalling Nkd1, and of noncanonical WNT-5B were increased in aged as compared to young animals. Gene expression levels of Nkd1, Dkk2, WNT-5A, and WNT-5B correlated with p16, confirming that the noncanonical WNT signalling is ageing-related. These findings are supported by the observation that aged mice lacking the WNT-5B receptor FZD8 demonstrate reduced p16 gene expression levels (Figure S1). Our findings are similar to other studies demonstrating that noncanonical WNT signalling is increased during ageing (23, 24, 46). Interestingly, in young mice stimulation with recombinant WNT-5B decreased gene expression levels of alveolar Type I and II makers to a similar extent as elastase treatment. This implies that WNT-5B, similar to elastase, induces COPD-like pathophysiology in young mice, as alveolar type I and II gene expression is reduced in COPD (38, 39).

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 \textit{ex vivo} tissue cultures (47). As noncanonical WNT signalling inhibits canonical signalling, it might be that the ageing-related upregulation of noncanonical WNT signalling is at least partly responsible for the observed increased elastin degradation in aged mice. Hence, noncanonical WNT signalling may contribute to the observed increased basal airway contraction in aged mice. As mentioned above, elastase treatment failed to increase Lmi in aged mice. Interestingly, elastase treatment does increase Lmi in aged FZD8-/- mice (Figure S2). This may indicate that elastin fiber expression is increased or organized better in these mice, and this would support the
Noncanonical WNT signalling increases during ageing and induces alveolar damage

Noncanonical WNT signalling is involved in elastin degradation. To confirm a role for noncanonical WNT signalling in elastin degradation, future research is needed. The PCLS model would be a suitable tool for this purpose, as elastin fiber expression and organization could be assessed in PCLS obtained from young mice exposed to WNT-5B or in PCLS obtained from FZD8-/- mice. Taken together, these results indicate that noncanonical WNT signalling negatively affects alveolar cells in young animals, increases during ageing, and that it may be involved in elastin regulation.

Noncanonical WNT signalling is increased during ageing and expression levels of WNT-5A, WNT-5B and FZD8 are even further increased in COPD (16, 19 (chapter 2)). In addition, dysregulation of noncanonical WNT signalling increases inflammatory processes, fibroblast activation, and TGFβ-induced profibrotic signalling (16, 18, 19 (chapter 2), 21). Therefore, targeting the noncanonical WNT pathway may represent a therapeutic target for the treatment of COPD. Our findings that WNT-5B treatment induces alveolar damage, and that WNT-5B expression is correlated with ageing confirm the role of noncanonical WNT signalling in lung tissue damage and ageing. In addition, these findings confirm the potential therapeutic value of targeting noncanonical WNT signalling in COPD. Our data show that WNT-5B has more effect on markers of alveolar repair in young than aged animals. This could indicate that noncanonical WNT-5B signalling is more detrimental during development than during aging. The lack of effect of exogenously added WNT-5B in aged mice could be due to the increased gene expression of WNT-5B in these animals. It may be possible that in aged mice, the maximum effect of WNT-5B is already reached. This study was performed in healthy animals, and it might be that pulmonary pathology increases the susceptibility to the impairing effects of noncanonical WNT signalling during ageing. Indeed, previous work demonstrates that WNT-5B-mediated CXCL-8 release is higher in pulmonary fibroblasts from COPD patients than from non-COPD controls (chapter 2) (19). This suggests that pulmonary disease pathology indeed increases the sensitivity of the lungs to the effects of noncanonical WNT signalling. Therefore, even though noncanonical WNT might not have detrimental effects in healthy aged lung tissue, it is likely that this pathway has impairing effects on lung tissue affected by COPD. In order to have a better understanding of the precise role of
noncanonical WNT signalling in ageing-related lung alterations and pathology, further research is needed. In the current set-up we used mice of 12 months old for the aged group. This does not exactly cover the whole range of ageing. Therefore, It would be interesting to study noncanonical WNTs and FZDs, markers of alveolar repair, and airway function in mice at multiple subsequent timepoints ranging from young to old (up to 24 months of age), and assess whether there is a causal relationship between noncanonical WNT signalling and lung ageing. Notably, it would be interesting to assess this relationship in both healthy ageing mice and ageing COPD model mice to further determine the effect of noncanonical WNT signalling on the development of lung pathology. Taken together, our findings confirm a role for noncanonical WNT signalling in ageing. In addition, these results stress the value of this pathway as a possible therapeutic target for the treatment of COPD.

In summary, these results demonstrate that elastase treatment induces an aged phenotype in PCLS from young mice, mimicking structural and functional aspects of COPD pathophysiology. In addition, these findings demonstrate that PCLS from aged mice are less suitable to study the relation between lung structure, lung function, and lung repair processes as elastase is less effective in altering parenchymal and airway structure and function. Furthermore, we show that noncanonical WNT signalling increases during ageing, and induces alveolar damage in young mice similar to damage observed in COPD. This effect was not observed in aged mice. Taken together, our findings support the hypothesis that noncanonical WNT signalling is altered in ageing, and likely contributes to pulmonary tissue damage. Targeting this pathway may therefore be a promising therapeutic target in the treatment of COPD.

**Grants**

We would like to thank the Netherlands Organization for Scientific Research and the Netherlands Lung Foundation for financial support (Vidi grant: 016.116.309 and 6.1.14.009, respectively).
Noncanonical WNT signalling increases during ageing and induces alveolar damage

References


Noncanonical WNT signalling increases during ageing and induces alveolar damage


Chapter Five


