Chapter 4

A pro-inflammatory role for the Frizzled-8 receptor in chronic bronchitis


Anita I.R. Spanjer
Mark H. Menzen
Akkelies E. Dijkstra
Maarten van den Berge
H. Marike Boezen
David C. Nickle
Don D. Sin
Yohan Bossé
Corry-Anke Brandsma
Wim Timens
Dirkje S. Postma
Herman Meurs
Irene H. Heijink
Reinoud Gosens
Chapter 4

Abstract

Rationale
We have previously shown increased expression of the Frizzled-8 receptor of the WNT signalling pathway in COPD. Here, we investigated if the Frizzled-8 receptor has a functional role in airway inflammation associated with chronic bronchitis.

Methods
Acute cigarette smoke-induced airway inflammation was studied in wild-type and Frizzled-8 deficient mice. Genetic association studies and lung expression quantitative trait loci (eQTL) analyses for Frizzled-8 were performed to evaluate polymorphisms in FZD8 and their relationship to tissue expression in chronic bronchitis. Primary human lung fibroblasts and primary human airway epithelial cells were used for in vitro studies.

Results
Cigarette smoke exposure induced airway inflammation in wild-type mice, which was prevented in Frizzled-8 deficient mice, suggesting a crucial role for Frizzled-8 in airway inflammation. Furthermore, we found a significant genetic association (p=0.009) between single nucleotide polymorphism (SNP) rs663700 in the FZD8 region and chronic mucus hypersecretion, a characteristic of chronic bronchitis, in a large cohort of smoking individuals. We found SNP rs663700 to be a cis-eQTL regulating Frizzled-8 expression in lung tissue. Functional data link mesenchymal Frizzled-8 expression to inflammation as its expression in COPD-derived lung fibroblasts was regulated by pro-inflammatory cytokines in a genotype dependent manner. Moreover, Frizzled-8 regulates inflammatory cytokine secretion from human lung fibroblasts, which in turn promoted MUC5AC expression by differentiated human airway epithelium.

Conclusions
These findings indicate an important pro-inflammatory role for Frizzled-8 and suggest that its expression is related to chronic bronchitis. Furthermore, our findings indicate an unexpected role for fibroblasts in regulating airway inflammation in COPD.

KEY MESSAGES
What is the key question?
What is the functional role for the Frizzled-8 receptor in airway inflammation in COPD?

What is the bottom line?
We show that the Frizzled-8 receptor is associated with chronic bronchitis and is involved in cytokine secretion from human pulmonary fibroblasts as well as acute cigarette smoke-induced inflammation in a mouse model.

Why read on?
Our findings concerning the pro-inflammatory role for Frizzled-8 in airway inflammation in COPD provide a rationale for further exploration of the therapeutic potential of the Frizzled-8 receptor in COPD.
A pro-inflammatory role for the FZD8 receptor in chronic bronchitis

Introduction
Chronic obstructive pulmonary disease (COPD) is a complex lung disease, characterized by airflow limitation that is not fully reversible. Airflow limitation is usually progressive and associated with chronic inflammation, remodelling of the small airways, and emphysema development in some patients [1, 2].

The heterogeneity of COPD is represented by different phenotypes within the patient population, chronic bronchitis being one of them. Chronic bronchitis is defined by chronic cough and idiopathic sputum production for at least three months per year for two consecutive years [1]. Inflammatory signals can cause mucus cell metaplasia, resulting in more mucus by both an increased production of mucus by goblet cells and decreased elimination from the airways [3]. This is defined in patients as chronic mucus hypersecretion (CMH). CMH is associated with bronchial inflammation, accelerated lung function decline [4], increased mortality [5], and is a risk factor for COPD [6].

COPD treatment is not curative nor directed at different COPD phenotypes, including chronic bronchitis. At present, the pathophysiology and underlying mechanisms of chronic bronchitis are poorly understood. This hampers the development of targeted drugs. Therefore, a better understanding of the mechanisms behind chronic bronchitis is needed.

The wingless/integrase-1 (WNT) signalling pathway plays an important role in lung development [7]. Recent studies indicate involvement of the WNT signalling pathway in remodelling [8, 9] and inflammation [10, 11] in the lung. WNT ligands bind to transmembrane Frizzled (FZD) receptors, thereby controlling cell differentiation, growth and polarity [12]. Our previous results showed that FZD8 expression is higher in pulmonary fibroblasts of moderate (GOLD grade 2) and severe (GOLD grade 4) COPD patients after stimulation with transforming growth factor (TGF)-β compared to control fibroblasts [8]. However, the functional role for FZD8 in COPD is as yet unknown. WNT-3A [10], WNT-4 [13] and WNT-5A [14] have recently been shown to function as pro-inflammatory stimuli in the lung. Importantly, we previously showed that WNT-5A is a ligand for FZD8 in airway smooth muscle cells [9], reinforcing the possibility that the increased FZD8 expression in COPD fibroblasts may play a role in pro-inflammatory signalling in the lung.

Here, we aimed to investigate the link between FZD8 and airway inflammation in COPD. We studied the role of FZD8 in acute cigarette smoke-induced airway inflammation in vivo using a mouse model and in vitro in human lung fibroblasts obtained from COPD patients with and without CMH. Furthermore, we investigated the association between single nucleotide polymorphism (SNP)s in the FZD8 region and CMH, and their roles as cis-expression quantitative trait loci (eQTL), regulating FZD8 expression in lung tissue.

Methods
Ethics statement
For the genetics study on CMH approval by the local medical ethics committee and written informed consent from all patients was obtained. At Laval, lung specimens were collected from patients undergoing lung cancer surgery and stored at the “Institut universitaire de cardiologie et de pneumologie de Québec” (IUCPQ) site of the Respiratory Health Network Tissue Bank of the “Fonds de recherche du Québec – Santé” (www.tissuebank.ca).
Written informed consent was obtained from all subjects and the study was approved by the IUCPQ ethics committee. At Groningen, lung specimens were provided by the local tissue bank of the Department of Pathology and the study protocol was consistent with the Research Code of the University Medical Center Groningen and Dutch national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; http://www.federa.org). At Vancouver, the lung specimens were provided by the James Hogg Research Center Biobank at St. Paul’s Hospital and subjects provided written informed consent. The study was approved by the ethics committees at the UBC-Providence Health Care Research Institute Ethics Board. All animal experiments were performed according to the national guidelines and approved by the University of Groningen Animal Ethical Committee (committee approval number 5912B).

**Animal studies**

Heterozygous, inbred, specified-pathogen-free breeding colonies FZD8+/− mice (C57BL/6;129P2-FZD8tm1Dgen/J), showing no obvious phenotype, were obtained from the Jackson Laboratory (USA). After breeding, homozygous FZD8+/− mice and wild-type+/− (WT) littermates were used for experiments. Animals were housed under a 12 hour light-dark cycle and received food and water ad libitum. Male FZD8+/− and WT mice were subjected for four successive days to fresh air or cigarette smoke from Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) by whole body exposure, as described previously [22]. In brief, cigarette smoke was directly circulated into a 6-liter Perspex box. After removing the filter, each cigarette was smoked in five minutes at a rate of 5L/hour in a ratio of 60L/hour air using a peristaltic pump (45 rpm, Watson Marlow 323 E/D, Rotterdam, the Netherlands). On the first day, mice were exposed to one cigarette in the morning and three cigarettes in the afternoon. On the second to fourth day, mice were exposed to five cigarettes in the morning and five cigarettes in the afternoon. Control animals were exposed at the same time intervals to fresh air. Eight mice were included in each group. Sixteen hours after the last cigarette smoke exposure, mice were euthanized by subcutaneous injection with a mixture of medetomidine (0.5 mg/kg Dormitor®, Orion Pharma, Mechelen, Belgium) and ketamine (40.0 mg/kg, Alfasan, Woerden, the Netherlands) followed by exsanguination. The lungs were lavaged five times with 1 mL PBS. The bronchial alveolar lavage fluid (BALF) fractions were pooled. From these, cytospins were prepared to determine total and inflammatory cell numbers. Cytospins were stained with May-Grünwald and Giemsa (Sigma, St. Louis, MO, USA). Differential cell count was performed by counting 400 cells in duplicate in a blinded manner. Preceding immunohistochemistry, the upper right lung lobe was taken up in formalin and paraffin-embedded. Preceding mRNA isolation, the post caval lobe was snap frozen, mechanically crushed under liquid nitrogen and taken up in lysis buffer.

**Genetic association**

Genetic association studies for FZD8 and CMH were performed in the NELSON cohort; a large cohort of smoking individuals with and without COPD which was originally set-up to detect lung cancer [15]. After quality control, 717 heavy smokers with CMH (cases) and 1,795 heavy smokers without CMH (controls) all with ≥ 20 pack-years were included for the analysis as previously published [16]. Six SNPs were tested using multivariate logistic regression analysis under an additive model, with adjustment for ex- or current smoking and the two population sites (Groningen and Utrecht).
Expression quantitative trait loci (eQTL) analysis in lung tissue
The lung eQTL analysis was performed in a large dataset of lung tissue samples where both genotype and genome-wide gene expression data is available through a collaboration of three universities (Laval University, Quebec City, Canada; University of British Columbia, Vancouver, Canada; University of Groningen, Groningen, the Netherlands), and Rosetta/Merck Sharpe and Dohme as has been described previously, including patient characteristics [17]. After quality control, 1,095 patients out of 1,111 were included in the analysis. A cis-eQTL analysis was performed to determine the association between SNP rs663700 and the regulation of gene expression of genes within 100 kb [17]. In short, the association between genotype and expression of cis-eQTL genes was analyzed using linear regression on imputed data, first in the three cohorts separately, followed by a meta-analysis [18, 19].

Fibroblast cell culture
Human airway and parenchymal lung fibroblasts from ex-smoking GOLD grade 4 COPD patients with and without CMH were isolated from transplanted lungs as has been described previously [20]. Presence of CMH was defined by patient records. Patient characteristics are shown in table 1 and showed no differences in age, gender, smoking status, lung function or medication use. At the same time, we determined that none of the patients used for the study in fibroblasts was diagnosed with bronchiectasis, excluding the possible influence on the data.

Table 1 - Patient characteristics GOLD grade 4 COPD patients.

<table>
<thead>
<tr>
<th></th>
<th>GOLD grade 4 COPD without CMH</th>
<th>GOLD grade 4 COPD with CMH</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of subjects</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Age (range)</td>
<td>58 (57-62)</td>
<td>54 (48-61)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Pack-years (range)</td>
<td>44.9 (30-72)</td>
<td>31.3 (15-40)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>ex-smoker</td>
<td>ex-smoker</td>
</tr>
<tr>
<td>% predicted FEV₁ (range)</td>
<td>19.2 (12.23-25.69)</td>
<td>14.6 (13.12-20.12)</td>
</tr>
<tr>
<td>FEV₁/FVC (range)</td>
<td>0.30 (0.19-0.66)</td>
<td>0.23 (0.16-0.29)</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Age, pack-years, predicted FEV₁ and FEV₁/FVC are represented as mean (range).

MRC-5 human lung fibroblasts [21] were obtained from Sigma (St. Louis, MO, USA). Primary human airway and parenchymal lung fibroblasts and primary MRC-5 human lung fibroblasts were cultured in Ham’s F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 1.5 μg/ml amphotericin B. Prior to the experiment, cells were grown to confluence in 6-well or 24-well cluster plates and placed in Ham’s F12 medium with 0.5% (v/v) FBS for 24 hours. Subsequently, cells were stimulated with either 2 ng/ml recombinant human transforming growth factor (TGF)-β1, 1 ng/ml recombinant human interleukin (IL)-1β, 10 ng/ml recombinant human tumor necrosis factor (TNF)-α, 10 ng/ml recombinant human epidermal growth factor (EGF) or 5% cigarette smoke extract (CSE) for several time points. To prepare CSE, two cigarettes were smoked sequentially using a peristaltic pump (323 E/D; Watson Marlow, Rotterdam, the Netherlands) at 45 rpm through 25 ml of Ham’s
F12 medium supplemented with 0.5% (v/v) FBS, referred to as 100% CSE. 100% CSE was diluted to a working concentration of 5% CSE. CSE was freshly prepared before each experiment.

**Air liquid interface (ALI) cell culture**

Human airway epithelial cells were isolated after incubating tracheal tissue from donor lungs for 2 hours at 37°C in Protease IX (Sigma, St. Louis, MO, USA) and plated on coated culture dishes. Coating consisted of 10 μg/ml bovine serum albumin, 10 μg/ml fibronectin (both from Sigma, St. Louis, MO, USA) and 30 μg/ml collagen (PureCol®, Advanced Biomatrix, San Diego, CA, USA) in phosphate buffered saline (PBS). Human airway epithelial cells were grown to approximately 70% confluence in keratinocyte serum-free medium (KSFM) supplemented with 25 μg/ml streptomycin, 25 U/ml penicillin, 1 μM isoproterenol, 0.2 ng/ml EGF, 25 μg/ml bovine pituitary extract. For the first week after isolation, KSFM medium was additionally supplemented with 1.5 μg/ml amphotericin B and 5 μg/ml ciproxin. Cells were plated on a 0.4 μm polyester membrane 12 mm inserts (Transwell® Permeable Supports, Corning, NY, USA) coated well in 1:1 Bronchial Epithelial Cell Growth Medium (Lonza, Walkersville, MD, USA)/Dulbecco’s Modified Eagle’s Medium (Gibco, Grand Island, NY, USA) (BEGM/DMEM) medium supplemented with 25 μg/ml streptomycin, 25 U/ml penicillin, 5.5 mg/ml sodium pyruvate (all from Gibco, Grand Island, NY, USA) 0.4% (w/v) bovine pituitary extract, 0.5 ng/ml EGF, 5 μg/ml insulin, 10 μg/ml transferrin, 1 μM hydrocortisone, 6.5 ng/ml T3, 0.5 μg/ml epinephrine (all from Lonza, Walkersville, MD, USA), 15 ng/ml retinoic acid and 1.5 μg/ml bovine serum albumin (Sigma, St. Louis, MO, USA). When confluence was reached, cells were air-exposed and allowed to differentiate into mucociliary epithelium for 14 days. Subsequently, cells were serum deprived for 16 hours in BEGM + ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium) and stimulated basolaterally for 24 hours with 10 ng/ml recombinant human IL-6 or 10 ng/ml recombinant human chemokine ligand (CXCL)8 to measure MUC5AC gene expression and to perform immunohistochemistry for goblet cells.

**Immunohistochemistry**

Inserts of ALI cell culture were taken up in formalin and paraffin-embedded according to the protocol ‘Preparation of Costar® Transwell® Inserts for Histology’. Transverse cross-sections of 5 μm thick were used for morphometric analyses. Paraffin-embedded sections were stained for goblet cells with Periodic Schiff’s (PAS, Sigma-Aldrich, Zwijndrecht, the Netherlands). PAS-positive cells were counted and expressed per mm basement membrane. After sacrificing the mice, the upper right lung lobe was taken up in formalin and paraffin-embedded. Transverse cross-sections of 5 μm thick were used for morphometric analyses. Paraffin-embedded sections were stained for α-smooth muscle(sm)-actin using rabbit anti-α-sm-actin antibody (Abcam, Cambridge, UK) and visualized using a goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) and diaminobenzidine (Sigma-Aldrich, Zwijndrecht, the Netherlands). The presence of α-sm-actin around the airway was quantified using ImageJ [chapter 2 ref 25]. The surface of positively stained tissue was expressed as mm² per mm² basement membrane. MUC5AC positive cells were stained in paraffin-embedded sections using a MUC5AC antibody staining (Neomarkers, Fremont, CA, USA).
**Small interfering (si)RNA transfection**

Human fibroblasts were grown to 90% confluence and transfected with specific siRNA against the FZD8 transcript to knockdown FZD8. Cells were transfected in serum-free Ham’s F12 medium without supplements using 100 pmol FZD8-targeted siRNA or non-targeting control siRNA and Lipofectamine® 2000 Transfection Reagent. After 6 hours, the medium was changed to medium supplemented with 10% (v/v) FBS for 18 hours and subsequently to medium supplemented with 0.5% (v/v) FBS for 24 hours. Cells were stimulated with either recombinant human IL-1β or EGF in increasing concentrations (0.03-3 ng/ml IL-1β and 0.1-10 ng/ml EGF) for 24 hours. Knockdown was considered successful when FZD8 gene expression was reduced by 60%.

**Cytokine release from fibroblasts**

24 hours after stimulation, culture medium was collected for the determination of cytokines using enzyme-linked immunosorbent assay (ELISA) and Milliplex®. We used Milliplex® ( MILLIPLEX MAP Human Cytokine/Chemokine - Premixed 26 Plex, Millipore, Billerica, MA, USA) to screen for primary MRC-5 human lung fibroblast cytokine release. We specifically measured concentrations of secreted IL-6 and CXCL8 in primary MRC-5 human lung fibroblasts by ELISA according to the manufacturer’s instructions (#M1916 (IL-6), #M1918 (CXCL8); Sanquin, Amsterdam, the Netherlands).

**mRNA isolation and real-time PCR analysis**

Total mRNA from primary MRC-5 human lung fibroblasts and from mice lung tissue was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total mRNA from primary human airway and parenchymal lung fibroblasts was extracted using the miRNeasy Micro Kit (Qiagen, Venlo, the Netherlands). Total mRNA from differentiated primary human airway epithelial cells was extracted using Tri Reagent® Solution (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, the Netherlands). The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific™, Wilmington, DE, USA). Equal amounts of mRNA (1 µg) were then reverse transcribed using the Reverse Transcription System (Promega Benelux b.v., Leiden, the Netherlands) and the cDNA was stored at -20°C till further use. mRNA expression was determined using real-time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands). Primer sets are listed in table 2-3.
Chapter 4

Table 2 - Human primers used for the determination of specific genes of interest.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZD2 Forward</td>
<td>5’ CCC GACT TCAC GGT CTA CAT 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CTG TTG GTG AGG CGA GTG TA 3’</td>
</tr>
<tr>
<td>FZD6 Forward</td>
<td>5’ TTG TTG GCA TCT CTG CTG TC 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CCA TGG ATT TGG AAA TGA CA CC 3’</td>
</tr>
<tr>
<td>FZD8 Forward</td>
<td>5’ GAC ACT TGA TGG GCT GAG GT 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’CAA ATC TCG GTG TCT GGA AA 3’</td>
</tr>
<tr>
<td>WNT-5A Forward</td>
<td>5’ GGG TGG GAA CCA AGA AAA AT 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’TGG AAC CTA CCC ATC CCA TA 3’</td>
</tr>
<tr>
<td>WNT-5B Forward</td>
<td>5’ ACG CTG GAG ATC TCT GAG GA 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CGA GGT TGA AGC TGA GTT CC 3’</td>
</tr>
<tr>
<td>WNT-16 Forward</td>
<td>5’ GCT CCT GTG TGA AAA CA 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ ACC TCT TGA TGT ACG GTT GC 3’</td>
</tr>
<tr>
<td>MUC5AC Forward</td>
<td>5’ ATT TTT TTC CCA CTC CTG ATG 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ AAG ACA ACC CAC TCC CAA CC 3’</td>
</tr>
<tr>
<td>18S rRNA Forward</td>
<td>5’ CGC CGC TAG AGG TGA AAT TC 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ TTG GCA AAT GCT TTC GCT C 3’</td>
</tr>
</tbody>
</table>

Table 3 - Mouse primers used for the determination of specific genes of interest.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL2 Forward</td>
<td>5’ AAG TTT GCC TTG ACC CTG AA 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ AGG CAC ATG AGG TAC GAT CC 3’</td>
</tr>
<tr>
<td>CXCL5 Forward</td>
<td>5’ GAA AGC TAA GCG GAA TGC AC 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ GGG ACA ATG GTT TCC CTT TT 3’</td>
</tr>
<tr>
<td>KC Forward</td>
<td>5’ GCT GGG ATT CAC CTC AAG AA 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ AGG TGC CAT CAG AGC AGT CT 3’</td>
</tr>
<tr>
<td>FZD8 Forward</td>
<td>5’ TCC GTT CAG TCA TCA AGC AG 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CGG TTG TGC TGC TCA TAG AA 3’</td>
</tr>
<tr>
<td>WNT-5A Forward</td>
<td>5’ CAA ATA GGC AGC CGA GAG AC 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CTC TAG CGT CCA CGA ACT CC 3’</td>
</tr>
<tr>
<td>WNT-5B Forward</td>
<td>5’ GGT TCC ACT GGT GTT GCT TT 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ AGA CTT TTG TGA GGC GGA GA 3’</td>
</tr>
<tr>
<td>MUC5AC Forward</td>
<td>5’ GAG ATG GAG CAT CTG GGT GA 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ GCA GAA GCA GGG AGT GGT AG 3’</td>
</tr>
<tr>
<td>18S rRNA Forward</td>
<td>5’ AAA CGG CTA CCA CAT CCA AG 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CCT CCA ATG GAT CCT CGT TA 3’</td>
</tr>
</tbody>
</table>

Materials and reagents
Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA), recombinant human IL-1β, recombinant human TNF-α and recombinant human EGF were obtained from Sigma (St. Louis, MO, USA). Recombinant human IL-6 and recombinant human CXCL8 were obtained from ImmunoTools (Friesoythe, Germany). For CSE, Kentucky 3R4F research cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) were used. FZD8 targeted siRNA was obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). non-targeting control siRNA from Qiagen (Venlo, the Netherlands). Lipofectamine® 2000 Transfection Reagent was obtained from Invitrogen (Paisley, UK). All other chemicals were of analytical grade.

Statistical analysis
Statistical analysis of in vitro and in vivo experiments was performed with SigmaPlot™ software (Systat Software Inc., San Jose, CA, USA). All real-time PCR data were log
transformed before analysis. For comparison between two conditions the Student’s t-test
(α = 0.05, tested two-tailed) was used. Differences in FZD8 gene expression in fibroblasts
between two genotypes were tested using a one-tailed Mann-Whitney test, based on
the results of the eQTL analysis. To test differences between different patient groups,
one-way ANOVA on ranks was used. For comparison between multiple conditions within
the primary MRC-5 human lung fibroblast experiments and within the different groups
of mice, two-way ANOVA was used. Tests used for the analyses of the genetic studies are
described in the appropriate sections. Where appropriate, ANOVA was followed by a post
hoc analysis using the Student-Newman-Keuls multiple comparisons test. P < 0.05 was
considered significant.

Results

FZD8 regulates cigarette smoke-induced airway inflammation in mice

Inflammation in COPD follows a characteristic pattern of increased numbers of
macrophages, lymphocytes and neutrophils [2]. To investigate a possible role for FZD8
in inflammation, we used FZD8−/− mice and studied acute cigarette smoke-induced airway
inflammation, as described previously [22]. FZD8−/− mice were born according to normal
Mendelian ratio and survived after birth with no differences compared to WT mice. Baseline
lung structure was not different for the FZD8−/− mice and WT mice in terms of
gross morphological structure and expression of α-sm-actin around the airways and
vessels (figures 1A-C). Baseline presence of inflammatory cells and pro-inflammatory
cytokines was slightly higher in FZD8−/− mice compared to WT mice (figures 2A-G) and this
was significant for neutrophil number (p < 0.004) and CXCL5 (p < 0.005) gene expression
when using a Student’s t-test on the air exposed animals only.

Four days of cigarette smoke exposure induced an increase in the number of
lymphocytes and neutrophils in the bronchial alveolar lavage fluid (BALF) of WT mice
(figures 2A-B). No effect of cigarette smoke was found on total cells and macrophages
(figures 2C-D). Notably, cigarette smoke did not increase the number of lymphocytes and
neutrophils in FZD8−/− mice and neutrophil number in cigarette smoke exposed FZD8−/−
mice was significantly lower compared to cigarette smoke exposed WT mice (figure 2B).
Keratinocyte-derived cytokine (KC), CXCL2 and CXCL5 are chemotactic for neutrophils and
the gene expression of all three cytokines was increased upon cigarette smoke exposure
in WT mice (figure 2E-G). KC and CXCL2 tended to be reduced in FZD8−/− mice, whereas
for CXCL5, the cigarette smoke-induced increase was completely absent in FZD8−/− mice.
Gene expression of FZD8 and its putative ligands WNT-5A and WNT-5B was also measured
in whole lung homogenates and was not different between groups, although small, non-
significant increases in cigarette smoke exposed WT mice were apparent for all three
genes (figures 1D-F). MUC5AC gene expression significantly increased after cigarette
smoke exposure in WT mice but not in FZD8−/− mice (figure 1G). However, consistent with
previous studies [22, 23] we did not find any cells positive for MUC5AC protein expression
in lung tissue sections before and after cigarette smoke exposure in WT and FZD8−/− mice.
Together, these results suggest that FZD8 is involved in acute cigarette smoke-induced
airway inflammation in mice in vivo.
Figure 1: characterization of FZD8⁻/⁻ mice. WT and FZD8⁻/⁻ mice were exposed to PBS and paraffin-embedded sections were stained for α-sm-actin. (A) Quantification of the area positive for α-sm-actin around the airways in WT and FZD8⁻/⁻ mice. (B) Quantification of the area positive for α-sm-actin around the vessels in WT and FZD8⁻/⁻ mice. Data represent mean ± s.e.m. of 10 mice per group. (C) Representative staining for α-sm-actin in WT and FZD8⁻/⁻ mice. WT and FZD8⁻/⁻ mice were exposed to cigarette smoke for 4 days. mRNA expression was determined in whole lung homogenates. (D) FZD8 gene expression in whole lung homogenates of WT mice. (E) WNT-5A gene expression in whole lung homogenates of WT and FZD8⁻/⁻ mice. (F) WNT-5B gene expression in whole lung homogenates of WT and FZD8⁻/⁻ mice. (G) MUC5AC gene expression in whole lung homogenates of WT and FZD8⁻/⁻ mice. Data represent mean ± s.e.m. of 8 mice per group. * p < 0.05 compared to air exposed WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).
A pro-inflammatory role for the FZD8 receptor in chronic bronchitis

Figure 2: Acute cigarette smoke-induced airway inflammation is dependent on FZD8. WT and FZD8\(^{-/-}\) mice were exposed to cigarette smoke for 4 days. (A) Lymphocyte numbers in BALF; \(p = 0.068\) for the interaction of smoke exposure and genotype (two-way ANOVA). (B) Neutrophil numbers in BALF; \(p = 0.035\) for the interaction of smoke exposure and genotype (two-way ANOVA). (C) Total cell number in BALF; \(p = 0.079\) for the interaction of smoke exposure and genotype (two-way ANOVA). (D) Macrophage numbers in BALF; \(p = 0.105\) for the interaction of smoke exposure and genotype (two-way ANOVA). (E) CXCL2 gene expression in whole lung homogenates; \(p = 0.343\) for the interaction of smoke exposure and genotype (two-way ANOVA). (F) CXCL5 gene expression in whole lung homogenates; \(p = 0.021\) for the interaction of smoke exposure and genotype (two-way ANOVA). (G) KC gene expression in whole lung homogenates. Data represent mean ± s.e.m. of 8 mice per group. * \(p < 0.05\) ** \(p < 0.01\) *** \(p < 0.001\) compared to air exposed WT mice # \(p < 0.05\) compared to smoke exposed WT mice $ \(p < 0.05\) compared to air exposed FZD8\(^{-/-}\) mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test). ^^ \(p < 0.01\) compared to air exposed FZD8\(^{-/-}\) mice (two-tailed Student’s \(t\)-test).
A genetic association between FZD8 and CMH

In view of this pro-inflammatory role, we next studied the role of FZD8 in chronic bronchitis and found a genetic association between SNP rs663700 in the FZD8 region and CMH (p = 0.009; OR = 0.8059; 0.684-0.9495) in a large cohort of smoking individuals with and without COPD, implying that FZD8 may play a role in the development of CMH (figure 3A). Notably, we found that SNP rs663700 was associated with CMH already in the healthy smokers of the cohort (n = 1348; p = 0.002), indicating that the SNP links to CMH specifically and is independent of the presence of COPD. The protective C allele is associated with lower risk of having CMH and the susceptibility allele T with higher risk of having CMH. The five other SNPs tested in the FZD8 region were less or not associated with CMH (table 4).

Table 4 - OR for CMH for the 6 tested SNPs in the FZD8 region.

<table>
<thead>
<tr>
<th>SNP (Chromosome 10)</th>
<th>position (base pair)</th>
<th>OR</th>
<th>SE</th>
<th>p-value</th>
<th>left gene</th>
<th>right gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs640827</td>
<td>35941721.08499</td>
<td>0.07413</td>
<td>0.735</td>
<td>2.82 * 10^-02</td>
<td>GJD4</td>
<td>FZD8</td>
</tr>
<tr>
<td>rs10827519</td>
<td>35952397.07996</td>
<td>0.08744</td>
<td>0.6737</td>
<td>1.05 * 10^-02</td>
<td>GJD4</td>
<td>FZD8</td>
</tr>
<tr>
<td>rs663700</td>
<td>35956378.08095</td>
<td>0.08367</td>
<td>0.684</td>
<td>9.89 * 10^-03</td>
<td>GJD4</td>
<td>FZD8</td>
</tr>
<tr>
<td>rs596642</td>
<td>35976759.08652</td>
<td>0.07958</td>
<td>0.7403</td>
<td>6.89 * 10^-02</td>
<td>FZD8</td>
<td>LOC439954</td>
</tr>
<tr>
<td>rs11010252</td>
<td>35987557.1143</td>
<td>0.1164</td>
<td>0.9102</td>
<td>2.49 * 10^-01</td>
<td>FZD8</td>
<td>LOC439954</td>
</tr>
<tr>
<td>rs618443</td>
<td>35988716.1128</td>
<td>0.1019</td>
<td>0.9238</td>
<td>2.37 * 10^-01</td>
<td>FZD8</td>
<td>LOC439954</td>
</tr>
</tbody>
</table>

Next, we performed an eQTL analysis in a large dataset of lung tissue samples of non-, current- and ex-smokers with and without COPD [17] to determine the association between SNP rs663700 and the lung mRNA expression levels of genes within 100 kb, as has been described previously [17]. The eQTL analysis showed that SNP rs663700 has a cis-eQTL effect on FZD8 (p = 4.58 * 10^-11) and CCNY (Cyclin Y; p = 4.72 * 10^-4) gene expression in lung tissue (n = 1,095). The strongest eQTL effect was on FZD8 where the protective C allele is associated with lower expression and the susceptibility allele T with higher expression of FZD8 (results from the meta-analysis across the three cohorts: p = 4.94 * 10^-10; Spearman r = 0.19; figures 3B-E). The allele frequency within the study population was 65.8% for the homozygous CC, 30.3% for the heterozygous CT and 3.9% for the homozygous TT.

FZD8 expression in lung fibroblasts is associated with CMH

We previously established that FZD8 is expressed in fibroblasts and that its expression is increased in COPD [8]. To link these previous findings with the current observation that FZD8 regulates inflammation and is involved in chronic bronchitis, we studied the pro-inflammatory role of FZD8 in human airway and parenchymal lung fibroblasts from ex-smoking GOLD grade 4 COPD patients with and without CMH. We found that interleukin (IL)-1β and epidermal growth factor (EGF) induced a strong increase in FZD8 gene expression in lung fibroblasts. Interestingly, this normalized increase in FZD8 gene expression was stronger in primary airway and parenchymal lung fibroblasts of GOLD grade 4 COPD patients with CMH than in those without CMH (figures 4A,C). The mean basal FZD8 gene expression did not differ with respect to CMH status. No differences in gene expression with respect to CHM status were observed for the effects of IL-1β or EGF on WNT-5A, WNT-5B, WNT-16, FZD2 and FZD6 gene expression in parenchymal
A pro-inflammatory role for the FZD8 receptor in chronic bronchitis

Figure 3: Genetic association between FZD8 and CMH. (A) Genetic association between SNP rs663700 in the FZD8 region and CMH within a cohort of heavy smokers with and without COPD (n = 717 with CMH and n = 1,795 without CMH); p = 0.009; OR = 0.8059; 0.684 - 0.9495. FZD8 and SNP rs663700 are located on chromosome 10. C is the WT allele and protective, and T is the variant, susceptibility allele of SNP rs663700. CEU stands for Caucasian population in the HapMap project. (B) eQTL analysis results from the Groningen cohort (n=363). Boxplots show the relationship between gene expression and genotype; p = 6.85 * 10^{-05}. (C) eQTL analysis results from the Laval cohort (n=409). Boxplots show the relationship between gene expression and genotype; p = 3.43 * 10^{-05}. (D) eQTL analysis results from the UBC cohort (n=339). Boxplot show the relationship between gene expression and genotype UBC; p = 0.009. (E) eQTL analysis results from the meta-analysis across the three cohorts. Boxplots show the relationship between gene expression and genotype; p = 4.94 * 10^{-10}. 
lung fibroblasts (figures 5A-B), indicating a specific effect on FZD8. Although TGF-β did augment FZD8 gene expression, there was no significant difference between the two patient groups regarding CMH status, whereas tumor necrosis factor (TNF)-α and cigarette smoke extract did not augment FZD8 gene expression (figure 5C). This indicates a specific effect for IL-1β and EGF. Interestingly, receptor expression of EGFR was higher in airway fibroblasts and pulmonary fibroblasts of GOLD grade 4 COPD patients with CMH than without CMH, as well as IL1R1 expression in airway fibroblasts of GOLD grade 4 COPD patients with CMH than without CMH (figures 4B,D), suggesting a link between receptor expression and functional FZD8 induction by IL-1β and EGF.
A pro-inflammatory role for the FZD8 receptor in chronic bronchitis

Figure 4: IL-1β- and EGF-induced FZD8 expression is associated with CMH. Primary airway and parenchymal lung fibroblasts of GOLD grade 4 COPD patients with and without CMH were stimulated for 4 hours with IL-1β (1 ng/ml) or EGF (10 ng/ml) to study FZD8 gene expression. Basal delta Cq-values did not differ between groups: parenchymal fibroblasts GOLD grade 4 COPD: 16.29±0.61, airway fibroblasts GOLD grade 4 COPD: 16.28±0.28, parenchymal fibroblasts GOLD grade 4 COPD with CMH: 15.96±0.38, airway fibroblasts GOLD grade 4 COPD with CMH: 15.99±0.51. (A) IL-1β-induced FZD8 gene expression. (B) Basal IL1R1 gene expression. (C) EGF-induced FZD8 gene expression. (D) Basal EGFR gene expression. Data represent induction of gene expression of 8 patients per group. * p < 0.05 ** p < 0.01 (one-way ANOVA with Student-Newman-Keuls multiple comparisons test).
Figure 5: WNT/FZD gene expression in primary human parenchymal lung fibroblasts. Primary parenchymal lung fibroblasts of GOLD grade 4 COPD patients with and without CMH were stimulated for 4 hours with 5% CSE, IL-1β (1 ng/ml), TNF-α (10 ng/ml), TGF-β (2 ng/ml) or EGF (10 ng/ml) to study WNT-5A, WNT-5B, WNT-16, FZD2, FZD6 and FZD8 gene expression. (A) IL-1β-induced gene expression. Basal delta Cq-values did not differ between groups: parenchymal fibroblasts GOLD grade 4 COPD: 16.29±0.61, airway fibroblasts GOLD grade 4 COPD: 16.28±0.28, parenchymal fibroblasts GOLD grade 4 COPD with CMH: 15.66±0.38, airway fibroblasts GOLD grade 4 COPD with CMH: 15.66±0.51. (B) EGF-induced gene expression. (C) CSE, IL-1β, TNF-α, TGF-β and EGF effects on FZD8 gene expression. Data represent mean ± s.e.m. of induction of FZD8 gene expression of 8 patients per group. *** p < 0.001 compared to control (one-way ANOVA with Student-Newman-Keuls multiple comparisons test).
FZD8 gene expression in lung fibroblasts is regulated by SNP rs663700

We further analyzed the genetic basis of FZD8 gene expression in airway and parenchymal lung fibroblasts of GOLD grade 4 COPD patients with and without CMH. The fold induction of FZD8 gene expression after stimulation with IL-1β and EGF was found to be dependent on the genotype of the patients with respect to SNP rs663700. In line with the results of our lung eQTL analysis, airway fibroblasts of GOLD grade 4 COPD patients with the CT variant showed a higher induction of FZD8 gene expression than did patients with the CC variant after IL-1β but not after EGF stimulation (figures 6A-B). In parenchymal lung fibroblasts, this effect was less pronounced (figure 7A-B). The low allele frequency (3.9%) of the TT variant precluded us from obtaining sufficient numbers of donors to analyze the impact of the TT variant on gene expression in these studies. Our results suggest that variants in FZD8 gene expression in lung fibroblasts play a role in COPD patients who have CMH.

Figure 6: IL-1β- and EGF-induced increase of FZD8 gene expression in GOLD grade 4 COPD patients with and without CMH is regulated by SNP rs663700. Relationship between the fold induction in FZD8 gene expression in airway fibroblasts of GOLD grade 4 COPD patients after stimulation with IL-1β or EGF and the FZD8 genotype of the patients, with regard to SNP rs663700. (A) IL-1β-induced FZD8 gene expression. (B) EGF-induced FZD8 gene expression. The experiment was performed for 8 patients per group, 4 of which had a known genotype. Data represent 4 patients with CC and 4 patients with CT. * p < 0.05 (one-tailed Mann-Whitney test).

Figure 7: IL-1β- and EGF-induced increase of FZD8 gene expression in parenchymal lung fibroblasts of GOLD grade 4 COPD patients: correlation to SNP rs663700. Correlation of the fold induction in FZD8 gene expression in parenchymal lung fibroblasts of GOLD grade 4 COPD patients after stimulation with IL-1β or EGF to the genotype of the patients, concerning SNP rs663700. (A) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with IL-1β and patient genotype. (B) Correlation between the fold induction in FZD8 gene expression after stimulation of parenchymal lung fibroblasts with EGF and patient genotype. Data represent 5 patients with CC and 6 patients with CT; (one-tailed Mann-Whitney test).
Chapter 4

FZD8 regulates IL-6 and CXCL8 secretion by lung fibroblasts
In view of our findings using the FZD8<sup>+/−</sup> mice, our next aim was to investigate in what way the changed expression in FZD8 gene expression in lung fibroblasts may affect pro-inflammatory responses. We investigated which cytokines are produced by primary MRC-5 human lung fibroblasts in response to IL-1β and EGF. Stimulation with IL-1β induced granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, chemokine ligand CXCL8, interferon gamma-induced protein (IP)-10 and monocyte chemotactic protein (MCP)-1 secretion (figure 8A). Stimulation with EGF induced GM-CSF, IL-6 and CXCL8 secretion (figure 8B). We hypothesized that FZD8 may regulate the IL-1β- and EGF-induced release of IL-6 and CXCL8 from fibroblasts, as these cytokines are increased in COPD and contribute largely to the inflammation seen in COPD [24]. Moreover, in accordance with a previous study [25] we observed that IL-6 and CXCL8 induce MUC5AC gene expression and CXCL8 induces PAS positive mucin protein expression indicative of goblet cell differentiation in differentiated primary human airway epithelial cells from healthy donors (figures 8C-D), further linking these cytokines to CMH. We were, however, not able to detect MUC5AC protein release. We found that IL-1β dose-dependently increased both IL-6 and CXCL8 secretion from primary MRC-5 human lung fibroblasts (figures 9A-B), whereas EGF dose-dependently increased CXCL8 secretion (figure 9C), but not IL-6 secretion (figure 10A). FZD8 knockdown by specific siRNA (knockdown efficiency 65% on average; see figures 10B-C) significantly reduced IL-1β-induced IL-6 secretion (p = 0.037 for the interaction of IL-1β stimulation and siRNA knockdown (two-way ANOVA) and CXCL8 secretion (p = 0.008 for the interaction of IL-1β stimulation and siRNA knockdown (two-way ANOVA)) (figures 9A-B). EGF-induced CXCL8 secretion was also reduced by FZD8 knockdown (p < 0.001 for the interaction of EGF stimulation and siRNA knockdown (two-way ANOVA) (figure 9C). These data show that FZD8 is involved in the IL-1β-induced secretion of IL-6 and CXCL8 as well as in the EGF-induced secretion of CXCL8. Furthermore, these data stress the importance of the fibroblast in the inflammatory response as seen in chronic bronchitis in COPD.
A pro-inflammatory role for the FZD8 receptor in chronic bronchitis

Figure 8: IL-6 and CXCL8 are released by primary MRC-5 human lung fibroblasts and induce MUC5AC gene expression in ALI-cultured primary human airway epithelial cells. Primary MRC-5 human lung fibroblasts were stimulated for 24 hours with IL-1β (1 ng/ml) or EGF (10 ng/ml). A Milliplex® was performed to screen for 26 cytokines to investigate which cytokines were produced. (A) IL-1β-induced cytokine secretion from primary MRC-5 human lung fibroblasts. (B) EGF-induced cytokine secretion from primary MRC-5 human lung fibroblasts. Data represent mean ± s.e.m. of 5 independent experiments. * p < 0.05 ** p < 0.01 *** p < 0.001 compared to basal conditions (two-tailed Student’s t-test). Primary human airway epithelial cells were stimulated for 24 hours with IL-6 (10 ng/ml) and CXCL8 (10 ng/ml) to study MUC5AC gene expression. (C) IL-6- and CXCL8-induced MUC5AC gene expression in differentiated primary human airway epithelial cells. Data represent mean ± s.e.m. of 5 independent experiments. (D) IL-6- and CXCL8-induced number of goblet cells per mm basement membrane (BM) in paraffin-embedded sections of differentiated primary human airway epithelial cells. Data represent mean ± s.e.m. of 4 independent experiments. * p < 0.05 compared to basal conditions (two-tailed Student’s t-test).
Chapter 4

Figure 9: FZD8 regulates IL-1β- and EGF-induced IL-6 and CXCL8 secretion. Primary MRC-5 human lung fibroblasts were transfected with specific FZD8 siRNA or non-targeting siRNA and stimulated for 24 hours with increasing concentrations of IL-1β (0.03-3 ng/ml) and EGF (0.1-10 ng/ml). (A) IL-1β-induced IL-6 secretion by primary MRC-5 human lung fibroblasts; p = 0.037 for the interaction of IL-1β stimulation and siRNA knockdown (two-way ANOVA). (B) IL-1β-induced CXCL8 secretion by primary MRC-5 human lung fibroblasts; p = 0.008 for the interaction of IL-1β stimulation and siRNA knockdown (two-way ANOVA). (C) EGF-induced CXCL8 secretion by primary MRC-5 human lung fibroblasts; p < 0.001 for the interaction of EGF stimulation and siRNA knockdown (two-way ANOVA). Data represent mean ± s.e.m. of 5 (CXCL8) or 4 (IL-6) independent experiments. ** p < 0.01 *** p < 0.001 compared to control # p < 0.05 compared to stimulated control siRNA (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).
Figure 10: EGF effects on IL-6 secretion by MRC-5 human lung fibroblasts.
Primary MRC-5 human lung fibroblasts were transfected with specific FZD8 siRNA or non-targeting siRNA and stimulated for 24 hours with increasing concentrations of EGF (0.1-10 ng/ml). (A) EGF-induced IL-6 secretion by primary MRC-5 human lung fibroblasts. Data represent mean ± s.e.m. of 4 independent experiments. (B) FZD8 knockdown for the experiments shown in figures 9A-B. Data represent mean ± s.e.m. of 5 independent experiments. (C) FZD8 knockdown for the experiments shown in figure 9C and 10A. Data represent mean ± s.e.m. of 4 independent experiments. * p < 0.05 compared to control siRNA # p < 0.05 ## p < 0.01 compared to stimulated control siRNA (two-way ANOVA with Student-Newman-Keuls multiple comparisons test).
Chapter 4

Discussion

This study shows for the first time that FZD8 plays a critical role in inflammatory processes involved in chronic bronchitis, including CMH, and reveals an unexpected role for the fibroblast in this process. We found that acute cigarette smoke-induced airway inflammation in an in vivo mouse model is partly FZD8 dependent. In FZD8−/− mice, cigarette smoke did not induce neutrophilic inflammation, which was accompanied by the reduced expression of CXCL5 and to a lesser extent of CXCL2 and KC compared to cigarette smoke exposed WT mice. Furthermore, we show that there is an association between pro-inflammatory cytokine-induced FZD8 gene expression in airway and parenchymal lung fibroblasts of GOLD grade 4 COPD patients and the presence of CMH. This is explained in part by increased EGFR and IL1R1 receptor expression in fibroblasts of GOLD grade 4 COPD patients with CMH compared to patients without CMH and in part by a polymorphism in the FZD8 region. SNP rs663700 in the FZD8 region is associated with CMH in a cohort of smokers and additionally is a cis-eQTL in lung tissue, regulating FZD8 expression. Moreover, SNP rs663700 is associated with increased FZD8 gene expression in fibroblasts of GOLD grade 4 COPD patients with CMH. Upon stimulation with IL-1β and EGF, primary MRC-5 human lung fibroblasts secrete IL-6 and CXCL8, a process which is regulated by FZD8, and we show that both of these cytokines are able to initiate MUC5AC gene expression and CXCL8 induces goblet cells in differentiated primary human airway epithelial cells.

COPD is a heterogeneous disease characterized by different patient subpopulations [26]. We investigated the potential role for FZD8 in chronic bronchitis, and found a clear association between the cytokine-induced expression of FZD8 gene expression and CMH, a clinical expression which is a consequence of the pathologic process in chronic bronchitis. Of importance is to emphasize that the genetic association was independent of the presence of COPD, indicating that FZD8 likely plays a role in CMH in healthy smokers as well. CMH is associated with bronchial inflammation, accelerated lung function decline, COPD morbidity and increased mortality [4-6]. The association of the genetic variance in FZD8 with CMH is the first evidence to support a genetic link between the WNT signalling pathway and a phenotype of COPD. Interestingly, we also found a specific role for FZD8 in the chronic bronchitis phenotype, reinforcing the contention that phenotypes of COPD may be characterized by specific pathophysiological mechanisms.

While the communication of both damaged and intact epithelium to the fibroblasts is widely recognized in the airways [27], we now find indications that fibroblasts play an additional crucial role in inflammation and epithelial mucus production. Upon damage, the epithelium secretes inflammatory cytokines such as EGF, IL-1β and IL-1α. IL-1α has been shown to induce IL-6 and CXCL8 in fibroblasts [28]. Importantly, IL-6 and CXCL8 secretion from primary MRC-5 human lung fibroblasts are increased upon co-culturing these cells with human bronchial epithelial (16-HBE) cells. This process is mediated via IL-1α [29]. IL-1α and IL-1β are known to function via the same receptor, IL1R1. IL-1β and EGF stimulated fibroblasts to produce IL-6 and CXCL8 via FZD8 in our study. We propose that in this way FZD8 contributes to the inflammation present in chronic bronchitis. Our data support this hypothesis, as neutrophil and lymphocyte recruitment in the BALF are reduced in FZD8−/− mice as well as mRNA expression of the neutrophil attractants CXCL5 and KC in whole lung homogenates, whereas IL-6 and CXCL8 induced mucus production in differentiated primary human airway epithelial cells. Both cytokines are secreted by
human lung fibroblasts upon stimulation with IL-1β and EGF, and we demonstrate that this process is regulated by FZD8. In addition, we show that expression of IL1R1 and the EGFR is increased in fibroblasts of GOLD grade 4 COPD patients with CMH compared to patients without CMH. This shows that IL-1β and EGF lead to increased FZD8 expression in CMH in part via increased expression of their receptors, and in part based on FZD8 genotype.

For many WNT ligands, it is either not known or highly context dependent via which FZD receptor they exert their effect. FZD8 is a known receptor for WNT-5A [9] and WNT-5B [30] in airway smooth muscle cells and pulmonary fibroblasts. However, based on the crystal structure of the cysteine rich domain of FZD8, it must be assumed that most if not all WNT ligands are potential agonists [31], whereas R-spondins have also been described as FZD8 agonists [32]. Conversely, connective tissue growth factor [33] and insulin-like growth factor-binding protein-4 [34] may antagonize FZD8 by direct binding. WNT-5A and WNT-5B are highly expressed in fibroblasts [8], and a role of FZD8 in their responses has been described [9, 30] Furthermore, the regulation by WNT-5A of CXCL8 release from human neutrophils has been described [14] Therefore, WNT-5A and WNT-5B are potential ligands for FZD8 in chronic bronchitis, which is supported by our findings that WNT-5A and WNT-5B induce IL-6 and CXCL-8 release from human lung fibroblasts [35]. Clearly, a better appreciation of the ligands involved in FZD8 signalling, their cellular sources and their role in chronic bronchitis require further studies.

COPD is currently treated symptomatically and not phenotype specific. The awareness that COPD patients need treatment according to their phenotype stimulated the investigation of new drug targets [24]. We found IL-6 and CXCL8 to be important in inducing mucus production. There are no biologicals used in COPD that target IL-6. Blocking CXCL8 by using a blocking antibody only or by blocking the CXCR2 receptor has been reported not to have a clinical impact [36]. Our data show that targeting FZD8 on fibroblasts could prevent both the secretion of IL-6 and CXCL-8, leading to a reduction in inflammatory processes and mucus secretion. Therefore, FZD8 may provide a rational drug target for chronic bronchitis and the development of FZD8 receptor antagonists may be warranted.

In conclusion, our results indicate that FZD8 plays an important pro-inflammatory role in chronic bronchitis. Interestingly, the fibroblast appears to play a major role herein. We provide genetic and functional data in vitro and in vivo supporting the role of FZD8 in pro-inflammatory cytokine production, inflammatory cell recruitment and mucus hypersecretion. These findings show a potential important role for WNT signalling via FZD8 in fibroblasts in chronic bronchitis. Therefore, targeting FZD8 is a strategy worth pursuing for the treatment of chronic bronchitis.

Acknowledgments

The authors would like to thank L.L.G. Šebek and I.S.T. Bos at the University of Groningen and the research staff at the Respiratory Health Network Tissue Bank of the FRQS for their valuable assistance.
References


5 Ramos FL, Krahne JS, Kim V. Clinical issues of mucus accumulation in COPD. *Int J Chron Obstruct Pulmon Dis* 2014;9:139-150.


28 Fuchigami T, Kibe T, Koyama H, et al. Regulation of IL-6 and IL-8 production by reciprocal cell-to-cell interactions between tumor cells and stromal fibroblasts through IL-1alpha in ameloblastoma. *Biochem Biophys Res Commun* 2014.

29 Osei ET, Brandsma CA, Noordhoek JA, et al. Crosstalk between epithelium and fibroblasts; implications for COPD. *Eur Respir J* 2014;38:

30 Spanjer AIR, Jansen SR, Baarsma HA, et al. TGF-β-induced extracellular matrix protein expression in human lung fibroblasts requires autocrine WNT signaling via the Frizzled-8 receptor. *Am J Respir Crit Care Med* 2012;185:


