Chapter 5

A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk

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Chapter 5

Abstract

Rationale

Epithelial-fibroblast communication is an important aspect in the pathophysiology of chronic obstructive pulmonary disease (COPD). We previously showed that epithelial cells drive pro-inflammatory responses in fibroblasts. Here, we investigated if and which WNT ligands are involved in the inflammatory response that is induced upon co-culturing fibroblasts with epithelial cells.

Methods

MRC-5 human lung fibroblasts were co-cultured with 16HBE bronchial epithelial cells, and primary airway fibroblasts of ex-smoking COPD grade 4 patients were co-cultured with differentiated primary airway epithelial cells of healthy donors. We determined the expression of WNT ligands, FZD receptors and the inflammatory cytokines interleukin (IL)-6 and chemokine ligand CXCL8. Functional effects of WNT ligands were studied in a co-culture model of MRC-5 human lung fibroblasts and differentiated primary airway epithelial cells by inhibiting all WNT secretion using IWP-2 and specific WNTs using neutralizing antibodies. mRNA and protein expression were determined using PCR and ELISA.

Results

WNT-4 and WNT-7B were predominantly expressed in epithelial cells, while WNT-5A and WNT-5B were most strongly expressed in fibroblasts. Upon co-culturing primary airway fibroblasts of COPD patients with differentiated airway epithelial cells, we found an increase in WNT-4 and WNT-7B mRNA expression in epithelial cells, while WNT-5A and WNT-5B mRNA expression in fibroblasts was not changed. This was accompanied by an increase in IL-6 and an even stronger increase in CXCL8 protein secretion in the basolateral medium. Moreover, we found IL-6 and CXCL8 mRNA expression to be increased in fibroblasts upon co-culture. Inhibition of WNT secretion using the porcupine inhibitor IWP-2 further increased IL-6 protein secretion and did not affect CXCL8 protein secretion in co-culture. Furthermore, anti-WNT-4, but not anti-WNT-7B, anti-WNT-5A or anti-WNT-5B further increased IL-6 protein secretion upon co-culturing MRC-5 human lung fibroblasts with differentiated primary airway epithelial cells.

Conclusion

These data show that WNT-4 suppresses IL-6 protein secretion that is induced upon co-culturing fibroblasts with epithelial cells.
Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible and usually progressive. Chronic inflammation, remodelling of the small airways and parenchymal destruction all contribute to this airflow limitation [1-3]. Abnormal tissue damage and repair responses lead to airway epithelial changes and mesenchymal activation and are thought to contribute to the pathophysiology of COPD [4-6].

Epithelial cells are the first line of defence against exposure to harmful substances in the air. Especially upon damage, epithelial cells secrete cytokines and growth factors that can have their effect on the underlying fibroblasts. Fibroblasts contribute to remodelling by producing extracellular matrix proteins or by differentiating into more contractile myofibroblasts [7, 8]. In addition, fibroblasts have an important pro-inflammatory role in COPD [chapter 4]. It was recently reported that in the communication between epithelial cells and fibroblasts, epithelial cells drive the pro-inflammatory response of fibroblasts [9]. When placed in co-culture, the epithelial production of the cytokine interleukin (IL)-1α causes fibroblasts to produce more chemokine ligand CXCL8 [9].

The wingless/integrase-1 (WNT) signalling pathway plays an essential role in lung development [10]. Recent studies also indicate the involvement of the WNT signalling pathway in remodelling [11, 12] and inflammatory processes [13, 14] in the lungs. For the secretion of WNT ligands, acetylation by the endoplasmic reticulum transmembrane protein porcupine is necessary, upon which WNT ligands can have paracrine or autocrine effects [15]. WNT ligands bind to transmembrane Frizzled (FZD) receptors and can together with different co-receptors regulate cell differentiation, growth and polarity in a variety of cell systems [16, 17]. WNT/FZD combinations activate either the canonical WNT pathway, which signals via β-catenin, or one of the noncanonical pathways, which mainly signal to calcium (WNT/Ca²⁺ pathway) or to RhoA/c-Jun N-terminal kinase (JNK; WNT/planar cell polarity (PCP) pathway) [16, 18, 19]. WNT ligands and FZD receptors are expressed by virtually all cells. The effect of a WNT ligand depends on the FZD and co-receptors that are expressed by the specific target cell type [20]. WNT-3A [13], WNT-4 [21, 22], WNT-5A [23-26], WNT-5B [26], FZD5 [23] and FZD8 [chapter 4] have recently been shown to exert a pro-inflammatory role in the lungs. However, it is currently unknown whether WNT ligands contribute to pro-inflammatory processes that are directed by the communication between epithelial cells and fibroblasts.

We previously found increased expression of WNT-5B and FZD8 in pulmonary fibroblasts of GOLD grade 2 and grade 4 patients compared to fibroblasts of non-COPD controls [27], increased expression of WNT-5A in whole lung homogenates of GOLD grade 2 and 4 COPD patients compared to those of non-COPD controls [26] and increased expression of WNT-4 in epithelial cells of COPD patients compared to control smokers [21]. Moreover, we showed that FZD8 regulates IL-6 and CXCL8 protein secretion in human lung fibroblasts and that these cytokines induce MUC5AC mRNA expression in differentiated epithelial cells [chapter 4]. In addition, WNT-4 [21, 22], WNT-5A and WNT-5B [26] have been shown to regulate pro-inflammatory responses by the epithelium and fibroblasts. This indicates that WNT ligands may be involved in the dysregulated communication between fibroblasts and epithelial cells, thereby contributing to pathological features observed in COPD.
Here, we investigated whether WNT signalling contributes to pro-inflammatory processes that have been implicated in COPD and can occur in the epithelial-fibroblast cross-talk. We studied WNT and FZD expression in human bronchial epithelial (16HBE14o-) and lung fibroblast (MRC-5) cell lines as well as in a co-culture of differentiated primary airway epithelial cells from controls with primary airway fibroblasts of COPD patients. Furthermore, we studied functional WNT signalling interactions in a co-culture of differentiated primary airway epithelial cells from controls with MRC-5 human lung fibroblasts and found an unexpected suppressive role for WNT-4 on IL-6 protein secretion.

Methods

Cell culture

Fibroblast cell culture
Primary airway fibroblasts from ex-smoking GOLD grade 4 COPD patients (n = 16) were isolated from residual donor lung tissue from transplant procedures as has been described previously [28]. Patient characteristics are shown in table 1. Primary MRC-5 human lung fibroblasts [29] were obtained from Sigma (St. Louis, MO, USA). Primary human airway fibroblasts and MRC-5 human lung fibroblasts were cultured in Ham’s F12 medium supplemented with 10% (v/v) foetal 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 12-well cluster plates.

### Table 1 - Patient characteristics GOLD grade 4 COPD patients.

<table>
<thead>
<tr>
<th>number of subjects</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>56 (48-62)</td>
</tr>
<tr>
<td>Seks</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>7</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
</tr>
<tr>
<td>Pack-years (range)</td>
<td>38.1 (15-72)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>ex-smoker</td>
</tr>
<tr>
<td>% predicted FEV₁ (range)</td>
<td>16.9 (12.23-25.69)</td>
</tr>
<tr>
<td>FEV₁/FVC (range)</td>
<td>0.27 (0.16-0.66)</td>
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</tbody>
</table>

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

Air liquid interface (ALI) epithelial 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 and 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.
A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk

(Transwell® Permeable Supports, Corning, NY, USA) coated well in 1:1 Bronchial Epithelial Cell Growth Medium (BEGM; Lonza, Walkersville, MD, USA)/Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY, USA) 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 triiodothyronine (T3), 0.5 μg/ml noradrenaline (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.

Co-culture of 16HBE14o- and MRC-5 cells
16HBE14o- cells [30] were obtained from Dr. Gruenert (University of California, San Francisco, CA, USA). Cells were first grown separately to confluence on 24-well plates (MRC-5 cells) or on collagen-coated 6.5 mm inserts (16HBE14o- cells) in Eagle’s minimum essential medium (EMEM) + 10% foetal calf serum. Inserts with or without 16HBE14o-cells were then placed in wells with or without MRC-5 fibroblasts. After 72 hours, cells were serum-deprived for 24 hours and incubated in serum-free medium for another 24 hours. Subsequently, cells were harvested to determine mRNA expression. Basolateral and apical medium was collected for protein analysis.

Co-culture of differentiated airway epithelial cells and primary airway fibroblasts or MRC-5 fibroblasts
Preceding co-culture, primary human airway epithelial cells of controls were differentiated and cultured separately as described above. After differentiation, cells were serum-deprived for 16 hours in BEGM + ITS (ITS: 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenium). Differentiated primary human airway epithelial cells were then cultured with or without primary airway fibroblasts of GOLD grade 4 COPD patients or MRC-5 human lung fibroblasts for 24 hours in BEGM + ITS. Cells cultured separately were used as a control. In some experiments, co-culture with MRC-5 human lung fibroblasts was in the presence or absence of IWP-2 (100nM), control-IGG (4 μg/ml), anti-WNT-4 (4 μg/ml), anti-WNT-5A (4 μg/ml), anti-WNT-5B (1:250) or anti-WNT-7B (4 μg/ml). Basolateral medium was collected to determine protein secretion and cells were harvested for mRNA analysis.

Cytokine secretion
IL-6 and CXCL8 protein secretion was determined in cell-free basolateral and apical medium using enzyme-linked immunosorbent assay (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 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.

<table>
<thead>
<tr>
<th>Table 2 - Human primers used for the determination of specific genes of interest.</th>
<th>Primer sequence</th>
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<tr>
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<td>Reverse 5’</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Reverse 5’</td>
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</tr>
<tr>
<td>FZD8 Forward 5’</td>
<td>GAC ACT TGA TGG GCT GAG GT 3’</td>
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<tr>
<td>Reverse 5’</td>
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<td>CCG TGT GTG TGT GTG TGT GT 3’</td>
</tr>
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<tr>
<td>Reverse 5’</td>
<td>TGG AAC TTA CCC ATC CCA TA 3’</td>
</tr>
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<tr>
<td>Reverse 5’</td>
<td>TTG GCA AAT GCT TTC GCT C 3’</td>
</tr>
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</table>

Materials and reagents
Human WNT-4 polyclonal antibody (AF6076), human/mouse WNT-5A monoclonal antibody (Clone 442625; MAB645) and human WNT-7B polyclonal antibody (AF3460) were obtained from R&D Systems (Minneapolis, MN, USA). IWP-2 (I0536; N-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-4-oxo-3-phenylthieno[3,2-d]pyrimidin-2-yl)thio]-acetamide) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit WNT-5B polyclonal antibody was obtained from Abcam (ab93134; Cambridge, UK). All other chemicals were of analytical grade.

Statistical analysis
Statistical analyses were performed with Microsoft® Excel™ (2010). For comparison between two conditions a t-test assuming equal variances or a t-test assuming unequal variances (α = 0.05, tested two-tailed) was used as indicated in the figure legends. Synergism in figure 3 was determined by comparing the level of cytokine secretion in coculture to the sum of the levels of cytokine secretion in separately cultured cells. P < 0.05 was considered significant.
Results

WNT ligands and FZD receptors are differentially expressed in 16HBE14o- human bronchial epithelial cells and MRC-5 human lung fibroblasts.

To investigate whether WNTs and FZDs contribute to epithelial-mesenchymal communication, we first determined the WNT ligands and FZD receptors that are most abundantly expressed by each cell type. We assessed the mRNA expression of WNT-4, WNT-5A, WNT-5B, WNT-7B, WNT-10B and WNT-11 as well as FZD2, FZD6 and FZD8 based on our previous observations showing abundant expression of these WNT ligands and receptors in fibroblasts and epithelial cells [21, 27]. We found that both cell types expressed these WNTs and FZDs, however with a distinct profile (figure 1A). Human bronchial epithelial 16HBE14o- cells more abundantly expressed FZD6 (p < 0.01), WNT-4 (p < 0.01), WNT-7B (p < 0.001) and WNT-10B (p < 0.01) compared to MRC-5 human lung fibroblasts, while MRC-5 fibroblasts displayed a stronger expression of FZD8 (p < 0.001), WNT-5A (p < 0.001) and WNT-5B (p < 0.01; figure 1A) in comparison to 16HBE14o- cells. Although expression of WNT-10B was significantly higher in epithelial cells (p < 0.01), expression in both cell types was very low. WNT-11 mRNA expression was not different between epithelial cells and fibroblasts. We therefore focused our further studies on WNT-4 and WNT-7B as epithelial derived WNTs and on WNT-5A and WNT-5B as fibroblast derived WNTs.

IL-6 secretion is increased in a co-culture of 16HBE14o- human bronchial epithelial cells and MRC-5 human lung fibroblasts.

Data from our group previously showed that the co-culture of 16HBE14o- epithelial cells and MRC-5 human lung fibroblasts results in a strong increase of the inflammatory cytokine CXCL8, which is mainly produced by the fibroblasts and regulated by IL-1α [9]. Here, we found that the secretion of the pro-inflammatory cytokine IL-6 is also up-regulated in this co-culture at both 24 and 72 hours of co-culture (figure 1B).

Figure 1: WNT ligands and FZD receptors are differentially expressed in 16HBE14o- human bronchial epithelial cells and MRC-5 human lung fibroblasts. We determined mRNA expression in 16HBE14o- and MRC-5 cells as described in the methods section. (A) mRNA expression of FZD2, FZD6, FZD8, WNT-4, WNT-5A, WNT-5B, WNT-7B, WNT-10B and WNT-11 in 16HBE14o- and MRC-5 cells. High Ct-values respond to low copy numbers. Data represent mean ± s.e.m. of 3 independent experiments. * p < 0.05 ** p < 0.01 *** p < 0.001 MRC-5 cells compared to 16HBE14o- cells (two-tailed t-test assuming equal variances). (B) IL-6 protein secretion in mono- and co-cultured 16HBE14o- and MRC-5 cells after 24 hours and 72 hours. Data represent mean ± s.e.m. of 3 independent experiments. * p < 0.05 compared to mono-culture of the same time point $ p < 0.05 $$ p < 0.01$ compared to the same culture condition of different time points (two-tailed t-test assuming unequal variances).
**IL-6, CXCL8, WNT-4 and WNT-7B expression are increased in a co-culture model of normal differentiated primary epithelial cells and primary fibroblasts from COPD patients**

We previously found that increased WNT signalling in fibroblasts of COPD patients contributes to inflammation [27, chapter 4]. Therefore, we cultured normal primary human airway epithelial cells from residual donor lung tissue from transplant procedures with primary airway fibroblasts of GOLD grade 4 COPD patients. We studied the mRNA expression of WNT-4 and WNT-7B in the epithelial cells and the mRNA expression of WNT-5A, WNT-5B, IL-6 and CXCL8 in the fibroblasts. Although there was a high interpatient variance, WNT-4 (p < 0.05) and WNT-7B (p < 0.05) expression in the epithelium significantly increased in differentiated normal primary human airway epithelial cells upon co-culture with primary airway fibroblasts of GOLD grade 4 COPD patients (figure 2A). Similar to the effects in cell lines, IL-6 (p < 0.05) and CXCL8 (p < 0.05) mRNA expression increased upon co-culture in the fibroblasts, whereas WNT-5A and WNT-5B did not change upon co-culture (figure 2A). In line with this finding, CXCL8 (p < 0.05) and to a lesser extent IL-6 (p < 0.01) protein secretion also increased upon co-culturing these cells (figure 2B).

![Figure 2](image_url)

**Figure 2: Co-culture of differentiated primary human airway epithelial cells and primary human airway fibroblasts increases WNT and inflammatory cytokine expression.** Differentiated primary airway epithelial cells and primary airway fibroblasts of GOLD grade 4 COPD patients were co-cultured for 24 hours as described in the methods section. (A) mRNA expression of WNT-4 and WNT-7B in epithelial cells and mRNA expression of WNT-5A, WNT-5B, IL-6 and CXCL8 in fibroblasts. Data represent data points of 16 individual patients and their mean. * p < 0.05 compared to separately cultured epithelial cells or fibroblasts (two-tailed t-test assuming unequal variances). (B) IL-6 and CXCL8 apical protein secretion of separately cultured fibroblasts and basolateral protein secretion in co-culture. Data represent mean ± s.e.m. of 16 individual patients. * p < 0.05 ** p < 0.01 compared to separately cultured fibroblasts (two-tailed t-test assuming unequal variances).
WNT-4 suppresses IL-6 secretion in a co-culture of differentiated primary airway epithelial cells and MRC-5 human lung fibroblasts

Next, we investigated whether secreted WNTs are involved in regulating IL-6 and CXCL8 protein secretion upon co-culturing fibroblasts with epithelial cells. Similar to previous results [9] and our data above, we found that in a co-culture of differentiated primary airway epithelial cells and MRC-5 human lung fibroblasts, IL-6 and CXCL8 increased upon co-culture (figures 3A-B). We found an additive effect of separately cultured fibroblasts and separately cultured epithelial cells for IL-6, and a synergistic effect for CXCL8 (p < 0.01; figure 3B). IL-6 and CXCL8 are mainly secreted by the fibroblasts, indicated by the higher mRNA levels in fibroblasts compared to the levels in epithelial cells in co-culture (figures 3C-D).

Figure 3: IL-6 and CXCL8 protein secretion is increased in a co-culture of differentiated primary human airway epithelial cells and MRC-5 human lung fibroblasts. Differentiated primary airway epithelial cells and MRC-5 human lung fibroblasts were co-cultured for 24 hours as described in the methods section. (A) IL-6 and (B) CXCL8 protein secretion in separately cultured and co-cultured differentiated primary airway epithelial cells and MRC-5 human lung fibroblasts. The increase in CXCL8 protein secretion in co-culture is synergistic (p = 0.006), determined by comparing the level of cytokine secretion in co-culture to the sum of the levels of cytokine secretion in separately cultured cells. (C) IL-6 and (D) CXCL8 mRNA expression in separately cultured and co-cultured differentiated primary airway epithelial cells and MRC-5 human lung fibroblasts. Data represent mean ± s.e.m. of 6 independent experiments. * p < 0.05 ** p < 0.01 *** p < 0.001 compared as indicated in the figure (two-tailed t-test assuming equal (D) or unequal (A;B;C) variances).
Chapter 5

To investigate whether WNT ligands contribute to the increase in IL-6 and CXCL8 protein secretion upon co-culture, we studied the effect of the porcupine inhibitor IWP-2 to block all WNT secretion from cells [31]. We found that in separately cultured epithelial cells and fibroblasts, IWP-2 did not affect IL-6 protein secretion. Of interest, in the co-culture model, inhibition of WNT secretion caused a further increase in IL-6 protein secretion, indicating a negative regulatory effect of WNTs on IL-6 protein secretion in this set-up (figure 4A). While we did not find this effect for CXCL8 protein secretion in co-culture, CXCL8 protein secretion in separately cultured epithelial cells was increased by adding IWP-2 (figure 4B).

To pinpoint which WNT has a regulatory function on IL-6 protein secretion, we added specific antibodies against WNT-4, WNT-5A, WNT-5B and WNT-7B in the basolateral compartment just prior to placing the cells in co-culture. Anti-WNT-4 up-regulated IL-6 protein secretion in co-culture, while anti-WNT-5A, anti-WNT-5B and anti-WNT-7B had no effect (figure 4C). None of the antibodies had an effect on CXCL8 protein secretion in the co-culture (figure 4D). Together, these data suggest that WNT-4 derived from epithelial cells has a negative regulatory role on IL-6, but not CXCL8 protein secretion by fibroblasts.

Discussion

This study shows for the first time that WNT ligands play a suppressive regulatory role in inflammatory cytokine secretion in a co-culture of epithelial cells and fibroblasts. More specifically, our data indicate that the release of WNT-4 from epithelial cells reduces the secretion of IL-6 upon co-culturing with fibroblasts. We found that WNT-4 and WNT-7B are predominantly expressed in epithelial cells, while WNT-5A and WNT-5B are abundantly expressed in fibroblasts, suggesting that these WNT ligands are secreted from these cells and can have paracrine effects on other cell types. Co-culturing differentiated primary airway epithelial cells isolated from residual donor lung tissue from transplant procedures with primary airway fibroblasts of severe (GOLD grade 4) COPD patients increased epithelial WNT-4 and WNT-7B mRNA expression together with IL-6 and CXCL8 protein secretion in the basolateral compartment. In a co-culture of differentiated airway epithelial cells with MRC-5 human lung fibroblasts we demonstrated that inhibiting WNT secretion using IWP-2 further increases IL-6, but not CXCL8 protein secretion. Using neutralizing antibodies, we observed that WNT-4 is responsible for this effect, indicating a specific anti-inflammatory role for WNT-4, regulating IL-6 protein secretion from fibroblasts upon co-culture with epithelial cells.

WNT-4 has recently been shown to be pro-inflammatory in epithelial cells from patients with COPD [21, 22]. Additionally, WNT-4 expression was increased in epithelial cells of COPD patients, but downregulated after stimulation with cigarette smoke extract [21, 22]. Durham et al. showed that increased WNT-4 expression is associated with increased IL8 expression in bronchial biopsy samples [22]. Similarly, we previously showed that WNT-4 up-regulates CXCL8 protein secretion via noncanonical signalling in bronchial epithelial cells from COPD patients; an effect that was aggravated in cells exposed to cigarette smoke extract [21]. In this study, we found that CXCL8 protein secretion in separately cultured epithelial cells is negatively regulated by WNTs. In addition, we found a protective role for WNT-4 in the production of the pro-inflammatory cytokine IL-6,
Figure 4: WNT-4 negatively regulates IL-6 protein secretion in a co-culture of differentiated primary human airway epithelial cells and MRC-5 human lung fibroblasts. Differentiated primary human airway epithelial cells were co-cultured with MRC-5 human lung fibroblasts for 24 hours in the presence or absence of IWP-2 (100nM), control-IGG (4 μg/ml), anti-WNT-4 (4 μg/ml), anti-WNT-5A (4 μg/ml), anti-WNT-5B (1:250) or anti-WNT-7B (4 μg/ml) as described in the methods section. (A) The effect of IWP-2 on IL-6 protein secretion in mono- and co-culture. Break: 10 – 100 pg/ml. (B) The effect of IWP-2 on CXCL8 protein secretion in mono- and co-culture. Break: 25 – 500 pg/ml. (C) The effect of different antibodies on IL-6 and (D) CXCL8 protein secretion in co-culture. Data represent mean ± s.e.m. of 5 independent experiments. * p < 0.05 compared to basal conditions (two-tailed t-test assuming equal (A) or unequal (B,C,D) variances).
without affecting CXCL8 protein secretion in co-cultured fibroblasts. One explanation for this contrast with our previous findings could be that the pro- or anti-inflammatory role of WNT-4 is dependent on the activation of canonical anti-inflammatory or noncanonical pro-inflammatory WNT signalling [24]. It was recently suggested by showing functional selectivity in the binding capacities of WNTs and FZDs that the downstream signalling cascades activated upon their binding are dependent on the presence of the different subtypes of FZD receptors [20]. Mammalian WNTs and FZDs are preserved between species and WNT-4 has been shown to be able to bind FZD6 in canine kidney cells [32], FZD7 and FZD8 in mouse thymocytes [33], and human FZD5 and FZD8 cysteine rich domains (CRDs) [20], while it does not bind to human FZD1 [20, 34], FZD2, FZD4 and FZD7 [20]. Analysis of FZD receptor expression revealed that both FZD6 and FZD8 are expressed by fibroblasts, although FZD6 was more abundantly expressed by epithelial cells. Whether FZD6 is involved in the regulation of IL-6 and CXCL8 production by fibroblasts is not known, but we previously showed that FZD8 regulates IL-1β- and epidermal growth factor (EGF)-induced IL-6 and CXCL8 protein secretion in human lung fibroblasts [chapter 4] and that WNT-5A [35] and WNT-5B [chapter 2] function as noncanonical ligands for this receptor. In line, other studies from our lab showed that WNT-5B and to a lesser extent WNT-5A can induce both IL-6 and CXCL8 from primary pulmonary fibroblasts via noncanonical WNT signalling [26] whereas Lyons et al. showed that the WNT-4/FZD6 pair activates canonical WNT signalling [32]. It is tempting to speculate that in our co-culture model, WNT-4 binds to FZD6 to activate canonical WNT signalling, thereby suppressing IL-6 protein secretion.

We found a synergistic increase for CXCL8 upon co-culturing epithelial cells and fibroblasts and an additive increase for IL-6 protein secretion. This difference could be explained by the observed inhibitory effect of WNT-4 on IL-6 but not on CXCL8 protein secretion. While we previously showed that FZD8 regulates both IL-6 and CXCL8 protein secretion from fibroblasts [chapter 4] and WNT-5A and WNT-5B induce both IL-6 and CXCL8 protein secretion from fibroblasts [26], we did not find a role for WNTs in the increased CXCL8 protein secretion in co-culture. An explanation for this discrepancy between the regulation of IL-6 and CXCL8 and the lack of a role for WNT signalling in the regulation of CXCL8 in our co-culture model could be that other factors, including IL-1α [9] strongly regulate CXCL8 protein secretion, with WNT signalling only exerting a minor effect.

Together, our previous and current data indicate that there is both a pro-inflammatory and an anti-inflammatory role for WNT-4 in airway pathology; a pro-inflammatory role for WNT-4 has been demonstrated in airway epithelial cells [21, 22], while in the current study we show an anti-inflammatory effect of WNT-4 on fibroblasts. Up-regulation of WNT-4 has been found in COPD patients [21, 22] and in line with the data presented in our study, this could serve as a protective mechanism, where acute cigarette smoking downregulates WNT-4 expression and chronic exposure to cigarette smoke, like in COPD patients, causes a protective up-regulation of WNT-4 with respect to IL-6 release by fibroblasts.

IL-6 levels are increased in sputum of COPD patients compared to healthy controls [36] and IL-6 levels are negatively correlated to forced expiratory volume in 1 second (FEV1), a measure of airway obstruction [37]. In addition, three SNPs in IL-6 have been reported to be associated with a decline in FEV1 and five SNPs in IL-6 are associated with susceptibility for COPD [38]. Negative regulation of IL-6 by WNT-4 may thus have a
A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk

A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk

... protective effect on the progression of lung function decline in COPD. An IL-6 inhibitor (tocilizumab) is already used for inhibiting inflammation in rheumatoid arthritis and currently multiple IL-6 inhibitors are tested for effectiveness in inflammatory diseases, including COPD [39-41].

In conclusion, our findings show the importance of identifying WNT-FZD pairs in the interaction between different cell systems as present in the lungs and indicate that WNT signalling plays an important role in airway epithelium-mesenchymal cross-talk, specifically with respect to the regulation of IL-6. Interestingly, WNT-4 has a negatively regulatory role on IL-6 protein secretion by fibroblasts in a co-culture with epithelial cells, and in this way may contribute to the pathogenesis of COPD.

Acknowledgments

The authors would like to thank J. Abma, M.H. Menzen and J.-P. Ng-Blichfeldt at the University of Groningen for their technical assistance.
Chapter 5

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A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk


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A role for WNT-4 in the regulation of pro-inflammatory responses driven by epithelial-mesenchymal cross-talk