Non-canonical WNT-5B signalling induces inflammatory responses in human lung fibroblasts.

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Running title: WNT-5A/B-induced inflammatory responses in human lung fibroblasts

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Abstract

Background: COPD is a progressive chronic lung disease characterized by pulmonary inflammation. Several recent studies indicate aberrant expression of WNT ligands and Frizzled receptors in the disease. For example, WNT-5A/B ligand expression was recently found increased in lung fibroblasts of COPD patients. However, possible effects of WNT-5A and WNT-5B on inflammation have not been investigated yet. In this study we assessed the regulation of inflammatory cytokine release in response to WNT-5A/B signalling in human lung fibroblasts.

Methods: Primary human fetal lung fibroblasts (MRC-5), and primary lung fibroblasts from COPD patients and non-COPD controls were treated with recombinant WNT-5A or WNT-5B to assess IL-6 and CXCL8 cytokine secretion and gene expression levels.

Results: Following WNT-5B, and to a lesser extent WNT-5A stimulation, fibroblasts showed increased IL-6 and CXCL8 cytokine secretion and mRNA expression. WNT-5B-mediated IL-6 and CXCL8 release was higher in fibroblasts from COPD patients than in non-COPD controls. In MRC-5 fibroblasts, WNT-5B-induced CXCL8 release was mediated primarily via the Frizzled-2 receptor and TAK1-signalling, whereas canonical β-catenin signalling was not involved. In further support of non-canonical signalling, we showed activation of JNK, p38 and p65 NF-κB by WNT-5B. Furthermore, inhibition of JNK and p38 prevented WNT-5B-induced IL-6 and CXCL8 secretion, whereas IKK inhibition prevented CXCL8 secretion only, indicating distinct pathways for WNT-5B-induced IL-6 and CXCL8 release.

Conclusion: WNT-5B induces IL-6 and CXCL8 secretion in pulmonary fibroblasts. WNT-5B mediates this via Frizzled-2 and TAK1. As WNT-5 signalling is increased in COPD, this WNT-5-induced inflammatory response could represent a therapeutic target.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a life-threatening disease characterized by progressive airflow limitation, which is associated with an abnormal inflammatory response of the lungs to noxious particles or gases. Long-term exposure to cigarette smoke is a major risk factor for the development of COPD (26, 27). Important pathophysiological features of COPD that contribute to the progressive loss of lung function are inflammation, airway wall remodelling, bronchoconstriction, mucus hypersecretion, and emphysema (5).

There is increasing recognition of the potential importance of mesenchymal cells in inflammation (1). Recently it has been demonstrated that pulmonary fibroblasts and airway smooth muscle cells may participate in the dysregulated inflammatory response seen in COPD (6, 31, 34). Pulmonary fibroblasts are potent producers of several inflammatory mediators, including cytokines and chemokines (11, 24, 28), which are of relevance in COPD. For example, it has been shown that airway fibroblasts of COPD patients express higher levels of interleukin 6 (IL-6) and chemokine (C-X-C motif) ligand 8 (CXCL8) than airway fibroblasts of controls (34). The inflammatory mediators produced by pulmonary fibroblasts influence the type and quantity of inflammatory cells that infiltrate airway and lung tissue in chronic respiratory disease (1). Taken together, alterations in fibroblast function likely play an important role in COPD.

Upon inflammation and tissue damage in the lung the Wingless/integrase-1 (WNT) signalling pathway can be activated, as has been reviewed (12, 21). In this pathway, autocrine or paracrine secreted WNT ligands activate Frizzled receptors (FZD) and subsequently induce various downstream signalling pathways in the cell. Canonical WNT/β-catenin signalling involves WNT ligand binding to FZD and lipoprotein receptor-related protein (LRP) cell surface receptors, followed by cytosolic stabilization and nuclear translocation of β-catenin.
This nuclear translocation results in transcription of WNT target genes involved in tissue repair and remodelling (7), including growth factors (e.g., vascular endothelial growth factor; VEGF), extracellular matrix (ECM) proteins (e.g., fibronectin), matrix metalloproteinases (e.g., MMP-9) and cytokines (e.g., CXCL8). Non-canonical WNT signalling is mediated via intracellular calcium and c-Jun N-terminal kinase (JNK). These pathways regulate cell motility and gene transcription (25, 29) and antagonize canonical, β-catenin dependent gene transcription.

Non-canonical WNT signalling has been implicated in inflammation. For example, non-canonical WNT-5A is able to induce Toll-like receptor 4 (TLR4) dependent cytokine production from murine C57BL/6 macrophages (18), and it stimulates chemotactic migration and chemokine production in human neutrophils (18). Dysregulated expression of non-canonical WNT-4, WNT-5A and WNT-5B has been observed in fibroblasts and airway epithelium of COPD patients as compared to controls (2, 13). In airway epithelium, WNT-4 appears to play a role in cytokine secretion (9, 13). In addition, WNT-5B regulates fibroblast activation via non-canonical signaling (32). Furthermore, WNT-5B protein expression is significantly higher in the airway epithelium from smokers with COPD than non-smokers as well as control smokers (14). In addition, this study demonstrates that WNT-5B protein expression levels do not differ between non-smokers and control smokers, indicating that the higher WNT-5B expression in COPD is not smoking, but disease-related (14). However, the effect of non-canonical WNT-5A/B signalling on inflammation in COPD is not well understood.

In the present study, we investigated the effect of WNT-5A/B on the inflammatory response in human lung fibroblasts and in primary lung fibroblasts of individuals with and without COPD. Furthermore, we investigated the receptors and molecular mechanisms involved in this response by examining the participation of various non-canonical WNT activated
pathways.
Materials and methods

Reagents: Recombinant human/mouse WNT-5A and human WNT-5B were from R&D systems (Abingdon, UK). Small interfering RNAs (siRNAs) specific for human FZD2, human FZD8, human related to receptor tyrosine kinase (RYK), and human transforming growth factor β activated kinase-1 (TAK1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and rabbit anti-p65 were purchased from Santa Cruz Biotechnology. Rabbit anti-phospho-Thr183/Tyr185-SAPK/JNK antibody and rabbit anti-phospho-p44/42 mitogen-activated protein kinases (MAPK) (ERK) antibody were obtained from Cell Signalling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody and HRP-conjugated goat anti-rabbit antibody were obtained from Sigma (St. Louis, MO, USA). Human β-catenin and non-targeting siRNA were procured from Qiagen (Venlo, the Netherlands). Lipofectamine 2000 transfection® reagent was obtained from Invitrogen. LL-Z1640-2 was obtained from Bioaustralis (Smithfield, NSW, Australia), SP600125 and U0126 were obtained from Tocris (Bristol, UK), BIRB0796 was obtained from Axon Medchem (Groningen, the Netherlands).

Cell culture: MRC-5 human lung fibroblasts (4, 17) (ATCC CCL 171) and primary lung fibroblasts from individuals with and without COPD were cultured in Ham’s F12 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/l streptomycin, 1.4 µg/ml fungizone and 100 U/ml penicillin. Unless otherwise specified, for each experiment cells were grown to confluence and subsequently culture medium was substituted with Ham’s F12 medium supplemented with 0.5% (v/v) FBS, 2 mM L-glutamine, 100 mg/l streptomycin, 1.4 µg/ml fungizone and 100 U/ml penicillin for a period of 24 hours. Cells were stimulated for different time-points with recombinant human/mouse WNT-5A or human WNT-5B in Ham’s F12 medium supplemented with 0.5% FBS, L-glutamine and
antibiotics. According to the manufacturer (R&D systems), the EC50 for recombinant WNT-5A should be in the range of 100-500 ng/ml whereas that of WNT5B should be in the range of 30-150 ng/ml. In that respect, the concentrations that we chose (5-500 ng/ml) are in the EC50 to submaximal range and should not be considered supramaximal. When applied, pharmacological inhibitors (i.e. BIRB0796 (1 µM) (4), SP600125 (10 µM) (20), JNK6o (10 µM) (20), LL-Z1640-2 (500 nM) (23)) were added 30 minutes before the addition of WNT-5B.

Whole lung tissue: Whole lung tissue of COPD and non-COPD patients was kindly provided by Dr. Jan Stolk (Leiden University Medical Center).

siRNA transfection: MRC-5 fibroblasts were grown to 90% confluence in 6-well cluster plates and transiently transfected with siRNA’s specific for human β-catenin, human FZD2, human FZD8, or human RYK. Cells were transfected in serum-free Ham’s F12 without any supplements using 200 pmol of siRNA in combination with Lipofectamine 2000 transfection® reagent. Control transfections were performed using a non-silencing control siRNA. After 6 hours of transfection, medium was changed to Ham’s F12 supplemented with 10% FBS, L-glutamine and antibiotics. Cells were incubated overnight followed by a culture period of 24 hours in Ham’s F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Medium was then refreshed before cells were used for experimentation.

Preparation of cell lysates: Cells were lysed in ice-cold sodiumdodecylsulphate (SDS) buffer (composition: 62.5 mM Tris, 2% w/v SDS, 1 mM NaF, 1 mM Na3VO4, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 7 mg/ml pepstatin A, pH 6.8). Viscosity of lysates was reduced by using 0.66 mm needles and protein concentration was determined using Pierce® protein determination according to the manufacturer’s instructions. Lysates were stored at -20°C till further use.
**Western Blot analysis:** Equal amounts of protein (10–20 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were subsequently recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using TotalLabTM software (Nonlinear dynamics; Newcastle, UK) or ImageJ software (open source, public domain) (30).

**mRNA isolation and real-time PCR:** Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Duren, Germany) 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. Real-time PCR data was analyzed using the comparative cycle threshold (Cq: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA and/or GAPDH (ΔCq). Relative differences were determined using the equation $2^{(-\Delta\Delta Cq)}$. Primer sets used to analyze gene expression are shown in Table 1.

**ELISA:** Confluent MRC-5 human lung fibroblasts were washed twice with warm (37°C) Hank’s balanced salt solution (HBSS; composition (mg/l): KCl 400, KH$_2$PO$_4$ 60, NaCl 8000, NaHCO$_3$ 350, Na$_2$HPO$_4$,1H$_2$O 50, glucose 1000; pH 7.4) followed by a period of 24h in Ham’s F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Consecutively,
medium was refreshed and cells were stimulated with recombinant WNT-5B (500 ng/ml) in the presence or absence of the selective I kappa B kinase (IKK) inhibitor SC514 (10 µM), the selective IKK inhibitor TPCA-1 (10 µM), the selective TAK1 inhibitor LL-Z1640-2 (500 nM), the selective p38 inhibitor BIRB0796 (1 µM), the selective JNK inhibitor SP600125 (10 µM), or the selective JNK inhibitor JNK6o (10 µM). Cell supernatants were harvested 24h after stimulation and stored at -20°C until assayed for IL-6 or CXCL8. Cytokine levels were determined by specific ELISA according to the manufacturer’s instructions (IL-6 and CXCL8 kit Sanquin, Amsterdam, the Netherlands).

**Immunocytochemistry:** Lung fibroblasts were plated on Lab-Tek™ borosilicate chamber slides and treated with recombinant WNT-5B (500 ng/ml) for 30 min, 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). Cells were then permeabilized by incubation for 5 min at 4°C in CB containing 3% PFA and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were first blocked for 2 hours 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. Incubation with primary antibody (p65, diluted 1:20) occurred overnight at 4°C in Cyto-TBS containing 0.1% Tween 20 (Cyto-TBST). Incubation with FITC-conjugated secondary antibody was for 2 h at room temperature in Cyto-TBST. Nuclei were stained with Hoechst 33342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and analysed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

**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
Bonferroni or Tukey multiple comparisons test, where appropriate. Differences were considered to be statistically significant when $p<0.05$. 

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Results

WNT-5B induces IL-6 and CXCL8 secretion and mRNA expression.

We first investigated whether WNT-5A and WNT-5B were able to induce an inflammatory response. Following WNT-5B, and to a lesser extent WNT-5A stimulation, a clear dose-dependent increase in IL-6 and CXCL8 cytokine secretion was seen (Figures 1A-D). This was associated with a specific increase in IL-6 and CXCL8 mRNA expression. Following WNT-5B (500 ng/ml) stimulation for 4 hours, gene expression levels of IL-6 and CXCL8, but not GM-CSF, were enhanced (Figure 1E). To confirm the physiological relevance of this WNT-5A- and-B-driven inflammatory response, we stimulated primary fibroblasts taken from COPD patients and non-COPD controls with WNT-5A and WNT-5B. The clinical characteristics of the subject groups are represented in Table 2. Following WNT-5A and WNT-5B stimulation, a clear dose-dependent increase in IL-6 and CXCL8 secretion was seen in the primary fibroblasts. This increase was significant following stimulation with 500 ng/ml of both WNT-5A and WNT-5B (Figures 1F-G). Notably, the increase in IL-6 and CXCL8 following WNT-5B, but not WNT-5A, was significantly higher in fibroblasts from COPD patients than in fibroblasts from non-COPD controls (Figures 1H-K). This difference was only visible when the data was expressed as percentage of basal levels, as there was a high variability in basal IL-6 and CXCL8 secretion between COPD and non-COPD cells. Collectively, the above-mentioned results show that both WNT-5A and WNT-5B are able to induce an inflammatory response in both MRC-5 human lung fibroblasts and primary human lung fibroblasts.

WNT-5B-induced IL-6/CXCL8 release is mediated via non-canonical FZD2 signalling.

Next, we investigated the underlying signalling mechanisms of the inflammatory responses observed. As the WNT-5A-induced cytokine release was less clear than the response caused
by WNT-5B, we chose to focus on the mechanisms underlying the WNT-5B-induced inflammatory response. First, to confirm that WNT-5B-mediated cytokine release resulted from non-canonical signalling, we employed β-catenin specific siRNA. Transfection of MRC-5 cells with β-catenin siRNA significantly repressed β-catenin transcripts to ~40% of the baseline expression in comparison to non-targeting siRNA transfected cells (Figure 2A). In addition, active β-catenin protein expression was reduced significantly following β-catenin siRNA transfection (2B). β-catenin knockdown did not at all attenuate WNT-5B-induced IL-6 or CXCL8 release, showing that cytokine release was not mediated by canonical WNT signalling (Figure 2C-D). In further support, we quantified the protein expression of active (non-phospho) β-catenin following 30 minutes of WNT-5B stimulation in MRC-5 cells. In agreement with the results mentioned above, WNT-5B did not increase the expression of active β-catenin (data not shown).

Next, we targeted key receptors in non-canonical WNT signalling to identify the signalling cascades involved. FZD2, FZD8 and the coreceptor RYK were previously found to be abundantly expressed in pulmonary fibroblasts (2), hence we employed FZD2, FZD8 and RYK-specific siRNA. Transfection of MRC-5 cells with FZD2 siRNA, FZD8 siRNA or RYK siRNA significantly repressed transcripts to 40% of baseline expression (Figure S1). Following transfection, we observed that knockdown of FZD2, and to a lesser extent FZD8 and RYK, inhibited the WNT-5B mediated CXCL8, but not the IL-6 response (figure 3A-D). In order to investigate whether knockdown of one FZD receptor would cause a compensatory increase of the other receptor, we investigated whether FZD2 knockdown affected FZD8 knockdown and vice versa. Knockdown of FZD2 did not significantly affect FZD8 mRNA expression levels, and knockdown of FZD8 did not affect FZD2 mRNA expression levels (data not shown). In addition, we tried to perform a double FZD2/FZD8 knockdown.
However, following double knockdown, MRC-5 cells were not viable anymore and clearly showed detachment from the culture plate surface.

**Non-canonical WNT mRNA expression is increased in COPD**

To further study the pathophysiological relevance of the WNT ligands and receptors under investigation, we quantified the mRNA expression levels of WNT-5A, WNT-5B, FZD2, FZD8, IL-6 and CXCL8 in lung tissue homogenates from COPD patients and non-COPD controls. As compared to basal non-COPD, the mRNA expression levels of WNT-5A, FZD2, IL-6 and CXCL8 were found to be increased significantly (Figure 4).

**TAK1 signalling mediates WNT-5B–induced IL-6 and CXCL8 expression.**

As TAK1 is involved in non-canonical WNT signalling and regulates downstream pathways involved in inflammation (8, 33), we employed pharmacological inhibition of TAK1 using LL-Z1640-2. 500 nM LL-Z1640-2 reduced WNT-5B-induced IL-6 and CXCL8 to basal levels, indicating that TAK1 mediates WNT-5B-mediated IL-6 and CXCL8 production (Figures 5A-B). In addition, we employed TAK1-specific siRNA. Following transfection, WNT-5B-mediated CXCL8 secretion was inhibited whereas IL-6 secretion was not inhibited (Figure 5C-D). This was in contrast to the TAK-1 inhibitor LL-Z1640-2, which was able to inhibit both WNT-5B-mediated CXCL8 and IL-6 secretion. This difference is likely explained by the low knockdown efficiency. Compared to control, TAK-1 siRNA reduced TAK-1 mRNA expression levels with only 40 percent, whereas LL-Z1640-2 inhibits maximally (Figure 5E).

Next, we sought to determine the signalling mechanisms downstream of TAK1 activation. As TAK1 activates JNK, p38, ERK and NF-κB pathways in multiple systems (8), we investigated the role of these pathways. We found that WNT-5B induced activation of JNK and p38, as indicated by their increased phosphorylation status (Figures 6A-B). In addition,
immunocytochemical staining for the NF-κB subunit p65 indicated that stimulation with WNT-5B (30 min) significantly induced the nuclear translocation of p65, which is an indication of activation of the NF-κB pathway (Figure 6C). WNT-5B did not induce activation of ERK as indicated by its lack of changes in phosphorylation status (Figure 6D). Furthermore, inhibition of TAK1 by LL-Z1640-2 reduced activation of JNK and p38 in both basal and WNT-5B-stimulated conditions (Figures 6E-F). Remarkably, TAK1 inhibition increased basal p65 nuclear translocation (data not shown). In agreement with the above mentioned findings, pharmacological inhibition of p38 with the inhibitor BIRB0796 reduced WNT-5B-induced IL-6 and CXCL8 to basal levels (Figures 7A-B), whereas pharmacological inhibition of JNK with the inhibitor SP600125 (10 µM) reduced WNT-5B-induced CXCL8, but not IL-6 levels (Figures 7C-D). Remarkably, JNK inhibition increased basal IL-6 secretion. Similar data were observed using the JNK inhibitor JNK6o (10 µM) (data not shown). Furthermore, the pharmacological inhibition of IKK with the selective inhibitor SC514 (10 µM) reduced WNT-5B-mediated CXCL8, but not IL-6 levels (Figure 7E-F). In addition, inhibition of IKK by the selective inhibitor TPCA-1 (10 µM) reduced both basal and WNT-5B-mediated IL-6 and CXCL8 secretion levels (data not shown). Collectively, this indicates that TAK1 signalling and its downstream effects on p38, JNK and NF-κB mediate cytokine secretion by WNT-5B, with differential roles on IL-6 versus CXCL-8 secretion.
**Discussion**

It is increasingly recognized that lung fibroblasts play an important role in the regulation of inflammatory responses in COPD (6, 31, 34). To our knowledge, this is the first study demonstrating that non-canonical signalling by WNT-5B and WNT-5A may play an important role in inflammatory responses in human lung fibroblasts. We showed that in both MRC-5 human lung fibroblasts and patient-derived fibroblasts WNT-5B increased IL-6 and CXCL8 cytokine secretion and mRNA expression. Following WNT-5A stimulation, the same responses were observed, albeit to a lesser extent. Although WNT-5A had smaller responses compared to WNT-5B in both MRC-5 and primary fibroblasts, the effect of WNT-5A was higher in primary fibroblasts than in MRC-5 cells. Therefore we cannot exclude a pro-inflammatory role of WNT-5A completely. FZD2 primarily mediated WNT-5B-induced release of CXCL8, but not IL-6, with possible smaller roles for FZD8 and RYK. The response was independent of β-catenin but involved TAK1 and p38, JNK, and p65 activation. The role of these pathways was different for WNT-5B-induced release of IL-6 and CXCL8 with IL-6 being less sensitive to NF-κB inhibition. The pro-inflammatory role of WNT-5A and WNT-5B may be of importance to COPD, as the FZD2 receptor was found increased in COPD.

Apart from IL-6 and CXCL8 other cytokines are involved in the pathophysiology of COPD. It may be possible that WNT-5A and WNT-5B affect these other cytokines as well. However, previous screens from our lab showed that only a limited number of cytokines could be detected and of these, IL-6 and CXCL8 were by far the most abundant. Further studies into GM-CSF, CXC10, CSF3 and CCL2 did not show regulation by WNT5B, indicating that at least in lung fibroblasts, these effects of WNT5B appear to be relatively specific for IL-6 and CXCL8. Although we cannot exclude the possibility that WNT-5B might affect other
cytokines than IL-6 and CXCL8, the MRC-5 fibroblast might not be the right model to investigate this.

In an investigation of the receptors involved in WNT-5B-mediated cytokine production, we found that the secretion of CXCL8, but not IL-6, was completely blocked following knockdown of FZD2, suggesting distinct mechanisms for WNT-5B-induced release of IL-6 and CXCL8. This contention was also supported by the observation that NF-κB has differential roles in IL-6 and CXCL8 secretion. The knockdown of FZD8 and RYK had smaller, non-significant, effects on IL-6 and CXCL8 secretion, suggesting that the involvement of these receptors cannot be excluded. Indeed, in parallel studies we observed a pro-inflammatory role for the FZD8 receptor in IL-1β signalling, reinforcing the possibility that WNT mediated pro-inflammatory signalling is regulated by multiple rather than a single FZD receptor (31).

To investigate the pathways involved in WNT-5B stimulated cytokine production, we found that the production of both IL-6 and CXCL8 was completely inhibited following TAK1 inhibition. TAK1 can be activated by FZD2, as demonstrated in HEK293 cells (16). P38, JNK, NF-κB and MEK are downstream targets of TAK1. Of these, p38, JNK and NF-κB were significantly activated by WNT-5B. Inhibition of p38 reduced both the WNT-5B-induced IL-6 and CXCL8 secretion, whereas inhibition of JNK and NF-κB signalling significantly reduced the CXCL8 secretion only, suggesting distinct pathways for WNT-5B-mediated IL-6 and CXCL8 release.

Other studies have demonstrated similar mechanisms by which non-canonical WNT signalling leads to inflammation. For example, it has been demonstrated that WNT-5A participates in dental pulp inflammation in a MAPK-(p38, JNK and ERK) and NF-κB-dependent manner (35). Another study showed that upon WNT-5A stimulation, ERK, p38 and
JNK were phosphorylated, leading to chemotactic migration and chemokine production in human neutrophils (18). Together, these studies and our present study indicate that the MAPK pathway and the NFκB pathway mediate non-canonical WNT signalling leading to inflammation in several systems.

The present study demonstrates that WNT-5A and WNT-5B play a role in COPD, as shown by the increased expression of WNT-5A and the WNT-5B receptor FZD2 in whole lung tissue of COPD patients, and by their pro-inflammatory effects in MRC-5 and primary fibroblasts. Notably, the pro-inflammatory effects of WNT-5B were found to be more pronounced in fibroblasts from COPD patients than in controls. Recently, several studies have demonstrated a switch from canonical to non-canonical WNT signalling in several systems, including COPD. Targeting this switch in the WNT pathway may represent a therapeutic target for the treatment of COPD. For example, it has been shown that non-canonical WNT-5A was up-regulated at early stages in both elastase- and cigarette smoke-induced animal models of COPD (3), which fits our data showing increased WNT-5A expression in whole lung tissue of COPD patients. In the study performed by Baarsma et al., fibroblast-derived WNT-5A was a negative regulator of canonical WNT signalling in alveolar epithelial cells, leading to an attenuated WNT-3A-induced proliferation of A549 cells. In the same study, WNT-5A led to decreased β-catenin expression in primary mouse ATII cell cultures that spontaneously differentiate into ATI cells upon active canonical WNT/β-catenin signalling (3). The non-canonical WNT-4 has also been associated with COPD in two separate studies, both showing expression in airway epithelial cells and its involvement in pro-inflammatory cytokine expression (9, 13). Another study showed that ageing of mouse hematopoietic stem cells (HSCs) was associated with a shift from canonical to non-canonical WNT signalling due to elevated expression of WNT-5A (10). In that study, WNT-5A treatment of young HSCs induced ageing whereas WNT-5A haploinsufficiency attenuated HSC ageing, and stem-cell-
intrinsic reduction of WNT-5A expression resulted in functionally rejuvenated aged HSCs. Therefore, in addition to the possible importance of a switch between canonical and non-canonical WNT signalling in COPD, this switch could also be a general mechanism involved in ageing-related diseases.

Our results agree with previous studies showing the role of pulmonary fibroblasts in the dysregulated inflammatory response seen in COPD (6, 11, 24, 28, 31, 34). Our current findings show increased inflammatory responses following WNT-5A and WNT-5B stimulation in fibroblasts from COPD patients as compared to controls. In addition to their contribution to inflammatory responses, inflammatory mediators produced by pulmonary fibroblasts may also influence the functioning and remodelling of airway wall smooth muscle in the small airways (6). As the degree of airflow limitation in COPD is directly related to the degree of airway wall thickness (15), targeting the remodelling of the airway wall is of importance. We previously reported a role for WNT-5A in ECM production by airway smooth muscle (22), and for WNT-5B in ECM production by fibroblasts (32), demonstrating that non-canonical WNT signalling may be involved in airway remodelling in addition to being involved in inflammation. Targeting non-canonical WNT-5B and WNT-5A signalling may therefore be of therapeutic relevance in treating COPD.

It has not yet been shown whether WNT-5B is a negative regulator of canonical signalling in the lung. However, as WNT-5A and WNT-5B share signalling characteristics (19), it seems likely that WNT-5B could act in a similar way. Although WNT-5B expression was not increased in whole lung tissue of COPD patients, its expression is increased in fibroblasts of COPD patients (2). Moreover, in the present study we showed the increased expression of the WNT-5B receptor FZD2 in whole lung tissue of COPD patients, as well as an enhanced effect of WNT-5B on IL-6 and CXCL8 secretion in fibroblasts taken from COPD patients. These finding support our hypothesis that WNT-5B signalling may contribute to COPD, as
knockdown of FZD2 in MRC-5 cells prevented the WNT-5B-induced inflammatory cytokine release. The available expression and functional data on the increase of WNT-5A, WNT-5B and FZD2 in COPD therefore indicate enhanced activation of non-canonical WNT signalling in COPD.

In conclusion, WNT-5B is able to induce the inflammatory cytokines IL-6 and CXCL8 via non-canonical FZD2 signalling, mediating its effect via TAK1 and downstream MAPK and NF-κB signalling pathways. This inflammatory response may be of importance in COPD and may therefore form a relevant therapeutic target.
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Figure legends

**Figure 1.** Effect of WNT-5A and WNT-5B stimulation on the secretion of IL-6 and CXCL8 and on the mRNA expression of IL-6, CXCL8 and GM-CSF. (A-D) MRC-5 cells were exposed to WNT-5A or WNT-5B for 24 hours. Supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=6). (E) MRC-5 cells were exposed to WNT-5B (500 ng/ml) for 4 h. RNA was isolated and mRNA expression levels of IL-6, CXCL8 and GM-CSF were measured by RT-PCR. Expression was related to the expression of the housekeeping gene 18S. mRNA levels are expressed as fold change compared to the unstimulated control value (2^{−ΔΔCt}, n=3). (F-G) Primary pulmonary fibroblasts (n=19) were exposed to WNT-5A or WNT-5B for 24 h. Supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA. (H-K) Primary pulmonary fibroblasts from COPD patients (n=12) or controls (n=7) were exposed to WNT-5A or WNT-5B for 24 h. Supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA. The statistical significance of differences between means was determined on log transformed data by repeated measures ANOVA (A-D), Student’s t-test (E, H-K), or by 2-way ANOVA (F-G), followed by Tukey multiple comparisons test, where appropriate. Data represent mean±SEM, *p<0.05 compared to basal control.

**Figure 2.** Effect of WNT-5B stimulation on the secretion of IL-6 and CXCL8 following β-catenin knockdown. (A) MRC-5 cells were transfected with β-catenin-specific siRNA or a non-targeting siRNA as control. Subsequently, cells were stimulated with WNT-5B (500 ng/ml) for 24 h. Expression of β-catenin mRNA was expressed relative to non-targeting siRNA transfected, untreated control. mRNA levels are expressed as fold change compared to the unstimulated control value (2^{−ΔΔCt}, n=6). (B) MRC-5 cells were transfected with β-
catenin-specific siRNA or a non-targeting siRNA as control. Total cell lysates were prepared and active β-catenin was detected by Western Blot. Equal loading protein was verified by the analysis of GAPDH (n=4). (C-D) MRC-5 cells were transfected with β-catenin-specific siRNA or a non-targeting siRNA as control. Subsequently, cells were stimulated with WNT-5B (500 ng/ml). Following 24 h, supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=6). The statistical significance of differences between means was determined on log transformed data by Student’s t-test (B), or by 2-way ANOVA (A,C), followed by Tukey multiple comparisons test, where appropriate. Data represent mean±SEM, *p<0.05 compared to basal control.

**Figure 3. Receptors involved in the WNT-5B-induced inflammatory response.**

(A-F) MRC-5 cells were transfected with FZD2, FZD8 or RYK-specific siRNA or a non-targeting siRNA as a control. Subsequently, cells were stimulated with WNT-5B (500 ng/ml). Following 24 h, supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=4-7). The statistical significance of differences between means was determined on log transformed data by 2-way ANOVA (A-F), followed by Tukey multiple comparisons test. Data represent mean±SEM. *p<0.05 compared to basal control, #p<0.05 compared to FZD2 siRNA.

**Figure 4. Non-canonical WNT pathway gene expression is increased in COPD.** RNA was isolated from lung tissue homogenates from COPD patients or non-COPD controls (n=16 and n=5, respectively) and gene expression levels of WNT-5A, WNT-5B, FZD2, FZD8, IL-6, and CXCL8 were measured by Real-time PCR. Expression was related to the expression of the housekeeping genes 18S and GAPDH (mean). mRNA levels are expressed as fold change compared to the group-average of the non-COPD control group value (2^{ΔΔCt}). The statistical
significance of differences between means was determined on log transformed data by Student’s $t$-test. Data represent mean±SEM, *$p<0.05$ compared to basal control.

Figure 5. WNT-5B induces IL-6 and CXCL8 secretion via TAK1. (A, B) MRC-5 cells were treated with the selective TAK1 inhibitor LL-Z1640-2 (500 nM). Subsequently, cells were stimulated with WNT-5B (500 ng/ml). Following 24 h, supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=5). (C,D) MRC-5 cells were transfected with TAK1-specific siRNA or a non-targeting siRNA as a control. Subsequently, cells were stimulated with WNT-5B (500 ng/ml). Following 24 h, supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=6). (E) Expression of TAK1 mRNA was expressed relative to non-targeting siRNA transfected, untreated control. mRNA levels are expressed as fold change compared to the unstimulated control value ($2^{-\Delta\Delta Ct}$, n=6). The statistical significance of differences between means was determined on log transformed data by 2-way ANOVA (A,B,C,D), followed by Tukey multiple comparisons test, or by Student’s $t$-test (E). Data represent mean±SEM. *$p<0.05$ compared to basal control, #$p<0.05$ compared to WNT-5B.

Figure 6. JNK, p38 and p65 signalling mediates WNT-5B–induced IL-6 and CXCL8 expression. (A) MRC-5 cells were exposed to WNT-5B (500 ng/ml) for 30 and 60 minutes. Total cell lysates were prepared and phosphorylated JNK was detected by Western Blot. Equal protein loading was verified by the analysis of GAPDH (n=8). (B) MRC-5 cells were exposed to WNT-5B (500 ng/ml) for 30 and 60 minutes. Total cell lysates were prepared and phosphorylated p38 was detected by Western Blott. Equal protein loading was verified by the analysis of GAPDH (n=4). (C) Evaluation of nuclear translocation of p65 in MRC-5 cells after WNT-5B stimulation (30 min). Following stimulation, the cells were subsequently fixed
and permeabilized. Cells were stained for p65 (FITC; green) and nucleus (Hoechst 33342; blue). Pictures were taken at 400 x magnification. (D) MRC-5 cells were exposed to WNT-5B (500 ng/ml) for 30 and 60 minutes. Total cell lysates were prepared and phosphorylated ERK was detected by Western Blot. Equal protein loading was verified by the analysis of GAPDH (n=5). (E) MRC-5 cells were treated with the selective TAK1 inhibitor LL-Z1640-2 (500 nM). Subsequently, cells were stimulated with WNT-5B (500 ng/ml) for 30 minutes. Total cell lysates were prepared and phosphorylated JNK was detected by Western Blot. Equal protein loading was verified by the analysis of GAPDH (n=4). (F) MRC-5 cells were treated with the selective TAK1 inhibitor LL-Z1640-2 (500 nM). Subsequently, cells were stimulated with WNT-5B (500 ng/ml) for 30 minutes. Total cell lysates were prepared and phosphorylated p38 was detected by Western Blot. Equal protein loading was verified by the analysis of GAPDH (n=4). The statistical significance of differences between means was determined on log transformed data by repeated measures ANOVA (A,D), Student’s t-test (C,B), or 2-way ANOVA (E,F) followed by Tukey multiple comparisons test, where appropriate. Data represent mean±SEM. *p<0.05 compared to basal control, #p<0.05 compared to WNT-5B.

Figure 7. JNK, p38 and p65 signalling mediates WNT-5B–induced IL-6 and CXCL8 expression, whereas IKK mediates only CXCL8 secretion. (A-F) MRC-5 cells were treated with the selective p38 inhibitor BIRB0796 (1 µM), the selective JNK inhibitor SP600125 (10 µM), or with the selective IKK inhibitor SC514 (10 µM). Subsequently, cells were exposed to WNT-5B (500 ng/ml) for 24 h. Following 24 h, supernatant was collected and secretion of the inflammatory cytokines IL-6 and CXCL8 was measured by ELISA (n=3-6). The statistical significance of differences between means was determined on log transformed data by repeated measures ANOVA (A,D), Student’s t-test (B,C) or by 2-way ANOVA (E,F), followed by Tukey multiple comparisons test, where appropriate. Data represent mean±SEM. *p<0.05 compared to basal control, #p<0.05 compared to WNT-5B.
### Table 1. Primers used for RT-PCR analysis.

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Table 2. Clinical characteristics of the subjects involved in the studies.

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All values are represented as median values with ranges in parentheses. FEV1 % predicted = Forced Expiratory Volume in 1 second as percentage of predicted value; FVC = Forced Vital Capacity. Stage means severity of COPD according to GOLD criteria. Statistical significance was determined by a kruskall-wallis ANOVA followed by a two-way Student’s t-test for unpaired observations. *p<0.05 compared to basal control.
Figure 1. A, B, C, D, E, F, G, H, I, J, K
Figure 2. A, B, C, D
CXCL8 protein expression (% control)

IL-6 protein expression (% control)

Non-targeting siRNA      FZD2 siRNA

Non-targeting siRNA      FZD8 siRNA

Non-targeting siRNA      RYK siRNA

Figure 3
mRNA expression (% basal non-COPD average)

- CXCL8
- IL-6
- WNT-5B
- WNT-5A
- FZD8
- FZD2
Figure 5. A, B, C, D, E
Figure 6. A, B, C, D, E, F
IL-6 protein expression (% control)

A

CXCL8 protein expression (% control)

B

C

D

E

F

Basal              BIRB796
Basal              SP600125
Basal                SC514