The WNT receptor Frizzled-8 in pulmonary remodelling and inflammation

Spanjer, Anita Indra Radha

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

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

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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

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

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Chapter 2

Transforming growth factor-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor Frizzled-8


Anita I.R. Spanjer
Hoeke A. Baarsma
Lisette M. Oostenbrink
Sepp R. Jansen
Christine C. Kuipers
Michael Lindner
Dirkje S. Postma
Herman Meurs
Irene H. Heijink
Reinoud Gosens*
Melanie Königshoff*

* both authors contributed equally
Abstract

Background and purpose
Transforming growth factor (TGF)-β is important in lung injury and remodelling processes. TGF-β and WNT signalling are interconnected, however the WNT ligands/receptor complexes involved, are unknown. Here, we aimed to identify Frizzled (FZD) receptors that mediate TGF-β-induced pro-fibrotic signalling.

Basic procedures
MZC-5 and primary human lung fibroblasts were stimulated with TGF-β1, WNT-5A or WNT-5B in the presence and absence of specific pathway inhibitors. Specific small interfering RNA was used to knock-down FZD8. In vivo studies using bleomycin-induced lung fibrosis were performed in wild-type and FZD8 deficient mice.

Main findings
TGF-β1 induced FZD8 specifically via Smad3 dependent signalling in MRC-5 and primary human lung fibroblasts. Importantly, FZD8 knockdown reduced TGF-β1-induced collagen Iα1, fibronectin, versican, α-smooth muscle(sm)-actin and connective tissue growth factor. Moreover, bleomycin-induced lung fibrosis was attenuated in FZD8 deficient mice in vivo. While inhibition of canonical WNT signalling did not affect TGF-β1-induced gene expression in vitro, noncanonical WNT-5B mimicked TGF-β1-induced fibroblast activation. FZD8 knockdown reduced both WNT-5B-induced gene expression of fibronectin and α-sm-actin as well as WNT-5B-induced changes in cellular impedance.

Principal conclusions
Collectively, our findings demonstrate a role for FZD8 in TGF-β-induced pro-fibrotic signalling, and imply WNT-5B as the ligand for FZD8 in these responses.
Introduction

Fibroblasts in the parenchyma and airways are the primary cells contributing to extracellular matrix (ECM) deposition and turnover in the lung. In this way, fibroblasts are important mediators influencing physiological repair processes and tissue homeostasis. However, in pathological conditions, e.g. in case of airway and pulmonary fibrosis, fibroblast function is altered, leading to myofibroblast differentiation and abnormal changes in ECM expression and composition [1, 2].

A key player in fibroblast activation is transforming growth factor (TGF)-β. TGF-β is a pro-fibrotic growth factor that activates fibroblasts, thereby stimulating ECM production and initiating myofibroblast differentiation [1, 3, 4]. Myofibroblasts are characterized by a more contractile profile than fibroblasts and show an increased expression of markers such as α-smooth muscle (sm)-actin, connective tissue growth factor (CTGF), type III collagen and plasminogen activator inhibitor (PAI)-1 [5-7].

Recent findings indicate similar key roles for the wingless/integrase-1 (WNT) signalling pathway in tissue homeostasis and remodelling in many organ systems, including the lung [8-12]. WNT ligands bind to transmembrane Frizzled (FZD) receptors and can thereby control cell differentiation, growth and polarity in a variety of cell systems. FZD receptors activate either the canonical pathway, which signals to 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/PCP (planar cell polarity) pathway) [13-16].

We previously demonstrated that TGF-β signalling increases the expression of WNT ligands and FZD receptors in human lung fibroblasts, of which the induction of FZD8 was most profound. We furthermore showed that TGF-β-induced fibronectin and α-sm-actin expression was dependent on β-catenin [10, 11]. This underscores the role of WNT signalling in fibroblast activation and remodelling as well as the cross-talk that exists between TGF-β and WNT signalling in these responses. However, the exact functional roles of individual WNT ligands and FZD receptors such as FZD8 in these processes are still largely unknown. In the present study, we therefore investigated the functional role of FZD8 in TGF-β signalling in vitro as well as in vivo. We show that FZD8 is involved in TGF-β signalling in bleomycin-induced fibrosis using wild-type and FZD8 deficient mice. In addition, we demonstrate that FZD8 plays a role in TGF-β-induced ECM turnover and myofibroblast differentiation and provide evidence that WNT-5B is its ligand in these responses.

Materials and methods

Ethics statement

Primary human lung fibroblasts were obtained from resected human lung tissue from the Asklepios bio bank for lung diseases at the Comprehensive Pneumology Center (CPC) and isolated as previously described [17]. All participants gave written informed consent and the study was approved by the local ethics committee of Ludwig-Maximilians University of Munich, Germany. The animal experiments were done in accordance with the guidelines of the Ethics committee of the Helmholtz Zentrum München, as approved by the Regierungspräsidium Oberbayern, Germany.
Chapter 2

Cell culture
MRC-5 human lung fibroblasts [18] 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 at 37°C with 5% CO₂. Prior to stimulation, cells were grown to confluence in 6-well cluster plates and serum deprived in supplemented medium with 0.5% (v/v) FBS for 24 hours. Primary human lung fibroblasts were cultured in DMEM / Ham’s F12 (1:1) with 20% (v/v) foetal calf serum (FCS) and 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C with 5% CO₂. Prior to stimulation, cells were grown to confluence and serum deprived for 24 hours in medium with 0.1% FCS and 100 μg/ml streptomycin and 100 U/ml penicillin.

Cell stimulation
Pulmonary fibroblasts were stimulated with either 2 ng/ml recombinant TGF-β, 1 μg/ml recombinant WNT-5A or 1 μg/ml recombinant WNT-5B. The inhibitors Dickkopf (DKK)-1 (0.3 μg/ml), SIS3 (3μM), U-0126 (3 μM), Y-27632 (1 μM), PKF 115–584 (0.1 μM), and LY-294002 (3 μM), were added 30 minutes prior to stimulation. The concentrations of these inhibitors were chosen based on their selectivity profiles described in literature [19-23] and effectiveness against their respective targets in our cell system (unpublished observations).

siRNA transfection
Cells were grown to 90% confluence and transfected with specific small interfering (si)RNA against the FD8 transcript. Cells were transfected in serum-free Ham’s F12 without supplements using 100 pmol FD8-targeted siRNA or non-targeting control siRNA and Lipofectamine® 2000 Transfection Reagent (Invitrogen, Paisley, UK). After 6 hours the medium was changed to supplemented medium with 10% (v/v) FBS for 18 hours and subsequently to supplemented medium with 0.5% (v/v) FBS for 24 hours. Cells were stimulated as described above.

mRNA isolation and Real-Time PCR analysis
Total mRNA of MRC-5 human lung fibroblasts was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total mRNA of primary human lung fibroblasts and of the mouse lungs was extracted using peqGold Total RNA Kit including the DNase digestion (PeqLab, Erlangen, Germany). The eluted mRNA was quantified using spectrophotometry (Nanodrop, Thermo Scientific™, Wilmington, DE, USA). Equal amounts of mRNA (1 μg) were then reverse transcribed according to the Reverse Transcription System (Promega Benelux b.v., Leiden, the Netherlands) and the cDNA was stored at -20°C until further use. 5 μl ABSolute™ Blue SYBR Green Supermix, containing fluorescein to comprise well to well variation, 1 μM of gene-specific forward and reverse primer and 1 μl of cDNA sample, were used in a total volume of 10 μl in a 48 well plate. The sequences of the primers used for determining genes of interest are listed in tables 1 and 2. Gene expression was determined using Real-Time PCR, which was performed with the Illumina Eco Personal QPCR System (Westburg, Leusden, the Netherlands). Real-time PCR data were analysed using the comparative cycle threshold (Ct) method.
Table 1 - Primers used for the determination of specific human genes of interest.

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZD1</td>
<td>5' TCG ACT TCC TGA AGC TGG AT</td>
<td>3'</td>
</tr>
<tr>
<td>FZD2</td>
<td>5' CCC GACT TCAC GGT CTA CAT</td>
<td>3'</td>
</tr>
<tr>
<td>FZD3</td>
<td>5' TCT CTT TGG CCC TTG ACT G</td>
<td>3'</td>
</tr>
<tr>
<td>FZD4</td>
<td>5' CCA GGA TTC CTT CCA AGT CA</td>
<td>3'</td>
</tr>
<tr>
<td>FZD5</td>
<td>5' AGC TAA AAT GGC CAG AGC AA</td>
<td>3'</td>
</tr>
<tr>
<td>FZD6</td>
<td>5' TTG TTG GCA TCT CTC TTG TC</td>
<td>3'</td>
</tr>
<tr>
<td>FZD7</td>
<td>5' CGA CGC TCT TTA CCG TTC TC</td>
<td>3'</td>
</tr>
<tr>
<td>FZD8</td>
<td>5' GAC ACT TGA TGG GCT GAG GT</td>
<td>3'</td>
</tr>
<tr>
<td>FZD9</td>
<td>5' AGA CCA TCG TCA TCC TGA CC</td>
<td>3'</td>
</tr>
<tr>
<td>FZD10</td>
<td>5' CCT CCA AGA CTC TGC AGT CC</td>
<td>3'</td>
</tr>
<tr>
<td>collagen Iα1</td>
<td>5' AGC CAG CAG ATC GAG AAC AT</td>
<td>3'</td>
</tr>
<tr>
<td>type III collagen</td>
<td>5' CCA TGA ATG GTG TTG TTC AG</td>
<td>3'</td>
</tr>
<tr>
<td>fibronectin</td>
<td>5' TCG AGG AGG AAA TTC CAA TG</td>
<td>3'</td>
</tr>
<tr>
<td>versican</td>
<td>5' ACA CAC GTG CAC CTC ATC AT</td>
<td>3'</td>
</tr>
<tr>
<td>α-sm-actin</td>
<td>5' GAC CCT GAA GTA CCC GAT AGA AC</td>
<td>3'</td>
</tr>
<tr>
<td>CTGF</td>
<td>5' GGA AAA GAT TCC GAC CCA AT</td>
<td>3'</td>
</tr>
<tr>
<td>PAI-1</td>
<td>5' CGC CAG AGC AGG ACG AA</td>
<td>3'</td>
</tr>
<tr>
<td>WNT-5A</td>
<td>5' GGG TGG GAA CCA AGA AAA AT</td>
<td>3'</td>
</tr>
<tr>
<td>WNT-5B</td>
<td>5' ACG CTG GAG ATC TCT GAG GA</td>
<td>3'</td>
</tr>
<tr>
<td>Axin2</td>
<td>5' CCT GCC ACC AAG ACC TAC AT</td>
<td>3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>5' TGG AAC CTA CCC ATC CCA TA</td>
<td>3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5' CGC CGC TAG AGG TGA AAT TC</td>
<td>3'</td>
</tr>
</tbody>
</table>
Table 2 - Primers used for the determination of specific mouse genes of interest.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FZD8</td>
<td>Forward 5‘ GCA AGG AGG CCC AAC TAA GAC 3‘</td>
</tr>
<tr>
<td></td>
<td>Reverse 5‘ GAG GCC CAA GCG GAT CA 3‘</td>
</tr>
<tr>
<td>Axin2</td>
<td>Forward 5‘ AGC AGA GGG ACA GGA ACC A 3‘</td>
</tr>
<tr>
<td></td>
<td>Reverse 5‘ CAC TTG CCA GTT TCT TTG GCT 3‘</td>
</tr>
<tr>
<td>fibronectin</td>
<td>Forward 5‘ GGT GTA GCA CAA CTT CCA ATT ACG 3‘</td>
</tr>
<tr>
<td></td>
<td>Reverse 5‘ GGA ATT TCC GCC TCG AGT CT 3‘</td>
</tr>
<tr>
<td>HPRT</td>
<td>Forward 5‘ CCT AAG ATG AGC GCA AGT TGA A 3‘</td>
</tr>
<tr>
<td></td>
<td>Reverse 5‘ CCA CAG GAC TAG AAC ACC TGC TAA 3‘</td>
</tr>
</tbody>
</table>

Smad binding element-4 activity

MRC-5 human lung fibroblasts were plated on 6-wells plates and transfected as described above with 0.5 µg plasmid DNA encoding Smad binding element (SBE)-4-firefly luciferase and with 0.12 µg plasmid DNA encoding CMV-Renilla luciferase. SBE-4 activity is a measure of activation of TGF-β/SMAD signalling. Transfected cells were then stimulated with TGF-β1 (2 ng/ml) in the presence or absence of SIS3 (3µM) as described above. After 24 hours, cells were lysed. Subsequently, firefly luciferase and Renilla luciferase activity were measured using a Luminometer (Luminoskan Ascent, Thermo Electron Corporation, Waltham, MA, USA) and using the Dual-Luciferase® Reporter Assay (Promega Benelux b.v., Leiden, the Netherlands). Firefly luciferase activity was normalized against Renilla luciferase activity.

Western blot analysis

To obtain whole cell lysates, cells were washed once with ice-cold (4°C) Hank’s balanced salt solution (HBSS; composition [mg/l]: KCl 400, KH₂PO₄ 60, NaCl 8000, NaHCO₃ 350, Na₂HPO₄.1H₂O 50, glucose 1000; pH 7.4) then lysed in ice cold sodium dodecyl sulfate (SDS) buffer (composition: 62.5 mM Tris, 2% w/v SDS, 1 mM NaF, 1 mM Naᵥ₂VO₄, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 7 mg/ml pepstatin A; pH 6.8). Protein concentration was determined in whole cell lysates and whole lung homogenates using a Pierce® BCA protein determination assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Lysates were stored at -20°C until further use. Equal quantities of protein (10-20 µg/lane) were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. These were analysed for proteins of interest using specific primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin or β-tubulin were used as a loading control. By using chemiluminescence reagents, bands were recorded in the ‘G:BOX iChem i gel documentation system’ equipped with GeneSnap image acquisition software (Syngene, Cambridge, UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene, Cambridge, UK).

Immunocytochemistry

MRC-5 human lung fibroblasts were grown to confluence on Lab-Tek™ borosilicate chamber slides and changed to supplemented medium with 0.5% (v/v) FBS for 24 hours prior to stimulation. Cells were stimulated with 2 ng/ml recombinant TGF-β1, 1 µg/ml recombinant WNT-5A or 1 µg/ml recombinant WNT-5B for 48 hours, fixed for
TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8

15 min at room temperature (RT) in cytoskeletal buffer (CB; composition: 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂ and 5 mM glucose; pH 6.1) containing 3% paraformaldehyde (PFA). Cells were then permeabilised by incubation for 5 min at RT in CB containing 3% PFA and 0.3% Triton X-100. Fixed cells were then blocked for 2 hours at RT in Cyto-tris buffered saline (TBS) buffer (20 mM Tris base, 154 mM NaCl, 2.0 mM EGTA and 2.0 mM MgCl₂; pH 7.2) containing 1% bovine serum albumin (BSA) and 2% normal donkey serum. Incubation with primary antibody (mouse anti-α-sm-actin, diluted 1:100) happened overnight at 4°C in Cyto-TBS containing 0.1% Tween 20 (Cyto-TBST). Incubation with Cy3-conjugated secondary antibody was done for 2 hours at RT in Cyto-TBST. Nuclei were stained with Hoechst 33342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen, Paisley, UK) and analyzed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

Impedance measurement
WNT-5B mediated cellular activation was assessed using the xCELLigence system (Roche Applied Science, Penzberg, Germany), using E-plates (ACEA Biosciences Inc., San Diego, CA, USA). Impedance was measured by integrated microelectronic sensor arrays in the bottom of the E-plates. Electrode impedance can be affected by changes in intracellular mass redistribution and provides a means to assess activation of ligand-receptor complexes in living cells in real-time [24]. Changes in impedance were measured and converted to the relative and dimensionless cell index (CI), using the Real-Time Cell Analyzer (RTCA) software (Roche Applied Science, Penzberg, Germany; ACEA Biosciences Inc., San Diego, CA, USA). CI represents the impedance change divided by a background value. CI = (Zi - Z0) / 15 Ω. The change in impedance is represented by the impedance at an individual time point (Zi) during the experiment minus the background electrical resistance measured in the absence of cells prior to the experiment (Z0). MRC-5 human lung fibroblasts were transfected as described above with specific FZD8-targeted siRNA or non-targeting control siRNA. 24 hours after transfection, cells were trypsinized and plated in 16 well E-plates in supplemented medium with 0.5% (v/v) FBS. E-plates were then placed into the xCELLigence station and maintained in 5% CO₂ at 37°C while CI was measured real-time. CI was measured every 15 minutes for 16 hours. CI increased while cells attached. After 16 hours, prior to the experiment, CI was stable. If cells respond to stimulation, CI will change immediately. Therefore prior to stimulation with ligands, CI was measured at 1 second intervals. Cells were stimulated with recombinant WNT-5B (1 μg/ml). After stimulation, time intervals for CI measurement were sequentially 1 second for 300 seconds, 5 minutes for 1 hour and 15 minutes for 24 hours. Delta CI was determined using the CI value just prior to WNT-5B addition and after stabilization.

Preparation of WNT-3A-conditioned medium
Mouse L-cells (subcutaneous fibroblasts; ATCC®, Wesel, Germany) stably expressing WNT-3A were used to obtain WNT-3A-conditioned medium. Conditioned medium obtained from parental L-cells was used as a control. Conditioned medium was prepared as per ATCC® guidelines.
Chapter 2

Animal studies
Heterozygous, inbred, specified-pathogen-free breeding colonies of Fzd8<sup>−/−</sup> mice (C57BL/6;129P2-Fzd8<sup>tm1Dgen</sup>/J), showing no obvious phenotype, were obtained from the Jackson Laboratory (USA). After breeding, homozygous Fzd8<sup>−/−</sup> mice and WT littermates were used for experiments. Mice (8-12 weeks) were sedated intraperitoneally with a combination of 0.2 mg/ml medetomidine (Orion Pharma, Hamburg, Germany), 2.0 mg/ml midazolam (Roche Pharma, Mannheim, Germany) and 0.02 mg/ml phentanyl (Janssen-Cliag, Neuss, Germany), according to the body weight. 50 μl bleomycin (Sigma Aldrich, Taufkirchen, Germany) in phosphate buffered saline (PBS; 3U/kg) or PBS was intratracheally instilled through a MicroSprayer 20 G INTROCAN (Penn Century, Wyndmoor, PA, USA), consisting of a high pressure syringe and an intratracheal aerosolizer. After installation, the narcosis was antagonized by a combination of 0.29 mg/ml atipamezole (Orion Pharma, Hamburg, Germany), 0.059 mg/ml flumazenil (Hexal, Holzkirchen, Germany) and 0.14 mg/ml naloxone (Actavis, Munich, Germany) according to body weight. On day 14 after bleomycin installation, the mice were sedated according to body weight with 0.14% (v/v) ketamine (Pharma Partner Hamburg, Germany) and 0.03% (v/v) xylazine (Proxylaz, Bela Pharm, Vechta, Germany) in 0.9% sodium chloride. Lung lobes were snap frozen and stored in -80°C prior to mRNA isolation and protein determination and one lobe was filled with 4% paraformaldehyde for histology. Paraffin-embedded sections were stained with haematoxylin-eosin and Masson-Goldner (Carl Zeiss, Jena, Germany) to evaluate collagen deposition. Staining intensity was quantified using ImageJ [25].

Antibodies and reagents
Recombinant human TGF-β1, recombinant human DKK-1, recombinant human/mouse Wnt-5A and recombinant mouse Wnt-5B were obtained from R&D systems (Abingdon, UK). 6,7-dimethyl-2-{[2E]-3-(1-methyl-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-prop-2-enoyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride (SIS3) was obtained from Merck (Darmstadt, Germany). 1,4-diamino-2,3-dicyano-1,4-bis-[2-aminophenylthio]butadiene (U-0126), (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632), 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) were obtained from Tocris Cookson (Bristol, UK). PKF 115-584 was obtained from Novartis Pharma AG (Basel, Switzerland). Fzd8 siRNA was obtained from Santa Cruz Biotechnology Inc. (Heidelberg, Germany), non-silencing control siRNA from Qiagen (Venlo, the Netherlands).

Statistical analysis
All Real-Time PCR data were log-transformed before statistical analysis. For comparison between two conditions, a Student’s t-test or Mann-Whitney test was used where appropriate as indicated in the legends. For comparisons between multiple conditions,
TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8

Results

TGF-β1-induced FZD8 gene and protein expression in human lung fibroblasts

We previously showed that TGF-β1 stimulation induced FZD8 gene expression in primary human lung fibroblasts [11]. Here, we extended these findings and investigated the regulation of FZD8 by TGF-β1 in more detail. We observed that TGF-β1 strongly and specifically induces FZD8 gene and protein expression in MRC-5 and primary human lung fibroblasts with little to no effect on other FZD receptor subtypes (figures 1A-F). The effect on FZD8 gene expression was already visible after 4 hours of stimulation and more pronounced after 24 hours (figure 1B). The induction of FZD8 gene expression was concentration dependent, with an EC50 for TGF-β1 of 0.26 ng/ml and a maximal response at 2 ng/ml (18.5 ± 3.2 fold increase compared to untreated control; p < 0.001; figure 1C). TGF-β1 also increased FZD8 gene expression in primary human lung fibroblasts (figures 1E-F). Parallel with the induction of FZD8, TGF-β1 increased the gene expression of the ECM components collagen Iα1, fibronectin and versican as well as the differentiation markers α-sm-actin, PAI-1 and CTGF (figure 2A). TGF-β1 dose dependently increased the expression of fibronectin, versican and CTGF, which are WNT responsive genes [11, 23-29], with an EC50 of 0.14 ng/ml, 0.30 ng/ml and 2.52 ng/ml, respectively (figure 2B). Based on these data, we used a concentration of 2 ng/ml TGF-β1 and the 24-hour time point for gene expression analysis in subsequent experiments.

TGF-β1-induced FZD8 gene expression in human lung fibroblasts is Smad3 dependent

TGF-β signals via several intracellular pathways involving Smad, mitogen activated protein kinase (MAPK)/extracellular signal regulated kinases (ERK), Rho kinase, β-catenin/T-cell factor (TCF) and phosphoinositide (PI3)-kinase [30]. To investigate via which pathway the induction of FZD8 by TGF-β1 is established, we used specific inhibitors of Smad3 (SIS3 - 3 μM), MAPK ERK kinase (MEK)1/2 (U-0126- 3μM), Rho kinase (Y-27632- 1 μM), β-catenin/TCF (PKF 115-584- 0.1 μM) and PI3-kinase (LY-294002- 3μM). The concentrations of these inhibitors were chosen to provide (sub)maximal and selective inhibition of their respective targets in airway mesenchymal cells ([19-23] and unpublished observations). Importantly, the TGF-β1-induced expression of FZD8 was attenuated by the Smad3 inhibitor (57%), but not affected by the inhibition of MEK1/2, Rho-kinase, β-catenin/TCF or PI3-kinase signalling (figure 1G). These results demonstrate that TGF-β1-induced FZD8 expression in MRC-5 human lung fibroblasts is Smad3-dependent. Smad3 inhibition also blocked TGF-β1-induced SBE-4 activity, which was studied as a measure of activation of TGF-β/SMAD signalling (figure 2C), and impaired TGF-β1-induced expression of fibronectin, versican and CTGF (figures 2D-F).
Chapter 2
TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8

Figure 1: TGF-β-induced FZD8 mRNA and protein expression in human lung fibroblasts is Smad3 dependent. MRC-5 human lung fibroblasts and primary human lung fibroblasts were stimulated with TGF-β1 for 4 or 24 hours (mRNA) or 48 hours (protein). Specific inhibitors were used to block individual TGF-β1 signalling pathways. (A) Gene expression of FZD receptors in MRC-5 human lung fibroblasts after 24 hours of stimulation with TGF-β1 (2 ng/ml). Of note: high Ct-values correspond to low copy numbers. Data represent mean ± s.e.m. of 3-6 independent experiments. (B) FZD8 gene expression in MRC-5 human lung fibroblasts after stimulation with TGF-β1 (2 ng/ml) at different time points. Data represent mean ± s.e.m. of 5 independent experiments. (C) Concentration dependent increase of FZD8 gene expression in MRC-5 human lung fibroblasts after 24 hours of stimulation with increasing concentrations of TGF-β1 (EC50: 0.26 ng/ml). Data represent mean ± s.e.m. of 5-8 independent experiments. (D) FZD8 protein expression in MRC-5 human lung fibroblasts after 48 hours of stimulation with TGF-β1. Data represent mean ± s.e.m. of 10 independent experiments. (E) FZD8 gene expression in primary human lung fibroblasts after 24 hours of stimulation with TGF-β1 (2 ng/ml). Data represent mean ± s.e.m. of 4 patients. (F) FZD8 protein expression in primary human lung fibroblasts after 48 hours of stimulation with TGF-β1 (2 ng/ml). Data represent mean ± s.e.m. of 4 patients. (G) Involvement of specific signalling cascades in the TGF-β1-induced gene expression of FZD8. The inhibitors were used to inhibit the following pathways: SIS3 (3μM) – Smad3, U-0126 (3 μM) – MEK1/2, Y-27632 (1 μM) – Rho kinase, PKF 115–584 (0.1 μM) – β-catenin/TCF, and LY-294002 (3 μM) – PI3-kinase. Data represent mean ± s.e.m. of 8-10 independent experiments. *** p < 0.001 ** p < 0.01 * p < 0.05 compared to basal conditions (A-D: two-tailed Student’s t-test; E-F: two-tailed Mann-Whitney test), # p < 0.05 compared to the stimulated control (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test).
Figure 2: TGF-β1 induces the gene expression of different ECM proteins and myofibroblast differentiation markers in a Smad3 dependent fashion. MRC-5 human lung fibroblasts were stimulated with TGF-β1 (2 ng/ml) for 24 hours. SIS3 (3μM) was used to block Smad3 signalling. (A) The effect of TGF-β1 on the gene expression of different ECM proteins and myofibroblast differentiation markers. Data represent mean ± s.e.m. of 5 independent experiments. (B) Concentration dependent increase of fibronectin (EC50: 0.14 ng/ml), versican (EC50: 0.30 ng/ml) and CTGF (EC50: 2.52 ng/ml) after 24 hours of stimulation with increasing concentrations of TGF-β1. Data represent mean ± s.e.m. of 4-8 independent experiments. (C) The effect of inhibiting Smad3 on SBE4 activity. Data represent mean ± s.e.m. of 6 independent experiments. (D) The effect of inhibiting Smad3 on the TGF-β1-induced gene expression of fibronectin. Data represent mean ± s.e.m. of 4 independent experiments. (E) The effect of inhibiting Smad3 on the TGF-β1-induced gene expression of versican. Data represent mean ± s.e.m. of 5 independent experiments. (F) The effect of inhibiting Smad3 on the TGF-β1-induced gene expression of CTGF. Data represent mean ± s.e.m. of 5 independent experiments. *** p < 0.001 ** p < 0.01 * p < 0.05 compared to basal conditions (Student’s t-test), ### p < 0.001 ## p < 0.01 # p < 0.05 compared to the stimulated control (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test).
TGF-β-induced ECM expression and myofibroblast differentiation is regulated by FZD8

TGF-β activates fibroblasts, resulting in an enhanced ECM turnover and myofibroblast differentiation [2, 6, 7, 31, 32]. We show that parallel with the induction of FZD8, TGF-β increased the gene expression of the ECM components collagen Iα1, fibronectin and versican as well as the differentiation markers α-sm-actin, PAI-1 and CTGF (figure 2A). To study a possible role for FZD8 in this process, we used specific FZD8-targeting siRNA. As a control, FZD8 knockdown reduced the gene expression of this receptor by 56% at baseline and almost completely prevented the induction of FZD8 gene expression in response to TGF-β (from 4.5 ± 1.5 fold to 0.6 ± 0.2 fold of baseline FZD8 expression in control siRNA transfected cells; figure 3). FZD8 knockdown significantly reduced the TGF-β-induced expression of collagen Iα1, fibronectin, versican, α-sm-actin and CTGF, but not of PAI-1 (figure 3) and type III collagen (data not shown). The expression of these genes was differentially attenuated, versican expression being most affected (85% reduction), followed by CTGF (70%), fibronectin (60%), α-sm-actin (53%) and collagen Iα1 (38%). These results show that FZD8 is partly involved in the TGF-β-induced gene expression of several ECM proteins and myofibroblast differentiation markers.

**Figure 3: TGF-β-induced ECM expression is regulated by FZD8.** MRC-5 human lung fibroblasts were stimulated with TGF-β (2 ng/ml) for 24 hours. The expression of FZD8 was silenced with specific FZD8-targeting siRNA using non-targeting siRNA as a control. Figure shows the effect of FZD8 knockdown on TGF-β-induced gene expression of ECM components and myofibroblast differentiation markers. Data represent mean ± s.e.m. of 7 independent experiments. *** p < 0.001 ** p < 0.01 * p < 0.05 compared to control siRNA, ### p < 0.001 ## p < 0.01 # p < 0.05 compared to the stimulated control (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test).
Figure 4: Bleomycin-induced fibrosis in mice is partially dependent on FZD8. FZD8−/− mice and WT littermates were instilled with bleomycin to induce fibrosis and followed up after 14 days as described in the methods section. (A) FZD8 gene expression in whole lung tissue. Data represent mean ± s.e.m. of 5-8 mice per group. *** p < 0.001 compared to control WT mice ### p < 0.001 compared to bleomycin-instilled WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test). (B) Fibronectin gene expression and (C) fibronectin protein expression in whole lung tissue. Data represent mean ± s.e.m. of 3-6 mice per group. ** p < 0.01 compared to control WT mice ### p < 0.001 and ## p < 0.01 compared to bleomycin-instilled WT mice $ p < 0.05 compared to PBS treated FZD8−/− mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test). (D) Histologic assessment of fibrotic areas in the lung using the Masson-Goldner staining. Data represent mean ± s.e.m. of 5-8 mice per group. *** p < 0.001 ** p < 0.01 compared to control WT mice, # p < 0.05 compared to bleomycin-instilled WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test). (E) Representation for the quantification of the staining shown in figure 3D.
**Bleomycin-induced fibrosis in mice is partially dependent on FZD8**

Next, we investigated whether FZD8 also contributes to the development of lung fibrosis *in vivo*. To this end, FZD8-/− mice and WT littermates were instilled with bleomycin to induce fibrosis, a process highly dependent on TGF-β signalling [33]. We first confirmed that FZD8 gene expression was reduced in FZD8-/− mice compared to WT mice (figure 4A). Single bleomycin instillation caused strong fibrotic alterations within the lung after 14 days, characterized by increased fibronectin and collagen deposition in the lungs of WT mice. Notably, collagen deposition was reduced by 34% (p < 0.05) and fibronectin protein expression by 57% (p < 0.05) in the lungs of bleomycin-subjected FZD8-/− mice compared to WT mice subjected to bleomycin (figures 4B-E).

**TGF-β1-induced ECM expression and myofibroblast differentiation is not regulated via canonical WNT signalling**

WNT signalling can occur via the canonical β-catenin dependent pathway and via the noncanonical, β-catenin independent signalling pathways [13-15]. Our previous studies showed that TGF-β1-induced α-sm-actin, collagen Iα1, fibronectin and versican expression are regulated by β-catenin signalling [10, 11]. We thus hypothesized that activation of FZD8 mediates canonical WNT signalling which might potentiate TGF-β1-induced ECM turnover and myofibroblast differentiation. We therefore studied the effects of endogenous canonical WNT signalling inhibition in the presence of TGF-β signalling. We applied the well-known WNT antagonist DKK-1 that targets the interaction of FZDs with the co-receptor low-density lipoprotein receptor-related protein (LRP)5/6. This co-receptor is indispensable for canonical WNT signalling [13]. Interestingly, DKK-1 (0.3 μg/ml) had no effect at all on TGF-β1-induced gene expression, suggesting that FZD8 signals through noncanonical WNT signalling (figure 6A). As a positive control, DKK-1 inhibited the expression of the canonical WNT target gene Axin2, which was induced by WNT-3A-conditioned medium (figure 5). In line with this, the expression of the canonical WNT target gene Axin2 was increased at basal levels in the FZD8-/− mice (figure 6B) and a similar trend was observed for activated β-catenin expression (data not shown), suggesting that FZD8 does not target the canonical pathway, as its presence functions inhibitory. In further support, TGF-β1 had no effect on ser1490 LRP5/6 phosphorylation in MRC-5 human lung fibroblasts, an event necessary for canonical WNT signalling (figure 6C).

---

**Figure 5: DKK-1 regulates WNT-3A-induced gene expression of Axin2.** MRC-5 human lung fibroblasts were stimulated with WNT-3A-conditioned medium for 24 hours. The canonical WNT signalling pathway was inhibited with DKK-1 (0.3 μg/ml). Figure shows the effect of DKK-1 on WNT-3A-induced Axin2 gene expression. Data represent mean ± s.e.m. of 3 independent experiments.
Chapter 2

Figure 6: TGF-β-induced ECM expression and myofibroblast differentiation is not regulated via canonical WNT signalling. MRC-5 human lung fibroblasts were stimulated with TGF-β1 (2 ng/ml) for 24 hours (mRNA) or for different time points (protein) as indicated. The canonical WNT signalling pathway was inhibited with DKK-1 (0.3 μg/ml). (A) The effect of the canonical WNT signalling pathway on TGF-β-induced gene expression of ECM proteins and myofibroblast differentiation markers. Data represent mean ± s.e.m. of 5 independent experiments. *** p < 0.001 ** p < 0.01 compared to basal conditions (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test). (B) FZD8⁻/⁻ mice and WT littermates were instilled with bleomycin for 14 days as described in the methods section. Figure shows Axin2 gene expression in whole lung tissue. Data represent mean ± s.e.m. of 5-8 mice per group. ** p < 0.01 * p < 0.05 compared to control WT mice, ### p < 0.001 compared to bleomycin-instilled WT mice (two-way ANOVA with Student-Newman-Keuls multiple comparisons test). (C) Analysis of active β-catenin and ser1490 LRP5/6 phosphorylation by TGF-β1.
Noncanonical WNT-5B is a ligand for FZD8

We previously demonstrated that TGF-β induces specific expression of the noncanonical ligand WNT-5B in human lung fibroblasts [11]. In view of these and our current results, we investigated the gene expression of two typical noncanonical ligands, WNT-5A and WNT-5B, in MRC-5 human lung fibroblasts in response to TGF-β. Indeed, TGF-β induced the gene expression of both ligands in a concentration dependent manner with an EC50 of 0.28 ng/ml and 0.26 ng/ml respectively (figures 7A-B). Similar to FZD8 gene expression, the TGF-β-induced WNT-5B gene expression was Smad3-dependent, whereas WNT-5A gene expression was not (figures 7C-D). Functional studies showed that stimulation with recombinant WNT-5B mimicked the TGF-β-induced gene expression of ECM components and differentiation markers; WNT-5B significantly induced the gene expression of fibronectin, versican, α-sm-actin and CTGF, whereas WNT-5A did not (figure 7E). We confirmed these findings at the protein level for fibronectin and α-sm-actin (figures 7F-G). For these experiments, we used stimulation with recombinant WNT-5A as a control (figures 7F-G). This observation might imply that WNT-5B is a ligand for FZD8-mediated fibroblast activation. Thus, we studied the impact of FZD8 knockdown on WNT-5B-induced dynamic mass redistribution as a measure of ligand dependent receptor activation. First, we showed that WNT-5B-induced changes in cellular impedance, a measure for ligand dependent receptor activation, are partially prevented by FZD8 knockdown (41%), confirming that WNT-5B engages FZD8 receptor signalling for fibroblast activation (figures 8A-B). Next, we studied the effect of FZD8 knockdown on WNT-5B-induced gene expression in MRC-5 human lung fibroblasts, focusing on the WNT-5B responsive genes fibronectin, versican, α-sm-actin and CTGF. Indeed, the WNT-5B-induced expression of fibronectin (34%) and α-sm-actin (38%) was significantly downregulated by FZD8 knockdown, indicating that WNT-5B is dependent on FZD8 for the induction of these genes (figure 8C). Collectively, these data show that noncanonical WNT-5B is a ligand for FZD8 and that FZD8 regulates TGF-β-induced ECM gene expression and myofibroblast differentiation in human lung fibroblasts.
Chapter 2

Figure 5

A

B

C

D

E

- 50 -
Figure 5: Noncanonical WNT-5B mimics the effects of TGF-β on fibroblast activation. MRC-5 human lung fibroblasts were stimulated with TGF-β1 (2 ng/ml), WNT-5A (1µg/ml) or WNT-5B (1µg/ml) for 24 hours (mRNA) or for 48 hours (protein and immunocytochemistry). (A) Concentration dependent increase of WNT-5A gene expression after stimulation with increasing concentrations of TGF-β1 (EC50: 0.28 ng/ml). Data represent mean ± s.e.m. of 5-8 independent experiments. (B) Concentration dependent increase of WNT-5B gene expression after stimulation with increasing concentrations of TGF-β1 (EC50: 0.26 ng/ml). Data represent mean ± s.e.m. of 5-8 independent experiments. (C) The effect of inhibiting Smad3 on the TGF-β1-induced gene expression of WNT-5A. Data represent mean ± s.e.m. of 5 independent experiments. (D) The effect of inhibiting Smad3 on the TGF-β1-induced gene expression of WNT-5B. Data represent mean ± s.e.m. of 5 independent experiments. (E) Recombinant TGF-β1-, WNT-5A- and WNT-5B-induced effects on ECM gene expression and myofibroblast differentiation. Data represent mean ± s.e.m. of 3-7 independent experiments. (F) The effect of recombinant TGF-β1, WNT-5A and WNT-5B on protein expression of fibronectin and α-sm-actin. Data represent mean ± s.e.m. of 3-5 independent experiments. (G) The effect of TGF-β1, WNT-5A and WNT-5B stimulation on sm-actin stress fiber formation. Cells were stained for α-sm-actin (red) and DNA (Hoechst 33342; blue). Pictures were taken at 100× magnification. *** p < 0.001 ** p < 0.01 * p < 0.05 compared to basal conditions compared to basal conditions (Student’s t-test; figures A-B, E-F). ** p < 0.01 * p < 0.05 compared to basal conditions ## p < 0.01 compared to the stimulated control $$$ p < 0.01 compared to inhibited control (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test; figures C-D).
Figure 8: WNT-5B functions as a ligand for FZD8. WNT-5B mediated cellular activation was assessed using the xCELLigence system (Roche Applied Science, Penzberg, Germany), as described in the methods section. MRC-5 human lung fibroblasts were transfected as described above with specific FZD8-targeted siRNA or non-targeting control siRNA. Cells were stimulated with recombinant WNT-5B (1 μg/ml). Delta CI was determined using the CI value just prior to WNT-5B addition and after stabilization.

(A) Tracing shows representative example measurement of the effect of FZD8 knockdown on WNT-5B-induced changes in impedance as a measure of receptor-mediated fibroblast activation. CI values are normalized against Time = 0 min. (B) The effect of FZD8 knockdown on WNT-5B-induced changes in impedance as a measure of receptor-mediated fibroblast activation. Data represent mean ± s.e.m. of 5 independent experiments. (C) MRC-5 human lung fibroblasts were stimulated with WNT-5B (1 μl/ml) for 24 hours. The expression of FZD8 was silenced with specific FZD8-targeting siRNA using non-targeting siRNA as a control. Figure shows the effect of FZD8 knockdown on WNT-5B-induced gene expression of ECM components and myofibroblast differentiation markers. Data represent mean ± s.e.m. of 6 independent experiments. *** p < 0.001 * p < 0.05 compared to basal conditions, ### p < 0.001 # p < 0.05 compared to the stimulated control (one-way repeated measures ANOVA with Student-Newman-Keuls multiple comparisons test).
Discussion

We show for the first time that the FZD8 receptor in human lung fibroblasts is required in part for the effects of TGF-β on the expression of various ECM proteins and myofibroblast differentiation in vitro as well as for bleomycin-induced fibrosis in vivo. Furthermore, we demonstrate that these effects are established via noncanonical WNT signalling and that specifically WNT-5B functions as a ligand for FZD8 to mediate the effects of TGF-β on fibroblast activation. These findings provide supportive evidence that a signalling axis involving FZD8 and WNT-5B plays a role in TGF-β-induced ECM turnover and repair mechanisms.

Fibroblasts contribute to tissue repair and remodelling by regulating ECM turnover and by their differentiation into myofibroblasts [1, 6, 7]. TGF-β activates fibroblasts, thereby stimulating ECM production and initiating myofibroblast differentiation leading to a more contractile phenotype [3, 7, 31, 32, 34]. Epithelial cells and inflammatory cells can secrete TGF-β, which can act upon fibroblasts to induce ECM production and myofibroblast differentiation [35, 36]. We observe here that TGF-β regulates the expression of ECM proteins and myofibroblast markers, i.e. collagen Iα1, fibronectin, versican, α-sm-actin and CTGF, in a FZD8 dependent manner in human lung fibroblasts. The induction of PAI-1 however, was unaffected by FZD8 knockdown. In addition, not all genes were attenuated by FZD8 knockdown to the same extent. Whereas versican and CTGF were nearly completely inhibited, collagen Iα1, fibronectin and α-sm-actin were only partially inhibited. In vivo, bleomycin exposure induced the expression of both collagen and fibronectin, which were partially inhibited in FZD8 deficient mice. This indicates that signalling via FZD8 is to some degree specific for individual ECM components and that other mechanisms (e.g. Smad3-dependent gene transcription) play additional regulatory roles in the TGF-β-induced expression of these components. Of importance, we show that WNT-5A and WNT-5B are induced in response to TGF-β with a similar EC50 as observed for FZD8 induction, but TGF-β induced the expression of FZD8 and WNT-5B, but not WNT-5A, in a Smad3 dependent manner.

Our findings strongly suggest that WNT-5B can function as an endogenous ligand for the FZD8 receptor. This is supported by several observations. First, we confirmed our previous findings showing that WNT-5A and WNT-5B are highly expressed in human lung fibroblasts [11]. However, only WNT-5B expression was Smad3 dependent and only WNT-5B mimicked the functional effects of TGF-β on the induction of fibronectin, versican, α-sm-actin and CTGF gene expression, as well as fibronectin and α-sm-actin protein expression. In addition, WNT-5B-induced changes in cellular impedance were partially prevented by FZD8 knockdown, indicating that WNT-5B engages FZD8 receptor signalling. Finally, our results showed that FZD8 is required for the induction of fibronectin and α-sm-actin gene expression by WNT-5B. Collectively, this implicates that TGF-β activates a WNT-5B/FZD8 ligand/receptor complex that is required for the induction of fibronectin and α-sm-actin and that this complex plays a regulatory role in the induction of collagen Iα1, versican and CTGF gene expression.

Whereas these data suggest a primary role for WNT-5B, we do not wish to completely rule out a role for WNT-5A in ECM production in general, as WNT-5A is known to regulate fibroblast proliferation [37] and our previous findings indicate that in airway smooth muscle, TGF-β induces the expression of both WNT-5A and FZD8. In this study, we showed that both WNT-5A and FZD8 knockdown reduced ECM production in response to
TGF-β [12]. Thus, the role of WNT-5A may be cell-specific, or context specific as the WNT ligand and receptor profile expressed by airway smooth muscle cells and fibroblasts do not completely overlap [11, 12].

The WNT signalling pathway operates through canonical β-catenin dependent and noncanonical β-catenin independent signalling routes [13-16]. Although TGF-β is known to activate β-catenin [11], our current data show that the canonical WNT signalling pathway is not involved in TGF-β-induced ECM gene expression. We blocked the canonical pathway using DKK-1, which interferes with the interaction between the FZD receptor and the LRP5/6 co-receptor necessary for canonical pathway activation [13]. Inhibiting the canonical pathway had no effect at all on the TGF-β-induced effects, whereas it did inhibit WNT-3A-induced Axin2 gene expression. Furthermore, stimulation with TGF-β did not result in LPR5/6 phosphorylation, an event necessary for canonical WNT signalling, whereas TGF-β did promote the expression of noncanonical WNT-5B. In turn, WNT-5B mimicked the TGF-β effects via the FZD8 receptor. In vivo, expression of the canonical WNT signalling target gene Axin2 and activated β-catenin were not reduced in the FZD8 deficient mice, or even significantly increased. This indicates that the signalling downstream of TGF-β/FZD8 is not via canonical WNT signalling, which is in apparent contrast to previous findings from us and others showing that TGF-β activates β-catenin in vitro and in vivo [10, 11, 38-44] and to the findings that β-catenin is required for TGF-β-induced ECM gene expression [10]. However, in addition to canonical WNT signalling, Smad2/3 is also known to activate β-catenin gene expression [39, 40] and β-catenin can physically interact with Smad2/3/4 proteins to regulate target gene expression [45, 46]. Together with previous publications [11, 47], our current data indicate therefore that TGF-β can activate β-catenin directly via PI3-kinase/protein kinase B (Akt)/glycogen synthase kinase (GSK)-3 dependent effects on β-catenin protein stability and ERK1/2 dependent effects on β-catenin gene expression, without the requirement for canonical WNT signalling. This notion is supported by findings showing that DKK-1 has no effect on TGF-β-induced ECM protein production or β-catenin expression in human airway smooth muscle cells [12] and that TGF-β does not promote the expression of classical canonical WNT target genes such as Axin2 in human lung fibroblasts [11]. Taken all results together, this implies that FZD8 partly regulates WNT-5B- and TGF-β-induced effects on fibroblast activation via noncanonical WNT signalling and that this signalling branch operates parallel to TGF-β-induced, WNT independent, β-catenin signalling to promote gene expression.

Recent studies indicate the involvement of WNT signalling in tissue remodelling in many organ systems including the lung [8, 11, 37]. Chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) or idiopathic pulmonary fibrosis (IPF) are progressive and devastating diseases, characterized by a lung function that is compromised by underlying inflammatory and remodelling mechanisms. To date, only limited treatment options that target the underlying lung injury and remodelling are available. Remodelling in COPD occurs mainly in the small airways, while in IPF the parenchymal lung tissue is mostly affected. However, both diseases are characterized by TGF-β-induced fibroblast activation and remodelling [48, 49]. Our current data show that TGF-β-induced fibroblast activation in vitro and bleomycin-induced fibrosis in vivo, a process dependent on TGF-β signalling, is regulated via FZD8. Furthermore, WNT-5B contributes to TGF-β-induced fibroblast activation as a ligand that signals via FZD8. In line with these findings, it was recently reported that high FZD8 gene expression in lung
TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8

tissue of IPF patients correlates with more rapid disease progression [44]. In addition, our published findings showed that the TGF-β-induced expression of WNT-5B and FZD8 is higher in fibroblasts of COPD patients compared to fibroblasts of non-COPD controls [11]. Collectively, our data provide evidence that signalling via the WNT-5B/FZD8 ligand/receptor complex may play an important role in ECM turnover and repair mechanisms in lung diseases. This pathway may play a role in chronic lung diseases, providing a rationale to further explore the therapeutic potential of this pathway.

Acknowledgements

The authors would like to thank Dr. Esther Schmitt (Novartis Pharma AG) for the generous gift of PKF 115-584. This project is funded by the Netherlands Lung Foundation (NLF grant 3.2.10.042). Hoeke A. Baarsma is supported by a postdoctoral fellowship from the European Respiratory Society (Fellowship LTRF 79-2012) and a fellowship from the Helmholtz Zentrum Germany (PFP PD-135). Melanie Königshoff is supported by a European Research Council Starting Grant (ERC-StG-LS7, Grant No. 261302). Mark Menzen and Anastasia van den Berg are acknowledged for excellent technical assistance.
Chapter 2

References


TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8.


TGF-β-induced pro-fibrotic signalling is regulated in part by the WNT receptor FZD8