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TGF- β -induced profibrotic signaling is regulated in part by the WNT receptor Frizzled-8

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ABSTRACT TGF- β is important in lung injury and remodeling processes. TGF- β and Wntless/integrin-1 (WNT) signaling are interconnected; however, the WNT ligand-receptor complexes involved are unknown. Thus, we aimed to identify Frizzled (FZD) receptors that mediate TGF- β -induced profibrotic signaling. MRC-5 and primary human lung fibroblasts were stimulated with TGF- β ₁, 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. TGF- β ₁ induced *FZD8* specifically via Smad3-dependent signaling in MRC-5 and primary human lung fibroblasts. It is noteworthy that *FZD8* knockdown reduced TGF- β ₁-induced collagen I α 1, 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*. Although inhibition of canonical WNT signaling did not affect TGF- β ₁-induced gene expression *in vitro*, noncanonical WNT-5B mimicked TGF- β ₁-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. Collectively, our findings demonstrate a role for *FZD8* in TGF- β -induced profibrotic signaling and imply that WNT-5B may be the ligand for *FZD8* in these responses.—Spanjer, A. I. R., Baarsma, H. A., Oostenbrink, L. M., Jansen, S. R., Kuipers, C. C., Lindner, M., Postma, D. S., Meurs, H., Heijink, I. H., Gosens, R., Königshoff, M. TGF- β -induced profibrotic signaling is regulated in part by the WNT receptor Frizzled-8. *FASEB J.* 30, 1823–1835 (2016). www.fasebj.org

Key Words: airway remodeling • extracellular matrix • myofibroblast differentiation • WNT signaling

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 physiologic repair processes and tissue homeostasis. However, in pathologic conditions, as in the 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 TGF- β . TGF- β is a profibrotic 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 Wntless/integrin-1 (WNT) signaling pathway in tissue homeostasis and remodeling 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 non-canonical pathways, which signal mainly to calcium (WNT/Ca²⁺ pathway) or to RhoA/JNK (WNT/planar cell polarity pathway) (13–16).

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Abbreviations: CI, cell index; COPD, chronic obstructive pulmonary disease; CTGF, connective tissue growth factor; Cyto-TBST, cyto-TBS containing 0.1% Tween 20; DKK, Dickkopf; ECM, extracellular matrix; FBS, fetal bovine serum; FZD, Frizzled; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; LRP, lipoprotein receptor-related
(continued on next page)

We previously demonstrated that TGF- β signaling increased 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 signaling in fibroblast activation and remodeling as well as the cross-talk that exists between TGF- β and WNT signaling 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 signaling both *in vitro* and *in vivo*. We show that *FZD8* is involved in TGF- β signaling 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 we 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 biobank for lung diseases at the Comprehensive Pneumology Center and isolated as previously described (17). All participants gave written informed consent, and the study was approved by the Ludwig-Maximilians University Ethics Committee. 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.

Cell culture

MRC-5 human lung fibroblasts (18) were cultured in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 μ g/ml streptomycin, 50 U/ml penicillin, and 1.5 μ g/ml amphotericin B at 37°C with 5% CO₂. Before 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 h. Primary human lung fibroblasts were cultured in DMEM/Ham's F12 (1:1) with 20% (v/v) fetal calf serum and 100 μ g/ml streptomycin and 100 U/ml penicillin at 37°C with 5% CO₂. Before stimulation, cells were grown to confluence and serum deprived for 24 h in medium with 0.1% fetal calf serum and 100 μ g/ml streptomycin and 100 U/ml penicillin.

Cell stimulation

Pulmonary fibroblasts were stimulated with either 2 ng/ml recombinant TGF- β ₁, 1 μ g/ml recombinant WNT-5A, or 1 μ g/ml recombinant WNT-5B. The inhibitors Dickkopf (DKK)-1 (0.3 μ g/ml), SIS3 (3 μ M), U-0126 (3 μ M), Y-27632 (1 μ M), PKF

115-584 (0.1 μ M), and LY-294002 (3 μ M) were added 30 min before stimulation. The concentrations of these inhibitors were chosen on the basis of their selectivity profiles as described in literature (19–23) and their effectiveness against their respective targets in our cell system (unpublished results).

Small interfering RNA transfection

Cells were grown to 90% confluence and transfected with specific small interfering (si)RNA against the *FZD8* transcript. Cells were transfected in serum-free Ham's F12 without supplements using 100 pmol *FZD8*-targeted siRNA or nontargeting control siRNA and Lipofectamine 2000 Transfection Reagent (Invitrogen, Paisley, United Kingdom). After 6 h the medium was changed to supplemented medium with 10% (v/v) FBS for 18 h and subsequently to supplemented medium with 0.5% (v/v) FBS for 24 h. 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, 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 μ g) were then reverse transcribed according to the Reverse Transcription System (Promega Benelux, Leiden, The Netherlands), and the cDNA was stored at –20°C until further use. A total of 5 μ l Absolute Blue SYBR Green Supermix, containing fluorescein to comprise well-to-well variation, a 1 μ M concentration of gene-specific forward and reverse primer, and 1 μ l of cDNA sample were used in a total volume of 10 μ l 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 analyzed using the comparative cycle threshold method.

Smad binding element-4 activity

MRC-5 human lung fibroblasts were plated on 6-well plates and transfected as described above with 0.5 μ g plasmid DNA encoding Smad binding element (SBE)-4 firefly luciferase and 0.12 μ g plasmid DNA encoding CMV-*Renilla* luciferase. SBE-4 activity is a measure of activation of TGF- β /SMAD signaling. Transfected cells were then stimulated with TGF- β ₁ (2 ng/ml) in the presence or absence of SIS3 (3 μ M) as described above. After 24 h, cells were lysed. Subsequently, firefly luciferase and *Renilla* luciferase activity were measured using a Luminometer (Luminoskan Ascent; Thermo Electron, Waltham, MA, USA) and using the Dual-Luciferase Reporter Assay (Promega Benelux). 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) HBSS [composition (mg/L): 400 KCl, 60 KH₂PO₄, 8000 NaCl, 350 NaHCO₃, 50 Na₂HPO₄·1H₂O, 1000 glucose; pH 7.4], then lysed in ice-cold sodium dodecyl sulfate buffer (composition: 62.5 mM Tris, 2% w/v sodium dodecyl sulfate, 1 mM NaF, 1 mM

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protein; PAI, plasminogen activator inhibitor; SBE, Smad binding element; siRNA, small interfering RNA; sm, smooth muscle; TBS, Tris-buffered saline; TCF, T-cell factor; WNT, Wingless/integrase-1; WT, wild-type

TABLE 1. Primers used for determination of specific human genes of interest

Gene	Primer sequence, 5'–3'	
	Forward	Reverse
<i>FZD1</i>	TCG ACT TCC TGA AGC TGG AT	AAG GTG GGA GAA GGG AGT GT
<i>FZD2</i>	CCC GACT TCAC GGT CTA CAT	CTG TTG GTG AGG CGA GTG TA
<i>FZD3</i>	TCT CTT TGG CCC TTG ACT G	ACA AAG AAA AGG CCG GAA AT
<i>FZD4</i>	CCA GGA TTC CTT CCA AGT CA	CCA TGT CCT TGT GGC CTA CT
<i>FZD5</i>	AGC TAA AAT GGC CAG AGC AA	AAT TCC CCC TGG GAA CTA TG
<i>FZD6</i>	TTG TTG GCA TCT CTG CTG TC	CCA TGG ATT TGG AAA TGA CC
<i>FZD7</i>	CGA CGC TCT TTA CCG TTC TC	GCC ATG CCG AAG AAG TAG AG
<i>FZD8</i>	GAC ACT TGA TGG GCT GAG GT	CAA ATC TCG GGT TCT GGA AA
<i>FZD9</i>	AGA CCA TCG TCA TCC TGA CC	CCA TGA GCT TCT CCA GCT TC
<i>FZD10</i>	CCT CCA AGA CTC TGC AGT CC	GAC TGG GCA GGG ATC TCA TA
Collagen Iα1	AGC CAG CAG ATC GAG AAC AT	TCT TGT CCT TGG GGT TCT TG
Type III collagen	CCA TGA ATG GTG GTT TTC AG	GTG TTT AGT GCA ACC ATC
Fibronectin	TCC AGG AGG AAA TTC CAA TG	ACA CAC GTG CAC CTC ATC AT
Versican	GGG AAC CTG GTG AAG AAA CA	CTT CCA CAG TGG GTG GTC TT
α-sm-Actin	GAC CCT GAA GTA CCC GAT AGA AC	GGG CAA CAC GAA GCT CAT TG
<i>CTGF</i>	GGA AAA GAT TCC GAC CCA AT	TGC TCC TAA AGC CAC ACC TT
<i>PAI-1</i>	CGC CAG AGC AGG ACG AA	GGA CAC ATC TGC ATC CTG AAG T
<i>WNT-5A</i>	GGG TGG GAA CCA AGA AAA AT	TGG AAC CTA CCC ATC CCA TA
<i>WNT-5B</i>	ACG CTG GAG ATC TCT GAG GA	CGA GGT TGA AGC TGA GTT CC
<i>Axin2</i>	CCT GCC ACC AAG ACC TAC AT	CTT CAT TCA AGG TGG GGA GA
<i>HPRT</i>	AAG GAC CCC ACG AAG TGT TG	GGC TTT GTA TTT TGC TTT TCC A
18S rRNA	CGC CGC TAG AGG TGA AAT TC	TTG GCA AAT GCT TTC GCT C

Na₃VO₄, 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, Rockford, IL, USA). Lysates were stored at –20°C until further use. Equal quantities of protein (10–20 µg per lane) were subjected to electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. These were analyzed 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 was used as a loading control. By using chemiluminescence reagents, bands were recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngene, Cambridge, United Kingdom). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene).

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 h before 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 h, then fixed for 15 min at room temperature in cytoskeletal buffer (composition: 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose, pH 6.1) containing 3% paraformaldehyde. Cells were then permeabilized by incubation for 5 min at room temperature in cytoskeletal buffer containing 3% paraformaldehyde and 0.3% Triton X-100. Fixed cells then were blocked for 2 h at room temperature in Cyto-Tris-buffered saline (TBS) buffer (20 mM Tris base, 154 mM NaCl, 2.0 mM EGTA, 2.0 mM MgCl₂, pH 7.2) containing 1% bovine serum albumin 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 h at room temperature in Cyto-TBST. Nuclei were stained with Hoechst dye 33342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and analyzed using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens; Olympus, Tokyo, Japan).

Impedance measurement

WNT-5B-mediated cellular activation was assessed using the xCELLigence system (Roche Applied Science, Penzberg, Germany) using E-plates (ACEA Biosciences, San Diego, CA, USA).

TABLE 2. Primers used for determination of specific mouse genes of interest

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

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 software (Roche Applied Science; ACEA Biosciences). CI represents the impedance change divided by a background value: $CI = (Z_i - Z_0) / 15 \Omega$. The change in impedance is represented by the impedance at an individual time point (Z_i) during the experiment minus the background electrical resistance measured in the absence of cells before the experiment (Z_0). MRC-5 human lung fibroblasts were transfected as described above with specific *FZD8*-targeted siRNA or nontargeting control siRNA. At 24 h 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 in real time. CI was measured every 15 min for 16 h. CI increased while cells attached. After 16 h, before the experiment, CI was stable. If cells respond to stimulation, CI will change immediately. Therefore, before stimulation with ligands, CI was measured at 1 s intervals. Cells were stimulated with recombinant WNT-5B (1 µg/ml). After stimulation, time intervals for CI measurement were sequentially 1 s for 300 s, 5 min for 1 h, and 15 min for 24 h. The change in CI was determined using the CI value just before WNT-5B addition and after stabilization.

Preparation of WNT-3A-conditioned medium

Mouse L cells [subcutaneous fibroblasts; American Type Culture Collection (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.

Animal studies

Heterozygous, inbred, specific-pathogen-free breeding colonies of *FZD8*^{+/-} mice (C57BL/6;129P2-*FZD8*^{tm1Dgen}/J), showing no obvious phenotype, were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). After breeding, homozygous *FZD8*^{-/-} mice and wild-type (WT) littermates were used for experiments. Mice (8–12 wk) 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 animal's body weight. A total of 50 µl bleomycin (Sigma-Aldrich, Taufkirchen, Germany) in PBS (3 U/kg) or PBS was intratracheally instilled through a MicroSprayer Aerosolizer 20 G INTROCAN (Penn Century, Wyndmoor, PA, USA). After installation, the narcosis was antagonized by a combination of 0.29 mg/ml atipamezole (Orion Pharma), 0.059 mg/ml flumazenil (Hexal, Holzkirchen, Germany), and 0.14 mg/ml naloxone (Actavis, Munich, Germany) according to body weight. On d 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 at -80°C before mRNA isolation and protein determination, and 1 lobe was filled with 4% paraformaldehyde for histology. Paraffin-embedded sections were stained with hematoxylin and eosin and Masson-Goldner (Carl Roth, Karlsruhe, Germany) to evaluate collagen deposition. Staining intensity was quantified by 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 (Minneapolis, MN, USA). 6,7-Dimethyl-2-[(2*E*)-3-(1-methyl-2-phenyl-1*H*-pyrrolo [2,3-*b*]pyridin-3-yl-prop-2-enoyl)-1,2,3,4-tetrahydroisoquinoline hydrochloride (SIS3) was obtained from Merck GmbH (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), and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY-294002) were obtained from Tocris Cookson (Bristol, United Kingdom). PKF 115-584 was obtained from Novartis Pharma AG (Basel, Switzerland). *FZD8* siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and nonsilencing control siRNA from Qiagen (Germantown, MD, USA). Lipofectamine 2000 Transfection Reagent and Hoechst 33342 were obtained from Invitrogen (Carlsbad, CA, USA). Mouse anti-α-sm-actin, mouse anti-β-actin, HRP-conjugated goat anti-mouse antibody, and HRP-conjugated rabbit anti-goat antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-fibronectin (H-300) antibody, goat anti-fibronectin (C20) antibody, and mouse anti-GAPDH antibody were obtained from Santa Cruz Biotechnology. Rabbit anti-phospholipoprotein receptor-related protein (LRP) 5/6 (Ser1490) antibody and rabbit anti-β-tubulin were obtained from Cell Signaling (Bioké, Leiden, The Netherlands). All other chemicals were of analytical grade.

Statistical analysis

All real-time PCR data were log transformed before statistical analysis. For comparison between 2 conditions, a Student's *t* test or Mann-Whitney *U* test was used, as appropriate. For comparisons between multiple conditions, 1-way repeated-measures ANOVA was used, followed by a *post hoc* analysis using the Student-Newman-Keuls multiple-comparisons test. For comparisons of the *in vivo* experiments, 2-way ANOVA was used, followed by a *post hoc* analysis using the Student-Newman-Keuls multiple-comparisons test. *P* < 0.05 was considered significant.

RESULTS

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

We previously showed that TGF-β₁ stimulation induced *FZD8* 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 (Fig. 1A–F). The effect on *FZD8* expression was already visible after 4 h of stimulation and was more pronounced after 24 h (Fig. 1B). The induction of *FZD8* expression was concentration dependent, with an EC₅₀ of 0.26 ng/ml for TGF-β₁ and a maximal response at 2 ng/ml (18.5 ± 3.2-fold increase compared with untreated control; *P* < 0.001; Fig. 1C). TGF-β₁ also increased *FZD8* expression in primary human lung fibroblasts (Fig. 1E, F). Parallel with the induction of *FZD8*, TGF-β₁ increased the expression of the ECM components collagen Iα1, fibronectin, and versican as well as

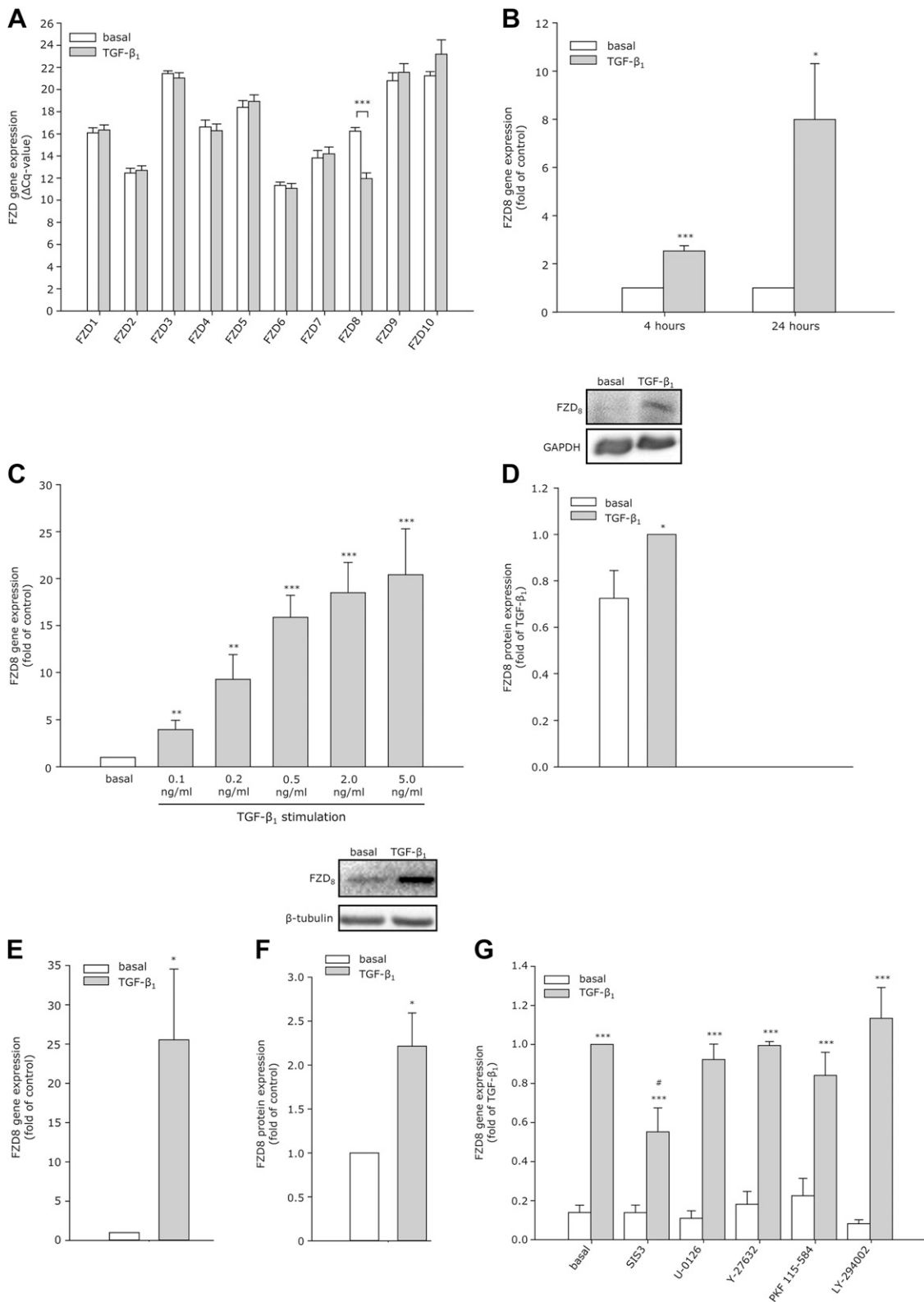


Figure 1. TGF-β₁-induced FZD8 mRNA and protein expression in human lung fibroblasts is Smad3 dependent. MRC-5 human lung fibroblasts and primary human lung fibroblasts were stimulated with TGF-β₁ for 4 or 24 h (mRNA) or 48 h (protein). Specific inhibitors were used to block individual TGF-β₁ signaling pathways. **A**) Gene expression of FZD receptors in MRC-5 human lung fibroblasts after 24 h of stimulation with TGF-β₁ (2 ng/ml). Of note, high cycle threshold (*C_t*) values correspond to low copy numbers. Data represent the means ± SEM of 3–6 independent experiments. **B**) *FZD8* expression in MRC-5 human lung fibroblasts after stimulation with TGF-β₁ (2 ng/ml) at different time points. Data represent the means ± SEM of 5 independent experiments. **C**) Concentration-dependent increase of *FZD8* expression in MRC-5 human lung fibroblasts after 24 h of (continued on next page)

the differentiation markers α -sm-actin, *PAI-1*, and *CTGF* (Supplemental Fig. S1A). TGF- β_1 dose-dependently increased the expression of fibronectin, versican, and *CTGF*, which are WNT-responsive genes (11, 26–29), with an EC₅₀ of 0.14, 0.30, and 2.52 ng/ml, respectively (Supplemental Fig. S1B). On the basis of these data, we used a concentration of 2 ng/ml TGF- β_1 and the 24 h time point for gene expression analysis in subsequent experiments.

TGF- β_1 -induced *FZD8* expression in human lung fibroblasts is Smad3 dependent

TGF- β signals *via* several intracellular pathways involving Smad, MAPK/ERK, Rho kinase, β -catenin/T-cell factor (TCF), and phosphoinositide 3-kinase (PI3K) (30). To investigate *via* which pathway the induction of *FZD8* by TGF- β_1 is established, we used specific inhibitors of Smad3 (SIS3, 3 μ M), MEK1/2 (U-0126, 3 μ M), Rho kinase (Y-27632, 1 μ M), β -catenin/TCF (PKF 115-584, 0.1 μ M), and PI3K (LY-294002, 3 μ M). The concentrations of these inhibitors were chosen to provide (sub)maximal and selective inhibition of their respective targets in airway mesenchymal cells (19–23) (unpublished results). It is noteworthy that the TGF- β_1 -induced expression of *FZD8* was attenuated by the Smad3 inhibitor (57%) but was not affected by the inhibition of MEK1/2, Rho-kinase, β -catenin/TCF, or PI3K signaling (Fig. 1G). These results demonstrate that TGF- β_1 -induced *FZD8* expression in MRC-5 human lung fibroblasts is Smad3 dependent. Smad3 inhibition also blocked TGF- β_1 -induced SBE-4 activity, which was studied as a measure of activation of TGF- β /SMAD signaling (Supplemental Fig. S1C) and which impaired TGF- β_1 -induced expression of fibronectin, versican, and *CTGF* (Supplemental Fig. S1D–F).

TGF- β_1 -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_1 increased the gene expression of the ECM components collagen I α 1, fibronectin, and versican as well as the differentiation markers α -sm-actin, *PAI-1*, and *CTGF* (Supplemental Fig. S1A). 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* expression in response to TGF- β_1 (from 4.5 ± 1.5 - to 0.6 ± 0.2 -fold of baseline *FZD8* expression in control siRNA-transfected cells; Fig. 2). *FZD8* knockdown significantly reduced the TGF- β_1 -induced expression of collagen I α 1, fibronectin, versican, α -sm-actin, and *CTGF* but not *PAI-1* (Fig. 2) and type III collagen (data not shown). The expression of these genes was differentially attenuated, with versican expression being the most affected (85% reduction), followed by *CTGF* (70%), fibronectin (60%), α -sm-actin (53%), and collagen I α 1 (38%). These results show that *FZD8* is partly involved in the TGF- β_1 -induced gene expression of several ECM proteins and myofibroblast differentiation markers.

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 signaling (33). We first confirmed that *FZD8* expression was reduced in *FZD8*^{-/-} mice compared with WT mice (Fig. 3A). Single bleomycin instillation caused strong fibrotic alterations within the lung after 14 d, 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 with WT mice subjected to bleomycin (Fig. 3B–E).

TGF- β_1 -induced ECM expression and myofibroblast differentiation is not regulated *via* canonical WNT signaling

WNT signaling can occur *via* the canonical β -catenin-dependent pathway and *via* the noncanonical β -catenin-independent signaling pathways (13–15). Our previous studies showed that TGF- β_1 -induced α -sm-actin, collagen I α 1, fibronectin, and versican expression are regulated by β -catenin signaling (10, 11). We thus hypothesized that activation of *FZD8* mediates canonical WNT signaling that might potentiate TGF- β_1 -induced ECM turnover and myofibroblast differentiation. We therefore studied the effects of endogenous canonical WNT signaling inhibition in the presence of TGF- β signaling. We applied the well-known WNT antagonist DKK-1, which targets the interaction of *FZD*s with the coreceptor low-density LRP5/6.

stimulation with increasing concentrations of TGF- β_1 (EC₅₀, 0.26 ng/ml). Data represent means \pm SEM of 5–8 independent experiments. D) *FZD8* protein expression in MRC-5 human lung fibroblasts after 48 h of stimulation with TGF- β_1 . Data represent means \pm SEM of 10 independent experiments. E) *FZD8* expression in primary human lung fibroblasts after 24 h of stimulation with TGF- β_1 (2 ng/ml). Data represent means \pm SEM of 4 patients. F) *FZD8* protein expression in primary human lung fibroblasts after 48 h of stimulation with TGF- β_1 (2 ng/ml). Data represent the means \pm SEM of 4 patients. G) Involvement of specific signaling cascades in TGF- β_1 -induced gene expression of *FZD8*. Inhibitors were used to inhibit following pathways: SIS3 (3 μ M), Smad3; U-0126 (3 μ M), MEK1/2; Y-27632 (1 μ M), Rho kinase; PKF 115-584 (0.1 μ M), β -catenin/TCF; LY-294002 (3 μ M), PI3K. Data represent the means \pm SEM of 8–10 independent experiments. *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$ compared with basal conditions (A–D, 2-tailed Student's *t* test; E, F, 2-tailed Mann-Whitney *U* test), # $P < 0.05$ compared with stimulated control (1-way repeated-measures ANOVA with Student-Newman-Keuls multiple-comparisons test).

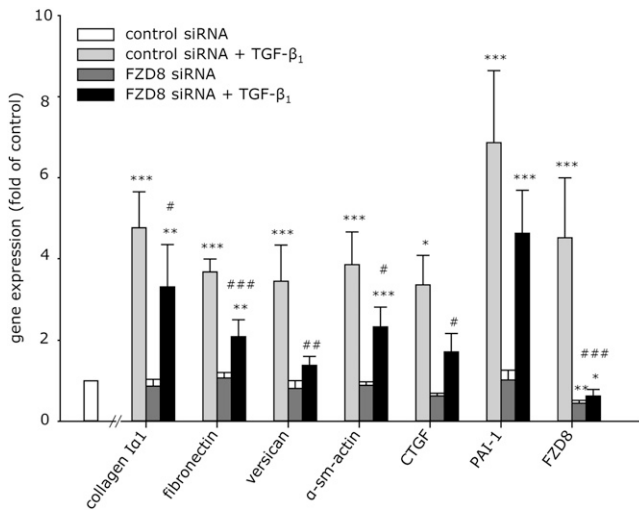


Figure 2. TGF-β₁-induced ECM expression is regulated by FZD8. MRC-5 human lung fibroblasts were stimulated with TGF-β₁ (2 ng/ml) for 24 h. Expression of *FZD8* was silenced with specific *FZD8*-targeting siRNA using nontargeting siRNA as control. Shown is effect of *FZD8* knockdown on TGF-β₁-induced gene expression of ECM components and myofibroblast differentiation markers. Data represent the means ± SEM of 7 independent experiments. ****P* < 0.001, ***P* < 0.01, **P* < 0.05 compared with control siRNA, ###*P* < 0.001, ##*P* < 0.01, #*P* < 0.05 compared with stimulated control (1-way repeated-measures ANOVA with Student-Newman-Keuls multiple-comparisons test).

This coreceptor is indispensable for canonical WNT signaling (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 signaling (Fig. 4A). As a positive control, DKK-1 inhibited the expression of the canonical WNT target gene *Axin2*, which was induced by WNT-3A-conditioned medium (Supplemental Fig. S2). In line with this, the expression of the canonical WNT target gene *Axin2* was increased at basal levels in the *FZD8*⁻⁷⁻ mice (Fig. 4B), 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 as inhibitory. Further, TGF-β₁ had no effect on ser1490 LRP5/6 phosphorylation in MRC-5 human lung fibroblasts, an event necessary for canonical WNT signaling (Fig. 4C).

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 expression of *WNT-5B* in MRC-5 human lung fibroblasts in response to TGF-β₁. Indeed, TGF-β₁ induced the expression of *WNT-5B* in a concentration-dependent manner, with an EC₅₀ of 0.26 ng/ml (Fig. 5A). Similar to *FZD8* expression, the TGF-β₁-induced *WNT-5B* expression was Smad3 dependent (Fig. 5B). Functional studies showed that stimulation with recombinant WNT-5B mimicked the TGF-β₁-induced gene expression of ECM components and differentiation markers; WNT-5B significantly induced the expression of

fibronectin, versican, α-sm-actin, and *CTGF* (Fig. 5C). We confirmed these findings at the protein level for fibronectin and α-sm-actin (Fig. 5D, E). For these experiments, we used stimulation with recombinant WNT-5A as a control (Fig. 5C–E). 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-mediated cellular 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 signaling for fibroblast activation (Fig. 6A, 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 down-regulated by *FZD8* knockdown, indicating that WNT-5B is dependent on FZD8 for the induction of these genes (Fig. 6C). Collectively, these data show that noncanonical WNT-5B is a ligand for FZD8 and that FZD8 regulates TGF-β₁-induced ECM gene expression and myofibroblast differentiation in human lung fibroblasts.

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 signaling 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 signaling axis involving FZD8 and WNT-5B plays a role in TGF-β-induced ECM turnover and repair mechanisms.

Fibroblasts contribute to tissue repair and remodeling 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 on fibroblasts to induce ECM production and myofibroblast differentiation (35, 36). We observed here that TGF-β regulates the expression of ECM proteins and myofibroblast markers 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 signaling *via* FZD8 is to some degree specific for individual ECM components and that other mechanisms (*e.g.*, Smad3-dependent gene transcription) play

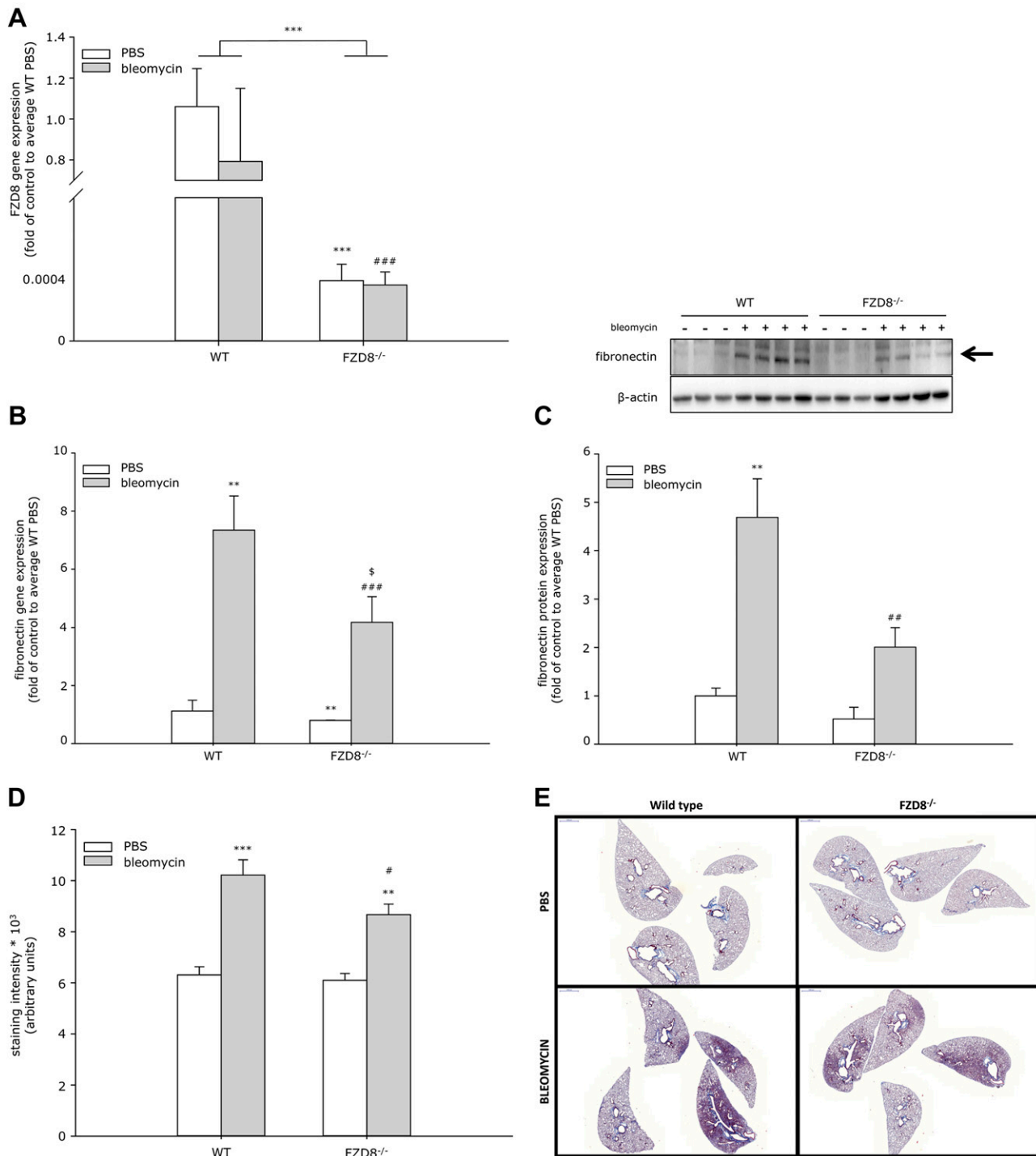


Figure 3. Bleomycin-induced fibrosis in mice is partially dependent on FZD8. *FZD8*^{-/-} mice and WT littermates were instilled with bleomycin to induce fibrosis and followed up after 14 d as described in Materials and Methods. *A*) *FZD8* expression in whole lung tissue. Data represent the means \pm SEM of 5–8 mice per group. ****P* < 0.001 compared with control WT mice, ###*P* < 0.001 compared with bleomycin-instilled WT mice (2-way ANOVA with Student-Newman-Keuls multiple-comparisons test). *B*, *C*) Fibronectin gene expression (*B*) and fibronectin protein expression (*C*) in whole lung tissue. Data represent the means \pm SEM of 3–6 mice per group. ***P* < 0.01 compared with control WT mice, ###*P* < 0.001 and ##*P* < 0.01 compared with bleomycin-instilled WT mice, \$*P* < 0.05 compared with PBS-treated *FZD8*^{-/-} mice (2-way ANOVA with Student-Newman-Keuls multiple-comparisons test). *D*) Histologic assessment of fibrotic areas in lung using Masson-Goldner staining. Data represent the means \pm SEM of 5–8 mice per group. ****P* < 0.001, ***P* < 0.01 compared with control WT mice, #*P* < 0.05 compared with bleomycin-instilled WT mice (2-way ANOVA with Student-Newman-Keuls multiple-comparisons test). *E*) Representation for quantification of staining shown in (*D*).

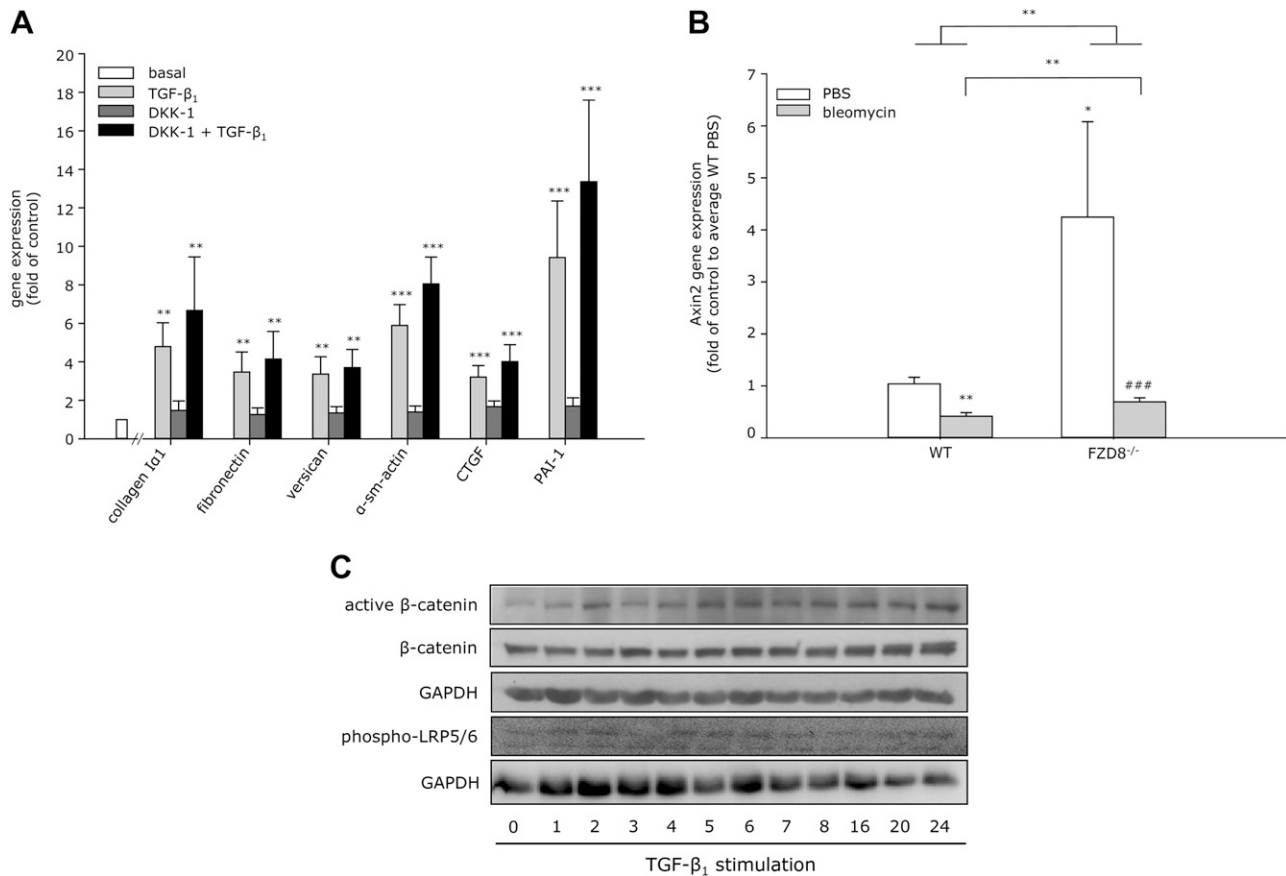


Figure 4. TGF- β_1 -induced ECM expression and myofibroblast differentiation is not regulated *via* canonical WNT signaling. MRC-5 human lung fibroblasts were stimulated with TGF- β_1 (2 ng/ml) for 24 h (mRNA) or for different time points (protein) as indicated. Canonical WNT signaling was inhibited with DKK-1 (0.3 μ g/ml). A) Effect of canonical WNT signaling pathway on TGF- β_1 -induced gene expression of ECM proteins and myofibroblast differentiation markers. Data represent the means \pm SEM of 5 independent experiments. *** P < 0.001, ** P < 0.01 compared with basal conditions (1-way repeated-measures ANOVA with Student-Newman-Keuls multiple-comparisons test). B) *FZD8*^{-/-} mice and WT littermates were instilled with bleomycin for 14 d as described in Materials and Methods. Shown is *Axin2* expression in whole lung tissue. Data represent the means \pm SEM of 5–8 mice per group. ** P < 0.01, * P < 0.05 compared with control WT mice, ### P < 0.001 compared with bleomycin-instilled WT mice (2-way ANOVA with Student-Newman-Keuls multiple-comparisons test). C) Analysis of active β -catenin and ser1490 LRP5/6 phosphorylation by TGF- β_1 .

additional regulatory roles in the TGF- β -induced expression of these components. Of importance, TGF- β induced the expression of both *FZD8* and *WNT5B* in a Smad3-dependent manner. We also show that *WNT5B* is induced in response to TGF- β with a similar EC₅₀ as that observed for *FZD8* induction.

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 *WNT5B* is highly expressed in human lung fibroblasts (11). *WNT5B* expression was also Smad3 dependent, and it mimicked the functional effects of TGF- β on the induction of fibronectin, versican, α -sm-actin, and *CTGF* expression as well as fibronectin and α -sm-actin expression. In addition, WNT-5B-induced changes in cellular impedance were partially prevented by *FZD8* knockdown, indicating that WNT-5B engages FZD8 receptor signaling. Finally, our results showed that FZD8 is required for the induction of fibronectin and α -sm-actin expression by WNT-5B. Collectively, this implies 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 I α 1, versican, and *CTGF* expression. We did not observe functional effects of WNT-5A on ECM production in our current study; however, 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 that study, we showed that both *WNT5A* 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 signaling pathway operates through canonical β -catenin-dependent and noncanonical β -catenin-independent signaling routes (13–16). Although TGF- β is known to activate β -catenin (11), our current data show that the canonical WNT signaling pathway is not involved in TGF- β -induced ECM gene expression. We blocked the

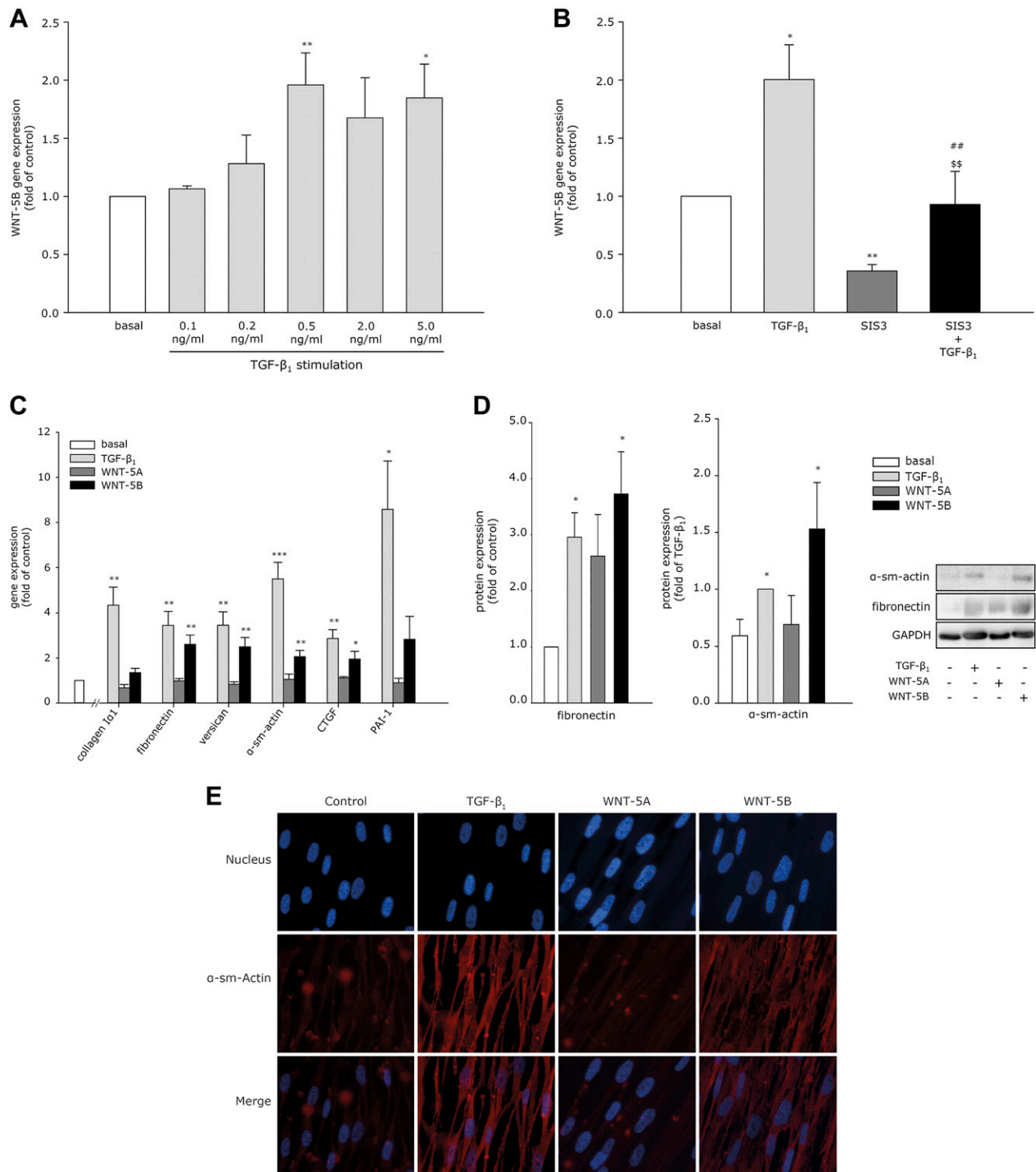


Figure 5. Noncanonical WNT-5B mimics effects of TGF- β_1 on fibroblast activation. MRC-5 human lung fibroblasts were stimulated with TGF- β_1 (2 ng/ml), WNT-5A (1 μ g/ml), or WNT-5B (1 μ g/ml) for 24 h (mRNA) or for 48 h (protein and immunocytochemistry). **A)** Concentration-dependent increase of *WNT-5B* expression after stimulation with increasing concentrations of TGF- β_1 (EC_{50} , 0.26 ng/ml). Data represent means \pm SEM of 5–8 independent experiments. **B)** Effect of inhibiting Smad3 on TGF- β_1 -induced gene expression of *WNT-5B*. Data represent the means \pm SEM of 5 independent experiments. **C)** Recombinant TGF- β_1 , WNT-5A-, and WNT-5B-induced effects on gene expression of ECM components and myofibroblast differentiation markers. Data represent the means \pm SEM of 3–7 independent experiments. **D)** Effect of recombinant TGF- β_1 , WNT-5A, and WNT-5B on protein expression of fibronectin and α -sm-actin. Data represent the means \pm SEM of 3–5 independent experiments. **E)** Effect of TGF- β_1 , WNT-5A, and WNT-5B stimulation on α -sm-actin stress fiber formation. Cells were stained for α -sm-actin (red) and DNA (Hoechst 33342; blue). Original magnification, $\times 100$. *** P < 0.001, ** P < 0.01, * P < 0.05 compared with basal conditions (Student's t test; A, C, D). ** P < 0.01, * P < 0.05 compared with basal conditions, ## P < 0.01 compared with stimulated control, \$\$\$ P < 0.01 compared with inhibited control (1-way repeated-measures ANOVA with Student-Newman-Keuls multiple-comparisons test; B).

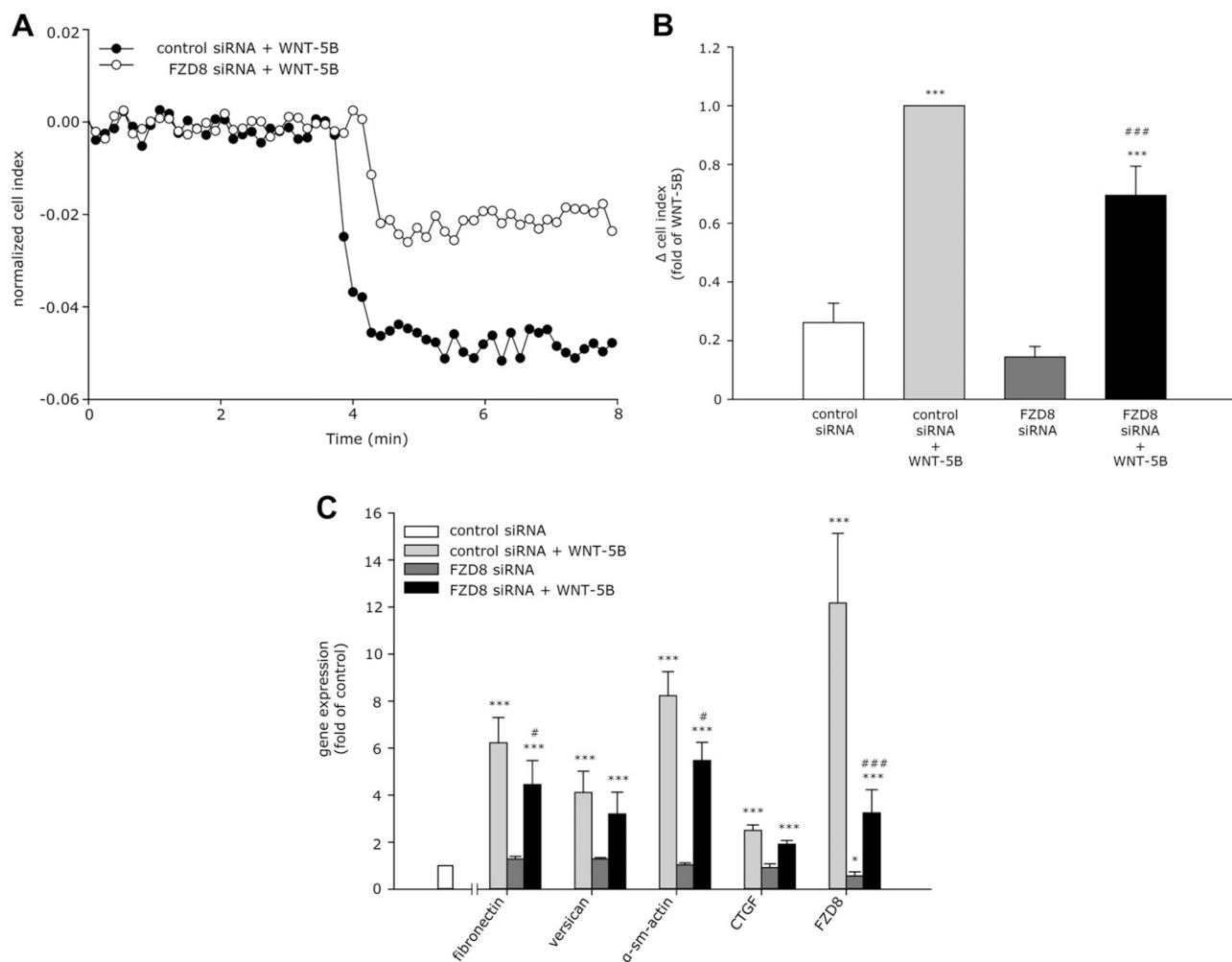


Figure 6. WNT-5B functions as ligand for FZD8. WNT-5B mediated cellular activation was assessed as described in Materials and Methods. MRC-5 human lung fibroblasts were transfected as described with specific *FZD8*-targeted siRNA or nontargeting control siRNA. Cells were stimulated with recombinant WNT-5B (1 μ g/ml). Change in CI was determined using CI value just before WNT-5B addition and after stabilization. **A)** Tracing shows representative example measurement of effect of *FZD8* knockdown on WNT-5B-induced changes in impedance as measure of receptor-mediated fibroblast activation. CI values are normalized against time = 0 min. **B)** Effect of *FZD8* knockdown on WNT-5B-induced changes in impedance as measure of receptor-mediated fibroblast activation. Data represent the means \pm SEM of 5 independent experiments. **C)** MRC-5 human lung fibroblasts were stimulated with WNT-5B (1 μ l/ml) for 24 h. Expression of *FZD8* was silenced with specific *FZD8*-targeting siRNA using nontargeting siRNA as control. This figure shows the effect of *FZD8* knockdown on WNT-5B-induced gene expression of ECM components and myofibroblast differentiation markers. Data represent the means \pm SEM of 6 independent experiments. *** P < 0.001, * P < 0.05 compared with basal conditions, ### P < 0.001, # P < 0.05 compared with stimulated control (1-way repeated-measures ANOVA with Student-Newman-Keuls multiple-comparisons test).

canonical pathway using DKK-1, which interferes with the interaction between the FZD receptor and the LRP5/6 coreceptor 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* expression. Furthermore, stimulation with TGF- β did not result in LRP5/6 phosphorylation, an event necessary for canonical WNT signaling, 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 signaling target gene *Axin2* and activated β -catenin were not reduced in the *FZD8*-deficient mice, or even significantly increased. This indicates that the signaling downstream of TGF- β /FZD8 is not *via* canonical WNT signaling,

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 signaling, Smad2/3 is also known to activate β -catenin 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* PI3K/PKB/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 signaling. 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 classic canonical WNT target genes such as *Axin2* in human lung fibroblasts (11). When all results are taken together, this implies that FZD8 partly regulates WNT-5B- and TGF- β -induced effects on fibroblast activation *via* noncanonical WNT signaling and that this signaling branch operates parallel to TGF- β -induced, WNT-independent β -catenin signaling to promote gene expression.

Recent studies indicate the involvement of WNT signaling in tissue remodeling in many organ systems including the lung (8, 11, 37). Chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) or idiopathic pulmonary fibrosis, are progressive and devastating diseases, characterized by lung function compromised by underlying inflammatory and remodeling mechanisms. To date, only limited treatment options that target the underlying lung injury and remodeling are available. Remodeling in COPD occurs mainly in the small airways, while in idiopathic pulmonary fibrosis the parenchymal lung tissue is mostly affected. However, both diseases are characterized by TGF- β -induced fibroblast activation and remodeling (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 signaling, 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* expression in lung tissue of patients with idiopathic pulmonary fibrosis 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 patients with COPD compared with fibroblasts of non-COPD control subjects (11). Collectively, our data provide evidence that signaling *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. **[F]**

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