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Reduced Frizzled Receptor 4 Expression Prevents WNT/ β -Catenin-driven Alveolar Lung Repair in Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Chronic obstructive pulmonary disease (COPD), in particular emphysema, is characterized by loss of parenchymal alveolar tissue and impaired tissue repair. Wingless and INT-1 (WNT)/ β -catenin signaling is reduced in COPD; however, the mechanisms thereof, specifically the role of the frizzled (FZD) family of WNT receptors, remain unexplored.

Objectives: To identify and functionally characterize specific FZD receptors that control downstream WNT signaling in impaired lung repair in COPD.

Methods: FZD expression was analyzed in lung homogenates and alveolar epithelial type II (ATII) cells of never-smokers, smokers, patients with COPD, and two experimental COPD models by quantitative reverse transcriptase–polymerase chain reaction, immunoblotting, and immunofluorescence. The functional effects of cigarette smoke on FZD4, WNT/ β -catenin signaling, and elastogenic components were investigated in primary ATII cells *in vitro* and in three-dimensional lung tissue cultures *ex vivo*. Gain- and loss-of-function approaches were applied to determine

the effects of FZD4 signaling on alveolar epithelial cell wound healing and repair, as well as on expression of elastogenic components.

Measurements and Main Results: FZD4 expression was reduced in human and experimental COPD lung tissues as well as in primary human ATII cells from patients with COPD. Cigarette smoke exposure down-regulated FZD4 expression *in vitro* and *in vivo*, along with reduced WNT/ β -catenin activity. Inhibition of FZD4 decreased WNT/ β -catenin-driven epithelial cell proliferation and wound closure, and it interfered with ATII-to-ATI cell transdifferentiation and organoid formation, which were augmented by FZD4 overexpression. Moreover, FZD4 restoration by overexpression or pharmacological induction led to induction of WNT/ β -catenin signaling and expression of elastogenic components in three-dimensional lung tissue cultures *ex vivo*.

Conclusions: Reduced FZD4 expression in COPD contributes to impaired alveolar repair capacity.

Keywords: emphysema; regeneration; smoking; cigarette smoke; Frizzled 4 receptor

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At a Glance Commentary

Scientific Knowledge on the

Subject: WNT/ β -catenin signaling is decreased in chronic obstructive pulmonary disease (COPD) and emphysema, but the mechanisms behind tissue destruction, impaired tissue repair, and particularly involvement of specific WNT receptors (frizzled [FZD]) remain unknown.

What This Study Adds to the

Field: The WNT receptor FZD4 is decreased in human and experimental COPD. This study demonstrates, for the first time to our knowledge, that FZD4 down-regulation leads to loss of WNT/ β -catenin signal activity and subsequently to reduced alveolar epithelial cell wound repair capacity and impaired expression of elastogenic components, two major features of COPD pathogenesis. Thus, FZD4 might represent a potential therapeutic target for COPD.

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of death worldwide and is associated with a poor outcome and high socioeconomic burden (1). COPD is characterized by chronic airway inflammation and remodeling as well as loss of parenchymal alveolar tissue, called emphysema (2, 3). COPD initiation and development has been linked to a variety of genetic and environmental insults, with cigarette smoking being one of the major risk factors (4). The mechanisms driving disease pathogenesis include oxidative stress, a protease-antiprotease imbalance, and altered innate immune response, leading to pulmonary inflammation, cell death, and alveolar destruction (5–7). More recent data suggest that impaired endogenous lung tissue maintenance and, in particular, damaged alveolar epithelial cell repair processes further contribute to emphysema development and progression; however, the molecular mechanisms are not fully understood (6–9). The identification of novel therapeutic targets that are suitable for inducing lung repair is of major interest because to date no causal therapy is available for this devastating disease.

Signaling pathways that govern normal lung development represent potential targets. Wingless and INT-1 (WNT)/ β -catenin signaling is known to be critical for lung morphogenesis (10, 11). The WNT pathway consists of at least 19 different WNT ligands, which are secreted glycoproteins that can bind to 10 distinct Frizzled (FZD) receptors and low-density lipoprotein-related protein (LRP)5/6 coreceptors (10, 12). WNT signaling can be divided into canonical (i.e., β -catenin-dependent) and noncanonical signaling. In the case of canonical WNT/ β -catenin signaling, ligand-receptor binding prevents β -catenin phosphorylation and thus also degradation. β -catenin accumulates in the cytoplasm and translocates to the nucleus, where it binds to T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF). This in turn leads to the expression of various target genes involved, among other processes, in cell proliferation (12).

We and others have demonstrated that canonical WNT/ β -catenin signaling is reduced in the lung epithelium of emphysematous lung tissue from patients with COPD (13–16). Activation of WNT/ β -catenin by a glycogen synthase kinase-3 β inhibitor (lithium chloride) led to reversal of experimental emphysema *in vivo* (13) and, importantly, to alveolar epithelial cell activation in three-dimensional lung tissue cultures (3D-LTCs) derived from patients with COPD (14). The mechanism underlying WNT/ β -catenin signal reduction in COPD, however, has not yet been explored. In the present study, we investigated the role of FZD receptors that control downstream WNT signaling. We demonstrate that FZD4 is down-regulated by cigarette smoke (CS) in experimental and human COPD, preventing WNT/ β -catenin signal activity and thus alveolar tissue repair in COPD. These data suggest that the induction of canonical WNT signaling by targeting the WNT receptor FZD4 in the lung might lead to improved alveolar epithelial cell function and thus might represent a potential beneficial approach for tissue repair in patients with COPD.

Methods

Human Lung Tissue Study Population

Lung resection specimens were obtained from 92 subjects; 78 of these specimens were

from surgery for solitary pulmonary tumors (Ghent University Hospital, Ghent, Belgium) and 14 were from explant lungs of patients with end-stage COPD undergoing lung transplant surgery (University Hospital Gasthuisberg, Leuven, Belgium). Lung tissue from the resection specimen was harvested by a pathologist at maximum distance from the tumor. The cohort of 92 patients was divided into four subgroups: 18 never-smokers, 26 smokers without airflow limitation, 34 patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II COPD, and 14 patients with GOLD stages III–IV COPD (Table 1). Patients were considered ex-smokers when they had ceased smoking for more than 1 year. COPD diagnosis and severity were defined using preoperative spirometry according to the GOLD classification. All patients with COPD in the study had stable disease because patients with exacerbations within 2 months before the study were excluded. Other exclusion criteria were chemotherapy or radiotherapy in the last 6 months, diagnosis of mesothelioma or asthma, and infection of the upper or lower respiratory tract in the preceding 4 weeks.

Our study was approved by the medical ethics committees of the Ghent University Hospital (2011/114) and the University Hospital Gasthuisberg (S51577). All subjects provided written informed consent. Primary human alveolar epithelial type II (phAII) cells were isolated from non-COPD ($n = 7$) or COPD ($n = 3$) lung tissue biopsies from the Comprehensive Pneumology Center cohort at the University Hospital Grosshadern of Ludwig Maximilian University. Participants provided written informed consent to participate in this study in accordance with approval by the local ethics committee of Ludwig Maximilian University, Munich, Germany (project 333-10, 455-12).

Animals

Six- to 8-week-old pathogen-free female C57BL/6N mice were obtained from Charles River (Wilmington, MA) and housed in rooms in conditions of constant temperature and humidity with 12-hour light/dark cycles. Mice had free access to water and rodent chow. For the mouse model of elastase-induced emphysema, pancreatic porcine elastase was dissolved in sterile phosphate-buffered saline (PBS) and applied orotracheally (80 U/kg body weight) in a total volume of 80 μ l. Control mice

Table 1. Characteristics of Study Population (Reverse Transcriptase–Polymerase Chain Reaction Study) (n = 92)

| | Never-Smokers | Smokers without COPD | GOLD Stage II COPD | GOLD Stages III–IV COPD |
|--|---------------|--------------------------|-----------------------------|------------------------------|
| Number of subjects | 18 | 26 | 34 | 14 |
| Sex ratio, male/female | 6/12* | 19/7* | 31/3* | 8/6* |
| Age, yr | 65 (56–70) | 63 (55–70) | 66 (58–69) [†] | 56 (54–60) ^{†‡§} |
| Current/ex-smoker | — | 16/10 | 22/12 | 0/14 |
| Smoking history, pack-years | 0 (0–0) | 28 (15–45) [‡] | 45 (40–60) ^{†‡} | 30 (25–30) ^{‡§} |
| FEV ₁ , after bronchodilator, L | 2.7 (2.3–3.2) | 2.7 (2.3–3.3) | 2.0 (1.8–2.4) ^{†‡} | 0.7 (0.7–0.9) ^{†‡§} |
| FEV ₁ , after bronchodilator, % predicted | 102 (92–116) | 95 (93–112) | 68 (61–75) ^{†‡} | 26 (20–32) ^{†‡§} |
| FEV ₁ /FVC, after bronchodilator, % | 78 (75–83) | 75 (71–79) [‡] | 56 (53–60) ^{†‡} | 32 (27–35) ^{†‡§} |
| DL _{CO} , % predicted | 90 (80–105) | 80 (61–102) | 67 (51–87) [‡] | 35 (33–41) ^{†‡§} |
| K _{CO} , % predicted | 103 (88–123) | 91 (68–107) [‡] | 87 (62–108) [‡] | 59 (50–65) ^{†‡§} |
| ICS, yes/no | 0/18* | 1/25* | 15/19* | 13/1* |

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; DL_{CO} = diffusing capacity of the lung for carbon monoxide; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroid.

Data are presented as median (interquartile range).

**P* < 0.001 by Fisher's exact test.

[†]*P* < 0.05 versus smokers without COPD by Mann-Whitney *U* test.

[‡]*P* < 0.05 versus never-smokers by Mann-Whitney *U* test.

[§]*P* < 0.05 versus GOLD stages I–II COPD by Mann-Whitney *U* test.

received 80 µl of sterile PBS. For the mouse model of CS-induced emphysema, animals were exposed either to filtered air (FA) or to mainstream CS generated from 3R4F research cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY) twice per day for 50 minutes for the indicated times, as described previously (17). Mice were anesthetized and killed at the indicated times after elastase instillation or CS exposure. Lung tissue was flushed, excised, snap-frozen in liquid nitrogen, and stored at –80°C until further analyses. All animal studies were conducted according to strict governmental and international guidelines and approved by the local government for the administrative region of Upper Bavaria (project 55.2-1-54-2532-129-14).

Generation of Human and Mouse *Ex Vivo* 3D-LTCs

Generation of the 3D-LTCs was performed as previously described (14). In brief, for the patient-derived 3D-LTCs, lung segments were cannulated through the bronchus and filled with warm agarose (3%, A9414; Sigma-Aldrich, St. Louis, MO). Segments were cut with a vibratome (Hydrax V55; Carl Zeiss, Jena, Germany) into 500-µm-thick slices (speed, 6–10 µm/s; frequency, 100 Hz; amplitude, 1.2 mm). Slices were treated with FzM1 (2.5 µM) or the respective dimethyl sulfoxide control for 24 hours or 72 hours in sterile medium (Gibco Dulbecco's modified Eagle medium/Ham's F-12 [Life Technologies, Carlsbad, CA]

supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B [Sigma-Aldrich]). For murine 3D-LTCs, healthy 6- to 8-week-old pathogen-free female C57BL/6N mice were used. Lungs were flushed via the heart with sterile sodium chloride solution and filled with warm, low-gelling-temperature agarose (2%) in sterile medium (Dulbecco's modified Eagle medium/Ham's F-12). Further, lobes were cut with a vibratome into 300-µm-thick slices (speed, 10–12 µm/s; frequency, 80 Hz; amplitude, 1 mm). 3D-LTCs were treated with FzM1 (2.5 µM), valproic acid (VA) (0.5 mM, P4543; Sigma-Aldrich), a combination of FzM1 and VA, or the respective dimethyl sulfoxide control for 24 hours or 72 hours in sterile cultivation medium containing 0.1% fetal calf serum. RNA was isolated, and gene expression was analyzed by quantitative polymerase chain reaction. Supernatants were collected and stored at –80°C before analysis. Detailed description of the materials and methods, including CS extract (CSE) preparation, cell culture, and functional readouts can be found in the METHODS section in the online supplement.

Results

FZD4 Is Expressed by the Alveolar Epithelium and Decreased in COPD

We first analyzed the expression of all FZD receptors in the mouse model of

elastase-induced emphysema, which has previously been shown to exhibit reduced WNT/β-catenin signaling (13) (Figure 1A). We found that *Fzd4* and *Fzd7* expression in particular was significantly decreased. *Fzd4* and *Fzd7* transcripts were also highly expressed in primary mouse alveolar epithelial type II (pmATII) cells, and we detected FZD4 protein in cultured pmATII cells (see Figures E1A and E1B in the online supplement). To elucidate the potential clinical relevance for altered FZD expression, we further screened for FZD expression in available microarray datasets from whole lung homogenates (GEO accession number GSE47460), as well as in phATII cell isolates from patients with COPD and patients without COPD (18). In both datasets, *FZD4*, but not *FZD7*, was significantly reduced (Figures E1C and E1D, respectively). We detected FZD4 protein expression on healthy phATII cells (Figure 1B) and confirmed *FZD4* reduction in phATII cells obtained from lung tissue explants from patients with COPD compared with donors (Figure 1C) (relative *FZD4* mRNA expression [mean ± SD], non-COPD, –1.2 ± 0.38; COPD, –2.36 ± 0.35; *P* = 0.106). Notably, we confirmed *FZD4* reduction in a large cohort of patients with COPD (n = 48) compared with never-smokers (n = 18) as well as in patients with severe COPD (n = 14) compared with smokers without COPD (n = 26) (Figure 1D). *FZD4* mRNA expression further positively correlated with lung

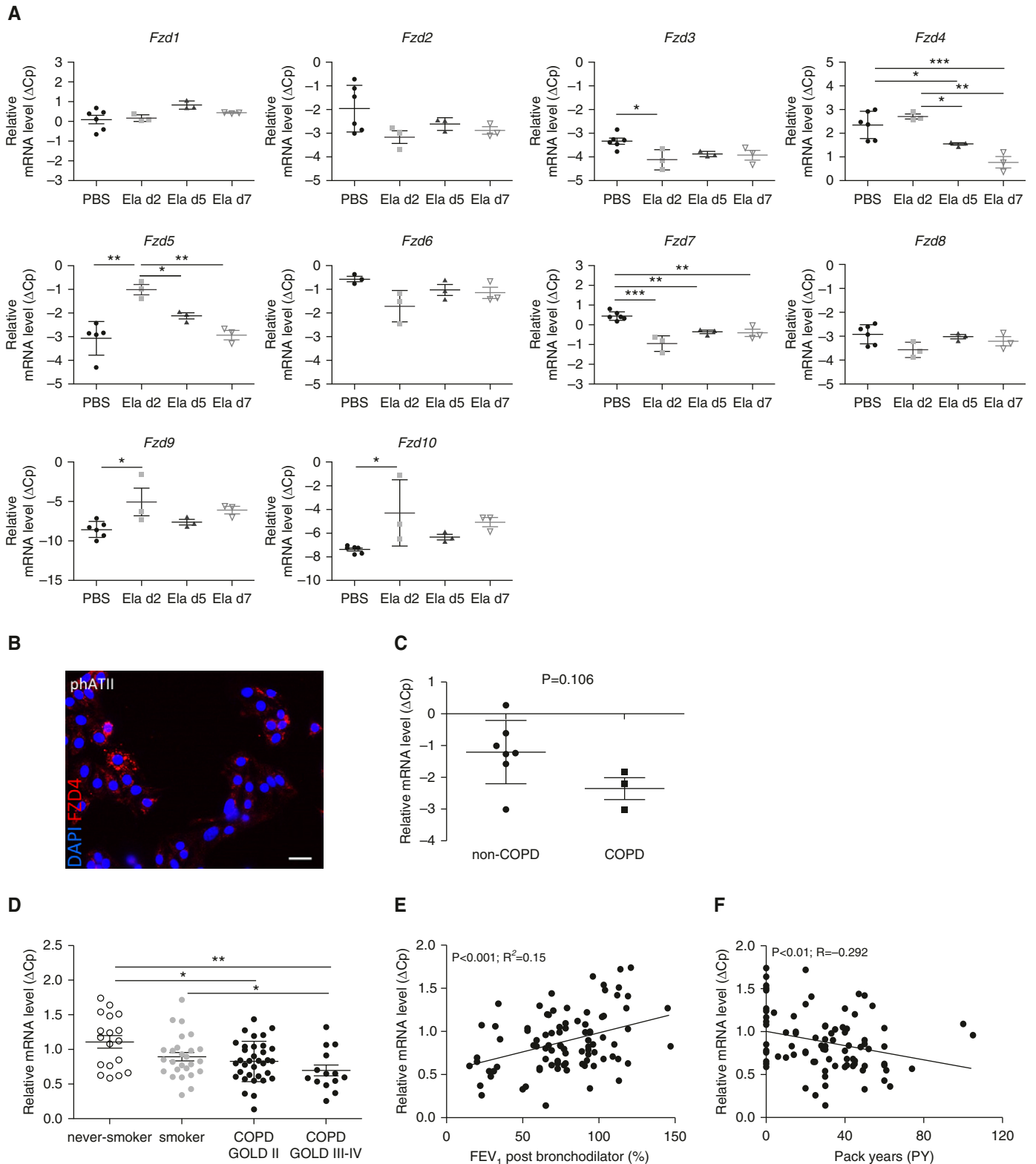


Figure 1. Frizzled receptor 4 (FZD4) is expressed in the alveolar epithelium and decreased in chronic obstructive pulmonary disease (COPD). (A) Frizzled (*Fzd*) expression in whole lung homogenate obtained at Day 2 (d2), Day 5 (d5), or Day 7 (d7) after phosphate-buffered saline (PBS) or elastase (Ela) instillation in C57BL/6 mice; relative gene expression, expressed as crossing point change ($\Delta C_p = C_p$ for *Hprt* - C_p for gene of interest), is presented as mean \pm SD (n=3–6). Means were compared using one-way analysis of variance followed by the Newman-Keuls multiple comparison test. (B) Immunofluorescent staining for FZD4 protein expression in primary human alveolar epithelial type II (phATII) cells. The scale bar represents 20 μ m.

function (FEV₁ percent predicted after bronchodilator) (Figures 1E and E2A) and negatively correlated with smoking pack-years (Figure 1F) and aging (Figure E2E) in this cohort (even when excluding samples from patients with GOLD stage IV COPD without cancer, as presented in Figure E2). In line with this, we observed that oxidative stress-induced senescence reduced *Fzd4* in epithelial cells *in vitro* (Figure E1E). We thus focused our further studies on FZD4 as a potential receptor contributing to impaired tissue repair observed in COPD pathogenesis.

FZD4 Expression Is Down-regulated by CS *In Vivo* and *In Vitro*

Given that cigarette smoking is an important risk factor for COPD, and on the basis of our data suggesting a link between CS and FZD4 expression (Figure 1D and 1F), we next addressed if FZD4 is regulated by CS *in vivo* and *in vitro*. We found reduced *Fzd4* receptor mRNA expression in lung homogenates from mice exposed to CS compared with FA at early (3 d) as well as late (4 mo) time points (Figure 2A) (relative *Fzd4* mRNA expression [mean ± SD], FA 3 d, 2.13 ± 0.1; CS 3 d, 1.35 ± 0.19; FA 4 mo, 1.49 ± 0.09; CS 4 mo, 0.5 ± 0.14). At baseline, *Fzd4* mRNA expression was also significantly reduced over time (Figure 2A), as well as in old versus young mice (Figure E3A), suggesting a potential aging-associated reduction. In agreement with the mRNA expression, we found reduced FZD4 protein as early as 3 days upon CS exposure as analyzed by immunoblotting, suggesting an immediate effect of CS on FZD4 expression, which was accompanied by reduced active β-catenin (ABC) protein expression (Figure 2B). Similarly, we found reduced FZD4 expression in the alveolar epithelium in tissue specimens of smokers compared with nonsmokers (Figure E3B). To investigate the effect of CS in more detail, we exposed pmATII cells directly to CSE (Figures 2D and 2E). CSE led to induction of the well-known marker

Cyp1a1 as well as reduction of *Sparc* expression (19) in pmATII cells (Figure 2D). Moreover, WNT/β-catenin target gene *Axin2* and *Fzd4* mRNA expression was decreased upon stimulation with CSE in pmATII cells and mouse lung epithelial (MLE12) cells (Figures 2D and E3C, respectively) (fold change relative mRNA expression [mean ± SD], *Axin2*, -1.44 ± 0.25; *Fzd4*, -1.66 ± 0.71; *P* < 0.01). FZD4 was also reduced on a protein level in pmATII cells on CSE exposure as assessed by immunofluorescent staining (Figure 2E) (FZD4 expression relative to 4',6-diamidino-2-phenylindole, as a percentage of 0% CSE [100%] [mean ± SD], 25% CSE, 76 ± 10%; *P* < 0.05) and immunoblotting (Figure E3D).

FZD4 Is a Positive Regulator of Canonical WNT Signaling

Given that CS exposure reduced FZD4 expression along with decreased WNT/β-catenin signal activity in alveolar epithelial cells, we next aimed to elucidate if FZD4 expression directly influences WNT/β-catenin signal activity. To this end, we used the recently described inhibitor FzM1, which is an allosteric ligand of FZD4 described to hamper the FZD4–Dishevelled complex that is required for β-catenin nuclear translocation (20). Treatment of pmATII cells with FzM1 led to decreased WNT/β-catenin signaling, as analyzed by target gene expression (*Axin2*, *Lgr5*) (Figure 3A) (logarithmic fold change [mean ± SD], *Axin2*, -1.76 ± 0.59; *Lgr5*, -2.12 ± 0.82; *P* < 0.05). In line with these findings, FzM1-mediated inhibition of FZD4 led to the attenuation of WNT3A-induced TCF/LEF-dependent gene transcription in MLE12 cells (Figure 3B) (TOP/FOP activation, fold of control [mean ± SD], FzM1, 1.46 ± 0.8; WNT3A, 8.55 ± 2.3; FzM1 + WNT3A, 2 ± 1.2; *P* < 0.01). Furthermore, FzM1 decreased ABC level and reduced LRP6 phosphorylation upon WNT3A treatment in MLE12 cells as assessed by immunoblotting

(Figure 3C). Moreover, we also found decreased *AXIN2* expression by FzM1 in human 3D-LTCs *ex vivo* (Figure E4A). We further corroborated our findings using a gain-of-function approach (Figures 3D and E5). Overexpression (OE) of FZD4 led to enhanced WNT/β-catenin signal activity upon WNT3A stimulation in MLE12 cells (Figure 3D) (TOP/FOP activation FZD4 OE compared with empty vector [EV] [mean ± SD], OE, 1.3 ± 0.11; EV + WNT3A, 3.5 ± 1.6; OE + WNT3A, 12.1 ± 2.5; *P* < 0.001). Altogether, these data suggest that FZD4 is a direct regulator of canonical WNT signaling in the mouse and human alveolar epithelium.

FZD4 Expression Regulates Alveolar Epithelial Wound Healing and Repair

Canonical WNT signaling has been implicated in lung repair; therefore, we next investigated the role of FZD4 on alveolar epithelial cell function. We assessed cellular proliferation and wound healing, crucial processes involved in tissue repair upon gain and loss of function of FZD4. Inhibition of FZD4 signaling by FzM1 led to decreased cell viability (Figure 4A) (mean ± SD FzM1 compared with control [100%], 73 ± 7%; *P* < 0.001), decreased total cell counts (Figure 4B) (mean ± SD FzM1 compared with control [100%], 62 ± 17.6%; *P* < 0.05), and reduction of Ki67 in MLE12 cells (Figure 4D) (percentage of Ki67-positive cells [mean ± SD], control, 78.8 ± 0.01%; FzM1, 67.6 ± 3%; *P* < 0.05). In addition, FzM1 significantly delayed wound closure in a scratch assay (Figure 4F) (closed scratch in percent [mean ± SD], control, 73.1 ± 2.9%; FzM1, 44.3 ± 6.5%; *P* < 0.05). These data were further confirmed by FZD4 OE, which led to increased cell viability (Figure 4C) (FZD4 OE vs. EV [mean ± SD], 123.6 ± 11.6%; *P* < 0.01), percentage of Ki67-positive cells (Figure 4E) (percent of Ki67-positive cells [mean ± SD], EV, 45.5 ± 0.9%;

Figure 1. (Continued). (C) *FZD4* gene expression in pmATII cells isolated from patients without COPD and patients with COPD; relative gene expression, expressed as ΔC_p, is presented as mean ± SD (non-COPD, n = 7; COPD, n = 3). Means were compared using Student's *t* test. (D) *FZD4* expression in lung resection specimens from 92 subjects, comprising 18 never-smokers, 26 smokers without airflow limitation, 34 patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II COPD, and 14 patients with GOLD stages III–IV COPD; relative gene expression, expressed as ΔC_p (corrected by a normalization factor that was calculated based on the expression of the three reference genes), is presented as mean ± SEM. Means were compared using the Mann-Whitney *U* test. (E) Correlation between *FZD4* expression in the lung and the lung function parameter FEV₁ percent predicted. (F) Correlation between *FZD4* expression in the lung and pack-years of smoking. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. DAPI = 4', 6-diamidino-2-phenylindole.

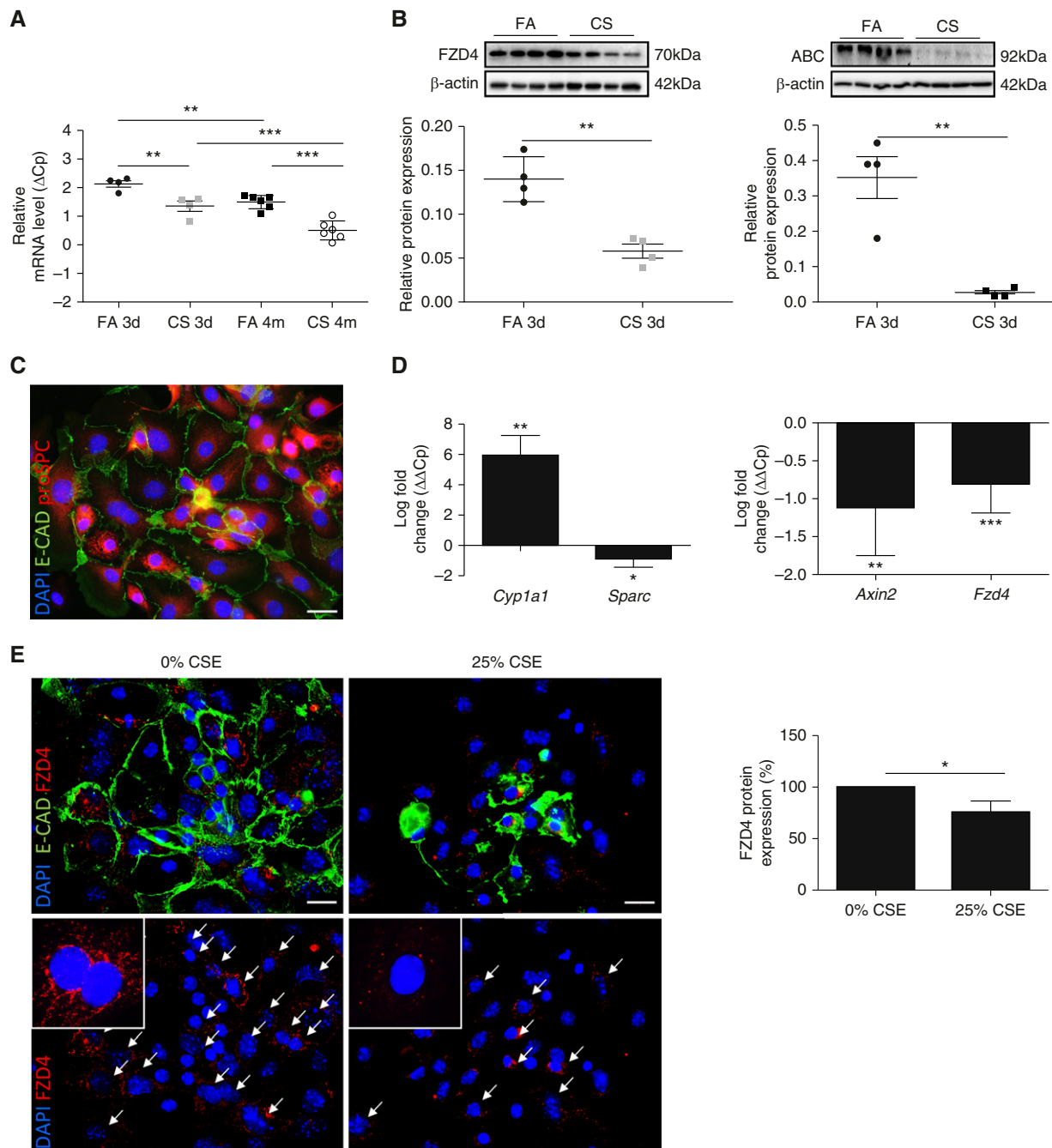


Figure 2. Frizzled receptor 4 (FZD4) expression is down-regulated by cigarette smoke (CS) *in vivo* and *in vitro*. (A) *Fzd4* gene expression in whole lung homogenate obtained from C57BL/6 mice exposed to filtered air (FA) or CS for 3 days or 4 months; relative gene expression, expressed as crossing point change (ΔC_p), is presented as mean \pm SD (n = 4–6). Means were compared using one-way analysis of variance followed by the Newman-Keuls multiple comparison test. (B) Representative immunoblot showing FZD4 and active β -catenin protein expression in whole lung homogenate obtained from C57BL/6 mice exposed to FA or CS for 3 days, quantified relative to β -actin expression; data are presented as mean \pm SD (n = 4). Means were compared using Student's *t* test. (C) Representative FZD4 immunofluorescent staining of primary mouse alveolar epithelial type II (pmATII) cells at Day 2 of culture. The scale bar represents 20 μ m. (D) Gene expression in pmATII cells exposed to 0% or 25% cigarette smoke extract (CSE) for 24 hours. Gene expression comparative crossing point change ($\Delta\Delta C_p$) is presented as mean \pm SD (n = 4). Means were compared using a paired Student's *t* test. (E) Representative images of immunofluorescent staining for FZD4 (arrows indicate FZD4-positive cells) and CDH1 (E-CAD) in pmATII cells exposed to 0% or 25% CSE for 24 hours. Overall expression of the FZD4 protein was quantified in comparison to the number of cells with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining, with data presented as mean \pm SD (n = 4); 0% CSE serves as 100%. Means were compared using a paired Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ABC = active β -catenin; proSPC = prosurfactant protein C.

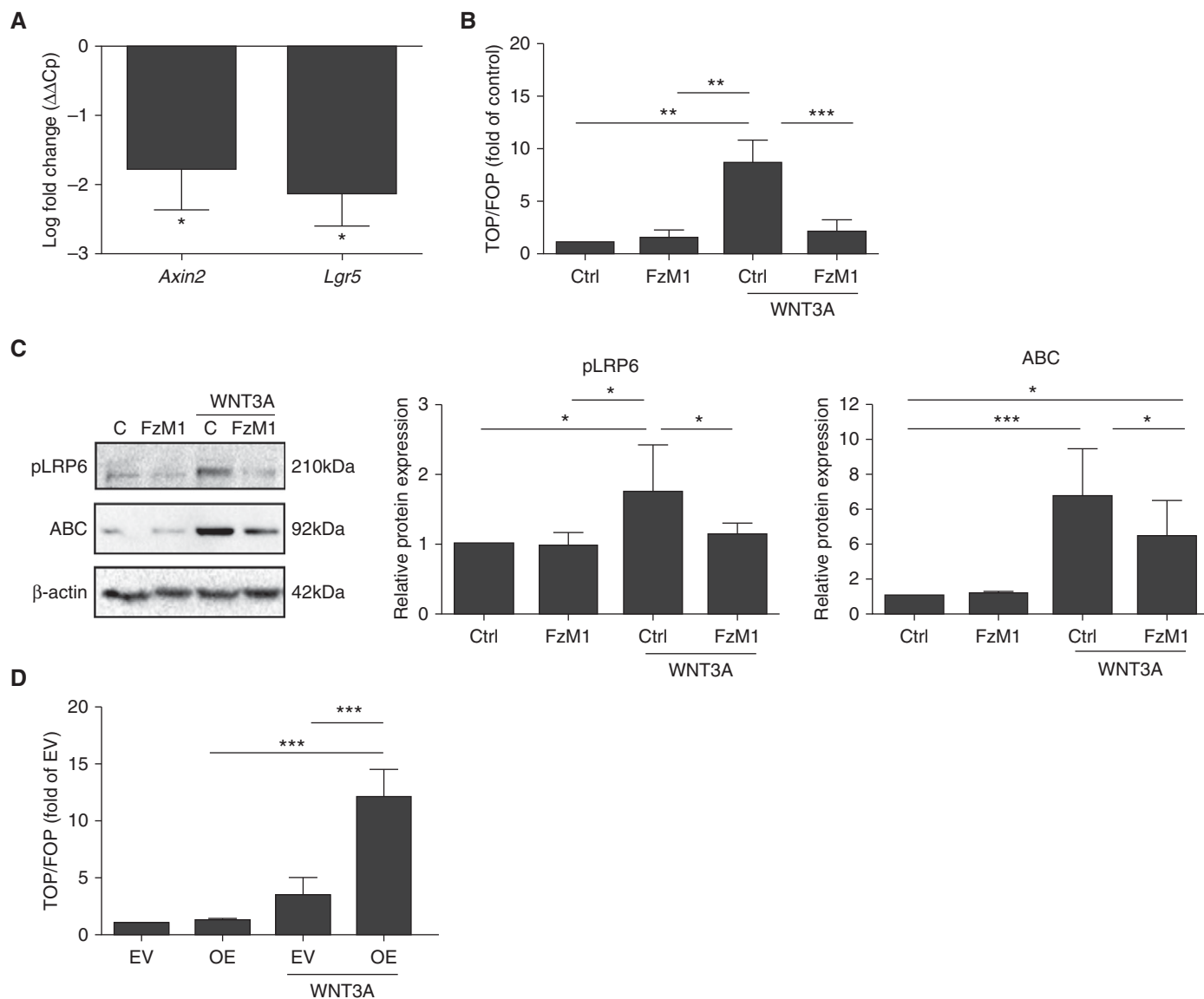


Figure 3. Frizzled receptor 4 (FZD4) is a positive regulator of canonical WNT signaling. (A) mRNA expression of canonical WNT target genes *Axin2* and *Lgr5* in primary mouse alveolar epithelial type II cells treated with FzM1 (2.5 μ M) for 24 hours. Gene expression comparative crossing point change ($\Delta\Delta C_p$) is presented as mean \pm SD (n = 3). Means were compared using a paired Student's *t* test. (B) Determination of β -catenin–dependent gene transcription (TOP/FOP flash assay) in mouse lung epithelial (MLE12) cells pretreated with FzM1 (10 μ M) and stimulated with WNT3A (100 ng/ml); data are presented as mean \pm SD (n = 3). Means were compared using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison test. (C) Representative immunoblot showing active β -catenin (ABC) protein level and low-density lipoprotein-related protein 6 phosphorylation (pLRP6) in MLE12 cells pretreated with FzM1 for 20 minutes, stimulated with WNT3A for 3 hours, and quantified relative to β -actin expression; data are presented as mean \pm SD (n = 5). Means were compared using one-way ANOVA followed by the Newman-Keuls multiple comparison test. (D) Determination of β -catenin–dependent gene transcription in MLE12 cells transfected with empty vector (EV) or FZD4-overexpressing plasmid (OE) and treated with WNT3A (100 ng/ml); data are presented as mean \pm SD (n = 3). Means were compared using one-way ANOVA followed by the Newman-Keuls multiple comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Ctrl = control.

OE, 56.7 \pm 6.4%; *P* < 0.05) as well as accelerated wound closure (Figure 4G) (closed scratch in percent [mean \pm SD], EV, 74.9 \pm 10.3%; OE, 80.7 \pm 10.4%; *P* < 0.01).

ATII cells are capable of self-renewal and exert progenitor function for ATI cells upon alveolar epithelial injury. We next elucidated whether FZD4 expression

impacts ATII cell marker expression, transdifferentiation, and organoid formation. FZD4 inhibition by FzM1 led to a reduction of alveolar epithelial cell marker transcripts, such as *E-cadherin* and the ATI cell marker *T1 α* (Figure E6A). Moreover, in the well-characterized *in vitro* model, in which ATII cells spontaneously activate WNT/ β -catenin signaling and

transdifferentiate into ATI-like cells during primary culture *in vitro* (21, 22), we observed that blocking of FZD4 by FzM1 led to a significant decrease of the ATI cell marker *T1 α* on the protein level. This was accompanied by decreased ABC and LRP6 phosphorylation (both indicators of WNT/ β -catenin activity) (Figure 5), indicating that loss of FZD4 activity results

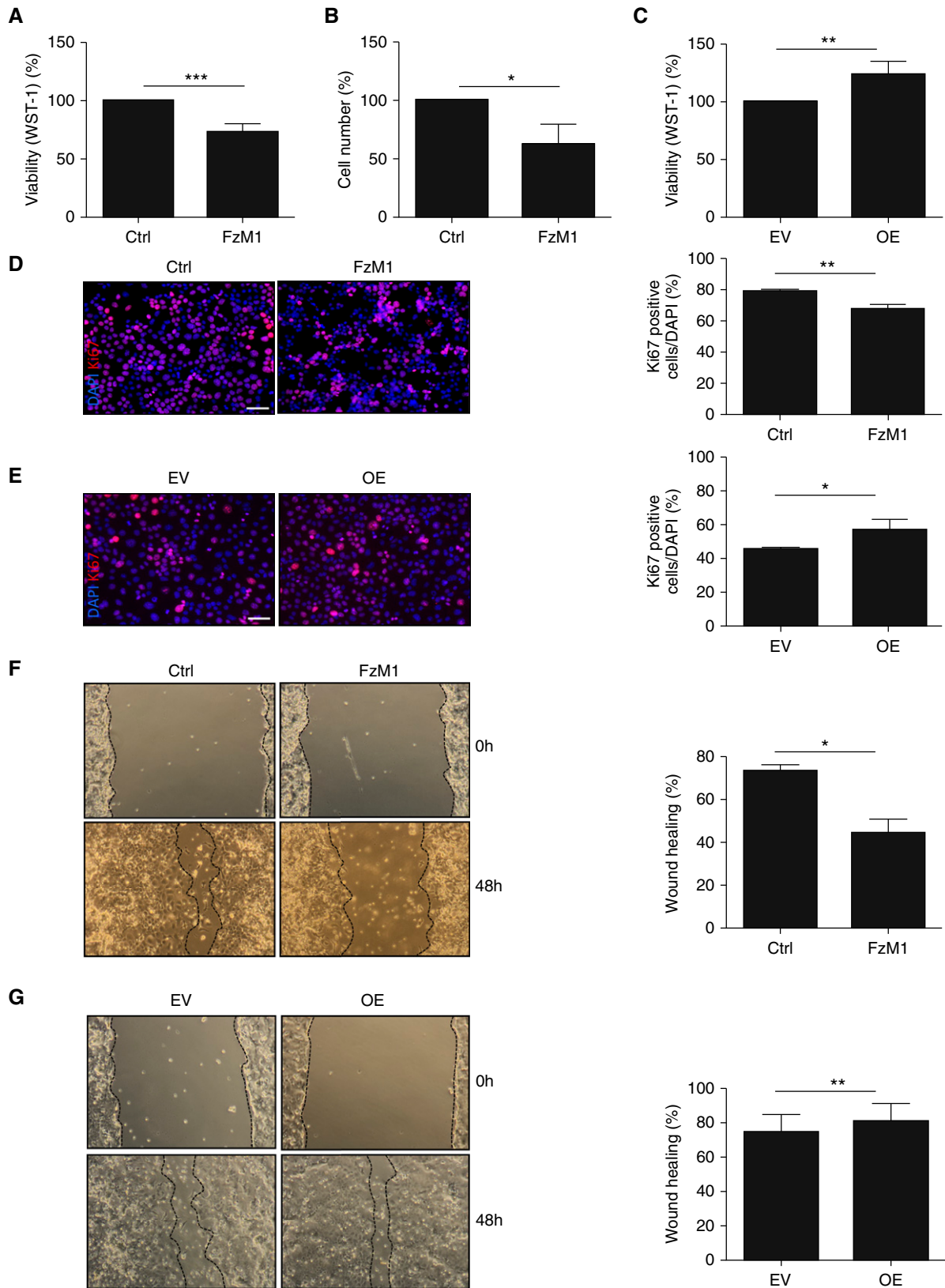


Figure 4. Frizzled receptor 4 (FZD4) expression regulates alveolar epithelial cell proliferation and wound closure. (A) Influence of FZD4 expression on the viability of lung epithelial cells determined by WST-1 assay in mouse lung epithelial (MLE12) cells treated with FzM1 (5 μ M) for 24 hours; data are presented as mean \pm SD (n = 4). (B) Number of MLE12 cells after 24-hour treatment without or with FzM1 (5 μ M); data are presented in comparison with

in a defect of ATII-to-ATI cell transdifferentiation, which is presumably mediated by reduced canonical WNT signaling. In addition, we observed a significantly lower number of organoids formed by ATII cells upon FzM1 treatment (Figure E6B). Altogether, these results indicate that FZD4 acts as a positive regulator of lung epithelial wound healing, (trans)differentiation, and organoid formation and thus might be a potential regulator of tissue repair in the lung.

FZD4 Regulates WNT/ β -Catenin-driven Repair and Elastogenic Components

WNT/ β -catenin signaling has recently been linked to extracellular and elastin remodeling (14, 23). Loss of elastic fibers from small airways and alveolar walls represents a main feature of COPD, and elastin, which constitutes the main component of elastic fibers (24), has been shown to be regulated by CS (25). Notably, we detected decreased elastin (*Eln*) gene expression induced by CS both in whole lung homogenate *in vivo* and in epithelial cells *in vitro* (Figures 6A–6C). Although lung epithelial cells most likely do not present the main source of elastin, protein was also detected in cultured ATII cells (Figure E7A), suggesting that the latter cells might contribute to the elastic matrix upon injury. We further analyzed expression of elastogenic components and found that both CSE and FZD4 inhibition by FzM1 reduced gene expression of elastin fiber genes such as *Eln*, insulin-like growth factor 1 (*Igf1*), lysyl oxidase (*Lox*), lysyl oxidase-like 1 (*Loxl1*), fibulin-1 (*Fbln1*), and fibulin-5 (*Fbln5*) (26) (Figures 6C and 6D, respectively) in pmATII cells. Remarkably, protein levels of IGF1, which has been implicated in elastogenesis processes and shown to be secreted by ATII cells in previous studies (27, 28), were also significantly reduced upon CSE

as well as FZD4 inhibition in pmATII cells (Figure 6E and 6F). Furthermore, we confirmed decreased expression of both *Eln* and *Igf1* in *ex vivo* 3D-LTCs upon CSE and FzM1 treatment (Figures 6G, 6H, and E8A).

Finally, we aimed to restore FZD4 function and found that FZD4 OE in MLE12 cells induced *Eln* transcript and was able to rescue the CSE-induced elastin reduction (Figure 6I); however, *Igf1* expression was below the detection limit in this cell line. As such, we used pmATII cells as well as VA, which has been reported to induce FZD4 expression (29). Treatment of pmATII cells with VA led to a significant increase in *Fzd4* (but not *Fzd5* or *Fzd8*) transcript (Figure E9A), along with *Axin2* induction (Figure E9A). As expected, induction of WNT/ β -catenin signaling by VA was diminished by cotreatment with the FZD4 inhibitor as measured by TOP/FOP flash activity (Figure E9B). Accordingly, we found an increase in *Fzd4* and *Axin2* by VA treatment in *ex vivo* mouse 3D-LTCs (Figure 6J), and whereas *Igf1* transcript was not significantly induced at this time point, we detected a significant increase in IGF1 protein upon VA treatment *ex vivo* (Figure 6K). Taken together, these data support the notion that FZD4 signaling is involved in elastic matrix composition.

Discussion

We report reduced expression of the FZD4 receptor in patients with COPD as well as in two independent animal models of COPD. We demonstrate, for the first time to our knowledge, that down-regulation of FZD4 within the alveolar epithelium is mediated by CS *in vitro* and *in vivo*, leading to loss of WNT/ β -catenin signal activity and subsequently to reduced lung tissue repair. We show that FZD4 reduction diminishes alveolar epithelial cell wound repair capacity and contributes to reduced

elastogenic component expression, features of COPD pathogenesis, and thus might represent a potential therapeutic target (Figure 7).

Loss of functional parenchymal lung tissue is a main feature of COPD; however, the underlying mechanisms are still poorly understood. Altered WNT signal activity has been linked to COPD (13–16, 30, 31). It has been shown that WNT/ β -catenin signaling is reduced in the lung epithelium in COPD and that this contributes to diminished tissue repair (13, 14) as well as airway inflammation (32). Notably, we recently demonstrated that activation of WNT/ β -catenin is feasible in patient-derived human 3D-LTCs and induces alveolar epithelial cell activation (14). The cause of WNT/ β -catenin signal reduction in emphysema and a potential involvement of specific WNT/ β -catenin receptors, however, remain elusive.

WNT signaling is regulated by a large number of different, yet structurally similar, WNT ligands and FZD receptors. At least 19 WNT ligands and 10 membrane-bound FZD receptors are described to transduce intracellular WNT signaling via a number of coreceptors (12). WNT–FZD interactions are proposed to be highly context dependent; however, knowledge of specific WNT–FZD pairs remains limited (33). To date, there are no studies investigating the functional influence of FZD4 on WNT signaling in either the lung, or in general. FZD4 has been reported to be involved in angiogenesis in the eye, with FZD4 mutation in humans being responsible for incomplete retinal vascularization in familial exudative vitreoretinopathy (34, 35). In the present study, we initially found FZD4 expression to be enriched in the alveolar epithelium, and we showed that FZD4 expression is altered by CS *in vitro* and *in vivo*. Similarly, Guo and colleagues recently reported reduced *FZD4* mRNA in a CS-exposed bronchial epithelial cell line *in vitro* (30). Notably, expression analysis in

Figure 4. (Continued). control (100%) as mean \pm SD (n = 3). Means were compared using Student's *t* test. (C) Influence of FZD4 expression on the viability of lung epithelial cells determined by WST-1 assay in MLE12 cells transfected with empty vector (EV) or FZD4-overexpressing plasmid (OE); data are presented as mean \pm SD (n = 4). (D) Representative images and quantification of Ki67 immunofluorescent staining of MLE12 cells treated with dimethyl sulfoxide (DMSO; Ctrl) or FzM1; data are presented as mean \pm SD (n = 3). The scale bar represents 50 μ m. (E) Representative images and quantification of Ki67 immunofluorescent staining of MLE12 cells transfected with EV or OE; data are presented as mean \pm SD (n = 3). Note the reduced baseline proliferation upon transfection in comparison to DMSO control in (D). The scale bar represents 50 μ m. (F) Representative images and quantification of wound size in MLE12 cells treated with DMSO or FzM1; data are presented as mean \pm SD (n = 3). (G) Representative images and quantification of wound size in MLE12 cells transfected with EV or OE; data are presented as mean \pm SD (n = 4). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. DAPI = 4',6-diamidino-2-phenylindole.

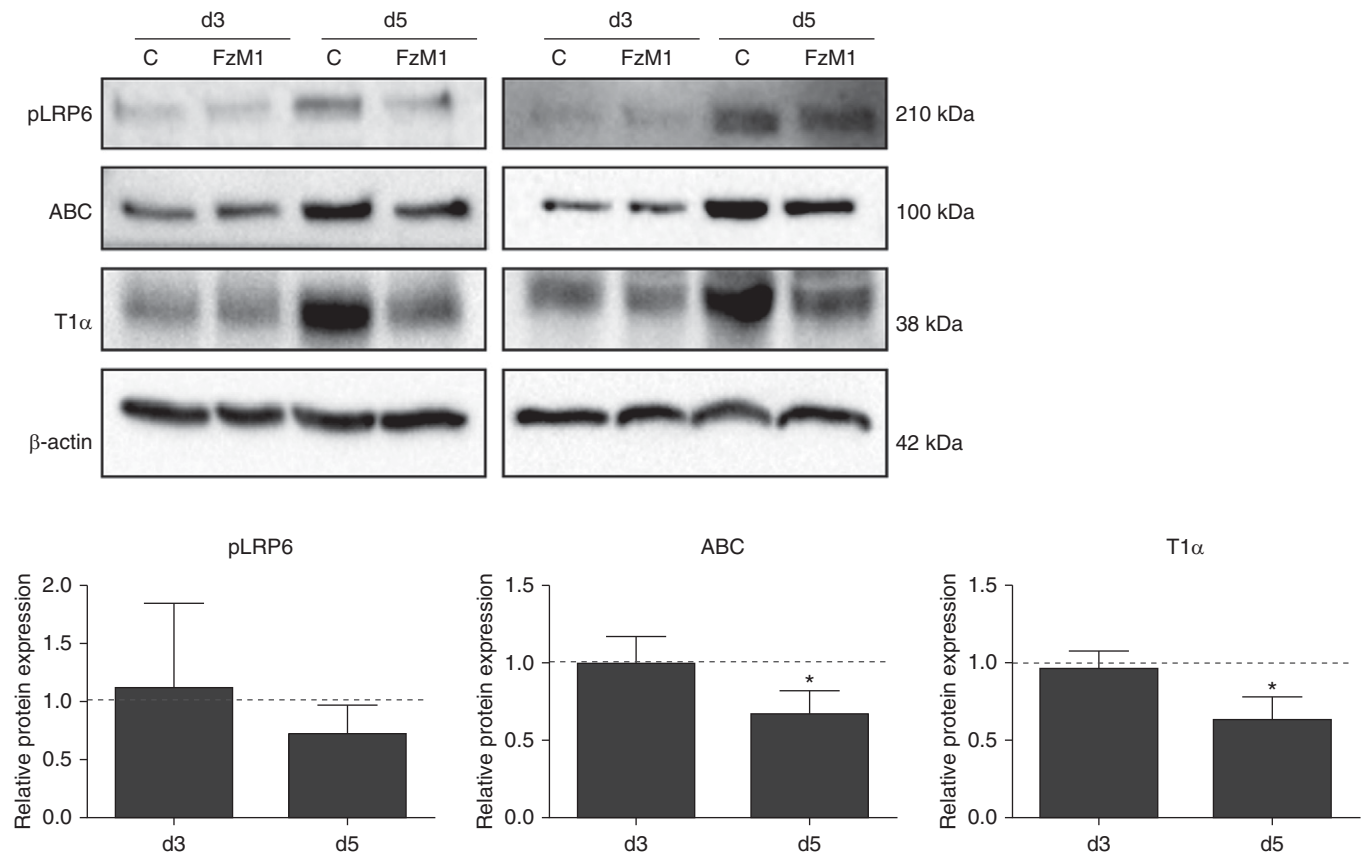


Figure 5. Frizzled receptor 4 alters alveolar epithelial type II (ATII)-to-ATI cell transdifferentiation processes. (Top) Representative immunoblot showing protein levels of active β -catenin (ABC), low-density lipoprotein-related protein 6 phosphorylation (pLRP6), and T1 α (ATI epithelial marker) in primary mouse alveolar epithelial type II cells treated with dimethyl sulfoxide (control [C]) or FzM1 (2.5 μ M) for 24 hours (Day 3 [d3] of culture) or 72 hours (Day 5 [d5] of culture). (Bottom) Quantification relative to β -actin expression (left, pLRP6; middle, ABC; right, T1 α); data are presented as mean \pm SD relative to respective control ($n = 3-4$). Means were compared using Student's t test. * $P < 0.05$.

our cohort of never-smokers, smokers, and patients with COPD revealed that FZD4 expression is even further decreased in patients with late-stage COPD compared with smokers, suggesting additional COPD-related mechanisms driving FZD4 reduction.

COPD has been proposed to be a disease of accelerated aging (36). Interestingly, FZD4 expression correlated with age in the human and murine lung, further indicating that WNT signaling also impacts aging processes and might be involved in cellular senescence (36, 37). We recently reported a canonical-to-noncanonical WNT signal switch in COPD (38) that might be driven by aging (39). We provide several lines of evidence that FZD4 mainly mediates canonical WNT/ β -catenin signaling, which is in line with previous reports (33, 40), thus suggesting that FZD4 reduction might contribute to a WNT signal

shift by inhibiting WNT/ β -catenin signaling. Nevertheless, we cannot rule out that FZD4 reduction also impacts noncanonical WNT signaling, and further studies are needed to shed light onto WNT receptor-mediated noncanonical WNT signaling in alveolar epithelial cell repair.

ATII cells play a major role in lung homeostasis and also can serve as a progenitor pool for ATII and ATI cells after alveolar injury (41, 42). Gain and loss of function of FZD4 not only modulated WNT/ β -catenin signaling in alveolar epithelial cells but also further led to several functional effects, such as wound healing, differentiation of the alveolar epithelium, and organoid formation. These data are in line with previous findings describing an imbalance of alveolar epithelial cell apoptosis/proliferation in end-stage emphysema (43) and strongly indicate

that FZD4 signaling is relevant for alveolar epithelial cell repair.

Breakdown of elastic fibers in the lung is one of the main features of COPD, and overall alveolar fiber content has been shown to positively correlate with lung function parameters (44). Elastic fibers are insoluble extracellular matrix assemblies that consist of an elastin core and a mantle of fibrillin-rich microfibrils (45). Cross-linking of tropoelastin monomers, a critical step in generating an insoluble elastin polymer, is initiated by the Lox family of enzymes (Lox and Lox1-Lox4). Inflammatory mediators such as macrophage elastase (matrix metalloproteinase-12) can modulate elastin gene expression and subsequently decrease accumulation of elastic fibers in tissues (6, 7). CS is known to increase the elastolytic activity of macrophages (25, 46), impair the production of Lox, and alter elastin resynthesis in elastase-induced

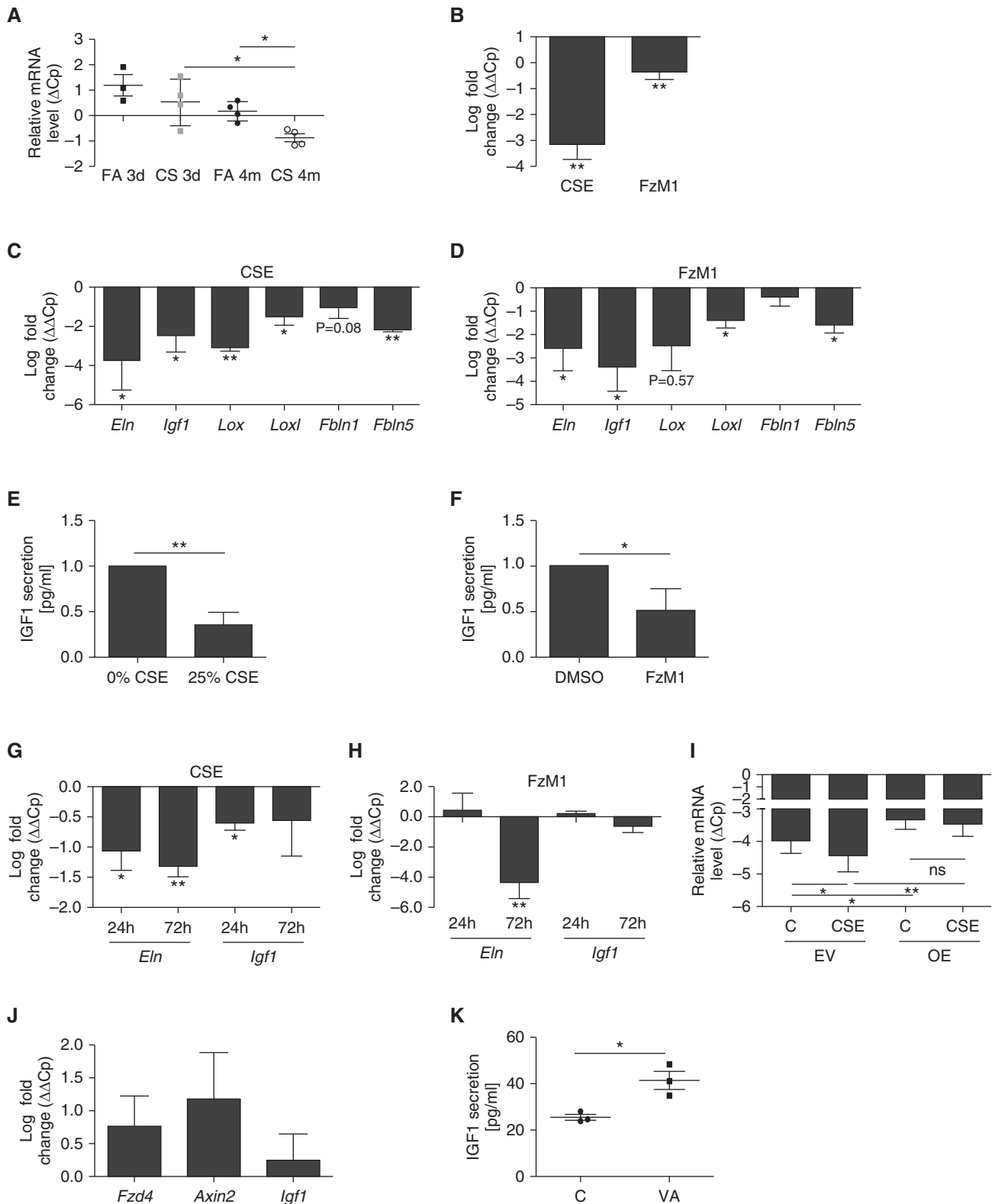


Figure 6. Frizzled receptor 4 (FZD4) expression influences elastogenic components. (A) *Eln* gene expression in whole lung homogenate of cigarette smoke-exposed (CSE) mice. Relative gene expression, expressed as crossing point change (ΔC_p), is presented as mean \pm SD (n=3-5). Means were compared using one-way analysis of variance followed by the Newman-Keuls multiple comparison test. (B) *Eln* gene expression in mouse lung epithelial (MLE12) cells after CSE or treated with FzM1. Gene expression, expressed as comparative crossing point ($\Delta\Delta C_p$), is presented as mean \pm SD (n=3-4).

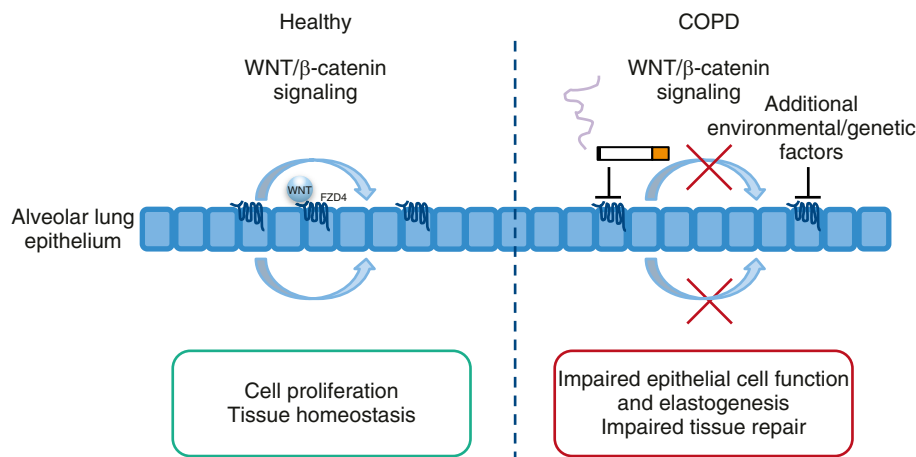


Figure 7. Schematic representation of the proposed contribution of frizzled receptor 4 (FZD4) signaling to impaired tissue repair in chronic obstructive pulmonary disease (COPD). Healthy lung (*left*): In the presence of FZD4 receptor in the alveolar lung epithelium, WNT/β-catenin signaling maintains cell proliferation and tissue repair. COPD lung (*right*): Decreased expression of FZD4 receptor induced by cigarette smoke and additional environmental/genetic factors lead to reduced WNT/β-catenin signaling and inhibition of epithelial cell function and components of the elastic matrix, thus contributing to impaired tissue repair and emphysema development.

emphysema (47), and it has been shown to decrease protein elastin content *in vivo* (48). Similarly, we observed that CS decreased elastin expression in a COPD model *in vivo* and down-regulated expression of elastogenic components, such as *Loxl*, *in vitro*. Notably, blocking of FZD4 mimicked this alteration in alveolar epithelial cells *in vitro*. Moreover, IGF1, which is an epithelial cell-derived proelastogenic cytokine reduced in COPD (49–51), was also reduced upon both CSE and FZD4 blockade. To evaluate the potential role for FZD4 signaling to enhance lung repair processes, we applied two different approaches, FZD OE *in vitro* and induction of FZD4 by VA *ex vivo*. FZD4 OE was able to rescue the CSE-induced decrease in elastin expression; however, we could not

detect all elastogenic components in the used cell line. VA is a U.S. Food and Drug Administration–approved drug for seizures and for manic or mixed episodes associated with bipolar disorder. It has been linked to increases in FZD4 expression (29) and could be used for treatment of primary cells and tissue. VA treatment led to FZD4-dependent induction of WNT signal activity and increased expression of IGF1, which are decreased in patients with COPD (49–51). However, we cannot exclude that VA additionally acts on other signaling pathways contributing to the observed effects.

FZD4 is the first WNT receptor that we identified to be involved in alveolar epithelial cell repair. Recently, it has been reported that FZD8 is expressed by lung fibroblasts and

exhibits a proinflammatory role in chronic bronchitis (31). Moreover, we found that FZD8 on fibroblasts also mediates profibrotic transforming growth factor-β signaling in lung fibrosis (52). Our data suggest that other FZD receptors are also differentially regulated in COPD and thus might very well be involved in mechanisms of lung injury and repair. One can hypothesize that these FZDs might be enriched and/or regulated in other cell types, such as mesenchymal or inflammatory cells, that are further needed for proper lung repair. Distinct WNT/FZD signals seem to be cell specific, thus highlighting the potential of these membrane-bound receptors as therapeutic targets.

In the present study, we identified reduced FZD4 signaling as an important contributor to impaired lung repair in COPD using several different experimental setups *in vivo*, *ex vivo*, and *in vitro*. Certain limitations of the study, however, require further investigation. COPD is a very complex disease with several factors contributing to the pathogenesis. In this study, we focused mainly on the impact of the main risk factor CS on FZD4 expression and subsequent alveolar epithelial cell repair; however, the role of FZD4 needs to be explored further in the context of other hallmarks of COPD, such as inflammation and tissue remodeling. We confirmed that FZD4 impacts WNT/β-catenin signaling and elastogenic mediators in *ex vivo* 3D-LTCs from precision-cut lung slices, which enabled investigation within the intact three-dimensional lung architecture and several structural cell types (14). Moreover, this novel method represents an important alternative to *in vivo*

Figure 6. (Continued). Means were compared using a paired Student's *t* test. (C) *Eln*, *Igf1*, *Lox*, *Loxl*, *Fbln1*, and *Fbln5* gene expression in primary mouse alveolar epithelial type II (pmATII) cells treated with FzM1. Gene expression, expressed as $\Delta\Delta C_p$, is presented as mean \pm SD ($n = 3$). Means were compared using a paired Student's *t* test. (D) *Eln*, *Igf1*, *Lox*, *Loxl*, *Fbln1*, and *Fbln5* gene expression in pmATII cells exposed to CSE. Gene expression, expressed as $\Delta\Delta C_p$, is presented as mean \pm SD ($n = 3$). Means were compared using a paired Student's *t* test. (E) Relative insulin-like growth factor 1 (IGF1) protein secretion in pmATII cells exposed to CSE. Data presented as mean \pm SD ($n = 4$). Means were compared using a paired Student's *t* test. (F) Relative IGF1 protein secretion in pmATII cells treated with FzM1. Data are presented as mean \pm SD ($n = 5$). Means were compared using a paired Student's *t* test. (G) *Eln* and *Igf1* gene expression in mouse precision-cut lung slices (PCLSs) after CSE for 24 hours or 72 hours. Gene expression, expressed as $\Delta\Delta C_p$, is presented as mean \pm SD ($n = 3$). Means were compared using a paired Student's *t* test. (H) *Eln* and *Igf1* gene expression in mouse PCLSs treated with FzM1 for 24 hours or 72 hours. Gene expression, expressed as $\Delta\Delta C_p$, is presented as mean \pm SD ($n = 3$). Means were compared using a paired Student's *t* test. (I) *Eln* gene expression in MLE12 cells transfected with overexpressed (OE) FZD4 and with CSE. Gene expression, expressed as $\Delta\Delta C_p$, is presented as mean \pm SD ($n = 3$ –5). Means were compared using one-way analysis of variance followed by the Newman-Keuls multiple comparison test. (J) Gene expression upon valproic acid (VA) treatment in mouse three-dimensional lung tissue cultures (3D-LTCs). $\Delta\Delta C_p$ is presented as mean \pm SD ($n = 3$). (K) Relative IGF1 protein secretion in mouse 3D-LTCs. Data are presented as mean \pm SD ($n = 3$). Means were compared using a paired Student's *t* test. * $P < 0.05$, ** $P < 0.01$. C = control; CS = cigarette smoke; DMSO = dimethyl sulfoxide; EV = empty vector; FA = filtered air; ns = not significant.

animal models. The application of 3D-LTCs holds promise to reduce overall animal experiments, thus fulfilling the “3 Rs” (replacement, reduction, and refinement) recommendations of the European Parliament (53). Nevertheless, studies genetically modifying FZD4 signaling in mice *in vivo* will be of high interest to further elucidate features of lung tissue repair, including changes in

elastic fiber composition, and to corroborate FZD4 as a potential therapeutic target for COPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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