Activation of the WNT/β-catenin pathway attenuates experimental emphysema

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
APC	adenomatosis polyposis coli
AQP5	aquaporin 5
A1AT	alpha 1-antitrypsin
BAL	bronchoalveolar lavage
BASC	bronchoalveolar stem cells
BMP4	bone morphogenetic protein 4
CDH1	cadherin-1
Col1a1	collagen, type I, alpha 1
COPD	chronic obstructive pulmonary disease
EMT	Epithelial-mesenchymal transition
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FEV1	forced expiratory volume in one second
FGFR2	Fibroblast growth factor receptor 2
FOXP2	forkhead box P2
FVC	forced expiratory vital capacity
FZD	frizzled
GOLD	global initiative for chronic obstructive lung disease
GSK-3β	glycogen synthase kinase-3β
HGF	hepatocyte growth factor
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	hematopoietic stem cells
KGF	keratinocyte growth factor
IL-2	interleukine-2
IPF	Idiopathic pulmonary fibrosis
LEF	lymphoid enhancer-binding factor
LiCl	lithium chloride
LRP	low density lipoprotein receptor-related proteins
LTBP	latent TGF-beta binding protein
MCL	mean chord length
mRNA	messenger ribonucleic acid

MMP-12	matrix metalloproteinase-12
MIP-2	macrophage inflammatory protein
NRF2	nuclear erythroid-related factor 2
NSCLC	non-small cell lung cancer
qRT-PCR	quantitative real time polymerase chain reaction
PBGD	porphobilinogen deaminase
RT	room temperature
SAD	small airway disease
SA-β-gal	senescence-associated β-galactosidase
SMP-30	Senescence marker protein-30
SIRT-1	sirtuin I
SP-C	surfactant protein-C
SPF	specific pathogen-free
TCF	T-cell-specific transcription factor
TNF-α	tumor necrosis factor-α
TGF-β	transforming growth factor, beta
TTF	thyroid transcription factor 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organization
WISP1	WNT1-inducible-signaling pathway protein 1
β-gal	β-galactosidase
4-HNE	4-hydroxy-2-nonenal

SUMMARY

Rationale*:* Chronic obstructive pulmonary disease (COPD) is a devastating and poorly understood disease. Currently, no causal therapy for COPD is available. The objectives of this study were, to characterize WNT/β-catenin signaling in COPD in humans and elucidate its potential role as a preventive and therapeutic target in experimental emphysema in mice.

Methods*:* The expression, localization, and activity of WNT/β-catenin signaling was assessed in 12 human COPD and 12 transplant donor samples using quantitative RT-PCR, immunohistochemistry, and Western blotting. The role of WNT/β-catenin signaling was assessed in elastase-induced emphysema and therapeutic modulation thereof was assessed in elastase-induced emphysema in TOPGAL reporter and wild type mice *in vivo*.

Measurements and Main Results*:* WNT/β-catenin signaling components were largely expressed in alveolar epithelium in human COPD lungs. In COPD, no activation was observed and immunohistochemical analysis revealed reduced nuclear β-catenin staining. Similarly, WNT/β-catenin signaling was downregulated in the experimental emphysema model. Preventive, as well as therapeutic, WNT/β-catenin activation by lithium chloride attenuated experimental emphysema, as assessed by decreased airspace enlargement, improved lung function, reduced collagen content, and elevated expression of alveolar epithelial cell markers upon WNT activation.

Conclusion*:* Decreased WNT/β-catenin signaling is involved in parenchymal tissue destruction and impaired repair capacity in emphysema. These data indicate a crucial role of WNT/β-catenin signaling in lung repair mechanisms *in vivo*, and highlight WNT/β-catenin activation as a future therapeutic approach for emphysema.

ZUSAMMENFASSUNG

Hintergrund: Die chronisch obstruktive Lungenerkrankung (COPD) ist eine häufige und schwere Erkrankung, für die aufgrund der unklaren Pathogenese keine kausale Therapie zur Verfügung steht. Das Ziel dieser Arbeit war es, den WNT/β-catenin Signalweg in humanen COPD-Proben zu charakterisieren, und die Rolle des Signalwegs als präventives und therapeutisches Target im Elastase-induzierten Emphysemmodel in Mäusen zu untersuchen.

Methoden: Die Expression, Lokalisation, und Aktivität des WNT/β-catenin Signalwegs wurde im Gewebe von 12 humanen COPD Patienten und 12 Transplantdonoren mittels quantitativer RT-PCR, Immunohistochemie und Western Blot untersucht. Die Rolle des WNT/β-catenin Signalwegs als therapeutisches Ziel wurde im Elastase-induzieretn Emphysemmodel in TOPGAL Reporter- und wildtyp Mäusen *in vivo* untersucht.

Resultate: Die Komponenten des WNT/β-catenin Signalwegs wurden vorwiegend im Alveolarepithel humaner COPD Lungen exprimiert. In diesen Lungen konnte keine erhöhte Aktivität festgestellt werden und die Anzahl der β-catenin positiven Zellen war vermindert. Auch im Tiermodel war der WNT/β-catenin Signalweg runterreguliert. Präventive, als auch therapeutische Aktivierung des WNT/β-catenin Signalwegs mittels Lithiumchlorid führte zu einer Milderung des experimentell induzierten Emphysems, belegt durch eine Normalisierung der Alveolargröße, verbesserte Lungenfunktion, reduzierten Kollagengehalt und erhöhter Expression von Epithelzellmarkern durch WNT/β-catenin Aktivierung.

Zusammenfassung: Der Parenchymverlust in emphysematösen Lungen ist mit einer verminderten Aktivität des WNT/β-catenin Signalwegs sowie eingeschränkten Reparaturkapazität in emphysematösen Lungen assoziiert. Diese Daten lassen eine wichtige Rolle des WNT/β-catenin Signalwegs in Reparaturprozessen der Lunge *in vivo* vermuten, und heben die Aktivierung des WNT/β-catenin Signalwegs als therapeutische Option in Lungenemphysem hervor.

INTRODUCTION

According to the latest estimates from World Health Organisation (WHO) about chronic respiratory diseases, currently 210 million people have chronic obstructive pulmonary disease (COPD) (1). COPD is an emerging serious global health problem, expected to become the third leading cause of death by 2020 (2), although COPD is often not diagnosed. Nearly half of the world population lives in or near areas with poor air quality (1).

Due to its frequency and poor outcome, COPD is associated with a tremendous socioeconomic and individual disease burden (3). Currently, no causal therapy for COPD is available. Optimal therapeutic management of patients suffering from COPD requires a multidisciplinary approach. Pharmacological therapy with bronchodilators and glucocorticoids, long term oxygen therapy, pulmonary rehabilitation, and surgery are the cornerstones of COPD management. Nevertheless, the avoidance of the major risk factor cigarette smoke exposure is the only effective strategy to slow down COPD progression (4, 5). Importantly, COPD can also develop in never smokers (6) and has been associated with a variety of endogenous and exogenous risk factors, such as coal dust or air pollution (7, 8).

COPD is a very heterogeneous disease which is nowadays more and more regarded as a syndrome instead of a single disease. COPD is effecting all compartments of the lung and is known to have systemic effects which are associated with a number of co-morbidities. The two airflow limiting lesions in COPD are small airway disease (SAD) and pulmonary emphysema. SAD, also termed obstructive bronchiolitis, includes airway inflammation with increased mucous production, airway wall remodeling and peribronchiolar fibrosis (9-11). Emphysema is defined as destruction of the alveolar architecture due to distal airspace enlargement (12, 13) (Figure 1). Lungs affected with centrilobular emphysema, which is mainly caused by cigarette smoking develop airspace enlargement mainly in the upper lobe. Persons with alpha 1-antitrypsin (A1AT) deficiency in contrast usually develop emphysema in the lower lobes of the lung, which is known as panlobular emphysema (14).

The degree of airflow limitation can be easily assessed by spirometry (15-17). The severity of COPD is spirometrically classified according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria using the ratio of forced expiratory volume in one second (FEV1) / forced vital capacity (FVC) (12).

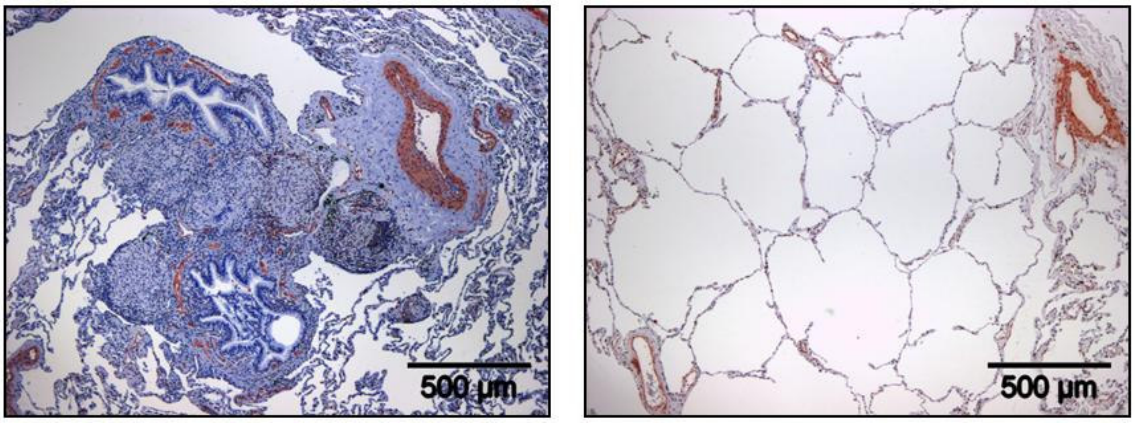


Figure 1. Small airway disease (left panel) and emphysema (right panel) characterizing COPD. Immunohistochemical staining was performed on tissue sections derived from COPD Gold IV lungs. Sections were stained α-smooth muscle actin. Representative pictures of two independent experiments using four different transplant donor or COPD lung tissues, respectively. Magnification is indicated in the pictures.

The above mentioned systemic manifestations and co-morbidities of COPD are following: cardiovascular disease, diabetes, cancer, skeletal muscle dysfunction, or weight loss (18-22). These are proposed to result from the systemic effects of smoking and ongoing systemic inflammation (23, 24). Systemic inflammation in COPD has been clearly demonstrated in past years, assessed by increased oxidative stress (25, 26), activated inflammatory cells (27-29), or increased cytokine levels in the systemic circulation (30-32).

Due to the limited number of symptomatic treatment and the lack of diseases modifying agents, the pathobiology of COPD has received a lot of attention over the last decade. Currently the following hypotheses are believed to be involved in the pathogenesis of COPD:

Extracellular Matrix Proteolysis: Protease/Antiprotease Imbalance

The first indications supporting the protease/antiprotease hypothesis came from Laurell and Eriksson who, in 1963, described five patients with deficiency of A1AT, the primary inhibitor of the neutral serine proteinase neutrophil elastase who had emphysema (33). Subsequently, investigators instilled a variety of proteinases, like papain (34) or pancreatic elastase into the lungs of rodents which caused emphysema (35-38), supporting the idea that an imbalance of proteases and antiproteases is responsible for emphysema development. A1AT is produced in the liver, and as mentioned above, one of its functions is

to protect the lungs from the neutrophil elastase enzyme, which can disrupt connective tissue (39). Patients with mutations in the A1AT gene (especially with the PiZZ phenotype) have deficiency in this enzyme and develop subsequently early onset emphysema. Cigarette smoke is especially harmful to individuals with A1AT deficiency. In addition to increasing the inflammatory reaction in the airways, cigarette smoke directly inactivates A1AT by oxidizing essential methionine residues to sulfoxide forms, decreasing the enzyme activity (39). These observations firmly established neutrophil elastase as the proteinase most likely responsible for tissue destruction in emphysema. Animal experiments were performed supporting the need for a protease/antiprotease balance. Neutrophil elastase-null mice were significantly protected against chronic cigarette smoke-induced emphysema (40). Furthermore, macrophages are the primary inflammatory cell in the lower airspace under normal conditions, and particularly in response to long-term cigarette smoking (41). Macrophages also contain elastolytic enzymes. Because it degrades elastin and is predominantly produced by alveolar macrophages, matrix metalloproteases (MMP)-12 activation has been another leading candidate proteinase responsible for pulmonary emphysema. Shapiro and colleagues demonstrated that MMP-12-null mice are protected from the development of cigarette smoke-induced emphysema (41). MMP-$12^{-/-}$ mice also failed to recruit monocytes into their lungs in response to cigarette smoke. This subsequently led to the finding that cigarette smoke causes constitutive macrophages to produce MMP-12, which, in turn, cleaves elastin into fragments chemotactic for monocytes.

So far, there have been no long-term clinical trials of synthetic proteinase inhibitors agents in COPD patients due mainly to the high cost of such trials.

Oxidative Stress

There is considerable evidence for increased oxidative stress due to an imbalance between oxidants and antioxidants in the lungs of patients with COPD. Studies have documented increased expression of markers of oxidative stress in the lungs of patients with COPD, compared with healthy subjects or smokers with a similar smoking history who have not developed COPD (42). A key mediator of oxidant-induced cell signaling and apoptosis is 4-Hydroxy-2-nonenal (4-HNE), a highly reactive diffusible product of lipid peroxidation has been found to be present in greater quantities in airway epithelial and endothelial cells in the lungs of patients with COPD, compared with smokers with a similar smoking history who have not developed the disease (43).

There are many actions of oxidative stress that can potentially play a role in the pathogenic mechanisms in COPD. These include the inactivation of antiproteases (such as A1AT or secretory leukoprotease inhibitor) (44) or activation of metalloproteases (45) by oxidants, which in turn results in a protease/antiprotease imbalance in the lungs (46). Oxidants can directly damage components of the lung matrix (e.g., elastin and collagen) and can also interfere with elastin synthesis and repair (42).

Furthermore, molecular mechanisms such as transcription factor activation and chromatin remodeling, as a result of increased oxidative stress, may be responsible for perpetuating inflammation in COPD (47, 48). The master antioxidant transcription factor nuclear erythroid-related factor 2 (Nrf2), whose activity is decreased in COPD lungs (49), appears to be a central player in the susceptibility to emphysema (50). The protective role of Nrf2 in emphysema is underscored by the increased susceptibility to Nrf2-null mice to cigarette smoke– (50, 51) and elastase-induced emphysema (52). Furthermore, enhancement of Nrf2 expression using a small molecule activator protected wild-type mice against cigarette smoke–induced emphysema (53). In these mice, the protection afforded by the Nrf2 activator correlated with decreased alveolar cell death rather than inflammation.

Alveolar Cell Apoptosis and Proliferation

Apoptosis programmed cell death or necrosis is a consequence of protease/antiprotease imbalance. However, studies have recently shown that apoptosis can occur independently of the preceding matrix protease degradation (54). Apoptosis was shown to occur predominantly in endothelial cells in the alveolar walls, compared with lungs from normal subjects or from smokers without COPD (55). Rodents with loss of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR), respectively had apoptosis-dependent emphysema (56, 57). Furthermore, in subsequent experimental studies cigarette smoke was shown to reduced expression levels of VEGFR2 supporting this idea (58). Intratracheal instillation of active caspase-3 caused acute apoptosis and alveolar enlargement supporting the idea that apoptosis could initiate alveolar enlargement. These alterations were reversible one week after the initial caspase-3 instillation (59). Apoptotic cells in turn may release intracellular proteases, oxidants, and inflammatory mediators and also express several caspases on their cell surface (60). The exact apoptotic pathways involved in cigarette smoke-induced emphysema have not been elucidated thus far.

The net result of alveolar cell death on alveolar structure depends also on the lung's ability to undergo cell proliferation, which is pivotal for the maintenance of normal tissue homeostasis. Calabrese et al. observed a marked alveolar apoptosis/proliferation imbalance in favor of apoptosis in end-stage pulmonary emphysema (61).

Aging and Senescence

As the lung ages, there is a progressive air space enlargement, which has been considered as the result of a nondestructive process opposed to a destructive process as seen in cigarette-smoke-induced emphysema (62). However, this observation gave rise to the idea that premature aging of the lung is involved in the pathogenesis of emphysema. The cellular equivalent of aging is senescence, which is characterized by a nonproliferative state in which cells are metabolically active and apoptosis-resistant. Studies reported the presence of cellular senescence in the emphysematous lungs and in COPD patients. Peripheral blood mononuclear cells of patients with COPD have decreased telomere length, a hallmark of senescing cells (63). Furthermore, enhanced expression of markers of cell senescence including $p16^{Ink4a}$ and $p21^{CIP1/WAF1/Sdi1}$ and telomere shortening in alveolar epithelial and endothelial cells of emphysema patients when compared with smokers without emphysema and normal individuals were observed (64). Cigarette smoke extract leads to increased senescence-associated β-galactosidase (SA–β-gal) expression in cultured alveolar type II cells (65) or lung fibroblasts (66), which has also been shown in lung fibroblasts from emphysematous lungs (67).

Senescence marker protein-30 (SMP-30) knockout mice developed aging-related changes in the lung, including alveolar enlargement that resembled emphysema (68, 69) which was aggravated when mice were additionally exposed to cigarette smoke (70) suggesting a synergistic effect in the pathogenesis of emphysema.

Sirtuin I (SIRT-1) is essential for maintaining silent chromatin via the deacetylation of histones and has been shown to be decreased in lung cells from patients with COPD, compared with smokers who have not developed the disease, as a result of post-translational oxidative modification (71). This would accelerate the process of aging and also enhance inflammation.

The finding that decreased VEGF signaling caused experimental emphysema (56, 57) and the evidence that COPD lungs have decreased expression of VEGF and VEGFR-2 led to the concept that alveolar maintenance was required for structural preservation of the lung. Cigarette-smoke would then disrupt this maintenance program, causing emphysema (72). As mentioned above it has been demonstrated that an increase of endothelial cell apoptosis correlated with reduced expression of these molecules in human tissues (55), and it was shown that VEGF levels in induced sputum of patients with COPD (73), and bronchoalveolar lavage (BAL) from healthy smokers were decreased (74). Although the emphysema caused by VEGFR2 blockade is a model independent of inflammation, inhibition of VEGF and VEGFR2 signaling was mediated by oxidative stress in rodents (75, 76).

Hepatocyte growth factor (HGF) is another multifunctional factor which has been implicated in the pathogenesis of COPD. HGF is known to act on repairing lung injury and promoting angiogenesis (77, 78). Reduced levels of HGF correlated with progressive emphysematous changes and deterioration in pulmonary physiology in elastase-induced emphysema (79). HGF likely induced proliferation of bone marrow derived cells and resident endothelial cells in alveolar walls of mice treated with intratracheal instillation of pancreatic elastase (80). However, a role of HGF in the pathogenesis of COPD remains still controversial.

The transforming growth factor (TGF)-β superfamily constitutes more than 40 members, which are essential during organ development, a process often recapitulated in chronic diseases (81, 82). Emerging interest in the role of TGF-β in the pathogenesis of COPD has recently evolved (83), particularly since genetic studies have demonstrated an association of gene polymorphisms of the TGF-β superfamily with COPD (84, 85). In addition, increased expression of TGF-β1 in COPD lungs and primary cells, such as epithelial cells, macrophages, or fibroblasts isolated from COPD specimens, was reported, suggesting an impact of TGF-β signaling on the development and progression of COPD (86-88). Furthermore, latent TGF-β binding proteins (LTBPs)-3 (89) and -4 knock out (90), as well as Smad3-null mice (91) showed air space enlargement, indicating emphysema. These mice had reduced expression of TGF-β and TGF-β activation based on decreased expression of phospho-smad 2 and/or 3.

Developmentally active signaling pathways are thought to be reactivated during disease state to turn on repair processes taking place in the lungs of affected individuals, whereas this is the case in patients with COPD remains controversial.

Since emphysema, one main feature of COPD, is characterized by alveolar airspace enlargement, parenchymal tissue destruction, and impaired pulmonary regeneration (13), signaling pathways active during lung development exhibit potential therapeutic relevance. In this respect, WNT/β-catenin signaling is one of the most prominent signaling pathways, as a lack of WNT2A/2B activity results in complete lung agenesis (92). Furthermore, recent evidence has detected increased WNT/β-catenin signaling in pulmonary fibrosis and cancer (93-96), diseases associated with lung epithelial hyperplasia.

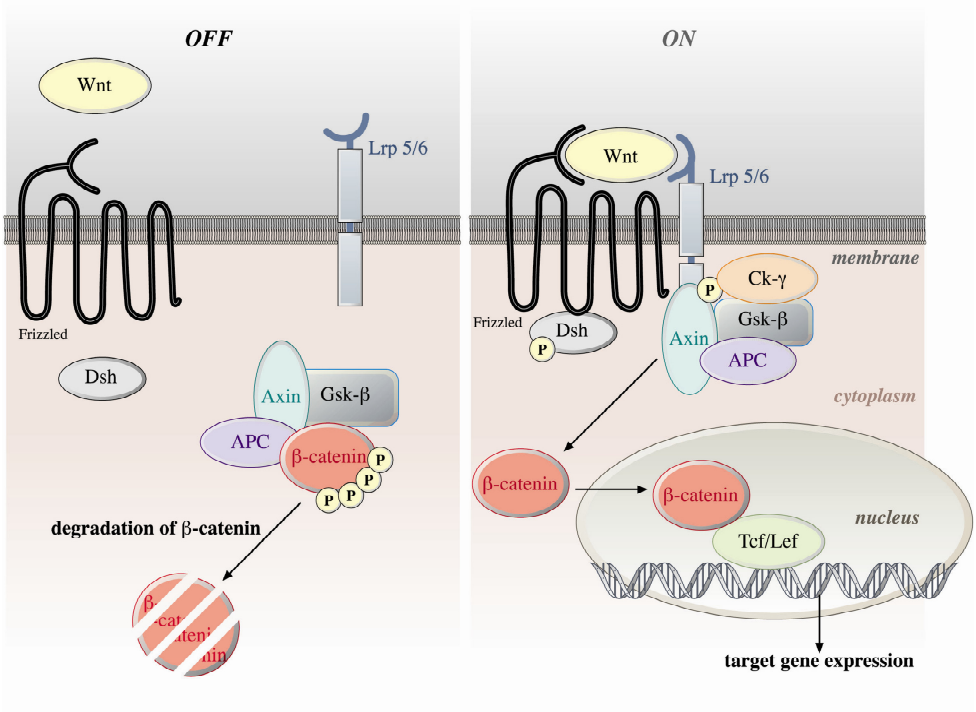
In non–small cell lung cancer (NSCLC) increased expression of WNT proteins, along with decreased expression of WNT regulators, is likely involved in the initial phase of tumorigenesis, as well as in the ongoing, multistep process of lung cancerogenesis (97). WNT signaling seems to be reactivated in response to an unknown injurious stimulus in idiopathic pulmonary fibrosis (IPF), most likely as ‘‘attempted regeneration’’ of lung epithelium. This may explain the findings of hyperplastic epithelium and epithelial to mesenchymal transition (EMT) in IPF lungs. Unfortunately, this ‘‘attempted regeneration’’ is not only insufficient to restore normal lung architecture in IPF, but, moreover, further drives fibrogenesis and IPF progression by paracrine actions on fibroblasts (94, 97, 98).

Emphysema is one main feature of COPD, characterised by alveolar airspace enlargement and parenchymal tissue destruction (13, 99). Lung tissue maintenance is believed to be a balanced process of lung injury and repair, which is impaired in COPD (100, 101).

WNT/β-catenin signaling

As mentioned above, recent evidence revealed that increased WNT/β-catenin signaling, which is essential for lung morphogenesis, is linked with parenchymal lung diseases, particularly with cancer and pulmonary fibrosis (93-96, 102). As shown in Figure 2, WNT/β-catenin signaling involves WNT ligand binding to cell surface receptors and cytosolic stabilization and nuclear translocation of β-catenin for target gene expression (103, 104). In the absence of active WNT ligands, β-catenin is constitutively phosphorylated by its interaction with axin, adenomatosis polyposis coli (APC), and glycogen synthase kinase (GSK)-β (called “beta-catenin destruction complex”), and subsequently degraded. In the presence of WNT ligands, two distinct membrane receptors, the frizzled (FZD) or the low

density lipoprotein receptor-related proteins (LRP) 5 and 6, are phosphorylated and activated upon ligand binding, leading to disruption of the β-catenin destruction complex. Cytosolic β-catenin then accumulates and undergoes nuclear translocation, where it regulates target gene expression through interaction with members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family.



Königshoff M, Am J Respir Cell Mol Biol. 2010

Figure 2. Canonical WNT/β-catenin signaling. The best characterized WNT signaling pathway is the β-catenin-dependent, or canonical, WNT signaling pathway. Here, in the absence of active Wnt ligands, β-catenin is constitutively phosphorylated by its interaction with axin, adenomatosis polyposis coli (APC), and glycogen synthase kinase (Gsk)-3β, and subsequently degraded (left panel). In the presence of Wnt ligands, two distinct membrane receptors, the frizzled (Fzd) or the low density lipoprotein receptor-related proteins (Lrp) 5 and 6, are activated upon ligand binding (left panel). In detail, Wnt stimulation leads to phosphorylation of Lrp6 by Gsk-3β and casein kinase γ in its cytoplasmic region, which leads to the recruitment of axin. Subsequently, β-catenin phosphorylation is attenuated, its degradation inhibited, and accumulated β-catenin undergoes nuclear translocation, where it regulates target gene expression through interaction with members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor (Tcf/Lef) family (right panel).

Importantly, WNT/β-catenin signaling is involved in lung epithelial injury and repair processes (102, 105, 106). Canonical WNT/β-catenin activation has been demonstrated to lead to increased proliferation of lung epithelial cells, in concert with upregulation of WNT target genes, suggesting a role of active WNT signaling in epithelial cell repair mechanism *in vitro* and *in vivo* (94, 96, 98, 107). Furthermore, inhibition of the WNT target gene WISP1 led to decreased alveolar epithelial proliferation and attenuation of experimental lung fibrosis (94). The impact of WNT/β-catenin signaling on the pathogenesis of COPD, however, has not been investigated thus far.

AIM OF THE STUDY

The aim of this study was to test whether disturbed WNT/β-catenin signaling is involved in impaired parenchymal tissue repair and destruction in emphysema. WNT/β-catenin activation may thus participate in lung regeneration by initiating alveolar epithelial cell repair processes.

To address this aim the following experimental setup was applied

1) the expression (qRT-PCR), activity (immunoblot) and localization (immunohistochemistry) of WNT/β-catenin signaling components were assessed in human COPD
2) the expression (qRT-PCR), activity (TOPGAL reporter mice) and localization (immunohistochemistry) of WNT/β-catenin signaling components were assessed in experimental emphysema in mice
3) the lung structure (immunohistochemistry and lung morphology) and lung function (dynamic compliance and resistance) were assessed in a preventive and therapeutic approach of WNT/β-catenin activation in experimental lung emphysema
4) the functional impact of WNT/β-catenin activation on extracellular matrix deposition and alveolar epithelial cell marker expression (qRT-PCR and immunoblot) was assessed after activation of WNT/β-catenin in experimental lung emphysema

MATERIALS AND METHODS

Equipment

ABI PRISM 7500 Detection System	Applied Biosystems, USA
Bioanalyzer 2100	Agilent Technologies, USA
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Developing machine X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader Packard	Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
GS-800TM Calibrated Densitometer	Bio-Rad, USA
Light microscope Olympus BX51	Olympus, Germany
Microscope LEICA AS MDW	Leica, Germany
Mini spin centrifuge	Eppendorf, Germany
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Multipette® plus	Eppendorf, Germany
Nanodrop®	Peqlab, Germany
PCR-thermocycler	MJ Research, USA
Pipetboy	Eppendorf, Germany
Pipetman: P10, P20, P100, P200, P1000	Gilson, France
Pipette tip: 200, 1000 µl,	Sarstedt, Germany
Pipette tip: 10 µl, 20 µl, 100 µl	Gilson, USA
Quantity One software	Bio-Rad, USA
Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Thermo-Fast® 96 PCR Plate	Thermo Scientific, USA

Western Blot Chambers: Mini Trans-Blot	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
Vacuum centrifuge	Eppendorf, Germany

Antibodies and reagents

The following antibodies were used in this study:

active β-catenin	(#05-665)	Cell Signaling Technology, Beverly, MA
Surfactant Protein C (SP-C)	(#3786)	Cell Signaling Technology, Beverly, MA
total β-catenin	(#9562)	Cell Signaling Technology, Beverly, MA
phospho-S9-GSK-3β	(#9336)	Cell Signaling Technology, Beverly, MA
total GSK-3β and	(#9315)	Cell Signaling Technology, Beverly, MA
phospho-LRP6	(#2568)	Cell Signaling Technology, Beverly, MA
total LRP6	(#2560)	Cell Signaling Technology, Beverly, MA
WNT1	(#ab15251)	Abcam, Cambridge, UK
WNT3a	(#38-2700)	Zymed Laboratories/Invitrogen, CA
α-smooth muscle actin	(#A2547)	Sigma-Aldrich, Saint Louis, MO
Aquaporin V	(#ab52054)	Abcam, Cambridge, UK

Antibodies for immunohistochemistry:

Surfactant Protein C (SP-C)	(#ab28744)	Abcam, Cambridge, UK
β-catenin	(#ab32572)	Abcam, Cambridge, UK

The following reagents were used:

Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
CompleteTM Protease inhibitor	Roche, Germany
DNA Ladder (100 bp, 1kb)	Promega, USA
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria

Ethanol absolute Riedel-de	Haën, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Glycine	Roth, Germany
GoTaq® Flexi DNA polymerase	Promega, USA
Hydrochloric acid	Sigma-Aldrich, Germany
Lithium chloride, ≥ 99%	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich, Germany
MuLV Reverse Transcriptase	Applied Biosystems, USA
Quick StartTM Bradford Dye Reagent	Bio-Rad, USA
Random Hexamers (50 µM)	Applied Biosystems, USA
RNase inhibitor	Applied Biosystems, USA
RNaseZAP®	Sigma-Aldrich, Germany
pancreatic elastase from porcine pancreas	Sigma-Aldrich, Saint Louis, MO
PeqGOLD Total RNA Kit	Peqlab, Germany
Roti®-Quick-Kit	Roth, Germany
SircolTM, Soluble Collagen Assay	Biocolor, UK
SYBER® Green PCT Kit	Invitrogen, UK
Tris	Roth, Germany
Tween 20	Sigma-Aldrich, Germany
Triton X-100	Promega, USA

Human tissues

Lung tissue was obtained from 12 COPD patients classified as Global Initiative for Chronic Obstructive Lung Disease (GOLD) IV undergoing lung transplant due to their underlying COPD (5 females, 7 males; mean age = 56±5 years; mean FEV1= 16.1±2.8%; mean FEV1/FVC = 43.4±14.6%) and 12 control subjects (transplant donors; 6 females, 6 males; mean age 42±10 years). COPD samples were taken from the parenchyma with histological validation of emphysematous changes. The IPF tissue was obtained as described previously (94). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine. Informed consent was obtained in written form from each subject for the study protocol.

Animals

Six- to eight-week-old pathogen-free female C57BL/6N mice (Charles River Laboratories) were used throughout this study. All experiments were performed in accordance with the guidelines of the Ethics Committee of the University of Giessen School of Medicine and approved by the Regierungspräsidium Giessen, Hessen, Germany. Mice had free access to water and rodent laboratory chow.

Mouse model of Elastase-induced pulmonary emphysema

Pancreatic elastase was dissolved in sterile PBS and applied orotracheally (100U/kg BW). Control mice received 80 µl sterile PBS. Lung tissues were excised and snap-frozen or inflated with 4 % (m/v) paraformaldehyde in PBS (PAA Laboratories) at 21 cm H_2O pressure for histological analyses.

Mouse model of WNT activation

The TOPGAL mice were purchased from Jackson Laboratories. The derivation of TOPGAL mice has been described in detail previously (108). Mice were bred under specific pathogen-free (SPF) conditions. The following primers were used for identification of transgenic animals:

Lac(Z)-F 5-gttgcagtgcacggcagatacacttgctga-3´;
Lac(Z)-R5´-gccactggtgtgggccataattcattcgc-3´.

Six to eight week old mice were used for all experiments.

WNT/β-catenin pathway activation in vivo

The WNT/β-catenin signaling pathway in lungs from C57BL/6N mice was activated via intraperitoneal injection of lithium chloride (200mg/KG/BW/day) (92). Lithium chloride mimics members of the WNT family of signaling proteins by inhibiting the activity of GSK-3β, causing intracellular accumulation of β-catenin, a feature associated with the canonical WNT/β-catenin signaling pathway (109). Lithium chloride was diluted in sterile water and fresh stock was prepared for every injection. Mice were treated on a daily basis, from day 0

(the day of elastase instillation) to day 7 in the preventive regimen and from day 7 to 14 in the therapeutic regimen.

Quantitative morphometry

To assess air space enlargement, the mean chord length (MCL) was quantified by superimposing a line grid on the images of lung sections at a magnification of 200×. Points on the lines of the grid hitting the air spaces, and intercepts of the lines with alveolar septa were counted to calculate MCL according to the formula: MCL = Σ Pair × L(p) / (Isepta / 2) (μm), where Σ Pair is the sum of the points of the grid overlaid air spaces, L(p) is the line length per point and Isepta is the sum of the intercepts of alveolar septa with the lines of the grid. For the morphometric assessment of alveolar surface area per unit volume of lung parenchyma (Sv) were determined by counting the number of points that fell on alveolar septal tissue and alveolar space, and by counting the number of intercepts with alveolar septal surface at a magnification of 200× according to the following formulas: Sv = 2 × Isepta / (Σ Ppar x L(p)/3) (1/μm), where Σ Ppar is the sum of the points of hitting parenchyma, L(p) is the line length per test point in μm, and Isepta the sum of the intercepts with alveolar septa.

Reverse transcription and quantitative RT-PCR

Total RNA was extracted using Qiagen extraction kits according to the manufacturer's protocol, and cDNAs were generated by reverse transcription using SuperScriptTM II (Invitrogen). Quantitative (q)RT-PCR was performed using fluorogenic SYBR Green and the Sequence Detection System Fast 7500 (PE Applied Biosystems). HPRT1 and PBGD, ubiquitously and equally expressed genes free of pseudogenes, were used as a reference gene in all human and mouse qRT-PCR reactions, respectively. PCR was performed using the primers listed in Table 1 and 2, at a final concentration of 200 nM. Relative transcript abundance of a gene is expressed in ΔCt values ($\Delta Ct = Ct^{reference} - Ct^{target}$). Relative changes in transcript levels compared to controls are ΔΔCt values ($\Delta\Delta Ct = \Delta Ct^{treated} - \Delta Ct^{control}$). All ΔΔCt values correspond approximately to the binary logarithm of the fold change as mentioned in the text. When relative transcript abundance is of information, expression levels are given in ΔCt levels.

Table 1. Primer sequences and amplicon sizes for human tissues. All primer sets worked under identical quantitative PCR cycling conditions with similar efficiencies to obtain simultaneous amplification in the same run. Sequences were taken from GeneBank, all accession numbers are denoted.

Gene	Accession		Sequences (5′→3′)	Length	Amplicon
β-catenin	NM001904	for	*AAGTGGGTGGTATAGAGGCTCTTG*	24 bp	77 bp
		rev	*GATGGCAGGCTCAGTGATGTC*	21 bp	
FZD1	NM003505	for	*AGCGCCGTGGAGTTCGT*	17 bp	64 bp
		rev	*CGAAAGAGAGTTGTCTAGTGAGGAAAC*	27 bp	
FZD2	NM001466	for	*CACGCCGCGCATGTC*	15 bp	63 bp
		rev	*ACGATGAGCGTCATGAGGTATTT*	23 bp	
FZD3	NM017412	for	*GGTGTTCCTTGGCCTGAAGA*	20 bp	72 bp
		rev	*CACAAGTCGAGGATATGGCTCAT*	23 bp	
FZD4	NM012193	for	*GACAACTTTCACACCGCTCATC*	22 bp	164 bp
		rev	*CCTTCAGGACGGGTTCACA*	19 bp	
GSK-3β	NM002093	for	*CTCATGCTCGGATTCAAGCA*	20 bp	86 bp
		rev	*GGTCTGTCCACGGTCTCCAGTA*	22 bp	
LEF1	NM016269	for	*CATCAGGTACAGGTCCAAGAATGA*	24 bp	93 bp
		rev	*GTCGCTGCCTTGGCTTTG*	18 bp	
LRP6	NM002336	for	*GATTCAGATCTCCGGCGAATT*	21 bp	83 bp
		rev	*GGCTGCAAGATATTGGAGTCTTCT*	24 bp	
TCF3	NM031283	for	*ACCATCTCCAGCACACTTGTCTAATA*	26 bp	71 bp
		rev	*GAGTCAGCGGATGCATGTGA*	20 bp	
TCF4	NM030756	for	*GCGCGGGATAACTATGGAAAG*	21 bp	89 bp
		rev	*GGATTTAGGAAACATTCGCTGTGT*	24 bp	
WNT2	NM003391	for	*CCTGATGAATCTTCACAACAACAGA*	25 bp	78 bp
		rev	*CCGTGGCACTTGCACTCTT*	19 bp	
WNT3a	NM033131	for	*GCCCCACTCGGATACTTCTTACT*	23 bp	98 bp
		rev	*GAGGAATACTGTGGCCCAACA*	21 bp	
WNT7b	NM058238	for	*GCAAGTGGATTTTCTACGTGTTTCT*	25 bp	65 bp
		rev	*TGACAGTGCTCCGAGCTTCA*	20 bp	
WNT10b	NM003394	for	*GCGCCAGGTGGTAACTGAA*	19 bp	59 bp
		rev	*TGCCTGATGTGCCATGACA*	19 bp	
HPRT 1	NM000194	for	*AAGGACCCCACGAAGTGTTG*	20 bp	137 bp
		rev	*GGCTTTGTATTTTGCTTTTCCA*	22 bp	
Axin1	NM008312	for	*GAGGATGCGGAGAAGAACCA*	20 bp	112 bp
		rev	*GACAAGGGTCTGGAGTTCTCATG*	23 bp	

Axin2	NM008313	for	*AGAAATGCATCGCAGTGTGAAG*	22 bp	102 bp
		rev	*GGTGGGTTCTCGGGAAATG*	19 bp	

Table 2. Primer sequences and amplicon sizes for mouse tissues. All primer sets worked under identical quantitative PCR cycling conditions with similar efficiencies to obtain simultaneous amplification in the same run. Sequences were taken from GeneBank, all accession numbers are denoted.

Gene	Accession		Sequences (5′→3′)	Length	Amplicon
β-catenin	NM007614	for	*TCAAGAGAGCAAGCTCATCATTCT*	24 bp	115 bp
		rev	*CACCTTCAGCACTCTGCTTGTG*	22 bp	
AP5	NM009701	for	*CCTTATCCATTGGCTTGTCG*	20bp	115bp
		rev	*CTGAACCGATTCATGACCAC*	20bp	
CDH1	NM009864	for	*CCATCCTCGGAATCCTTGG*	19bp	89bp
		rev	*TTTGACCACCGTTCTCCTCC*	20bp	
Col1a1	NM007742	for	*CCAAGAAGACATCCCTGAAGTCA*	23bp	128bp
		rev	*TGCACGTCATCGCACACA*	18bp	
FZD1	NM021457	for	*AAACAGCACAGGTTCTGCAAAA*	22 bp	58 bp
		rev	*TGGGCCCTCTCGTTCTT*	18 bp	
FZD2	NM020510	for	*TCCATCTGGTGGGTGATTCTG*	21 bp	66 bp
		rev	*CTCGTGGCCCCACTTCATT*	19 bp	
GSK-3β	NM019827	for	*TTTGAGCTGGATCCCTAGGATGA*	23 bp	75 bp
		rev	*TTCTTCGCTTTCCGATGCA*	19 bp	
LEF1	NM010703	for	*GGCGGCGTTGGACAGAT*	17 bp	67 bp
		rev	*CACCCGTGATGGGATAAACAG*	21 bp	
LRP5	NM008513	for	*CAACGTGGACGTGTTTTATTCTTC*	24 bp	138 bp
		rev	*CAGCGACTGGTGGTGCTGTAGTCA*	21 bp	
LRP6	NM008514	for	*CCATTCCTCTCACTGGTGTCAA*	22 bp	146 bp
		rev	*GCCAAACTCTACCACATG TTCCA*	23 bp	
PBGD	NM013551	for	*GGTACAAGGCTTTCACGATCGC*	22bp	135bp
		rev	*ATGTCCGGTAACGGCGGC*	18bp	
SP-C	NM0011286	for	*TCTGCTCATGGGCCTCCAC*	19bp	116bp
		rev	*CGATGGTGTCTGCTCGCTC*	19bp	
TCF4	NM009333	for	*GTGGGAACTGCCCCGTTT*	18 bp	59 bp
		rev	*GTTCCTGATGAACCTTCACAAC*	23 bp	
TTF1	NM009442	for	*AGCTTCCGAAGCCGAAGTATC*	21bp	98bp
		rev	*AGAACGGAGTCGTGTGCTTTG*	21bp	
WNT2	NM023653	for	*AGCCCTGATGAACCTTCACAAC*	22 bp	78 bp
		rev	*TGACACTTGCATTCTTGTTTCAAG*	24 bp	
WNT3a	NM009522	for	*GCACCACCGTCAGCAACA*	18 bp	57 bp

		rev	*GGGTGGCTTTGTCCAGAACA*	20 bp	
WNT7b	NM009528	for	*TCGAAAGTGGATCTTTTACGTGTTT*	25 bp	67 bp
		rev	*TGACAATGCTCCGAGCTTCA*	20 bp	
WNT10b	NM011718	for	*TGGGACGCCAGGTGGTAA*	18 bp	60 bp
		rev	*CTGACGTTCCATGGCATTTG*	20 bp	
FGFR2	NM014183	for	*ACACAGATAGCTCCGAAGACGTTGT*	25 bp	140 bp
		rev	*CCCAGCCGGACAGCGGAACT*	20 bp	
BMP4	NM012159	for	*ATTCGGAGCGACGCACTGCC*	20 bp	146 bp
		rev	*ACGACCATCAGCATTCGGTTACCA*	24 bp	
TNF-α	NM021926	for	*CATCTTCTCAAAATTCGAGTGACAA*	25 bp	174 bp
		rev	*TGGGAGTAGACAAGGTACAACCC*	24 bp	
MIP-2	NM020310	for	*ATCCAGAGCTTGAGTGTGACGC*	22 bp	89 bp
		rev	*AAGGCAAACTTTTTGACCGCC*	21 bp	
IL-1β	NM016176	for	*AGTTGACGGACCCCAAAAGAT*	21 bp	57 bp
		rev	*GGACAGCCCAGGTCAAAGG*	19 bp	
Axin1	NM012005	for	*CGAAGGGAGATGCAGGAGAGTA*	22 bp	67 bp
		rev	*AAGTGCGAGGAATGTGAGGTAGA*	23 bp	
Axin2	NM012006	for	*TGCATCTCTCTCTGGAGCTG*	19 bp	118 bp
		rev	*ACAGCGAGTTATCCAGCGAC*	20 bp	

Immunohistochemistry

Lungs were placed in 4% (w/v) paraformaldehyde after explantation, and processed for paraffin embedding. Sections (3 µm) were cut, mounted on slides, subjected to antigen retrieval, and quenching of endogenous peroxidase activity using 3% (v/v) H_2O_2 for 20 min. Immune complexes were visualized using suitable peroxidase-coupled secondary antibodies, according to the manufacturer's protocol (Histostain Plus Kit; Zymed/Invitrogen).

Western blot analysis

Human lung tissue specimens were homogenized in extraction buffer [20 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, supplemented with

Complete™ Proteinase Inhibitor Cocktail (Merck Biosciences)] and whole proteins were extracted by centrifugation (12.000 × g) for 10 min at 4°C. Samples containing 25 mg of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gels. The separated proteins were transferred to nitrocellulose membranes (Invitrogen), blocked with 5% skim milk, and incubated with the indicated antibodies. Proteins were then visualized by enhanced chemiluminescence detection (ECL, Amersham Biosciences, Uppsala, Sweden), as reported. Prior to reprobing, nitrocellulose membranes were incubated with stripping stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7)] at 50°C for 30 min.

Collagen assay

Whole-mouse lung homogenates were used for *in vivo* analysis. Total collagen content was determined using the Sircol Collagen Assay kit (Biocolor). Equal amounts of protein lysates were added to 1 ml Sircol dye reagent, followed by 30 minutes of mixing. After centrifugation at 10.000 × g for 10 minutes, the supernatant was aspirated, and 1 ml of alkali reagent was added. Samples and collagen standards were then read at 540 nm in a spectrophotometer (Bio-Rad). Collagen concentrations were calculated using a standard curve with acid-soluble type I collagen.

Detection of β-galactosidase in TOPGAL mice

The β-galactosidase was detected using the X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) reporter gene staining kit from Sigma-Aldrich. Lung tissues were excised and immediately transferred to fixative containing 0.2% glutaraldehyde, 5mM EGTA, 100mM $MgCl_2$ in 0.1 M $NaPO_4$ (pH 7.3) for 4h at 4°C with one solution change. The samples were transferred to 15% sucrose in PBS for 4h and subsequently to 30% sucrose in PBS at 4°C overnight. Samples were embedded in Tissue-Tek O.C.T. and 15µm sections were cut. The sections were dried at RT for 2h before staining. For staining, the sections were washed twice with PBS and the X-Gal staining solution was incubated overnight at 37°C. Counterstain was performed with Nuclear Red.

Pulmonary function studies in mice

Mouse pulmonary function testings were performed and analyzed using a FinePointe RC invasive pulmonary function device (Buxco Research Systems; Wilmington, NC) as published previously (110). All mice were anesthetized i.p. with MMF (Medetomidin, Midazolam, Fentanyl), intubated and placed in a FinePointe RC system. In a heated plethysmograph chamber, mice were ventilated at an average rate of 150 breaths per minute, and flow, mouth and esophageal pressure and heart rate were monitored to measure resistance and dynamic compliance. After an initial acclimation period of three minutes, two subsequent one-minute measurements were performed and averaged.

Quantification nuclear localization of β-catenin in ATII cells

Double staining with SP-C (antibody: rabbit, Fast red as chromogen, and β-catenin: antibody mouse, grey SG as chromogen). Digital pictures of whole lobe were made by a Mirax system (Zeiss, Oberkochen, Germany) and printed at size of 25x18 cm (Approximately × 15 magnification). Using a square grid system B100 (111) points falling on the alveolar region were counted, and at least 8 sites were selected in volume-weighted random manner. Each site was analyzed at final magnification of ×870 on the screen or directly under light microscope using objective ×40. ATII cells with nuclear localization of β-catenin were counted when gray/dark blue staining was found in the nucleus (no counterstaing for the nuclear was done). Data are shown as percentage of number of cells with nuclear localization of β-catenin to total number of ATII cells analyzed. Four lung sections from donors and COPD patients were analyzed.

Statistical analysis

All ΔCt values obtained from real-time RT-PCR were analyzed for normal distribution using the Shapiro-Wilk test, using assignment of a normal distribution with $p > 0.05$. Normality of data was confirmed using quantile-quantile plots. The means of indicated groups were compared using two-tailed Student's *t*-test, or a one-way analysis of variance (ANOVA) with Tukey HSD post hoc test for studies with more than 2 groups. Results were considered statistically significant when $p < 0.05$.

RESULTS

WNT/β-catenin signaling in chronic obstructive pulmonary disease (COPD)

Initially, we sought to investigate whether components of the WNT/β-catenin signaling pathway are differentially regulated in COPD. The mRNA levels of the main WNT/β-catenin signaling components were quantified in lung tissue specimen of transplant donors and COPD patients (GOLD IV) using quantitative (q)RT-PCR. Samples underwent pathological examination and were taken from emphysematous areas. As depicted in Figure 3A, WNT ligands were variably expressed in the human adult lung, with low expression of *WNT10b*. In COPD lung specimens, only *WNT10b* mRNA levels were statistically significant upregulated (log-fold change of 2.23 ± 0.64). Next, we analyzed the expression of common WNT receptors and co-receptors. As shown in Figure 3B, the most abundant receptors in the human lung were frizzled (*FZD) 1* and *4*, and the co-receptor low density lipoprotein receptor-related protein (*LRP*) 6. Interestingly, all receptors exhibited similar expression pattern in COPD and transplant donor lungs. Likewise, the intracellular mediators glycogen synthase kinase (*GSK)*-3β and *β-catenin* were equally expressed in COPD and transplant donor lungs (Figure 3C). All members of the T-cell-specific transcription factor/lymphoid enhancer-binding factor TCF/LEF family of transcription factors, except *TCF1*, were expressed, but not differentially regulated, in COPD or transplant donor lungs (Figure 3C).

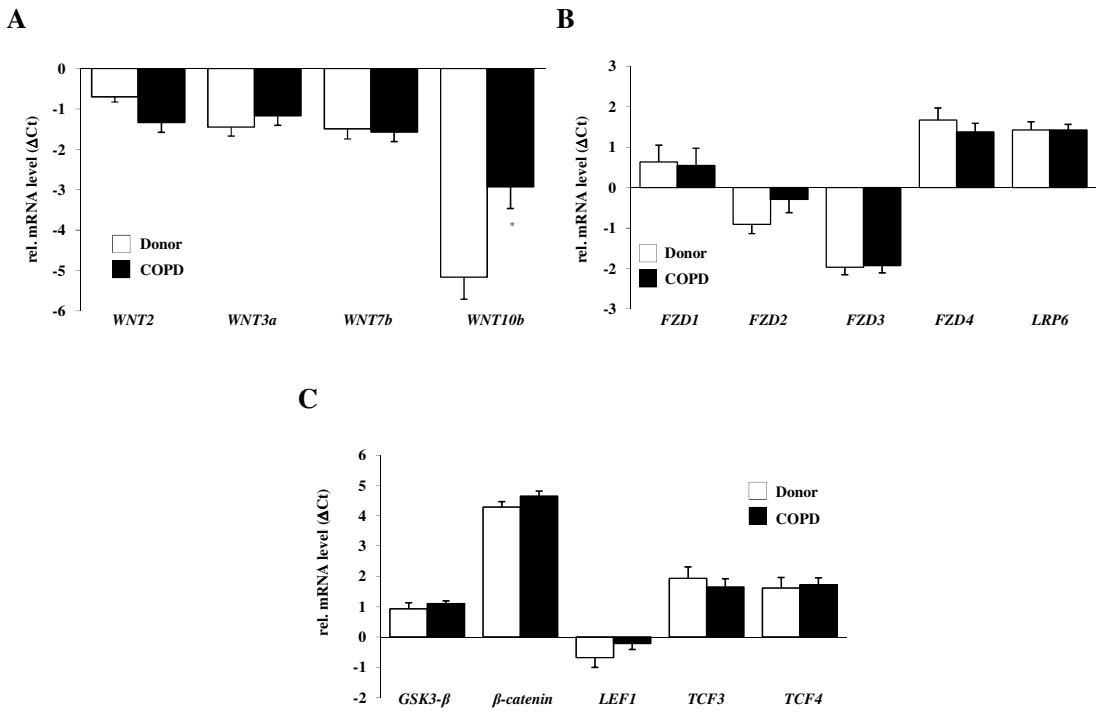


Figure 3. The expression and activity of WNT/β-catenin signaling components in COPD and transplant donor patients. The mRNA levels of (A) the WNT ligands *WNT2, 3a, 7b*, and *10b*, (B) the receptors frizzled (*FZD) 1–4*, low density lipoprotein-related protein *(LRP) 6*, and (C) the intracellular signal transducers glycogen synthase kinase *(GSK)-3β, β-catenin*, lymphoid enhancer-binding factor *(LEF) 1*, T-cell-specific transcription factor *(TCF) 3, TCF 4*, were assessed in transplant donor and COPD lung specimen by quantitative RT-PCR. Results are derived from 12 donors and 12 COPD patients and presented as mean ±s.e.m., * $p<0.05$.

We went on to specifically determine WNT/β-catenin signaling activity in COPD by phosphorylation analysis of LRP6 and GSK-3β, which has recently been demonstrated to be a sensitive indicator of WNT activity in tissue sections (112, 113). Western Blot analysis of phosphorylated GSK-3β and total GSK-3β, phosphorylated LRP6 and total LRP6, as well as active β-catenin and total β-catenin revealed that there was no difference between COPD and transplant donor samples on a tissue homogenate level (Figure 4A, for densitometry see Figure 4B).

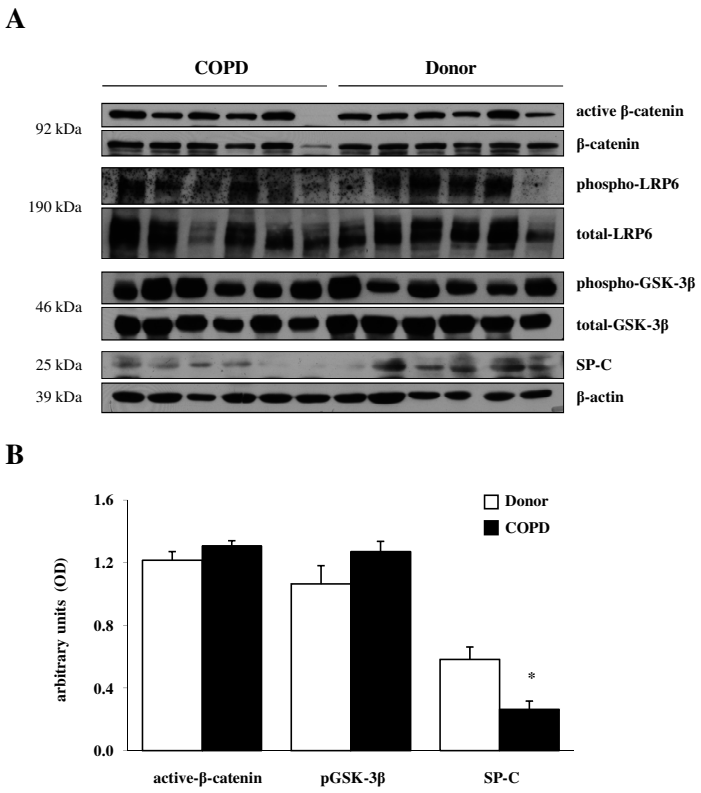


Figure 4. Activity of the WNT/β-catenin pathway in lung homogenates of COPD and transplant donor patients. (A) The expression of active WNT components in lung homogenates of transplant donor and COPD patients was analyzed by immunoblotting of phosphorylated and total GSK-3β and low density lipoprotein-related protein (LRP) 6, respectively, as well as active and total β-catenin. Blotting of total β-catenin GSK-3β, LRP6 and β-actin served as loading controls. Immunoblotting of surfactant protein-C (SP-C) was used as a positive control. (B) Results were confirmed by densitometry and presented as mean ±s.e.m., * $p<0.05$.

To further elucidate WNT/β-catenin signal activity in COPD lung tissue, we performed immunohistochemical staining of β-catenin, the main signal transducer of the WNT/β-catenin pathway. As demonstrated by co-localization with SP-C, β-catenin expression was detected in alveolar epithelial type II (ATII) cells in COPD and transplant donor lung tissue (Figure 5). Remarkably, β-catenin was mainly localized to the basolateral side of ATII cells in COPD and donor lungs, indicating inactive WNT/β-catenin signaling (Figure 5, arrows).

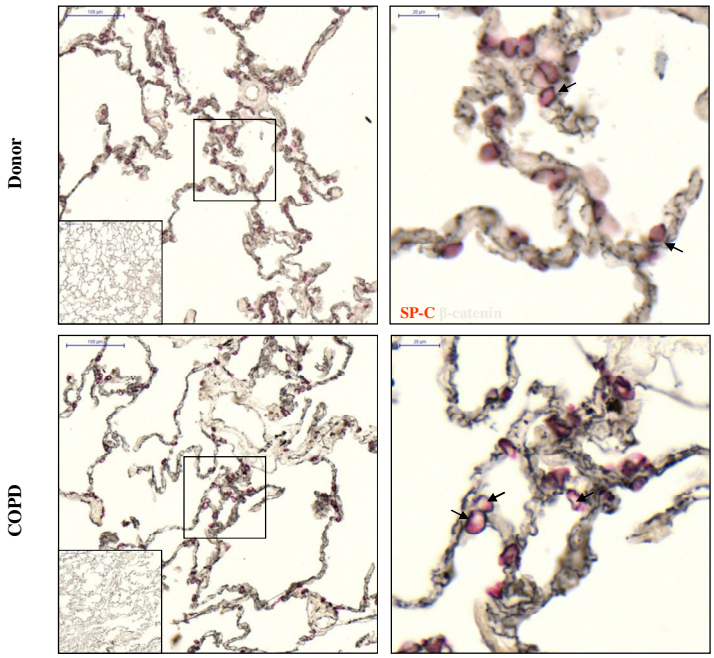


Figure 5. Expression and localization of β-catenin in lung tissues of COPD and transplant donor patients. Immunohistochemical staining was performed on tissue sections derived from transplant donor and COPD lungs. Sections with co-staining of SP-C (Fast red as chromogen) and β-catenin (grey SG as chromogen) were used to demonstrate co-localization of SP-C and β-catenin in alveolar epithelial type II (ATII) cells. Arrows indicate absence of nuclear β-catenin in tissue sections of COPD and donor lungs (right lower panel). Representative pictures of two independent experiments using four different transplant donor or COPD lung tissues, respectively. Magnification is indicated in the pictures.

Furthermore, immunofluorescence staining of β-catenin was performed (Figure 6). Nuclear β-catenin staining was only rarely detected in COPD lung tissue. As a positive control, we analyzed ATII cells in fibrotic lung tissue, demonstrating increased active WNT/β-catenin signaling in ATII cells (Figure 6, left panel, arrows), thereby confirming previous findings of enhanced WNT/β-catenin activity in lung fibrosis (98).

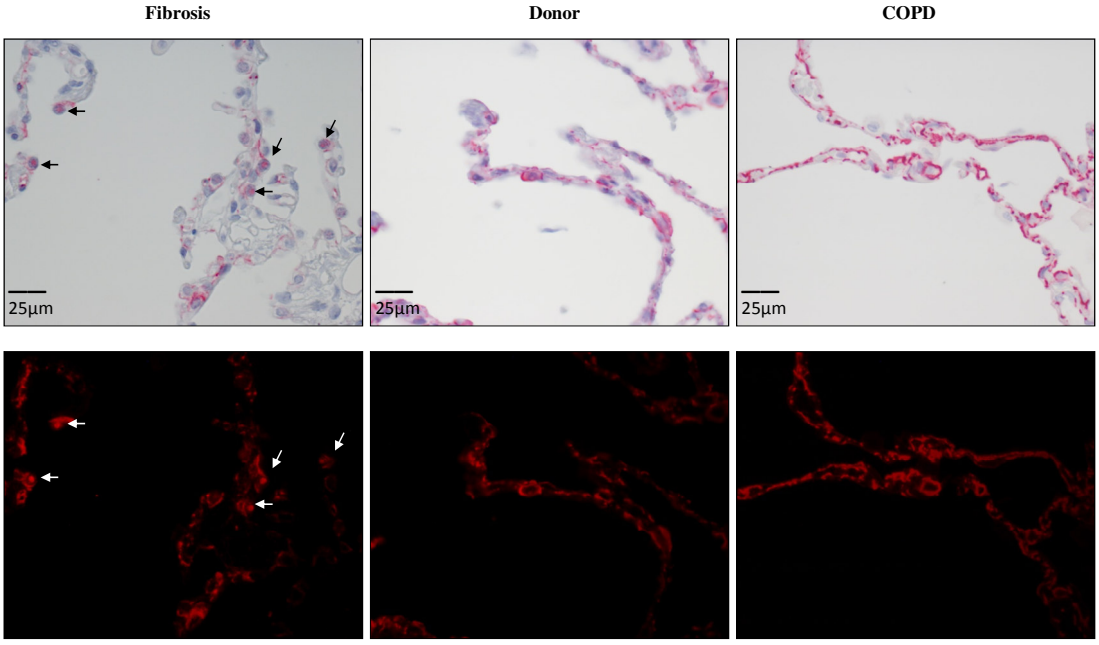


Figure 6. Expression and localization of β-catenin in lung tissues of COPD, fibrosis and transplant donor patients. Immunofluorescence staining of β-catenin was performed on tissue sections derived from transplant donor, IPF, or COPD lungs, respectively. Counterstaining was performed with hematoxylin to visualize cell nuclei. Nuclear β-catenin positive cells are indicated by arrows. Representative pictures of two independent experiments using three different transplant donor or COPD and two different IPF lung tissues, respectively. Magnification is indicated in the pictures.

Importantly, quantification of ATII cells exhibiting nuclear localization of β-catenin demonstrated decreased numbers of positive cells in COPD lung tissue compared with transplant donor lung tissue (donor: 19.0%±4.0 vs. COPD: 4.7%±1.5; $p<0.019$) (Figure 7).

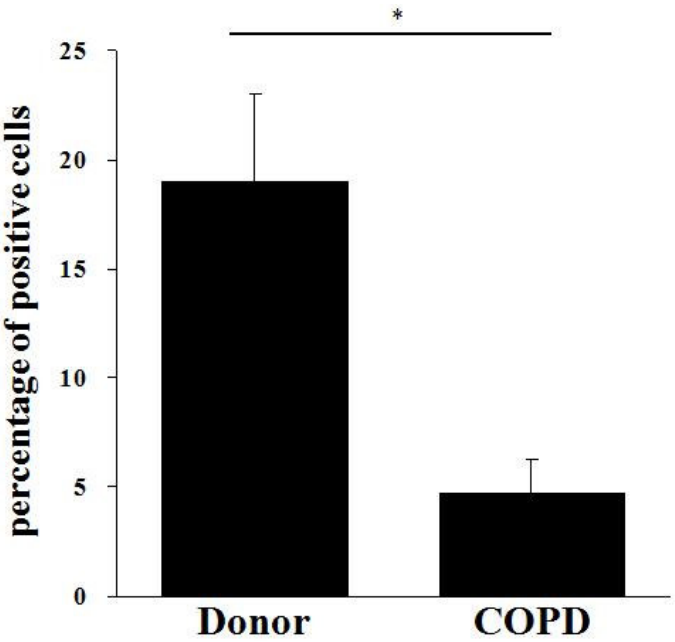


Figure 7. Numbers of β-catenin positive cells in transplant donor und COPD lung tissue. Quantification of β-catenin-positive cells was performed using four tissue sections derived from transplant donor and COPD lungs, as described in the materials and methods of this manuscript. Data are presented as mean ±s.e.m., * $p<0.05$.

In summary, WNT/β-catenin signaling components were largely expressed in alveolar epithelium in human COPD lungs. In COPD, quantification of nuclear β-catenin staining revealed reduced WNT/β-catenin signaling in COPD compared with transplant donors.

The results obtained thus far were obtained from human lung tissue derived from end-stage COPD patients. To gain a more comprehensive view about the role of WNT/β-catenin signaling and modulation thereof in earlier stages of emphysema, we preceded our studies using the mouse model of elastase-induced emphysema. Mice were subjected to a single orotracheal application of elastase and developed emphysema over a two-week time course (Figure 8).

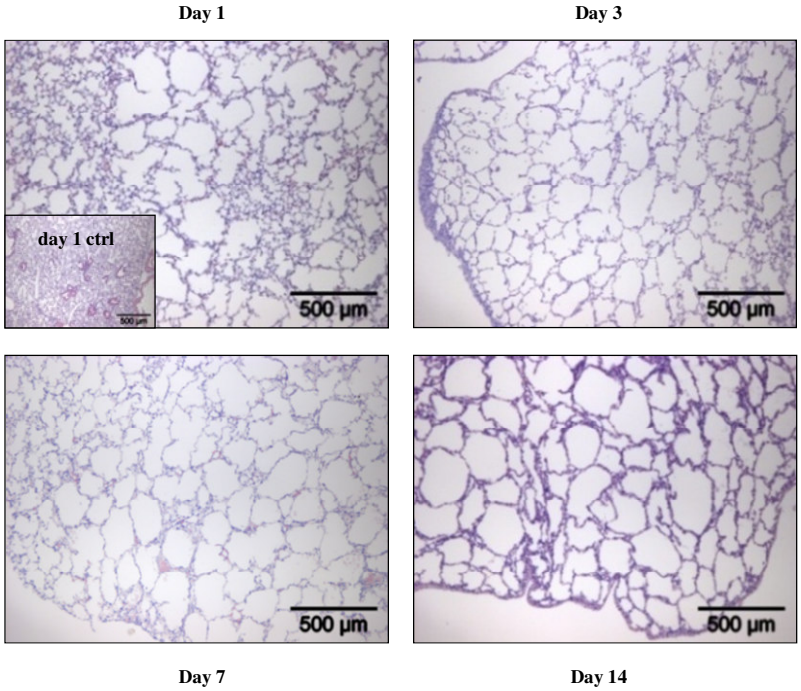


Figure 8. Histological assessment of lung structure in experimental emphysema. Mice were subjected to elastase instillation, and lungs were obtained on day 1, 3, 7, and 14 after challenge for immunohistochemistry. Stainings are representative of two independent experiments using at least three different elastase- or control-treated lung tissues for each time point.

As depicted in Figure 9A-C, we analyzed in detail the mRNA expression profile of the main WNT/β-catenin signaling components during emphysema development in elastase-treated mice compared with their respective controls. All investigated WNT/β-catenin signaling components were expressed in emphysematous lungs, and, most importantly,

exhibited mainly reduced expression in elastase-treated mice compared with their respective controls. Significant downregulation was observed for *WNT2* and *WNT10b* as early as 1 day after induction (log-fold change day 1 for *WNT2*: 1.33±0.10; p<0.0001 and *WNT10b*: -1.26±0.35; p=0.0064) (Figure 9A). Further, *LRP6* and *FZD1* exhibited significantly decreased expression after emphysema induction at day 1 and day 7, respectively (log-fold change day 1 *LRP6*: -1.04±0.22; p=0.0056, day 7 *FZD1*: -1.78±0.20; p=0.0043) (Figure 9B). In addition, the transcription factor *LEF1* was significantly downregulated as early as day 1 (log-fold change -1.85±0.37; p=0.0036), while *TCF4* was significantly downregulated on day 7 (log-fold change -1.58±0.32; p=0.0172, Figure 9C).

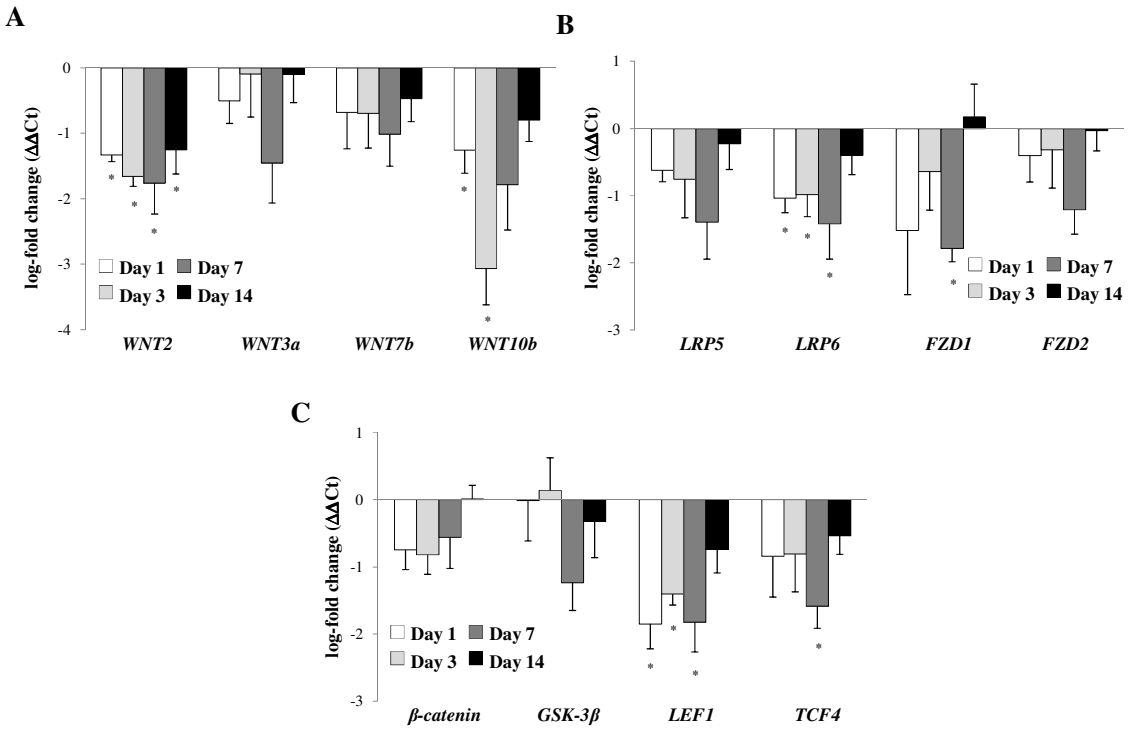


Figure 9. The mRNA expression profile of WNT/β-catenin signaling components in experimental emphysema in mice. Mice were subjected to elastase instillation, and lungs were obtained on day 1, 3, 7, and 14 after challenge for RNA isolation. The mRNA levels of (A) the WNT ligands *WNT2*, *3a*, *7b*, and *10b*, (B) the receptors *LRP5, 6, FZD1* and *2*, and (C) the intracellular signal transducers and transcription factors *GSK-3β, β-catenin, LEF1,* and *TCF4* were assessed in mice subjected to elastase after the indicated time points (n=4 each). At each time point, mice subjected to elastase were compared with 4 control littermates. Results are presented as log-fold change (mean ±s.e.m.), * p<0.05.

Importantly, reduced expression of WNT/β-catenin signaling components remained for up to 28 days after the initial challenge, accompanied by constant airspace enlargement and altered lung function (Figure 10A-C).

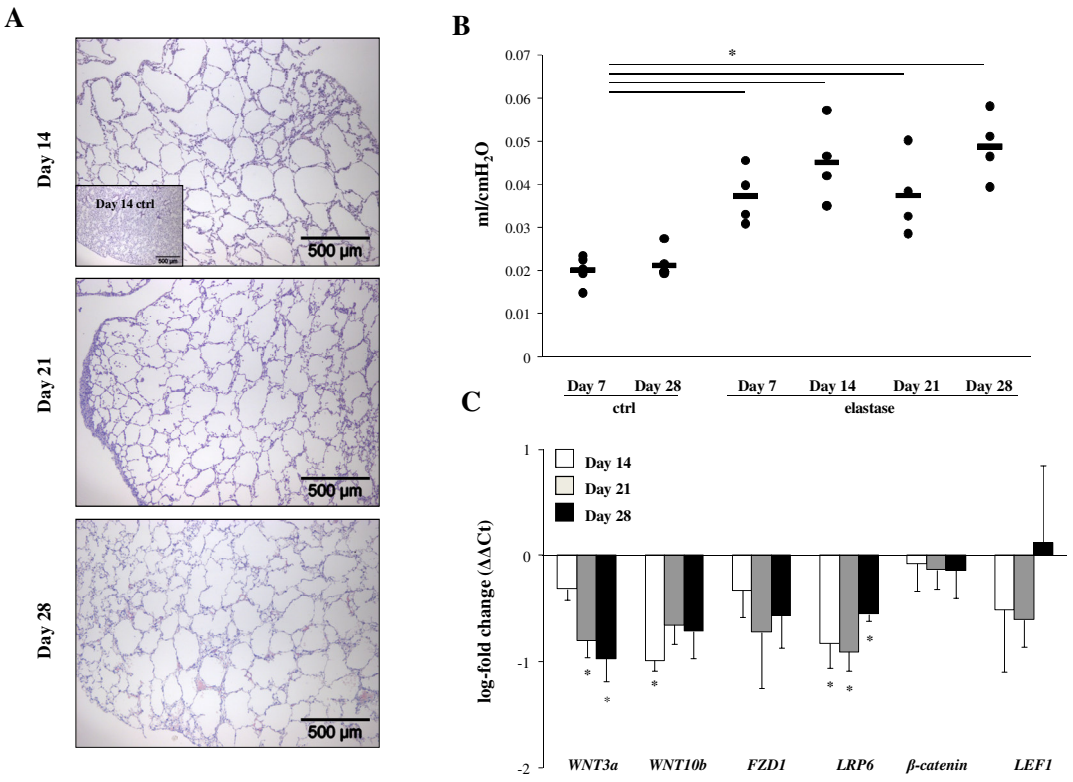


Figure 10. The late course mRNA expression profile of WNT/β-catenin signaling components and lung function in experimental emphysema. Mice were subjected to elastase instillation, and lungs were obtained on day 14, 21, or 28 days after challenge for immunohistochemistry and RNA isolation. Stainings (A) are representative of two independent experiments using at least three different elastase- or control-treated lung tissues for each time point. The dynamic compliance (B) was assessed by lung function analysis at different time points after elastase administration and compared with untreated mice on day 7 and day 28, respectively. The mRNA levels (C) of the WNT ligands *WNT3a* and *10b*, the receptors *FZD1* and *LRP6* and the intracellular signal transducers and transcription factors *β-catenin* and *LEF1* and *TCF4* were assessed in mice subjected to elastase after the indicated time points (n = 4 each). At each time point, mice subjected to elastase were compared with 4 control littermates. Results are presented as log-fold change (mean ±s.e.m.), * $p<0.05$.

Next, TOPGAL reporter mice were used to elucidate WNT/β-catenin signal activity during the development of experimental lung emphysema *in vivo.* Mice subjected to elastase

did not exhibit activation of WNT/β-catenin signaling during emphysema development, as indicated by the absence of β-galactosidase (β-Gal) staining (Figure 11).

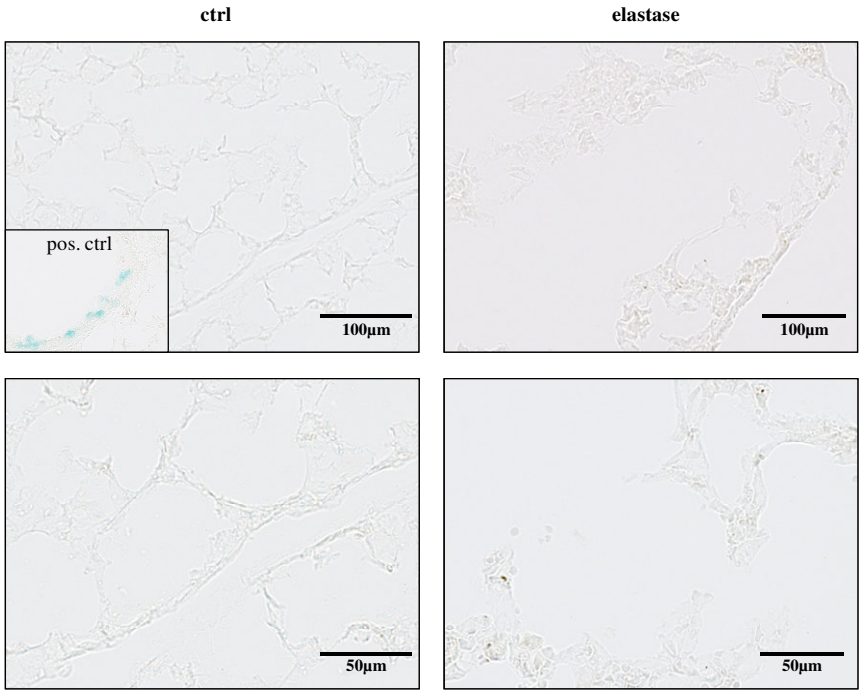


Figure 11. Activity of WNT/β-catenin signaling in experimental emphysema. TOPGAL reporter mice were treated orotracheally with elastase or vehicle orotracheally (n =3 each), as described in detail in methods. The absence of β-gal staining indicates no activation of WNT/β-catenin in the lungs in elatsase- or vehicle-treated mice. The positive control represents mice treated orotracheally with WNT3a to activated WNT/β-catenin signaling. Pictures are representative of at least 2 independent experiments, using at least three different lung tissues for each condition. Magnification is indicated in the pictures.

WNT/β-catenin activation as a therapeutic approach in experimental emphysema

Next, we assessed whether WNT/β-catenin activation represented an effective therapeutic option in emphysema. We used lithium chloride (LiCl), which mimics WNT signaling by inhibiting the activity of GSK-3β, causing intracellular accumulation of β-catenin and WNT target gene expression (109). To demonstrate that LiCl indeed leads to WNT/β-catenin activation in the lung, we treated TOPGAL reporter mice or C57BL/6N wild type

mice, both subjected to elastase or vehicle, with LiCl. We started LiCl treatment on the day of emphysema induction followed by daily application for 7 days (referred to as "preventive approach", detailed treatment scheme in Figure 12).

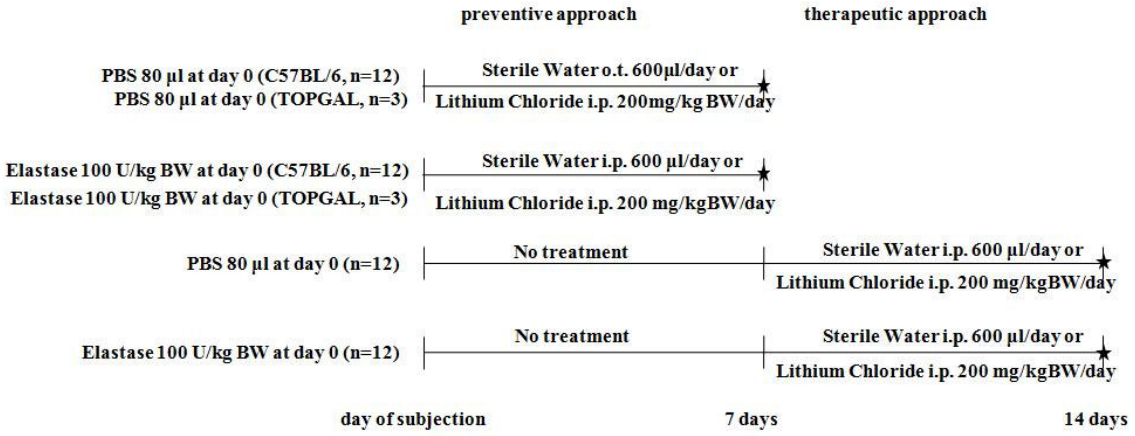


Figure 12. Treatment regimen for preventive and therapeutic activation of WNT/β-catenin signaling in experimental emphysema. Mice were subjected to a single orotracheal application of elastase, which led to the development of pulmonary emphysema from day 1 to 14. WNT/β-catenin activation by lithium chloride (LiCl) was initiated on the day of elastase subjection to day 7 (referred to as *preventive approach*), or from day 7 to day 14 (referred to as *therapeutic approach*), using the indicated concentrations (daily application intraperitonal (i.p.), C57BL/6: n=12, TOPGAL: n = 3 for each group)

Importantly, bronchial and alveolar epithelial cells regularly stained for β-Gal in response to LiCl (Figure 13), which is in accordance with β-Gal staining after WNT3a treatment in the lung (94). Interestingly, enhanced β-Gal staining in the alveolar epithelium was observed in the elastase-challenged mice, while in control mice, bronchial staining was predominant (Figure 13).

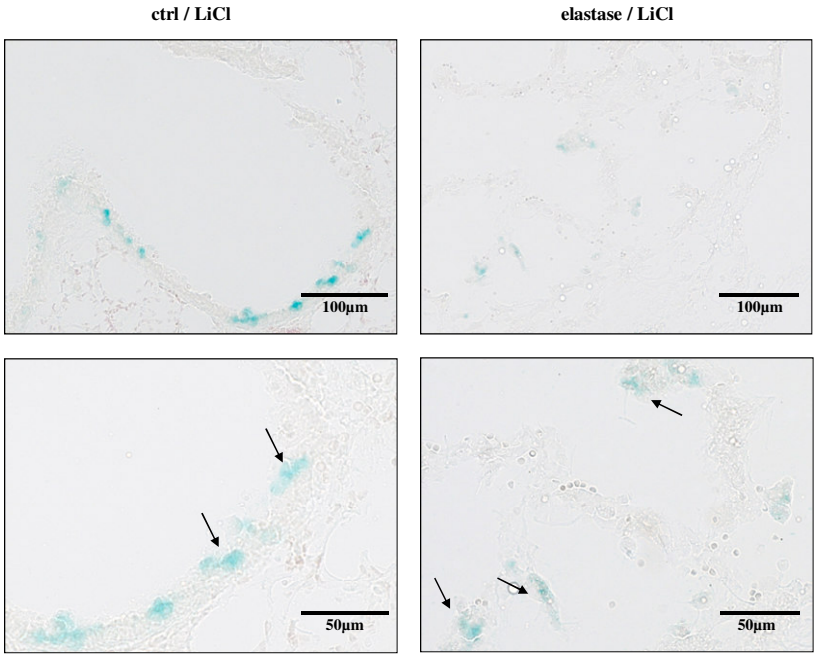


Figure 13. Activation of WNT/β-catenin signaling in experimental emphysema I. TOPGAL reporter mice were treated orotracheally with elastase followed either by LiCl or vehicle intraperitoneally (n =3 each), as described in detail in methods. The β-gal staining indicates WNT/β-catenin activity in the lungs in LiCl- or vehicle-treated mice (arrows). Pictures are representative of at least 2 independent experiments, using at least three different lung tissues for each condition. Magnification is indicated in the pictures.

Moreover, we observed increased expression of several WNT target genes and WNT components in LiCl-treated emphysematous lungs compared with vehicle-treated emphysematous lungs. As shown in Figure 14A and 14B the mRNA levels of the WNT target genes fibroblast growth factor receptor (FGFR) 2 (elastase/vehicle *vs.* elastase/LiCl: 2.44±0.25 *vs*. 3.31±0.075; p=0.029), Axin1 (-0.15±0.09 *vs.* 0.34±0.05; p=0.006) and Axin2 (-0.27±0.17 *vs.* 0.39±0.06; p=0,031) were increased in lung homogenates of emphysematous mice treated with LiCl compared with control mice, while bone morphogenetic protein (BMP) 4 did not reach statistical significance (1.22±0.20 *vs*. 1.49±0.10) (Figure 14A). The WNT components β-catenin, GSK-3β, LEF1 and TCF4 were upregulated in lung homogenates of emphysematous mice treated with LiCl compared with control mice with statistical

significance for LEF1 (-2.88±0.14 *vs.* -2.17±0.09; p=0.007) and TCF4 (0.18±0.13 *vs.* 0.84±0.11; p=0.019) (Figure 14B).

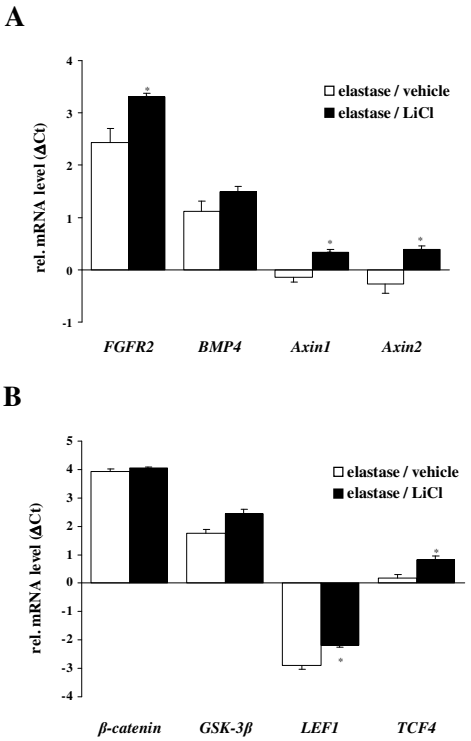
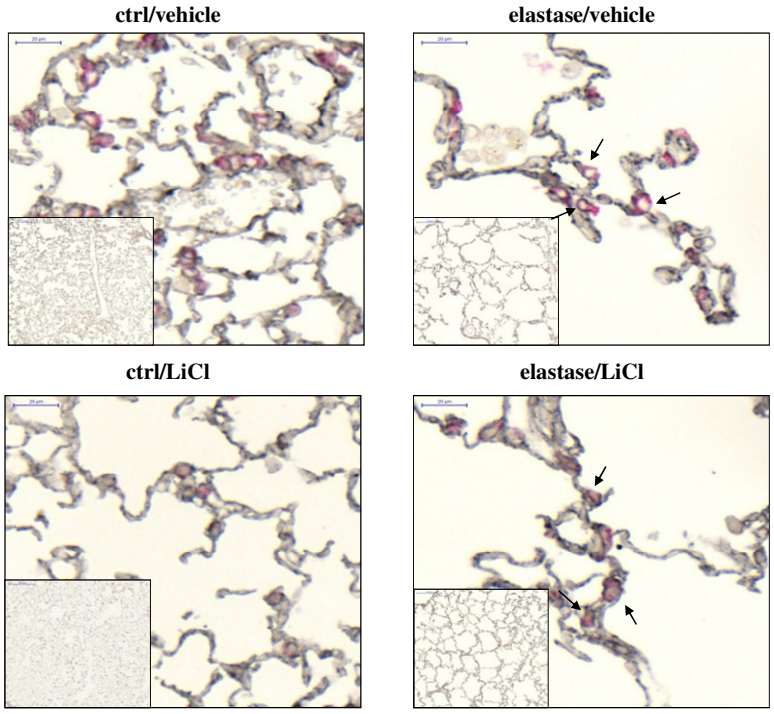


Figure 14. Activation of WNT/β-catenin signaling in experimental emphysema II. Mice were treated orotracheally with elastase followed either by LiCl or vehicle intraperitoneally (n =12 each), as described in detail in methods. (A) The mRNA levels of the WNT target genes fibroblast growth factor receptor (*FGFR*) 2, bone morphogenetic protein (*BMP*) 4, *Axin1* and *Axin2,* and (B) the WNT components *β-catenin, GSK-3β, LEF1,* and *TCF4* were assessed in lung homogenates of emphysematous mice treated with LiCl compared with control mice (n=6). Results are presented as mean ±s.e.m.; **p*< 0.05.

To further corroborate active WNT/β-catenin signaling upon LiCl treatment, we assessed nuclear β-catenin staining in ATII cells using double immunohistochemstry of SP-C and β-catenin (Figure 15A), as well as immunofluorescence staining of β-catenin (Figure 15B) in LiCl-or vehicle-treated lungs elastase-challenged or control lungs. Immunohistochemical analysis demonstrated increased staining of SP-C/β-catenin-positive cells, as well as nuclear β-catenin staining, compared with vehicle-treated mice (Figure 15A, 15B, lower panels, arrows).

A


ctrl/vehicle
elastase/vehicle
ctrl/LiCl
elastase/LiCl

B

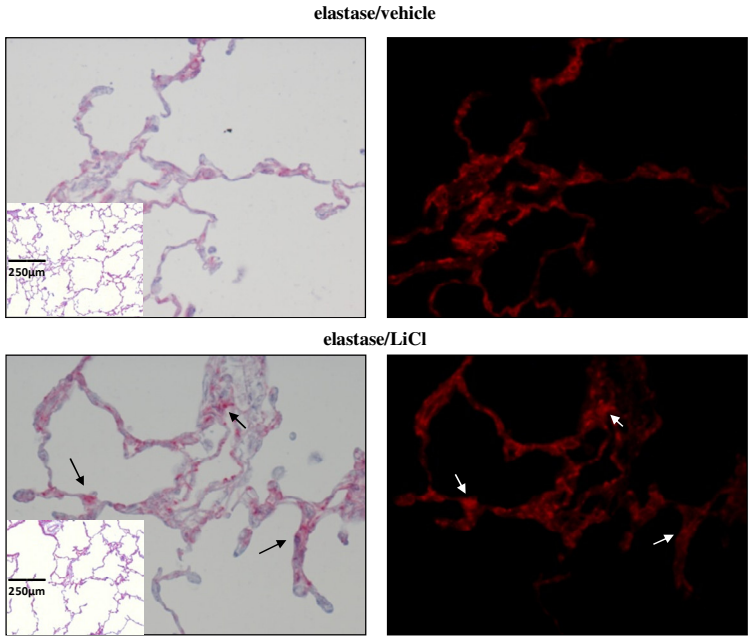

elastase/vehicle
250µm
elastase/LiCl
250µm

Figure 15. Expression and localization of β-catenin in experimental emphysema. (A) Immunohistochemical staining was performed on tissue sections derived from emphysematous or control mouse lungs treated with LiCl or vehicle, as indicated, and were used to demonstrate co-localization and nuclear β-catenin in alveolar epithelial type II (ATII) cells (gray/dark blue staining in the nucleus). Nuclear β-catenin-negative ATII cells (right upper panel) and nuclear β-catenin-positive cells (right lower panel) are indicated by arrows. (B) Furthermore, single immunofluorescence staining of β-catenin was performed on tissue sections derived from emphysematous mouse lungs treated with LiCl or vehicle, as indicated. Counterstaining was performed with hematoxylin to visualize cell nuclei. Nuclear β-catenin positive cells (lower panels) are indicated by arrows. Representative pictures of two independent experiments using at least three different mouse lung tissues, respectively. Magnification is indicated in the pictures. Low magnification pictures are provided in inlays in the respective pictures.

In order to elucidate the effects of WNT/β-catenin activation by LiCl on emphysema development, we analyzed key features of emphysema, such as destroyed lung architecture and surface, decreased ATII cells, or enhanced collagen deposition. Notably, histological assessment of the lung structure revealed marked attenuated emphysematous changes after preventive WNT/β-catenin activation in experimental lung emphysema (Figure 16A). Quantitative morphometric analysis of airspace enlargement confirmed beneficial effects of WNT/β-catenin activation by depicting improvement of elastase-induced changes. In LiCl-treated mice, decreased mean chord length (109.9±7.8 μm *vs.* 74.0±6.6 μm; p=0.0021, Figure 16B) and increased surface area (560.7±55.7 cm^2 *vs.* 317.8±27.5 cm^2; p=0.0067, Figure 16C) were observed compared with vehicle-treated mice.

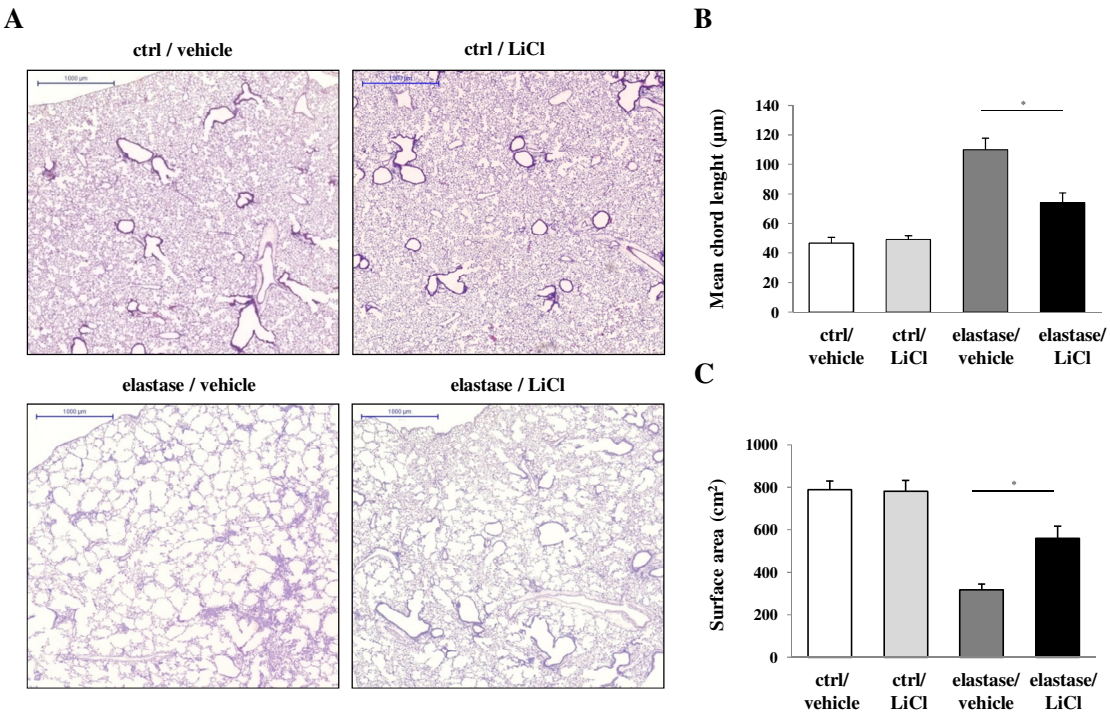


Figure 16. Assessment of lung structure after preventive WNT/β-catenin activation in experimental lung emphysema. Mice were subjected to elastase instillation, and treated with LiCl or vehicle, as described in Figure 12. (A) After seven days, lungs were processed for hematoxylin and eosin staining of lung sections (magnification as indicated). Stainings are representative of two independent experiments using three different elastase- or control-treated lung tissues. (B) The mean chord length and (C) surface area were assessed by morphometric analysis, as described in detail in the methods of this manuscript.

In addition, we analyzed clinically relevant variables, such as lung function parameters. Importantly, the dynamic compliance improved in emphysematous LiCl-treated mice compared with emphysematous vehicle-treated mice (0.0358±0.004 ml/cmH_2O *vs.* 0.0287±0.003 ml/cmH_2O; p=0.0374, Figure 17A), while no significant alterations were observed in lung resistance (1.463±0.365 cmH2O.s/L vs. 1.438±0.195 cmH2O.s/L; Figure 17B).

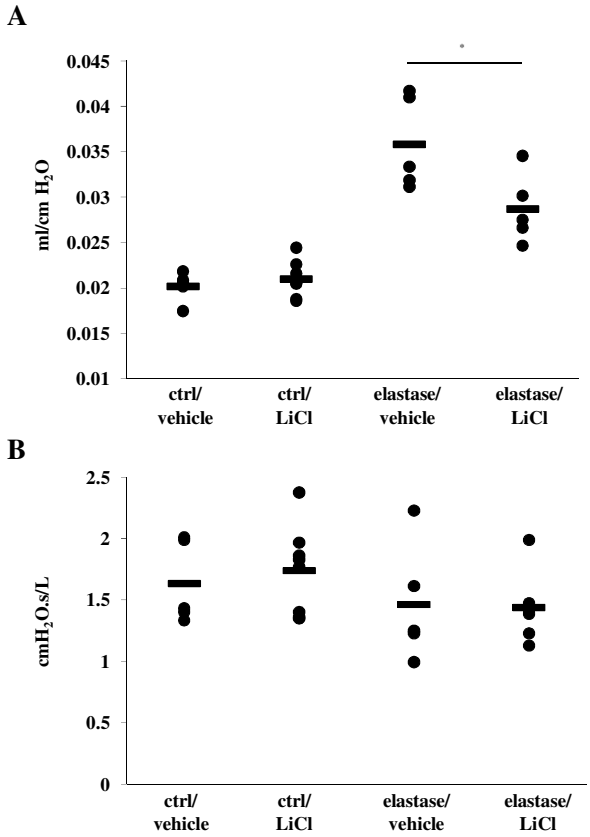


Figure 17. Assessment of lung function after preventive WNT/β-catenin activation in experimental lung emphysema. Mice were subjected to elastase instillation, and treated with LiCl or vehicle, as described in Figure 12. After seven days, dynamic compliance (A) and resistance (B) were assessed by lung function analysis, as described in detail in the methods of this manuscript.

Next, we went on to analyze alterations in collagen and alveolar epithelial marker expression. The mRNA level of type I collagen α1 was significantly reduced in LiCl-treated animals (log-fold change -0.86±0.33; p=0.0496, Figure 18A), which was further substantiated by decreased total collagen content in lung homogenates in emphysematous mice treated with LiCl compared with vehicle-treated mice (29.6µg/m±4.1 *vs.* 16.1µg/ml±3.9, p=0.042, Figure 18B). To further delineate the functional impact of WNT/β-catenin activation on alveolar structure, we assessed the mRNA levels of several alveolar epithelial cell markers (Figure 18A). The expression *SP-C* was increased in LiCl-treated mice (log-fold change -1.21±0.42; p=0.0475). Furthermore, elevated mRNA level of cadherin 1 (*cdh1*), thyroid transcription factor 1 (*ttf1*), forkhead box P2 (*foxp2*), and aquaporin 5 (*aqp5*) were observed after WNT/β-

catenin activation in experimental emphysema (log-fold change *cdh1* 0.97±0.20, p=0.003; *ttf1* 1.20±0.30, p=0.033; *foxp2* 1.38±0.59, p=0.146; and *aqp5* 0.63±0.18, p=0.025, Figure 18A).

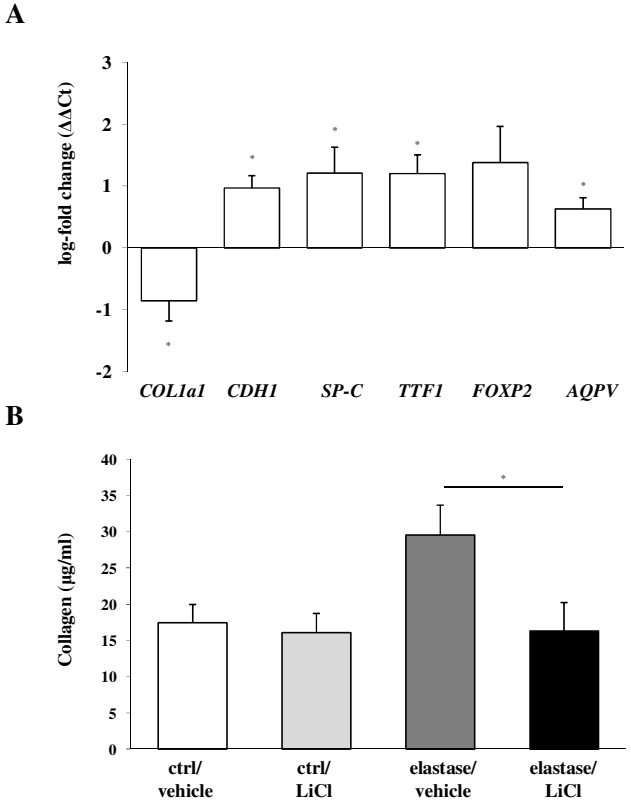


Figure 18. Quantitative analysis of lung structure after preventive WNT/β-catenin activation in experimental emphysema I. (A) The mRNA levels of the ECM component type I collagen α1 (*col1a1*), the epithelial cell marker cadherin 1 (*cdh1*), surfactant protein-C (*sp-c*), thyroid transcription factor 1 (*ttf1*), forkhead box P2 (*foxp2*), and aquaporin 5 (*aqp5*) were assessed by qRT-PCR. (B) The collagen content was measured in total lung homogenates using the Sircol Collagen Assay kit (Biocolor). Results are presented as log-fold change of mRNA levels in LiCl-treated versus untreated lungs or relative expression levels (mean ±s.e.m.), * $p<0.05$.

Alterations of the mRNA levels of SP-C and AQP5 were confirmed on the protein level, as depicted by increased protein expression LiCl-treated mice compared with vehicle-treated mice, suggesting an improved alveolar structure due to WNT/β-catenin activation in experimental emphysema (Figure 19A). To assess a possible effect of WNT/β-catenin activation on inflammation, we analyzed mRNA levels of the pro-inflammatory genes interleukin β (*IL1β*), tumor necrosis factor-α (*TNFα*), and macrophage inflammatory protein

(*MIP*) 2. For all investigated transcripts, no significant alterations were observed (Figure 19B).

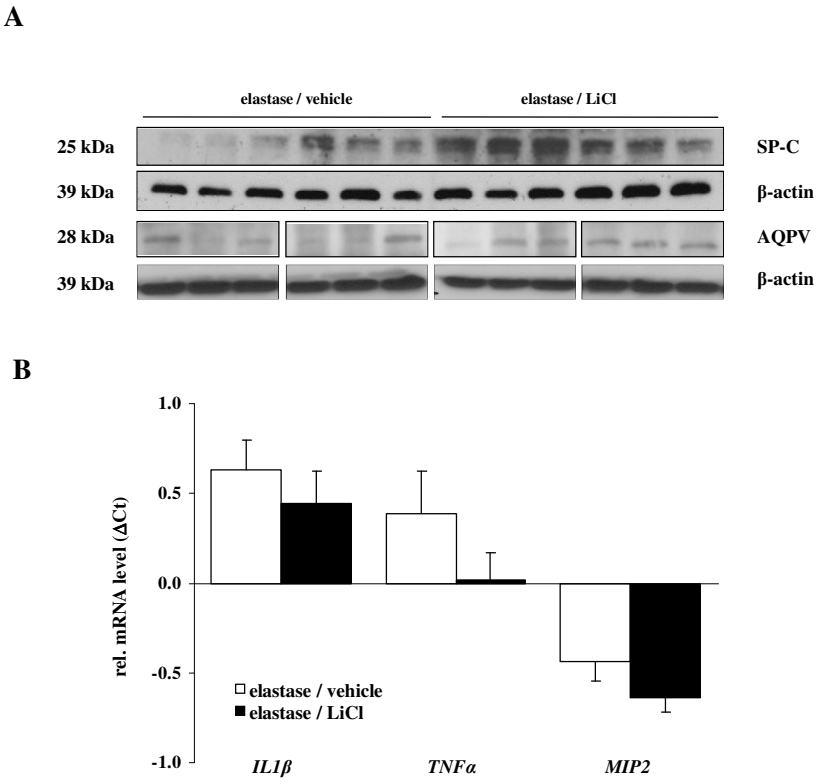


Figure 19. Quantitative analysis of lung structure after preventive WNT/β-catenin activation in experimental emphysema II. (A) The expression of SP-C and Aquaporin 5 (AQP5) in lung homogenates of emphysematous mice treated with LiCl compared with control mice, was analyzed by immunoblotting (n=6 each). (B) The mRNA levels of pro-inflammatory genes interleukin β (*IL1β*), tumor necrosis factor-α (*TNFα*), and macrophage inflammatory protein 2 (*MIP*) 2 were assessed by qRT-PCR. Results are presented as log-fold change of mRNA levels in LiCl-treated versus untreated lungs or relative expression levels (mean ±s.e.m.), * $p<0.05$.

In summary, preventive WNT/β-catenin activation led to an attenuation of experimental emphysema, as measured by decreased airspace enlargement, collagen content, improved lung function, and restored alveolar structure upon WNT/β-catenin activation.

Finally, we wanted to evaluate, whether WNT/β-catenin activation exhibit beneficial effects on established emphysema. Therefore, we initiated LiCl treatment on day 7 after emphysema induction followed by daily application until day 14 (referred to as "therapeutic

approach", detailed treatment scheme in Figure 12). Histological assessment of the lung structure revealed a marked attenuation of emphysema along with restored lung architecture (Figure 20A) and significantly decreased airspace enlargement after therapeutic WNT/β-catenin activation (mean chord length: 106.4±2.3 *vs.* 85.3±2.5; p=0.021, Figure 20B). Furthermore, WNT/β-catenin activation by LiCl was demonstrated by increased mRNA levels of the WNT target genes Axin1 and Axin2 (elastase/vehicle *vs.* elastase/LiCl, Axin1: 0.18±0.05 *vs.* 0.78±0.14; p=0.006), (Axin2: 0.09±0.11 *vs.* 0.91±0.17; p=0.003) in lung homogenates of emphysematous mice upon therapeutic LiCl treatment (Figure 20C).

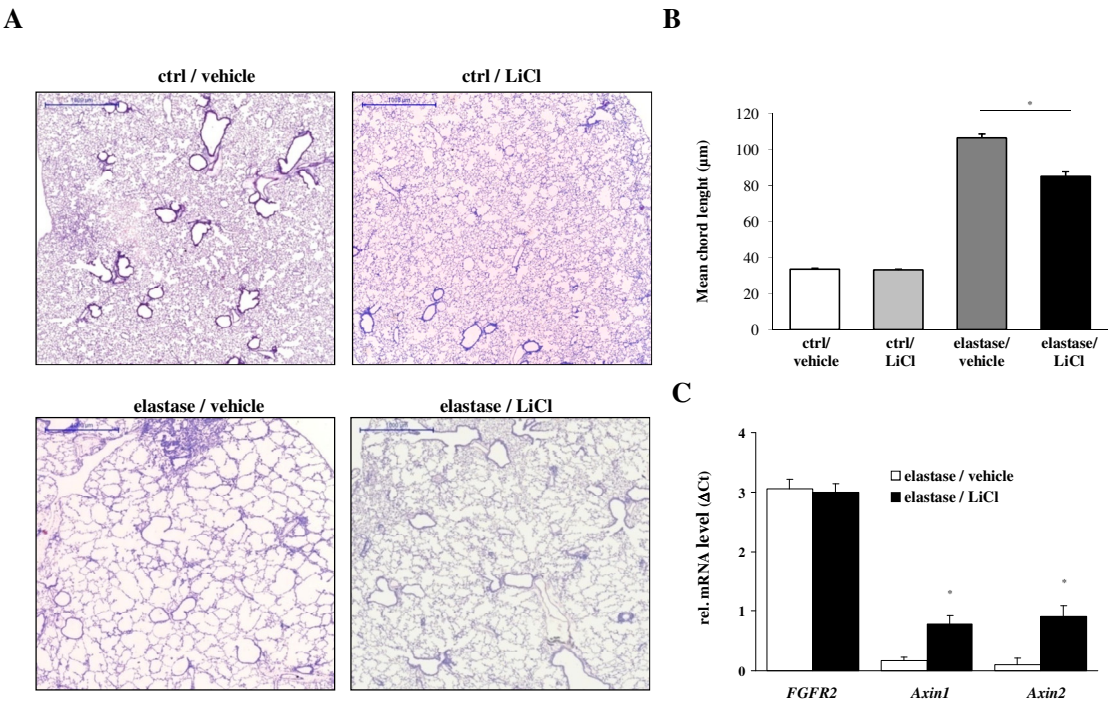


Figure 20. Assessment of lung structure after therapeutic WNT/β-catenin activation in experimental emphysema. Mice were subjected to elastase instillation and treated with LiCl or vehicle, as described in Figure 12. After 14 days, lungs were processed for hematoxylin and eosin staining of lung sections (magnification as indicated). Stainings are representative of two independent experiments using three different elastase- or control-treated lung tissues. (B) The mean chord length was assessed by morphometric analysis as described in detail in the methods of this manuscript. (C) The mRNA levels of the WNT target genes fibroblast growth factor receptor (*FGFR*) 2, *Axin1,* and *Axin2* and were assessed in lung homogenates of emphysematous mice treated with LiCl compared with control mice (n=6). Results are presented as mean ±s.e.m; **p*<0.05.

Moreover, analysis of lung function parameters revealed improved dynamic compliance in emphysematous LiCl-treated mice compared with emphysematous vehicle-treated mice (0.0381±0.003 ml/cmH_2O *vs*. 0.032±0.003 ml/cmH_2O; p=0.024, Figure 21A). The resistance remained unchanged (1.700±0.230 cmH2O.s/L *vs*. 2.337±0.568 cmH2O.s/L; Figure 21B).

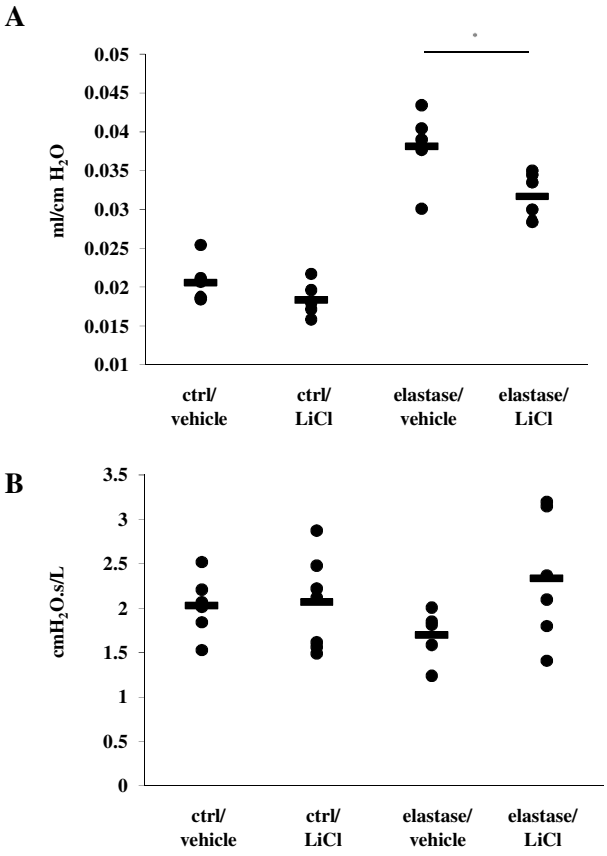


Figure 21. Assessment of lung function after therapeutic WNT/β-catenin activation in experimental emphysema. Mice were subjected to elastase instillation and treated with LiCl or vehicle, as described in Figure 12. After 14 days (A) dynamic compliance and (B) resistance were assessed by lung function analysis, as described in detail in the methods of this manuscript.

DISCUSSION

COPD is a progressive and devastating disease and patients diagnosed with COPD have only limited therapeutic options (4, 114). COPD is characterized by irreversible expiratory airflow limitation due to two main intrapulmonary features: small airway disease (SAD) and emphysema. SAD is characterized by airway inflammation with increased mucus production and peribronchiolar fibrosis, while emphysema is defined as destruction of the alveolar architecture due to distal airspace enlargement (115, 116). Cigarette smoking is the most important risk factor for COPD, and believed to activate several signaling cascades, which impair cellular and molecular maintenance, finally leading to destruction of alveolar structure (8, 117). Strong evidence supports a major role of inflammatory processes and protease / antiprotease imbalance in the development of emphysema, however, recent studies have also pointed out that additional or complimentary pathway abnormalities are involved in the development and progression of alveolar destruction (100, 118). Recently, Yildirim and colleagues demonstrated that activation of keratinocyte growth factor (KGF) by palifermin is able to induce alveolar maintenance programs in emphysematous lungs. Palifermin induced expression of TGF-β1 in and release of active TGF-β1 from primary mouse alveolar epithelial type 2 cells, in vitro recombinant TGF-β1 was able to induce elastin gene expression (119). These findings suggested that this effect of palifermin is linked to alveolar epithelial type II (ATII) cell-derived growth factors, such as TGF-β1, thus highlighting a novel target for emphysema therapy.

Along these lines, developmentally active pathways have been suggested to operate during lung development such as the WNT/β-catenin pathway (120-122). Importantly, a recent study demonstrated that mouse embryos deficient for WNT2/2b expression display complete lung agenesis, further highlighting the importance of this signal pathway in lung morphogenesis (92).

Here, we took a comprehensive approach investigating the quantitative assessment of canonical WNT/β-catenin signaling components at the mRNA and protein level, localization, as well as activity of WNT/β-catenin signaling in emphysematous COPD specimen. We demonstrated, for the first time, that active WNT/β-catenin signaling is decreased in emphysema.

Recently, the WNT/β-catenin pathway has been linked with parenchymal lung diseases, such as pulmonary fibrosis (96). Canonical WNT/β-catenin activation has been demonstrated to be activated in idiopathic pulmonary fibrosis (IPF), and, in particular, to be

involved in epithelial cell repair mechanism *in vitro* and *in vivo* (94-96, 98). The finding that WNT/β-catenin signaling is decreased in emphysema further substantiates a critical role of WNT/β-catenin signaling in alveolar epithelial cell homeostasis, and reveals a reasonable mechanism for alveolar tissue destruction in emphysema.

The investigated human COPD tissues were obtained from patients undergoing lung transplantation, representing end-stage diseased lungs (GOLD IV). At this stage, efforts of the lung to repair or reverse may be diminished or even extinct. Early intervention therapy is unquestioned and patients suffering from COPD would benefit from immediate therapy. However, some patients have only few complaints in early stages of disease, and symptoms, like dyspnea on exertion, cough and sputum production are often neglected or attributed to lack of exercise and smoking habits (123), therefore COPD is usually diagnosed in GOLD stage III-IV (124). Undoubtedly, it will be important to investigate the role of WNT/β-catenin signaling in earlier stages of COPD or even smokers in future studies.

To further evaluate the impact of WNT/β-catenin signaling during development of emphysema, and to be able to therapeutically intervene and analyze structural and functional differences in a reasonable time frame, we therefore focused our studies on the elastase-induced emphysema model in mice (125). In our experimental emphysema model, reduced expression of the WNT/β-catenin signaling pathway was observed, which was further confirmed using TOPGAL reporter mice that displayed no WNT/β-catenin activity upon elastase treatment.

One of the most frequently investigated hypothesis and supported by strong evidence is the protease/antiprotease imbalance hypothesis (40, 41). The hypothesis states that an excess of neutrophil elastase and matrix metalloproteases results in a destructive process of the collagen and elastin fibers in the lung parenchyma. Therefore, we decided to proceed with the elastase-induced emphysema in mice. In this model the administration of an elastolytic enzyme leads to a progression of airspace enlargement and loss of alveolar architecture over the course of one week after administration, thus enabling easy and economic investigations and interventions opposed to long-lasting models like the cigarette-smoke-induced model of COPD. However, the early and acute onset of this model is criticized because it is in contrast to the slow and chronic process seen in human COPD. Furthermore, it is believed that elastase-induced emphysema does not model the multifactorial process seen with cigarette-smoke exposure (125). On the other hand, the cigarette-smoke-induced model of emphysema is very time and money consuming. It usually takes up to eight months and the emphysematous changes produced in this model can be subtle. A limitation of elastase- and

smoke-induced emphysema is that both models lack small airway remodeling (126). It has been described that small airway walls of guinea pigs after long-term cigarette-smoke exposure are thickened (127) and might be therefore used as a model for small airway disease.

Importantly, our results are in accordance with a recent publication by Blacquiere *et al.,* reporting that maternal smoking during pregnancy decreases WNT signaling in mice. Although the elastase model does not reflect all of the events seen with smoke (125), these results suggest that reduced activity of WNT/β-catenin signaling is a common and relevant feature of emphysema development. It has to be stated, however, that a recent publication by Liu et al. reported activation of WNT/β-catenin upon cigarette smoke condensate exposure on lung epithelial cell lines *in vitro* (128). Future studies using different animal models, such as cigarette-smoke exposure, in combination with relevant *in vitro* studies, will extend our insight on WNT/β-catenin signaling in emphysema.

Following our initial observations, we aimed to assess whether WNT/β-catenin activation represented an effective therapeutic option in emphysema. To this end, we used lithium chloride (LiCl), which is a well-known activator of WNT/β-catenin signaling by inhibiting glycogen synthase kinase 3β (GSK-3β) (109). In addition to LiCl, several small molecule inhibitors, such as SB216763 or SB415286, are available and may be taken into account for future studies. LiCl may influence other signaling pathways besides WNT/β-catenin (112, 129). To assure this, we demonstrated that LiCl treatment led to 1) enhanced WNT/β-catenin signal activity in lung epithelial cells in TOPGAL reporter mice, 2) increased nuclear staining of β-catenin in ATII cells, and 3) induced WNT target gene expression in the lung. As such, we provide several lines of evidence that the main effects of LiCl reported in this study are mediated by active WNT/β-catenin signaling.

We decided to pursue two different approaches, preventive as well as therapeutic WNT/β-catenin activation in our emphysema model. Most importantly, WNT/β-catenin activation resulted in a marked attenuation of airspace enlargement and improved lung morphology in both treatment regimen. Moreover, improvement of clinically relevant parameters, such as dynamic lung compliance, further emphasized that WNT/β-catenin activation may be a suitable future therapeutic option for emphysema.

WNT/β-catenin activation led to a reduction of collagen on the protein as well as mRNA level. Higher collagen content in the elastase-induced emphysema model was initially found in hamsters and has been shown to correlate with the degree of parenchyma destruction (130, 131). Increased collagen level after elastase administration have been suggested to present inefficient tissue repair attempts, implicating that decreased collagen level after

WNT/β-catenin activation may be reflecting an improved tissue maintenance. Moreover, WNT/β-catenin activation led to a marked increase in several ATII and I cell marker, such as SP-C and TTF1 (ATII), as well as AQP5 (ATI), suggesting an increased maintenance of the alveolar structure due to WNT/β-catenin activation. Surfactant proteins play an important role in the maintenance of alveolar structure, thus impairment in surfactant metabolism in ATII cells may lead to enlargement and destruction of the alveolar space (100, 132). Increased expression of AQP5, which is a water channel that resides in the ATI cell apical plasma membrane (133), further underlines that alveolar structure is restored after WNT/β-catenin activation in experimental emphysema.

With respect to inflammatory processes involved in emphysema development, no significant impact of WNT/β-catenin activation on the inflammatory process has been observed in our model. The expression analysis, however, was performed in lung homogenate samples, which implies that the expression profiles are subject to the cellular composition of the samples used. Furthermore, it has been shown that active WNT/β-catenin signaling occurs in the fibroproliferative phase after acute lung inflammation in a mouse model of oxidant-induced injury (134), supporting an involvement of WNT/β-catenin signaling in the resolution and regeneration phase after lung injury.

Matrix degradation is a prominent feature in emphysema, which leads to destruction of the normal alveolar structure and damaged alveolar epithelial cells (117, 135, 136). We propose that the inability of activating WNT/β-catenin signaling as a survival signal in the alveolar epithelium in emphysema may represent a crucial mechanism, initiating or potentiating the loss of alveolar epithelial cells in emphysema. Therefore, WNT/β-catenin activation may be a future therapeutic tool to drive alveolar epithelial cell repair processes. In conditions different from emphysema.

Our results suggest that active WNT/β-catenin signaling affects extracellular matrix turnover and alveolar epithelial cell survival. Previous reports demonstrated that WNT/β-catenin activation, by recombinant WNT proteins as well as LiCl, led to enhanced epithelial cell proliferation and survival (94, 98, 137-139). Moreover, WNT/β-catenin activation also impacts fibroblast behavior and extracellular matrix production (98, 140, 141). Along these lines, it is most apparent that the detailed mechanisms, which underline the beneficial effects of WNT/β-catenin activation in emphysema involves several cell types. In this respect, different stem cell populations have been described with respect to lung repair and regeneration, such as bronchioalveolar stem cells (BASC) or bone-marrow-derived stem cells (142-145). An involvement of WNT/β-catenin signaling on BASC regulation has been

reported. Moreover, it has been demonstrated that WNT/β-catenin is critical for normal hematopoietic stem cell (HSC) maintenance (146), and may be an important regulator of organ regeneration (147). Future studies are therefore clearly needed to elucidate whether stem cells are involved in the beneficial effects of WNT/β-catenin activation in emphysema.

Finally, the role of WNT signaling in lung cancer needs to be assessed carefully, in particular for future therapeutic developments. COPD patients exhibit a 4.5 fold increased risk for the development of lung cancer (148). Several WNT proteins have been found to be differentially expressed in non-small cell lung carcinoma specimen, driving epithelial cell proliferation and apparently taken part in the multi-step oncogenic process (149). In patients at high risk to develop lung cancer, activation of WNT/β-catenin signaling may have devastating effects. Cigarette smoke is the main risk factor for both, COPD and lung cancer (150). In this respect, it has to be further explored how the same trigger can have two opposing effects on the alveolar epithelium. On the one hand, increased activity of WNT/β-catenin signaling is linked to lung cancer and on the other hand, as reported here, silenced WNT/β-catenin signaling is associated with emphysema. Understanding the diverse responses of the alveolar epithelium to cigarette-smoke in respect to WNT/β-catenin signaling activity will be necessary to use WNT/β-catenin signaling as therapeutic target for both.

Taken together, this study demonstrates decreased active WNT/β-catenin signaling in COPD specimen and in experimental emphysema. Preventive, as well as therapeutic WNT/β-catenin activation led to a significant reduction of experimental emphysema with restored alveolar epithelial structure and function.

To further investigate the role of WNT/β-catenin signaling in the pathogenesis of COPD further experimental evidence would be of great interest but were beyond the scope of this study. Some of this experiments could be as following: First, to study the expression and activity of WNT/β-catenin signaling components in lungs from COPD patients a) with different GOLD stages, b) with and without smoking history, and c) in subpopulations of COPD, like in patients with alpha-1 antitrypsin deficiency or patients with combined diseases (fibrosis and emphysema or lung cancer and emphysema). Second, to investigate the expression profile of WNT/β-catenin signaling components in different lung compartments, such as small airways (site of small airway disease), parenchyma (site of emphysematous changes), or vessels (site of vascular abnormalities), to overcome the limitation of using total lung homogenates. Thirdly, it would be interesting to test therapeutically activation of WNT/β-catenin signaling in other animal models of COPD, such as the cigarette-smoke-induced emphysema in mice. Further, genetically modified mice could be used and subjected

to elastase or cigarette-smoke. For example, gain-of-function of WNT/β-catenin signaling could be achieved by mutation of the phosphorylation sites of GSK-3β or β-catenin. Conditional gain-of-function mutations could be introduced in the ATII cells of the alveolar epithelium using a SP-C cre line.

In summary, future *in vitro* and *in vivo* studies will certainly provide more insights on the mechanistic principles underlying decreased WNT/β-catenin signaling in COPD, and whether therapeutic WNT/β-catenin activation will present as a future therapeutic tool in this devastating disease.

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DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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Place and Date Nikolaus Kneidinger

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