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Glycogen synthase kinase-3 (GSK-3) and β-catenin: potential novel therapeutic targets for COPD

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Chapter 1

General introduction

Preface

The primary objective of this thesis is to establish the functional roles of β-catenin and glycogen synthase kinase-3 (GSK-3) in the pathological processes that underpin chronic obstructive pulmonary disease (COPD). This general introduction focusses first on the pathophysiology of COPD, which is characterized by abnormalities in the lung and specifically in airway wall structure and the parenchyma. Subsequently, the complexity of canonical *Wingless/intregrase-1* (WNT) signaling pathway, of which β-catenin and GSK-3 are key components, will be discussed. This is followed by an overview of the physiological function and regulation of GSK-3, independent of canonical WNT signaling. The scope of this thesis and the specific aims are defined in the last part of the general introduction.

1. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is defined as a common preventable and treatable disease, which is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced inflammatory response in the airways and the lung to noxious particles and gases. Exacerbations and co-morbidities contribute to the overall severity in individuals patients [1]. An individual with COPD experiences shortness of breath (dyspnea), has productive cough (increased secretion of mucus) and may wheeze [1-3]. Based on the World Health Organization (WHO) estimates, COPD ranked as 5th leading cause of death in 2002 and it is anticipated that tobacco-attributable deaths, including COPD, will further increase in the next decades [4].

According to Global Initiative for Chronic Obstructive Lung Diseases (GOLD) criteria, the severity of COPD is classified in four different stages (table 1) [1, 3]. Spirometry is essential for the diagnosis of COPD and provides insight in the severity of pathological changes. The presence of airflow limitation is determined by measuring the forced expiratory volume in 1 second (FEV_1) and the forced vital capacity (FVC); the maximum volume of air that can be exhaled during a forced maneuver. The FEV_1 is a measurement which is influenced by factors as age, sex, height and ethnicity and therefore most often expressed as percentage of predicted. A ratio of FEV_1/FVC between 70-80% is considered normal in the general population, whereas a value <70% indicates airflow obstruction and the possibility of COPD [3]. Environmental and genetic factors have been shown to contribute to the development of COPD and cigarette smoke is the most significant associated risk factor. However, other risk factors, including occupational dust and chemicals as well as (indoor) air pollution have to be taken into account [2, 5, 6]. The chronic airflow limitation is the resultant of the small airway disease together with parenchymal tissue destruction (emphysema), two pathological features in COPD of which the relative severity can vary from individual to individual. Furthermore, inflammation is an eminent pathological characteristic of COPD; it is evident in mild disease and increases with disease severity.

Table 1: Classification of COPD severity according to GOLD criteria

Classification of COPD stage	Spirometry measurements and symptoms
COPD stage I (mild COPD)	Mild airflow limitation: FEV_1 / FVC <70%, FEV_1 ≥80% predicted *Symptoms*: cough and sputum* production may be present, but not always.
COPD stage II (moderate COPD)	Worsening of airflow limitation: FEV_1 / FVC <70%, 50% ≤ FEV_1 < 80% predicted *Symptoms*: shortness of breath on exertion cough and sputum production sometimes present.
COPD stage III (severe COPD)	Further worsening of airflow limitation: FEV_1 / FVC <70%, 30% ≤ FEV_1 < 50% predicted *Symptoms*: shortness of breath, reduced exercise capacity, fatigue and exacerbations
COPD stage IV (very severe COPD)	Severe airflow limitation: FEV_1 / FVC <70%, FEV_1 < 30% predicted, or FEV<50% with presence of chronic respiratory failure. *Symptoms*: general quality of life is impaired and exacerbations may be life threatening.

* Sputum: expectorated matter, especially mucus or mucopurulent matter expectorated in diseases of the respiratory tract

1.1 Chronic inflammation

Pathological changes characteristic of COPD are found throughout the respiratory tract, including the proximal and peripheral airways, the lung parenchyma and the pulmonary vasculature [7]. The pathophysiology is characterized by a persistent chronic inflammation with an influx of various innate and adaptive immune cells in the different compartments of the lungs. In general, the degree of pulmonary inflammation correlates to the severity of airflow limitation observed in COPD [8].

Innate and adaptive immune response

The predominant inflammatory cell types observed in the lungs of COPD patients include macrophages, dendritic cells and neutrophils, as well as T and B lymphocytes suggesting the contribution of an adaptive immune response [7, 9]. Macrophages and dendritic cells are important for the clearance of the lungs of foreign particles by phagocytosis and they secrete various cytokines and growth factors, which enhance the recruitment of inflammatory cells and activate structural cells in the airways [10]. Although the phagocytic action of alveolar macrophages may be impaired in COPD, they are still capable of producing pro-inflammatory cytokines and growth factors like interleukin-8 (IL-8, CXCL8), IL-1β, tumor necrosis factor (TNF-α) and transforming growth factor-β_1 (TGF-β_1) in response to different stimuli, including cigarette smoke [11-15]. Secreted cytokines, for example IL-8, act as potent chemo-attractants resulting in the increased influx of neutrophils to the respiratory tract. Subsequently, the neutrophils are activated, which results in the release of proteolytic enzymes and various cytokines and in oxidative stress [16]. The secreted proteolytic enzymes like neutrophil elastase, myeloperoxidases and several matrix metalloproteinases have been implicated in the development of emphysema by disturbing the protease/anti-protease balance resulting in increased breakdown of parenchymal lung tissue [16-19]. In addition, the neutrophilia contributes to the mucus hypersecretion, another important pathological feature in COPD pathogenesis [20]. In the course of COPD development, the neutrophils may have acquired altered cellular responses, resulting in enhanced recruitment and activation of these cells in response to pro-inflammatory cytokines [21]. In this context, also the gene expression profile of the neutrophils may have changed to a more pro-inflammatory profile, resulting in increased cytokine release [22]. This may have impact on exacerbations, which are episodes of acute worsening of symptoms at times accompanied by an impairment of lung function, and usually require additional medical attention. During an exacerbation, the inflammatory response is further enhanced with increased expression of pro-inflammatory cytokines like IL-8 and eotaxin. This may be accompanied by an increased recruitment of eosinophils, and in more severe exacerbations by more pronounced neutrophilia [23-26]. Collectively, the neutrophils play an important role in the pathogenesis of COPD by contributing to the development of emphysema, mucus hypersecretion and perpetuation of the inflammation.

The innate and adaptive immune responses are amongst others linked together by dendritic cells, which are specialized antigen presenting cells. In the epithelium, the

dendritic cells are capable of sensing foreign particles and take up antigens to process them. In turn, the dendritic cell presents these antigens to naive T-lymphocytes to promote $CD4^+$ cell differentiation and $CD8^+$ T-lymphocyte cytotoxicity [27, 28]. In response to cigarette smoke, the function of dendritic cells as well as the composition of the dendritic cell population is modulated, which may crucially contribute to COPD pathogenesis [27, 28]. Although both are increased in COPD, the $CD8^+$ T lymphocyte population is predominant over $CD4^+$ T lymphocytes in the airways and lung parenchyma [29]. The presence of $CD8^+$ T lymphocytes correlates to airflow obstruction in the larger airways (determined by FEV1 % predicted), and this association increases in the small airways and parenchyma [6, 30, 31]. The activated T-lymphocytes may directly cause tissue injury due to their cytolytic activity ($CD8^+$ lymphocytes) or indirectly by secreting pro-inflammatory cytokines and/or proteolytic enzymes [32, 33]. Indeed, activated $CD4^+$ and $CD8^+$ T-lymphocytes have been observed in COPD patients with emphysema, and showed enhanced production of cytokines and secreted proteolytic enzymes, as perforins and granzymes, which have been implicated in COPD pathogenesis [33-35]. In addition to T-lymphocytes, also B-lymphocytes have been found in lymphoid follicles present in the parenchyma and bronchial wall of COPD patients with emphysema [36]. The numbers of B-lymphocytes present in the airways increase with disease progression and correlate to airflow limitation in COPD [37]. The B-lymphocytes are oligoclonal in nature, indicating an antigen specific immune response [36]. Several antigens have been proposed to activate T- and B-lymphocytes, including cigarette smoke antigens, various microbial peptide antigens, elastin-related peptides, breakdown products of the extracellular matrix and (other) auto-antigens [32, 38]. However, to date the exact identity of the peptide antigen(s) has not yet been elucidated and consequently the (pathologic) role of the adaptive immune response in COPD pathogenesis is not yet fully established [38, 39]. Collectively, the chronic inflammation contributes to the airway disease and parenchymal destruction both contributing to airflow limitation COPD.

It is important to note that most of the inflammatory cells present in individuals with COPD are also observed in smokers without airflow limitations (asymptomatic smokers) [40]. Strikingly, about 15-20% of smokers develop clinically relevant COPD, indicating that genetic predisposition and additional environmental factors determine susceptibility of smokers to eventually develop disease [41]. Interestingly, smoking cessation has beneficial effects on the progression of lung function decline; nevertheless it appears that quitting smoking hardly affects the presence of inflammatory cells in the lungs of individuals with COPD and that the inflammatory process persists in these individuals [42-44].

1.2 Structural changes in the lungs

As mentioned, COPD is characterized by irreversible airflow limitation, due to the chronic inflammation causing structural changes of the airways and lung parenchyma. Although changes the large airway structure can be observed in COPD, the major alterations are present in the smaller airways, which undergo extensive remodeling. The parenchyma suffers from tissue destruction, resulting in the development of emphysema [2, 7, 37, 45].

Airway remodeling

The small airways (<2 mm in diameter) offer little resistance in normal lungs, but become the major site of obstruction in COPD due to extensive remodeling [37, 46]. There is a strong association between COPD progression and wall thickness of the smaller airways [37]. The remodeling process present in the bronchi and smaller airways encompasses abnormalities of the epithelial layer, the airway smooth muscle layer and the occurrence of airway wall fibrosis [37, 46].

The epithelium is an important physical barrier that protects the airways against inhaled pathogens and particles from the environment. In response to cigarette smoke, the epithelium secretes various soluble factors, as cytokines and growth factors, which direct and activate the immune system [47]. Chronic exposure to cigarette smoke damages the airway epithelium, causing epithelial remodeling and resulting in both squamous and mucous metaplasia [48, 49]. In squamous metaplasia the mucociliary clearance is impaired due to the substitution of columnar epithelium by squamous epithelium, whereas mucous metaplasia is the process in which mucous cells proliferate and mucous is overproduced in response to pro-inflammatory cytokines. Both processes significantly contribute to airflow obstruction in individuals with COPD [48, 49]. The epithelial derived growth factors may in turn affect the smooth muscle cells and fibroblasts lining the airway wall, thereby further contributing to small airway remodeling.

Increased airway smooth muscle mass is an important pathological feature of chronic inflammatory lung diseases [50, 51]. Yet, in contrast to asthma no alterations in smooth muscle mass in the large airways have been reported in COPD [52]. Conversely, particularly in more severe stages of COPD, the smaller airways show a significant increase in airway smooth muscle mass, that negatively correlates to FEV_1 [37]. In addition to an increased volume due to hypertrophy and/or hyperplasia, the peripheral airway smooth muscle layer of individuals with COPD may also display an increased contractile capacity, contributing to airflow obstruction by narrowing of the airway lumen [53]. Furthermore, an increased number of neutrophils within the smooth muscle layer has been observed in individuals with COPD, indicating mutual communication between structural cells of the airways and inflammatory cells [54]. Indeed, in addition to their contractile properties, the airway smooth muscle cells have an immunomodulatory function, as they are capable of producing a variety of cytokines, chemokines, growth factors, enzymes and other mediators in response to various stimuli [51, 55-57]. For

instance, the inflammatory cytokines IL-1β and/or TNF-α activate the airway smooth muscle cells to produce the neutrophil chemo-attractant IL-8, leukotactin-1 (CCL15) and eotaxin among many other cytokines [55, 56, 58]. In addition, growth factors, neurotransmitters, allergic mediators and (soluble components of) cigarette smoke can stimulate the synthetic function of airway smooth muscle cells in a synergistic fashion, thereby recruiting and activating several immune cells within the lungs, contributing to disease pathophysiology [55-57]. Collectively, the airway smooth muscle cells may contribute to the development of COPD by their contractile as well as their synthetic function.

Fibrosis of the airway wall is another key feature of small airway remodeling, which is primarily characterized by alterations in the extracellular matrix (ECM) deposition [59]. The factors driving small airway fibrosis and remodeling are currently poorly understood, however it is clear that fibroblasts are the primary cells regulating the ECM turnover in the lungs and aberrant activation of these cells may lead to tissue fibrosis [60]. It has been postulated that the inflammatory injury incited by cigarette smoke causes aberrant wound healing in the airways resulting in excessive connective tissue accumulation causing airway fibrosis, however the inflammatory response is not necessarily required [6, 61, 62]. In general, small airway remodeling in COPD is considered to be due to excessive fibrogenic growth factor release, in particular that of transforming growth factor β (TGF-β) [62, 63]. An emerging interest in the role of TGF-β in COPD pathogenesis has evolved, as single nucleotide polymorphisms (SNPs) in the *TGF-β* gene are associated with the development of COPD [64-68]. Moreover, several studies indicate an increased expression of this growth factor in smokers and COPD patients [64-68]. Cigarette smoke can directly activate latent TGF-β from lung fibroblasts and stimulate the secretion of various other pro-fibrotic factors from the airways [62, 69]. In agreement, pulmonary fibroblasts from individuals with COPD appear to be more responsive to cigarette exposure regarding ECM production than fibroblasts from individuals without COPD [70]. Members of the TGF-β superfamily activate cell surface serine-threonine kinase receptors, which in turn activate small phenotype and mothers against decapentaplegic related protein (smad) transcription factors. The intracellular regulation of smad signaling may be altered in COPD pathogenesis, indicated by reduced expression of the inhibitory smad6 and smad7 in this disease [71, 72]. In addition to smads, other signaling pathways can be activated by TGF-β, and collectively initiate the differentiation of fibroblast in more secretory active myofibroblasts [73, 74]. Accordingly, an association between myofibroblast-like cells and airway obstruction was recently demonstrated in large airway wall biopsies from individuals with COPD [75]. To date, however, no study has addressed the presence of myofibroblasts in the smaller airways in relation to COPD pathogenesis. Taken together, pulmonary fibroblasts contribute to the small airway fibrosis observed in individuals with COPD.

Emphysema
In the peripheral lung of individuals with COPD a variable degree of emphysema can be present, which may be due to pulmonary fibroblast dysfunction or the incapacity of the fibroblasts to inadequately repair the ongoing destructive response [76]. Emphysema is a major contributor to morbidity and mortality in individuals with COPD, and is defined by the loss of the alveolar structure due to chronic inflammation and tissue destruction, resulting in enlargement of the parenchymal airspaces [3]. The prognosis of smoking-related pulmonary emphysema is poor and the current possibilities of therapeutic intervention are sparse and frequently ineffective [77]. Several mechanisms focusing on the chronic injury induced by cigarette smoke have been postulated to contribute to emphysema, including an imbalance in proteases and anti-proteases, increased apoptosis of alveolar epithelial cells, ineffective phagocytosis function of macrophages thereby attenuating the immunological defense of the lungs, and increased oxidative stress due to mitochondrial dysfunction [77]. These mechanisms collectively contribute to the loss of functional lung tissue by degradation of elastic fibers and loss of alveolar attachments causing hyperinflation, air trapping and airflow obstruction [78].

In contrast to the exuberant fibrotic response developing in the smaller airways, emphysema could be considered to be a deficient repair process with impaired alveolar regeneration leading to failure of lung maintenance [77, 78]. In this context, cigarette smoke seems to impair the fibroblast function in the parenchyma creating an imbalance in the ECM turnover in the lungs [79-82]. Furthermore, the expression of fibroblast activating mediators in the airway and parenchyma may be different, as has recently been postulated and demonstrated in a mouse model of cigarette smoke-induced lung disease [83]. These findings support the concept that the repair mechanism in the parenchyma is impaired due to limited availability of growth factors and other mediators, resulting in the development of emphysema. Nevertheless, the contention that the expression of fibrogenic growth factors in the human emphysematous lung is decreased is not indisputably established [84, 85]. Studies assessing gene expression profiles of human lung tissue from individuals with (severe) emphysema are often not consistent or actually demonstrate an increased expression of genes encoding for various growth factors (including TGF-β) and genes involved in extracellular matrix synthesis [84, 85]. Although the emphysematous lung is predominantly characterized by destruction of functional lung tissue, there are also indications that a remodeling process affecting the connective ECM occurs within the alveolar wall, resulting in increased elastin and collagen content [86, 87]. This suggests that a repair process is activated during the development of emphysema, which seems to be inadequate to fully restore the tissue in response to the concurrent tissue destruction. The molecular mechanisms contributing to parenchymal tissue repair are not well defined, but may be important for restoring functional lung tissue and constitute a feasible target for improved therapeutic intervention of COPD pathophysiology.

1.3 Extrapulmonary effects of COPD

The prolonged and pervasive inflammation in COPD extends beyond the pulmonary system and causes extrapulmonary effects, which clarifies that COPD is a disease with systemic consequences [88]. Sometimes, these systemic effects are the major clinical problems affecting the patients. The systemic inflammation is responsible for the observed skeletal muscle weakness in COPD, which is characterized by a phenotype shift in myocytes, atrophy of specific muscle fibers and possibly increased apoptosis of myocytes [89, 90]. In addition to the muscular weakness, atherosclerotic vascular disease, depression, osteoporosis and disturbance of the electrolyte balance may all be part of COPD [88, 91].

1.4 Therapeutic intervention

Smoking cessation is by far the most important preventive and therapeutic intervention in COPD and is recommended by the GOLD guidelines [3]. Prevention of exposure to tobacco smoke (active or passive smoking) prevents up to 90% of the development of COPD in the Western world. Quitting smoking slows down the accelerated rate of lung function decline even in severe COPD and improves survival compared to individuals with COPD who continue with smoking [92].

The pharmacological treatment of COPD consists of bronchodilatory and anti-inflammatory therapy. The β_2-adrenergic receptor agonists and anticholinergics comprise the main groups of drugs for bronchodilation, whereas (inhaled) glucocorticosteroids are the main anti-inflammatory therapy used in COPD [93]. The therapeutic interventions are judged on their beneficial effects on lung function decline, frequency of exacerbations and quality of life. The widely used bronchodilators provide symptomatic relief to a certain extent and improve exacerbation frequency as well as quality of life [2, 94]. The corticosteroids also improve quality of life and reduce the number of exacerbations, however an effect of this class of drugs on the accelerated lung function decline appears to be either absent or only present in a small group of COPD patients [95-98]. Recently, a phosphodiesterase 4 (PDE4) inhibitor, a new group of anti-inflammatory drugs, has been registered for the treatment for COPD [99, 100]. No compelling evidence indicates that any of the current pharmaceutical therapies attenuates the progressive airflow obstruction and loss of lung function in COPD patients and the limitations of these therapeutic drugs underline that novel agents are required for the treatment of COPD.

2. Wingless/Integrase-1 (WNT) signaling pathway

Age-associated changes in the structure and function of the lungs may increase the pathogenetic susceptibility to COPD [101]. In this context, signaling pathways that are activated during lung development may be of potential therapeutic relevance, particularly the *Wingless/Integrase-1* (WNT) signaling pathway. The WNT signaling pathway is essential for proper lung development, as loss of the specific ligands activating this pathway cause complete lung agenesis [102, 103]. Furthermore, maternal smoking is a risk factor for developing lung disease later in life and in mice it was demonstrated that maternal smoking negatively influences gene expression of WNT pathway components in lung tissue of the offspring, which may cause aberrant lung development possibly contributing to the increased susceptibility to lung diseases [104, 105]. The expression of WNT pathway genes in the epithelium of asymptomatic smokers and smokers with COPD is decreased, contributing to the alterations of the airway epithelium observed in smoking-related diseases [106]. Furthermore, a recent study indicates that activation of WNT pathway signaling may attenuate experimental emphysema in mice [107]. Activation of WNT signaling is required for lung development and it contributes to tissue repair, whereas aberrant activation resulting in excessive WNT pathway signaling is associated with fibroproliferative diseases in various organs, including the lungs [108, 109].

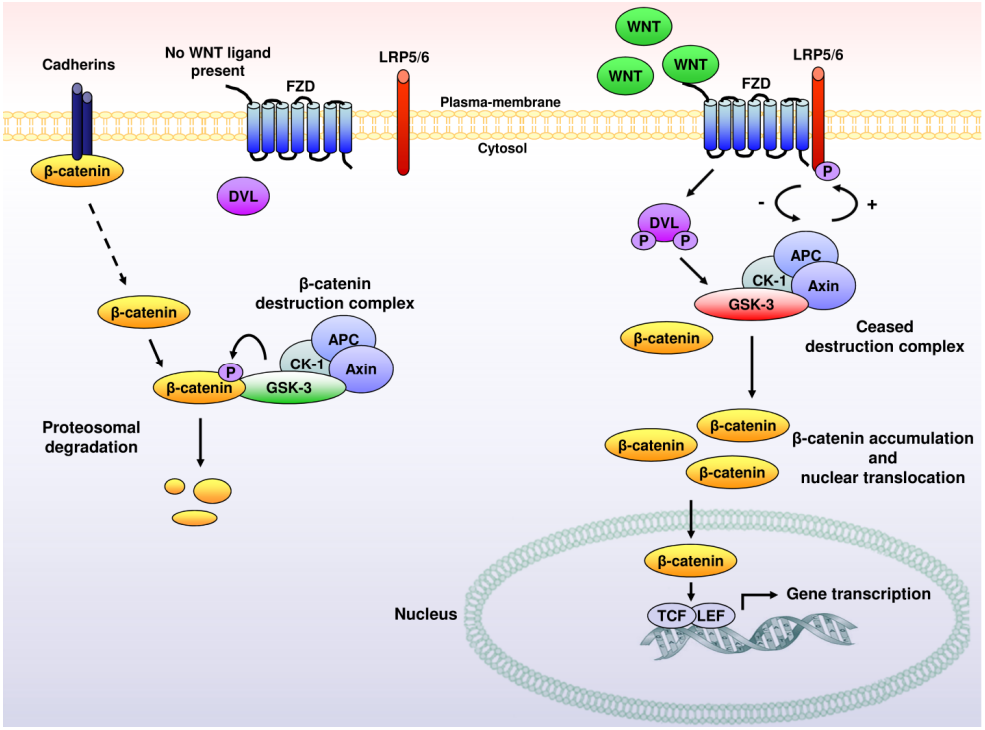
2.1 Overview of canonical and non-canonical WNT signaling pathway

The WNT signaling pathways are highly conserved amongst species, are essential in developmental processes, and may be reactivated during disease pathogenesis. A simplified and straight-lined representation of canonical (i.e. β-catenin-dependent) WNT signaling (figure 1) is that the pathway is activated by a secreted extracellular WNT ligand that binds to a seven transmembrane receptor, called Frizzled (FZD), and/or its co-receptors the low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6). The activated FZD receptor in turn switches on the intracellular signaling cascade by activating the dishevelled proteins (DVL), which cause disruption and thereby inactivation of a so called destruction complex consisting of adenomatosis polyposis coli (APC), axin, glycogen synthase kinase-3 (GSK-3) and casein kinase-1 (CK-1) [110-113]. In the absence of WNT ligands, the destruction complex phosphorylates cytosolic β-catenin, the key effector of canonical WNT signaling, and thereby targets it for proteosomal degradation. However, in the presence of WNT signaling the disruption and inhibition of the destruction complex allows free β-catenin to accumulate in the cytosol and subsequently translocate into the nucleus. The transcriptional co-activator β-catenin associates in the nucleus with the T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) family of transcription factors and induces gene transcription (figure 1).

In addition to the canonical WNT signaling, also various non-canonical WNT signaling pathways exist, which do not depend on β-catenin as downstream effector, but mainly activate c-Jun-N-terminal kinase (JNK)-dependent or Ca^{2+}-dependent signaling pathways [110-113]. Originally, the non-canonical Wnt

pathway was synonymous for the planar cell polarity (PCP) pathway. The PCP pathway regulates tissue morphogenesis and the synchronous polarity of sheets of cells. This specific pathway is associated with the downstream activation of JNK and proteins involved in cytoskeleton rearrangement [114, 115]. On the other hand, the WNT/Ca^{2+} signaling pathway is associated with the activation of phospholipase C (PLC), which lead to the formation of inositol 1,4,5-triphosphate (IP3) and 1,2 diacylglycerol (DAG) from the membrane-bound phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2). The formation of IP3 and DAG results in an increase in intracellular Ca^{2+} levels. Subsequently, Ca^{2+} causes the activation of calmodulin-dependent protein kinase II (CAMKII), protein kinase C (PKC) and the nuclear factor of activated T cells (NFAT) transcription factor as well as a variety of other transcription factors [114-116]. The WNT/Ca^{2+} signaling pathway is in general important for developmental processes; however it has recently also been linked to cancer development and it may contribute to inflammatory responses [116].

Over the last decades WNT signaling has been extensively investigated and here the focus will be on the components of canonical WNT/β-catenin signaling and their complex regulation by a variety of molecular mechanisms (figure 2). Despite the complexities, the canonical WNT pathway is still far better understood than the non-canonical WNT pathway.



▲ ***Figure 1: Schematic representation of canonical WNT signaling.***
In the absence of extracellular WNT ligands (left): cytosolic β-catenin is recognized by the destruction complex, which is composed of axin, adenomatous polyposis C (APC), casein kinase-1 (CK-1) and glycogen synthase kinase-3 (GSK-3). The destruction complex phosphorylates β-catenin, thereby targeting the protein for ubiquitination and subsequent proteosomal degradation. *In the presence of extracellular WNT ligands (right)*: the WNT ligands bind to the seven- transmembrane receptor Frizzled (FZD) and/or the low density lipoprotein receptor related proteins (LRP) 5 and 6. The activated receptors transduce their signal intracellularly via activation of disheveled (DVL) proteins. Activation of DVL in combination with the phosphorylation of LRP5/6 results in a disengaged or disrupted destruction complex, allowing cytosolic β-catenin to accumulate and thereafter to translocate to the nucleus. The transcriptional co-activator β-catenin associates with T-cell factor (TCF)/Lymphoid enhancer factor (LEF) family of transcription factors and induces gene transcription (see text for detailed description).

2.2 WNT ligands

WNT ligand genes encode evolutionary conserved secreted glycoproteins (table 2) that act as signaling molecules essential for a number of fundamental processes like embryonic patterning and cell-fate decisions in development. The name WNT is a fusion of the wingless (*Wg*) gene and the homologous vertebrate oncogene intergrase-1 (*int-1*) [117]. The canonical WNT pathway originates from 1973, when a mutation in the *Wg* gene in *Drosophila Melanogaster* was identified and the loss of function mutations resulted in impaired haltere development and wing deficient flies, demonstrating the physiological relevance of *Wg* in the development of *Drosophila Melanogaster* [118]. The proto-oncogene *int-1* was discovered in 1982, as an important gene in the development of mouse mammary tumor virus (MMTV)-induced breast cancers in mice [119]. In 1987 this gene was cloned and soon thereafter it was discovered that the *Drosophila Melanogaster* homolog of *int-1* was identical to the *Wg* gene [117, 120]. It became apparent that *int-1* encoded for a secreted glycoprotein and that this gene presented a paradigm for a group of related mammalian genes. Eventually in 1991 this resulted in a new nomenclature for *Wg* / *int-1* and the related genes; the WNT ligand family [117, 121]. Wg still acts as the exemplifying protein for WNT signaling to explore fundamental principles of this pathway; however, extrapolation of principles that apply for Wg to other individual WNT ligand proteins should be done with prudence. Currently the family of secreted WNT-glycoproteins in human is presented by 19 different ligands (table 2), which are historically defined by their amino-acid sequence rather than by their functional properties. This family of ligands share common characteristic structural features, which include a signal sequence required for secretion, several highly charged amino acids, various potential glycosylation sites and a cysteine-rich domain consisting of 22-25 cysteine residues [122-124]. The genes encoding for WNT ligands are present in both vertebrates and invertebrates, but the number of encoding genes varies significantly between species [123]. Nevertheless, in vertebrates the WNT ligand orthologs within different species share high homology, up to 98% identical sequences in WNT-1 (*Wg)* for instance [122-124].

In general, WNT ligands consist of ~350 amino acids and have a corresponding molecular weight of ~40 kDa and the multiple cysteine residues contribute to proper folding of the ligand [125, 126]. Immature WNT ligands are posttranslationally modified in the endoplasmic reticulum (ER) by the oligosaccharyl transferase complex (OST) and porcupine, a multipass transmembrane protein. The OST complex attaches oligosaccharides to the WNT ligand backbone (*N*-glycosylation) [125]. Loss of porcupine resembles lack of Wg/WNT signaling, whereas overexpression results in aberrant glycosylation and/or palmitoylation (i.e. covalent modification by a lipid moiety) of WNT ligands without increasing canonical WNT signaling [124, 127]. Therefore, it appears that porcupine is required for the appropriate lipid modifications of WNT before they are secreted. The function of *N*-glycosylation by porcupine or the OST complex is not yet elucidated [125]. The secretion of WNT ligands is subsequently mediated by the recently identified the multipass transmembrane protein Wntless/Evenness

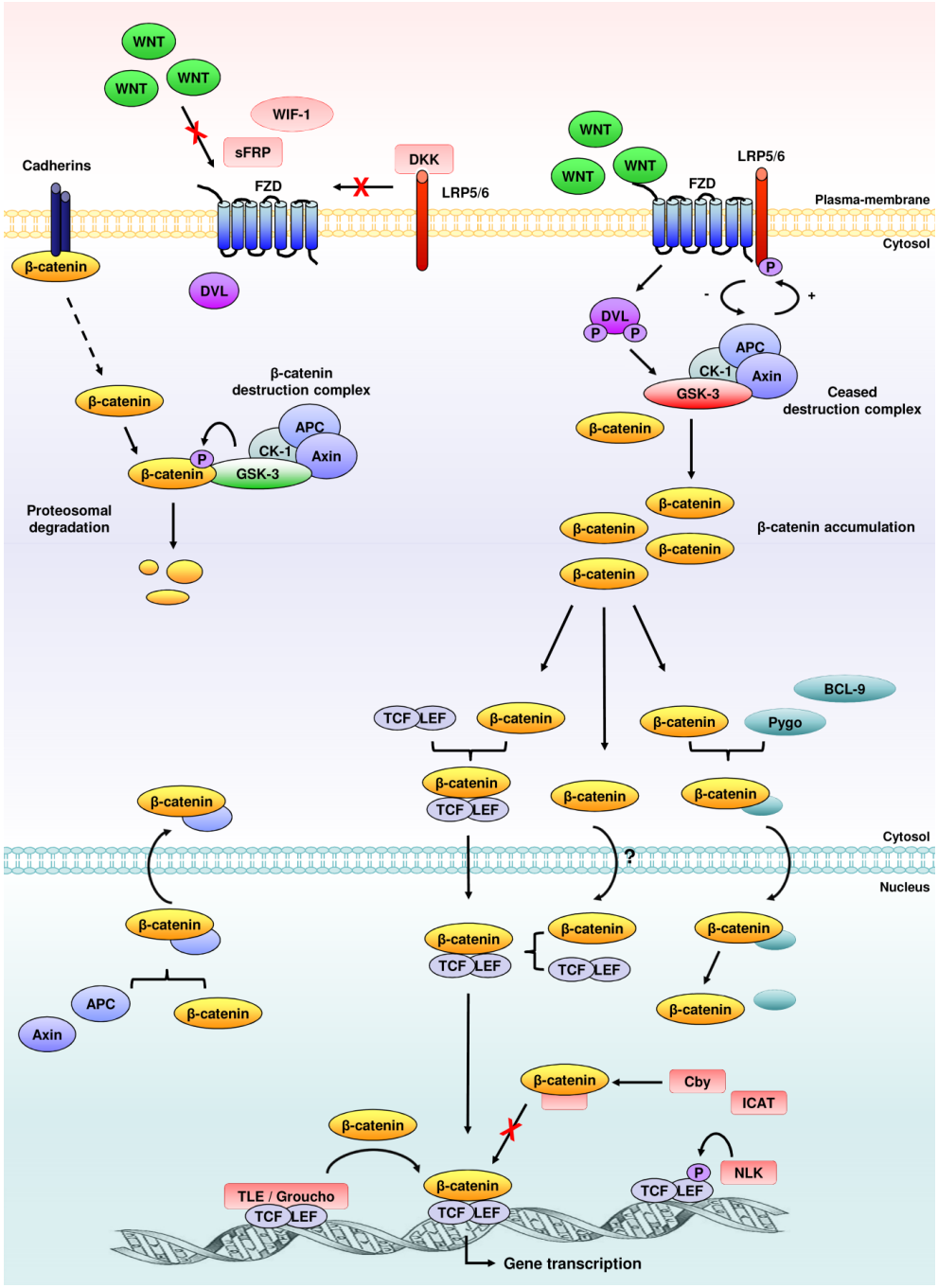
interrupted (Wls/Evi), which recognizes porcupine-mediated lipidation of specific amino residues within the WNT ligand [124, 125, 128]. Wls/Evi facilitates the cellular trafficking and alternatively controls specific modifications within the ligand required for secretion [125, 129, 130].

Table 2: WNT signaling pathway components and modulators

Components and function	Family members
WNT ligands	WNT-1 (Wg), WNT-2, WNT-2B, WNT-3, WNT-3A, WNT-4, WNT-5A, WNT-5B, WNT-6, WNT-7A, WNT-7B, WNT-8A, WNT-8B, WNT-9A, WNT-9B, WNT-10A, WNT-10B, WNT-11, WNT-16
Alternative ligands	Norrin and R-spondins
Extracellular modulators	Secreted Frizzled related proteins (sFRP), WNT-inhibitory factor (WIF-1), Dickkopfs (DKK)
FZD receptors	FZD_1, FZD_2, FZD_3, FZD_4, FZD_5, FZD_6, FZD_7, FZD_8, FZD_9, FZD_{10}
LRP receptors	Low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6)
Alternative receptors	Ror2 and Ryk
Signaling intermediates	Dishevelleds: DVL1, DVL2, DVL3
β-Catenin destruction complex	Axin, adenomatosis polyposis coli (APC), Glycogen synthase kinase-3 (GSK-3), Casein kinase-1 (CK-1), Protein phosphatases (PP1 and PP2A)
Cellular trafficking and distribution	Cadherins, pygopus (Pygo) and legless (Lgs/BCL-9)
Effector	β-catenin (Armadillo)
Transcription factors	T-cell factor (TCF1, TCF-3, TCF-4), Lymphoid enhancer factor (LEF-1)
Intracellular modulators	Nemo-like kinase (NLK), Groucho / Transducin-like enhancer of split (TLE) inhibitor of β-catenin and TCF4 (ICAT), Chibby (Cby)

The secreted WNT ligands are lipid modified glycoproteins therefore tightly associated with the cell surface and the surrounding extracellular matrix promoting short range signaling [131]. The extracellular matrix proteins, in particular glycosaminoglycan (GAG)-modified proteins, like the cell-surface heparan sulfate containing proteoglycans perlecan, syndecan and glypican or the small leucine-rich repeat proteoglycan biglycan have an important in role in regulating the WNT ligand concentration, distribution and activity [131-133]. Likewise, the less conventional heparan sulfate proteoglycan collagen XVIII is able to bind WNT ligands controlling extracellular movement and their readiness to induce signaling [134]. Nevertheless, WNT ligands may also extend their range of signaling, which demands morphological alterations in their molecular structure and/or requires a carrier as the prevalent lipid modifications may negatively affect the distribution of WNT ligands proteins in the aqueous extracellular fluid. For instance, several secreted Wg proteins can form a multimer complex in which the lipid chains are insulated, increasing its solubility [112, 135]. In addition, WNT ligand movement may be regulated by proteins involved in transcytosis: a process by which macromolecules are normally transported across the interior of a cell. The transcytosis process is mediated by a cluster of proteins called the retromer complex, which is hypothesized to promote the assembly of WNT ligands to other proteins like lipoprotein particles, which in turn function as chaperones to extend the signaling range of the specific WNT ligand [124, 125, 136].

As mentioned, WNT ligands can broadly activate two distinct signaling pathways subdivided in the canonical β-catenin pathway and the non-canonical pathway, in which the latter is further divided in the planar cell polarity pathway (PCP) and the WNT/Ca^{2+}-dependent pathway. The conception of distinct WNT ligands that mutually exclusively induce either canonical or non-canonical WNT signaling, originates from studies performed in *Xenopus Leavis* [137]. Ectopic expression of WNT ligands that activate canonical β-catenin-dependent WNT signaling, resulting in the induction of a secondary axis in early *Xenopus Leavis* embryos, were classified as canonical WNT ligands. Other WNT ligands are not axis inducing or act even inhibitory when misexpressed, but these ligands modulate cell adhesion and cell movement in these embryos indicating non-canonical signaling [137, 138]. These findings are the basis of classifying WNT-1 (Wg), WNT-3A, WNT-8 and WNT-8B as canonical WNT signaling ligands, whereas on the other hand WNT-4, WNT-5A and WNT-11 were classified as non-canonical WNT ligands. Nevertheless, more recent evidence indicates that this strict classification of WNT ligands to be purely activators of either canonical or non-canonical WNT signaling is not justified and activation of these signaling cascades is highly dependent on cell type and on the specific receptors expressed by the cells [137-140].


WNT
WNT
WNT
WNT
WIF-1
sFRP
DKK
Cadherins
FZD
LRP5/6
Plasma-membrane
Cytosol
β-catenin
DVL
LRP5/6
FZD
P
DVL
P
P
APC
CK-1
Axin
GSK-3
Ceased
destruction complex
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β-catenin
β-catenin accumulation
BCL-9
TCF LEF
β-catenin
β-catenin
Pygo
β-catenin
TCF LEF
β-catenin
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Nucleus
β-catenin
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APC
Axin
β-catenin
β-catenin
TCF LEF
β-catenin
TCF LEF
β-catenin
β-catenin
Cby
ICAT
β-catenin
TLE / Groucho
TCF LEF
β-catenin
TCF LEF
P
NLK
TCF LEF
Gene transcription

◄ ***Figure 2: Regulation of canonical WNT signaling.***
The activity of the (canonical) WNT signaling pathway is tightly regulated via endogenously expressed extracellular and intracellular modulators. *Extracellular WNT pathway modulators*: the secreted FZD related proteins (sFRPs) and members of the Dickkopf protein family (DKKs). The sFRPs affect both canonical as non-canonical WNT signaling via binding to WNT ligands, thereby preventing the interaction of the ligands with FZD receptors. WNT inhibitory factor-1 (WIF-1) is also appointed as a member of the sFRPs. The DKKs exclusively affect the canonical WNT signaling pathway by binding to the LRP receptors, hence preventing the formation of the WNT/FZD/LPR signaling complex, which is required for canonical WNT signaling. *Nuclear translocation of β-catenin*: WNT ligand-induced inhibition of the destruction complex allows accumulation of cytosolic β-catenin and subsequent nuclear translocation. β-Catenin lacks a specific nuclear translocation sequence (NLS) and the nuclear translocation is facilitated by TCF/LEF-1 transcription factors, pygopus (Pygo), BCL-9 (legless), or is achieved by direct binding of β-catenin to the nuclear pore via a process that is not yet fully elucidated. Nuclear export of β-catenin to the cytosol is facilitated by APC and Axin. *Modulators of WNT signaling in the nucleus*: β-catenin induces gene transcription via its interaction with TCF/LEF-1 transcription factors. In the absence of WNT signaling the transcriptional potency of TCF/LEF-1 is suppressed via the interaction with TLE (Groucho). In addition, TCF/LEF-1 can be phosphorylated by Nemo-like kinase (NLK) resulting in inhibition of the DNA binding. The transcriptional potential of β-catenin in is blocked via the interaction with chibby (Cby) or the polypeptide inhibitor of β-catenin and TCF-4 (ICAT) (see text for detailed description).

2.3 Cell surface receptors for WNT ligands: Frizzled (FZD) and low-density lipoprotein receptor related proteins (LRP) receptors

The interaction of WNT ligands with their receptors on the cellular surface is the first step in transducing the extracellular signal into an intracellular response. Two distinct receptor families are critical for (canonical) WNT signaling; the Frizzled family of seven transmembrane receptors (FZD receptors) and the low-density lipoprotein receptor related proteins 5 and 6 (LRP5/6 receptors; Arrow in *Drosophila Melanogaster*). The initial connection of WNT ligands binding to FZD receptors resulting in the activation of canonical WNT signaling was demonstrated in the mid 1990's [141, 142]. Experiments in *Drosophila Melanogaster* transfected with the FZD_2 receptor ortholog, demonstrated the interaction of the receptor with the ligand Wg and subsequent stabilization of *Armadillo* (the *Drosophila Melanogaster* ortholog of β-catenin) [142]. Additional evidence implying this interaction came from experiments performed in *Xenopus Leavis* [141]. Ectopic expression of the rat FZD_1 receptor in this species resulted in the expression of two canonical WNT responsive target genes, which was enhanced by co-expression of WNT-8, linking the FZD receptors to the WNT signaling pathway [141].

Currently, the human FZD receptor family consists of 10 distinct members (FZD_1 through FZD_{10}; table 2) [143]. The identified FZD receptors contain common structural features, which include a cysteine-rich domain (CRD) in the extracellular amino-terminus (N-terminus), a seven transmembrane domain and a short cytoplasmic tail at the carboxyl-terminus (C-terminus). The highly conserved CRD domain (10 cysteine residues) has been implicated to be a specific binding domain for WNT ligands. Indeed, specific WNT ligands were demonstrated to directly bind to this domain and induce downstream signaling activity [144, 145]. However, mutated FZD receptors that lack the CRD domain may still be able to induce signaling activity, although frequently less efficient than their wild type counterparts

[146, 147]. In addition, when these mutated FZD receptors are fused with either another WNT ligand binding domain or directly linked to a WNT ligand, they are able to completely restore the WNT signaling capacity [147-149]. Collectively, these findings indicate that the CRD domain functions as a binding domain for WNT ligands but happens to be not indispensable, possibly indicating that other parts of the FZD receptor require the interaction with a WNT ligand for signaling and that the CRD domain facilitates this interaction [148, 150]. The relationship between FZD receptors and WNT signaling has now been established; however, the specificity of this interaction has still not been completely elucidated. This is largely due the large number of WNT ligands and FZD receptors, the potential multitude of WNT/FZD interactions and because of the mutual functional redundancies in both the ligands and the receptors [151].

The intracellular domains of FZD receptors do not have enzymatic motifs and the signal transduction is mediated by the recruitment of intracellular signaling molecules [152]. Thus, WNT-dependent activation of FZD receptors results in the subsequent recruitment of the downstream signaling intermediate dishevelled (DVL; table 2). The name dishevelled DVL or Dsh in *Drosophila Melanogaster*) originates from the phenotype of disorientated hair and body development in *Drosophila Melanogaster* [153]. Three human homologues have been identified (DVL1 through DVL3) and DVLs have three well conserved domains: the DIX (DVL/axin), PDZ (postsynaptic density 95, disc large, zona occludens-1) and DEP (DV, Egl-10, pleckstrin) domains [154]. WNT signaling induces rapid polymerization of DVL at the plasma membrane which involves the DIX and PDZ domains, the latter being important for the direct interaction of DVL to FZD receptors and downstream effectors [154]. Furthermore, WNT signaling induces (hyper)phosphorylation of DVLs, which suggests that this may be important for their function [154-156]. Several kinases have been demonstrated to be involved in the phosphorylation of DVL, including casein kinase(CK)-1 (i.e. CK-1ε and CK-1δ), CK-2 and PAR-1 (*par-1* kinase or microtubule-affinity-regulating kinase; MARK) [154-156]. Nevertheless, it is not yet completely established what the functional consequence of DVL phosphorylation response to WNT signaling is. In addition, the members of the FZD receptor family show high resemblance with the family of G-protein coupled seven transmembrane receptors (GPCRs) and therefore G-proteins have been suggested to be the link between the FZD receptor and the downstream activation of DVLs. Accordingly, the requirement of G-proteins in the activation of canonical and non-canonical WNT signaling has been shown [157-159]. However, to date there is no evidence of the direct physical interaction of G-proteins to any of the identified FZD receptors and further studies are required to substantiate the understanding of the involvement of G-proteins in WNT signaling as well as of the signaling events directly downstream of FZD receptors [158].

Activation of DVLs is important in both canonical and non-canonical WNT signaling and DVLs have dynamic interactions with a wide range of downstream binding partners. This implies that an (additional) signal is required to discriminate between

both pathways. The FZD receptors can be considered as general WNT receptors, as these receptors are capable of activating both the canonical and the non-canonical WNT signaling pathways [148, 160, 161]. Selectivity of canonical and non-canonical pathway activation by WNT ligands appears to be mainly dependent on the recruitment and/or activation of LRP5/6 co-receptors (figure 1 and table 2), which are indispensable for the canonical signaling pathway (table 2). This was elegantly demonstrated by the fusion of WNT-5A (generally considered to be a non-canonical WNT ligand) to a part of dickkopf-2 (DKK-2; a modulator of WNT signaling) that interacts with LRP5/6. The fusion protein activated canonical WNT signaling and this could be antagonized by inhibition of either the FZD or the LRP5/6 (co-)receptor. As anticipated, the non-mutated WNT-5A was not able to activate β-catenin signaling. Taken together, this implicates that FZD and LRP5/6 can form a signaling complex together and that LRP5/6 determine the downstream signaling response to a WNT ligand [140]. Therefore it was suggested that non-canonical WNT ligands (e.g. WNT-5A) are less efficient in promoting the association of the FZD receptor with LRP5/6, and accordingly do not activate canonical signaling [140]. However, the existence of receptor signaling complexes composed of WNT/FZD/LRP5/6 (the so-called LRP signalosomes) has not yet been demonstrated under physiological conditions. The requirement of LRP receptors in canonical WNT signaling was demonstrated in experiments in *Drosophila Melanogaster.* Complementary evidence in mammalian cells showed that mutations in the LRP5 or LRP6 resulting in truncated proteins lacking the extracellular domain, induced a constitutively active canonical WNT signal, whereas the lack of the intracellular domain results in dominant negative receptors [162-164]. Loss of LRP6 in mice ($LRP6^{-/-}$ mice) induces severe developmental defects that are perinatally lethal; $LRP5^{-/-}$ mice are viable but develop osteoporosis and metabolic disorders, and double knock out ($LRP5^{-/-}$ and $LRP6^{-/-}$) mice die shortly after gastrulation [165-167]. These phenotypes are consistent with loss of WNT signaling and demonstrate that although LRP5 and LRP6 have overlapping functions, loss of LRP6 is more severe than loss of LRP5. WNT ligands bind with much lower affinity to LRP6 receptors when compared to the FZD receptors [163]. The transduction of the extracellular signal into the intracellular compartment is facilitated by the proposed LRP5/6 signalosomes [163, 168-170]. In the signalosomes LRP6 is phosphorylated at the intracellular domain. To be more specific, the serines/threonine in the P-P-[S/T]-P-X-S motifs (P: proline, S: serine, T: threonine and X: variable amino acid residue) are the targets for phosphorylation [170, 171]. Canonical WNT ligands induce sequential phosphorylation of LRP6 receptors predominantly mediated by GSK-3 (Zeste-White3 or Shaggy in *Drosophila Melanogaster)* and/or casein kinase-1 (CK-1) isoforms, and this dual phosphorylation promotes the engagement of LRP6 with the scaffolding protein axin at the plasma membrane [171]. Additional kinases have been described to facilitate the phosphorylation of LRP receptors, including protein kinase A (PKA), members of the cyclin-dependent protein kinases (i.e. Pftk-1) and G protein-coupled receptor kinases (Grk5/6) [170]. Interestingly, CK-1 and GSK-3 are both members of the destruction complex involved in the phosphorylation and

degradation of β-catenin, and it was demonstrated that it is not the cytosolic pool of GSK-3 that is responsible of LRP6 phosphorylation, but rather a membrane-associated pool of the kinase [112, 171, 172]. Also constitutive (i.e. WNT signaling independent) phosphorylation of LRP6 has been observed, though the kinases involved in this process may be different from those involved in WNT mediated LRP6 activation [170, 171]. GSK-3 is considered to be a constitutively active kinase and this raises the question of why this kinase contributes to WNT-mediated activation rather than the possible constitutive phosphorylation of LRP6 [173, 174]. The explanation could be that in the absence of WNT signaling the phosphorylation sites in the cytoplasmic domain of LRP6 are not accessible for GSK-3. This may indeed be the case, as WNT signals are able to induce a conformational change in LRP6 resulting in activation of the signaling pathway [164]. Much remains to be discovered regarding the exact mechanism(s) of conventional WNT receptor activation and their signaling at the plasma membrane in WNT ligand receiving cells.

2.4 Alternative ligands and receptors in WNT signaling

In addition to WNT ligands, also other proteins may either directly or indirectly activate FZD or LRP5/6 receptors, resulting in increased β-catenin stability and thereby mimicking canonical WNT signaling activation. For example, Norrin and R-spondins (the RSPO protein family, consisting of four members) are secreted proteins that are able to either directly bind to FZD receptors or to assemble a signaling complex containing the FZD receptor when they bind to the LGR4/5 receptors (leucine-rich repeat containing, G-protein coupled receptors 4 and 5), both resulting in WNT pathway activation [175-177]. Interestingly, R-spondins may have synergy with canonical WNT ligands in activating β-catenin. The expression of R-spondins is closely related to the expression of the canonical WNT ligands WNT-1 and WNT-3A, indicating a possible feed forward mechanism that enhances canonical WNT signaling [175]. Similarly, WNT ligands also have the option of activating non-conventional receptors with a WNT-ligand binding domain, leading to other modes of WNT signaling. Alternative receptors for WNT signaling may be members of the single span transmembrane tyrosine kinase receptors Ror and Ryk. Signaling by these receptors is initiated by direct binding of a WNT ligand to these receptors. In the case of Ror2 the ligand can bind to the CRD domain, which is also present in the FZD receptors, whereas Ryk (Derailed in *Drosophila Melanogaster)* does not contain a CRD but another WNT binding sequence called WNT inhibitory factor (WIF) domain, which was initially identified in the N-terminus of the secreted WNT pathway modulator WNT inhibitory protein-1 (WIF-1) [149, 178, 179]. Interestingly, Smoothened (Smo; another CRD-containing receptor) is important for sonic hedgehog (Shh) signaling, but has recently also been associated with WNT signaling [143, 180]. However, activation of Smo is considered to be independent of an extracellular ligand and therefore it is unlikely to be a direct receptor for WNT ligands [143, 181]. Nevertheless, the functional outcome of Ror2 activation shows high resemblance to WNT-5A signaling, implicating that this alternative receptor contributes to non-canonical WNT

signaling [182, 183]. On the other hand, activation of Ryk has been linked to both canonical and non-canonical WNT signaling [182]. The involvement of these alternative ligands and non-conventional receptors in the WNT signaling cascade and their contribution to (patho)physiological processes is still a largely unresolved area.

2.5 Modulators of WNT signaling

WNT signaling pathway activation is furthermore regulated via endogenously expressed extracellular and intracellular modulators, including secreted Frizzled related proteins (sFRPs), WNT inhibitory factor-1 (WIF-1), the Dickkopf protein family (DKK's), Nemo-like kinase (NLK) and β-catenin binding inhibitors (table 2, figure 2). Although most of these proteins have been identified predominantly by their capability of inhibiting WNT-signaling, more recently it has become apparent that they may also have other physiologically relevant signaling properties.

Secreted and extracellular WNT signaling modulators

The first main class of WNT signaling modulators is the secreted Frizzled related proteins (sFRP) which comprise a family of 5 members in humans: sFRP-1 through sFRP-5, of which sFRP-3 is also known as FrzB (Frizzled motif associated with bone development) [184]. Since their discovery, it was established that distinct sFRPs directly bind to specific WNT ligands, thereby attenuating WNT signaling and without making a distinction between WNT ligands that activate either canonical or non-canonical signaling (figure 2) [185-189]. Interestingly, the sFRPs contain an N-terminal cysteine-rich domain (CRD), which highly resemblances the CRD domain in FZD receptors, being the WNT-ligand binding domain [184, 186, 190]. However, whether WNT signaling is antagonized by sFRPs through the association of WNT ligands to this CRD domain is still matter of debate as also the C-terminal domain may be important for inhibiting WNT signaling [185, 191, 192]. In addition, sFRP-1 can also directly interact with FZD receptors [184]. Thus, the sFRPs may have a dual mechanism to antagonize WNT signaling, by either direct interaction with WNT ligands preventing them from binding to FZD receptors, or by forming a nonfunctional complex with the FZD receptors [184, 185, 191]. The physiological relevance of the sFRPs is even more complicated, as the expression of different sFRPs is associated with distinct cellular responses. This may be due to differences in binding affinity of distinct WNT ligands for specific sFRPs, the variety of WNT ligands present or may be dependent on the cellular expression of FZD receptors, as sFRPs may also act as chaperones guiding WNT ligands to cellular sites with relatively high expression of FZD receptors [185, 190, 191]. WNT-inhibitory factor (WIF-1), Klotho and Cerberus are also counted among the sFRPs class of WNT inhibitors as they are secreted proteins that directly bind specific WNT ligands and attenuate their activity, however with different efficiencies [149, 185]. In contrast to sFRPs and FZD receptors, WIF-1 does not contain the specific CRD domain, but is able to bind WNT ligands via a specific N-terminal domain (i.e. the WIF domain) [149, 178].

Another class of extracellular WNT signaling modulators is the Dickkopf (DKK) family of proteins. In 1998, Dickkopf-1 (DKK-1) was first of this class to be identified, as a regulator of canonical WNT signaling [193]. Shortly thereafter, three other members (i.e. DKK-2, DKK-3 and DKK-4) were identified, as well as a DKK-3-related protein named Soggy [185, 193, 194]. Unlike sFRPs, the secreted cysteine-rich DKK proteins are able to inhibit the WNT signaling pathway without direct binding to the WNT ligands, with the exception of DKK-2, which has a stimulatory effect on canonical WNT signaling [195-199]. The inhibitory DKK's specifically prevent canonical WNT signaling by binding to the LRP5/6 co-receptors thereby preventing the formation of WNT-FZD-LRP5/6 complex that is required for the activation of intracellular β-catenin signaling (figure 2) [195-198]. In addition, DKKs may bind with high affinity to the single transmembrane receptors Kremen 1 and 2 (Krem1 and krem2). For instance, DKK-1 can inhibit canonical WNT signaling by constituting a tertiary complex that further consists of krem2 and LRP6 [200]. This subsequently results in rapid endocytosis of the LRP6 receptor, decreasing the plasma-membrane expression of the receptor and thereby restricting the possibility of canonical WNT signaling activation [200]. Krem2-mediated internalization of LRP6 is not a general applied mechanism of DKK-1. It was recently demonstrated in several mammalian cell lines, that canonical WNT signaling was attenuated by DKK-1 via its interaction with LRP6; however no effect on either the cell surface expression or degradation of LRP6 was observed [201]. Interestingly, DKKs may be downstream targets of canonical WNT/β-catenin signaling, which suggests a negative feedback mechanism to govern WNT signaling [202-205]. Because the DKK proteins selectively and exclusively inhibit the canonical WNT signaling pathway and are available as recombinant proteins, they are very useful as molecular tools to distinguish whether WNT ligand-induced cellular responses are mediated via the canonical or non-canonical signaling pathway. In addition to DKK's, LRP5 and LRP6 are also the targets of several novel extracellular WNT signaling inhibitors/modulators including pigment epithelium-derived factor (PEDF), Sclerostin (SOST), Mesd, Serine proteinase inhibitor A3K (SERPINA3K) and Adenomatosis polyposis coli down-regulated protein-1 (APCDD1) [206-209]. The tight regulation of spatiotemporal expression of WNT ligands by the extracellularly expressed WNT modulators, like sFRPs and DKKs, is likely involved in the regulation of a variety of cellular functions and critical developmental processes by (canonical) WNT signaling.

2.6 β-Catenin structure and cellular function

The multi-functional protein β-catenin plays a crucial role both in cell adhesion, by being a component of cadherens based adherens junctions stabilizing cell-cell contacts, and in gene transcription as a transcriptional co-activator in canonical WNT signaling. The catenin family of proteins consists of three closely related members; α-catenin, β-catenin and γ-catenin [210]. Originally β-catenin and γ-catenin, the latter also known as plakoglobin, were described in 1989 as proteins linked to the transmembrane cadherins located at the intracellular surface of the plasma membrane [211]. Cadherins directly interact with these specific catenin

proteins, linking them to the cytoskeleton [210, 212]. A highly regulated and dynamic cytosolic pool of β-catenin is responsible for the signal transduction in canonical WNT signaling. Whether these two cellular functions of β-catenin are interconnected is still a controversial issue, as disengagement of the protein from the cadherins complex can contribute to cytosolic β-catenin expression, but may also be the result of a shift in the (continuous) cellular turnover (i.e synthesis and degradation) of β-catenin [172, 213-215].

Structural and biochemical studies revealed that β-catenin has a flexible N- and C-terminus domain and a characteristic central domain consisting of 12 armadillo repeats [216, 217]. The armadillo repeats are highly conserved and each repeat contains 42 amino acid residues and is arranged in three helixes. Conjointly the armadillo repeats form a superhelix with a positively charged groove, which is important for the interaction of β-catenin to other proteins [217]. The various binding partners of β-catenin show overlapping binding sites preventing simultaneous binding, implying spatial-temporal regulation of WNT signaling and function of the other binding proteins [216]. As do many other proteins, β-catenin undergoes posttranslational modifications, predominantly at the N- and/or C-terminal domain, of which phosphorylation and ubiquitination of the protein are the most well-known and additionally, it can also be acetylated [216, 218, 219]. These posttranslational modifications regulate the cellular expression, distribution and function of β-catenin.

The cytosolic pool of β-catenin is tightly regulated by a multi-protein complex, the so-called β-catenin destruction complex, and canonical WNT stimulation interferes with this complex resulting in increased β-catenin stability (figures 1 and 2).

2.7 The β-catenin destruction complex components

The β-catenin destruction complex plays a central role in the canonical WNT signaling pathway by tightly regulating cellular β-catenin expression and keeping the cytosolic β-catenin levels low in the absence of WNT signals. The composition of the destruction complex may be dynamic with proteins that form the central core of the complex, whereas other proteins transiently associate with the complex either in a WNT-dependent or independent fashion. Axin, APC, GSK-3 and CK-1 form the core proteins of the destruction complex and their function will be briefly discussed [111, 112, 172].

The scaffold protein axin links the other components together and brings them in close proximity to each other. Axin expression is considered rate limiting for the assembly of the destruction complex [220-222]. However, the closely related protein Axin2 (alias axin-like protein or conductin) is a target of β-catenin signaling and is able to assemble an (alternative) destruction complex comprising a negative feedback mechanism for canonical WNT signaling [220-222]. Adenomatous polyposis coli (APC) derives its name from observations that mutations in the gene result in the development of familial adenomatous polyposis, an inherited condition

in which numerous polyps form mainly in the epithelium of the large intestine [223]. The function of APC in the destruction complex has not completely been elucidated, as some researchers propose that it causes retention of β-catenin at the complex, while others suggest that it promotes the release of phosphorylated β-catenin from the destruction complex [172, 224, 225]. In addition, APC may also be involved in the nuclear shuttling of β-catenin, as will be discussed further on. Moreover, APC was originally classified as a tumor suppressor, which is mutated in 80% of the cancers resulting in constitutive complexes of β-catenin with downstream transcription factors in the nucleus [223, 226]. Taken together, the exact function of APC in the destruction complex is not yet fully established, but it is essential in the regulation of cellular β-catenin expression.

The main action of the destruction complex is to target β-catenin by phosphorylation in order to make it recognizable for the β-transducin repeat containing protein-1 (β-TrRC-1), a member of the SCF (Skp1/Cullin/F-box) ubiquitin ligase complex [227, 228]. CK-1α and GSK-3 are the kinases associated with the destruction complex and are responsible for the hierarchical phosphorylation of the N-terminal domain of β-catenin [172, 218, 229, 230]. The proposed mechanism of action is that β-catenin associates with either axin or APC in the destruction complex, thereby bringing the N-terminal domain of β-catenin in close proximity to CK-1, which in turn causes the serine 45 (ser45) phosphorylation of β-catenin [172]. GSK-3 favors substrates that have been primed in advance by phosphorylation and this ser45 phosphorylation makes β-catenin a primed substrate [173]. Consequently, GSK-3 phosphorylates β-catenin from the C-terminal to the N-terminal domain at threonine 41 (thr41), ser37 and ser33 in addition to ser45 [173, 218]. Moreover, GSK-3 also alternately phosphorylates axin and APC, which is required for proper handling of β-catenin within the destruction complex [172, 218]. The phosphorylated β-catenin leaves the destruction complex, a process possibly mediated by APC, to be ubiquitinated and subsequently degraded [218, 225]. WNT signaling reduces the N-terminal phosphorylation of β-catenin, thereby allowing cytosolic accumulation of the protein. Although there seems to be a general consensus that WNT signaling inhibits the GSK-3 mediated phosphorylation of β-catenin, the effect on the CK-1-induced phosphorylation is still a point of debate [231, 232].

As mentioned, the composition of the destruction complex is dynamic and the presence of protein phosphatases may influence the function of the destruction complex [172, 233, 234]. For instance, protein phosphatase 1 (PP1) regulates the assembly of such a functional β-catenin degradation complex. Inhibition of PP1 results in increased association of GSK-3 to axin, due to increased phosphorylation of the latter [233]. Controversial results have been published concerning the role of protein phosphatase 2A (PP2A) in WNT signaling. This phosphatase is able to dephosphorylate the GSK-3-mediated phosphorylation of axin and APC, thereby disrupting the destruction complex and facilitating β-catenin activation. In addition, the phosphorylation of β-catenin can be reversed by PP2A, which furthermore

supports canonical WNT pathway activation. Conversely, it has been implied that PP2A influences GSK-3 activity by regulating the phosphorylation status of the kinase and thereby decreasing β-catenin activation [172, 234].

2.8 Canonical WNT ligand mediated inhibition of the β-catenin destruction complex

Central in the transmission of canonical WNT signaling is the functional inhibition of the β-catenin destruction complex. The direct downstream events of canonical WNT ligand binding to the cellular surface receptors are the activation of DVL, which act downstream of FZD receptors, and the phosphorylation of the intracellular domain of LRP5/6. Therefore, how WNT signaling affects the destruction complex requires understanding of the function of DVL proteins and LRP5/6 receptors and their relation to the destruction complex.

The subcellular distribution of DVLs is highly dynamic and in response to a WNT signal the majority of cellular expressed DVL is recruited to the plasma membrane, whereas a small fraction translocates to the nucleus, which correlates to the activation of canonical WNT signaling [154, 235]. DVLs are signaling intermediates that do not contain enzymatic motifs but various putative protein-protein interaction domains [152]. At the plasma membrane, DVL guides the clustering of the FZD receptor and LRP6 as well as facilitation of the phosphorylation of the latter [154, 168, 236]. Furthermore, activated DVL causes the relocation of axin and/or GSK-3 to the plasma membrane and inhibits their function [154, 237]. DVL proteins are important for the clustering of protein complexes, however, not necessarily at the plasma membrane as mutated DVL proteins that lack the membrane association domain can still activate WNT signaling [238]. The functional inhibition of GSK-3 was proposed to be most likely due to DVL-mediated conformational changes in axin, thereby limiting substrate accessibility and not via direct inhibition of the kinase [237]. This indirect inhibitory mechanism is all the more likely, since WNT ligands do not seem to induce serine 9/21 phosphorylation of GSK-3, a general mechanism applied by other growth factors to inhibit the kinase [239-241]. In addition, DVL has also been implicated to facilitate the displacement of GSK-3 from the destruction complex, more particularly the dissociation from axin. This process has been proposed to be mediated by the GSK-3 binding protein (GBP) and the closely related protein frequently rearranged in T-cell lymphoma (FRAT-1). DVL binds to GBP/FRAT-1 at the N-terminal domain and this will lead to the additional association to GSK-3 at a domain, which is also required for binding of the kinase to axin [242, 243]. In addition, DVL-3 is capable of forming a complex with K-homology splicing regulator protein (KSRP), which negatively affects the mRNA stability of β-catenin at basal conditions [244].This can be reversed upon WNT-3A stimulation, indicating that DVL can also modulate canonical WNT signaling independently of the destruction-complex [244]. Although extensively studied, the biochemical pathways leading to DVL activation and downstream signaling events resulting in inhibition of the destruction complex are still not completely understood.

Simultaneously, the LRP5/6 receptor activation contributes to WNT signaling by regulating the composition and activity of the destruction complex. An important consequence of WNT-induced activation is the recruitment of both axin and GSK-3 to LRP5/6 at the cell-membrane [151, 163, 245]. Activation of LRP5/6 has been proposed to inhibit the formation of the destruction complex and/or cause disruption of this complex by promoting the degradation of axin [246, 247]. As noted, the assembly of a destruction complex is critically dependent on the expression of axin as it is the lowest expressed core component [222]. Therefore, the degradation of axin provides a vigorous mechanism for increasing β-catenin stability. Nevertheless, increased stabilization and accumulation of β-catenin precedes the degradation of axin [248, 249]. Moreover, canonical WNT signaling also occurs in the absence of axin degradation, suggesting an additional mechanism that regulates β-catenin stability [249]. An alternative could be that activated LRP receptors directly inhibit the destruction complex. Indeed, the phosphorylated cytoplasmic domain of LRP6 receptors is able to directly inhibit GSK-3 by acting as a (pseudo) substrate for the kinase and this has consequences for proper functioning of the destruction complex [250]. First of all, β-catenin is not directly phosphorylated anymore and secondly the alternately GSK-3-mediated phosphorylation of axin and APC is also prevented. Subsequently, axin can be dephosphorylated by PP1 resulting in the dissociation both GSK-3 and β-catenin from axin [218, 251]. Recently, a distinct cell-biological model was proposed by which WNT signaling intervenes with the destruction complex. This model postulates that WNT ligands induce the uptake of GSK-3 into multivesicular bodies (MVBs), which is mediated by LRP5/6 and axin [252, 253]. The MVBs sequester the enzyme away from cytosolic β-catenin, allowing the latter to accumulate [253].

Collectively, the WNT signal induced recruitment of axin and GSK-3 to the LRP receptors may disrupt the organized structure of destruction complex, preventing the complex to function effectively to target β-catenin. The molecular mechanisms mentioned here, have been reported to contribute to canonical WNT signaling and disengage the destruction complex. Nevertheless much remains to be clarified concerning the spatiotemporal regulation of the transcriptional co-activator β-catenin by the destruction complex.

2.9 Translocation of β-catenin to the nucleus

Accumulation of β-catenin in the cytoplasm, due to decreased proteosomal degradation, coincides with the translocation of β-catenin into the nucleus via currently not completely established mechanisms (figure 2). For instance, β-catenin lacks a specific nuclear localization sequence (NLS), in contrast to various other proteins that contain this sequence and translocate to the nucleus via the importin-α/β receptor pathway [254, 255]. Furthermore, the nuclear translocation of solely β-catenin does not seem to be dependent on either importin-β or Ran-mediated protein import systems [255-257]. Another proposed mechanism involves specific transcription factor-mediated nuclear translocation, as these proteins bind β-catenin, contain a NLS sequence and therefore possibly act as chaperones guiding

β-catenin into the nucleus via a conventional nuclear import pathway [255, 258-260]. Indeed, overexpression of β-catenin or TCF/LEF-1 transcription factors results in increased nuclear import of β-catenin in the absence of WNT signaling [261-263]. Accordingly, under physiological circumstances the nuclear translocation of β-catenin is mediated by LEF-1 which requires an additional WNT-dependent signal, however [264]. Moreover, the TCF/LEF-1 family of transcription factors also inhibits nuclear export, resulting in nuclear retention of β-catenin [265-267]. Nonetheless, specific mutations in the TCF/LEF-1 interaction regions of *Armadillo* (the *Drosophila Melanogaster* ortholog of β-catenin), resulted in a preferential nuclear expression of protein without having transcriptional activity, however [268, 269]. These findings indicate that there should be alternative mechanisms for nuclear translocation of β-catenin. Similarly to the TCF/LEF-1-mediated mechanism, the recently discovered proteins pygopus (Pygo) and legless (Lgs or BCL-9 in mammals) have been shown to cooperatively regulate nuclear localization of β-catenin by shuttling between cytosol and the nucleus (figure 2) [270, 271]. In addition, in response to a WNT signal a small fraction of cellular DVL translocates to the nucleus, which is essential for canonical WNT signaling [235]. The three distinct DVL proteins all contain a NLS domain and have been found to be able to interact with β-catenin [235, 272, 273]. Therefore it is plausible that DVLs also act as a chaperone guiding β-catenin into the nucleus, however no direct evidence of this cellular transport mechanism has been published so far.

By contrast, there is evidence indicating that β-catenin translocation can be independent of chaperones. Indeed, β-catenin may also bind directly to the nuclear pore machinery and the subsequent nuclear import requires a not yet identified GTPase and is analogous to that of importin-β [255, 257, 274]. These similarities in cellular trafficking of both these proteins are likely explained by the fact that the armadillo (arm) repeats in β-catenin and the HEAT repeats in importin-β are structurally related and compete for the same docking component in the nuclear pore [255, 275, 276]. Recently a study proposed that the Rac1 GTPase may be required for the nuclear accumulation of β-catenin in response to WNT ligand stimulation [277]. Yet, activation of small GTPases like Rac1 is a particular event observed in non-canonical (i.e β-catenin independent) WNT signaling and therefore future studies will be required to substantiate the involvement of Rac1 in this process [115, 278]. In addition to nuclear import, nuclear export is also a dynamic process and an important determinant in the ability of β-catenin to be transcriptionally active in the nucleus [257]. The first evidence of nuclear export of β-catenin originates from 1999 and soon thereafter the scaffold protein APC was implicated as an important regulator of β-catenin signaling and cellular localization [279-282]. APC shuttles between the nucleus and cytoplasmic compartment, thereby escorting β-catenin to the cytosol, where it is targeted by phosphorylation for ubiquitination and ultimately proteosomal degradation (figure 2) [276, 279, 283, 284]. Axin, also a negative regulator of WNT signaling, can shuttle in and out of the nucleus similarly to APC, and thereby affecting β-catenin retention in the nucleus.

However, the physiological relevance of axin-dependent modulation of nuclear β-catenin is not yet established [285].

The associations of β-catenin with specific interaction partners determine to a large extend the cellular localization of the transcriptional co-activator. The association of β-catenin with TCF/LEF-1 predominantly mediates nuclear retention, while cadherins mediate retention at plasma-membrane and axin may facilitate cytosolic localization (figure 2) [266, 276]. Collectively, this indicates that the cellular localization of β-catenin is regulated by complex, dynamic processes and that extracellular WNT signals are important in orchestrating this process resulting in increased nuclear expression of the protein.

2.10 Nuclear β-catenin signaling

In the nucleus the canonical WNT signaling induces gene transcription via the activation of TCF/LEF-1 family of transcription factors (i.e. TCF-1, LEF-1, TCF-3 and TCF-4), which were discovered as lymphoid specific DNA binding proteins and share an almost identical high mobility (HMG) domain [286-288]. In the absence of WNT/β-catenin, the TCF/LEF-1 transcription factors are bound to so-called WNT responsive elements in the DNA, but they act as active suppressors of gene transcription [289, 290]. This repression of WNT target genes requires transducin-like enhancer of split (TLE), Groucho in *Drosophila Melanogaster*, a chromatin repressor that functions with histone deacetylases (HDACs) to facilitate chromatin compression and inhibition of transcription [291-294]. Already in the cytosol or once in the nucleus, β-catenin associates with the members of the TCF/LEF-1 family of transcription factors and forms a bipartite transcriptional complex. Importantly, the transmission of WNT signaling is performed by N-terminally dephosphorylated β-catenin, which is also referred to as active β-catenin [295]. How β-catenin causes the molecular switch of gene suppression to active gene transcription by binding to these transcription factors has long been an unacquainted issue. The more so, since it was a general premise that β-catenin and TLE/Groucho bind to distinct sites of TCF/LEF-1 [289]. However, in 2005 it became clear that β-catenin directly replaces TLE/Groucho from TCF/LEF-1 by binding to an additional and previously unidentified site within the transcription factor, which overlaps with the binding site of suppressor [296]. The function of the TCFs are tightly regulated in the nucleus by a variety of mechanisms, and these posttranslational modifications include phosphorylation, sumoylation, ubiquitination and acetylation, which potentially contribute to the interaction of the transcription factors with other proteins or the DNA [297]. For instance, the Nemo-like kinase (NLK) antagonizes the action of TCF/LEF by phosphorylation of a domain distinct from the HMG-DNA binding domain; however, this modification does inhibit DNA binding [298]. In addition, the transcriptional potential of β-catenin can be blocked by Chibby (Cby), which interacts with the C-terminal activation domain of β-catenin and competes with TCF/LEF [299, 300]. Similarly, the polypeptide inhibitor of β-catenin and TCF-4 (ICAT) prevents gene transcription by binding to β-catenin (figure 2) [301].

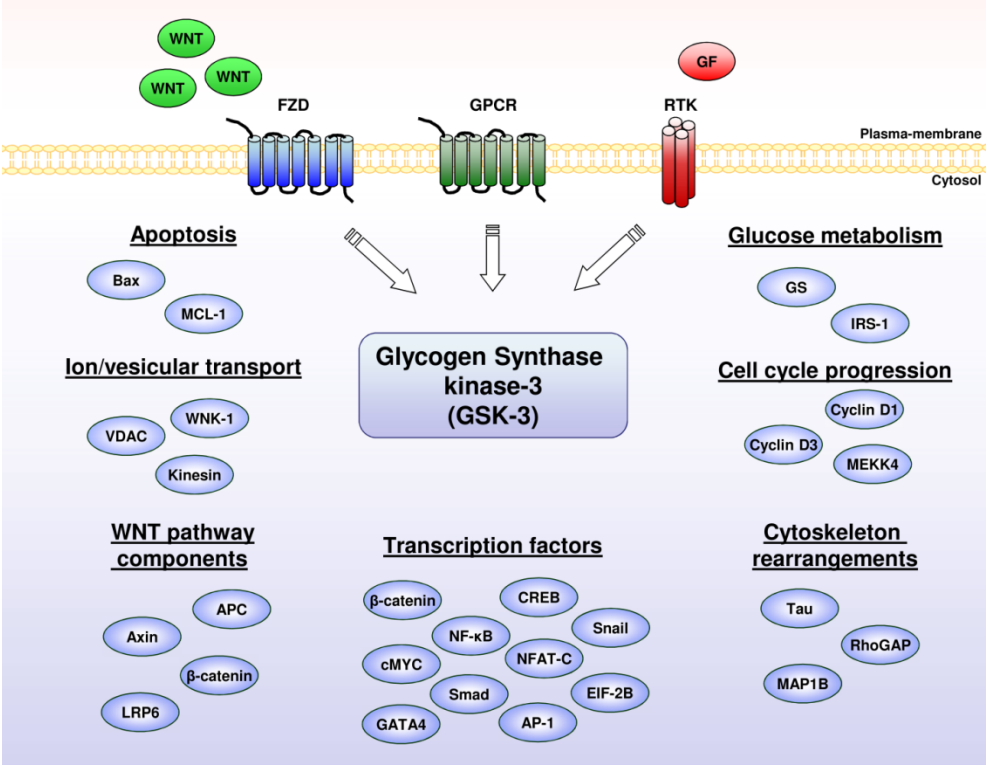
The central armadillo repeats within β-catenin are important for inducing gene transcription and these are required for the interaction with the transcription factors, in particular armadillo repeats 3-10 (R3-R10) form the core of the TCF/LEF-1 interaction. When TCF/LEF-1 occupies these repeats, the C- and N-terminal domains of β-catenin will recruit additional factors that modulate the DNA chromatin structure and that are involved in the regulation of RNA polymerase II [291]. The β-catenin/TCF/LEF-1 complex recruits factors with intrinsic histone acetyltransferase (HAT) activity, like cAMP responsive element binding protein (CREB) binding protein (CREBBP or CBP) or p300 [291]. The HMG domain in the TCFs induces a strong bending of the DNA upon binding, bringing specific but distant DNA sequences together, allowing accessibility to HATs and inducing gene transcription [291, 302]. The list of WNT target genes is growing and over a hundred genes have been demonstrated to be dependent on β-catenin and to require the interaction with TCF/LEF-1 transcription factors [303]. The genes regulated by β-catenin/TCF/LEF-1 are involved in a broad range of (cellular) functions important in embryonic development and tissue homeostasis. The wide array of β-catenin-dependent target genes encompasses various transcription factors (e.g. members of the Sox family, c-myc, n-myc, LEF-1 and TCF), cell-cycle regulatory proteins (e.g. cyclin D1), growth factors (e.g. VEGF, TGF-β and FGF18), extracellular matrix proteins (e.g. versican and fibronectin), pro-inflammatory cytokines and enzymes (e.g. IL-8 and COX-2) as well as proteinases, including several matrix metalloproteinases (i.e. MMP-2, MMP-7, MMP-12) [303]. WNT signaling is an autoregulated signaling pathway, signifying that also positive and negative regulators of the pathway (e.g. Wg, FZD_7, DKK-1 and sFRP-2) are target genes that are controlled by β-catenin and TCF/LEF-1 transcription factors [303]. Aberrant activation of canonical WNT signaling can result in development of cancers and contributes to various fibroproliferative diseases [111, 304].

3. Glycogen synthase kinase-3 (GSK-3) signaling

As mentioned, GSK-3 is a critical regulator of canonical WNT signaling by phosphorylating β-catenin, thereby making β-catenin recognizable for the ubiquitin ligase complex and subsequent proteosomal degradation [227, 228]. Yet, GSK-3 was first discovered based on its ability to regulate glycogen metabolism, as the enzyme that phosphorylates and thereby inactivates glycogen synthase, for obvious reasons explaining the name of the kinase [305]. However, the name does not adequately describe the quantity of substrates and cellular functions attributed to GSK-3 [306]. The enzyme has been associated with a wide variety of cellular processes, including next to glycogen synthesis, insulin signaling, microtubule dynamics and cytoskeletal rearrangement, cell differentiation, protein synthesis, cell motility, intracellular vesicular transport, as well as cell proliferation and cell survival (figure 3) [174, 306]. GSK-3 appears to be a cellular and molecular processor, integrating various signaling pathways by utilizing over 50 structural proteins, intracellular signaling proteins and transcription factors, including β-catenin, tau (τ), neurofilaments, components of the nuclear factor kappa B (NF-κB) pathway, cAMP responsive element binding protein (CREB), smads, several

members of the cyclin protein family, activator protein-1 (AP-1) and eukaryotic initiation factor 2B (eIF2B) [307].

GSK-3 is a ubiquitously expressed serine/threonine kinase occurring in two closely related isoforms; GSK-3α (located on chromosome: 19q13.2, MW: ~51 kDa) and GSK-3β (chromosome 3q13.33, MW: ~46 kDa). The two isoforms share high homology, with up to 97-98% homology in their kinase domain [308, 309]. Recently, it was demonstrated that the *GSK-3β* gene could be alternatively spliced resulting in a GSK-3β variant (called GSK-3β' or GSK-3β2), which is predominantly or exclusively present in the brain and neuronal tissues [310, 311]. GSK-3α and GSK-3β have similar substrate specificity and overlapping functions; however, they are not completely functionally redundant. This became unambiguously evident in GSK-3 knockout mice. GSK-3α$^{-/-}$ mice are viable and revealed increased glucose tolerance and insulin sensitivity compared to wild type mice, whereas deletion of GSK-3β (GSK-3β$^{-/-}$ mice) causes apoptosis of hepatocytes and results in embryonic lethality [312, 313]. Interestingly, the GSK-3α$^{-/-}$ mice do not show increased β-catenin stabilization or a higher prevalence of tumorigenesis [306]. This indicates that the loss of GSK-3α could be compensated for by GSK-3β; however, evidently not the other way around.



▲ **Figure 3: Cellular processes regulated by GSK-3.** Glycogen synthase kinase-3 (GSK-3) is a multifunctional kinase regulating cellular responses by integration of various signaling pathways downstream of FZD receptors, various G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). GSK-3 exerts its actions by phosphorylation of over 50 substrates involved in glucose metabolism, cell cycle progression, cytoskeletal rearrangements, apoptosis, ion transport and intracellular vesicular transport. Moreover, it is critically involved in canonical WNT signaling and regulates the potency of a wide variety of transcription factors (see text for detailed description).

3.1 Regulation of GSK-3 activity

A wide variety of cellular processes is dependent on GSK-3 signaling and therefore it is crucial that the enzyme activity is tightly regulated. GSK-3 is an unconventional enzyme as it is considered to be a constitutively active kinase, that means high kinase activity at basal conditions, which is altered in response to a variety of endogenous and exogenous signals [306, 314]. As mentioned, the kinase function is disrupted by WNT signaling due to sequestration of the kinase away from the substrate, by disruption of the β-catenin destruction complex or by its interaction with phosphorylated LRP5/6 receptors [242, 243, 250, 252, 253]. In addition, the activity of GSK-3 is differentially regulated by phosphorylation, dependent on which specific amino acid residues are phosphorylated within the enzyme. Tyrosine (tyr) phosphorylation of GSK-3 (i.e. tyr279 GSK-3α and tyr216 GSK-3β) has been demonstrated to increase the enzymatic activity of the kinase [315]. To date, three tyrosine kinases have been identified that are able to induce tyrosine phosphorylation of the GSK-3.These are Fyn, a protein kinase member of the Src family, PYK2, a member of the focal adhesion kinase family, and the mitogen-activated protein kinase kinase (MAPKK) MEK1 [315-318]. In addition to the modest increase in enzyme activity, the phosphorylation of GSK-3 at these specific tyrosine residues may be of additional physiological relevance, as these tyrosine residues are also important for determining the cellular localization of GSK-3 [319].

On the contrary, phosphorylation of specific serine residues in the N-terminus, i.e. ser21 on GSK-3α and ser9 on GSK-3β, negatively influences the kinase activity [320, 321]. Insulin is the exemplifying hormone for inducing cellular signaling via GSK-3 inhibition by phosphorylation of these serine residues. Initially, it was demonstrated that insulin induces dephosphorylation of glycogen synthase, at amino acid residues targeted by GSK-3 [322]. Soon thereafter, evidence showed that insulin directly inhibited GSK-3 via the induction of serine phosphorylation, which was mediated by protein kinase B (PKB/Akt) [320]. Thus, in response to insulin, GSK-3 activity is attenuated resulting in decreased glycogen synthase phosphorylation and consequently increased glycogen synthesis. In addition to PKB (Akt), several other kinases including p70 S6 kinase (p70S6K), p90 ribosomal S6 kinase (p90 Rsk), PKA, Integrin-like kinase (ILK) as well as several PKC isoforms can induce the inhibitory serine phosphorylation of GSK-3 [323, 324]. The phosphorylation of these specific serine residues turns the N-terminal domain of GSK-3 into a pseudosubstrate for the kinase. The phospho-serines occupy a binding pocket within GSK-3, which is distinct from the kinase's catalytic site. Nevertheless, the binding pocket is also the target of primed (i.e. pre-phosphorylated) substrates and upon auto-inhibition by the phospho-serines the accessibility of other (primed) substrates to the catalytic domain of the kinase is competitively prevented [173, 314, 325]. Calpain, a protein belonging to the family of non-lysosomal cysteine proteases, is capable of cleaving the inhibitory N-terminal domain of GSK-3 and thereby prevents the possibility of auto-inhibition of the enzyme [326]. Besides insulin, a variety of other growth factors and stimuli are capable of inhibiting GSK-3 by serine 9 and 21 phosphorylation, important for the

metabolic effects of these mediators [320, 321]. Growth factors and WNT signaling both induce inhibition of GSK-3; however, the mechanisms of inhibition are dissimilar and it appears that this may lead to differential downstream signaling and consequently distinct cellular responses [241, 327]. Moreover, whether growth factor-induced GSK-3 inhibition contributes to increased stability of β-catenin is still a point of debate [213, 241, 241, 327, 328].

Furthermore, cellular signaling by GSK-3 is determined by substrate preference, as the enzyme favors substrates that have been primed in advance by phosphorylation at amino residues positioned four amino acids C-terminally from the serine (ser) of threonine (thr) residue to be targeted by GSK-3 [173, 314, 325, 329]. For clarification, the consensus sequence for GSK-3 substrates is ser/thr-X-X-X-ser-p/thr-p, where the first ser or thr is the target residue for GSK-3, the X represents variable amino acid residues, and the last ser-p/thr-p is the site of priming phosphorylation. The crystal structure of GSK-3 reveals that the substrate binding domain contains several charged residues. These charged residues permit interaction with the phosphate group of the primed substrate, thereby facilitating substrate binding and accessibility to the catalytic domain of GSK-3 [173, 330]. The specific priming sequence can be found for instance in glycogen synthase, CREB, Cyclin E, τ (tau) and β-catenin [174, 325, 329]. Accordingly, several priming kinases have been identified including casein kinases (i.e. CK-1 and CK-2) and PKA [174, 325, 329]. Although GSK-3 favors the primed substrates, priming of the substrate is not necessarily required.

Collectively, GSK-3 contributes to an extensive array of cellular responses and the activity of the enzyme is tightly regulated by protein-protein interactions (WNT signaling), the phosphorylation status of specific amino acid residues within the kinase, and substrate preferentiality. The functional relevance of GSK-3 in cellular signaling has been extensively studied both *in vitro* and *in vivo*, using small molecule inhibitors of the enzyme, like lithium ions (Li^+) and a wide variety of specific synthetic inhibitors [331-334].

3.2 GSK-3 as potential therapeutic target

Because of the wide variety of putative substrates, GSK-3 signaling has been associated with several pathophysiological conditions, especially the metabolic disorder diabetes mellitus and the neurodegenerative Alzheimer's disease [335]. As previously stated, GSK-3 is critically involved in glucose metabolism in response to insulin by regulating glycogen synthase activity. The expression and, more importantly, the activity of the GSK-3 appears to be upregulated in skeletal muscle of individuals with type II diabetes mellitus suggesting that the kinase could contribute to insulin insensitivity and hence development of diabetes mellitus [334]. Furthermore, in Alzheimer's disease the neurofibrillary tangles, important in the neuropathology, are composed of hyperphosphorylated τ (tau, a microtubule-associated protein), which is attributed to GSK-3 [336, 337]. Accordingly, differences in protein expression and kinase activity were found in specific brains

regions of individuals with Alzheimer's disease post-mortem [337]. Furthermore, during the last decade it has become apparent that GSK-3 is possibly capable of regulating the activation of the NF-κB pathway or that it influences the transcriptional response of this pathway [312, 338]. Accordingly, several inhibitors of GSK-3 have been demonstrated both *in vitro* and *in vivo* to influence NF-κB mediated cellular responses [338]. Activation of NF-κB signaling plays an important role in inflammation and is associated with various chronic pathological conditions including cancer, arthritis, colitis and chronic inflammatory lung diseases [339-341].

Ergo, GSK-3 could be a potential target for treatment of several chronic (inflammatory) diseases. However, a potential drawback of using GSK-3 inhibitors may be the increased activation of β-catenin signaling [306, 329, 338]. Interestingly, inhibition of GSK-3 is often, but not necessarily, the primary step in increasing cellular β-catenin expression. Therefore, (pharmacological) inhibition of GSK-3 as an independent event does not always result in increased β-catenin levels and/or TCF-dependent gene transcription, unless it is accompanied by an additional signal [332, 342, 343]. Furthermore, the degree of inhibition of kinase activity may be an important determinant of the cellular response. For instance, attenuation of GSK-3 activity up to 50% does not result in increased β-catenin but may have metabolic effects, whereas it requires over 70-75% reduction of GSK-3 enzymatic activity to observe substantial differences in the expression of this transcriptional co-activator [344]. In general, clinically used therapeutics reduce the activity of proteins and kinases, rather than completely abrogating activity. The therapeutic concentrations of lithium, which is used as the primary treatment of bipolar mood disorders, are sufficient to inhibit GSK-3 *in vivo* [329]. Epidemiological evidence indicates that long-term treatment of patients with lithium does not increase cancer morbidity and mortality, but is rather associated with a beneficial effect on cancers and mortality [345]. These findings are favorable to further investigate the opportunity of using selective GSK-3 inhibitors for treatment of several (chronic) diseases. Thus, GSK-3 may be a potential target for therapeutic intervention and small molecule inhibitors of GSK-3 are currently investigated (pre)clinically for the treatment of Alzheimer's disease and diabetes mellitus [335, 346, 347]. Nevertheless, the role of GSK-3 and the possible effects of small molecule inhibitors of the enzyme on development and progression of chronic inflammatory lung diseases are currently to a large extent an unresolved field or research, in particular in relation to COPD pathogenesis.

4. Scope of thesis

Collectively, β-catenin signaling and GSK-3 are often interconnected, but can also function independently of one another. Both proteins are importantly involved in a wide variety of cellular processes and aberrant regulation of both has been associated with several chronic diseases. COPD is a pathological condition of the lungs characterized by chronic inflammation as well as structural alterations in lung architecture. The molecular mechanisms contributing to the pathophysiology of COPD have not yet been fully elucidated. The primary objective of this thesis is to establish the functional roles of β-catenin and GSK-3 in the pathological processes that underpin COPD. To this aim, we performed experiments both *in vitro* and *in vivo* by using human airway smooth muscle cells, human pulmonary fibroblasts and an established guinea pig model of lipopolysaccharide (LPS)-induced COPD [348].

As stated before, airway remodeling is a major pathological feature of COPD and an aberrant ECM deposition has been found in and surrounding the smooth muscle layer of the airways of individuals with COPD [45]. **Chapter 2** describes the activation of β-catenin signaling in response to the profibrotic growth factor TGF-β_1 and the contribution of the transcriptional co-activator β-catenin to ECM deposition by human airway smooth muscle cells. The contribution of β-catenin signaling to cellular responses in these cells was investigated by silencing of the protein by specific siRNA and by pharmacological inhibition of its signaling capacity, using the small molecule PKF115-584. In addition, the relevance of β-catenin signaling was studied by expressing a transcriptionally active, non-degradable β-catenin mutant (S33Y-β-catenin).

In addition to airway remodeling with excessive ECM deposition, COPD is characterized by the occurrence of emphysema with loss of parenchymal tissue and a possibly insufficient repair process. Fibroblasts play an important role in tissue repair in the lungs and fibroblast function may be altered in emphysema. In **chapter 3** we characterized the expression of WNT pathway components in pulmonary fibroblasts and explored the participation of β-catenin signaling to ECM production and myofibroblast differentiation. Furthermore, we determined whether the expression of specific WNT pathway components and the activation of β-catenin in response to TGF-β_1 were different in primary human lung fibroblasts of individuals without or with moderate/very severe COPD. Moreover, we established the role of GSK-3 in the differentiation of these pulmonary fibroblasts into more active myofibroblasts (**chapter 4**). Myofibroblast differentiation was initiated by stimulating pulmonary fibroblasts with TGF-β_1 and the role of GSK-3 was investigated by pharmacological inhibition of the kinase by the selective inhibitor SB216763. Downstream target proteins regulating TGF-β_1 signaling were analyzed for alterations in activity status due to GSK-3 inhibition.

Chronic inflammation is another hallmark feature of COPD pathogenesis and accumulating evidence indicates the involvement of GSK-3 in regulating

inflammation. The airway smooth muscle, due to its synthetic function, is a possible source of pro-inflammatory cytokines. The studies described in **chapter 5** investigate the involvement of GSK-3 in IL-8, eotaxin and vascular endothelial growth factor (VEGF) secretion in human airway smooth muscle. To activate the secretion of these cytokines and the growth factor, the airway smooth muscle cells were stimulated with cigarette smoke extract (CSE) or IL-1β, two stimuli relevant in COPD pathogenesis. The potential involvement of GSK-3 in the cytokine and growth factor secretion was investigated using the two distinct small molecule inhibitors SB216763 and CT/CHIR99021.

To further elaborate the *in vitro* findings we investigated the possibility of GSK-3 as a potential therapeutic target *in vivo* in a guinea pig model of LPS-induced COPD (**chapter 6**). The animals were instilled intranasally with saline or LPS, twice weekly for 12 weeks, and pretreated with saline or the selective GSK-3 inhibitor SB216763. The effects of GSK-3 inhibition on LPS-induced inflammation and structural alterations in the lungs were investigated.

References

1. J.Vestbo, A.G.Augusti, A.Anzueto, P.J.Barnes, L.M.Fabbri, P.Jones, et al. Global initiative for Chronic Obstructive Lung Diseases (GOLD); Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease (revised 2011). 2011.
2. Molfino NA, Jeffery PK (2007) Chronic obstructive pulmonary disease: histopathology, inflammation and potential therapies. Pulm Pharmacol Ther 20: 462-472.
3. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, et al. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 176: 532-555.
4. Mathers CD, Loncar D (2006) Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 3: e442.
5. Eisner MD, Anthonisen N, Coultas D, Kuenzli N, Perez-Padilla R, et al. (2010) An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 182: 693-718.
6. Jeffery PK (2004) Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 1: 176-183.
7. Hogg JC (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 364: 709-721.
8. Di SA, Capelli A, Lusuardi M, Balbo P, Vecchio C, et al. (1998) Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am J Respir Crit Care Med 158: 1277-1285.
9. Barnes PJ (2008) Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 8: 183-192.
10. Barnes PJ (2004) Alveolar macrophages as orchestrators of COPD. COPD 1: 59-70.
11. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M (2003) Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. Immunol Cell Biol 81: 289-296.
12. Vignola AM, Chanez P, Chiappara G, Merendino A, Zinnanti E, et al. (1996) Release of transforming growth factor-beta (TGF-beta) and fibronectin by alveolar macrophages in airway diseases. Clin Exp Immunol 106: 114-119.
13. Keatings VM, Collins PD, Scott DM, Barnes PJ (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am J Respir Crit Care Med 153: 530-534.
14. Culpitt SV, Rogers DF, Shah P, De MC, Russell RE, et al. (2003) Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 167: 24-31.
15. Janson RW, Hance KR, King TE, Jr. (1995) Human alveolar macrophages produce predominantly the 35-kD pro-forms of interleukin-1 alpha and interleukin-1 beta when stimulated with lipopolysaccharide. Am J Respir Crit Care Med 151: 1613-1620.
16. Beeh KM, Beier J (2006) Handle with care: targeting neutrophils in chronic obstructive pulmonary disease and severe asthma? Clin Exp Allergy 36: 142-157.
17. Shapiro SD (2003) Proteolysis in the lung. Eur Respir J Suppl 44: 30s-32s.
18. Sharafkhaneh A, Hanania NA, Kim V (2008) Pathogenesis of emphysema: from the bench to the bedside. Proc Am Thorac Soc 5: 475-477.
19. Roghanian A, Sallenave JM (2008) Neutrophil elastase (NE) and NE inhibitors: canonical and noncanonical functions in lung chronic inflammatory diseases (cystic fibrosis and chronic obstructive pulmonary disease). J Aerosol Med Pulm Drug Deliv 21: 125-144.
20. Nadel JA (2000) Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. Chest 117: 386S-389S.
21. Noguera A, Batle S, Miralles C, Iglesias J, Busquets X, et al. (2001) Enhanced neutrophil response in chronic obstructive pulmonary disease. Thorax 56: 432-437.
22. Oudijk EJ, Nijhuis EH, Zwank MD, van de Graaf EA, Mager HJ, et al. (2005) Systemic inflammation in COPD visualised by gene profiling in peripheral blood neutrophils. Thorax 60: 538-544.

23. Wedzicha JA, Donaldson GC (2003) Exacerbations of chronic obstructive pulmonary disease. Respir Care 48: 1204-1213.
24. Bocchino V, Bertorelli G, Bertrand CP, Ponath PD, Newman W, et al. (2002) Eotaxin and CCR3 are up-regulated in exacerbations of chronic bronchitis. Allergy 57: 17-22.
25. Qiu Y, Zhu J, Bandi V, Atmar RL, Hattotuwa K, et al. (2003) Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 168: 968-975.
26. Saetta M, Di SA, Maestrelli P, Turato G, Ruggieri MP, et al. (1994) Airway eosinophilia in chronic bronchitis during exacerbations. Am J Respir Crit Care Med 150: 1646-1652.
27. Brusselle GG, Joos GF, Bracke KR (2011) New insights into the immunology of chronic obstructive pulmonary disease. Lancet 378: 1015-1026.
28. Van Pottelberge GR, Bracke KR, Joos GF, Brusselle GG (2009) The role of dendritic cells in the pathogenesis of COPD: liaison officers in the front line. COPD 6: 284-290.
29. Saetta M, Di SA, Turato G, Facchini FM, Corbino L, et al. (1998) CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 157: 822-826.
30. Lams BE, Sousa AR, Rees PJ, Lee TH (2000) Subepithelial immunopathology of the large airways in smokers with and without chronic obstructive pulmonary disease. Eur Respir J 15: 512-516.
31. O'Shaughnessy TC, Ansari TW, Barnes NC, Jeffery PK (1997) Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. Am J Respir Crit Care Med 155: 852-857.
32. Gadgil A, Duncan SR (2008) Role of T-lymphocytes and pro-inflammatory mediators in the pathogenesis of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 3: 531-541.
33. Urbanowicz RA, Lamb JR, Todd I, Corne JM, Fairclough LC (2010) Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients. Respir Res 11: 76.
34. Grumelli S, Corry DB, Song LZ, Song L, Green L, et al. (2004) An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. PLoS Med 1: e8.
35. Chrysofakis G, Tzanakis N, Kyriakoy D, Tsoumakidou M, Tsiligianni I, et al. (2004) Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. Chest 125: 71-76.
36. van der Strate BW, Postma DS, Brandsma CA, Melgert BN, Luinge MA, et al. (2006) Cigarette smoke-induced emphysema: A role for the B cell? Am J Respir Crit Care Med 173: 751-758.
37. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, et al. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 350: 2645-2653.
38. Brusselle GG, Demoor T, Bracke KR, Brandsma CA, Timens W (2009) Lymphoid follicles in (very) severe COPD: beneficial or harmful? Eur Respir J 34: 219-230.
39. Brandsma CA, Kerstjens HA, Geerlings M, Kerkhof M, Hylkema MN, et al. (2011) The search for autoantibodies against elastin, collagen and decorin in COPD. Eur Respir J 37: 1289-1292.
40. Isajevs S, Taivans I, Svirina D, Strazda G, Kopeika U (2011) Patterns of inflammatory responses in large and small airways in smokers with and without chronic obstructive pulmonary disease. Respiration 81: 362-371.
41. Cigarette smoking and health. American Thoracic Society (1996) Am J Respir Crit Care Med 153: 861-865.
42. Turato G, Di SA, Maestrelli P, Mapp CE, Ruggieri MP, et al. (1995) Effect of smoking cessation on airway inflammation in chronic bronchitis. Am J Respir Crit Care Med 152: 1262-1267.
43. Rutgers SR, Postma DS, Ten Hacken NH, Kauffman HF, van Der Mark TW, et al. (2000) Ongoing airway inflammation in patients with COPD who Do not currently smoke. Chest 117: 262S.
44. Miller M, Cho JY, Pham A, Friedman PJ, Ramsdell J, et al. (2011) Persistent airway inflammation and emphysema progression on CT scan in ex-smokers observed for 4 years. Chest 139: 1380-1387.

45. Kranenburg AR, Willems-Widyastuti A, Moori WJ, Sterk PJ, Alagappan VK, et al. (2006) Enhanced bronchial expression of extracellular matrix proteins in chronic obstructive pulmonary disease. Am J Clin Pathol 126: 725-735.
46. van den BM, Ten Hacken NH, Cohen J, Douma WR, Postma DS (2011) Small airway disease in asthma and COPD: clinical implications. Chest 139: 412-423.
47. Tam A, Wadsworth S, Dorscheid D, Man SF, Sin DD (2011) The airway epithelium: more than just a structural barrier. Ther Adv Respir Dis 5: 255-273.
48. Papi A, Casoni G, Caramori G, Guzzinati I, Boschetto P, et al. (2004) COPD increases the risk of squamous histological subtype in smokers who develop non-small cell lung carcinoma. Thorax 59: 679-681.
49. Williams OW, Sharafkhaneh A, Kim V, Dickey BF, Evans CM (2006) Airway mucus: From production to secretion. Am J Respir Cell Mol Biol 34: 527-536.
50. Lazaar AL, Panettieri RA, Jr. (2005) Airway smooth muscle: a modulator of airway remodeling in asthma. J Allergy Clin Immunol 116: 488-495.
51. Chung KF (2005) The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. Proc Am Thorac Soc 2: 347-354.
52. Pare PD, Wiggs BR, James A, Hogg JC, Bosken C (1991) The comparative mechanics and morphology of airways in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis 143: 1189-1193.
53. Opazo Saez AM, Seow CY, Pare PD (2000) Peripheral airway smooth muscle mechanics in obstructive airways disease. Am J Respir Crit Care Med 161: 910-917.
54. Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, et al. (2004) Neutrophilic infiltration within the airway smooth muscle in patients with COPD. Thorax 59: 308-312.
55. Oltmanns U, Chung KF, Walters M, John M, Mitchell JA (2005) Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle. Respir Res 6: 74.
56. Oenema TA, Kolahian S, Nanninga JE, Rieks D, Hiemstra PS, et al. (2010) Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle. Respir Res 11: 130.
57. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, et al. (2009) Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. Eur Respir J .
58. Joubert P, Lajoie-Kadoch S, Wellemans V, Letuve S, Tulic MK, et al. (2011) Expression and regulation of CCL15 by human airway smooth muscle cells. Clin Exp Allergy .
59. Salazar LM, Herrera AM (2011) Fibrotic response of tissue remodeling in COPD. Lung 189: 101-109.
60. McAnulty RJ (2007) Fibroblasts and myofibroblasts: their source, function and role in disease. Int J Biochem Cell Biol 39: 666-671.
61. Jeffery PK (2001) Remodeling in asthma and chronic obstructive lung disease. Am J Respir Crit Care Med 164: S28-S38.
62. Churg A, Tai H, Coulthard T, Wang R, Wright JL (2006) Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. Am J Respir Crit Care Med 174: 1327-1334.
63. Beasley MB (2010) Smoking-related Small airway disease--a review and update. Adv Anat Pathol 17: 270-276.
64. van Diemen CC, Postma DS, Vonk JM, Bruinenberg M, Nolte IM, et al. (2006) Decorin and TGF-beta1 polymorphisms and development of COPD in a general population. Respir Res 7: 89.
65. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl 34: 50s-59s.
66. de Boer WI, van SA, Sont JK, Sharma HS, Stolk J, et al. (1998) Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 158: 1951-1957.
67. Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, et al. (2001) Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). Am J Respir Crit Care Med 163: 1476-1483.

68. Konigshoff M, Kneidinger N, Eickelberg O (2009) TGF-beta signaling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimen. Swiss Med Wkly 139: 554-563.
69. Wang H, Liu X, Umino T, Kohyama T, Zhu YK, et al. (2003) Effect of cigarette smoke on fibroblast-mediated gel contraction is dependent on cell density. Am J Physiol Lung Cell Mol Physiol 284: L205-L213.
70. Krimmer DI, Burgess JK, Wooi TK, Black JL, Oliver BG (2011) Matrix Proteins from Smoke Exposed Fibroblasts are Pro-proliferative. Am J Respir Cell Mol Biol .
71. Springer J, Scholz FR, Peiser C, Groneberg DA, Fischer A (2004) SMAD-signaling in chronic obstructive pulmonary disease: transcriptional down-regulation of inhibitory SMAD 6 and 7 by cigarette smoke. Biol Chem 385: 649-653.
72. Zandvoort A, Postma DS, Jonker MR, Noordhoek JA, Vos JT, et al. (2008) Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. Respir Res 9: 83.
73. Bartram U, Speer CP (2004) The role of transforming growth factor beta in lung development and disease. Chest 125: 754-765.
74. Scotton CJ, Chambers RC (2007) Molecular targets in pulmonary fibrosis: the myofibroblast in focus. Chest 132: 1311-1321.
75. Lofdahl M, Kaarteenaho R, Lappi-Blanco E, Tornling G, Skold MC (2011) Tenascin-C and alpha-smooth muscle actin positive cells are increased in the large airways in patients with COPD. Respir Res 12: 48.
76. Plantier L, Boczkowski J, Crestani B (2007) Defect of alveolar regeneration in pulmonary emphysema: role of lung fibroblasts. Int J Chron Obstruct Pulmon Dis 2: 463-469.
77. Chung KF, Adcock IM (2008) Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. Eur Respir J 31: 1334-1356.
78. Horowitz JC, Martinez FJ, Thannickal VJ (2009) Mesenchymal cell fate and phenotypes in the pathogenesis of emphysema. COPD 6: 201-210.
79. Nakamura Y, Romberger DJ, Tate L, Ertl RF, Kawamoto M, et al. (1995) Cigarette smoke inhibits lung fibroblast proliferation and chemotaxis. Am J Respir Crit Care Med 151: 1497-1503.
80. Carnevali S, Nakamura Y, Mio T, Liu X, Takigawa K, et al. (1998) Cigarette smoke extract inhibits fibroblast-mediated collagen gel contraction. Am J Physiol 274: L591-L598.
81. Noordhoek JA, Postma DS, Chong LL, Vos JT, Kauffman HF, et al. (2003) Different proliferative capacity of lung fibroblasts obtained from control subjects and patients with emphysema. Exp Lung Res 29: 291-302.
82. Noordhoek JA, Postma DS, Chong LL, Menkema L, Kauffman HF, et al. (2005) Different modulation of decorin production by lung fibroblasts from patients with mild and severe emphysema. COPD 2: 17-25.
83. Churg A, Zhou S, Preobrazhenska O, Tai H, Wang R, et al. (2009) Expression of profibrotic mediators in small airways versus parenchyma after cigarette smoke exposure. Am J Respir Cell Mol Biol 40: 268-276.
84. Spira A, Beane J, Pinto-Plata V, Kadar A, Liu G, et al. (2004) Gene expression profiling of human lung tissue from smokers with severe emphysema. Am J Respir Cell Mol Biol 31: 601-610.
85. Ning W, Li CJ, Kaminski N, Feghali-Bostwick CA, Alber SM, et al. (2004) Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. Proc Natl Acad Sci U S A 101: 14895-14900.
86. Vlahovic G, Russell ML, Mercer RR, Crapo JD (1999) Cellular and connective tissue changes in alveolar septal walls in emphysema. Am J Respir Crit Care Med 160: 2086-2092.
87. Lang MR, Fiaux GW, Gillooly M, Stewart JA, Hulmes DJ, et al. (1994) Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs. Thorax 49: 319-326.
88. Huertas A, Palange P (2011) COPD: a multifactorial systemic disease. Ther Adv Respir Dis 5: 217-224.
89. Reid WD, Rurak J, Harris RL (2009) Skeletal muscle response to inflammation--lessons for chronic obstructive pulmonary disease. Crit Care Med 37: S372-S383.

90. Agusti AG, Sauleda J, Miralles C, Gomez C, Togores B, et al. (2002) Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 166: 485-489.
91. Spurzem JR, Rennard SI (2005) Pathogenesis of COPD. Semin Respir Crit Care Med 26: 142-153.
92. Godtfredsen NS, Lam TH, Hansel TT, Leon ME, Gray N, et al. (2008) COPD-related morbidity and mortality after smoking cessation: status of the evidence. Eur Respir J 32: 844-853.
93. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS (2001) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. Am J Respir Crit Care Med 163: 1256-1276.
94. Lapperre TS, Snoeck-Stroband JB, Gosman MM, Jansen DF, van SA, et al. (2009) Effect of fluticasone with and without salmeterol on pulmonary outcomes in chronic obstructive pulmonary disease: a randomized trial. Ann Intern Med 151: 517-527.
95. Barnes PJ (2010) Inhaled corticosteroids in COPD: a controversy. Respiration 80: 89-95.
96. Burge S (2001) Should inhaled corticosteroids be used in the long term treatment of chronic obstructive pulmonary disease? Drugs 61: 1535-1544.
97. Celli BR, Thomas NE, Anderson JA, Ferguson GT, Jenkins CR, et al. (2008) Effect of pharmacotherapy on rate of decline of lung function in chronic obstructive pulmonary disease: results from the TORCH study. Am J Respir Crit Care Med 178: 332-338.
98. Yang IA, Fong KM, Sim EH, Black PN, Lasserson TJ (2007) Inhaled corticosteroids for stable chronic obstructive pulmonary disease. Cochrane Database Syst Rev : CD002991.
99. Rabe KF (2011) Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. Br J Pharmacol 163: 53-67.
100. Sturton G, Fitzgerald M (2002) Phosphodiesterase 4 inhibitors for the treatment of COPD. Chest 121: 192S-196S.
101. Fukuchi Y (2009) The aging lung and chronic obstructive pulmonary disease: similarity and difference. Proc Am Thorac Soc 6: 570-572.
102. Goss AM, Tian Y, Tsukiyama T, Cohen ED, Zhou D, et al. (2009) Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. Dev Cell 17: 290-298.
103. Goss AM, Morrisey EE (2010) Wnt signaling and specification of the respiratory endoderm. Cell Cycle 9: 10-11.
104. Blacquiere MJ, Timens W, van den BA, Geerlings M, Postma DS, et al. (2010) Maternal smoking during pregnancy decreases Wnt signalling in neonatal mice. Thorax 65: 553-554.
105. Pattenden S, Antova T, Neuberger M, Nikiforov B, De SM, et al. (2006) Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure. Tob Control 15: 294-301.
106. Wang R, Ahmed J, Wang G, Hassan I, Strulovici-Barel Y, et al. (2011) Down-regulation of the canonical Wnt beta-catenin pathway in the airway epithelium of healthy smokers and smokers with COPD. PLoS One 6: e14793.
107. Kneidinger N, Yildirim AO, Callegari J, Takenaka S, Stein MM, et al. (2011) Activation of the WNT/beta-catenin pathway attenuates experimental emphysema. Am J Respir Crit Care Med 183: 723-733.
108. Bowley E, O'Gorman DB, Gan BS (2007) Beta-catenin signaling in fibroproliferative disease. J Surg Res 138: 141-150.
109. Konigshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, et al. (2008) Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One 3: e2142.
110. Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810.
111. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480.
112. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17: 9-26.
113. Angers S, Moon RT (2009) Proximal events in Wnt signal transduction. Nat Rev Mol Cell Biol 10: 468-477.
114. Semenov MV, Habas R, MacDonald BT, He X (2007) SnapShot: Noncanonical Wnt Signaling Pathways. Cell 131: 1378.

115. McNeill H, Woodgett JR (2010) When pathways collide: collaboration and connivance among signalling proteins in development. Nat Rev Mol Cell Biol 11: 404-413.
116. De A (2011) Wnt/Ca2+ signaling pathway: a brief overview. Acta Biochim Biophys Sin (Shanghai) 43: 745-756.
117. Korzh V (2008) Winding roots of Wnts. Zebrafish 5: 159-168.
118. Sharma RP, Chopra VL (1976) Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev Biol 48: 461-465.
119. Nusse R, Varmus HE (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31: 99-109.
120. Rijsewijk F, Schuermann M, Wagenaar E, Parren P, Weigel D, et al. (1987) The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell 50: 649-657.
121. Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, et al. (1991) A new nomenclature for int-1 and related genes: the Wnt gene family. Cell 64: 231.
122. Nusse R, Varmus HE (1992) Wnt genes. Cell 69: 1073-1087.
123. Miller JR (2002) The Wnts. Genome Biol 3: REVIEWS3001.
124. Mikels AJ, Nusse R (2006) Wnts as ligands: processing, secretion and reception. Oncogene 25: 7461-7468.
125. Coudreuse D, Korswagen HC (2007) The making of Wnt: new insights into Wnt maturation, sorting and secretion. Development 134: 3-12.
126. Tanaka K, Kitagawa Y, Kadowaki T (2002) Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. J Biol Chem 277: 12816-12823.
127. Kadowaki T, Wilder E, Klingensmith J, Zachary K, Perrimon N (1996) The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. Genes Dev 10: 3116-3128.
128. Herr P, Basler K (2011) Porcupine-mediated lipidation is required for Wnt recognition by Wls. Dev Biol .
129. Banziger C, Soldini D, Schutt C, Zipperlen P, Hausmann G, et al. (2006) Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell 125: 509-522.
130. Ching W, Nusse R (2006) A dedicated Wnt secretion factor. Cell 125: 432-433.
131. Reichsman F, Smith L, Cumberledge S (1996) Glycosaminoglycans can modulate extracellular localization of the wingless protein and promote signal transduction. J Cell Biol 135: 819-827.
132. Berendsen AD, Fisher LW, Kilts TM, Owens RT, Robey PG, et al. (2011) Modulation of canonical Wnt signaling by the extracellular matrix component biglycan. Proc Natl Acad Sci U S A 108: 17022-17027.
133. Lin X (2004) Functions of heparan sulfate proteoglycans in cell signaling during development. Development 131: 6009-6021.
134. Quelard D, Lavergne E, Hendaoui I, Elamaa H, Tiirola U, et al. (2008) A cryptic frizzled module in cell surface collagen 18 inhibits Wnt/beta-catenin signaling. PLoS One 3: e1878.
135. Katanaev VL, Solis GP, Hausmann G, Buestorf S, Katanayeva N, et al. (2008) Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of Wingless and Hedgehog in Drosophila. EMBO J 27: 509-521.
136. Panakova D, Sprong H, Marois E, Thiele C, Eaton S (2005) Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature 435: 58-65.
137. Du SJ, Purcell SM, Christian JL, McGrew LL, Moon RT (1995) Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos. Mol Cell Biol 15: 2625-2634.
138. Kuhl M (2002) Non-canonical Wnt signaling in Xenopus: regulation of axis formation and gastrulation. Semin Cell Dev Biol 13: 243-249.
139. van AR, Mikels A, Nusse R (2008) Alternative wnt signaling is initiated by distinct receptors. Sci Signal 1: re9.
140. Liu G, Bafico A, Aaronson SA (2005) The mechanism of endogenous receptor activation functionally distinguishes prototype canonical and noncanonical Wnts. Mol Cell Biol 25: 3475-3482.

141. Yang-Snyder J, Miller JR, Brown JD, Lai CJ, Moon RT (1996) A frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr Biol 6: 1302-1306.
142. Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, et al. (1996) A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382: 225-230.
143. Schulte G (2010) International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors. Pharmacol Rev 62: 632-667.
144. Hsieh JC, Rattner A, Smallwood PM, Nathans J (1999) Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. Proc Natl Acad Sci U S A 96: 3546-3551.
145. Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, et al. (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. Nature 412: 86-90.
146. Chen CM, Strapps W, Tomlinson A, Struhl G (2004) Evidence that the cysteine-rich domain of Drosophila Frizzled family receptors is dispensable for transducing Wingless. Proc Natl Acad Sci U S A 101: 15961-15966.
147. Povelones M, Nusse R (2005) The role of the cysteine-rich domain of Frizzled in Wingless-Armadillo signaling. EMBO J 24: 3493-3503.
148. Cadigan KM, Liu YI (2006) Wnt signaling: complexity at the surface. J Cell Sci 119: 395-402.
149. Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, et al. (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398: 431-436.
150. Bejsovec A (2005) Wnt pathway activation: new relations and locations. Cell 120: 11-14.
151. He X, Semenov M, Tamai K, Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. Development 131: 1663-1677.
152. Dale TC (1998) Signal transduction by the Wnt family of ligands. Biochem J 329 (Pt 2): 209-223.
153. Fahmy OG, Fahmy MJ (1959) Differential Gene Response to Mutagens in Drosophila Melanogaster. Genetics 44: 1149-1171.
154. Gao C, Chen YG (2010) Dishevelled: The hub of Wnt signaling. Cell Signal 22: 717-727.
155. Peters JM, McKay RM, McKay JP, Graff JM (1999) Casein kinase I transduces Wnt signals. Nature 401: 345-350.
156. Sun TQ, Lu B, Feng JJ, Reinhard C, Jan YN, et al. (2001) PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. Nat Cell Biol 3: 628-636.
157. Kilander MB, Dijksterhuis JP, Ganji RS, Bryja V, Schulte G (2011) WNT-5A stimulates the GDP/GTP exchange at pertussis toxin-sensitive heterotrimeric G proteins. Cell Signal 23: 550-554.
158. Schulte G, Bryja V (2007) The Frizzled family of unconventional G-protein-coupled receptors. Trends Pharmacol Sci 28: 518-525.
159. Liu T, DeCostanzo AJ, Liu X, Wang H, Hallagan S, et al. (2001) G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. Science 292: 1718-1722.
160. Medina A, Reintsch W, Steinbeisser H (2000) Xenopus frizzled 7 can act in canonical and non-canonical Wnt signaling pathways: implications on early patterning and morphogenesis. Mech Dev 92: 227-237.
161. Bhat KM (1998) frizzled and frizzled 2 play a partially redundant role in wingless signaling and have similar requirements to wingless in neurogenesis. Cell 95: 1027-1036.
162. Wehrli M, Dougan ST, Caldwell K, O'Keefe L, Schwartz S, et al. (2000) arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature 407: 527-530.
163. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, et al. (2000) LDL-receptor-related proteins in Wnt signal transduction. Nature 407: 530-535.
164. Liu G, Bafico A, Harris VK, Aaronson SA (2003) A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. Mol Cell Biol 23: 5825-5835.
165. Kelly OG, Pinson KI, Skarnes WC (2004) The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. Development 131: 2803-2815.
166. Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, et al. (2002) Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol 157: 303-314.
167. Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC (2000) An LDL-receptor-related protein mediates Wnt signalling in mice. Nature 407: 535-538.

168. Bilic J, Huang YL, Davidson G, Zimmermann T, Cruciat CM, et al. (2007) Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science 316: 1619-1622.
169. Cong F, Schweizer L, Varmus H (2004) Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. Development 131: 5103-5115.
170. Niehrs C, Shen J (2010) Regulation of Lrp6 phosphorylation. Cell Mol Life Sci 67: 2551-2562.
171. Zeng X, Tamai K, Doble B, Li S, Huang H, et al. (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438: 873-877.
172. Kimelman D, Xu W (2006) beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene 25: 7482-7491.
173. Doble BW, Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 116: 1175-1186.
174. Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359: 1-16.
175. Kamata T, Katsube K, Michikawa M, Yamada M, Takada S, et al. (2004) R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochim Biophys Acta 1676: 51-62.
176. de LW, Barker N, Low TY, Koo BK, Li VS, et al. (2011) Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476: 293-297.
177. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q (2011) R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci U S A 108: 11452-11457.
178. Liepinsh E, Banyai L, Patthy L, Otting G (2006) NMR structure of the WIF domain of the human Wnt-inhibitory factor-1. J Mol Biol 357: 942-950.
179. Xu YK, Nusse R (1998) The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases. Curr Biol 8: R405-R406.
180. Arimura S, Matsunaga A, Kitamura T, Aoki K, Aoki M, et al. (2009) Reduced level of smoothened suppresses intestinal tumorigenesis by down-regulation of Wnt signaling. Gastroenterology 137: 629-638.
181. Nusse R (2003) Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development 130: 5297-5305.
182. Nusse R (2008) Wnt signaling and stem cell control. Cell Res 18: 523-527.
183. Yoda A, Oishi I, Minami Y (2003) Expression and function of the Ror-family receptor tyrosine kinases during development: lessons from genetic analyses of nematodes, mice, and humans. J Recept Signal Transduct Res 23: 1-15.
184. Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, et al. (1999) Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J Biol Chem 274: 16180-16187.
185. Kawano Y, Kypta R (2003) Secreted antagonists of the Wnt signalling pathway. J Cell Sci 116: 2627-2634.
186. Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, et al. (1997) Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. Proc Natl Acad Sci U S A 94: 6770-6775.
187. Leyns L, Bouwmeester T, Kim SH, Piccolo S, De Robertis EM (1997) Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell 88: 747-756.
188. Wang S, Krinks M, Lin K, Luyten FP, Moos M, Jr. (1997) Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. Cell 88: 757-766.
189. Hoang BH, Thomas JT, bdul-Karim FW, Correia KM, Conlon RA, et al. (1998) Expression pattern of two Frizzled-related genes, Frzb-1 and Sfrp-1, during mouse embryogenesis suggests a role for modulating action of Wnt family members. Dev Dyn 212: 364-372.
190. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, et al. (1997) SARPs: a family of secreted apoptosis-related proteins. Proc Natl Acad Sci U S A 94: 13636-13641.
191. Chien AJ, Conrad WH, Moon RT (2009) A Wnt survival guide: from flies to human disease. J Invest Dermatol 129: 1614-1627.

192. Bhat RA, Stauffer B, Komm BS, Bodine PV (2007) Structure-function analysis of secreted frizzled-related protein-1 for its Wnt antagonist function. J Cell Biochem 102: 1519-1528.
193. Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, et al. (1998) Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391: 357-362.
194. Krupnik VE, Sharp JD, Jiang C, Robison K, Chickering TW, et al. (1999) Functional and structural diversity of the human Dickkopf gene family. Gene 238: 301-313.
195. Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, et al. (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol 11: 951-961.
196. Brott BK, Sokol SY (2002) Regulation of Wnt/LRP signaling by distinct domains of Dickkopf proteins. Mol Cell Biol 22: 6100-6110.
197. Mao B, Wu W, Li Y, Hoppe D, Stannek P, et al. (2001) LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature 411: 321-325.
198. Ahn VE, Chu ML, Choi HJ, Tran D, Abo A, et al. (2011) Structural Basis of Wnt Signaling Inhibition by Dickkopf Binding to LRP5/6. Dev Cell .
199. Wu W, Glinka A, Delius H, Niehrs C (2000) Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. Curr Biol 10: 1611-1614.
200. Mao B, Wu W, Davidson G, Marhold J, Li M, et al. (2002) Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature 417: 664-667.
201. Semenov MV, Zhang X, He X (2008) DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation. J Biol Chem 283: 21427-21432.
202. Gonzalez-Sancho JM, Aguilera O, Garcia JM, Pendas-Franco N, Pena C, et al. (2005) The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer. Oncogene 24: 1098-1103.
203. Niida A, Hiroko T, Kasai M, Furukawa Y, Nakamura Y, et al. (2004) DKK1, a negative regulator of Wnt signaling, is a target of the beta-catenin/TCF pathway. Oncogene 23: 8520-8526.
204. Bazzi H, Fantauzzo KA, Richardson GD, Jahoda CA, Christiano AM (2007) The Wnt inhibitor, Dickkopf 4, is induced by canonical Wnt signaling during ectodermal appendage morphogenesis. Dev Biol 305: 498-507.
205. Pendas-Franco N, Garcia JM, Pena C, Valle N, Palmer HG, et al. (2008) DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1alpha,25-dihydroxyvitamin D3. Oncogene 27: 4467-4477.
206. Filipovich A, Gehrke I, Poll-Wolbeck SJ, Kreuzer KA (2011) Physiological inhibitors of Wnt signaling. Eur J Haematol 86: 453-465.
207. Semenov M, Tamai K, He X (2005) SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. J Biol Chem 280: 26770-26775.
208. Park K, Lee K, Zhang B, Zhou T, He X, et al. (2011) Identification of a novel inhibitor of the canonical Wnt pathway. Mol Cell Biol 31: 3038-3051.
209. Zhang B, Abreu JG, Zhou K, Chen Y, Hu Y, et al. (2010) Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in the serine proteinase inhibitor family. Proc Natl Acad Sci U S A 107: 6900-6905.
210. Shapiro L, Weis WI (2009) Structure and biochemistry of cadherins and catenins. Cold Spring Harb Perspect Biol 1: a003053.
211. Nagafuchi A, Takeichi M (1989) Transmembrane control of cadherin-mediated cell adhesion: a 94 kDa protein functionally associated with a specific region of the cytoplasmic domain of E-cadherin. Cell Regul 1: 37-44.
212. Jansen SR, Van Ziel AM, Baarsma HA, Gosens R (2010) {beta}-Catenin regulates airway smooth muscle contraction. Am J Physiol Lung Cell Mol Physiol 299: L204-L214.
213. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, et al. (2010) De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. FASEB J 24: 757-768.
214. Klingelhofer J, Troyanovsky RB, Laur OY, Troyanovsky S (2003) Exchange of catenins in cadherin-catenin complex. Oncogene 22: 1181-1188.

215. Uglow EB, Slater S, Sala-Newby GB, guilera-Garcia CM, Angelini GD, et al. (2003) Dismantling of cadherin-mediated cell-cell contacts modulates smooth muscle cell proliferation. Circ Res 92: 1314-1321.
216. Xu W, Kimelman D (2007) Mechanistic insights from structural studies of beta-catenin and its binding partners. J Cell Sci 120: 3337-3344.
217. Huber AH, Nelson WJ, Weis WI (1997) Three-dimensional structure of the armadillo repeat region of beta-catenin. Cell 90: 871-882.
218. Verheyen EM, Gottardi CJ (2010) Regulation of Wnt/beta-catenin signaling by protein kinases. Dev Dyn 239: 34-44.
219. Wolf D, Rodova M, Miska EA, Calvet JP, Kouzarides T (2002) Acetylation of beta-catenin by CREB-binding protein (CBP). J Biol Chem 277: 25562-25567.
220. Kikuchi A (1999) Modulation of Wnt signaling by Axin and Axil. Cytokine Growth Factor Rev 10: 255-265.
221. Yamamoto H, Kishida S, Uochi T, Ikeda S, Koyama S, et al. (1998) Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3beta and beta-catenin and inhibits axis formation of Xenopus embryos. Mol Cell Biol 18: 2867-2875.
222. Lee E, Salic A, Kruger R, Heinrich R, Kirschner MW (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol 1: E10.
223. Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, et al. (1991) Identification and characterization of the familial adenomatous polyposis coli gene. Cell 66: 589-600.
224. Ha NC, Tonozuka T, Stamos JL, Choi HJ, Weis WI (2004) Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. Mol Cell 15: 511-521.
225. Xing Y, Clements WK, Le T, I, Hinds TR, Stenkamp R, et al. (2004) Crystal structure of a beta-catenin/APC complex reveals a critical role for APC phosphorylation in APC function. Mol Cell 15: 523-533.
226. Korinek V, Barker N, Morin PJ, van WD, de WR, et al. (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 275: 1784-1787.
227. Kitagawa M, Hatakeyama S, Shirane M, Matsumoto M, Ishida N, et al. (1999) An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. EMBO J 18: 2401-2410.
228. Wu G, Xu G, Schulman BA, Jeffrey PD, Harper JW, et al. (2003) Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. Mol Cell 11: 1445-1456.
229. Peifer M, Sweeton D, Casey M, Wieschaus E (1994) wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. Development 120: 369-380.
230. Yost C, Torres M, Miller JR, Huang E, Kimelman D, et al. (1996) The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev 10: 1443-1454.
231. Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, et al. (2002) Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev 16: 1066-1076.
232. Liu C, Li Y, Semenov M, Han C, Baeg GH, et al. (2002) Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108: 837-847.
233. Luo W, Peterson A, Garcia BA, Coombs G, Kofahl B, et al. (2007) Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex. EMBO J 26: 1511-1521.
234. Janssens V, Goris J (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem J 353: 417-439.
235. Itoh K, Brott BK, Bae GU, Ratcliffe MJ, Sokol SY (2005) Nuclear localization is required for Dishevelled function in Wnt/beta-catenin signaling. J Biol 4: 3.
236. Zeng X, Huang H, Tamai K, Zhang X, Harada Y, et al. (2008) Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. Development 135: 367-375.

237. Seidensticker MJ, Behrens J (2000) Biochemical interactions in the wnt pathway. Biochim Biophys Acta 1495: 168-182.
238. Park TJ, Gray RS, Sato A, Habas R, Wallingford JB (2005) Subcellular localization and signaling properties of dishevelled in developing vertebrate embryos. Curr Biol 15: 1039-1044.
239. Pansters NA, van d, V, Kelders MC, Laeremans H, Schols AM, et al. (2011) Segregation of myoblast fusion and muscle-specific gene expression by distinct ligand-dependent inactivation of GSK-3beta. Cell Mol Life Sci 68: 523-535.
240. Oloumi A, Syam S, Dedhar S (2006) Modulation of Wnt3a-mediated nuclear beta-catenin accumulation and activation by integrin-linked kinase in mammalian cells. Oncogene 25: 7747-7757.
241. Ding VW, Chen RH, McCormick F (2000) Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. J Biol Chem 275: 32475-32481.
242. Dajani R, Fraser E, Roe SM, Yeo M, Good VM, et al. (2003) Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex. EMBO J 22: 494-501.
243. Ferkey DM, Kimelman D (2002) Glycogen synthase kinase-3 beta mutagenesis identifies a common binding domain for GBP and Axin. J Biol Chem 277: 16147-16152.
244. Bikkavilli RK, Malbon CC (2010) Dishevelled-KSRP complex regulates Wnt signaling through post-transcriptional stabilization of beta-catenin mRNA. J Cell Sci 123: 1352-1362.
245. Mao J, Wang J, Liu B, Pan W, Farr GH, III, et al. (2001) Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol Cell 7: 801-809.
246. Kofron M, Birsoy B, Houston D, Tao Q, Wylie C, et al. (2007) Wnt11/beta-catenin signaling in both oocytes and early embryos acts through LRP6-mediated regulation of axin. Development 134: 503-513.
247. Tolwinski NS, Wehrli M, Rives A, Erdeniz N, DiNardo S, et al. (2003) Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity. Dev Cell 4: 407-418.
248. Liu X, Rubin JS, Kimmel AR (2005) Rapid, Wnt-induced changes in GSK3beta associations that regulate beta-catenin stabilization are mediated by Galpha proteins. Curr Biol 15: 1989-1997.
249. Cselenyi CS, Jernigan KK, Tahinci E, Thorne CA, Lee LA, et al. (2008) LRP6 transduces a canonical Wnt signal independently of Axin degradation by inhibiting GSK3's phosphorylation of beta-catenin. Proc Natl Acad Sci U S A 105: 8032-8037.
250. Piao S, Lee SH, Kim H, Yum S, Stamos JL, et al. (2008) Direct inhibition of GSK3beta by the phosphorylated cytoplasmic domain of LRP6 in Wnt/beta-catenin signaling. PLoS One 3: e4046.
251. Willert K, Shibamoto S, Nusse R (1999) Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. Genes Dev 13: 1768-1773.
252. Metcalfe C, Bienz M (2011) Inhibition of GSK3 by Wnt signalling - two contrasting models. J Cell Sci 124: 3537-3544.
253. Taelman VF, Dobrowolski R, Plouhinec JL, Fuentealba LC, Vorwald PP, et al. (2010) Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell 143: 1136-1148.
254. Eastman Q, Grosschedl R (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr Opin Cell Biol 11: 233-240.
255. Fagotto F, Gluck U, Gumbiner BM (1998) Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. Curr Biol 8: 181-190.
256. Hagen T, Sethi JK, Foxwell N, Vidal-Puig A (2004) Signalling activity of beta-catenin targeted to different subcellular compartments. Biochem J 379: 471-477.
257. Yokoya F, Imamoto N, Tachibana T, Yoneda Y (1999) beta-catenin can be transported into the nucleus in a Ran-unassisted manner. Mol Biol Cell 10: 1119-1131.
258. Prieve MG, Guttridge KL, Munguia J, Waterman ML (1998) Differential importin-alpha recognition and nuclear transport by nuclear localization signals within the high-mobility-group DNA binding domains of lymphoid enhancer factor 1 and T-cell factor 1. Mol Cell Biol 18: 4819-4832.

259. Zhang N, Wei P, Gong A, Chiu WT, Lee HT, et al. (2011) FoxM1 Promotes beta-Catenin Nuclear Localization and Controls Wnt Target-Gene Expression and Glioma Tumorigenesis. Cancer Cell 20: 427-442.
260. Bowman A, Nusse R (2011) Location, Location, Location: FoxM1 Mediates beta-Catenin Nuclear Translocation and Promotes Glioma Tumorigenesis. Cancer Cell 20: 415-416.
261. Huber O, Korn R, McLaughlin J, Ohsugi M, Herrmann BG, et al. (1996) Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech Dev 59: 3-10.
262. Ki H, Jung HC, Park JH, Kim JS, Lee KY, et al. (2006) Overexpressed LEF-1 proteins display different nuclear localization patterns of beta-catenin in normal versus tumor cells. Cell Biol Int 30: 253-261.
263. Hsu HT, Liu PC, Ku SY, Jung KC, Hong YR, et al. (2006) Beta-catenin control of T-cell transcription factor 4 (Tcf4) importation from the cytoplasm to the nucleus contributes to Tcf4-mediated transcription in 293 cells. Biochem Biophys Res Commun 343: 893-898.
264. Hsu SC, Galceran J, Grosschedl R (1998) Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol 18: 4807-4818.
265. Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, et al. (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382: 638-642.
266. Krieghoff E, Behrens J, Mayr B (2006) Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. J Cell Sci 119: 1453-1463.
267. Henderson BR, Galea M, Schuechner S, Leung L (2002) Lymphoid enhancer factor-1 blocks adenomatous polyposis coli-mediated nuclear export and degradation of beta-catenin. Regulation by histone deacetylase 1. J Biol Chem 277: 24258-24264.
268. Willert K, Nusse R (1998) Beta-catenin: a key mediator of Wnt signaling. Curr Opin Genet Dev 8: 95-102.
269. Orsulic S, Peifer M (1996) An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. J Cell Biol 134: 1283-1300.
270. Townsley FM, Cliffe A, Bienz M (2004) Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat Cell Biol 6: 626-633.
271. Townsley FM, Thompson B, Bienz M (2004) Pygopus residues required for its binding to Legless are critical for transcription and development. J Biol Chem 279: 5177-5183.
272. Gao C, Chen YG (2010) Dishevelled: The hub of Wnt signaling. Cell Signal 22: 717-727.
273. Gan XQ, Wang JY, Xi Y, Wu ZL, Li YP, et al. (2008) Nuclear Dvl, c-Jun, beta-catenin, and TCF form a complex leading to stabilization of beta-catenin-TCF interaction. J Cell Biol 180: 1087-1100.
274. Koike M, Kose S, Furuta M, Taniguchi N, Yokoya F, et al. (2004) beta-Catenin shows an overlapping sequence requirement but distinct molecular interactions for its bidirectional passage through nuclear pores. J Biol Chem 279: 34038-34047.
275. Malik HS, Eickbush TH, Goldfarb DS (1997) Evolutionary specialization of the nuclear targeting apparatus. Proc Natl Acad Sci U S A 94: 13738-13742.
276. Henderson BR, Fagotto F (2002) The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep 3: 834-839.
277. Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, et al. (2008) Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell 133: 340-353.
278. Rao SS, Go JT (2010) Update on the management of constipation in the elderly: new treatment options. Clin Interv Aging 5: 163-171.
279. Henderson BR (2000) Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol 2: 653-660.
280. Clevers H (2000) Armadillo takes the APC shuttle. Nat Cell Biol 2: E177-E178.
281. Neufeld KL, Zhang F, Cullen BR, White RL (2000) APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. EMBO Rep 1: 519-523.
282. Neufeld KL, Nix DA, Bogerd H, Kang Y, Beckerle MC, et al. (2000) Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm. Proc Natl Acad Sci U S A 97: 12085-12090.
283. Rosin-Arbesfeld R, Townsley F, Bienz M (2000) The APC tumour suppressor has a nuclear export function. Nature 406: 1009-1012.

284. Rosin-Arbesfeld R, Cliffe A, Brabletz T, Bienz M (2003) Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription. EMBO J 22: 1101-1113.
285. Wiechens N, Heinle K, Englmeier L, Schohl A, Fagotto F (2004) Nucleo-cytoplasmic shuttling of Axin, a negative regulator of the Wnt-beta-catenin Pathway. J Biol Chem 279: 5263-5267.
286. Travis A, Amsterdam A, Belanger C, Grosschedl R (1991) LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. Genes Dev 5: 880-894.
287. van de WM, Oosterwegel M, Dooijes D, Clevers H (1991) Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J 10: 123-132.
288. Korinek V, Barker N, Willert K, Molenaar M, Roose J, et al. (1998) Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. Mol Cell Biol 18: 1248-1256.
289. Eastman Q, Grosschedl R (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr Opin Cell Biol 11: 233-240.
290. Novak A, Dedhar S (1999) Signaling through beta-catenin and Lef/Tcf. Cell Mol Life Sci 56: 523-537.
291. Mosimann C, Hausmann G, Basler K (2009) Beta-catenin hits chromatin: regulation of Wnt target gene activation. Nat Rev Mol Cell Biol 10: 276-286.
292. Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, et al. (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 395: 608-612.
293. Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, et al. (1998) Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature 395: 604-608.
294. Levanon D, Goldstein RE, Bernstein Y, Tang H, Goldenberg D, et al. (1998) Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. Proc Natl Acad Sci U S A 95: 11590-11595.
295. Staal FJ, Noort MM, Strous GJ, Clevers HC (2002) Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. EMBO Rep 3: 63-68.
296. Daniels DL, Weis WI (2005) Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol 12: 364-371.
297. Kikuchi A, Kishida S, Yamamoto H (2006) Regulation of Wnt signaling by protein-protein interaction and post-translational modifications. Exp Mol Med 38: 1-10.
298. Ishitani T, Ninomiya-Tsuji J, Matsumoto K (2003) Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. Mol Cell Biol 23: 1379-1389.
299. Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, et al. (2003) Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. Nature 422: 905-909.
300. Takemaru K, Fischer V, Li FQ (2009) Fine-tuning of nuclear-catenin by Chibby and 14-3-3. Cell Cycle 8: 210-213.
301. Gottardi CJ, Gumbiner BM (2004) Role for ICAT in beta-catenin-dependent nuclear signaling and cadherin functions. Am J Physiol Cell Physiol 286: C747-C756.
302. Arce L, Yokoyama NN, Waterman ML (2006) Diversity of LEF/TCF action in development and disease. Oncogene 25: 7492-7504.
303. Nusse R. The WNT homepage. http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes . 2011.
304. Gosens R, Meurs H, Schmidt M (2008) The GSK-3/beta-catenin-signalling axis in smooth muscle and its relationship with remodelling. Naunyn Schmiedebergs Arch Pharmacol 378: 185-191.
305. Embi N, Rylatt DB, Cohen P (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem 107: 519-527.
306. Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A (2009) Glycogen synthase kinase 3: more than a namesake. Br J Pharmacol 156: 885-898.
307. Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29: 95-102.

308. Woodgett JR (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 9: 2431-2438.
309. Woodgett JR (1991) cDNA cloning and properties of glycogen synthase kinase-3. Methods Enzymol 200: 564-577.
310. Wood-Kaczmar A, Kraus M, Ishiguro K, Philpott KL, Gordon-Weeks PR (2009) An alternatively spliced form of glycogen synthase kinase-3beta is targeted to growing neurites and growth cones. Mol Cell Neurosci 42: 184-194.
311. Schaffer B, Wiedau-Pazos M, Geschwind DH (2003) Gene structure and alternative splicing of glycogen synthase kinase 3 beta (GSK-3beta) in neural and non-neural tissues. Gene 302: 73-81.
312. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, et al. (2000) Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 406: 86-90.
313. Macaulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, et al. (2007) Glycogen synthase kinase 3alpha-specific regulation of murine hepatic glycogen metabolism. Cell Metab 6: 329-337.
314. Cohen P, Frame S (2001) The renaissance of GSK3. Nat Rev Mol Cell Biol 2: 769-776.
315. Hughes K, Nikolakaki E, Plyte SE, Totty NF, Woodgett JR (1993) Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J 12: 803-808.
316. Lesort M, Jope RS, Johnson GV (1999) Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3beta and Fyn tyrosine kinase. J Neurochem 72: 576-584.
317. Takahashi-Yanaga F, Shiraishi F, Hirata M, Miwa Y, Morimoto S, et al. (2004) Glycogen synthase kinase-3beta is tyrosine-phosphorylated by MEK1 in human skin fibroblasts. Biochem Biophys Res Commun 316: 411-415.
318. Hartigan JA, Xiong WC, Johnson GV (2001) Glycogen synthase kinase 3beta is tyrosine phosphorylated by PYK2. Biochem Biophys Res Commun 284: 485-489.
319. Bhat RV, Shanley J, Correll MP, Fieles WE, Keith RA, et al. (2000) Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. Proc Natl Acad Sci U S A 97: 11074-11079.
320. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378: 785-789.
321. Stambolic V, Woodgett JR (1994) Mitogen inactivation of glycogen synthase kinase-3 beta in intact cells via serine 9 phosphorylation. Biochem J 303 (Pt 3): 701-704.
322. Parker PJ, Caudwell FB, Cohen P (1983) Glycogen synthase from rabbit skeletal muscle; effect of insulin on the state of phosphorylation of the seven phosphoserine residues in vivo. Eur J Biochem 130: 227-234.
323. Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65: 391-426.
324. Persad S, Troussard AA, McPhee TR, Mulholland DJ, Dedhar S (2001) Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. J Cell Biol 153: 1161-1174.
325. Frame S, Cohen P, Biondi RM (2001) A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol Cell 7: 1321-1327.
326. Goni-Oliver P, Lucas JJ, Avila J, Hernandez F (2007) N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation. J Biol Chem 282: 22406-22413.
327. Ng SS, Mahmoudi T, Danenberg E, Bejaoui I, de LW, et al. (2009) Phosphatidylinositol 3-kinase signaling does not activate the wnt cascade. J Biol Chem 284: 35308-35313.
328. Cheon SS, Nadesan P, Poon R, Alman BA (2004) Growth factors regulate beta-catenin-mediated TCF-dependent transcriptional activation in fibroblasts during the proliferative phase of wound healing. Exp Cell Res 293: 267-274.
329. Eldar-Finkelman H (2002) Glycogen synthase kinase 3: an emerging therapeutic target. Trends Mol Med 8: 126-132.
330. Dajani R, Fraser E, Roe SM, Young N, Good V, et al. (2001) Crystal structure of glycogen synthase kinase 3 beta: structural basis for phosphate-primed substrate specificity and autoinhibition. Cell 105: 721-732.
331. Ryves WJ, Harwood AJ (2001) Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. Biochem Biophys Res Commun 280: 720-725.

332. Cohen P, Goedert M (2004) GSK3 inhibitors: development and therapeutic potential. Nat Rev Drug Discov 3: 479-487.
333. Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, et al. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. Chem Biol 7: 793-803.
334. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, et al. (2007) The selectivity of protein kinase inhibitors: a further update. Biochem J 408: 297-315.
335. Martinez A (2008) Preclinical efficacy on GSK-3 inhibitors: towards a future generation of powerful drugs. Med Res Rev 28: 773-796.
336. Jope RS, Yuskaitis CJ, Beurel E (2007) Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. Neurochem Res 32: 577-595.
337. Hooper C, Killick R, Lovestone S (2008) The GSK3 hypothesis of Alzheimer's disease. J Neurochem 104: 1433-1439.
338. Dugo L, Collin M, Thiemermann C (2007) Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. Shock 27: 113-123.
339. Wong ET, Tergaonkar V (2009) Roles of NF-kappaB in health and disease: mechanisms and therapeutic potential. Clin Sci (Lond) 116: 451-465.
340. Edwards MR, Bartlett NW, Clarke D, Birrell M, Belvisi M, et al. (2009) Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. Pharmacol Ther 121: 1-13.
341. Baker RG, Hayden MS, Ghosh S (2011) NF-kappaB, inflammation, and metabolic disease. Cell Metab 13: 11-22.
342. Steinbrecher KA, Wilson W, III, Cogswell PC, Baldwin AS (2005) Glycogen synthase kinase 3beta functions to specify gene-specific, NF-kappaB-dependent transcription. Mol Cell Biol 25: 8444-8455.
343. Staal FJ, Burgering BM, van de WM, Clevers HC (1999) Tcf-1-mediated transcription in T lymphocytes: differential role for glycogen synthase kinase-3 in fibroblasts and T cells. Int Immunol 11: 317-323.
344. Lange C, Mix E, Frahm J, Glass A, Muller J, et al. (2011) Small molecule GSK-3 inhibitors increase neurogenesis of human neural progenitor cells. Neurosci Lett 488: 36-40.
345. Cohen Y, Chetrit A, Cohen Y, Sirota P, Modan B (1998) Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. Med Oncol 15: 32-36.
346. Rey JP, Ellies DL (2010) Wnt modulators in the biotech pipeline. Dev Dyn 239: 102-114.
347. Martinez A, Gil C, Perez DI (2011) Glycogen synthase kinase 3 inhibitors in the next horizon for Alzheimer's disease treatment. Int J Alzheimers Dis 2011: 280502.
348. Pera T, Zuidhof A, Valadas J, Smit M, Schoemaker RG, et al. (2011) Tiotropium inhibits pulmonary inflammation and remodelling in a guinea pig model of COPD. Eur Respir J 38: 789-796.

Chapter 2

β-Catenin is required for TGF-β_1-induced extracellular matrix production by airway smooth muscle cells

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Abstract

Chronic inflammatory airway diseases like asthma and COPD are characterized by airway remodeling with altered extracellular matrix (ECM) deposition. Transforming growth factor-β_1 (TGF-β_1) is upregulated in asthma and COPD, and contributes to tissue remodeling in the airways by driving ECM production by structural cells, including airway smooth muscle. In this study, we investigated the activation of β-catenin signaling and its contribution to ECM production by airway smooth muscle cells in response to TGF-β_1. Stimulation of airway smooth muscle cells with TGF-β_1 resulted in a time-dependent increase of total and non-phosphorylated β-catenin protein expression via induction of β-catenin mRNA and inhibition of GSK-3. In addition, the TGF-β_1-induced β-catenin activated TCF/LEF-dependent gene transcription, as determined by the β-catenin sensitive TOP-flash luciferase reporter assay. Furthermore, TGF-β_1 stimulation increased mRNA expression of collagen Iα1, fibronectin, versican and PAI-1. Pharmacological inhibition of β-catenin by PKF115-584 or down regulation of β-catenin expression by specific siRNA substantially inhibited TGF-β_1-induced expression of the ECM genes. Fibronectin protein deposition by airway smooth muscle cells in response to TGF-β_1 was also inhibited by PKF115-584 and β-catenin siRNA. Moreover, transfection of airway smooth muscle cells with a non-degradable β-catenin mutant (S33Y β-catenin) was sufficient for inducing fibronectin protein expression. Collectively, these findings indicate that β-catenin signaling is activated in response to TGF-β_1 in airway smooth muscle cells, which is required and sufficient for the regulation of ECM protein production. Targeting β-catenin-dependent gene transcription may therefore hold promise as a therapeutic intervention in airway remodeling in both asthma and COPD.

Key words: Fibronectin, collagen, versican, asthma, airway remodelling

Introduction

Chronic obstructive pulmonary disease (COPD) and asthma are obstructive airway diseases characterized by structural changes and thickening of the airway wall (3; 58). These pathological features, referred to as airway remodeling, include increased airway smooth muscle mass and altered extracellular matrix (ECM) profile in the airways, which may contribute cooperatively to airway hyperresponsiveness and the airflow obstruction observed in both diseases (3; 26; 38; 58). Increased airway smooth muscle mass is associated with decreased lung function in severe asthma and may contribute to COPD pathogenesis, particularly in more severe states of disease (35; 41; 55). Altered expression of ECM proteins within and surrounding the smooth muscle bundle has also been observed in asthma, which contributes to disease pathogenesis (1; 21; 57). The mechanisms leading to the development and progression of airway remodeling are not well understood, but airway smooth muscle cells may contribute to the process through cell proliferation and by producing and releasing various inflammatory mediators, growth factors and ECM proteins (39; 46; 57).

The ECM is an intricate structure of macromolecules that acts as mechanical support for maintenance of airway function and is produced by a variety of mesenchymal cells in the airways, including fibroblasts and airway smooth muscle cells (25; 51). The matrix is a dynamic network that has the potential to influence cellular functions such as proliferation, migration, cytokine secretion and differentiation of various resident cell types in the airways (21; 22; 38). In the airway wall of asthmatics the ECM profile is altered with increased expression of specific collagens, fibronectin, tenascin, hyaluronan, versican and laminin, whereas deposition of other components such as decorin are decreased (1; 38). COPD patients exhibit increased bronchial deposition of ECM proteins, including fibronectin, laminin and collagens I, III and IV, and diminished decorin expression peribronchially which collectively contribute to deteriorated lung function and airway remodeling (45; 65). Growth factors play an important role in the development of airway remodeling and are released during the chronic airway inflammation. In particular, transforming growth factor-β (TGF-β) is a multifunctional cytokine, which is increased in airways of asthmatics and COPD patients and is stored in the ECM in its latent, inactive form (12; 16; 25). Cleaving of latent TGF-β results in its activation, thereby allowing it to stimulate various structural and inflammatory cells in the lung resulting in ECM production and airway remodeling (12; 25; 42; 44).

Accumulating evidence indicates that activation of β-catenin signaling is associated with various fibroproliferative diseases, which implies a functional role for β-catenin in tissue remodeling (5; 10; 13). β-Catenin is a member of the Armadillo family of proteins and is associated with the cadherin/catenin complexes at adherens junctions where it stabilizes cell-cell contacts (13). We have previously shown that β-catenin regulates active tension development by airway smooth muscle by its capacity to stabilize these adherens junctions (37). In addition, β-catenin serves a role in the Wnt signaling pathway by regulating T-cell factor (TCF) / Lymphoid enhancer factor (LEF)-mediated gene transcription (13). Cellular β-catenin levels are tightly regulated by the constitutively active enzyme glycogen synthase kinase-3 (GSK-3), which phosphorylates and thereby targets cytosolic β-catenin for proteosomal degradation (13; 40). Recently, various growth factors, including TGF-β, have been demonstrated to activate β-catenin signaling through GSK-3 inhibition, which cooperates with smad signaling to induce gene transcription (9; 33; 53). Stabilized (non-phosphorylated) β-catenin activates several target genes, including matrix metalloproteinases (MMP's), growth factors, ECM proteins as well as pro-inflammatory mediators and enzymes (6; 14; 17; 24; 32; 36; 49; 60; 67). Accordingly, we previously demonstrated a role for β-catenin in airway smooth muscle cell proliferation (28; 53). However, the role of β-catenin in ECM protein production by airway smooth muscle is still unclear. Insight in β-catenin signaling in airway smooth muscle may be of benefit to better understand development and progression of airway remodeling. Therefore, in the present study, we investigated the activation of β-catenin signaling and its contribution to ECM production by airway smooth muscle cells in response to TGF-β_1.

Material and Methods

Cell culture

Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), from three different donors were used for all experiments. The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared, as we have previously described (31), from macroscopically healthy segments of 2nd-to-4th generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (Dr. H Unruh, Section of Thoracic Surgery, University of Manitoba, Canada). All procedures were approved by the Human Research Ethics Board of the University of Manitoba. As previously described in detail (31), each cell line was thoroughly characterized to passage 10 and higher, and was shown to express a number of smooth muscle (sm) contractile phenotype marker proteins (e.g. sm-myosin heavy chain (sm-MHC), sm-α-actin, and desmin). For all experiments, myocytes were grown on uncoated plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (50 U/mL streptomycin, 50 µg/mL penicillin, 1.5 µg/mL amphotericin B) and 10 % (v/v) foetal bovine serum (FBS). Unless differently specified, *c*ells were serum-starved for 1 day in DMEM supplemented with antibiotics and ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium). For TGF-β_1 stimulation, cells were washed twice with warm (37°C) phosphate buffered saline (PBS; composition: 140.0 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) and subsequently subjected to stimulation with TGF-β_1 in serum-free DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor PKF115-584 was added 30 minutes before the addition of TGF-β_1.

TOP/FOP-flash assay

For the TOP/FOP-flash luciferase assay, cells were grown to ~95% confluence on 100 mm dishes and then transfected with TOP-flash plasmid DNA or the negative control FOP-flash plasmid DNA (Upstate Biotechnology, Charlottesville, VA, USA) using Lipofectamine 2000^{TM} in serum- and antibiotics-free DMEM. After 6 hours, medium was changed to DMEM supplemented with antibiotics and 10 % FBS, in which cells were grown for another 18 hours. Cells were then trypsinized and re-plated in 96 well plate format and grown in DMEM supplemented with antibiotics and 10 % FBS for another 8 hours, after which cells were serum-deprived for 24 hours. Cells were then subjected to TGF-β_1 (2 ng/ml) stimulation in DMEM supplemented with antibiotics for 16 hours and luciferase activity was assayed using the Promega luciferase assay system (Madison, Wisconsin, USA). TOP-flash activity was normalized to FOP-flash activity. The transfection efficiencies of the TOP-flash plasmid and FOP-flash plasmid were similar, as determined by co-

transfection with eGFP and subsequent measurement of eGFP positive airway smooth muscle cells by fluorescence microscopy (data not shown).

β-Catenin siRNA transfection
Airway smooth muscle cells were grown to ~90% confluence in 6-well cluster plates and transfected with a 21-bp, double-stranded siRNA targeted against the β-catenin transcript (Qiagen, Venlo, The Netherlands). Cells were transfected in serum-free DMEM without any supplements using 200 pmol of siRNA in combination with lipofectamine 2000™ transfection reagent. Control transfections were performed using a non-silencing control siRNA (Qiagen, Venlo, The Netherlands). After 6 hours of transfection, cells were washed once with warm (37°C) PBS followed by a period of 48 hours in DMEM supplemented with antibiotics. Consecutively, medium was refreshed and cells were stimulated with TGF-β_1 (2 ng/ml) for 24 or 48 hours.

Mutant S33Y β-Catenin plasmid transfection
Airway smooth muscle cells were grown to ~90% confluence in 6-well cluster plates and transfected with β-catenin-S33Y plasmid DNA (AddGene plasmid 19286, AddGene public repository, Cambridge, MA) (43). Cells were transfected in serum-free DMEM without any supplements using 0.1-1 µg of plasmid in combination with lipofectamine 2000 transfection reagent. Control transfections were performed using 2 µg Green Fluorescent Protein (GFP) expression vector. After 6 hours of transfection, cells were washed once with warm (37°C) PBS followed by a period of 48 hours in DMEM supplemented with 10% FBS and antibiotics.

Isolation of mRNA and real-time PCR analysis
Total mRNA was extracted using the RNeasy mini kit (Qiagen, Venlo, the Netherlands). Briefly, cells were harvested in RNA*later* stabilization buffer and homogenized by passing the lysate 10 times through a 20 gauge needle. Lysates were then mixed with an equal volume of 70% ethanol, and total mRNA was purified using RNeasy mini spin columns. The eluted mRNA was quantified using spectrophotometry (Nanodrop, ThermoScientific, Wilmington, USA). Equal amounts of total mRNA (1 µg) were then reverse transcribed and stored at -20 °C until further use. cDNA was subjected to real-time PCR, which was performed with an Illumina Eco™ Personal QPCR System (Westburg, Leusden, The Netherlands). In short, 5 µl absolute blue QPCR SYBR green mix, containing fluorescein to account for well to well variation, 0.1 µM of gene-specific forward and reverse primer (listed in Table 1) and 1 µl of 1:2 diluted cDNA sample were used in a total volume of 10 µl and added to a 48 well plate. Real-time PCR data were analyzed using the comparative cycle threshold (C_q: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S

ribosomal RNA (designated as ΔC_q). Relative differences were determined using the equation $2^{-(\Delta\Delta Cq)}$. Cycle parameters were: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles followed by 5 minutes at 72°C.

Preparation of cell lysates

To obtain whole cell lysates, cells were washed once with ice-cold (4°C) PBS then lysed in ice-cold sodiumdodecylsulphate (SDS) buffer (composition: 62.5 mM Tris, 2 % w/v SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, pH 6.8). Lysates were then sonicated and protein concentration was determined according to Pierce protein determination according to the manufacturer's instructions. Lysates were stored at -20 ºC till further use.

Western blot analysis

Equal amounts of protein (10-20 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene; Cambridge; UK).

Antibodies and reagents

Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, HRP-conjugated goat anti-rabbit antibody and HRP-conjugated rabbit anti-goat antibody were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-GSK-3 antibody, goat anti-fibronectin (C20) antibody and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-phospho-Ser9/21-GSK-3 antibody was from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-total β-catenin antibody was from BD Biosciences (San Jose, CA, USA). Mouse anti-non-phosphorylated-β-catenin antibody (ABC, clone 8E7) was from Millipore (Amsterdam, the Netherlands). GFP expressing vector was kindly provided by Dr. B. van Water and Dr. S.E. Le Dévédec from the division of Toxicology, Leiden Amsterdam Center for Drug Research (64). Lipofectamine 2000 transfection reagent was from Invitrogen (Paisley, UK). Recombinant human TGF-β_1 was from R&D systems (Abingdon, UK). All other chemicals were of analytical grade.

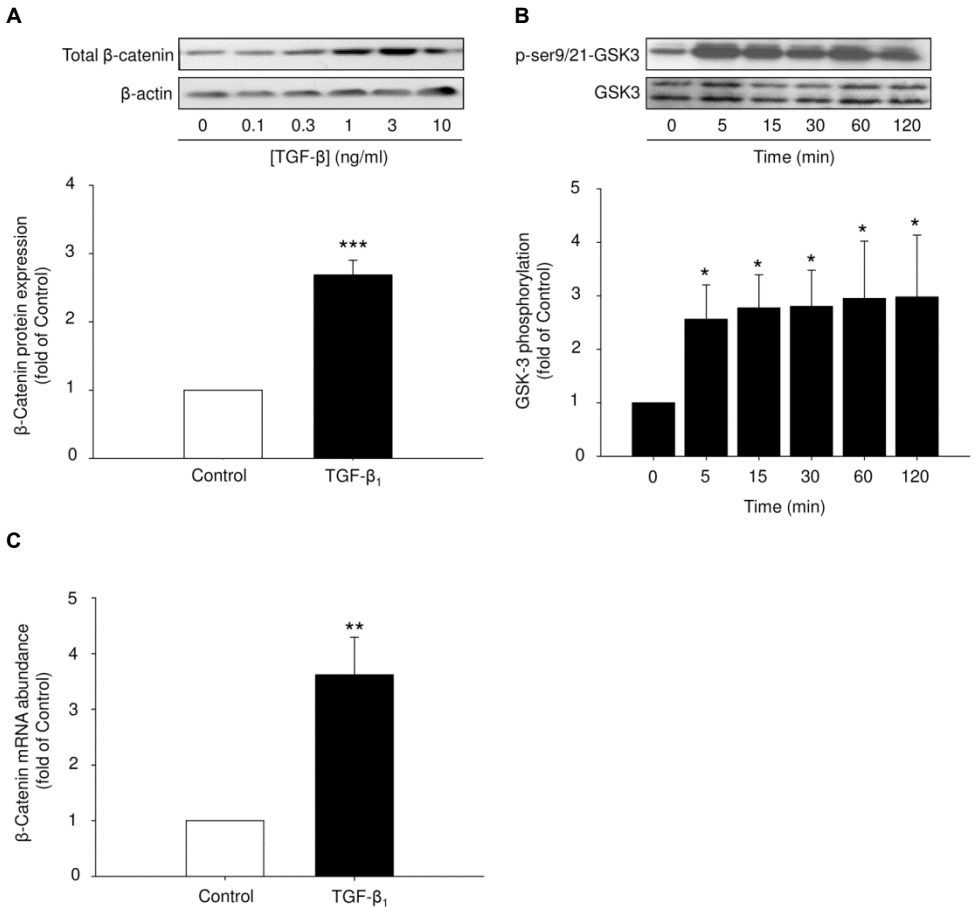
Results

TGF-β_1 induces β-catenin expression in airway smooth muscle cells.

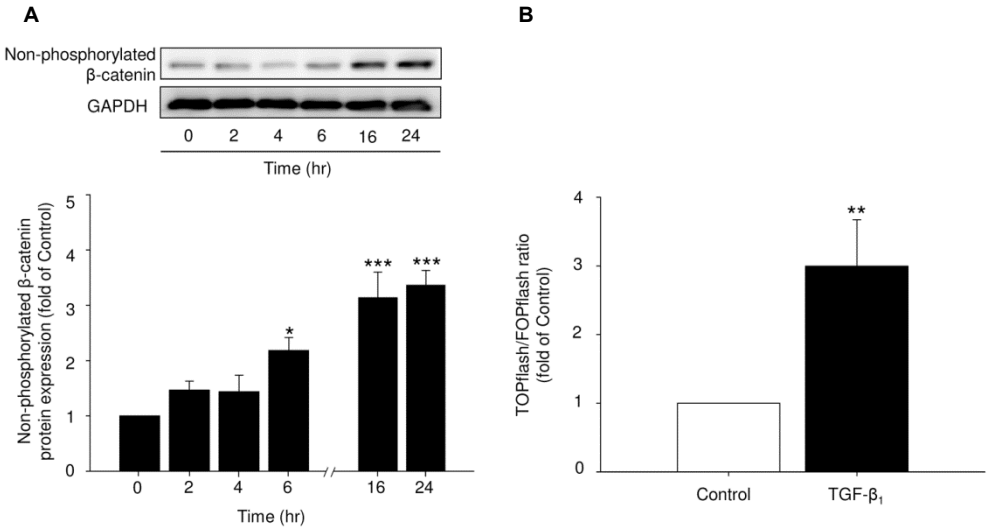
First we aimed to determine if TGF-β_1 regulates β-catenin expression by airway smooth muscle cells. Airway smooth muscle cells were stimulated with increasing concentrations of TGF-β_1 for 24 hours, which resulted in a concentration-dependent increase in total β-catenin protein expression. The induced β-catenin expression reached a maximum using TGF-β_1 between 1 and 3 ng/ml (Figure 1A). β-Catenin expression and localization are tightly regulated by a cytosolic multiprotein complex containing GSK-3, which in its unphosphorylated form targets free cytosolic β-catenin for intracellular breakdown (13; 40). Consistent with this contention, stimulation of airway smooth muscle cells for different time-points with TGF-β_1 (2 ng/ml) induced a profound and sustained inhibitory ser9/21 phosphorylation of GSK-3 (e.g. ser21 of GSK-3α and ser9 of GSK-3β isoform) (Figure 1B). In line with our previous observations (28), TGF-β_1 also increased β-catenin mRNA abundance in airway smooth muscle cells (Figure 1C).

TGF-β_1-induced β-catenin is transcriptionally active.

We next determined whether TGF-β_1-induced β-catenin was coupled to the activation of gene transcription. Stimulation of airway smooth muscle for up to 24 hours with TGF-β_1 (2 ng/ml) resulted in a marked, time-dependent increase in the expression of non-phosphorylated β-catenin (Figure 2A). This increase was linked with activation of TCF/LEF-dependent gene transcription, as assayed using the β-catenin sensitive TOP-flash luciferase assay (Figure 2B). Collectively, these findings indicate that TGF-β_1 induces β-catenin mRNA and protein expression in airway smooth muscle, which subsequently activates TCF/LEF-dependent gene transcription.



▲ ***Figure 1: Induction of β-catenin expression by TGF-β_1 in airway smooth muscle cells.***
(A) Airway smooth muscle cells were stimulated with increasing concentrations of TGF-β_1 (0.1 - 10 ng/ml) for 24 hours. Expression of total β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of β-actin. Responses of TGF-β_1 (2 ng/ml) on total β-catenin expression were quantified by densitometry and normalized to β-actin expression, representing mean ± s.e.m. of 3 independent experiments. (B) Airway smooth muscle cells were stimulated with TGF-β_1 (2 ng/ml) for up to 2 hours. Induction of ser9/21 phosphorylation of GSK-3 was evaluated by immunoblotting using a phospho-specific antibody. Equal protein loading was verified by the analysis of total GSK-3. Responses of TGF-β_1 on GSK-3 phosphorylation were quantified by densitometry and normalized to total GSK-3 expression, representing mean ± s.e.m. of 3 independent experiments. (C) qRT-PCR analysis of β-catenin mRNA expression after 24 hours of TGF-β_1 (2 ng/ml) stimulation. Expression of β-catenin by TGF-β_1 is expressed relative to untreated airway smooth muscle cells. Data represents mean ± s.e.m. of 4 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to untreated airway smooth muscle cells. Statistical significance determined by two-tailed student's *t*-test for paired observations.



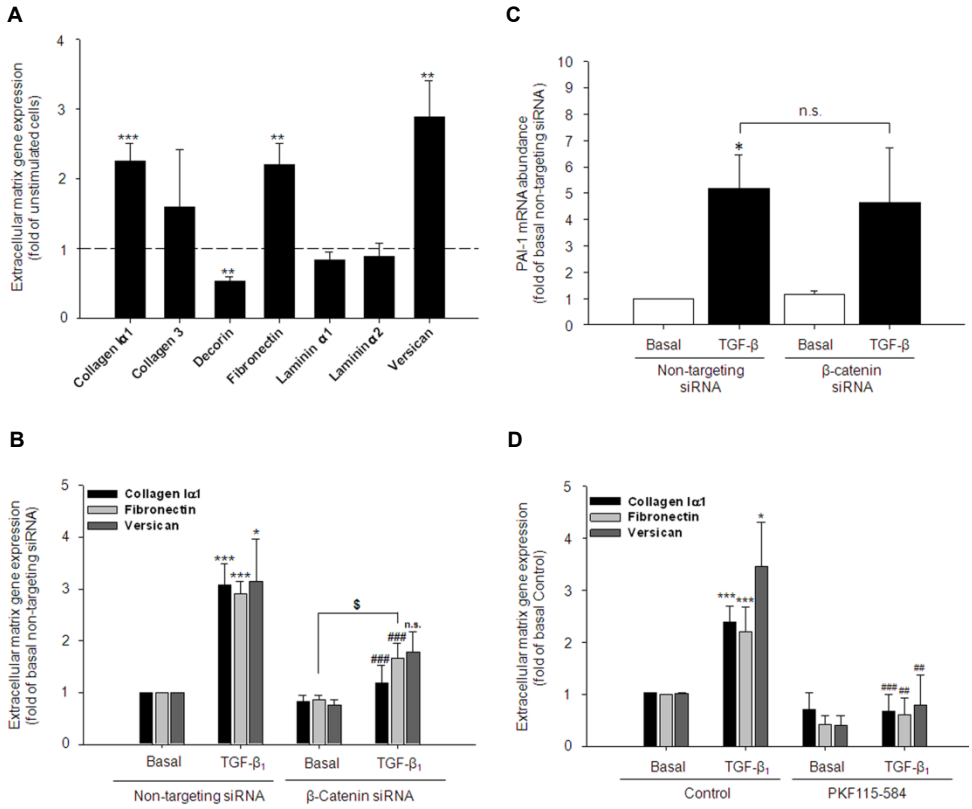
▲ ***Figure 2: Induction of non-phosphorylated β-catenin in human airway smooth muscle in response to TGF-β_1.*** (A) Airway smooth muscle cells were stimulated with TGF-β_1 (2 ng/ml) for up to 24 hours. Expression of non-phosphorylated β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Non-phosphorylated β-catenin expression was quantified by densitometry and normalized to GAPDH, representing mean ± s.e.m. of 5 independent experiments. *p<0.05 and ***p<0.001 compared to untreated airway smooth muscle cells. Statistical significance determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test. (B) TGF-β_1 (2 ng/ml; 16 hr.) induces β-catenin-dependent gene transcription, determined by the TOP-flash luciferase assay. TOP-flash luciferase activity was normalized to FOP-flash luciferase activity and expressed as a percentage of control representing mean ± s.e.m. of 4 independent experiments. **p<0.01 two-tailed student's *t*-test for paired observations.

Functional role of β-catenin signaling in TGF-β_1-induced extracellular matrix expression.

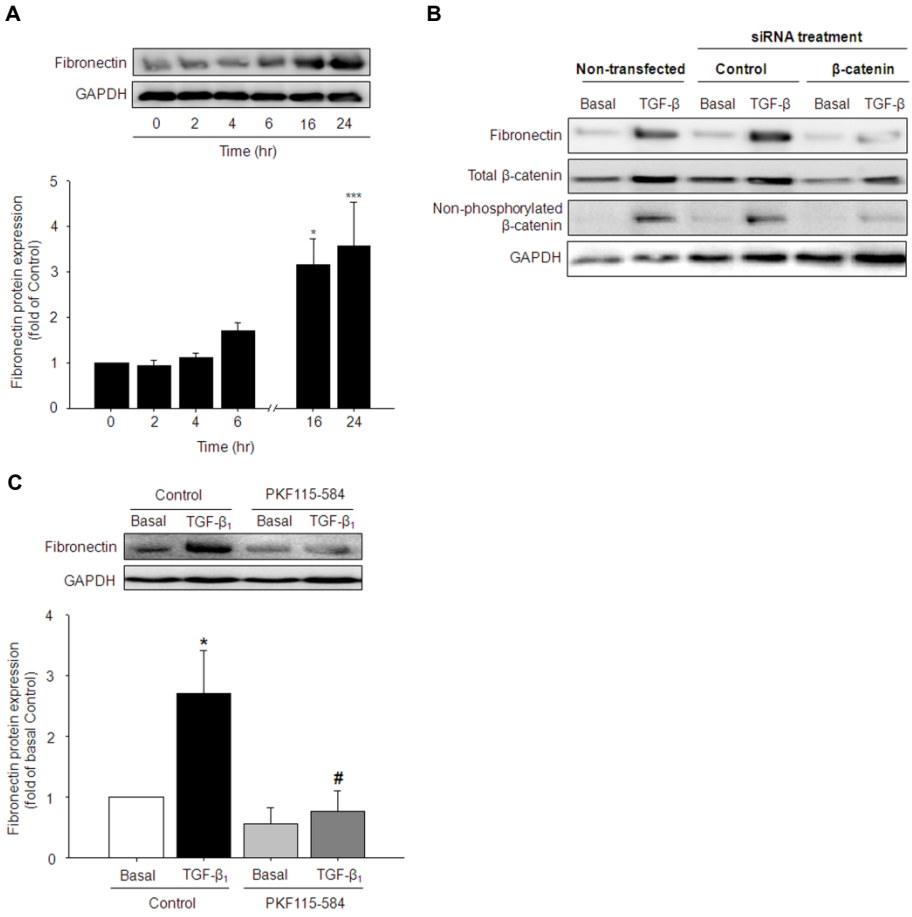
Given the importance of TGF-β_1 in regulation of ECM production by airway smooth muscle cells, we examined the role of β-catenin in this process. Stimulation of airway smooth muscle cells for 24 hours with TGF-β_1 (2 ng/ml) induced mRNA expression of the ECM proteins collagen Iα1 (collagen, type 1, alpha 1), fibronectin and versican (Figure 3A). In contrast, mRNA abundance of collagen III, laminin α1 and laminin α2 did not change significantly, whereas decorin mRNA abundance was attenuated by TGF-β_1 stimulation (Figure 3A).

Next, we investigated the effect of β-catenin silencing on TGF-β_1-induced ECM gene expression. Initial experiments indicated that after transfection of the cells with β-catenin siRNA, optimal reduction in the expression of β-catenin was achieved at 48-72 hours of treatment (53). Therefore, airway smooth muscle cells were transfected with specific β-catenin siRNA or non-targeting siRNA with concomitant serum deprivation and treated with TGF-β_1 (2 ng/ml) for 24 hours thereafter to ensure optimal β-catenin knock down during the window of TGF-β_1 treatment. In non-targeting siRNA treated airway smooth muscle cells TGF-β_1 induced the expression of collagen Iα1, fibronectin and versican (Figure 3B). Silencing of β-catenin by specific siRNA did not affect basal mRNA abundance of collagen Iα1, fibronectin and versican (Figure 3B). However, the TGF-β_1-induced expression of collagen Iα1 and fibronectin was attenuated when β-catenin was down regulated (Figure 3B). Though silencing β-catenin expression appeared to decrease in TGF-β_1-induced versican expression, differences were not statistically significant (Figure 3B). β-Catenin has a dual function in cellular signaling, by regulating TCF/LEF-dependent gene transcription and secondly it stabilizes cell-cell contacts by being a component of cadherin-based adherens junctions (13; 37). Down regulation of β-catenin by siRNA disrupts cell-cell contracts and this possibly affects TGF-β receptor signaling. Therefore, we investigated the effect of β-catenin siRNA on the mRNA expression of the smad-dependent gene plasminogen activator inhihitor-1 (PAI-1)(15; 56). Stimulation of airway smooth muscle cells with TGF-β_1 resulted in a 5-fold induction of PAI-1 mRNA expression. Silencing of β-catenin expression did not affect the basal or TGF-β_1-induced expression of PAI-1 (Figure 3C). These finding implicate that the responsiveness of smooth muscle cells to TGF-β_1 is not altered by down regulation of β-catenin expression.

To further verify the functional role of β-catenin/TCF interactions in TGF-β-induced ECM gene expression, we pharmacologically inhibited β-catenin signaling by PKF115-584 a compound that disrupts the interaction of the transcriptionally active (non-phosphorylated) β-catenin/T-cell factor-4 (TCF-4) complex (4; 47; 52). In line with the findings obtained with β-catenin siRNA, PKF115-584 did not significantly affect basal expression of collagen Iα1 ($p=0.432$), fibronectin ($p=0.693$) or versican ($p=0.394$), but largely attenuated the TGF-β_1-induced expression of the ECM genes (Figure 3D). Although pharmacological inhibitors may be less specific than siRNA, these data show that β-catenin signaling can be directly targeted using a small molecule. Taken together, these data indicate that β-catenin signaling activated by TGF-β_1 regulates the expression of specific ECM genes in airway smooth muscle.



▲ ***Figure 3: β-Catenin signaling regulates TGF-β$_1$-induced extracellular matrix gene expression in airway smooth muscle cells.*** (A) qRT-PCR analysis of extracellular matrix gene expression in airway smooth muscle cells stimulated with TGF-β$_1$ (2 ng/ml) for 24 hours. Expression of extracellular matrix genes by TGF-β$_1$ is expressed relative to untreated airway smooth muscle cells. Data represent mean ± s.e.m. of 4-9 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 determined by two-tailed student's t-test for paired observations. (B-D) Contribution of β-catenin signaling to TGF-β$_1$-induced gene expression of extracellular matrix proteins and plasminogen activator inhibitor-1 (PAI-1). Airway smooth muscle cells were stimulated for 24 hours with TGF-β$_1$ (2 ng/ml) and functional role of β-catenin was determined by (B-C) silencing of β-catenin expression by specific siRNA as described in materials and methods or (D) pharmacological inhibition by PKF115-584 (100 nM), which inhibits the nuclear β-catenin/TCF-4 interactions. Data represents mean ± s.e.m. of 5 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to unstimulated/basal control. $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to TGF-β$_1$ stimulation. $^{\$}$p<0.05 compared to basal of β-catenin siRNA treated airway smooth muscle cells. Statistical significance determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test.

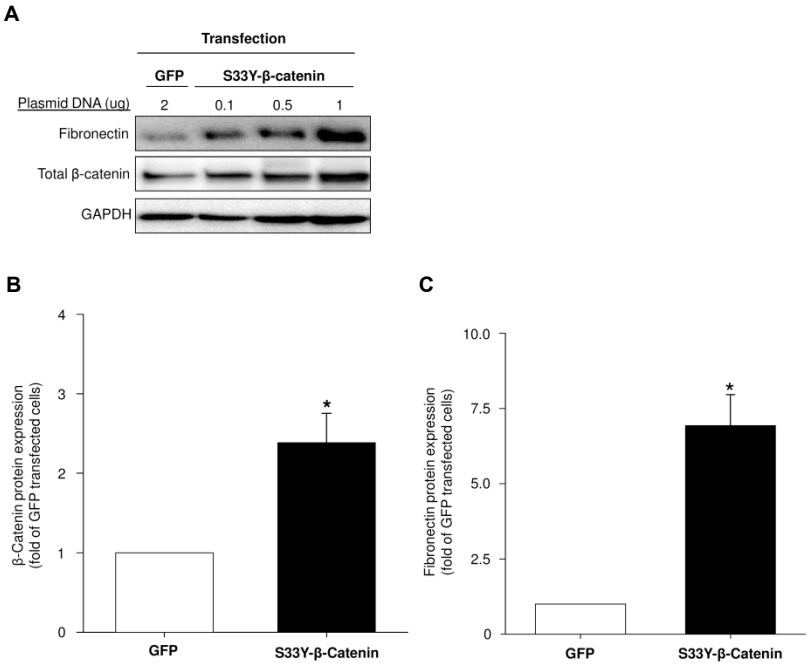


▲ ***Figure 4: β-Catenin signaling required for TGF-β$_1$-induced fibronectin protein expression by airway smooth muscle cells.*** (A) Airway smooth muscle cells were stimulated with TGF-β$_1$ (2 ng/ml) for up to 24 hours. Expression of fibronectin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Fibronectin deposition was quantified by densitometry and normalized to GAPDH expression, representing mean ± s.e.m. of 5 independent experiments. *p<0.05 and ***p<0.001 compared to untreated airway smooth muscle cells. Statistical significance determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test. (B) Airway smooth muscle cells were transfected with a siRNA against the β-catenin transcript or a non-targeting siRNA control as described in the materials and methods. Subsequently, cells were stimulated with TGF-β$_1$ (2 ng/ml) for 48 hours. Expression of total β-catenin, non-phosphorylated β-catenin and fibronectin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. A representative immunoblot of 3 independent experiments is shown. (C) Airway smooth muscle cells were stimulated with TGF-β$_1$ (2 ng/ml) for 48 hours in the absence or presence of the pharmacological inhibitor PKF115-584 (100 nM). Expression of fibronectin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Fibronectin expression was quantified by densitometry, representing mean ± s.e.m. of 4 independent experiments. *p<0.05 compared to untreated control, $^{\#}$p<0.05 compared to TGF-β$_1$ stimulation. Statistical significance determined by one-way ANOVA followed by a Newman-Keuls multiple comparison test.

To confirm the significance of β-catenin signaling in regulating ECM protein expression by airway smooth muscle cells, we assessed the role of β-catenin in TGF-β_1-induced fibronectin protein expression. Stimulation of airway smooth muscle cells with TGF-β_1 induced fibronectin protein expression in a time-dependent manner (Figure 4A). Silencing of β-catenin markedly reduced basal and TGF-β_1-induced expression of total and non-phosphorylated β-catenin (Figure 4B). Importantly, down regulation of β-catenin also attenuated TGF-β_1-induced fibronectin protein expression in the airway smooth muscle cells (figure 4B). To corroborate these findings we used PKF115-584 to inhibit β-catenin signaling pharmacologically. In agreement with the findings using β-catenin siRNA, PKF115-584 prevented the increased fibronectin expression induced by TGF-β_1 (Figure 4B). Collectively, these data indicate that induction of transcriptionally active β-catenin by TGF-β_1 is required for ECM mRNA and protein expression by airway smooth muscle cells.

β-Catenin activation is sufficient for fibronectin expression by airway smooth muscle.

Next we hypothesized that increased expression of β-catenin was sufficient to induce fibronectin expression by airway smooth muscle cells. To test this hypothesis, airway smooth muscle cells were transiently transfected with a constitutively active β-catenin mutant (S33Y-β-catenin). This S33Y-β-catenin mutant has a serine to tyrosine substitution at position 33 and is therefore insensitive to GSK-3 mediated phosphorylation and proteasomal degradation (43). Airway smooth muscle cells were transfected with increasing amounts of the S33Y-β-catenin mutant (0.1 – 1 μg), whereas control cultures were transfected with green fluorescent protein (GFP) (figure 5A). The transfection with the S33Y-β-catenin mutant resulted in an increased expression of total β-catenin, which was optimal when cells were transfected with 1.0 μg of plasmid DNA (Figure 5A and 5B). The transfection with the constitutively active S33Y-β-catenin mutant (1 μg) also resulted in a strong increase in fibronectin expression by airway smooth muscle cells (Figure 5A and 5C), whereas the expression of the smad regulated gene PAI-1 was not affected (data not shown). These data indicate that an increase in transcriptionally active β-catenin is sufficient to increase the expression of fibronectin by airway smooth muscle cells.



▲ ***Figure 5: Increased β-catenin expression is sufficient for stimulating fibronectin production by airway smooth muscle cells***. (A) Airway smooth muscle cells were transiently transfected with increasing amounts of β-catenin-S33Y plasmid (0.1 – 1 µg) or with a GFP containing vector (2 µg), which was used as a control. Expression of fibronectin and total β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. The effect of β-catenin-S33Y plasmid (1 µg) on (B) total β-catenin and (C) fibronectin protein expression was quantified by densitometry, representing mean ± s.e.m. of 3-4 independent experiments. *p<0.05 compared to GFP control. Statistical significance determined by two-tailed student's *t*-test for paired observations.

Discussion

In the present study, we demonstrate that β-catenin signaling plays an important role in the regulation of ECM production by airway smooth muscle. We show that in response to TGF-β_1 stimulation β-catenin protein abundance is stabilized, which subsequently results in increased TCF/LEF-dependent gene transcription. Down regulation of β-catenin expression or pharmacological inhibition of the nuclear β-catenin/TCF-4 complex, attenuates TGF-β_1-induced expression of collagen Iα1 and fibronectin, indicating a requirement of β-catenin signaling in this process. Furthermore, expression of a degradation-resistant β-catenin mutant (S33Y-β-catenin) strongly activates the production of fibronectin, implying that increased expression of β-catenin is sufficient to activate ECM protein production in airway smooth muscle cells. Collectively, these findings indicate that β-catenin signaling is both required and sufficient for the regulation of ECM production by airway smooth muscle.

Our current and previously published data indicate a key role for β-catenin in smooth muscle biology. In its role as an adherens junction associated protein that links to the actin cytoskeleton, β-catenin is important for contractile force generation of airway smooth muscle cells, presumably by stabilizing cell-cell contacts, allowing force transmission between neighboring cells (37). Moreover, growth factors that stimulate airway smooth muscle growth induce nuclear accumulation of β-catenin, which activates TCF/LEF-dependent gene transcription and subsequent induction of cell proliferation (28; 30; 53). Our current findings indicate that matrix protein expression by airway smooth muscle is also regulated by β-catenin dependent gene transcription. These data are consistent with findings in vascular smooth muscle cells, in which growth factor-induced β-catenin activation and subsequent induction of TCF/LEF-dependent gene transcription, regulates smooth muscle cell proliferation, vascular endothelial growth factor (VEGF) secretion and extracellular matrix protein production, including fibronectin and versican (14; 18; 59; 60; 62; 63). Collectively, these findings indicate a central role for β-catenin in many cellular responses that underpin smooth muscle remodeling in both the airways and the vasculature (30).

Cytosolic β-catenin levels are tightly regulated by the constitutively active enzyme GSK-3β. A fraction of cellular GSK-3β forms a so called destruction complex with axin, casein kinase I (CK-I) and adenomatous polyposis coli (APC); this complex phosphorylates and subsequently targets cytosolic β-catenin for proteasomal degradation (13). This tight regulation explains the relatively low abundance of active (non-phosphorylated) β-catenin at baseline, even though total β-catenin protein expression is abundant at the plasma membrane. The activity of GSK-3β is

negatively regulated by serine phosphorylation, which can be induced by numerous stimuli, including growth factors (23; 28; 29; 53). Growth factors are believed to increase β-catenin stabilization in part via this inactivation of GSK-3. In line with this contention, we demonstrate that TGF-β_1 induces a strong and sustained phosphorylation of GSK-3 in airway smooth muscle cells, followed by increased expression of active, non-phosphorylated β-catenin. Accumulation of stabilized β-catenin contributes to the cellular expression of total β-catenin. Therefore, the overall cellular expression of β-catenin is also augmented by TGF-β_1 stimulation, however this increase distinct from the rise in active β-catenin expression as the plasma membrane associated pool of β-catenin is not induced in response to TGF-β_1 (28). We demonstrate that the expression of the stable S33Y-β-catenin mutant, which has a serine to tyrosine substitution at position 33 and therefore is insensitive to GSK-3-mediated phosphorylation and proteosomal degradation, is sufficient to activate fibronectin deposition by airway smooth muscle cells. This demonstrates the importance of rigorous control of β-catenin expression by GSK-3 in regulating cellular responses of the airway smooth muscle. In addition to GSK-3 dependent regulation of β-catenin, we found that TGF-β_1 induced an increased β-catenin mRNA expression, indicating that the *de novo* synthesis of the protein is also regulated by this growth factor. Indeed, we have previously shown that expression of a dominant negative H-RAS as well as pharmacological inhibition of ERK1/2 kinase (e.g. MEK) attenuates growth factor-induced β-catenin mRNA and protein expression (28). Taken together, these findings demonstrate that TGF-β_1 regulates β-catenin expression in part by increasing protein stability and in part by ERK1/2-dependent *de novo* synthesis of the protein in airway smooth muscle cells.

In the airways, TGF-β_1 is sequestered in inactive complex in the ECM and upon activation it stimulates cell surface serine-threonine receptor kinases leading to phosphorylation of smad (small phenotype and mothers against decapentaplegic related protein) family of intracellular signaling proteins. The activated smads translocate to the nucleus and associate with DNA binding partners and various transcriptional co-activators, thereby regulating gene transcription (2; 44). Smad phosphorylation is critical for TGF-β_1 signaling, yet simultaneously a variety of other pathways, including ERK1/2 and GSK-3/β-catenin signaling are activated by TGF-β_1 that in turn support functional TGF-β_1 driven responses (2; 33). Biochemical studies have shown that smad and β-catenin may cooperatively regulate TCF/LEF transcription factors, resulting in synergistic activation of gene transcription (14; 48). The TCF/LEF family of transcription factors are the downstream effectors of the canonical Wnt/β-catenin signaling pathway and consists of four members, i.e. TCF-1, LEF, TCF-3 and TCF-4, which all share homology in their DNA binding domain. Stabilized (unphosphorylated) cytosolic β-

catenin translocates to the nucleus were it associates and activates these TCF/LEF transcription factors (7; 13). The experiments performed with the pharmacological inhibitor PKF115-584 indicate that at least TCF-4 plays an important role in the regulation of versican, collagen Iα1 and fibronectin expression by airway smooth muscle cells, as this inhibitor disrupts the interaction between nuclear β-catenin and TCF-4 (4; 47; 52). This fits with observations that indicate abundant expression of the TCF-4 transcription factor by mesenchymal cells (7; 11). Transcription factors of the TCF family not only activate gene transcription, but may also be active suppressors of specific gene transcription (7). However, silencing of β-catenin expression by specific siRNA did not affect the decrease in decorin gene expression caused by TGF-β_1 (data not shown), indicating that this effect is not due to the activation of the TCF family of transcription factors. In the present study we further demonstrate that ECM deposition is attenuated by silencing of β-catenin signaling and, more specifically, that fibronectin deposition is increased in response to overexpression of β-catenin. These data unambiguously demonstrate the involvement of β-catenin signaling in TGF-β-induced ECM deposition by airway smooth muscle cells. Furthermore, TGF-β_1 stimulation induced the expression of the smad-dependent gene plasminogen activator inhibitor-1 (PAI-1), which was not affected by the siRNA against β-catenin. Similarly, expression of the transcriptionally active β-catenin mutant (S33Y-β-catenin) did not alter basal PAI-1 expression (data not shown). Collectively, these results show that TGF-β_1 signaling is directed to specific intracellular pathways by β-catenin.

Increased airway smooth muscle mass in concert with aberrant extracellular matrix deposition in the airways is thought to contribute to the pathogenesis of chronic inflammatory lung diseases, like asthma and COPD (35; 58). The expression of various ECM components in the airways is altered in these chronic inflammatory lung diseases (1; 3; 38; 45). More specifically, in asthma the expression of fibronectin, hyaluronan, versican, biglycan, lumican and collagen I are increased within as well as surrounding the airway smooth muscle (1; 57; 61). In COPD, changes in airway smooth muscle mass and altered ECM deposition are less pronounced compared to asthma, but may become more important in more severe stages of disease (35). In addition to increased bronchial ECM deposition, the expression of laminin β2 is increased in the airway smooth muscle bundle of COPD patients and inversely correlates to the forced expiratory volume in 1 second (FEV_1) (45). The expression and composition of ECM in the airways of patients with chronic inflammatory lung diseases may also strongly influence airway smooth muscle function and thereby contribute to disease pathogenesis. For instance, fibronectin and collagen I have been demonstrated to enhance airway smooth muscle proliferation, migration, cell survival, and negatively affect the contractile capacity of airway smooth muscle cells (22; 27; 34; 54). The

significance of the interaction between the ECM and airway smooth muscle *in vivo* has recently been established in an animal model of chronic allergic asthma. In this study it was demonstrated that inhibition of the interaction between the ECM proteins and their integrins attenuated allergen-induced airway smooth muscle remodelling (20). Corticosteroids used for control of asthma and COPD symptoms, do not seem to have a beneficial effect on ECM production by airway smooth muscle, and may under certain circumstances even increase the matrix production (19; 39; 50). Matrix protein production can be induced by growth factors that are upregulated in asthma and COPD, like TGF-β_1, connective tissue growth factor (CTGF) and VEGF (8; 12; 66). The underlying mechanisms by which these growth factors, in particular TGF-β_1, induce matrix production is therefore of major interest. We demonstrate that β-catenin signaling is activated in response to TGF-β_1 and plays an important regulatory role in ECM production by airway smooth muscle. This suggests that targeting β-catenin dependent gene transcription is a strategy worth pursuing in future studies, particularly since corticosteroids used for the control of asthma and COPD symptoms do not seem to have a beneficial effect on ECM production by airway smooth muscle (19; 39; 50).

Collectively, these findings indicate that β-catenin signaling is activated in response to TGF-β_1, which is required and sufficient for the regulation of ECM production by airway smooth muscle cells. The current data adds to the increasing evidence indicating the importance of β-catenin in airway smooth muscle function. The regulatory role of β-catenin in airway smooth muscle may be of importance in TGF-β_1 driven airway wall remodeling. Targeting β-catenin-dependent gene transcription may therefore hold promise as a therapeutic intervention.

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References

1. Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, Ferreira DS, Mulder A, Gomes HA, Fernezlian SM, James A and Mauad T. Extracellular matrix components and regulators in the airway smooth muscle in asthma. *Eur Respir J* 32: 61-69, 2008.
2. Attisano L and Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 296: 1646-1647, 2002.
3. Bara I, Ozier A, Tunon de Lara JM, Marthan R and Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. *Eur Respir J* 36: 1174-1184, 2010.
4. Barker N and Clevers H. Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* 5: 997-1014, 2006.
5. Bowley E, O'Gorman DB and Gan BS. Beta-catenin signaling in fibroproliferative disease. *J Surg Res* 138: 141-150, 2007.
6. Brabletz T, Jung A, Dag S, Hlubek F and Kirchner T. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am J Pathol* 155: 1033-1038, 1999.
7. Brantjes H, Barker N, van EJ and Clevers H. TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. *Biol Chem* 383: 255-261, 2002.
8. Burgess JK, Johnson PR, Ge Q, Au WW, Poniris MH, McParland BE, King G, Roth M and Black JL. Expression of connective tissue growth factor in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* 167: 71-77, 2003.
9. Cheon SS, Nadesan P, Poon R and Alman BA. Growth factors regulate beta-catenin-mediated TCF-dependent transcriptional activation in fibroblasts during the proliferative phase of wound healing. *Exp Cell Res* 293: 267-274, 2004.
10. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, Pedron S, Bertaso M, Scarpa A, Murer B, Cancellieri A, Maestro R, Semenzato G and Doglioni C. Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol* 162: 1495-1502, 2003.
11. Cho EA and Dressler GR. TCF-4 binds beta-catenin and is expressed in distinct regions of the embryonic brain and limbs. *Mech Dev* 77: 9-18, 1998.
12. Chung KF. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 34: 50s-59s, 2001.
13. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell* 127: 469-480, 2006.
14. Clifford RL, Deacon K and Knox AJ. Novel regulation of vascular endothelial growth factor-A (VEGF-A) by transforming growth factor (beta)1: requirement for Smads, (beta)-CATENIN, AND GSK3(beta). *J Biol Chem* 283: 35337-35353, 2008.
15. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y and Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. *Mol Cell Biol* 19: 2495-2504, 1999.
16. de Boer WI, van SA, Sont JK, Sharma HS, Stolk J, Hiemstra PS and van Krieken JH. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158: 1951-1957, 1998.
17. De Langhe SP, Sala FG, Del Moral PM, Fairbanks TJ, Yamada KM, Warburton D, Burns RC and Bellusci S. Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung. *Dev Biol* 277: 316-331, 2005.
18. de JP, V, Ali Z, Alastalo TP, Ikeno F, Sawada H, Lai YJ, Kleisli T, Spiekerkoetter E, Qu X, Rubinos LH, Ashley E, Amieva M, Dedhar S and Rabinovitch M. BMP promotes motility and represses growth of smooth muscle cells by activation of tandem Wnt pathways. *J Cell Biol* 192: 171-188, 2011.

19. de KJ, Schrumpf JA, Evertse CE, Sont JK, Roughley PJ, Rabe KF, Hiemstra PS, Mauad T and Sterk PJ. Bronchial matrix and inflammation respond to inhaled steroids despite ongoing allergen exposure in asthma. *Clin Exp Allergy* 35: 1361-1369, 2005.
20. Dekkers BG, Bos IS, Gosens R, Halayko AJ, Zaagsma J and Meurs H. The integrin-blocking peptide RGDS inhibits airway smooth muscle remodeling in a guinea pig model of allergic asthma. *Am J Respir Crit Care Med* 181: 556-565, 2010.
21. Dekkers BG, Maarsingh H, Meurs H and Gosens R. Airway structural components drive airway smooth muscle remodeling in asthma. *Proc Am Thorac Soc* 6: 683-692, 2009.
22. Dekkers BG, Schaafsma D, Nelemans SA, Zaagsma J and Meurs H. Extracellular matrix proteins differentially regulate airway smooth muscle phenotype and function. *Am J Physiol Lung Cell Mol Physiol* 292: L1405-L1413, 2007.
23. Doble BW and Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116: 1175-1186, 2003.
24. Doyle JL and Haas TL. Differential role of beta-catenin in VEGF and histamine-induced MMP-2 production in microvascular endothelial cells. *J Cell Biochem* 107: 272-283, 2009.
25. Duvernelle C, Freund V and Frossard N. Transforming growth factor-beta and its role in asthma. *Pulm Pharmacol Ther* 16: 181-196, 2003.
26. Ebina M, Takahashi T, Chiba T and Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis* 148: 720-726, 1993.
27. Freyer AM, Johnson SR and Hall IP. Effects of growth factors and extracellular matrix on survival of human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 25: 569-576, 2001.
28. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, Meurs H and Schmidt M. De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. *FASEB J* 24: 757-768, 2010.
29. Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H and Halayko AJ. Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 293: L1348-L1358, 2007.
30. Gosens R, Meurs H and Schmidt M. The GSK-3/beta-catenin-signalling axis in smooth muscle and its relationship with remodelling. *Naunyn Schmiedebergs Arch Pharmacol* 378: 185-191, 2008.
31. Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J and Halayko AJ. Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 291: L523-L534, 2006.
32. Gradl D, Kuhl M and Wedlich D. The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. *Mol Cell Biol* 19: 5576-5587, 1999.
33. Guo X and Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* 19: 71-88, 2009.
34. Hirst SJ, Twort CH and Lee TH. Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* 23: 335-344, 2000.
35. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO and Pare PD. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 350: 2645-2653, 2004.
36. Howe LR, Subbaramaiah K, Chung WJ, Dannenberg AJ and Brown AM. Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. *Cancer Res* 59: 1572-1577, 1999.
37. Jansen SR, Van Ziel AM, Baarsma HA and Gosens R. {beta}-Catenin regulates airway smooth muscle contraction. *Am J Physiol Lung Cell Mol Physiol* 299: L204-L214, 2010.

38. Johnson PR. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin Exp Pharmacol Physiol* 28: 233-236, 2001.
39. Johnson PR, Black JL, Carlin S, Ge Q and Underwood PA. The production of extracellular matrix proteins by human passively sensitized airway smooth-muscle cells in culture: the effect of beclomethasone. *Am J Respir Crit Care Med* 162: 2145-2151, 2000.
40. Jope RS, Yuskaitis CJ and Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res* 32: 577-595, 2007.
41. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzo H, Lemiere C, Olivenstein R, Ernst P, Hamid Q and Martin J. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol* 124: 45-51, 2009.
42. Kang HR, Cho SJ, Lee CG, Homer RJ and Elias JA. Transforming growth factor (TGF)-beta1 stimulates pulmonary fibrosis and inflammation via a Bax-dependent, bid-activated pathway that involves matrix metalloproteinase-12. *J Biol Chem* 282: 7723-7732, 2007.
43. Kolligs FT, Hu G, Dang CV and Fearon ER. Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. *Mol Cell Biol* 19: 5696-5706, 1999.
44. Konigshoff M, Kneidinger N and Eickelberg O. TGF-beta signaling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimen. *Swiss Med Wkly* 139: 554-563, 2009.
45. Kranenburg AR, Willems-Widyastuti A, Moori WJ, Sterk PJ, Alagappan VK, de Boer WI and Sharma HS. Enhanced bronchial expression of extracellular matrix proteins in chronic obstructive pulmonary disease. *Am J Clin Pathol* 126: 725-735, 2006.
46. Lazaar AL and Panettieri RA, Jr. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin Immunol* 116: 488-495, 2005.
47. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW and Shivdasani RA. Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell* 5: 91-102, 2004.
48. Letamendia A, Labbe E and Attisano L. Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. *J Bone Joint Surg Am* 83-A Suppl 1: S31-S39, 2001.
49. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM and Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis* 8: 43-51, 2005.
50. Mauad T, Bel EH and Sterk PJ. Asthma therapy and airway remodeling. *J Allergy Clin Immunol* 120: 997-1009, 2007.
51. McAnulty RJ. Fibroblasts and myofibroblasts: their source, function and role in disease. *Int J Biochem Cell Biol* 39: 666-671, 2007.
52. Minke KS, Staib P, Puetter A, Gehrke I, Gandhirajan RK, Schlosser A, Schmitt EK, Hallek M and Kreuzer KA. Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. *Eur J Haematol* 82: 165-175, 2009.
53. Nunes RO, Schmidt M, Dueck G, Baarsma H, Halayko AJ, Kerstjens HA, Meurs H and Gosens R. GSK-3/beta-catenin signaling axis in airway smooth muscle: role in mitogenic signaling. *Am J Physiol Lung Cell Mol Physiol* 294: L1110-L1118, 2008.
54. Parameswaran K, Radford K, Zuo J, Janssen LJ, O'Byrne PM and Cox PG. Extracellular matrix regulates human airway smooth muscle cell migration. *Eur Respir J* 24: 545-551, 2004.
55. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, Ludwig MS, Martin JG and Hamid Q. Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 116: 544-549, 2005.
56. Piek E, Ju WJ, Heyer J, Escalante-Alcalde D, Stewart CL, Weinstein M, Deng C, Kucherlapati R, Bottinger EP and Roberts AB. Functional characterization of transforming growth factor

beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 276: 19945-19953, 2001.
57. Pini L, Hamid Q, Shannon J, Lemelin L, Olivenstein R, Ernst P, Lemiere C, Martin JG and Ludwig MS. Differences in proteoglycan deposition in the airways of moderate and severe asthmatics. *Eur Respir J* 29: 71-77, 2007.
58. Postma DS and Timens W. Remodeling in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 3: 434-439, 2006.
59. Quasnichka H, Slater SC, Beeching CA, Boehm M, Sala-Newby GB and George SJ. Regulation of smooth muscle cell proliferation by beta-catenin/T-cell factor signaling involves modulation of cyclin D1 and p21 expression. *Circ Res* 99: 1329-1337, 2006.
60. Rahmani M, Read JT, Carthy JM, McDonald PC, Wong BW, Esfandiarei M, Si X, Luo Z, Luo H, Rennie PS and McManus BM. Regulation of the versican promoter by the beta-catenin-T-cell factor complex in vascular smooth muscle cells. *J Biol Chem* 280: 13019-13028, 2005.
61. Roberts CR and Burke AK. Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. *Can Respir J* 5: 48-50, 1998.
62. Slater SC, Koutsouki E, Jackson CL, Bush RC, Angelini GD, Newby AC and George SJ. R-cadherin:beta-catenin complex and its association with vascular smooth muscle cell proliferation. *Arterioscler Thromb Vasc Biol* 24: 1204-1210, 2004.
63. Uglow EB, Slater S, Sala-Newby GB, guilera-Garcia CM, Angelini GD, Newby AC and George SJ. Dismantling of cadherin-mediated cell-cell contacts modulates smooth muscle cell proliferation. *Circ Res* 92: 1314-1321, 2003.
64. van de WB, Houtepen F, Huigsloot M and Tijdens IB. Suppression of chemically induced apoptosis but not necrosis of renal proximal tubular epithelial (LLC-PK1) cells by focal adhesion kinase (FAK). Role of FAK in maintaining focal adhesion organization after acute renal cell injury. *J Biol Chem* 276: 36183-36193, 2001.
65. van Straaten JF, Coers W, Noordhoek JA, Huitema S, Flipsen JT, Kauffman HF, Timens W and Postma DS. Proteoglycan changes in the extracellular matrix of lung tissue from patients with pulmonary emphysema. *Mod Pathol* 12: 697-705, 1999.
66. Zanini A, Chetta A, Imperatori AS, Spanevello A and Olivieri D. The role of the bronchial microvasculature in the airway remodelling in asthma and COPD. *Respir Res* 11: 132, 2010.
67. Zhang X, Gaspard JP and Chung DC. Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res* 61: 6050-6054, 2001.

Chapter 3

Activation of WNT/β-catenin signaling in pulmonary fibroblasts by TGF-β_1 is increased in chronic obstructive pulmonary disease

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterized by abnormal extracellular matrix (ECM) turnover. Recently, activation of the WNT/β-catenin pathway has been associated with abnormal ECM turnover in various chronic diseases. We determined WNT-pathway gene expression in pulmonary fibroblasts of individuals with and without COPD and disentangled the role of β-catenin in fibroblast phenotype and function.

Methods: We assessed the expression of WNT-pathway genes and the functional role of β-catenin, using MRC-5 human lung fibroblasts and primary pulmonary fibroblasts of individuals with and without COPD.

Results: Pulmonary fibroblasts expressed mRNA of genes required for WNT signaling. Stimulation of fibroblasts with TGF-β_1, a growth factor important in COPD pathogenesis, induced WNT-5B, FZD_8, DVL3 and β-catenin mRNA expression. The induction of WNT-5B, FZD_6, FZD_8 and DVL3 mRNA by TGF-β_1 was higher in fibroblasts of individuals with COPD than without COPD, whilst basal expression was similar. Accordingly, TGF-β_1 activated β-catenin signaling, as shown by an increase in transcriptionally active and total β-catenin protein expression. Furthermore, TGF-β_1 induced the expression of collagen1α1, α-sm-actin and fibronectin, which was attenuated by β-catenin specific siRNA and by pharmacological inhibition of β-catenin, whereas the TGF-β_1-induced expression of PAI-1 was not affected. The induction of transcriptionally active β-catenin and subsequent fibronectin deposition induced by TGF-β_1 were enhanced in pulmonary fibroblasts from individuals with COPD.

Conclusions: β-catenin signaling contributes to ECM production by pulmonary fibroblasts and contributes to myofibroblasts differentiation. WNT/β-catenin pathway expression and activation by TGF-β_1 is enhanced in pulmonary fibroblasts from individuals with COPD. This suggests an important role of the WNT/β-catenin pathway in regulating fibroblast phenotype and function in COPD.

Key words: Frizzled, Extracellular matrix, Lung, Fibronectin

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation, which is associated with an abnormal inflammatory response of the lungs to noxious particles or gases. Long-term exposure to cigarette smoke is the major risk factor for the development of COPD [1, 2]. Progressive loss of lung function can be caused by airway wall remodeling, bronchoconstriction, occlusion of the airway lumen by mucus and destruction of alveolar attachments of the airways within the lung (emphysema) [3]. Aberrant extracellular matrix (ECM) turnover contributes to both airway remodeling and pulmonary emphysema.

Fibroblasts play an important role in ECM turnover in the parenchyma and small airways by producing ECM constituents [4-6]. Transforming growth factor-β (TGF-β) is locally upregulated in COPD and is the key mediator stimulating ECM production by recruiting and activating fibroblasts and initiating their differentiation process into myofibroblasts [5, 7-9]. Airway fibroblasts may thus contribute to small airways remodeling in COPD. By contrast, in the peripheral lung with pulmonary emphysema, there is inadequate tissue repair and associated damage, which is perhaps due to fibroblast dysfunction [10, 11]. This discrepancy may be explained by insufficient activation of fibroblast in regions affected by emphysema to compensate for the tissue destruction by proteases. Furthermore, lung fibroblasts from patients with pulmonary emphysema show an aberrant proliferation capacity and differences in ECM synthesis [12-14]. Cigarette smoke can also affect a number of fibroblast functions implicated in alveolar regeneration and repair [11, 15]. Consequently, extrinsic and intrinsic dysregulation of fibroblast function in COPD along with phenotypically distinct fibroblast populations in the airways and parenchyma, may contribute to the development of both small airway fibrosis and emphysema [16, 17].

Recently, it was demonstrated that activation of the canonical WNT/β-catenin signaling pathway is associated with fibroblast activation, fibrosis and tissue repair [18, 19]. β-Catenin is an essential component of canonical WNT signaling, in which it serves a role in activating gene transcription [20]. In the presence of WNT-ligands, cytosolic β-catenin is stabilized, permitting it to serve as a transcriptional co-activator. In addition, various growth factors, including TGF-β, can activate β-catenin signaling either directly or via autocrine WNT ligand production [19, 21, 22]. Stabilized (non-phosphorylated) β-catenin activates several target genes including matrix metalloproteinases (MMP's), growth factors, ECM proteins and pro-inflammatory mediators and enzymes [23-31]. The role of the WNT/β-catenin pathway in COPD is largely unknown. However, in support of a role in tissue repair,

a recent study indicates that activation of WNT/β-catenin signaling protects against experimental emphysema in mice [32].

In the present study, we investigated the expression of WNT-pathway genes in human lung fibroblasts and determined the functional role of the transcriptional co-activator β-catenin in regulating TGF-β_1-induced human lung fibroblast phenotype and function. Furthermore, we compared the expression of WNT pathway genes and activation of β-catenin in primary pulmonary fibroblasts of individuals with and without COPD.

Materials and Methods

Ethics statement
The study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; htttp://www.federa.org).

Subjects
Primary lung fibroblasts were cultured from lung tissue obtained from 18 individuals with and without COPD. Classification of COPD severity was based on the Global initiative for chronic obstructive lung disease (GOLD) criteria [1]. Fibroblasts obtained from these individuals, were divided into three categories: fibroblasts from individuals with moderate (GOLD stage II, n=5), and severe COPD (stage IV, n=6), and from individuals with histologically normal lungs (n=7). Emphysema was assessed by routine histological examination of lung tissue, which was performed by an experienced pulmonary pathologist (WT). Fibroblasts were isolated from peripheral lung tissue of which areas with no macroscopically visible airways and blood vessels were used. Clinical characteristics of the groups are presented in table 1.

Tissue from the control group (median forced expiratory volume in one second (FEV_1) 96.9% predicted) was derived from noninvolved lung tissue of patients undergoing surgical resection for pulmonary carcinoma. Patients had no airway obstruction and no chronic airway symptoms, such as cough and sputum production. Material was always taken as far away as possible from the tumour, or from a noninvolved lobe. No histopathological lesions were present.

Tissue of GOLD stage II COPD patients (median FEV_1 52.6 % of predicted) was derived from noninvolved lung tissue from patients undergoing resection surgery for pulmonary carcinoma. Histopathologically emphysematous lesions were present, however, of limited and varying severity. Moderate forms of emphysema can be histopathologically demonstrated by finding isolated or free-lying segments of viable alveolar septal tissue or isolated cross sections of pulmonary vessels.

Tissue of GOLD stage IV COPD patients (median FEV_1 17.1 % predicted) was obtained from patients with COPD undergoing surgery for lung transplantation or lung volume reduction. All individuals had quitted smoking for at least 1 year before surgery. The resected tissue showed both macroscopically and microscopically severe emphysematous lesions, often accompanied by bullae. Subpleural fibrous areas were avoided.

Pulmonary fibroblast cultures were established from parenchymal lung tissue by means of an explant technique. Absence of mycoplasma contamination in the fibroblast cultures was confirmed with a mycoplasma detection kit (Roche Diagnostics, Almere, The Netherlands). Isolated cells were characterized as fibroblasts by morphological appearance and expression pattern of specific proteins [12]. All cells exhibited a characteristic staining pattern for vimentin, fibronectin, and the fibroblast marker prolyl-4-hydroxylase and lacked immunoreactivity for keratin. Five percent or less of the cells was positive for desmin and α-sm-actin.

Cell culture
MRC-5 lung fibroblasts [33] (ATCC CCL 171) and primary lung fibroblasts from individuals with and without COPD, were cultured in Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/l streptomycin and 100 U/ml penicillin. Unless otherwise specified, for each experiment cells were grown to confluence and subsequently culture medium was substituted with Ham's F12 medium supplemented with 0.5% (v/v) FBS, 2 mM L-glutamine, 100 µg/l streptomycin and 100 U/ml penicillin for a period of 24 hours. Cells were stimulated for different time-points with TGF-β_1 in Ham's F12 medium supplemented with 0.5% FBS, L-glutamine and antibiotics. When applied, pharmacological inhibitors (i.e. quercetin 40 µM or PKF115-584 100 nM) were added 30 minutes before the addition of TGF-β_1.

mRNA isolation and real-time PCR analysis
Total mRNA was extracted using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). Briefly, cells were harvested in RNA*later* stabilization buffer and homogenized by passing the lysate 10 times through a 20 gauge needle. Lysates were then mixed with an equal volume of 70% ethanol, and total mRNA was purified using RNeasy mini spin columns. The eluted mRNA was quantified using spectrophotometry (Nanodrop, ThermoScientific, Wilmington, USA). Equal amounts of total mRNA (1 µg) were then reverse transcribed and stored at -20 °C until further use.

cDNA was subjected to real-time PCR, which was performed with a MyiQ™ Single-Color detection system (Bio-Rad laboratories Inc. Life Science group, Hercules, CA, USA). In short, 12.5 µl iQ™ SYBR Green Supermix, containing fluorescein to account for well to well variation, 0.1 µM of gene-specific forward and reverse primer and 1 µl of 1:5 diluted cDNA sample were used in a total volume of 25 µl and added to a 96 well plate. The sequences of the primers used for determining WNT pathway components and WNT target genes are listed in the supporting information tables (table S1-S4).

Real-time PCR data were analyzed using the comparative cycle threshold (Cq: amplification cycle number) method. Cycle parameters were: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles followed by 5 minutes at 72°C. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA ($Cq_{\text{gene of interest}} - Cq_{18S\ rRNA}$; designated as ΔCq). Several housekeeping genes, including β2-microglobulin (B2M; NM_00408) and phospholipase A2 (YWAHZ; NM_003406), were tested for the influence of the experimental procedure on the expression [34]. The expression of both ribosomal protein S18 (18S rRNA) and β2-microglobulin was stable in the tested conditions. Phospholipase A2 (YWAHZ; NM_003406) expression fluctuated after TGF-β stimulation, however. Ribosomal protein S18 was chosen as most optimal household gene because gene expression was most stable under basal as well as stimulation conditions (figure S1). Relative differences in gene expression were determined using the equation $2^{-(\Delta\Delta Cq)}$.

siRNA transfection

MRC-5 fibroblasts were grown to ~90% confluence in 6-well cluster plates and transiently transfected with a 21-bp, double-stranded siRNA targeted against the β-catenin transcript (Qiagen, Venlo, The Netherlands). Cells were transfected in serum-free Ham's F12 without any supplements using 1.5 µg/ml of siRNA in combination with lipofectamine 2000 transfection reagent. Control transfections were performed using a non-silencing control siRNA (Qiagen, Venlo, The Netherlands). After 6 hours of transfection, cells were washed once with warm (37°C) Hank's Balanced Salt Solution (HBSS; composition [mg/l]: KCl 400, KH_2PO_4 60, NaCl 8000, $NaHCO_3$ 350, $Na_2HPO_4.1H_2O$ 50, glucose 1000, pH: 7.4) followed by a period of 24 hours in Ham's F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Consecutively, medium was refreshed and cells were stimulated with TGF-β_1 (2 ng/ml) for 48 hours.

Preparation of cell lysates

To obtain whole cell lysates, cells were washed once with ice-cold (4°C) HBSS then lysed in ice-cold sodiumdodecylsulphate (SDS) buffer (composition: 62.5 mM Tris, 2 % w/v SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 7 µg/ml pepstatin A, pH 6.8). Lysates were then sonicated and protein concentration was determined according to Pierce protein determination according to the manufacturer's instructions. Lysates were stored at -20 ºC till further use.

Nuclear extracts
Confluent MRC-5 fibroblasts were serum deprived in Ham's F12 medium supplemented with 0.5% (v/v) FBS, 2 mM L-glutamine, 100 µg/l streptomycin and 100 U/ml penicillin for a period of 24 hours. Subsequently cells were stimulated for 24 hour with TGF-β_1 (2 ng/ml) and nuclear extracts were prepared using the nuclear extract kit (40010, Active Motif) according to the manufacturer's instructions. Protein concentration in the nuclear extracts was determined according to the Bradford protein assay. Nuclear extracts were stored at -80 ºC till further use.

Western blot analysis
Equal amounts of protein (10-20 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were either subsequently visualized on film or recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisistion software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using Totallab^{TM} software (Nonlinear dynamics; Newcastle, UK) or GeneTools analysis software (Syngene; Cambridge; UK), respectively.

Immunocytochemistry
Lung fibroblasts were plated onto Lab-Tek^{TM} borosilicate chamber slides and treated with TGF-β_1 2 ng/ml for 48 hours, fixed for 15 min at 4 ^{o}C in cytoskeletal (CB) buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$ and 5 mM glucose at pH 6.1) containing 3 % paraformaldehyde (PFA). Cells were then permeabilized by incubation for 5 min at 4^{o}C in CB buffer containing 3% PFA and 0.3% Triton X-100. For immunofluorescence microscopy, fixed cells were first blocked for 2 hours at room temperature in Cyto-TBS buffer (20 mM Tris base, 154 mM NaCl, 2.0 mM EGTA and 2.0 mM $MgCl_2$ at pH 7.2) containing 1% bovine serum albumin (BSA) and 2% normal donkey serum. Incubation with primary antibody (i.e unphosphorylated-β-catenin, diluted 1:200) occurred overnight at 4^{o}C in Cyto-TBS containing 0.1 % Tween 20 (Cyto-TBST). Incubation with Cy3-conjugated secondary antibody was for 2h at room temperature in Cyto-TBST. Filamentous actin was stained with Alexa Fluor 488 phalloidin (15 minutes at RT) and nuclei with Hoechst 3342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and analyzed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

Antibodies and reagents

Mouse anti-α-sm-actin, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, HRP-conjugated goat anti-rabbit antibody and HRP-conjugated rabbit anti-goat antibody were purchased from Sigma (St. Louis, MO, USA). Goat anti-MMP-2 antibody was purchased from R&D systems (Minneapolis, MN, USA). Rabbit anti-GSK-3 antibody, goat anti-fibronectin (C20) antibody, Mouse anti-Lamin A/C antibody, mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and Rabbit-anti-Smad2/3 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-phospho-Ser9/21-GSK-3 antibody and rabbit anti-phospho-432/425-smad3 antibody were from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-total β-catenin antibody was from BD Biosciences (San Jose, CA, USA). Mouse anti-non-phosphorylated-β-catenin antibody (clone 8E7) was from Millipore (Amsterdam, the Netherlands). Cy3 conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA). Lipofectamine 2000 transfection reagent and alexa Fluor 488 phalloidin were from Invitrogen (Paisley, UK). Recombinant human TGF-β_1 was from R&D systems (Abingdon, UK). All other chemicals were of analytical grade.

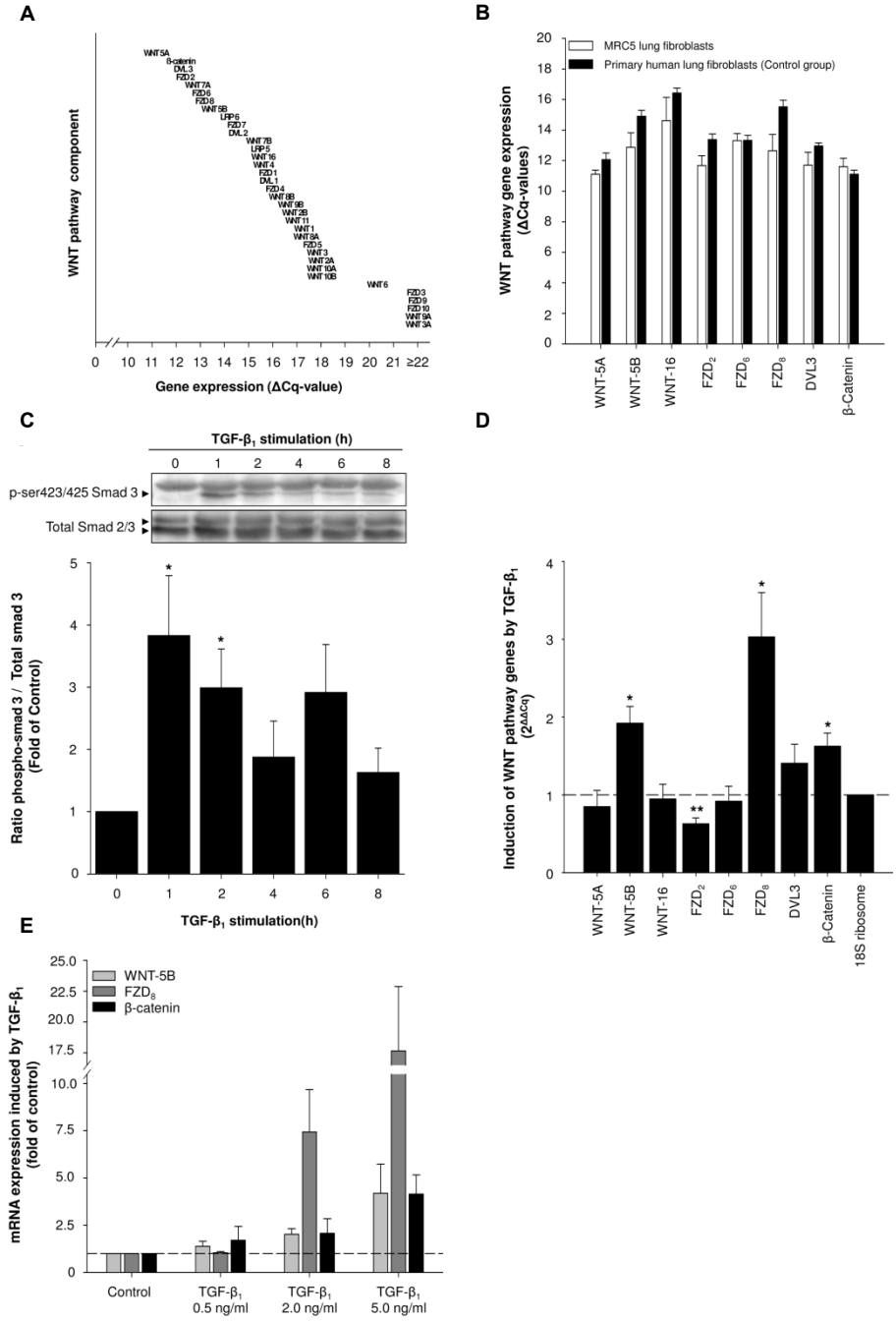
Results

Expression of genes required for functional WNT signaling by fibroblasts

We first investigated WNT pathway gene expression in MRC-5 human lung fibroblasts. A clear mRNA signal was observed for the majority of WNT pathway genes, but with considerable differences in the degree of expression (Figure 1A). The WNT-ligands WNT-5A, WNT-5B and WNT-16, the Frizzled (FZD) receptors FZD_2, FZD_6 and FZD_8 as well as the intracellular signaling protein dishevelled (DVL3) and the key-effector of canonical WNT signaling, β-catenin, were abundantly expressed (figure 1A and 1B). This subset of specific WNT pathway genes was selected for further studies based on their abundant expression at baseline, on previous findings indicating the regulation of these genes by TGF-β_1 in airway smooth muscle (unpublished data), and based on recent literature indicating the involvement of the selected WNT ligands, FZD receptors and intracellular signaling molecules in cellular processes relevant for fibroblasts function [35-37]. A role for additional WNT pathway genes in fibroblast function can, however, not be ruled out. To investigate if these genes were also highly expressed in primary human lung fibroblasts, we performed qRT-PCR analysis of these WNT pathway genes in fibroblasts of individuals without COPD (control) and compared them to the expression in MRC-5 fibroblasts, which produced similar results (figure 1B).

Recently, studies have suggested that activation of WNT signaling plays an important role in remodeling and repair in several organs and that it may show a cooperative interaction with the TGF-β_1/smad pathway [18, 38-42]. Smad signaling is key in TGF-β_1 induced cellular responses and therefore we investigated first the phosphorylation of smad3. MRC-5 fibroblasts were stimulated with TGF-β_1 (2 ng/ml) for various time-points resulting in a time-dependent increase in ser423/425-smad3 phosphorylation, which was most profound at the early time-points of 1-2 hours (figure 1C).

Next, we wondered if TGF-β_1 would affect the expression of the selected WNT pathway genes in human lung fibroblasts. Stimulation of MRC-5 human lung fibroblasts with TGF-β_1 (2 ng/ml; 4 hours) altered the expression profile of specific WNT-pathway genes (figure 1D). The expression of WNT-5B, FZD_8 and β-catenin was significantly increased in TGF-β_1 treated fibroblasts (fold-induction 1.92 ± 0.22, 3.03 ± 0.57 and 1.66 ± 0.16, respectively), whereas FZD_2 mRNA expression was significantly down-regulated to 0.63 ± 0.07-fold compared with untreated fibroblasts. The expression of WNT-5A, WNT-16, FZD_6 and DVL3 was unaltered after TGF-β_1 stimulation (figure 1D). Concentration-response curves with 0.5, 2 and 5 ng/ml of TGF-β_1 show that the expression of WNT-5B, FZD_8 and β-catenin in MRC-5 fibroblasts is concentration dependent (figure 1E).


A
WNT pathway component
Gene expression (ΔCq-value)
B
MRC5 lung fibroblasts
Primary human lung fibroblasts (Control group)
WNT pathway gene expression (ΔCq-values)
WNT-5A
WNT-5B
WNT-16
FZD2
FZD6
FZD8
DVL3
β-Catenin
C
TGF-β1 stimulation (h)
p-ser423/425 Smad 3
Total Smad 2/3
Ratio phospho-smad 3 / Total smad 3 (Fold of Control)
TGF-β1 stimulation(h)
D
Induction of WNT pathway genes by TGF-β1 (2^ΔΔCq)
18S ribosome
E
mRNA expression induced by TGF-β1 (fold of control)
WNT-5B
FZD8
β-catenin
Control
TGF-β1 0.5 ng/ml
TGF-β1 2.0 ng/ml
TGF-β1 5.0 ng/ml

◄ ***Figure 1: Quantitative expression of specific WNT pathway genes in human lung fibroblasts.*** (A) WNT pathway gene expression in MRC5 human lung fibroblasts. Data shown are average Cq-values corrected for 18s ribosomal RNA expression determined in triplicate by quantitative real-time PCR. Of note; a lower Cq-value corresponds with higher gene expression. (B) The WNT pathway genes WNT-5A, WNT-5B, WNT-16, FZD_2, FZD_6, FZD_8, DVL3 and β-catenin were analyzed by quantitative real-time PCR in MRC-5 fibroblasts and primary human lung fibroblasts. (C) Time-dependent activation of smad3 in response to TGF-β_1 (2 ng/ml). Phosphorylation of ser423/425-smad3 was evaluated in whole cell lysates by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of total smad2/3. Data represents mean ± s.e.m. of 6 independent experiments. *$p<0.05$ compared to untreated MRC-5 fibroblasts determined by a two-tailed student's *t*-test for paired observations. (D) qRT-PCR analysis of WNT-5A, WNT-5B, WNT-16, FZD_2, FZD_6, FZD_8, DVL3 and β-catenin in MRC-5 fibroblasts after 4h of TGF-β_1 (2 ng/ml) stimulation. Expression of WNT pathway genes by TGF-β_1 is corrected for 18S rRNA and expressed relative to untreated MRC-5 fibroblasts. Data represents mean ± s.e.m. of 5 independent experiments. *$p<0.05$, **$p<0.01$ compared to untreated MRC-5 fibroblasts determined by a two-tailed student's *t*-test for paired observations. (E) Effect of increasing concentrations TGF-β_1 on WNT-5B, FZD_8 and β-catenin gene expression. MRC-5 fibroblasts were stimulated with 0.5, 2.0 and 5.0 ng/ml TGF-β_1 for 24h. WNT-5B, FZD_8 and β-catenin expression was determined by qRT-PCR analysis, corrected for 18S rRNA and expressed relative to untreated MRC-5 fibroblasts (control). Data represents mean ± s.e.m. of 4-7 independent experiments. $p<0.05$ for dose-dependency of WNT-5B, FZD_8 and β-catenin gene expression in response to TGF-β_1 determined by a One-way ANOVA.

Table 1: Clinical characteristics of the subjects involved in the studies

	Subject groups		
	Control	**COPD stage II**	**COPD stage IV**
Number of subjects	7	5	6
Age (years)	58 (46-74)	73 (70-77) *	55 (52-59)
Body mass index (Kg/m^2)	26.5 ± 2.0 (n = 4)	25.5 ± 1.5 (n = 5)	21.0 ± 0.6 # (n = 6)
Sex			
Male	4	5	4
Female	3	0	2
Smoking status			
Ex-smoker	4	4	6
Current smoker	2	1	0
Non-smoker	1	0	0
Pack-years	36 (0-70)	42.5 (17.5-55)	30 (20-38)
FEV_1 % predicted	96.9 (75.9 - 118.0)	52.6 (38.0 – 66.9)	17.1 *** (14.0 - 18.5)
FEV_1 / FVC	76.0 (71.4 – 81.5)	49.3 (37.0 – 60.7)	27.7 ** (14.0 – 62.1)

All values are represented as median values with ranges in parentheses. Ex –smokers = not smoking for at least one year. FEV_1 % predicted = Forced Expiratory Volume in 1 second as percentage of predicted value; FVC = Forced Vital Capacity. Stage means severity of COPD according to GOLD criteria. Statistical significance determined by a kruskall-wallis ANOVA followed by Dunn's multiple comparisons test or a two-way student's *t*-test for unpaired observations. **p<0.05, **p<0.01, ***p<0.001 compared to Control group and #p<0.05 compared to individuals with COPD stage II.*

Differential WNT pathway gene expression in primary lung fibroblasts from individuals with and without COPD

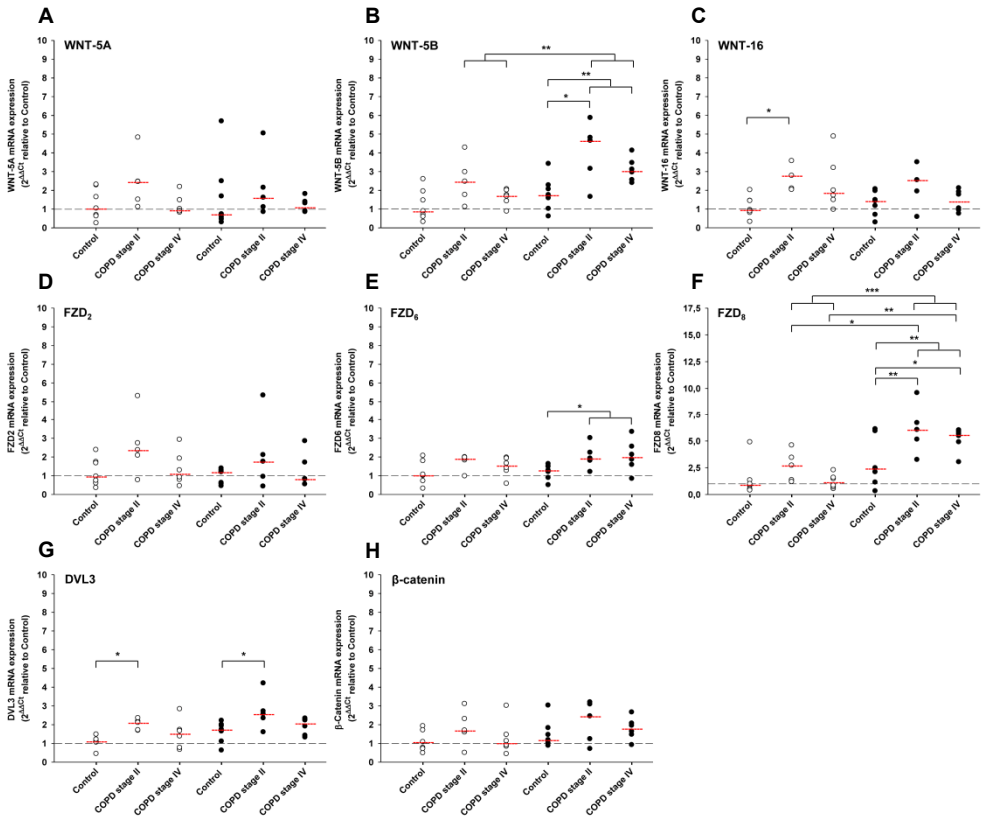
To investigate if WNT pathway gene expression was altered in COPD, we quantified the most abundant WNT signaling pathway genes by qRT-PCR in primary human lung fibroblasts from individuals with and without COPD at different stages of disease (COPD GOLD stage II or GOLD stage IV). The clinical characteristics of the subject groups are represented in table 1. The individuals with COPD stage II were significantly older and individuals with COPD stage IV had a lower body mass index (BMI). The smoking history (i.e. smoking status and pack-years) and gender distribution was similar in all groups.

WNT ligands

First, we determined the expression profile of the ligands WNT-5A, WNT-5B and WNT-16. No significant differences were observed in the basal expression of WNT-5A and WNT-5B in fibroblasts from individuals with COPD compared to individuals without COPD (controls), whilst WNT-16 expression was significantly higher in individuals with COPD stage II (figure 2A-C) In line with the MRC-5 fibroblasts, stimulation with TGF-β_1 had no effect on mRNA expression of WNT-5A and WNT-16, but induced mRNA expression of WNT-5B. Interestingly, WNT-5B mRNA expression in TGF-β_1 treated fibroblasts was higher in individuals with than without COPD (figure 2B). The mRNA expression of WNT-5A and WNT-16 in TGF-β_1 treated fibroblasts was not different in individuals with or without COPD (figures 2A and 2C).

Frizzled (FZD) receptors

Basal mRNA expression of the FZD-receptors FZD_2, FZD_6, and FZD_8 was similar in fibroblasts from individuals with and without COPD, independent of GOLD stage (figure 2D-F). In contrast to what we observed in MRC-5 cells, in fibroblasts from individuals without COPD, the expression of the FZD_2 receptors was unaltered in response to TGF-β_1 (figure 2D-F). Likewise, the expression of FZD_2 did not differ after TGF-β_1 stimulation in fibroblasts from individuals with COPD, independent of GOLD stage (Figure 2D). Conversely, FZD_6 mRNA expression in fibroblasts from individuals with COPD was upregulated in the presence of TGF-β_1. The total FZD_6 mRNA content was significantly higher (1.67 ± 0.18–fold) in fibroblasts from individuals with COPD than controls after TGF-β_1 stimulation (figure 2E). In addition, TGF-β_1 up regulated FZD_8 mRNA expression in fibroblasts from individuals with as well as without COPD. Total FZD_8 mRNA content in TGF-β_1 stimulated fibroblasts was higher in individuals with either COPD stage II or stage IV than in fibroblasts from controls (figure 2F).



▲***Figure 2: Differential WNT pathway gene expression in primary lung fibroblasts from individuals with and without COPD.*** Primary lung fibroblasts were isolated from individuals without (control) and with COPD (GOLD stage II and IV) as described in the materials en methods. qRT-PCR analysis of WNT-5A (A), WNT-5B (B), WNT-16 (C), FZD_2 (D), FZD_6 (E), FZD_8 (F), DVL3 (G) and β-catenin (H) mRNA of primary lung fibroblasts treated with or without TGF-β_1 (2 ng/ml) for 4h. Expression of WNT pathway genes is plotted relative to the mean expression in untreated fibroblasts from controls. Data are derived from 7 controls and 11 COPD patients (5 GOLD stage II and 6 GOLD stage IV). mRNA expression was determined both at baseline (open circles; ○) and after TGF-β stimulation (closed circles; ●). Median of each group is indicated by -----. *p<0.05, **p<0.01, ***p<0.001, two-tailed student's *t*-test for unpaired observations or a One-way ANOVA followed by a Newman-Keuls multiple comparison test.

DVL3 and β-catenin

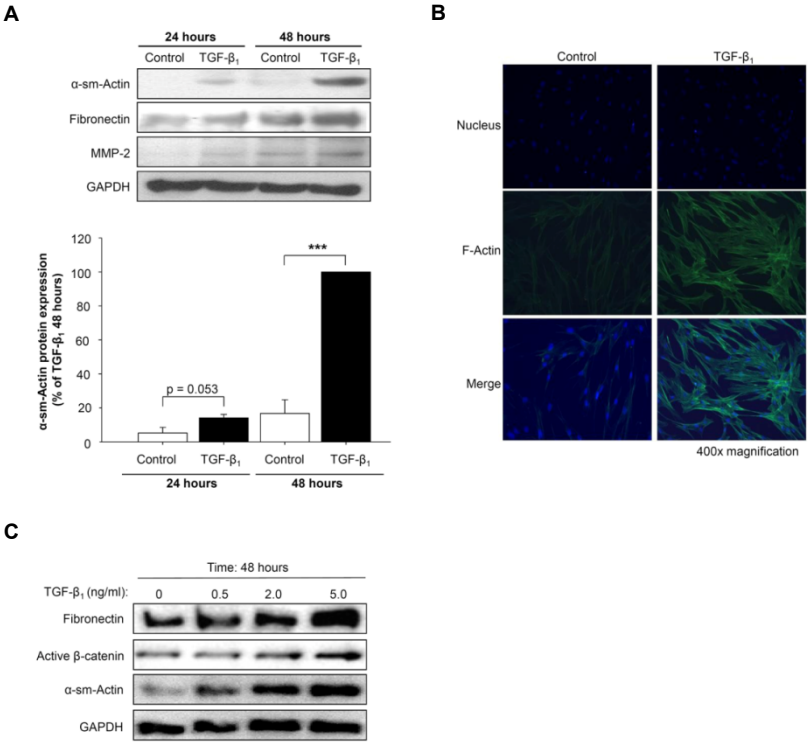
The mRNA expression at baseline of β-catenin, the key effector of WNT signaling, was comparable in fibroblasts from individuals with and without COPD, whereas the expression of the intracellular WNT signaling protein DVL3 was significantly higher in individuals with COPD stage II (figure 2G). However, no differences in DVL3 mRNA expression were observed between controls and individuals with COPD stage IV. TGF-β_1 stimulation resulted in an upregulation of both DVL3 and

β-catenin mRNA in fibroblasts from individuals with and without COPD (figure 2G-H). After TGF-β_1 stimulation, total DVL3 mRNA levels in fibroblasts from COPD patients with GOLD stage II were significantly higher than in controls (figure 2G).

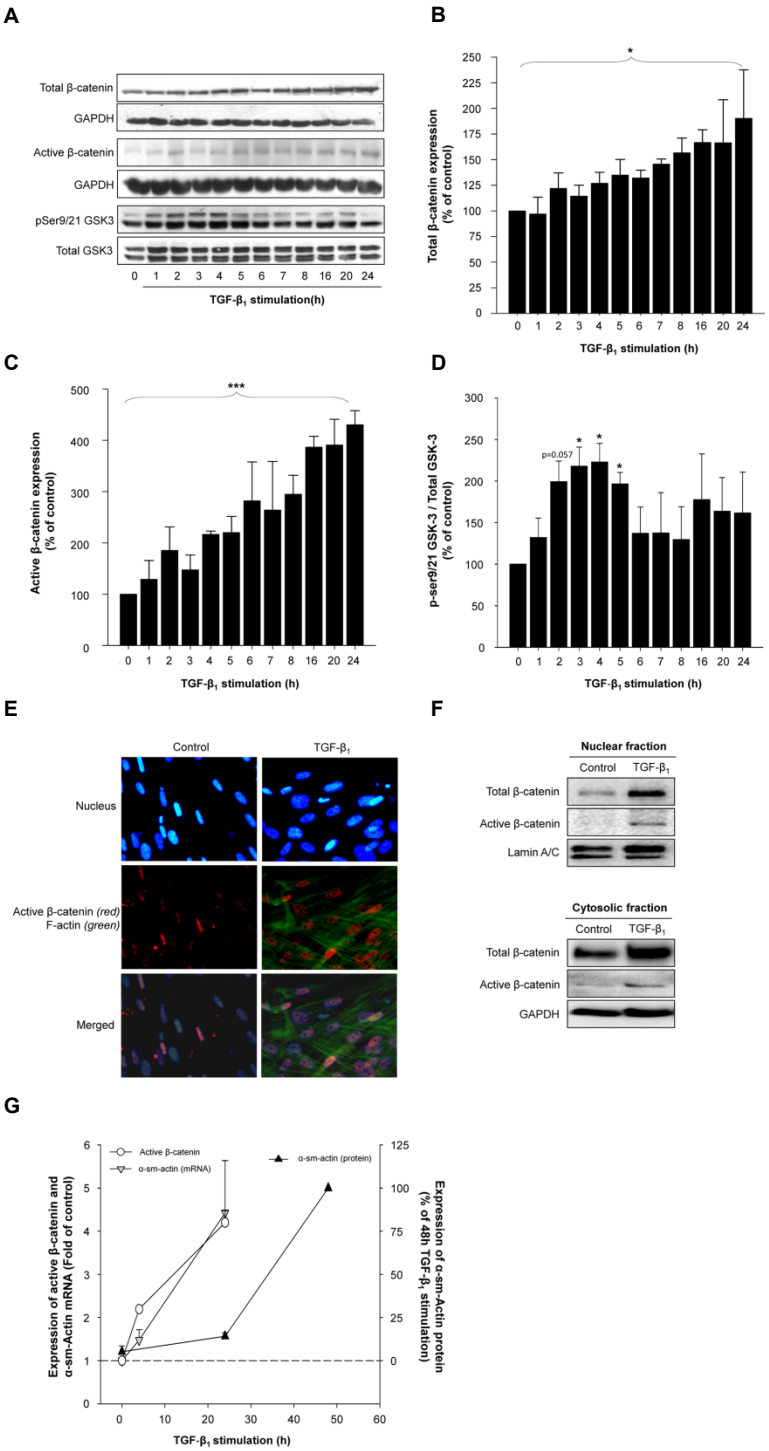
TGF-β_1 induces myofibroblast differentiation and activates β-catenin signaling

We next studied activation of the WNT effector β-catenin in response to TGF-β_1 and determined its functional role in myofibroblast differentiation. Treatment of MRC-5 human lung fibroblasts for 48 hours with TGF-β_1 (2 ng/ml) resulted in a significant increase in protein expression of the differentiation markers α-sm-actin, fibronectin and MMP-2 (figure 3A). Cytochemical staining for filamentous actin (F-actin) indicated that TGF-β_1 (48 hours) distinctively induced the formation of stress fibers (F-actin) in these cells, another indication of myofibroblast differentiation (figure 3B). Stimulation of MRC-5 fibroblasts with 0.5, 2 and 5 ng/ml of TGF- β_1 for 48 hours shows that the increase of fibronectin and α-sm-actin protein expression is concentration-dependent (figure 3C). Interestingly, the expression of active (unphosphorylated) β-catenin followed similar concentration dependence (figure 3C). Therefore, the activation of β-catenin in response to TGF-β_1 was investigated in more detail.

Treatment of fibroblasts with TGF-β_1 (2 ng/ml) resulted in a significant, time-dependent increase in total β-catenin protein expression after 24 hours of stimulation compared to untreated fibroblasts (figure 4A-B). Interestingly, TGF-β_1 induced an even more pronounced increase in the expression of the transcriptionally active (non-phosphorylated) β-catenin, with kinetics similar to the induction of total β-catenin (figure 4C). Glycogen synthase kinase-3 (GSK-3) is a major protein kinase involved in regulating β-catenin cellular expression and is negatively regulated by ser9 and ser21 (ser9 of GSK-3β and ser21 of GSK-3α) phosphorylation [43]. Therefore, the effect of TGF-β_1 on GSK-3 phosphorylation was also investigated. TGF-β_1 induced a strong inhibitory ser9/21 phosphorylation of GSK-3 (figure 4A and 4D). As would be expected, expression of the transcriptionally active (non-phosphorylated) β-catenin distinctively increased in the cytosolic and nuclear compartment after TGF-β_1 stimulation (figure 4E and 4F). Interestingly, activation of β-catenin signaling preceded myofibroblast differentiation (figure 4G).



▲ ***Figure 3: TGF-β1 induces myofibroblast differentiation of MRC-5 lung fibroblasts***. MRC-5 fibroblasts were grown to confluence and treated for 24h or 48h with 2 ng/ml of TGF-β_1. (A) Expression of the myofibroblasts markers α-sm-actin, fibronectin and MMP-2 was evaluated in whole cell lysates by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. Representative immunoblots of 5-8 independent experiments are shown. ***p<0.001, two-way student's *t*-test for paired observations. (B) Evaluation of stress fiber formation in MRC-5 lung fibroblasts after TGF-β_1 stimulation. MRC-5 lung fibroblasts were treated for 48h with TGF-β_1 (2 ng/ml) and subsequently fixed and permeabilized. Cells were stained for filamentous actin (488 phalloidin; green) and nucleus (Hoechst 3342; blue). Pictures were taken at 400x magnification. (C) Effect of increasing concentrations TGF-β_1 on myofibroblast differentiation. MRC-5 fibroblasts were stimulated with 0.5, 2.0 and 5.0 ng/ml TGF-β_1 for 48h. Expression of α-sm-actin, fibronectin and active β-catenin was evaluated in whole cell lysates by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. Representative immunoblots of 4 independent experiments are shown.


A
Total β-catenin
GAPDH
Active β-catenin
GAPDH
pSer9/21 GSK3
Total GSK3
0 1 2 3 4 5 6 7 8 16 20 24
TGF-β1 stimulation(h)
B
Total β-catenin expression (% of control)
TGF-β1 stimulation (h)
C
Active β-catenin expression (% of control)
***
TGF-β1 stimulation (h)
D
p-ser9/21 GSK-3 / Total GSK-3 (% of control)
p=0,057
TGF-β1 stimulation (h)
E
Control
TGF-β1
Nucleus
Active β-catenin (red)
F-actin (green)
Merged
F
Nuclear fraction
Control
TGF-β1
Total β-catenin
Active β-catenin
Lamin A/C
Cytosolic fraction
Control
TGF-β1
Total β-catenin
Active β-catenin
GAPDH
G
Active β-catenin
α-sm-actin (mRNA)
α-sm-actin (protein)
Expression of active β-catenin and α-sm-Actin mRNA (Fold of control)
Expression of α-sm-Actin protein (% of 48h TGF-β1 stimulation)
TGF-β1 stimulation (h)

◄***Figure 4: Treatment with TGF-β_1 increases β-catenin signaling in MRC-5 lung fibroblasts***. MRC-5 fibroblasts were grown to confluence and treated for up to 24h with TGF-β_1 (2 ng/ml). (A) Expression of total β-catenin, active (non-phosphorylated) β-catenin and ser9/21 phosphorylation of GSK-3 were evaluated by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH or total GSK-3, respectively. Responses of TGF-β_1 on total and active β-catenin expression (B and C) and ser9/21-GSK-3 phosphorylation (D) were quantified by densitometry, representing mean ± s.e.m. of 3 independent experiments. *p<0.05, ***p<0.001, two-tailed student's *t*-test for paired observations or repeated measures ANOVA followed by a Newman-Keuls multiple comparison test. (E) Evaluation of cellular localization of active (non-phosphorylated) β-catenin in MRC-5 lung fibroblasts stimulated with TGF-β_1 (2 ng/ml) for 48h. Fixed and permeabilized MRC-5 fibroblasts were (immuno)cytochemically stained for active (non-phosphorylated) β-catenin (Cy3; red) and stained for filamentous actin (488 phalloidin; green) and nucleus (Hoechst 33342; blue). Pictures were taken at 400x magnification. (F) Increased cytosolic and nuclear expression of β-catenin in response to TGF-β_1 stimulation. MRC-5 fibroblasts were stimulated with TGF-β_1 (2 ng/ml) for 24h. Subsequently cytosolic and nuclear extracts were prepared. Expression of total and active (non-phosphorylated) β-catenin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH and Lamin A/C, respectively. Representative immunoblots of 4 independent experiments are shown. (G) β-Catenin activation precedes myofibroblast differentiation. Expression of active β-catenin protein (open circles; ○), α-sm-actin mRNA (grey triangles; ▼) and α-sm-actin protein (black triangles; ▲) in response to TGF-β_1 (2 ng/ml) was determined by immunoblotting and quantitative real time PCR. Data represents mean ± s.e.m. of 3-8 independent experiments.

Functional role for β-catenin in TGF-β_1-induced myofibroblast differentiation

To determine the functional role of β-catenin in myofibroblast differentiation, we used specific small interfering RNA (siRNA) to silence β-catenin protein expression. After siRNA treatment, total β-catenin expression was reduced to 57 ± 7% in fibroblasts at baseline (figure 5A). TGF-β_1 stimulation after siRNA transfection, resulted in a significant increase in β-catenin protein expression in the non-targeting siRNA treated fibroblasts (control) (figure 5A-B), whereas this induction of total and - more importantly - transcriptionally active β-catenin was completely abrogated in fibroblasts treated with specific siRNA against β-catenin (figure 5B).

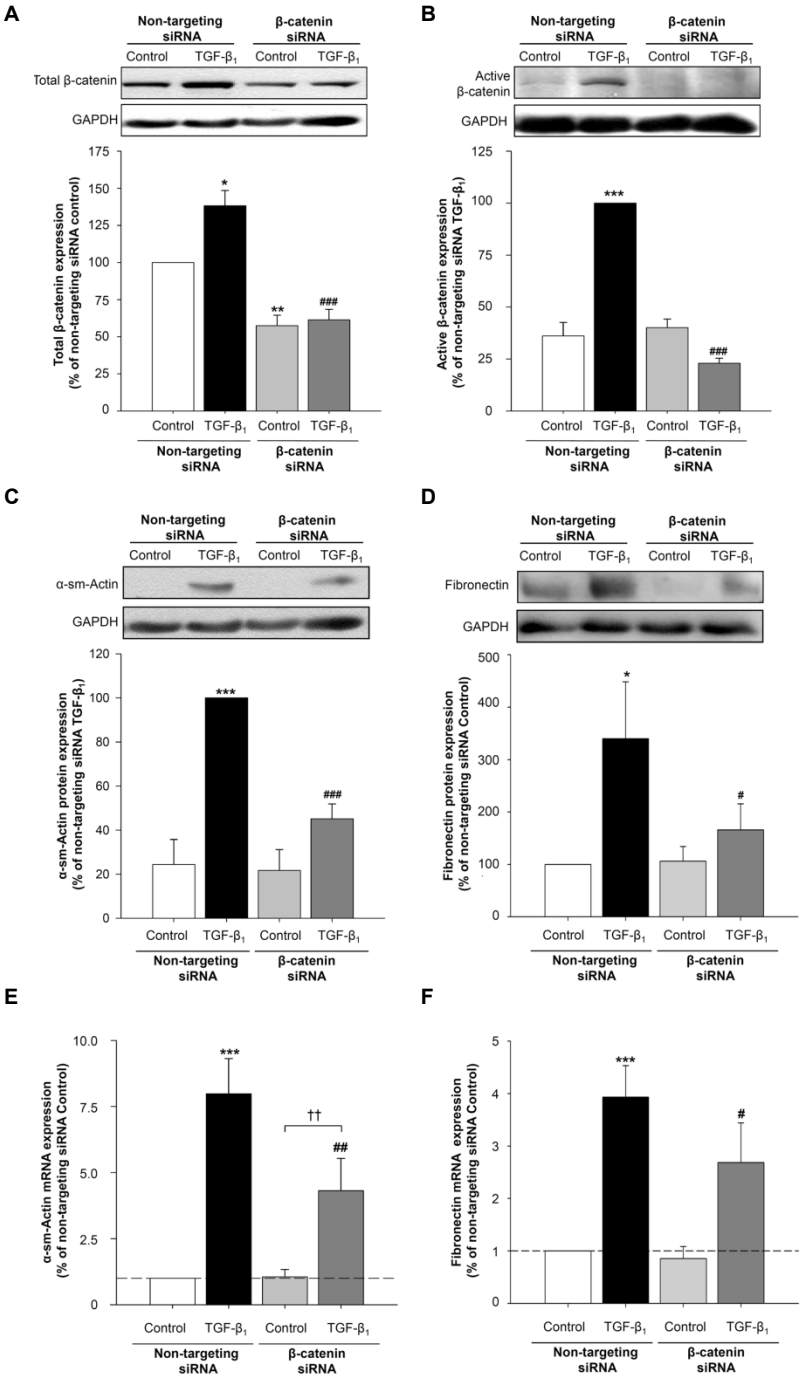
Next, we investigated the functional effects of β-catenin silencing on TGF-β_1-induced gene and protein expression. Non-targeting siRNA treated fibroblasts were stimulated with TGF-β_1 for 24 and 48 hours (for mRNA and protein determination, respectively), resulting in increased expression of α-sm-actin (figure 5C and 5E) and fibronectin (figure 5D and 5F). The induction of both α-sm-actin and fibronectin was largely attenuated in fibroblasts treated with specific siRNA against β-catenin (figure 5C-E). Silencing of β-catenin expression also reduced the TGF-β_1-induced collagen Iα1 mRNA expression, whereas the expression of plasminogen activator inhibitor-1 (PAI-1) was not affected (data not shown).

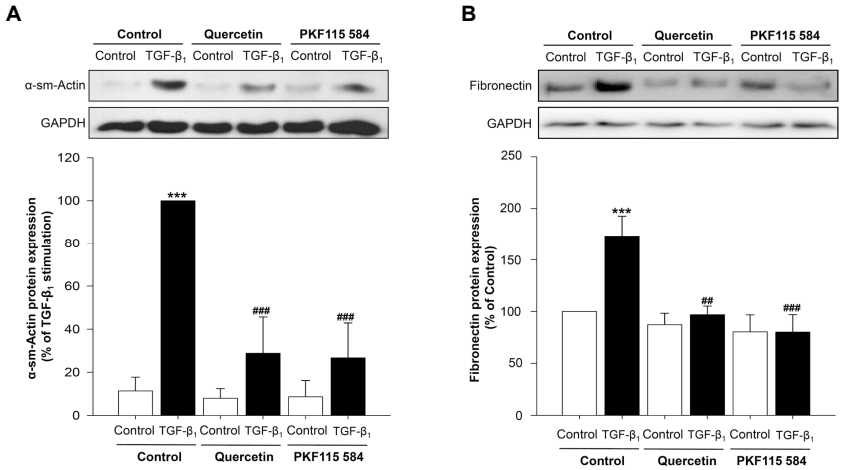
To further verify the functional role of β-catenin in lung fibroblasts we pharmacologically inhibited β-catenin signaling by either quercetin or PKF115-584, compounds that disrupt the interaction of the transcriptionally active β-catenin/T-cell factor-4 (TCF-4) complex [44-46]. Both pharmacological inhibitors greatly attenuated α-sm-actin induction and fully prevented the increased fibronectin deposition induced by TGF-β_1, without affecting basal expression of either α-sm-actin or fibronectin (figure 6A-B).

Fibroblasts of COPD patients show increased β-catenin activation and subsequent fibronectin deposition in response to TGF-β_1

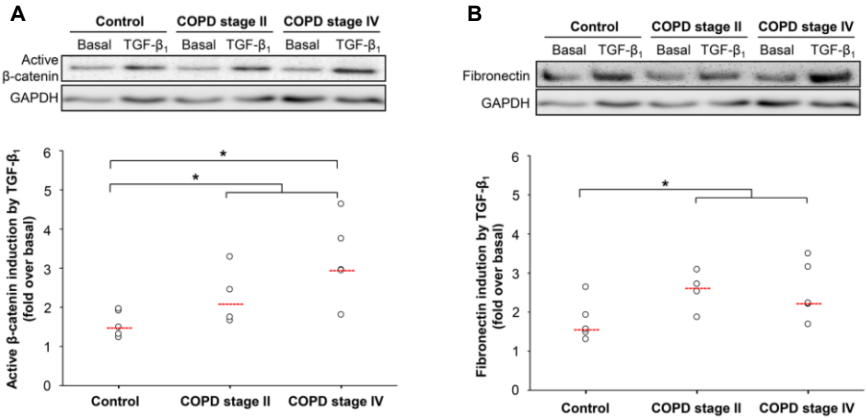
Fibroblasts from individuals with and without COPD had similar expression of active β-catenin at baseline. Stimulation with TGF-β_1 also resulted in a significant induction of active β-catenin in fibroblasts from individuals with and without COPD. Interestingly, the induction of active β-catenin was significantly higher in fibroblasts from individuals with COPD than those without COPD (figure 7A). In accordance with the increase in active β-catenin, fibronectin deposition was increased after TGF-β_1 stimulation and more so in fibroblasts from individuals with COPD (figure 7B). The expression of the myofibroblast marker α-sm-actin was also studied; however the fold induction of α-sm-actin by TGF-β_1 treatment could not be computed as basal expression of α-sm-actin in fibroblasts was occasionally not observed. However, no significant differences were observed for the TGF-β_1-induced α-sm-actin bands (GAPDH ratio) between individuals with and without COPD (data not shown). Thus, β-catenin activation and subsequent fibronectin deposition in response to TGF-β_1 is enhanced in lung fibroblasts from COPD patients compared to lung fibroblasts from controls.

► ***Figure 5: Silencing β-catenin expression by specific siRNA attenuates TGF-β_1-induced α-sm-actin and fibronectin expression***. Subconfluent MRC-5 lung fibroblast cultures were transfected with a siRNA against the β-catenin transcript. Control cultures were transfected with a non-targeting control siRNA. Transfected cells were treated with TGF-β_1 (2 ng/ml) for 48h. (A-B) The efficiency of β-catenin silencing was evaluated by immunoblotting the expression of (A) total β-catenin and (B) active β-catenin and GAPDH to correct for differences in protein loading. Data represent mean ± s.e.m. of 4-6 experiments. *p<0.05, **p<0.01 and ***p<0.001 compared to non-targeting siRNA control, $^{###}$p< 0.001 compared to non-targeting siRNA treated with TGF-β_1 determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test. (C-F) β-catenin siRNA attenuated TGF-β_1-induced α-sm-actin (C and E) and fibronectin (D and F) gene and protein expression. Expression or mRNA was determined by real-time PCR and normalized to 18S ribosomal mRNA expression. Protein expression was determined by immunoblotting and equal protein loading was verified by the analysis of GAPDH. Responses were quantified and normalized to the expression of 18S rRNA (gene) or GAPDH (protein). Data represent mean ± s.e.m. of 5-6 independent experiments. *p<0.05, ***p<0.001 compared to non-targeting siRNA control, $^{#}$p<0.05, $^{##}$p<0.01 $^{###}$p< 0.001 compared to non-targeting siRNA treated with TGF-β_1, ††p<0.01 compared to β-catenin siRNA control, one-way ANOVA followed by a Newman-Keuls multiple comparison test.


A
Non-targeting siRNA
β-catenin siRNA
Control
TGF-β1
Total β-catenin
GAPDH
Total β-catenin expression (% of non-targeting siRNA control)
B
Active β-catenin
Active β-catenin expression (% of non-targeting siRNA TGF-β1)
C
α-sm-Actin
α-sm-Actin protein expression (% of non-targeting siRNA TGF-β1)
D
Fibronectin
Fibronectin protein expression (% of non-targeting siRNA Control)
E
α-sm-Actin mRNA expression (% of non-targeting siRNA Control)
F
Fibronectin mRNA expression (% of non-targeting siRNA Control)



▲Figure 6: Pharmacological inhibition of β-catenin attenuates TGF-β1-induced α-sm-actin and fibronectin expression. Pharmacological inhibition of β-catenin/TCF_4 signaling by quercetin or PKF115-584. Confluent MRC-5 lung fibroblasts were treated with TGF-$β_1$ (2 ng/ml) for 48h in the absence or presence of either quercetin (40 µM) or PKF115-584 (100 nM). Expression of α-sm-actin (A) and fibronectin (B) was evaluated by immunoblotting using a specific antibody. Responses were quantified by densitometry and normalized to the expression of GAPDH. Data represent mean ± s.e.m. of 3 independent experiments. ***p< 0.001 compared untreated MRC-5 lung fibroblasts (control), $^{##}$p< 0.01; $^{###}$p< 0.001 to TGF-$β_1$ treated MRC-5 lung fibroblasts determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test.



▲Figure 7: Increased β-catenin activation and fibronectin deposition in fibroblast of individuals with COPD in response to TGF-β1. Primary lung fibroblasts were isolated from individuals without (control) and with COPD (GOLD stage II and IV) as described in the materials en methods. The fibroblasts were grown to confluence and treated for 48h with TGF-$β_1$ (2 ng/ml). Expression of active β-catenin (A) and fibronectin (B) was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Responses were quantified by densitometry and normalized to the expression of GAPDH. Data are derived from 5 controls and 9 COPD patients (4 GOLD stage II and 5 GOLD stage IV). Median of each group is indicated by -----. *P<0.05. Statistical differences between control and COPD were determined a two-tailed Mann-whitney test.

Discussion

Pulmonary fibroblasts play a pivotal role in COPD by regulating ECM turnover in the lungs [4, 11]. To our knowledge, this is the first study demonstrating that WNT/β-catenin signaling in pulmonary fibroblasts may play an important role in COPD. We show that pulmonary fibroblasts express genes required for functional WNT signaling, of which WNT-5B, FZD_8, DVL3 and β-catenin were significantly induced by TGF-β_1 in a concentration dependent manner in both MRC-5 and primary human lung fibroblasts. Interestingly, WNT-5B, FZD_6 and FZD_8 expression were significantly more upregulated in response to TGF-β_1 in primary fibroblasts from individuals with than without COPD. Furthermore, we also show that β-catenin, the key effector of canonical WNT signaling, regulates the induction of collagen1α1, α-sm-actin and fibronectin deposition by pulmonary fibroblasts in response to TGF-β_1, whereas the expression of PAI-1 is not regulated by β-catenin. Finally, we provide evidence that the induction of transcriptionally active β-catenin and subsequent fibronectin deposition induced by TGF-β_1 are significantly enhanced in lung fibroblasts from COPD patients.

An active and complex remodeling process is present in the peripheral lung when COPD develops, resulting in small airway fibrosis and a variable degree of emphysema. Fibroblasts are the primary cell type responsible for the production and maintenance of the extracellular matrix. Alterations in fibroblast function may therefore play an important role in COPD. In this respect, the canonical WNT/β-catenin signaling pathway is of particular interest, since this pathway has been linked to tissue repair and remodeling [20, 47]. Indeed, activation of canonical WNT/β-catenin signaling attenuates experimental emphysema in mice [32]. In that study, WNT pathway gene expression in lung tissue of COPD patients was also examined and, although the gene expression of specific WNT ligands and FZD receptors showed no notable changes in whole lung homogenate, alveolar type II cells had reduced β-catenin expression [32]. Our observations suggest that fibroblasts from the peripheral lung are more prone to TGF-β_1 stimulation in activating WNT signaling and regulating transcription of tissue repair genes such as fibronectin, despite the fact that the peripheral lung is the primary site of tissue destruction associated with pulmonary emphysema [2]. We also demonstrate that WNT-16 and DVL3 expression is higher at baseline in fibroblasts of individuals with COPD stage II, although curiously this did not result in increased baseline expression of either total or active β-catenin. Of interest is that this intrinsic difference in WNT pathway activation was seen for fibroblasts from both GOLD stage II and IV COPD patients compared to fibroblasts from controls.

We propose that during COPD pathogenesis, irrespective of GOLD stage, fibroblasts from the peripheral lung are promoted to repair tissue damage, but that

this repair response becomes insufficient in more advanced stages of disease. This can either be because of the development of intrinsic defects in the lung fibroblast (e.g. reduced proliferative capacity of fibroblasts in advanced stages of COPD [12] or altered intracellular Smad signaling [10]) or because of aberrant activation of fibroblasts by the locally expressed (pro-inflammatory) cytokines in the lung and / or the presence of cigarette smoke [5, 11, 48]. As a result, the destructive response in the lung may proceed and the tissue repair response by fibroblasts is deviant and not adequate. In line with this hypothesis the relative production of the proteoglycan versican as well as the expression of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2), both direct targets of WNT/β-catenin signaling [29, 49, 50], are higher in parenchymal fibroblasts from COPD patients than controls [15, 17]. Furthermore, versican expression is increased in pulmonary alveolar parenchyma of mild to moderate emphysematous COPD patients and is negatively correlated with FEV_1 [51]. These findings suggest that in COPD, parenchymal fibroblasts may have activated canonical WNT signaling, also in regions affected by emphysema, which regulates the subsequent synthesis of specific ECM components and enzymes.

The smoking histories (pack-years and smoking status) of the individuals with and without COPD were very similar, thus excluding that the observed differences between COPD and controls were primarily due to differences in smoking habits. No separate non-smoking control group (never smokers) was included in the study design. Therefore, this study does not provide insight into the effect of smoking on WNT pathway gene expression by fibroblasts. There is a statistically significant difference in age between individuals with COPD stage II (older) and individuals with either COPD stage IV or no COPD. Since the baseline expression of WNT pathway genes is higher in fibroblasts from COPD stage II compared to the other two groups (significant for WNT-16 and DVL3), we investigated the correlation between WNT pathway gene expression and age in the individuals without COPD (figure S2). We did not observe any significant correlation between age and WNT pathway gene expression, implying that these differences in gene expression at baseline are not primarily due to age.

Tissue repair by fibroblasts is a complex process involving the interplay of various growth factors and intracellular signaling pathways. Recently, crosstalk between WNT signaling pathway and growth factors in fibroblasts has been demonstrated [19, 52]. For instance, the WNT ligands WNT-3A and WNT-10B activate β-catenin signaling in NIH 3T3 fibroblasts resulting in an increased mRNA expression of connective tissue growth factor (CTGF, CCN2), endothelin-1 and TGF-β [53]. Moreover, the expression of WNT1-inducible signaling protein-1 (WISP-1), a member of the CCN family of secreted cysteine-rich matricellular proteins and a

direct target gene of WNT signaling, is increased in patients with idiopathic pulmonary fibrosis (IPF) and contributes to disease pathogenesis [41]. In dermal fibroblasts, both EGF and TGF-β increased β-catenin protein stability and induced β-catenin-mediated TCF-dependent transcriptional activity [19]. Current literature is limited concerning the signaling pathways involved in the regulation of WNT pathway gene expression by TGF-β. However, recently it was suggested that WNT gene expression may be regulated by smad proteins, as dickkopf (DKK) and casein kinase 1 (CSNK1A1) were predicted smad targets genes [54]. In the present study, we show that specific WNT genes are upregulated after 4 hours of TGF-β_1 treatment, which corresponds with the kinetics of activation of smad3. In addition, other signaling pathways such as ERK1/2 may be activated by TGF-β_1 that regulate the WNT pathway gene expression as demonstrated for β-catenin in our recent report [55]. Clearly, future studies are required to characterize the regulation of WNT pathway gene expression by TGF-β_1 in more detail, as we report that TGF-β_1 induces WNT and FZD mRNA expression in human lung fibroblasts and activates β-catenin signaling, which contributes to the fibroblast phenotype and function.

Our data suggest that TGF-β_1 induces β-catenin expression via several intracellular mechanisms. β-Catenin levels are tightly regulated by the constitutively active enzyme GSK-3. A fraction of cellular GSK-3 forms a complex with AXIN, casein kinase I (CK-I) and adenomatous polyposis coli (APC); this complex phosphorylates and subsequently targets β-catenin for proteosomal degradation [20]. The activity of both GSK-3 isoforms (e.g. GSK-3α and GSK-3β) is negatively regulated by serine (ser9 and ser21 of GSK-3β and GSK-3α, respectively) phosphorylation, which can be induced by numerous stimuli, including growth factors [21, 43, 55, 56]. We demonstrate that TGF-β_1 induces a transient time-dependent phosphorylation of both GSK-3 isoforms in MRC-5 fibroblasts, which might account for the initial increase of β-catenin stability. In addition, enhanced secretion of canonical WNT ligands by fibroblasts in response to TGF-β_1 may signal in an autocrine fashion, which then stabilizes β-catenin by disrupting the GSK-3/AXIN/CK-I/APC complex [20]. The increase in β-catenin protein expression after TGF-β_1 stimulation progresses even when GSK-3 phosphorylation has returned to basal levels, supporting such an autocrine signaling loop. In addition, β-catenin protein expression can be induced by growth factors by *de novo* synthesis of the protein [55]. The underlying mechanisms by which TGF-β_1 induces β-catenin expression in pulmonary fibroblasts are therefore not fully understood and require further exploration.

Surprisingly, TGF-β_1 did not or only modestly affect the mRNA expression of the canonical WNT target genes dickkopf-1 (DKK-1), vascular endothelial growth factor

(VEGF), interleukin-8 (IL-8) or MMP-2, and attenuated AXIN-2 mRNA expression in MRC-5 fibroblasts (figure S3), even though nuclear β-catenin was clearly induced. Thus, β-catenin contributes to the transcriptional activity induced by TGF-β_1, but this transcriptional activity may be different from that induced by canonical WNT ligands. Indeed, WNT-3A activates β-catenin signaling in fibroblasts but does not or very modestly activate the transcription of collagen-1 and α-sm-actin, whereas it potentiates the effect of TGF-β on these myofibroblasts markers [57, 58]. This implies that the interaction between TGF-β_1/smad and WNT/β-catenin signaling directs transcription to specific genes, which may be different from those activated by canonical WNT stimulation alone. In support, it was recently demonstrated smad proteins and β-catenin can directly interact, thereby (synergistically) activating the transcription of specific genes [27, 57]. Thus, further exploration of crosstalk between growth factors, in particular TGF-β_1, and the WNT-signaling pathway in lung fibroblasts is of major interest to understand tissue repair mechanisms.

Our results further show that TGF-β_1 induced collagen Iα1 and PAI-1 mRNA as well as α-sm-actin and fibronectin protein expression, indicative of fibroblast activation [59]. This activation of fibroblasts was accompanied by an increased expression of transcriptionally active β-catenin, which was primarily present in the nuclei of the fibroblasts. Silencing of β-catenin as well as pharmacological inhibition of β-catenin by either quercetin or PKF115-584, compounds that interrupt the β-catenin/TCF4 interaction [44-46], greatly attenuated the TGF-β_1-induced collagen Iα1, α-sm-actin and fibronectin expression. However, the induction of PAI-1 did not change, indicating that β-catenin directs TGF-β_1 signaling to specific intracellular pathways. Further, these data indicate that the responsiveness of the fibroblasts to TGF-β_1 was not affected by down regulation of β-catenin. Collectively, these data demonstrate that β-catenin signaling plays an important role in the activation process of pulmonary fibroblasts. This may contribute to COPD pathogenesis, because the activation of β-catenin signaling and subsequent fibronectin deposition in response to TGF-β_1 is higher in lung fibroblasts from patients with than without COPD.

In conclusion, our results indicate that the WNT/β-catenin signaling pathway is activated in pulmonary fibroblasts in response to the cytokine TGF-β_1. In primary fibroblasts of COPD patients, this activation is greatly enhanced compared to healthy controls, as is the induction of β-catenin. This suggests that WNT/β-catenin signaling plays an important role in tissue repair in the lung, and that targeting β-catenin-dependent gene transcription holds promise as a therapeutic intervention in COPD.

Acknowledgements

We would like to thank Dr. Esther Schmitt (Novartis Pharma AG) for the generous gift of PKF 115-584.

Supplement

Table S1: Primers used for determination of WNT ligands by qRT-PCR analysis

WNT ligand	NCBI accession number			Primer sequence	
WNT-1	NM_005430	Forward	5'	ACC CAA TCC CTC TCC ACT CT	3'
		Reverse	5'	GAT TCA AGG AAA AGC CAC CA	3'
WNT-2	NM_003391	Forward	5'	CAA GAA CGC TGA CTG GAC AA	3'
		Reverse	5'	TGA CTG CAG AAC ACC AGG AG	3'
WNT-2B	NM_024494	Forward	5'	ATT TCC CGC TCT GGA GAT TT	3'
		Reverse	5'	AAG CTG GTG CAA AGG AAA GA	3'
WNT-3	NM_030753	Forward	5'	TGT GAG GTG AAG ACC TGC TG	3'
		Reverse	5'	AAA GTT GGG GGA GTT CTC GT	3'
WNT-3A	NM_033131	Forward	5'	CCA CAC CGT CAG GTA CTC CT	3'
		Reverse	5'	TGT AGC TGG ATG GAG TGC AG	3'
WNT-4	NM_030761	Forward	5'	CAG GCA AGA AGA GGG AGATG	3'
		Reverse	5'	CCG TGT GTG TGT GTG TGT GT	3'
WNT-5A	NM_003392	Forward	5'	GGG TGG GAA CCA AGA AAA AT	3'
		Reverse	5'	TGG AAC CTA CCC ATC CCA TA	3'
WNT-5B	NM_030775	Forward	5'	ACG CTG GAG ATC TCT GAG GA	3'
		Reverse	5'	CGA GGT TGA AGC TGA GTT CC	3'
WNT-6	NM_006522	Forward	5'	GTC ACG CAG GCC TGT TCT AT	3'
		Reverse	5'	CGT CCA TAA AGA GCC TCG AC	3'
WNT-7A	NM_004625	Forward	5'	CCC ACC TTC CTG AAG ATC AA	3'
		Reverse	5'	ACA GCA CAT GAG GTC ACA GC	3'
WNT-7B	NM_058238	Forward	5'	GCC TGC AGG TCC TAG AAG TG	3'
		Reverse	5'	CTC CCA AAG TGC TGG GAT TA	3'
WNT-8A	NM_058244	Forward	5'	TGC AAG TTC CAG TTT GCT TG	3'
		Reverse	5'	ATC CTT TCC CCA AAT TCC AC	3'
WNT-8B	NM_003393	Forward	5'	CCA TGA ACC TGC ACA ACA AC	3'
		Reverse	5'	TGA GTG CTG CGT GGT ACT TC	3'
WNT-9A	NM_003395	Forward	5'	TGA GAA GAA CTG CGA GAG CA	3'
		Reverse	5'	CTG TGT GCA ATG CCT GTA CC	3'
WNT-9B	NM_003396	Forward	5'	GAG GAC TCA CCC AGC TTC TG	3'
		Reverse	5'	TAG GCC TAG TGC TTG CAG GT	3'
WNT-10A	AK315081	Forward	5'	AAG CTG CAC CGC TTA CAA CT	3'
		Reverse	5'	ATT CTC GCG TGG ATG TCT CT	3'
WNT-10B	NM_003394	Forward	5'	AAT GCG AAT CCA CAA CAA CA	3'
		Reverse	5'	GGG TCT CGC TCA CAG AAG TC	3'
WNT-11	NM_004626	Forward	5'	ACT CTG CTC AAG GAC CCT CA	3'
		Reverse	5'	GCT TCC AAG TGA AGG CAA AG	3'
WNT-16	NM_016087	Forward	5'	GCT CCT GTG CTG TGA AAA CA	3'
		Reverse	5'	ACC CTC TGA TGT ACG GTT GC	3'

Table S2: Primers used for determination of the dishevelled protein family by qRT-PCR analysis

Dishevelled	NCBI accession number			Primer sequence	
DVL1	NM_000421	Forward	5'	ACC CTG AAC CTC AAC AGT GG	3'
		Reverse	5'	CCC TTC ACT CTG CTG ACT CC	3'
DVL2	NM_004422	Forward	5'	CCC TTC ACT CTG CTG ACT CC	3'
		Reverse	5'	TGG AGG AGG AGG TCA CAT TC	3'
DVL3	NM_004423	Forward	5'	GAG GCT GAG GCA CAA GAA TC	3'
		Reverse	5'	GCA GGC AAG ATT GAG TCA CA	3'

Table S3: Primers used for determination of FZD receptors by qRT-PCR analysis

Frizzled	NCBI accession number			Primer sequence	
FZD_1	NM_003505	Forward	5'	TCG ACT TCC TGA AGC TGG AT	3'
		Reverse	5'	AAG GTG GGA GAA GGG AGT GT	3'
FZD_2	NM_001466	Forward	5'	CCC GACT TCAC GGT CTA CAT	3'
		Reverse	5'	CTG TTG GTG AGG CGA GTG TA	3'
FZD_3	NM_017412	Forward	5'	TCT CTT TGG CCC TTG ACT G	3'
		Reverse	5'	ACA AAG AAA AGG CCG GAA AT	3'
FZD_4	NM_012193	Forward	5'	CCA GGA TTC CTT CCA AGT CA	3'
		Reverse	5'	CCA TGT CCT TGT GGC CTA CT	3'
FZD_5	NM_003468	Forward	5'	AGC TAA AAT GGC CAG AGC AA	3'
		Reverse	5'	AAT TCC CCC TGG GAA CTA TG	3'
FZD_6	NM_003506	Forward	5'	TTG TTG GCA TCT CTG CTG TC	3'
		Reverse	5'	CCA TGG ATT TGG AAA TGA CC	3'
FZD_7	NM_003507	Forward	5'	CGA CGC TCT TTA CCG TTC TC	3'
		Reverse	5'	GCC ATG CCG AAG AAG TAG AG	3'
FZD_8	NM_031866	Forward	5'	GAC ACT TGA TGG GCT GAG GT	3'
		Reverse	5'	CAA ATC TCG GGT TCT GGA AA	3'
FZD_9	NM_003508	Forward	5'	AGA CCA TCG TCA TCC TGA CC	3'
		Reverse	5'	CCA TGA GCT TCT CCA GCT TC	3'
FZD_{10}	NM_007197	Forward	5'	CCT CCA AGA CTC TGC AGT CC	3'
		Reverse	5'	GAC TGG GCA GGG ATC TCA TA	3'

Table S4: Primers used for determination of LRP-coreceptors, WNT target genes and housekeeping genes by qRT-PCR analysis

Gene of interest	NCBI accession number	Primer sequence			
LRP5	NM_002335	Forward	5'	GCA GGA GGG GAA GCT CTA CT	3'
		Reverse	5'	GTA GAT GAA GTC CCC CAG CA	3'
LRP6	NM_002336	Forward	5'	CCC ATG CAC CTG GTT CTA CT	3'
		Reverse	5'	CCA AGC CAC AGG GAT ACA GT	3'
β-Catenin	NM_001904	Forward	5'	CCC ACT AAT GTC CAG CGT TT	3'
		Reverse	5'	AAT CCA CTG GTG AAC CAA GC	3'
Collagen1α1	NM_000088	Forward	5'	AGC CAG CAG ATC GAG AAC AT	3'
		Reverse	5'	TCT TGT CCT TGG GGT TCT TG	3'
α-sm-actin	NM_001141945	Forward	5'	GAC CCT GAA GTA CCC GATAGAAC	3'
		Reverse	5'	GGG CAA CAC GAA GCT CAT TG	3'
Fibronectin	NM_212482	Forward	5'	TCG AGG AGG AAA TTC CAA TG	3'
		Reverse	5'	ACA CAC GTG CAC CTC AT CAT	3'
MMP-2	NM_004530.4	Forward	5'	ACA TCA AGG GCA TTC AGG AG	3'
		Reverse	5'	GCC TCG TAT ACC GCA TCA AT	3'
VEGF	NM_001171623.1	Forward	5'	CTA CCT CCA CCA TGC CAA GT	3'
		Reverse	5'	TGG TGA TGT TGG ACT CCT CA	3'
IL-8	NM_000584.3	Forward	5'	TAG CAA AAT TGA GGC CAA GG	3'
		Reverse	5'	AAA CCA AGG CAC AGT GGA AC	3'
DKK-1	NM_12242.2	Forward	5'	ATG GAA CTC CCC TGT GAT TG	3'
		Reverse	5'	AAT AGG CAG TGC AGC ACC TT	3'

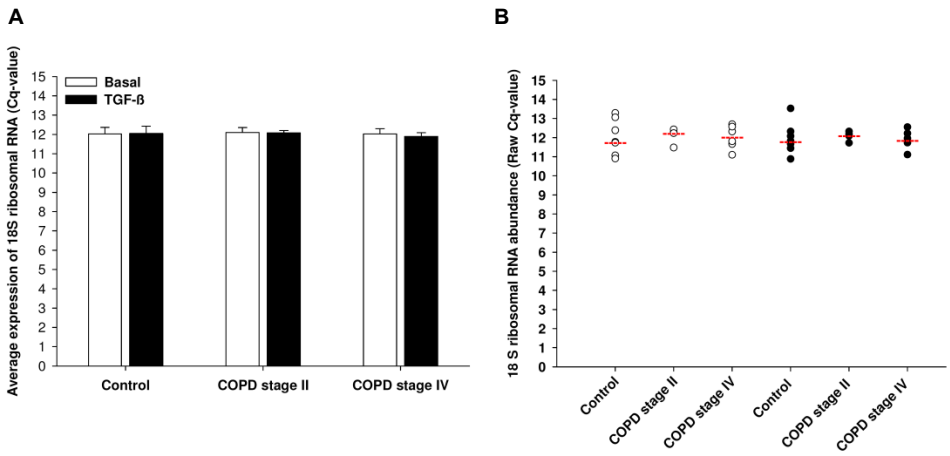


Figure S1: 18S ribosomal RNA abundance in primary fibroblasts from individuals without and with COPD. Primary lung fibroblast were isolated from individuals without (control) and with COPD (stage II and IV) as described in the methods. The lung fibroblasts were grown to confluence and treated for 4 hours with 2 ng/ml TGF-β_1. Analysis of 18S ribosomal RNA is perfomed by qRT-PCR analysis with 0.025 µg of cDNA as input. (A) average 18S rRNA expression in primary human lung fibroblasts and (B) raw Cq-values for all the individual subjects. 18S rRNA expression at baseline is indicated by open circles (○) and after TGF-β_1 stimulation (2 ng/ml; 4 hours) by closed circles (●). Median of each group is indicated by -----.

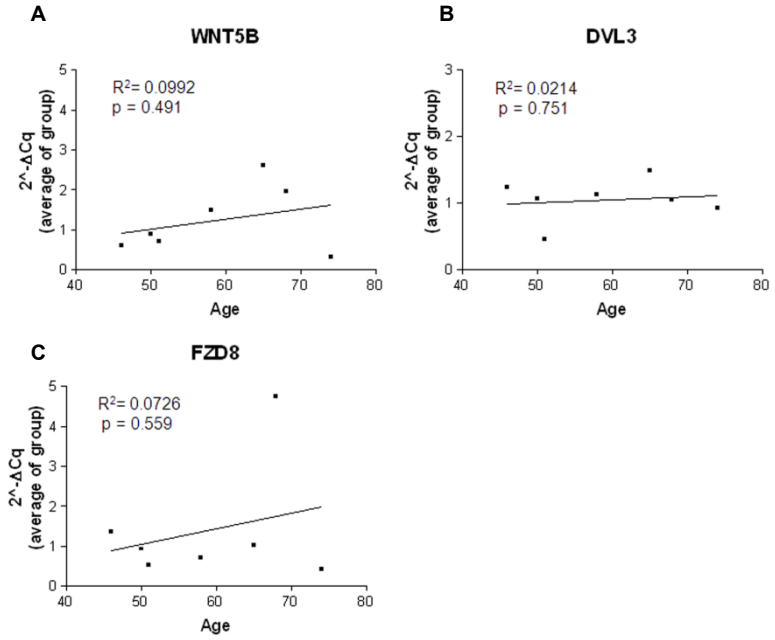


Figure S2: No age-dependent effects on WNT pathway gene expression in pulmonary fibroblasts of individuals without COPD. Expression of (A) WNT-5B, (B) DVL3 and (C) FZD_8 as a function of age of the individual primary lung fibroblasts isolated from individuals without COPD (control) as described in the methods. The fibroblasts were grown to confluence and subsequently mRNA was isolated. Analysis of WNT pathway gene expression is performed by qRT-PCR and corrected for 18S rRNA expression. The uninterrupted line indicates the linear regression.

A

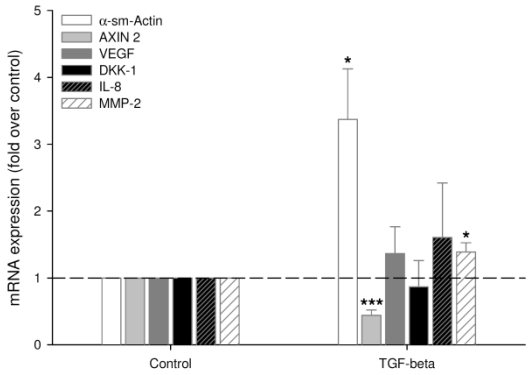


Figure S3: Effect of TGF-β stimulation on canonical WNT genes in human lung fibroblasts. qRT-PCR anaylsis of α-sm-actin (positve control), AXIN2, vascular endothelial growth factor (VEGF), Dickkopf-1 (DKK-1), interleukin-8 (IL-8) and matrix metalloproteinase-2 (MMP-2) in MRC-5 fibroblasts after 24 hours of TGF-β_1 (2 ng/ml) stimulation. Expression of canonical WNT target genes by TGF-β_1 is corrected for 18S rRNA and expressed relative to untreated MRC-5 fibroblasts (control). Data represents mean ± s.e.m. of 5-10 independent experiments. *p<0.05, ***p<0.001 compared to untreated MRC5 fibroblasts (two-tailed Student's *t*-test for paired observations).

References

1. Pauwels RA, Buist AS, Ma P, Jenkins CR, Hurd SS (2001) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. Respir Care 46: 798-825.
2. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, et al. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 176: 532-555.
3. Barnes PJ, Shapiro SD, Pauwels RA (2003) Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J 22: 672-688.
4. Konigshoff M, Kneidinger N, Eickelberg O (2009) TGF-beta signaling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimen. Swiss Med Wkly 139: 554-563.
5. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl 34: 50s-59s.
6. McAnulty RJ (2007) Fibroblasts and myofibroblasts: their source, function and role in disease. Int J Biochem Cell Biol 39: 666-671.
7. de Boer WI, van SA, Sont JK, Sharma HS, Stolk J, et al. (1998) Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 158: 1951-1957.
8. Bartram U, Speer CP (2004) The role of transforming growth factor beta in lung development and disease. Chest 125: 754-765.
9. Scotton CJ, Chambers RC (2007) Molecular targets in pulmonary fibrosis: the myofibroblast in focus. Chest 132: 1311-1321.
10. Zandvoort A, Postma DS, Jonker MR, Noordhoek JA, Vos JT, et al. (2008) Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. Respir Res 9: 83.
11. Plantier L, Boczkowski J, Crestani B (2007) Defect of alveolar regeneration in pulmonary emphysema: role of lung fibroblasts. Int J Chron Obstruct Pulmon Dis 2: 463-469.
12. Noordhoek JA, Postma DS, Chong LL, Vos JT, Kauffman HF, et al. (2003) Different proliferative capacity of lung fibroblasts obtained from control subjects and patients with emphysema. Exp Lung Res 29: 291-302.
13. Noordhoek JA, Postma DS, Chong LL, Menkema L, Kauffman HF, et al. (2005) Different modulation of decorin production by lung fibroblasts from patients with mild and severe emphysema. COPD 2: 17-25.
14. Holz O, Zuhlke I, Jaksztat E, Muller KC, Welker L, et al. (2004) Lung fibroblasts from patients with emphysema show a reduced proliferation rate in culture. Eur Respir J 24: 575-579.
15. Togo S, Holz O, Liu X, Sugiura H, Kamio K, et al. (2008) Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. Am J Respir Crit Care Med 178: 248-260.
16. Kotaru C, Schoonover KJ, Trudeau JB, Huynh ML, Zhou X, et al. (2006) Regional fibroblast heterogeneity in the lung: implications for remodeling. Am J Respir Crit Care Med 173: 1208-1215.
17. Hallgren O, Nihlberg K, Dahlback M, Bjermer L, Eriksson LT, et al. (2010) Altered fibroblast proteoglycan production in COPD. Respir Res 11: 55.
18. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, et al. (2003) Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. Am J Pathol 162: 1495-1502.
19. Cheon SS, Nadesan P, Poon R, Alman BA (2004) Growth factors regulate beta-catenin-mediated TCF-dependent transcriptional activation in fibroblasts during the proliferative phase of wound healing. Exp Cell Res 293: 267-274.

20. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480.
21. Nunes RO, Schmidt M, Dueck G, Baarsma H, Halayko AJ, et al. (2008) GSK-3/beta-catenin signaling axis in airway smooth muscle: role in mitogenic signaling. Am J Physiol Lung Cell Mol Physiol 294: L1110-L1118.
22. Guo X, Wang XF (2009) Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res 19: 71-88.
23. Gradl D, Kuhl M, Wedlich D (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. Mol Cell Biol 19: 5576-5587.
24. De Langhe SP, Sala FG, Del Moral PM, Fairbanks TJ, Yamada KM, et al. (2005) Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung. Dev Biol 277: 316-331.
25. Rahmani M, Read JT, Carthy JM, McDonald PC, Wong BW, et al. (2005) Regulation of the versican promoter by the beta-catenin-T-cell factor complex in vascular smooth muscle cells. J Biol Chem 280: 13019-13028.
26. Zhang X, Gaspard JP, Chung DC (2001) Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res 61: 6050-6054.
27. Clifford RL, Deacon K, Knox AJ (2008) Novel regulation of vascular endothelial growth factor-A (VEGF-A) by transforming growth factor (beta)1: requirement for Smads, (beta)-CATENIN, AND GSK3(beta). J Biol Chem 283: 35337-35353.
28. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J (2005) Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. Angiogenesis 8: 43-51.
29. Howe LR, Subbaramaiah K, Chung WJ, Dannenberg AJ, Brown AM (1999) Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. Cancer Res 59: 1572-1577.
30. Doyle JL, Haas TL (2009) Differential role of beta-catenin in VEGF and histamine-induced MMP-2 production in microvascular endothelial cells. J Cell Biochem 107: 272-283.
31. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T (1999) beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 155: 1033-1038.
32. Kneidinger N, Yildirim AO, Callegari J, Takenaka S, Stein MM, et al. (2010) Activation of the WNT/{beta}-Catenin Pathway Attenuates Experimental Emphysema. Am J Respir Crit Care Med 183: 723-733.
33. Jacobs JP, Jones CM, Baille JP (1970) Characteristics of a human diploid cell designated MRC-5. Nature 227: 168-170.
34. Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.
35. Hwang I, Seo EY, Ha H (2009) Wnt/beta-catenin signaling: A novel target for therapeutic intervention of fibrotic kidney disease. Arch Pharm Res 32: 1653-1662.
36. Laeremans H, Rensen SS, Ottenheijm HC, Smits JF, Blankesteijn WM (2010) Wnt/frizzled signalling modulates the migration and differentiation of immortalized cardiac fibroblasts. Cardiovasc Res 87: 514-523.
37. Pereira CP, Bachli EB, Schoedon G (2009) The wnt pathway: a macrophage effector molecule that triggers inflammation. Curr Atheroscler Rep 11: 236-242.
38. He W, Dai C, Li Y, Zeng G, Monga SP, et al. (2009) Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 20: 765-776.
39. Cheng JH, She H, Han YP, Wang J, Xiong S, et al. (2008) Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 294: G39-G49.
40. Konigshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, et al. (2008) Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One 3: e2142.

41. Konigshoff M, Kramer M, Balsara N, Wilhelm J, Amarie OV, et al. (2009) WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. J Clin Invest 119: 772-787.
42. Letamendia A, Labbe E, Attisano L (2001) Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. J Bone Joint Surg Am 83-A Suppl 1: S31-S39.
43. Doble BW, Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 116: 1175-1186.
44. Barker N, Clevers H (2006) Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov 5: 997-1014.
45. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, et al. (2004) Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell 5: 91-102.
46. Minke KS, Staib P, Puetter A, Gehrke I, Gandhirajan RK, et al. (2009) Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. Eur J Haematol 82: 165-175.
47. Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5: 691-701.
48. Schiller M, Javelaud D, Mauviel A (2004) TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. J Dermatol Sci 35: 83-92.
49. Read JT, Rahmani M, Boroomand S, Allahverdian S, McManus BM, et al. (2007) Androgen receptor regulation of the versican gene through an androgen response element in the proximal promoter. J Biol Chem 282: 31954-31963.
50. Rahmani M, Read JT, Carthy JM, McDonald PC, Wong BW, et al. (2005) Regulation of the versican promoter by the beta-catenin-T-cell factor complex in vascular smooth muscle cells. J Biol Chem 280: 13019-13028.
51. Merrilees MJ, Ching PS, Beaumont B, Hinek A, Wight TN, et al. (2008) Changes in elastin, elastin binding protein and versican in alveoli in chronic obstructive pulmonary disease. Respir Res 9: 41.
52. Sato M (2006) Upregulation of the Wnt/beta-catenin pathway induced by transforming growth factor-beta in hypertrophic scars and keloids. Acta Derm Venereol 86: 300-307.
53. Chen S, McLean S, Carter DE, Leask A (2007) The gene expression profile induced by Wnt 3a in NIH 3T3 fibroblasts. J Cell Commun Signal 1: 175-183.
54. Qin H, Chan MW, Liyanarachchi S, Balch C, Potter D, et al. (2009) An integrative ChIP-chip and gene expression profiling to model SMAD regulatory modules. BMC Syst Biol 3: 73.
55. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, et al. (2010) De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. FASEB J 24: 757-768.
56. Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, et al. (2007) Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. Am J Physiol Lung Cell Mol Physiol 293: L1348-L1358.
57. Shafer SL, Towler DA (2009) Transcriptional regulation of SM22alpha by Wnt3a: convergence with TGFbeta(1)/Smad signaling at a novel regulatory element. J Mol Cell Cardiol 46: 621-635.
58. Lam AP, Flozak AS, Russell S, Wei J, Jain M, et al. (2011) Nuclear {beta}-catenin is Increased in SSc Pulmonary Fibrosis and Promotes Lung Fibroblast Migration and Proliferation. Am J Respir Cell Mol Biol : 10.1165/rcmb.2010-0113OC.
59. Hinz B (2007) Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 127: 526-537.

Chapter 4

Glycogen synthase kinase-3 (GSK-3) regulates TGF-β_1-induced differentiation of pulmonary fibroblasts via suppression of CREB signaling

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Abstract

Background: Chronic lung diseases are characterized by abnormal extracellular matrix (ECM) turnover. Transforming growth factor-β (TGF-β) is a key mediator in stimulating ECM production by recruiting and activating lung fibroblasts and initiating their differentiation process into myofibroblasts. Glycogen synthase kinase-3 (GSK-3) regulates various intracellular signaling pathways; its role in TGF-β_1-induced myofibroblast differentiation is currently largely unknown.

Purpose: To determine the contribution of GSK-3 signaling to TGF-β_1-induced myofibroblast differentiation.

Experimental approach: We used MRC5 human lung fibroblasts and primary pulmonary fibroblasts of individuals with and without COPD. Protein and mRNA expression were determined by immunoblotting and RT-PCR analysis, respectively.

Results: Stimulation of MRC5 and primary human lung fibroblasts with TGF-β_1 resulted in a time- and dose-dependent increase of α-sm-actin, MMP-2 and fibronectin expression, indicative of myofibroblast differentiation. Pharmacological inhibition of GSK-3 by SB216763 dose-dependently attenuated TGF-β_1-induced expression of these myofibroblasts markers. Moreover, silencing of GSK-3 by siRNA or pharmacological inhibition by the structural unrelated GSK-3 inhibitors CT/CHIR99021 or LiCl inhibited TGF-β_1-induced expression of α-sm-actin and fibronectin. The effect of GSK-3 inhibition on α-sm-actin expression was similar in fibroblasts from individuals with and without COPD. Neither smad, NF-κB, nor ERK1/2 were involved in the inhibitory actions of GSK-3 inhibition by SB126763 on myofibroblast differentiation. Rather, SB216763 increased the phosphorylation of CREB, which in its phosphorylated form acts as a functional antagonist of TGF-β/smad signaling.

Conclusion and implication: We demonstrate that GSK-3 signaling regulates TGF-β_1-induced myofibroblast differentiation by regulating CREB phosphorylation. GSK-3 may constitute a useful target for treatment of chronic lung diseases.

Key words: cAMP (adenosine 3’5’ cyclic monophosphate) response element binding protein (CREB), Fibronectin, α-sm-actin, COPD, SB216763

Introduction

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed and constitutively active serine/threonine kinase occurring in two closely related isoforms GSK-3α and GSK-3β. GSK-3 was first discovered based on its ability to regulate glycogen metabolism, as the enzyme that phosphorylates and thereby inactivates glycogen synthase (Embi *et al.*, 1980; Doble & Woodgett, 2003; Jope & Johnson, 2004; Frame & Cohen, 2001). However, over the past few decades GSK-3 has been shown to contribute to various other signaling pathways that are involved in a wide variety of cellular functions including gene transcription, protein translation, apoptosis and cell cycle progression (Gosens *et al.*, 2007; Jope & Johnson, 2004; Frame & Cohen, 2001). GSK-3α and GSK-3β exert their cellular functions by regulating a variety of signaling proteins and transcription factors, including nuclear factor kappa-B (NF-κB), activator protein-1 (AP-1), cyclic AMP responsive element binding protein (CREB), members of the smad (small phenotype and mothers against decapentaplegic related protein) protein family, β-catenin and T-cell factor (TCF), among many others (Jope & Johnson, 2004; Gotschel *et al.*, 2008; Baarsma *et al.*, 2011a; Liang & Chuang, 2006). Distinct intracellular pools of GSK-3 have been implicated to regulate divergent signalling pathways simultaneously within the same cell, and the large number of putative substrates implies that GSK-3 is possibly a key regulator of cellular processes in fibroblasts (Gotschel *et al.*, 2008; Ding *et al.*, 2000). However, the role of GSK-3 signaling in human lung fibroblast function is currently largely unknown.

Fibroblasts are considered to be the primary cell type responsible for the extracellular matrix (ECM) maintenance in the lung and are implicated to play a major role in the aberrant ECM turnover seen in the pathogenesis of chronic lung diseases such as asthma, COPD and pulmonary fibrosis (Kranenburg *et al.*, 2006; McAnulty, 2007; Lofdahl *et al.*, 2011; Noordhoek *et al.*, 2003; Noordhoek *et al.*, 2005; Togo *et al.*, 2008). Growth factors released during the persistent chronic inflammation, in particular transforming growth factor-β (TGF-β), attract and activate pulmonary fibroblasts (Morty *et al.*, 2009; Chung, 2001). In addition, locally upregulated TGF-β_1 is capable of initiating the differentiation process of fibroblasts into more active myofibroblasts, spindle-shaped cells characterized by the expression of α-smooth muscle-actin containing stress fibers (Scotton & Chambers, 2007; Bartram & Speer, 2004). Members of the TGF-β superfamily are multifunctional proteins that regulate various cellular functions by binding to serine/threonine receptor kinases that transduce signals by intracellular smad proteins (Schiller *et al.*, 2004; Derynck *et al.*, 1998). Smad2 and smad3 are receptor-regulated smad proteins (R-smads), which are phosphorylated in the C-terminus domain by the type I receptor kinase upon TGF-β_1 binding. These

activated R-smads dissociate from the receptor and assemble a heterotrimeric complex, consisting of two R-smads with common smad4, which subsequently translocates to the nucleus where it regulates gene transcription through association with a variety of transcription factors together with the closely related co-activators p300 and/or CREB-binding protein (CBP) (Derynck *et al.*, 1998; Schiller *et al.*, 2004; Massague, 2000). In addition to smad-dependent signaling, smad-independent signaling cascades like mitogen activated protein kinases (MAPKs) and NF-κB are activated in response to TGF-β_1, which collectively define the cellular responses (Massague, 2000; Derynck & Zhang, 2003).

In the current study we assessed the contribution of GSK-3 signaling to myofibroblast differentiation of pulmonary fibroblasts. We report that GSK-3 signaling is critically involved in the TGF-β_1-induced myofibroblast differentiation by regulating CREB-dependent signaling.

Methods

Subjects

Primary lung fibroblasts were cultured from lung tissue obtained from 11 individuals with and without COPD. Classification of COPD severity was based on the Global initiative for Chronic Obstructive Lung disease (GOLD) criteria (Rabe *et al.*, 2007). Fibroblasts obtained from these individuals, were from individuals with moderate (GOLD stage II, n=3) or severe COPD (stage IV, n=4), and from individuals with histologically normal lungs (n=4). Emphysema was assessed by routine histological examination of lung tissue, which was performed by an experienced pulmonary pathologist (WT). Fibroblasts were isolated from peripheral lung tissue and areas without macroscopically visible airways and blood vessels were used. The study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies"; htttp://www.federa.org).

Cell culture

MRC5 lung fibroblasts and primary lung fibroblasts from individuals with and without COPD were cultured in Ham's F12 medium supplemented with 10% ($v.v^{-1}$) foetal bovine serum (FBS), 2 mM L-glutamine, 100 $\mu g.l^{-1}$ streptomycin and 100 $U.ml^{-1}$ penicillin. Unless otherwise specified, for each experiment cells were grown to confluence and subsequently culture medium was substituted with Ham's F12 medium supplemented with 0.5% ($v.v^{-1}$) FBS, 2 mM L-glutamine, 100 $\mu g.l^{-1}$ streptomycin and 100 $U.ml^{-1}$ penicillin for a period of 24 hours. Cells were stimulated for different time-points with TGF-β_1 (2 $ng.ml^{-1}$) or with 0.5, 2 and 5 $ng.ml^{-1}$ of TGF-β_1 for 48 hours. All experiments were performed in Ham's F12 medium supplemented with 0.5% FBS, L-glutamine and antibiotics. When applied, pharmacological inhibitors (i.e. SB216763 10 μM, CT/CHIR99021 1 μM, LiCL 10 mM, SIS3 3 μM, U0126 μM, SC-514 10-50 μM, PS1145 10 μM) or forskolin (2 μM) were added 30 minutes before the addition of TGF-β_1. The GSK-3 inhibitors (SB216763, CT/CHIR99021) had no effects on cell viability, which was verified by light microscopy, by analysis of total protein and by mitochondrial reduction assays (data not shown).

GSK-3 siRNA transfection

MRC-5 fibroblasts were grown to, 90% confluence in 6-well cluster plates and transiently transfected with double stranded siRNA targeted against the GSK-3 transcript, which targets both GSK-3α and GSK-3β (Santa Cruz biotechnology, CA, USA). Cells were transfected in serum-free Ham's F12 without any supplements using 200 pmol of siRNA in combination with lipofectamine 2000 transfection

reagent. Control transfections were performed using a non-silencing control siRNA (Qiagen, Venlo, The Netherlands). After 6 hours of transfection, cells were washed once with warm (37^{o}C) Hank's Balanced Salt Solution (HBSS; composition [mg/l]: KCl 400, KH_2PO_4 60, NaCl 8000, $NaHCO_3$ 350, $Na_2HPO_4.1H_2O$ 50, glucose 1000, pH: 7.4) followed by a period of 24 hours in Ham's F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Consecutively, medium was refreshed and cells were stimulated with TGF-β_1 (2 ng/ml) for 48 hours. The cells were lysed in ice-cold SDS buffer. Protein concentration was determined by Pierce protein determination according to the manufacturer's instructions.

mRNA isolation and real-time PCR analysis
Total mRNA was extracted using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). Briefly, cells were harvested in RNA*later* stabilization buffer and homogenized by passing the lysate 10 times through a 20 gauge needle. Lysates were then mixed with an equal volume of 70% ethanol, and total mRNA was purified using RNeasy mini spin columns. The eluted mRNA was quantified using spectrophotometry (Nanodrop, ThermoScientific, Wilmington, USA). Equal amounts of total mRNA (1 µg) were then reverse transcribed and stored at -20 °C until further use.

cDNA was subjected to real-time PCR, which was performed with a MyiQ™ Single-Color detection system (Bio-Rad laboratories Inc. Life Science group, Hercules, CA, USA). In short, 12.5 µl iQ™ SYBR Green Supermix, containing fluorescein to account for well to well variation, 0.1 µM of gene-specific forward and reverse primer and 1 µl of 1:5 diluted cDNA sample were used in a total volume of 25 µl and added to a 96 well plate. The sequences of the primers used are listed in the table 1. Real-time PCR data were analyzed using the comparative cycle threshold (Cq: amplification cycle number) method. Cycle parameters were: denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles followed by 5 minutes at 72°C. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA ($Cq_{\text{gene of interest}} - Cq_{18S\ rRNA}$; designated as ΔCq). Several housekeeping genes, including β2-microglobulin (B2M; NM_00408) and phospholipase A2 (YWAHZ; NM_003406), were tested for the influence of the experimental procedure on the expression (Vandesompele *et al.*, 2002). The expression of both ribosomal protein S18 (18S rRNA) and β2-microglobulin was stable in the tested conditions. Phospholipase A2 (YWAHZ; NM_003406) expression fluctuated after TGF-β_1 stimulation, however. Ribosomal protein S18 was chosen as most optimal household gene because gene expression was most stable under basal as well as stimulation conditions. Relative differences in gene expression were determined using the equation $2^{-(\Delta\Delta Cq)}$.

Table 1: Primers used for determination of myofibroblasts markers by qRT-PCR analysis

Gene of interest	NCBI accession number			Primer sequence	
Fibronectin	NM_212482	Forward	5'	TCGAGGAGGAAATTCCAATG	3'
		Reverse	5'	ACACACGTGCACCTCATCAT	3'
α-sm-actin	NM_001141945	Forward	5'	GACCCTGAAGTACCCGATAGAAC	3'
		Reverse	5'	GGGCAACACGAAGCTCATTG	3'
18S rRNA	NR_003286.2	Forward	5'	CGCCGCTAGAGGTGAAATTC	3'
		Reverse	5'	TTGGCAAATGCTTTCGCTC	3'
PAI-1	NG_013213.1	Forward	5'	CGCCAGAGCAGGACGAA	3'
		Reverse	5'	GGACACATCTGCATCCTGAAGT	3'
CTGF	NM_001901	Forward	5'	CCG TAC TCC CAA AAT CTC CA	3'
		Reverse	5'	GTA ATG GCA CGC ACA GGT CT	3'

Preparation of cell lysates

To obtain whole cell lysates, cells were washed once with ice-cold (4°C) HBSS then lysed in ice-cold sodiumdodecylsulphate (SDS) buffer (composition: 62.5 mM Tris, 2 % $w.v^{-1}$ SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 $\mu g.ml^{-1}$ aprotinin, 10 $\mu g.ml^{-1}$ leupeptin, 7 $\mu g.ml^{-1}$ pepstatin A, pH 6.8). Lysates were then sonicated and protein concentration was determined according to Pierce protein determination according to the manufacturer's instructions. Lysates were stored at -20 ºC till further use.

Western blot analysis

Equal amounts of protein (10-50 $\mu g.lane^{-1}$) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were either subsequently visualized on film or recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisistion software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using Totallab^{TM} software (Nonlinear dynamics; Newcastle, UK) or GeneTools analysis software (Syngene; Cambridge; UK), respectively.

Cytokine enzyme-linked immunosorbent assays (ELISA)

Confluent MRC5 human lung fibroblasts were washed twice with warm (37°C) HBSS followed by a period of 24 hours in Ham's F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Consecutively, medium was refreshed and cells were stimulated with TGF-β_1 (2 $ng.ml^{-1}$) or IL-1β (0.1 $ng.ml^{-1}$) in the presence or absence of the selective IKK inhibitor SC514 (10 μM) or PS-1145 (10 μM) for 24 hours (Bain *et al.*, 2007). Cell supernatants were harvested 24 hours after stimulation and stored at -20 °C until assayed for interleukin-8 (IL-8; CXCL8). Cytokine levels were determined by specific enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (IL-8 kit Sanquin, Amsterdam, The Netherlands).

Cytochemistry

MRC5 lung fibroblasts were plated onto Lab-Tek^{TM} borosilicate chamber slides and treated with TGF-β_1 (2 ng.ml^{-1}) for 48 hours, fixed for 15 min at 4 ^{o}C in cytoskeletal (CB) buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$ and 5 mM glucose at pH 6.1) containing 3 % paraformaldehyde (PFA). Cells were then permeabilized by incubation for 5 min at 4 ^{o}C in CB buffer containing 3 % PFA and 0.3% Triton X-100. Filamentous actin was stained with Alexa Fluor 488 phalloidin (15 minutes at room temperature) and nuclei with Hoechst 33342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and analyzed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens).

Antibodies and reagents

Mouse anti-α-sm-actin, horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, HRP-conjugated goat anti-rabbit antibody and HRP-conjugated rabbit anti-goat antibody were purchased from Sigma (St. Louis, MO, USA). Goat anti-fibronectin (C20) antibody and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-phospho-ser133-CREB antibody, rabbit anti-total CREB antibody, rabbit anti-VASP antibody, rabbit anti-phospho-Smad2 (Ser465/467), rabbit anti-phospho-Smad3 (Ser423/425, mouse anti-phospho-thr202/tyr204-ERK-1/2 antibody and mouse anti-total ERK-1/2 antibody were from Cell Signaling Technology (Beverly, MA, USA). Forskolin and 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763) were from Tocris bioscience (Bristol, UK). 6-(2-[4-(2,4-dichloro-phenyl)-5-(4-methyl-1H-imidazol-2-yl)-pyrimidin-2-ylamino]-ethylamino)-nicotinonitrile (CT/CHIR99021) was from Axon Medchem (Groningen, The Netherlands). Alexa Fluor 488 phalloidin and Hoechst 33342 were from Invitrogen (Paisley, UK). Recombinant human TGF-β_1 was from R&D systems (Abingdon, UK). All other chemicals were of analytical grade.

Results

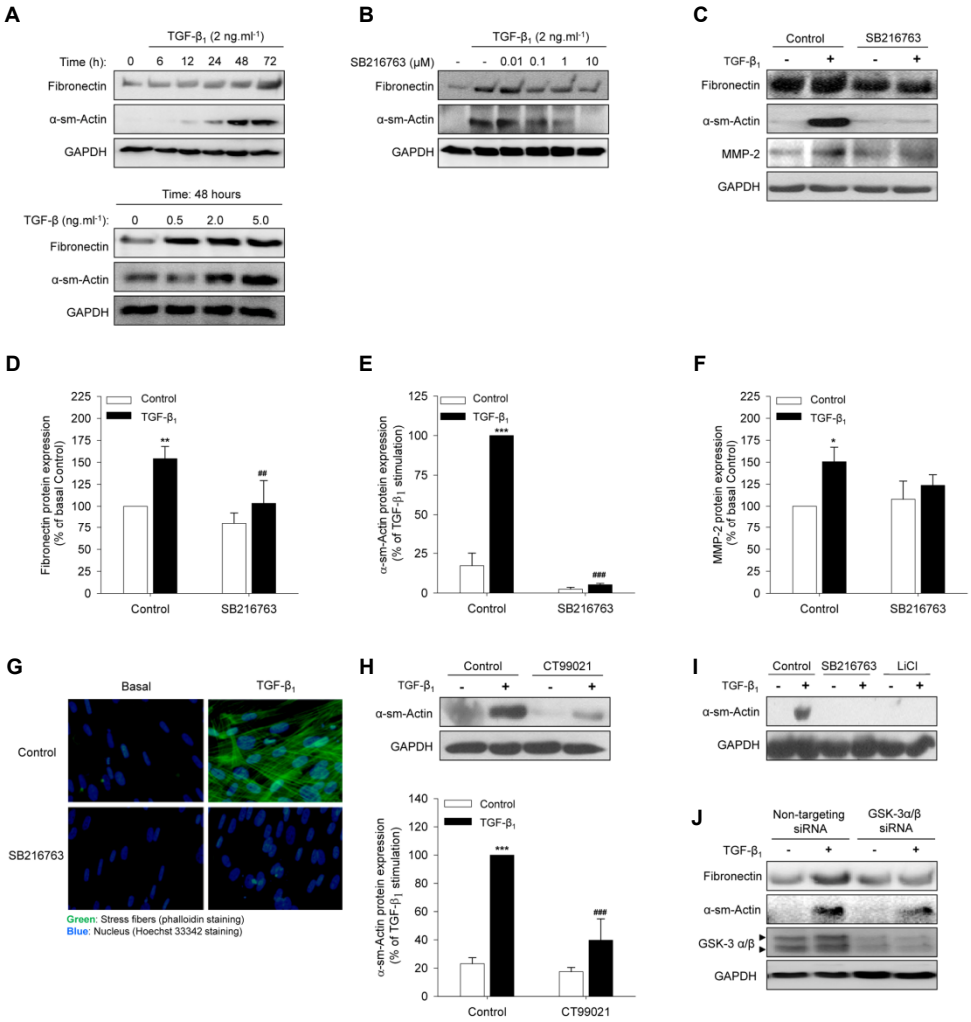
TGF-β_1 induces myofibroblast differentiation of human lung fibroblasts

MRC5 human lung fibroblasts were stimulated with TGF-β_1 (2 ng.ml^{-1}) for different time-points up to 72 hours to promote myofibroblast differentiation. TGF-β_1 stimulation resulted in a time-dependent up regulation of α-sm-actin expression as well as increased fibronectin deposition, which are two important markers of myofibroblast differentiation (figure 1A, top panel). Myofibroblast differentiation was evident after 48 hours of treatment (figure 1A, top panel). Stimulation of the fibroblasts with 0.5, 2 and 5 ng.ml^{-1} of TGF-β_1 showed a concentration dependent activation of the differentiation process (figure 1A, lower panel). Based on these initial findings, we selected the submaximal concentration of 2 ng.ml^{-1} TGF-β_1 and 48 hours of treatment for further experiments.

Inhibition of glycogen synthase kinase-3 (GSK-3) attenuates TGF-β_1-induced α-sm-actin expression and fibronectin deposition by human lung fibroblasts

Next, we next determined the contribution of GSK-3 signaling to TGF-β_1-induced expression of myofibroblast differentiation markers in lung fibroblasts. Pharmacological inhibition of GSK-3 by the selective inhibitor SB216763 dose-dependently prevented the induction of α-sm-actin and fibronectin protein by TGF-β_1 in human lung fibroblasts (figure 1B). DMSO did not affect myofibroblast differentiation (data not shown). The expression of fibronectin, α-sm-actin and matrix metalloproteinase-2 (MMP-2) in response to TGF-β_1 stimulation and GSK-3 inhibition by SB216763 was investigated in more detail (figure 1C-1F). TGF-β_1 significantly induced the expression of these myofibroblasts markers and pharmacological inhibition of GSK-3 by SB216763 (10 μM) prevented the TGF-β_1-induced α-sm-actin expression and fibronectin deposition (figure 1D and 1E). Though inhibition of GSK-3 appeared to attenuate TGF-β_1-induced MMP-2 expression, differences were not statistically significant (figure 1A and 1E). Cytochemical staining for filamentous actin in the pulmonary fibroblasts indicated that TGF-β_1 (2 ng.ml^{-1}, 48 h) distinctively induced the formation of stress fibers (filamentous actin; green) in these cells, another indication of myofibroblast differentiation (figure 1G). Similar to the effect on α-sm-actin and fibronectin expression, SB216763 (10 μM) prevented the formation of stress fibers in these cells. To confirm the requirement of GSK-3 in TGF-β_1-induced α-sm-actin expression, we used two other distinct and structurally unrelated GSK-3 inhibitors, namely CT/CHIR99021 (1 μM) and LiCl (10 mM). Of note, the three distinct small molecules used for inhibition of GSK-3 have dissimilar potential off-target effects (Bain *et al.*, 2007). In agreement with SB216763, CT/CHIR99021 and LiCl also greatly attenuated the induction of α-sm-actin by TGF-β_1 in MRC5 human lung fibroblasts, confirming the results obtained with SB216763 (figure 1H and 1I).

Moreover, silencing of GSK-3 expression by specific siRNA attenuated the TGF-β_1-induced expression of fibronectin and α-sm-actin (figure 1J). Taken together, these data indicate that GSK-3 inhibition attenuates TGF-β_1-induced responses in human lung fibroblasts.



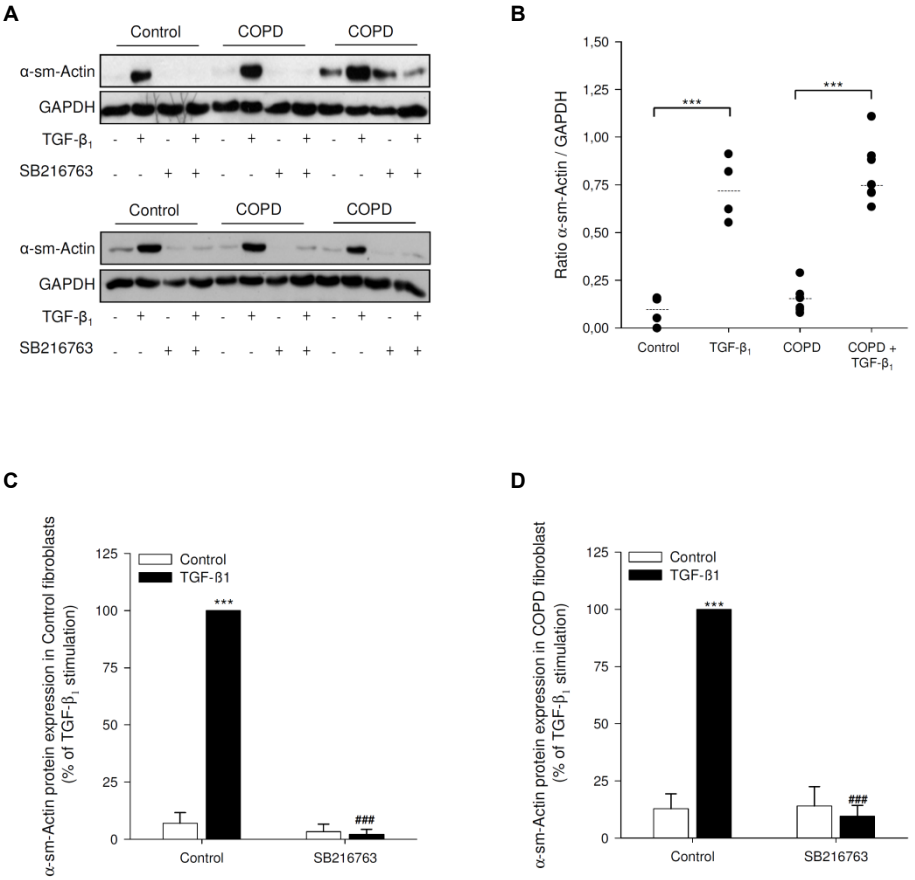
◄ ***Figure 1: TGF-β_1-induced myofibroblast differentiation of lung fibroblasts is attenuated by inhibition of GSK-3.*** (A) Time- and concentration dependent induction of the myofibroblasts markers α-sm-actin and fibronectin in response to TGF-β_1 stimulation. MRC5 human lung fibroblasts were stimulated either with 2 ng.ml^{-1} TGF-β_1 for the indicated time-point (upper panel) or with the indicated concentrations of TGF-β_1 for 48 hours (lower panel). Expression of the myofibroblast markers was evaluated in whole cell lysates by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. Representative immunoblots of 3 independent experiments are shown. (B) Pharmacological inhibition of GSK-3 by SB216763 dose-dependently prevents myofibroblast differentiation. MRC5 human lung fibroblasts were stimulated with 2 ng.ml^{-1} TGF-β_1 for 48 hours in the presence or absence of the selective GSK-3 inhibitor SB216763 (0.01 – 10 μM). Expression of the fibronectin and α-sm-actin was evaluated in whole cell lysates by immunoblotting using specific antibodies. (C) Inhibition of GSK-3 by SB216763 (10 μM) prevented the induction of the myofibroblast markers fibronectin, α-sm-actin and matrixmetalloproteinase-2 (MMP-2) in response to TGF-β_1 stimulation. Equal protein loading was verified by the analysis of GAPDH. Representative immunoblots of 3-8 independent experiments are shown. These responses of TGF-β_1 in the presence or absence of the GSK-3 inhibitor SB216763 on the expression of fibronectin (C and D), α-sm-actin (C and E) and MMP-2 (C and F) were quantified by densitometry. (G) Cytochemical evaluation of stress fiber formation in TGF-β_1-induced myofibroblast differentiation. MRC5 human lung fibroblasts were treated for 48 h with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of SB216763 and subsequently fixed and permeabilized. Cells were stained for filamentous actin (488 phalloidin; green) and nucleus (Hoechst 3342; blue). Pictures were taken at 400x magnification. (H-I) MRC5 human lung fibroblasts were grown to confluence and stimulated for 48 h with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the selective GSK-3 inhibitors or CT/CHIR99021 (1 μM), SB216763 (10 μM) or LiCl (10 mM). Expression of the α-sm-actin (upper panel) in the presence or absence of CT/CHIR99021 was evaluated in whole cell lysates by immunoblotting using specific antibodies and responses were quantified by densitometry (lower panel) representing mean ± s.e.m. of 4 independent experiments. (J) Silencing of GSK-3 by specific siRNA attenuates TGF-β_1-induced myofibroblast differentiation. Subconfluent MRC-5 lung fibroblast cultures were transfected with a siRNA against the GSK-3 (GSK-3α and GSK-3β) transcript and control cultures were transfected with a non-targeting control siRNA. Transfected cells were treated with TGF-β_1 (2 ng.ml^{-1}) for 48h. Expression of fibronectin, α-sm-actin and total GSK-3 was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. *p<0.05, **p<0.01 and ***p<0.001 compared to basal expression (control), $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to TGF-β_1 stimulation; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test.

We next used primary human lung fibroblasts, both from individuals with and without COPD (the clinical characteristics are presented in table 2). These primary human lung fibroblasts were stimulated with TGF-β_1 (2 ng.ml^{-1}, 48 h) in the absence or presence of SB216763 (10 μM). TGF-β_1 stimulation resulted in a clear induction of α-sm-actin in all primary human lung fibroblasts (figure 2A). The expression of α-sm-actin induced by TGF-β_1 was similar in lung fibroblasts from COPD patients and from individuals without COPD (figure 2A-B). As observed in the MRC-5 fibroblasts, SB216763 prevented the induction of α-sm-actin by TGF-β_1 in primary human lung fibroblasts (figure 2A-B). The effect of GSK-3 inhibition on α-sm-actin expression was similar in fibroblasts from individuals with and without COPD (figure 2C-D).

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

	Control	COPD
Number of subjects	4	7
Age (years)	55 (50-65)	59 (52-77)
Sex		
Male	2	5
Female	2	2
Smoking status		
Ex-smoker	0	7
Current smoker	3	0
Non-smoker	1	0
Pack-years	25 (0 - 70)	33.5 (22 - 55)
FEV_1 % predicted	100.9 (80.0 – 118.0)	17.2 ** (9.8 – 78.1)
FEV_1 / FVC %	75.3 (72.0 – 78.3)	37.0 ** (14.0 – 64.0)

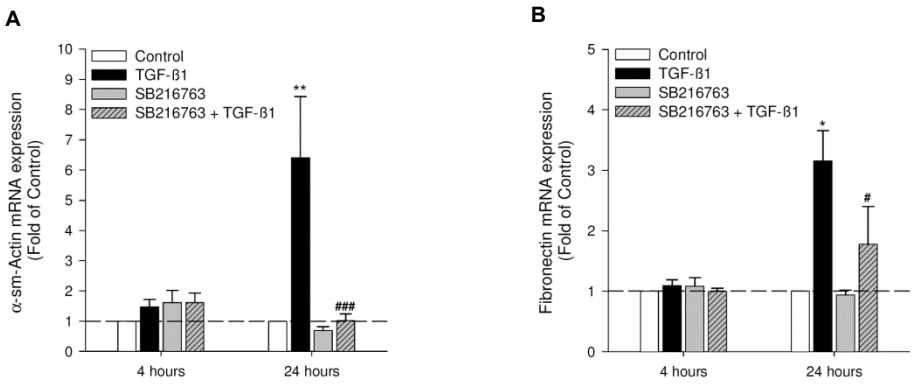
All values are represented as median values with ranges in parentheses. Ex –smokers = not smoking for at least one year. FEV_1 % predicted = Forced Expiratory Volume in 1 second as percentage of predicted value; FVC = Forced Vital Capacity. Statistical significance determined a two-tailed Mann-Whitney test. **$p<0.01$ compared to Control group.



▲ ***Figure 2: Myofibroblast differentiation of primary human lung fibroblasts of individuals with and without COPD is attenuated by GSK-3 inhibition***. Primary human lung fibroblasts of individuals with (n=7) and without COPD (n =4) were grown to confluence and stimulated for 48 h with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the selective GSK-3 inhibitor SB216763 (10 μM). (A-B) Expression of the myofibroblast marker α-sm-actin was evaluated in whole cell lysates by immunoblotting using a specific antibody. Equal protein loading was verified by the analysis of GAPDH. ***p<0.001 compared to basal expression (control); determined by two-tailed student's *t*-test for paired observations. Median α-sm-actin expression is indicated by ----. (C-D) Responses of TGF-β_1 in the presence or absence of the GSK-3 inhibitor on the expression of α-sm-actin were quantified by densitometry for fibroblast of individuals (C) without and (D) with COPD. ***p<0.001 compared to basal expression (control), $^{###}$p<0.001 compared to TGF-β_1 stimulation; determined by a One-way ANOVA followed by a Newman-Keuls multiple comparison test.

GSK-3 inhibition attenuates TGF-β_1-induced α-sm-actin and fibronectin mRNA expression

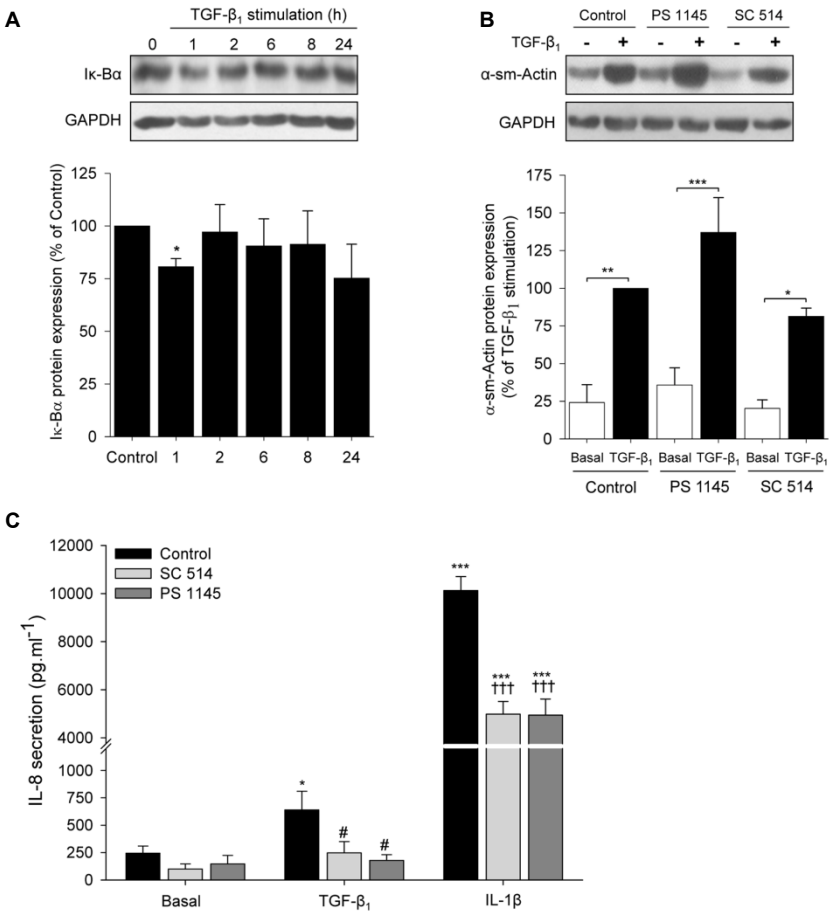
MRC5 human lung fibroblasts were stimulated with TGF-β_1 (2 ng.ml^{-1}) for 4 and 24 hours in the presence or absence of SB216763 (10 μM), after which mRNA levels of α-sm-actin and fibronectin were determined. Previous findings indicated these time-points to be optimal for the induction of gene expression by TGF-β_1 (Baarsma *et al.*, 2011b). A clear induction of both α-sm-actin and fibronectin mRNA was detected after 24 hours only (figure 3A-B). Pharmacological inhibition of GSK-3 by SB216763 completely prevented α-sm-actin mRNA induction and greatly attenuated the increase of fibronectin mRNA by TGF-β_1 (figure 3A-B). Inhibition of GSK-3 by CT99021 (1 μM) attenuated the induction of α-sm-actin and fibronectin mRNA to a similar extent (data not shown).



▲ ***Figure 3: Inhibition of GSK-3 attenuates TGF-β_1-induced α-sm-actin and fibronectin mRNA expression.*** MRC5 human lung fibroblasts were grown to confluence and stimulated for 4h and 24h with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the selective GSK-3 inhibitors SB216763 (10 μM). Gene expression of (A) α-sm-actin and (B) fibronectin was determined by qRT-PCR analysis, corrected for 18S rRNA and expressed relative to untreated MRC-5 fibroblasts (control). Data represents mean ± s.e.m. of 4 independent experiments. *p<0.05 and **p<0.01 compared to basal expression (control), $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 compared to TGF-β1 stimulation; determined by a One-way ANOVA followed by a Newman-Keuls multiple comparison test.

Activation of NF-κB signaling is not required for myofibroblast differentiation

A variety of cellular responses initiated by TGF-β_1 stimulation are mediated by the activation of the NF-κB signaling pathway (Arsura *et al.*, 2003; Gingery *et al.*, 2008). Moreover, in a diversity of cell types GSK-3 is involved in the regulation of NF-κB signaling by controlling the activation or transcriptional responses of this pathway (Baarsma *et al.*, 2011a; Dugo *et al.*, 2007; Hoeflich *et al.*, 2000). Therefore, we studied the activation of NF-κB signaling in response to TGF-β_1 and its contribution to myofibroblast differentiation. Activation of the NF-κB signaling pathway was determined by measuring the expression of the NF-κB inhibitory protein Iκ-Bα. We found a 19.3 ± 3.8% decrease of Iκ-Bα expression after 1 hour of TGF-β_1 stimulation, indicating that the pathway is activated in response to this growth factor (figure 4A). In line with this, the DNA binding activity of p65 NF-κB was modestly enhanced to 1.2-fold of basal at this time-point (data not shown). Next, we determined the contribution of NF-κB signaling to the myofibroblast differentiation. Pharmacological inhibition of IKK-2 by either PS-1145 or SC-514 did not affect the TGF-β_1-induced expression of α-sm-actin, although these selective inhibitors attenuated the IL-8 release in response to TGF-β_1 or IL-1β stimulation (figure 4B and C) (Bain *et al.*, 2007). These findings indicate that that TGF-β_1 modestly activates the NF-κB signaling pathway in MRC5 human lung fibroblasts. Activation of NF-κB contributes to cytokine release by the pulmonary fibroblast in response to TGF-β_1; however activation of the NF-κB signaling pathway is not required for myofibroblast differentiation, indicating that the effects of GSK-3 inhibition require distinct signaling intermediates.

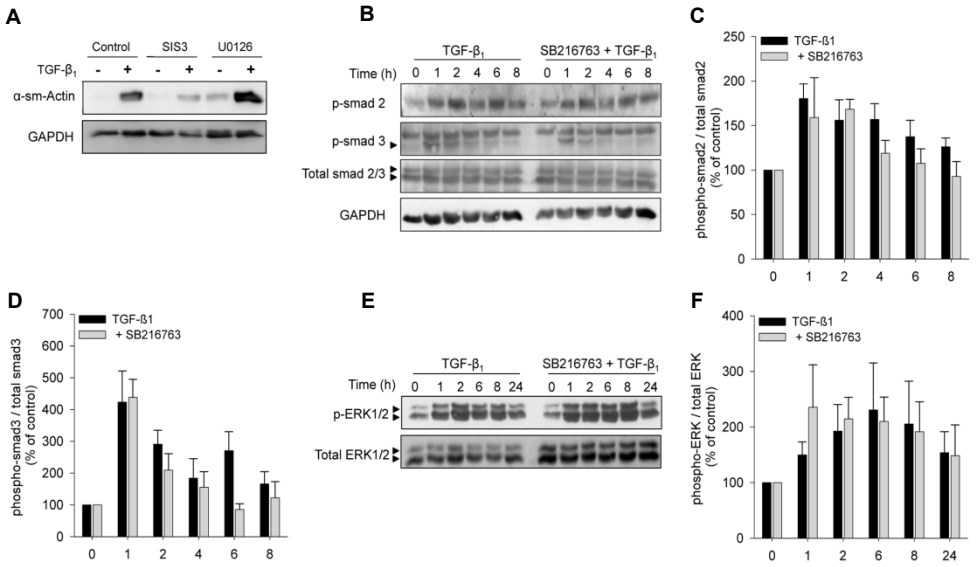


▲ ***Figure 4: Activation of NF-κB signaling by TGF-β1 is not required for myofibroblast differentiation.*** MRC5 human lung fibroblasts were grown to confluence and stimulated for various time-points with TGF-β_1 (2 ng.ml^{-1}). (A) Time-dependent decrease of the NF-κB-inhibitory protein Iκ-Bα. Expression of Iκ-Bα was evaluated by immunoblotting and responses were quantified by densitometry and normalized to the expression GAPDH. Data represents mean ± s.e.m. of 3 independent experiments. *p<0.05 compared to control (t = 0 h); determined by two-tailed student's *t*-test for paired observations. (B) Contribution of NF-κB signaling to TGF-β_1-induced myofibroblast differentiation. MRC5 human lung fibroblasts were stimulated for 48 hours with TGF-β1 in the presence or absence of either PS-1145 (10 μM) or SC-514 (50 μM), two distinct inhibitors of IκB-kinases (IKK). Expression of α-sm-actin was evaluated by immunoblotting and responses were quantified by densitometry and normalized to the expression GAPDH. Data represents mean ± s.e.m. of 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test. (C) Inhibition of IKK attenuates IL-8 secretion by human lung fibroblasts in response to TGF-β_1 and IL-1β stimulation. MRC5 human lung fibroblasts were grown to confluence and stimulated for 24 hours with TGF-β_1 (2 ng.ml^{-1}) or IL-1β (0.1 ng.ml^{-1}) in the presence of either PS-1145 (10 μM) or SC-514 (10 μM). The release of IL-8 by the fibroblasts was measured by ELISA. Responses shown represent mean ± s.e.m. of 3 independent experiments, each performed in duplicate.*p<0.05, and ***p<0.001 compared to basal (control), $^{\#}$p<0.05 compared to TGF-β_1 stimulation and †††p<0.001 compared to IL-1β stimulation; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test.

Smad and ERK-1/2 activation by TGF-β_1 are not attenuated by GSK-3 inhibition

We next investigated the activation of smad and the extracellular signal-regulated kinases 1/2 (ERK-1/2; p42/44 MAPK). These pathways may be involved in TGF-β_1-induced myofibroblast differentiation and are possibly regulated by GSK-3 (Sebe *et al.*, 2008; Ramirez *et al.*, 2006; Piek *et al.*, 2001; Guo *et al.*, 2008; Millet *et al.*, 2009). The involvement of the smad and ERK-1/2 signaling pathway in the differentiation process was investigated by using the specific inhibitor of smad3 (SIS3: 3 μM) and the selective MEK1/2 inhibitor U0126 (3 μM), respectively (Jinnin *et al.*, 2006; Bain *et al.*, 2007). Attenuation of smad signaling decreased the TGF-β_1 induced expression of a-sm-actin, indicating the requirement of smad signaling in the differentiation process (figure 5A). In line with these findings, stimulation with TGF-β_1 induced ser465/467-smad2 and ser423/425-smad3 phosphorylation in a time-dependent manner, without affecting the expression of total smad2 or smad3 (figure 5B). The phosphorylation of both smad2 and smad 3 was maximal after 1 hour of TGF-β_1 stimulation (figure 5B-D). However, inhibition of GSK-3 by SB216763 had no major effect on basal or TGF-β_1-induced phosphorylation or cellular expression of smad2 or smad3.

Pharmacological inhibition of ERK1/2 enhanced basal and TGF-β_1–induced expression of α-sm-actin, indicative of negative regulation by this pathway. Although TGF-β_1 activates ERK-1/2 by phosphorylation (figure 5E), inhibition of GSK-3 by SB216763 had no major effect on the basal or TGF-β_1-induced phosphorylation of ERK-1/2 (figure 5E and 5F). These data suggest that smad and ERK1/2 signaling are activated by TGF-β and that these signaling pathways have opposite effects on myofibroblast differentiation. GSK-3 signaling does not regulate the phosphorylation of either smad2/3 or ERK-1/2 in MRC5 fibroblasts, indicating that the effects of GSK-3 inhibition require distinct signaling intermediates.

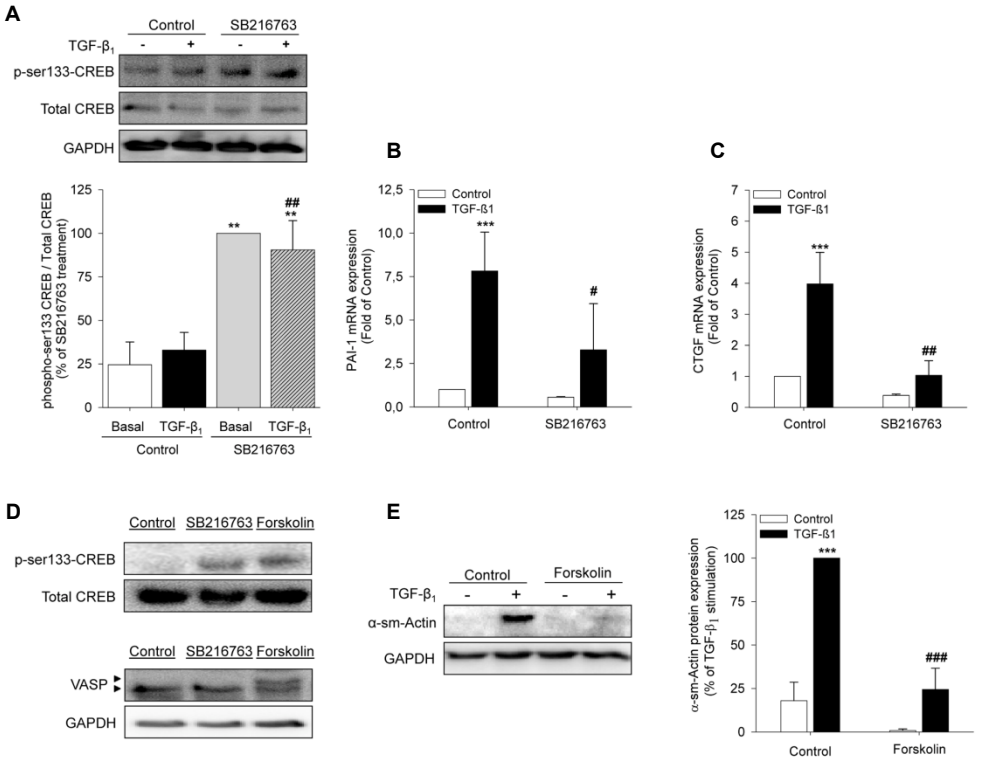


▲ Figure 5: Phosphorylation of R-smads and ERK-1/2 in response to TGF-β_1 is independent of GSK-3. (A) Contribution of smad and ERK1/2 signaling to myofibroblast differentiation**.** MRC5 human **l**ung fibroblasts were grown to confluence and stimulated for 48 hours with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the specific inhibitor of smad3 (SIS3; 3 µM) or the MEK1/2 inhibitor U0126 (3 µM). Expression of α-sm-actin was evaluated by immunoblotting. Equal protein loading was verified by the analysis of GAPDH. Representative immunoblots of 3 independent experiments are shown. (B-F) MRC5 human **l**ung fibroblasts were grown to confluence and stimulated for various time-points with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the selective GSK-3 inhibitors SB216763 (10 µM). (B) Time-dependent phosphorylation of R-smads in response to TGF-β_1 (2 ng.ml^{-1}). Expression of (C) phospho-ser465/467 smad2 (D) phospho-ser423/425 smad3. (E) Time-dependent phosphorylation of ERk1/2 in response to TGF-β_1 (2 ng.ml^{-1}). (F) Quantification of phospho-Thr202/Tyr204 ERK-1/2 expression evaluated by immunoblotting using specific antibodies. Responses were quantified by densitometry and normalized to the expression total smad2/3 and total ERK-1/2, respectively. Data represents mean ± s.e.m. of 3-6 independent experiments. $p<0.05$ for time-dependent activation of smad2, smad3 and ERK-1/2 in response to TGF-β_1 (determined by one-way ANOVA) and no significant effects of SB216763 on the TGF-β_1-induced activation of smad2, smad3 and ERK-1/2 (determined by two-way ANOVA).

GSK-3 inhibition promotes cAMP response element binding protein (CREB) activation in human lung fibroblasts

GSK-3 signaling has been demonstrated to have a modulatory effect on cyclic adenosine 3'5' monophosphate (cAMP) response element binding protein (CREB) signaling in various cell types (Tullai *et al.*, 2007; Grimes & Jope, 2001; Gotschel *et al.*, 2008). Phosphorylated CREB can act as a functional antagonist of smad signaling via competition for the common transcriptional co-activator CBP (CREB binding partner) (Schiller *et al.*, 2010; Ghosh *et al.*, 2000). Interestingly, we observed that inhibition of GSK-3 by SB216763 (10 μM, 1.5 hours) resulted in a significant increase in phosphorylated CREB (i.e. phosphorylated ser133-CREB) in MRC5 human lung fibroblasts, whereas the expression of total CREB was not affected by SB216763 (figure 6A). CREB phosphorylation was also increased by SB216763 in the presence of TGF-β_1, whereas stimulation with just TGF-β_1 (2 ng.ml^{-1}, 1 hour) did not result in increased ser133-CREB phosphorylation (figure 6A). In agreement with these findings, the expression of the canonical smad target gene PAI-1 and CTGF in response to TGF-β_1 was repressed by GSK-3 inhibition (figure 6B and 6C).

In view of these data, we further investigated the effect of CREB activation on myofibroblast differentiation. In order to activate CREB signaling in MRC5 human lung fibroblasts we used forskolin, an agent that elevates cellular cAMP levels by directly activating adenylyl cyclase. As expected, we found that stimulation with forskolin induced a strong phosphorylation of CREB on serine 133. Interestingly, the magnitude of this effect was similar to the phosphorylation induced by SB216763, which confirms that this effect of GSK-3 inhibition is robust (figure 6D). By using a vasodilator-stimulated phoshoprotein (VASP)-specific antibody that recognizes both phospho-VASP (upper band) and total VASP (lower band), we show that forskolin induced the ser157 phosphorylation of VASP, a protein kinase A (PKA) specific site (Smolenski *et al.*, 1998). SB216763 induced the phosphorylation of CREB, but did not affect VASP phosphorylation, indicating the involvement of a mechanism distinct from PKA (figure 6B). To reinforce that CREB signaling may be important in the differentiation process, we demonstrate that myofibroblast differentiation (i.e. α-sm-actin expression) was abrogated when MRC5 fibroblasts were pretreated with forskolin (figure 6E). These findings imply that CREB signaling is capable of producing a powerful inhibitory signal for myofibroblast differentiation, suggesting that phosphorylation of CREB by SB216763 is a plausible explanation for its anti-fibrotic effects.



▲***Figure 6: Activation of CREB due to GSK-3 inhibition attenuates myofibroblast differentiation.***(A) Phosphorylation of ser133-CREB in response to GSK-3 inhibition. Lung fibroblasts were grown to confluence and stimulated for 1 hour with TGF-β_1 (2 ng.ml^{-1}) in the presence or absence of the selective GSK-3 inhibitors SB216763 (10 µM). Of note, the pharmacological inhibitor was added 30 minutes prior to TGF-β_1. Expression of phospho-ser133-CREB was evaluated by immunoblotting using a specific antibody. Responses were quantified by densitometry and normalized to the expression total CREB. Data represents mean ± s.e.m. of 3-4 independent experiments. **p<0.01 compared to basal control, $^{###}$p<0.001 compared to just TGF-β1 stimulation; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test. (B-C) mRNA expression of canonical smad dependent genes (B) plasminogen activator inhibitor-1 (PAI-1) and (C) connective tissue growth factor (CTGF) in response to TGF-β (2 ng.ml^{-1}, 24 hours) in the presence or absence of SB216763 (10 µM) Data represent mean ± s.e.m. of 3-8 independent experiments. ***p<0.001 compared to basal control, $^{#}$p<0.05 and $^{##}$p<0.01 compared to TGF-β1 stimulation; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test. (D) Activation of CREB signaling by forskolin attenuates myofibroblast differentiation. *Top panel*: evaluation of CREB phosphorylation in response to SB216763 (10 µM, 1.5 h) or forskolin (2 µM, 1.5 h). *Lower panel*: evaluation of VASP phosphorylation in response to SB216763 (10 µM, 1.5 h) or forskolin (2 µM, 1.5 h). (E) Effect of forskolin on TGF-β_1-induced myofibroblast differentiation. Lung fibroblasts were stimulated for 48 h TGF-β_1 (2 ng.ml^{-1}) in the presence or absence forskolin (2 µM). Expression of α-sm-actin was evaluated by immunoblotting using specific antibodies. Responses on α-sm-actin were quantified by densitometry and normalized to the expression GAPDH. Data represents mean ± s.e.m. of 3 independent experiments. ***p<0.001 compared to basal control, $^{###}$p<0.001 compared to just TGF-β1 stimulation; determined by a one-way ANOVA followed by a Newman-Keuls multiple comparison test.

Discussion

We determined the contribution of GSK-3 signaling to myofibroblast differentiation and showed that pulmonary fibroblasts stimulated with TGF-β_1 time-dependently differentiate into myofibroblasts, as characterized by increased expression of α-sm-actin, fibronectin and MMP-2. Silencing of GSK-3 by siRNA or pharmacological inhibition of the kinase completely prevented the TGF-β_1-induced expression of these myofibroblasts markers at both the protein and mRNA level, implying that GSK-3 signaling is critically involved in the regulation of myofibroblast transdifferentiation induced by this growth factor. This inhibitory effect of GSK-3 inhibition was also present in primary human lung fibroblasts. Activation of NF-κB was not required for myofibroblast differentiation and GSK-3 was not involved in the phosphorylation of smads or ERK-1/2 by TGF-β_1. Rather, inhibition of GSK-3 resulted in increased levels of phosphorylated ser133-CREB, which acts as a functional antagonist of smad signaling and thereby prevents myofibroblast differentiation. Collectively these data indicate the GSK-3 regulates myofibroblast differentiation presumably by repressing CREB signaling.

Activation of fibroblasts is an important pathophysiological mechanism in chronic pulmonary diseases like idiopathic pulmonary fibrosis (IPF), characterized by interstitial (parenchymal) fibrosis, as well as in asthma and COPD where a variable degree of airway wall fibrosis is present and in COPD additionally insufficient parenchymal tissue repair, all contributing to disease development (Coward *et al.*, 2010; Hogg *et al.*, 2004). The airway obstruction observed in COPD is partially due to fibrosis of the central and peripheral airways and, in line with this, an association between the presence of myofibroblast-like cells and airway obstruction was recently demonstrated in airway wall biopsies from COPD patients (Hogg *et al.*, 2004; Lofdahl *et al.*, 2011). In contrast, in other areas of the lung COPD patient often develop emphysema, in which the fibroblast function may be inadequate or insufficient to restore tissue damage (Noordhoek *et al.*, 2003; Zandvoort *et al.*, 2008; Togo *et al.*, 2008; Rennard *et al.*, 2006). We here show that the differentiation process of lung fibroblasts is dependent on GSK-3 in MRC5 fibroblasts and also in primary peripheral lung fibroblasts of COPD patients and controls. This suggests that GSK-3 mediated ECM production is an important regulatory mechanism in peripheral lung fibroblasts that is still operative in COPD. Extrapolation of our findings in peripheral lung fibroblasts as a general applicable mechanism in pulmonary fibroblast function should be done with caution, however, since fibroblasts populations in the airways and parenchyma are phenotypically and functionally distinct (Zhou *et al.*, 2011; Hallgren *et al.*, 2010; Kotaru *et al.*, 2006; Noordhoek *et al.*, 2005; Noordhoek *et al.*, 2003).

TGF-β_1 is a multifunctional cytokine that regulates various cellular functions by acting on several signaling pathways (Derynck & Zhang, 2003; de Boer *et al.*, 1998). Stimulation of pulmonary fibroblasts with TGF-β_1 resulted in a modest decrease of the NF-κB-inhibitory protein Iκ-Bα, which may indicate that NF-κB signaling is activated to some extent. Inhibition of NF-κB signaling by two distinct IKK inhibitors (i.e. PS-1145 and SC-514) attenuated cytokine (e.g. IL-8) secretion induced by the growth factor in pulmonary fibroblasts. However, the IKK-inhibitors did not at all affect TGF-β_1-induced α-sm-actin expression, indicating that TGF-β_1 activated NF-κB signaling is not required for myofibroblast differentiation. Likewise, the phosphorylation of ERK-1/2 in response to TGF-β_1 was not affected by SB216763, showing that GSK-3 does not affect ERK-1/2 activation in pulmonary fibroblasts, despite reported findings that indicate modulation of ERK-1/2 signaling by GSK-3 (Wang *et al.*, 2006; Takada *et al.*, 2004). Smad phosphorylation is critical in canonical TGF-β_1 signaling and accordingly we demonstrate that smad2 and smad3 are phosphorylated at specific serine residues in the C-terminus (i.e. ser465/467 of smad2 and ser423/425 of smad3) by TGF-β_1. The C-terminus phosphorylation of R-smads is required for the interaction and recruitment of the two closely related co-activators p300 and CBP (Schiller *et al.*, 2004; Derynck *et al.*, 1998; Massague, 2000). Inhibition of GSK-3 did not affect the phosphorylation of R-smads, which indicates that GSK-3 does not directly intervene with smad activation. The significance of CBP/p300 as essential co-activators for smad driven gene-expression has comprehensively been studied and miscellaneous other transcription factors rely on their interaction with CBP/p300 resulting in signal-induced cooperative activation or repression of gene transcription (Janknecht *et al.*, 1998; Pouponnot *et al.*, 1998; Goodman & Smolik, 2000; Das *et al.*, 2008). CBP and p300 were initially identified as association partners for the transcription factors CREB and the oncoprotein E1A and activation of these transcription factors and subsequent association with CBP/p300 has been demonstrated to attenuate TGF-β_1-induced cellular responses in fibroblasts (Goodman & Smolik, 2000; Ghosh *et al.*, 2000; Schiller *et al.*, 2010). Phosphorylation of CREB on serine 133 results in increased activity as this phosphorylation is required for recruitment of the transcriptional co-activators (Goodman & Smolik, 2000). Several studies in various cell types have shown that GSK-3 regulates the phosphorylation and transcriptional activity of CREB, and although there is consensus on the regulation of CREB by GSK-3 the functional consequences of this modification are not yet fully established (Johannessen & Moens, 2007; Fiol *et al.*, 1994; Liang *et al.*, 2008; Tullai *et al.*, 2007; Martin *et al.*, 2005; Grimes & Jope, 2001). We show that inhibition of GSK-3 in pulmonary fibroblasts results in increased phosphorylation of ser133-CREB to a similar extent as observed with the cAMP-elevating agent forskolin, which also attenuated TGF-β_1-induced myofibroblast differentiation. Although this supports an inhibitory role for CREB in myofibroblast differentiation,

alternative mechanisms including inhibition of Rho, likely play additional roles in the forskolin effects (Akhmetshina *et al.*, 2008; Vardouli *et al.*, 2005). Forskolin induced the phosphorylation of VASP, indicating that PKA is activated. PKA is one of the main upstream activators of CREB as the kinase induces ser133-CREB phosphorylation (Johannessen *et al.*, 2004; Johannessen & Moens, 2007). Remarkably, SB216763 induced the ser133 phosphorylation of CREB, but did not alter the phosphorylation status of VASP. This indicates that PKA is not involved and suggests an alternative mechanism by which CREB is activated in response to GSK-3 inhibition. Possible alternative mechanisms could be that active GSK-3 suppresses another CREB kinase which is distinct from PKA, or that GSK-3 signaling is required for the activation of phosphatases which are involved in the dephosphorylation of CREB (Johannessen *et al.*, 2004; Johannessen & Moens, 2007). In human dermal fibroblasts activation of CREB signaling also attenuated smad-dependent signaling and expression of type 1 collagen and plasminogen activator inhibitor 1 (PAI-1) (Schiller *et al.*, 2010; Ghosh *et al.*, 2000). Further, SB216763 reduced the expression of the canonical smad target genes CTGF and PAI-1 in response to TGF-β_1. Collectively, this indicates that CREB signaling is capable of functionally antagonizing fibrotic responses, by reducing the transcriptional complexes of activated smads with CBP/p300. We show that inhibition of GSK-3 in pulmonary fibroblasts results in increased phosphorylation of ser133-CREB, which attenuates TGF-β_1-induced myofibroblast differentiation.

GSK-3 signaling contributes to many cellular responses by regulating a variety of transcription factors and transcriptional co-activators (Doble & Woodgett, 2003; Jope & Johnson, 2004). We previously demonstrated that β-catenin, one of the transcriptional co-activators regulated by GSK-3, is activated by TGF-β_1 via GSK-3 phosphorylation and contributes significantly to myofibroblast differentiation (Baarsma *et al.*, 2011b). Active GSK-3 prevents β-catenin signaling by phosphorylating cytosolic β-catenin thereby targeting it for proteosomal degradation (Doble & Woodgett, 2003; Jope & Johnson, 2004). Consequently, pharmacological inhibition of GSK-3 would be expected to result in increased β-catenin signaling and enhanced myofibroblast differentiation in response to TGF-β_1. This clearly does not occur in pulmonary fibroblasts, however, as several small molecule inhibitors of GSK-3 or silencing of the kinase by siRNA strongly attenuated myofibroblast differentiation. Compartmentalization of GSK-3 likely explains these differential functional effects. Only a fraction of total cellular GSK-3, bound in a cytosolic destruction complex with axin, casein kinase and APC regulates β-catenin levels, whereas other cellular pools of GSK-3 have additional, and even opposite functions, for example by regulating transcription factors such as NF-κB and CREB (Doble & Woodgett, 2003; Jope & Johnson, 2004; Voskas *et al.*, 2010). Compartmentalization of GSK-3 resulting in differential downstream

signaling has been described for insulin-induced compared to WNT-induced GSK-3 inhibition (Ding *et al.*, 2000). Pharmacological inhibitors affect all pools of GSK-3 within the cell and eliminate any selective signaling gained by the compartmentalization of the kinase. The resultant effects of GSK-3 inhibition are clearly inhibitory to myofibroblast activation, as the results of our study demonstrate.

Pharmacological inhibition of GSK-3 *in vivo* prevents inflammatory and fibrotic responses in the lung and it was suggested that these effects GSK-3 inhibition were due to suppression of NF-κB activation and subsequent inhibition of the recruitment and activation of inflammatory cells (Bao *et al.*, 2007; Cuzzocrea *et al.*, 2007; Cuzzocrea *et al.*, 2006). However, a recent study suggested that pharmacological inhibition of GSK-3 after the initial inflammation induced by bleomycin could also prevent the fibrotic process, suggesting that GSK-3 signaling may regulate the onset of fibrosis in the lung by directly acting on the deposition of extracellular matrix (ECM) proteins (Gurrieri *et al.*, 2010). Our previous and current findings suggest that GSK-3 inhibition can have direct anti-inflammatory and anti-fibrotic effects that require distinct (i.e. NF-κB and CREB, respectively) intracellular signaling pathways (Baarsma *et al.*, 2011a). This warrants further studies on the effectiveness of GSK-3 inhibitors in the inhibition of fibrotic responses, for example using animal models.

Collectively, we demonstrate in the current study that various responses of pulmonary fibroblast functioning that include ECM production are attenuated by GSK-3 inhibition via activation of CREB-dependent signaling. These data imply that GSK-3 inhibition may be beneficial in chronic lung diseases due to the inhibitory effects on inflammation and ECM production, which are regulated via distinct signaling pathways.

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References

1. Akhmetshina, A, Dees, C, Pileckyte, M, Szucs, G, Spriewald, BM, Zwerina, J *et al*. (2008). Rho-associated kinases are crucial for myofibroblast differentiation and production of extracellular matrix in scleroderma fibroblasts. *Arthritis Rheum* 58: 2553-2564.
2. Arsura, M, Panta, GR, Bilyeu, JD, Cavin, LG, Sovak, MA, Oliver, AA *et al*. (2003). Transient activation of NF-kappaB through a TAK1/IKK kinase pathway by TGF-beta1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. *Oncogene* 22: 412-425.
3. Baarsma, HA, Meurs, H, Halayko, AJ, Menzen, MH, Schmidt, M, Kerstjens, HA *et al*. (2011a). Glycogen synthase kinase-3 regulates cigarette smoke extract- and IL-1{beta}-induced cytokine secretion by airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 300: L910-L919.
4. Baarsma, HA, Spanjer, AI, Haitsma, G, Engelbertink, LH, Meurs, H, Jonker, MR *et al*. (2011b). Activation of WNT / beta-Catenin Signaling in Pulmonary Fibroblasts by TGF-beta(1) Is Increased in Chronic Obstructive Pulmonary Disease. *PLoS One* 6: e25450.
5. Bain, J, Plater, L, Elliott, M, Shpiro, N, Hastie, CJ, McLauchlan, H *et al*. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408: 297-315.
6. Bao, Z, Lim, S, Liao, W, Lin, Y, Thiemermann, C, Leung, BP *et al*. (2007). Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. *Am J Respir Crit Care Med* 176: 431-438.
7. Bartram, U & Speer, CP. (2004). The role of transforming growth factor beta in lung development and disease. *Chest* 125: 754-765.
8. Chung, KF. (2001). Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 34: 50s-59s.
9. Coward, WR, Saini, G & Jenkins, G. (2010). The pathogenesis of idiopathic pulmonary fibrosis. *Ther Adv Respir Dis* 4: 367-388.
10. Cuzzocrea, S, Crisafulli, C, Mazzon, E, Esposito, E, Muia, C, Abdelrahman, M *et al*. (2006). Inhibition of glycogen synthase kinase-3beta attenuates the development of carrageenan-induced lung injury in mice. *Br J Pharmacol* 149: 687-702.
11. Cuzzocrea, S, Genovese, T, Mazzon, E, Esposito, E, Muia, C, Abdelrahman, M *et al*. (2007). Glycogen synthase kinase-3beta inhibition attenuates the development of bleomycin-induced lung injury. *Int J Immunopathol Pharmacol* 20: 619-630.
12. Das, F, Ghosh-Choudhury, N, Venkatesan, B, Li, X, Mahimainathan, L & Choudhury, GG. (2008). Akt kinase targets association of CBP with SMAD 3 to regulate TGFbeta-induced expression of plasminogen activator inhibitor-1. *J Cell Physiol* 214: 513-527.
13. de Boer, WI, van, SA, Sont, JK, Sharma, HS, Stolk, J, Hiemstra, PS *et al*. (1998). Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158: 1951-1957.
14. Derynck, R, Zhang, Y & Feng, XH. (1998). Smads: transcriptional activators of TGF-beta responses. *Cell* 95: 737-740.
15. Derynck, R & Zhang, YE. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* 425: 577-584.
16. Ding, VW, Chen, RH & McCormick, F. (2000). Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J Biol Chem* 275: 32475-32481.
17. Doble, BW & Woodgett, JR. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116: 1175-1186.
18. Dugo, L, Collin, M & Thiemermann, C. (2007). Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. *Shock* 27: 113-123.
19. Embi, N, Rylatt, DB & Cohen, P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem* 107: 519-527.
20. Fiol, CJ, Williams, JS, Chou, CH, Wang, QM, Roach, PJ & Andrisani, OM. (1994). A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J Biol Chem* 269: 32187-32193.
21. Frame, S & Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 359: 1-16.

22. Ghosh, AK, Yuan, W, Mori, Y & Varga, J. (2000). Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators. *Oncogene* 19: 3546-3555.
23. Gingery, A, Bradley, EW, Pederson, L, Ruan, M, Horwood, NJ & Oursler, MJ. (2008). TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. *Exp Cell Res* 314: 2725-2738.
24. Goodman, RH & Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. *Genes Dev* 14: 1553-1577.
25. Gosens, R, Dueck, G, Rector, E, Nunes, RO, Gerthoffer, WT, Unruh, H *et al*. (2007). Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 293: L1348-L1358.
26. Gotschel, F, Kern, C, Lang, S, Sparna, T, Markmann, C, Schwager, J *et al*. (2008). Inhibition of GSK3 differentially modulates NF-kappaB, CREB, AP-1 and beta-catenin signaling in hepatocytes, but fails to promote TNF-alpha-induced apoptosis. *Exp Cell Res* 314: 1351-1366.
27. Grimes, CA & Jope, RS. (2001). CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. *J Neurochem* 78: 1219-1232.
28. Guo, X, Ramirez, A, Waddell, DS, Li, Z, Liu, X & Wang, XF. (2008). Axin and GSK-3{beta} control Smad3 protein stability and modulate TGF-{beta} signaling. *Genes Dev* 22: 106-120.
29. Gurrieri, C, Piazza, F, Gnoato, M, Montini, B, Biasutto, L, Gattazzo, C *et al*. (2010). 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), a glycogen synthase kinase-3 inhibitor, displays therapeutic properties in a mouse model of pulmonary inflammation and fibrosis. *J Pharmacol Exp Ther* 332: 785-794.
30. Hallgren, O, Nihlberg, K, Dahlback, M, Bjermer, L, Eriksson, LT, Erjefalt, JS *et al*. (2010). Altered fibroblast proteoglycan production in COPD. *Respir Res* 11: 55.
31. Hoeflich, KP, Luo, J, Rubie, EA, Tsao, MS, Jin, O & Woodgett, JR. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406: 86-90.
32. Hogg, JC, Chu, F, Utokaparch, S, Woods, R, Elliott, WM, Buzatu, L *et al*. (2004). The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 350: 2645-2653.
33. Janknecht, R, Wells, NJ & Hunter, T. (1998). TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev* 12: 2114-2119.
34. Jinnin, M, Ihn, H & Tamaki, K. (2006). Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression. *Mol Pharmacol* 69: 597-607.
35. Johannessen, M, Delghandi, MP & Moens, U. (2004). What turns CREB on? *Cell Signal* 16: 1211-1227.
36. Johannessen, M & Moens, U. (2007). Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases. *Front Biosci* 12: 1814-1832.
37. Jope, RS & Johnson, GV. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29: 95-102.
38. Kotaru, C, Schoonover, KJ, Trudeau, JB, Huynh, ML, Zhou, X, Hu, H *et al*. (2006). Regional fibroblast heterogeneity in the lung: implications for remodeling. *Am J Respir Crit Care Med* 173: 1208-1215.
39. Kranenburg, AR, Willems-Widyastuti, A, Moori, WJ, Sterk, PJ, Alagappan, VK, de Boer, WI *et al*. (2006). Enhanced bronchial expression of extracellular matrix proteins in chronic obstructive pulmonary disease. *Am J Clin Pathol* 126: 725-735.
40. Liang, MH & Chuang, DM. (2006). Differential roles of glycogen synthase kinase-3 isoforms in the regulation of transcriptional activation. *J Biol Chem* 281: 30479-30484.
41. Liang, MH, Wendland, JR & Chuang, DM. (2008). Lithium inhibits Smad3/4 transactivation via increased CREB activity induced by enhanced PKA and AKT signaling. *Mol Cell Neurosci* 37: 440-453.
42. Lofdahl, M, Kaarteenaho, R, Lappi-Blanco, E, Tornling, G & Skold, MC. (2011). Tenascin-C and alpha-smooth muscle actin positive cells are increased in the large airways in patients with COPD. *Respir Res* 12: 48.
43. Martin, M, Rehani, K, Jope, RS & Michalek, SM. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol* 6: 777-784.

44. Massague, J. (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1: 169-178.
45. McAnulty, RJ. (2007). Fibroblasts and myofibroblasts: their source, function and role in disease. *Int J Biochem Cell Biol* 39: 666-671.
46. Millet, C, Yamashita, M, Heller, M, Yu, LR, Veenstra, TD & Zhang, YE. (2009). A negative feedback control of transforming growth factor-beta signaling by glycogen synthase kinase 3-mediated Smad3 linker phosphorylation at Ser-204. *J Biol Chem* 284: 19808-19816.
47. Morty, RE, Konigshoff, M & Eickelberg, O. (2009). Transforming growth factor-beta signaling across ages: from distorted lung development to chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 6: 607-613.
48. Noordhoek, JA, Postma, DS, Chong, LL, Menkema, L, Kauffman, HF, Timens, W *et al*. (2005). Different modulation of decorin production by lung fibroblasts from patients with mild and severe emphysema. *COPD* 2: 17-25.
49. Noordhoek, JA, Postma, DS, Chong, LL, Vos, JT, Kauffman, HF, Timens, W *et al*. (2003). Different proliferative capacity of lung fibroblasts obtained from control subjects and patients with emphysema. *Exp Lung Res* 29: 291-302.
50. Piek, E, Ju, WJ, Heyer, J, Escalante-Alcalde, D, Stewart, CL, Weinstein, M *et al*. (2001). Functional characterization of transforming growth factor beta signaling in Smad2- and Smad3-deficient fibroblasts. *J Biol Chem* 276: 19945-19953.
51. Pouponnot, C, Jayaraman, L & Massague, J. (1998). Physical and functional interaction of SMADs and p300/CBP. *J Biol Chem* 273: 22865-22868.
52. Rabe, KF, Hurd, S, Anzueto, A, Barnes, PJ, Buist, SA, Calverley, P *et al*. (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 176: 532-555.
53. Ramirez, AM, Shen, Z, Ritzenthaler, JD & Roman, J. (2006). Myofibroblast transdifferentiation in obliterative bronchiolitis: tgf-beta signaling through smad3-dependent and -independent pathways. *Am J Transplant* 6: 2080-2088.
54. Rennard, SI, Togo, S & Holz, O. (2006). Cigarette smoke inhibits alveolar repair: a mechanism for the development of emphysema. *Proc Am Thorac Soc* 3: 703-708.
55. Schiller, M, Dennler, S, Anderegg, U, Kokot, A, Simon, JC, Luger, TA *et al*. (2010). Increased cAMP levels modulate transforming growth factor-beta/Smad-induced expression of extracellular matrix components and other key fibroblast effector functions. *J Biol Chem* 285: 409-421.
56. Schiller, M, Javelaud, D & Mauviel, A. (2004). TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 35: 83-92.
57. Scotton, CJ & Chambers, RC. (2007). Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Chest* 132: 1311-1321.
58. Sebe, A, Leivonen, SK, Fintha, A, Masszi, A, Rosivall, L, Kahari, VM *et al*. (2008). Transforming growth factor-beta-induced alpha-smooth muscle cell actin expression in renal proximal tubular cells is regulated by p38beta mitogen-activated protein kinase, extracellular signal-regulated protein kinase1,2 and the Smad signalling during epithelial-myofibroblast transdifferentiation. *Nephrol Dial Transplant* 23: 1537-1545.
59. Smolenski, A, Bachmann, C, Reinhard, K, Honig-Liedl, P, Jarchau, T, Hoschuetzky, H *et al*. (1998). Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. *J Biol Chem* 273: 20029-20035.
60. Takada, Y, Fang, X, Jamaluddin, MS, Boyd, DD & Aggarwal, BB. (2004). Genetic deletion of glycogen synthase kinase-3beta abrogates activation of IkappaBalpha kinase, JNK, Akt, and p44/p42 MAPK but potentiates apoptosis induced by tumor necrosis factor. *J Biol Chem* 279: 39541-39554.
61. Togo, S, Holz, O, Liu, X, Sugiura, H, Kamio, K, Wang, X *et al*. (2008). Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. *Am J Respir Crit Care Med* 178: 248-260.
62. Tullai, JW, Chen, J, Schaffer, ME, Kamenetsky, E, Kasif, S & Cooper, GM. (2007). Glycogen synthase kinase-3 represses cyclic AMP response element-binding protein (CREB)-targeted immediate early genes in quiescent cells. *J Biol Chem* 282: 9482-9491.

63. Vandesompele, J, De, PK, Pattyn, F, Poppe, B, Van, RN, De, PA *et al*. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034.
64. Vardouli, L, Moustakas, A & Stournaras, C. (2005). LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. *J Biol Chem* 280: 11448-11457.
65. Voskas, D, Ling, LS & Woodgett, JR. (2010). Does GSK-3 provide a shortcut for PI3K activation of Wnt signalling? *F1000 Biol Rep* 2: 82.
66. Wang, Q, Zhou, Y, Wang, X & Evers, BM. (2006). Glycogen synthase kinase-3 is a negative regulator of extracellular signal-regulated kinase. *Oncogene* 25: 43-50.
67. Zandvoort, A, Postma, DS, Jonker, MR, Noordhoek, JA, Vos, JT & Timens, W. (2008). Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. *Respir Res* 9: 83.
68. Zhou, X, Wu, W, Hu, H, Milosevic, J, Konishi, K, Kaminski, N *et al*. (2011). Genomic Differences Distinguish the Myofibroblast Phenotype of Distal Lung from Airway Fibroblasts. *Am J Respir Cell Mol Biol*.

Chapter 5

Glycogen synthase kinase-3 (GSK-3) regulates cigarette smoke extract- and IL-1β-induced cytokine secretion by airway smooth muscle

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Abstract

Glycogen synthase kinase-3 (GSK-3) is a constitutively active kinase that regulates multiple signaling proteins and transcription factors involved in inflammation. Its role in inflammatory lung diseases, including chronic obstructive pulmonary disease (COPD), is largely unknown. We investigated the role of GSK-3 in the secretion of chemokines and growth factors by human airway smooth muscle cells after exposure to cigarette smoke extract (CSE) or interleukin-1β (IL-1β), important factors involved in the development of COPD. Cultured human airway smooth muscle cells were exposed to increasing concentrations of CSE (1-15%) and IL-1β (0.01-1 ng/mL), which induced the secretion of VEGF-A and IL-8, whereas eotaxin secretion was induced by IL-1β only. Inhibition of GSK-3 by the selective inhibitor SB216763 or CHIR/CT99021 attenuated the cytokine and growth factor release induced by CSE and/or IL-1β, without affecting their basal release. Secretion of the cytokines by airway smooth muscle partially depends on NF-κB signaling and GSK-3 has been implicated in regulating multiple steps in activating the NF-κB signalling pathway. IL-1β treatment induced degradation of the NF-κB inhibitory protein IκB-α followed by nuclear translocation and DNA binding of p65 NF-κB, which were unaffected by inhibition of GSK-3. However, induction of NF-κB-dependent transcriptional activity by IL-1β and CSE was largely reduced upon GSK-3 inhibition by SB216763. Collectively, we demonstrate that CSE and IL-1β activate airway smooth muscle cells to secrete the pro-inflammatory cytokines IL-8, eotaxin and VEGF-A. Furthermore, we show that GSK-3 regulates the release of these cytokines induced by CSE and IL-1β by promoting NF-κB dependent gene transcription.

Keywords: VEGF-A; IL-8; Eotaxin; SB216763; NF-κB

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation, which is usually associated with an abnormal inflammatory response of the lungs to noxious particles or gases. Cigarette smoke is considered the major risk factor for the development of COPD (44). In addition to its role in bronchoconstriction, the airway smooth muscle, an important structural cell type in the airway wall, is considered to contribute to the inflammatory and remodelling processes observed in the pathogenesis of COPD (8).

The airway smooth muscle has a synthetic function by producing proteases, growth factors, chemokines and pro-inflammatory cytokines, including interleukin (IL)-6, IL-8, eotaxin, matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) (7; 8; 27; 33). Several pro-inflammatory cytokines, such as TNF-α and IL-1β, activate the synthetic capacity of airway smooth muscle (9). In addition, it has recently been demonstrated that cigarette smoke induces the secretion of a variety of different cytokines, chemokines and growth factors, which can be modulated by other pro-inflammatory mediators (3; 5; 40). For example, airway smooth muscle cells secrete IL-8 in response to cigarette smoke extract (CSE) exposure, which is strongly augmented by TNF-α or the muscarinic receptor agonist methacholine (21; 40). On the other hand, CSE inhibits TNF-α-induced eotaxin and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) release from airway smooth muscle (40). These cytokines, growth factors and angiogenic factors, which play an important role in the pathogenesis of COPD, depend on the nuclear factor (NF)-κB family of transcription factors for their synthesis and release from airway smooth muscle (19).

Accumulating evidence indicates a role for glycogen synthase kinase-3 (GSK-3) in regulating inflammatory responses (18). GSK-3 is an ubiquitously expressed constitutively active serine/threonine kinase that regulates multiple signalling pathways and thereby controlling a broad spectrum of cellular responses, including metabolism, gene transcription, protein translation, cell cycle regulation and apoptosis (17; 23). A pool of intracellular GSK-3 is part of a cytosolic multiprotein complex, further consisting of axin, adenomatous polyposis coli (APC) and casein kinase-1 (CK-1), that regulates intracellular stability of β-catenin (17; 25), an essential component of adherens junctions that can serve a role in activating gene transcription, when translocated to the nucleus (10). β-Catenin dependent, TCF/LEF-mediated gene transcription has been associated with the production of pro-inflammatory cytokines, enzymes and growth factors (e.g. IL-8, cyclooxygenase-2 and VEGF-A) (11; 28; 34; 36; 56). Moreover, GSK-3 signalling has, independently of β-catenin, direct effects on NF-κB signalling. Thus, Hoeflich

et.al (26) have shown that GSK-3 is required for the NF-κB-mediated survival response after TNF-α stimulation. The NF-κB pathway also plays a critical role in the regulation of inflammation by activating transcription of pro-inflammatory genes. Under basal conditions, the NF-κB heterodimer p65/p50 is predominantly bound to the NF-κB inhibitory protein IκB-α. Upon a variety of stimuli, IκB-α is phosphorylated by protein kinases (e.g. IKK) and targeted for intracellular breakdown. This allows the p65/p50 NF-κB subunits to translocate to the nucleus, where they are phosphorylated, interact with DNA and subsequently activate gene transcription (24). Several studies have proposed an essential role, particularly in inflammation, for GSK-3 in regulating multiple steps in the activation of NF-κB signalling, including degradation of IκB-α, p65 nuclear translocation, p65 phosphorylation and DNA binding (2; 15; 18; 30; 35; 41).

We previously demonstrated the importance of GSK-3/β-catenin signalling in airway smooth muscle proliferation (20; 38). Furthermore, GSK-3 appears to be an important regulator of airway smooth muscle hyperplasia and hypertrophy in a murine model of asthma (4; 14), however its role in cytokine and growth factor secretion is still unknown. In the present study, we investigated the contribution of GSK-3 to the secretion of the pro-inflammatory, chemoattractant cytokines eotaxin and IL-8 and the angiogenic growth factor VEGF-A induced by CSE and IL-1β from airway smooth muscle and demonstrate that the GSK-3-dependent modulation of their secretion relies on NF-κB signalling.

Methods

Cell culture

Human bronchial smooth muscle cell lines, immortalized by stable expression of human telomerase reverse transcriptase (hTERT), were used for all experiments. The primary cultured human bronchial smooth muscle cells used to generate each cell line were prepared, as we have previously described (22), from macroscopically healthy segments of 2nd-to-4th generation main bronchus obtained after lung resection surgery from patients with a diagnosis of adenocarcinoma (Dr. H Unruh, Section of Thoracic Surgery, University of Manitoba, Canada). All procedures were approved by the Human Research Ethics Board of the University of Manitoba.

As previously described in detail (22), each cell line was thoroughly characterized to passage 10 and higher, and was shown to express a number of smooth muscle (sm) contractile phenotype marker proteins (e.g. sm-myosin heavy chain (sm-MHC), sm-α-actin, and desmin). For all experiments, myocytes were grown on uncoated plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (50 U/mL streptomycin, 50 µg/mL penicillin, 1.5 µg/mL amphotericin B) and 10 % (v/v) foetal bovine serum (FBS).

Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by a standardized method by combusting two 3R4F research cigarettes (University of Kentucky, Lexington, USA); (filter removed) using a peristaltic pump (Watson Marlow, Falmouth Cornwall, England) and passing the smoke through 25 ml of FBS-free DMEM, supplemented with antibiotics, at a rate of one cigarette per 5 minutes. The obtained solution was designated 100% CSE and diluted to working concentration in DMEM supplemented with antibiotics. For all experiments, CSE was freshly prepared and used within 15 minutes after preparation.

Cytokine enzyme-linked immunosorbent assays

Cells were grown to confluence and serum-starved for 1 day in DMEM supplemented with antibiotics (50 U/mL streptomycin, 50 µg/mL penicillin, 1.5 µg/mL amphotericin B) and ITS (5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenium) before each experiment. Cells were washed twice with warm (37°C) phosphate buffered saline (PBS) (composition: 140.0 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) and subsequently subjected for 24 hours to different concentrations of CSE (0-15%) or IL-1β (0.01-1 ng/mL) in DMEM supplemented with antibiotics. When applied, pharmacological inhibitors (i.e. SB216763 or CHIR/CT99021) were added 30 minutes before the addition of CSE

or IL-1β. Cell supernatants were harvested 24 hours after stimulation and stored at -20 °C until assayed for vascular endothelial growth factor (VEGF-A; $VEGF_{121}$ and $VEGF_{165}$), eotaxin (CCL11) or interleukin-8 (IL-8; CXCL8). Cytokine levels were determined by specific enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (VEGF-A and eotaxin kit R&D systems Europe, Abingdon, UK and IL-8 kit Sanquin, Amsterdam, The Netherlands)

Preparation of cell lysates

Cells were grown to confluence and serum-starved for 1 day in DMEM supplemented with antibiotics and ITS before each experiment. Cells were then subjected to different treatments in DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor SB216763 (10 μM) was added 30 minutes before the addition of CSE or IL-1β. To obtain whole cell lysates, cells were washed once with ice-cold PBS (4°C) and then lysed in ice-cold radio immunoprecipation assay (RIPA) buffer (composition: 40 mM Tris, 150 mM NaCl, 1 % v/v Igepal CA-630, 1 % w/v deoxycholic acid, 1 mM NaF, 1 mM Na_3VO_4, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 7 μg/mL pepstatin A, pH 7.4). Subsequently, lysates were sonicated and protein concentration was determined according to Bradford. Lysates were stored at -20 ºC until further use.

Isolation of membrane- and nuclei-enriched fractions

Cells were grown to confluence on uncoated 100 mm dishes and serum-starved for 1 day in DMEM supplemented with antibiotics and ITS before each experiment. Cells were then subjected to treatments with CSE or IL-1β in DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor SB216763 (10 μM) was added 30 minutes before the addition of CSE or IL-1β. Cells were then washed with ice-cold PBS (4°C), and lysed for 10 minutes on ice in homogenization buffer (50 mM Tris (pH 7.4), supplemented with 1 mM Na_3VO_4, 1 mM NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 7 μg/mL pepstatin A). After 20 strokes in a Potter homogenizer, the homogenate was centrifuged for 5 minutes at 500 x *g*. The resulting nuclei-enriched pellet was resuspended in homogenization buffer supplemented with 1 % v/v Triton X-100 to solubilize the nuclei and to remove the insoluble cytoskeletal fraction by centrifugation (5 min at 16,100 x *g*). The supernatant obtained in the first centrifugation step was transferred to a new tube and centrifuged for 30 min at 16,100 x *g*. The membrane pellet was resuspended in 200 μl RIPA buffer, sonicated and protein concentration was determined according to Bradford. Samples were then stored at -20 ºC.

Western blot analysis
Equal amounts of protein (10-20 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. Bands were subsequently visualized on film using enhanced chemiluminescence reagents. Band intensities were quantified by densitometry using Totallab^{TM} software (Nonlinear dynamics; Newcastle, UK).

p65 NF-κB DNA binding capacity
Airway smooth muscle cells were grown to confluence on uncoated 100 mm dishes and serum-starved for 1 day in DMEM supplemented with antibiotics and ITS before each experiment. Cells were then subjected to treatments with IL-1β (1 ng/ml; 1 hr) in DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor SB216763 (10 µM) was added 30 minutes before the addition of IL-1β. Nuclear extracts were prepared with a nuclear extract kit (Active motif, Rixensart, Belgium) according to the manufacturers' instructions. The p65 NF-κB DNA binding capacity in 10 µg nuclear extract was assayed with the p65 NF-κB TransAM kit (Active motif, Rixensart, Belgium) and subsequently absorbance was measured at 450 nM.

NF-κB reporter gene assay
NF-κB transcriptional activity in airway smooth muscle cells was measured using the RapidReporter® pRR-High-NF-κB Gaussia luciferase reporter system (Active Motif, Rixensart, Belgium). The NF-κB plasmid vector contains the NF-κB tandem response element upstream of the Gaussia luciferase gene, while the control plasmid contains a non-inducible EF1α promoter driven positive control vector. Cells were grown on uncoated 100 mm dishes to ~80-90% confluence and washed twice with warm PBS (37°C). The cells were transfected in DMEM without serum and antibiotics for 6 hours using a mixture of Lipofectamine 2000 with either of pRR-High NF-κB-vector or pRR-high EF1α vector according to the manufacturers' instructions. After 6 hours, this DMEM medium was replaced by DMEM supplemented with antibiotics and 10 % (v/v) FBS. The next day, cells were replated in a 96-wells plate at a density of 30.000 cells/well and were grown for 24 hours in DMEM supplemented with antibiotics and 10 % (v/v) FBS. Next, the cells were serum-starved for 1 day in DMEM supplemented with antibiotics and ITS for another 24 hours. Cells were then subjected to treatments with CSE (15%) or IL-1β (1 ng/mL) in DMEM supplemented with antibiotics. When applied, the pharmacological inhibitor SB216763 (10µM) was added 30 minutes before the addition of CSE or IL-1β. After 1-4 hours of stimulation, cells were lysed using the lysis buffer supplemented with the Rapid Reporter Assay kit (Active Motif,

Rixensart, Belgium). Luciferase activity in lysates was measured using a luminometer (Wallac Victor^2 1420 multilabel counter)

Antibodies and reagents

Mouse monoclonal anti-β-actin antibody, HRP-conjugated rabbit anti-mouse antibody, interleukin-1β and HRP-conjugated rabbit anti-goat antibody were obtained from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal anti-lamin A/C antibody, mouse monoclonal anti-lamin A/C antibody, rabbit polyclonal anti-NF-κB p65 (C-20), Rabbit polyclonal anti IκB-α (C-15) and rabbit anti-caveolin-1 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-total β-catenin antibody was from BD Biosciences (San Jose, CA, USA). Mouse anti-unphosphorylated-β-catenin antibody (clone 8E7) was from Millipore (Amsterdam, the Netherlands). SB216763 was from Tocris Cookson (Bristol, UK). CT99021 was from Axon medchem (Groningen, The Netherlands). All other chemicals were of analytical grade.

Data analysis

Values reported for all data represent means ± standard error of the mean (s.e.m). The statistical significance of differences between means was determined by two-tailed Student's *t*-test for paired observations or a one-way ANOVA followed by a Newman-Keuls multiple comparison test, when appropriate. Differences were considered to be statistically significant when $p < 0.05$.

Results

GSK-3 inhibitors dose-dependently attenuate IL-1β-induced eotaxin release

Glycogen synthase kinase-3 (GSK-3) has been implicated in the regulation of multiple signalling proteins and transcription factors required for pro-inflammatory responses (30; 35). However, its role in regulating cytokine release by airway smooth muscle has not been reported. First, we assessed the effect of increasing concentrations of the selective GSK-3 inhibitor SB216763 on IL-1β-induced eotaxin release. Treatment of airway smooth muscle cells with the pro-inflammatory cytokine IL-1β (1 ng/mL) for 24 hours, resulted in a significant increase in eotaxin release. Inhibition of GSK-3 by SB216763 resulted in a dose-dependent decrease of eotaxin release by airway smooth muscle cells with an EC_{50} of 1.26 ± 0.40 μM (figure 1A). To confirm that the effects of SB216763 were primarily due to inhibition of GSK-3, we evaluated the effect of another selective and structurally unrelated GSK-3 inhibitor CHIR/CT99021 on IL-1β-induced eotaxin release (1). Inhibition of GSK-3 by CHIR/CT99021 resulted in a similar, dose-dependent attenuation of IL-1β-induced eotaxin release with an EC_{50} 0.68 ± 0.04 μM (figure 1B). Furthermore, as SB216763 is a direct ATP-competitive inhibitor of GSK-3, which does not affect the phosphorylation status of GSK-3 (29), we determined the effect of the SB216763 on β-catenin expression, a protein directly regulated by GSK-3. Treatment of airway smooth muscle cells with increasing concentrations of SB216763 resulted in an increased β-catenin expression with a corresponding EC_{50} of 4.80 ± 0.28 μM (figure 1C). Taken together, these data indicate that GSK-3 inhibition attenuates IL-1β-induced eotaxin release airway smooth muscle cells.

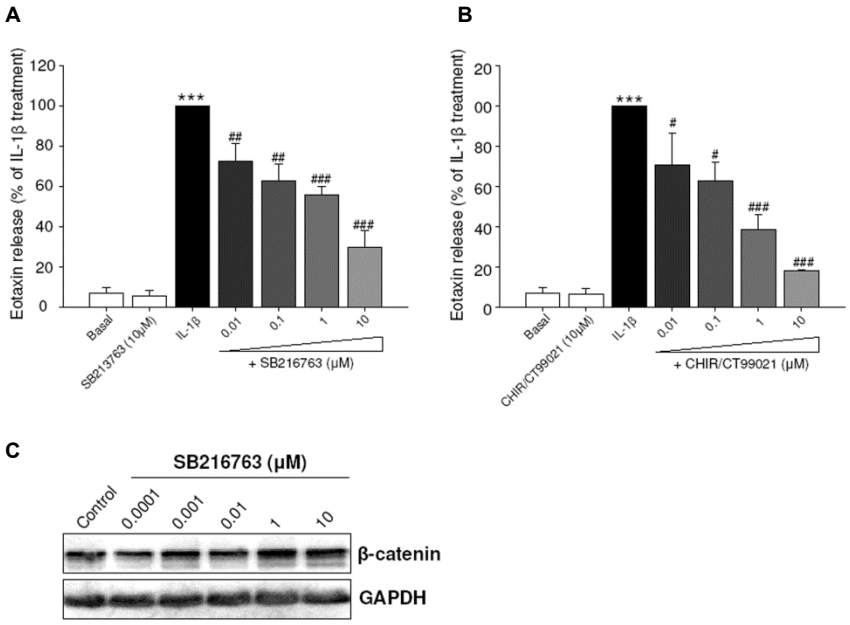

Figure 1: The GSK-3 inhibitors SB216763 and CHIR/CT99021 dose-dependently attenuate IL-1β-induced eotaxin release by airway smooth muscle cells. Airway smooth muscle cells were treated with IL-1β (1 ng/mL) for 24 hours in presence or absence of the selective GSK-3 inhibitor (A) SB216763 or (B) CHIR/CT99021. Both GSK-3 inhibitors were applied in concentrations of 0.01-10 μM. The release of eotaxin was measured by ELISA. Responses shown represent means ± s.e.mean of 3 independent experiments, each performed in duplicate. (C) Airway smooth muscle cells were treated for 16 hours with SB216763 in concentrations of 0.01-10 μM. β-Catenin protein expression was determined by immunoblotting using β-catenin-specific antibody. Equal protein loading was verified by analysis of GAPDH. Responses shown represent means ± s.e.mean of 3 independent experiments. ***p<0.001 compared to untreated airway smooth muscle cells (Basal), $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to IL-1β treated airway smooth muscle cells.

Differential effects of cigarette smoke extract and IL-1β on VEGF-A, eotaxin and IL-8 release

To determine if the effect of GSK-3 inhibition on IL-1β-induced eotaxin release was not only cytokine or stimulus specific, we investigated the release of vascular endothelial growth factor-A (VEGF-A) and IL-8 in response to IL-1β and cigarette smoke extract (CSE), important factors in the development of COPD. Accordingly, we assessed the contribution of GSK-3 signaling to VEGF-A and IL-8 secretion induced by IL-1β and CSE. Cultured airway smooth muscle cells release small quantities of VEGF-A (123 ± 8.5 pg/mL), eotaxin (9.8 ± 2.9 pg/mL) and IL-8 (39.0 ± 13.3 pg/mL) at baseline (figure 2A-C). Treatment of airway smooth muscle cells for 24 hours with increasing concentrations of CSE resulted in concentration-dependent increases in the release of VEGF-A (figure 2A). Only the highest concentration of CSE (15%) induced significant IL-8 (figure 2C) release, whereas

no effect of CSE was observed on eotaxin release (figure 1B). By contrast, treatment with IL-1β (0.01, 0.1 and 1 ng/mL) for 24 hours concentration-dependently induced the release of both eotaxin and IL-8 (figure 3B and 3C), whereas VEGF-A release was induced at the highest concentration of IL-1β only (figure 3A).

Pharmacological inhibition of GSK-3 by SB216763 (10 μM) resulted in an attenuation of CSE-induced cytokine and growth factor release, without affecting their baseline release (figure 2A-C). GSK-3 inhibition significantly reduced 5 and 15% CSE-induced VEGF-A release, by 47 ± 18% and 47 ± 14 %, respectively, and 15% CSE-induced IL-8 release by 33 ±17% (figure 2A and 2C). GSK-3 inhibition also attenuated the IL-1β-induced release of VEGF-A by 30 ± 18% and decreased eotaxin secretion caused by 0.1 and 1 ng/mL IL-1β by 58 ± 9% and 41 ± 8 %, respectively (figure 3A and 3B). Furthermore, SB216763 attenuated IL-1β-induced IL-8 release at the lowest concentration of IL-1β (0.01 ng/mL) applied, whereas at higher concentrations of IL-1β no effect of GSK-3 inhibition was observed (figure 3C). Collectively, these data clearly indicate that both CSE and IL-1β activate airway smooth muscle cells to secrete pro-inflammatory cytokines and chemokines; however, with different efficacy. Furthermore, it shows that GSK-3 activity is involved in the generation of a number of cytokines and growth factors in response to CSE or IL-1β treatment.

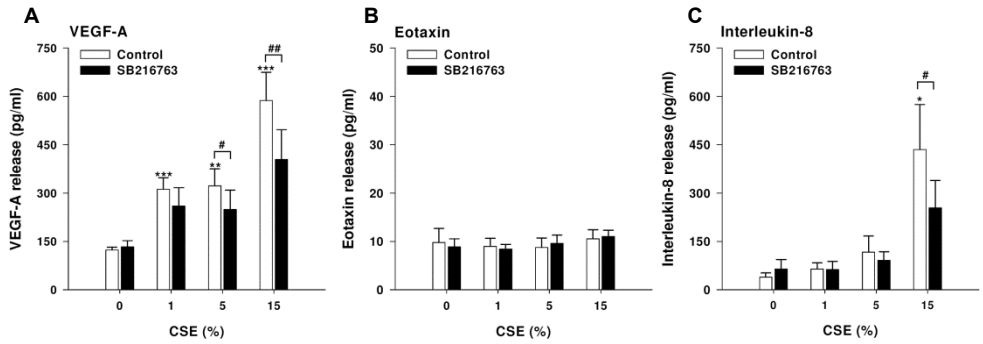

Figure 2: Cytokine and growth factor secretion induced by cigarette smoke extract (CSE) are attenuated by GSK-3 inhibition. Airway smooth muscle cells were treated with cigarette smoke extract (CSE; 1, 5 and 15%) for 24 hours in the presence or absence of the GSK-3 inhibitor SB216763 (10 μM). Subsequently, the release of (A) vascular endothelial growth factor (VEGF-A), (B) eotaxin and (C) interleukin-8 (IL-8) was measured by ELISA. Responses shown represent means ± s.e.mean of 4-6 independent experiments, each performed in duplicate. *p<0.05, ** p<0.01, ***p<0.001 compared to untreated airway smooth muscle cells, $^{\#}$p<0.05, $^{\#\#}$ p<0.01 compared to absence of SB216763.

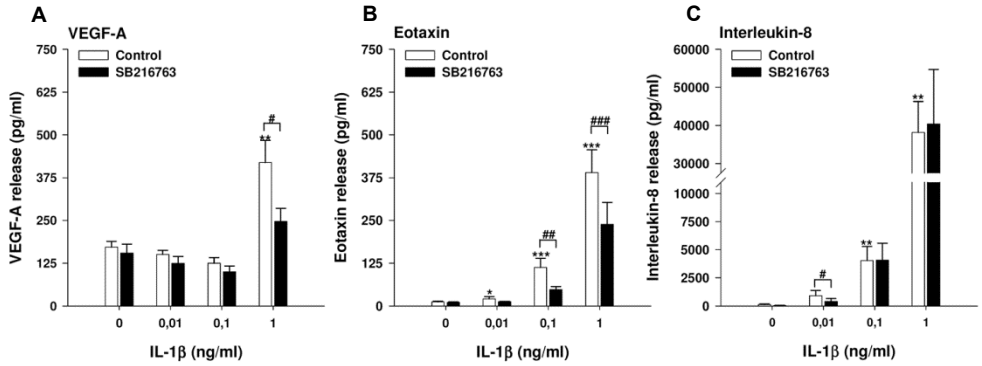

Figure 3: Cytokine and growth factor secretion induced by IL-1β are attenuated by GSK-3 inhibition. Airway smooth muscle cells were treated with IL-1β (0.01, 0.1 and 1 ng/mL) for 24 hours in presence or absence of the GSK-3 inhibitor SB216763 (10 μM). Subsequently, the release of (A) vascular endothelial growth factor (VEGF-A), (B) eotaxin and (C) interleukin-8 (IL-8) was measured by ELISA. Responses shown represent means ± s.e.mean of 4-7 independent experiments, each performed in duplicate. *p<0.05, **p<0.01, ***p<0.001 compared to untreated airway smooth muscle cells, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.01 compared to absence of SB216763.

Cigarette smoke extract and IL-1β do not activate β-catenin

β-Catenin is an essential component of cadherin-based adherens junctions, where it stabilizes cell-cell-contact. In addition, it can serve a role in activating gene transcription and β-catenin has been demonstrated to regulate VEGF and IL-8 synthesis and secretion by endothelial cells (36; 50). For this reason the regulation of β-catenin in response to CSE and IL-1β in airway smooth muscle cells was studied. After 24 hours of stimulation with 15 % CSE or 1 ng/mL IL-1β, we did not observe any differences in either the total expression of β-catenin or the nuclear expression of β-catenin (figure 4A). Moreover, the abundance of the transcriptionally active, non-phosphorylated form of β-catenin was also not altered by CSE (5 and 15 %) or IL-1β (0.1 and 1 ng/mL) treatment (figure 4B). As a positive control, treatment with 10% FBS induced the expression of total β-catenin by 1.8-fold and nuclear β-catenin and the abundance of transcriptionally active, non-phosphorylated β-catenin form by 2.8-fold (figure 4C). Collectively, these results demonstrate that β-catenin signalling is not activated in airway smooth muscle cells by either CSE or IL-1β.

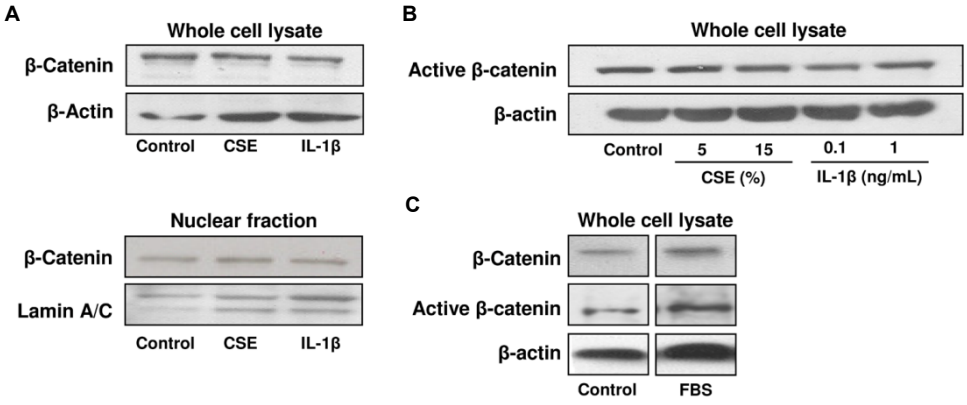

Figure 4: Treatment with cigarette smoke extract (CSE) or IL-1β does not activate the β-catenin pathway in airway smooth muscle cells. (A) Airway smooth muscle cells were treated with 15% cigarette smoke extract (CSE) or 1 ng/mL IL-1β for 24 hours. β-Catenin expression was determined in whole cell lysate and in nuclear fractions by using a β-catenin-specific antibody. Equal protein loading was verified by analysis of β-actin (whole cell lysate) and lamin A/C (nuclear fractions). Representative immunoblots of 3 independent experiments are shown. (B) Expression of the transcriptionally active (unphosphorylated) form of β-catenin was evaluated in whole cell lysates after 24 hours of treatment with the indicated concentrations CSE or IL-1β, by using a non-phospho-β-catenin-specific antibody. Equal protein loading was verified by analysis of β-actin. Representative immunoblots of 3 independent experiments are shown. (C) Expression of β-catenin and the transcriptionally active (unphosphorylated) form of β-catenin after 24 hours of 10% FBS treatment was evaluated in whole cell lysates by using a β-catenin-specific antibody and a non-phospho-β-catenin-specific antibody. Equal protein loading was verified by analysis of β-actin.

Role of GSK-3 in NF-κB signalling

GSK-3 signalling has been implicated in regulating multiple steps in activation of the NF-κB signalling pathway. Therefore, we investigated if GSK-3 signalling was involved in the degradation of the NF-κB inhibitory protein IκB-α in airway smooth muscle cells induced by IL-1β. Treatment with IL-1β (1 ng/mL) resulted in a significant, time-dependent decrease in IκB-α expression (figure 5A). After 30 minutes of IL-1β stimulation a slight decrease in IκB-α abundance was observed (13 ± 10 %), which reached a maximum after 60 minutes (54 ± 10 %) of IL-1β treatment (figure 5A and 5C). Pharmacological inhibition of GSK-3 by SB216763 did not alter the kinetics or magnitude of IL-1β-induced IκB-α degradation (figure 5B and 5C).

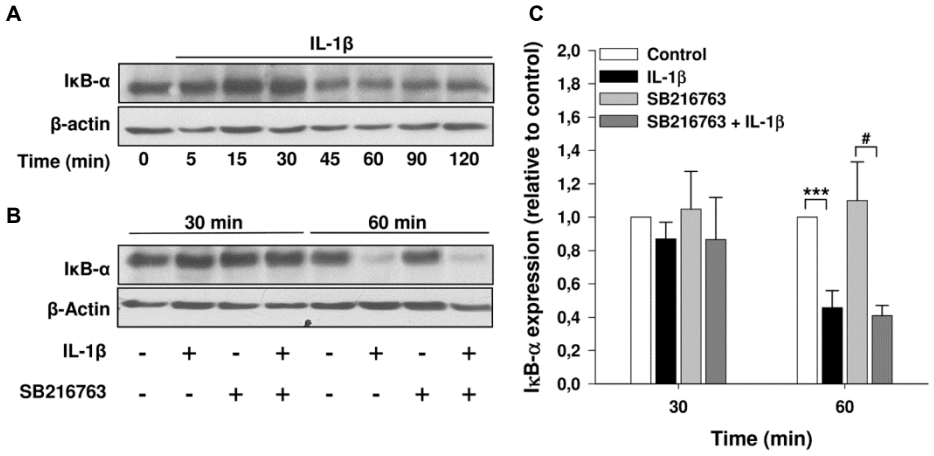

Figure 5: IL-1β-induced IκB-α degradation is not affected by GSK-3 inhibition. (A) IL-1β induces time-dependent IκB-α breakdown. Airway smooth muscle cells were treated with IL-1β (1 ng/mL) for the indicated time points. Expression of IκB-α was determined in whole cell lysates by using an IκB-α specific antibody. Equal protein loading was verified by analysis of β-actin.(B) IL-1β induced IκB-α breakdown is not affected by GSK-3 inhibition. Airway smooth muscle cells were treated with IL-1β (1 ng/mL) for the indicated time points in the presence or absence of the GSK-3 inhibitor SB216763 (10 μM). Expression of IκB-α was determined in whole cell lysates by using an IκB-α specific antibody. Equal protein loading was verified by analysis of β-actin. Responses were quantified by densitometry (panel C), representing means ± s.e.mean of 4 independent experiments. ***p<0.001 compared to control, # p<0.05 compared to SB216763.

Nuclear translocation and subsequent DNA binding of the p65/p50 NF-κB subunits is required for NF-κB dependent gene transcription (24) and is possibly regulated by GSK-3 (18). We therefore addressed whether GSK-3 is involved in the nuclear translocation of p65 NF-κB in airway smooth muscle cells. In line with the observed degradation of IκB-α, we found a significant, time-dependent increase of p65 NF-κB expression in the nuclei induced by IL-1β (1 ng/mL) (figure 6A-C). SB216763 did not influence IL-1β-induced p65 NF-κB nuclear translocation at any time point measured (figure 6A and 6B) and did not reduce the area under the curve (AUC) of the total time course (figure 6C). Further, we investigated whether the DNA binding capacity of p65 NF-κB was affected by SB216763. The DNA binding capacity of p65 NF-κB was significantly induced after treatment of IL-1β (1 ng/mL; 1h); however SB216763 did not affect the binding capacity (figure 6D).

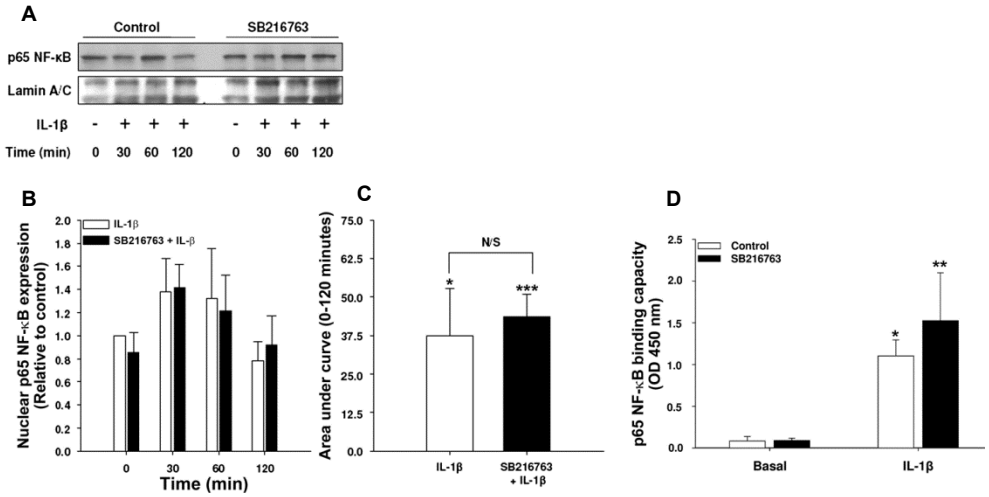

Figure 6: IL-1β-induced nuclear translocation and DNA binding capacity of p65 NF-κB is independent of GSK-3. (A-B) Airway smooth muscle cells were treated with IL-1β (1 ng/mL) for the indicated time-points in the presence or absence of the GSK-3 inhibitor SB216763 (10 μM). p65 NF-κB expression was evaluated in nuclear fractions by using a p65 NF-κB-specific antibody. Equal protein loading was verified by analysis of lamin A/C. (C) Area under the curve of time-dependent, IL-1β-induced p65 NF-κB nuclear translocation in the presence or absence of SB216763 (10 μM). Responses were quantified by densitometry, representing means ± s.e.mean of 5 independent experiments. *p<0.05, ***p<0.001 compared to basal nuclear p65 NF-κB expression. (D) Airway smooth muscle cells were treated with IL-1β (1 ng/mL) for 1 hour in the presence or absence of the GSK-3 inhibitor SB216763 (10 μM). The DNA binding capacity of p65 NF-κB was determined in 10 μg nuclear extract and assayed with the p65 NF-κB TransAM kit according to the manufacturers' instructions. Responses were quantified by densitometry, representing means ± s.e.mean of 3 independent experiments. *p<0.05, **p<0.01 compared to basal nuclear p65 NF-κB binding capacity.

Since GSK-3 inhibition did not alter the rate of IκB-α degradation, p65 NF-κB nuclear translocation or DNA binding capacity in airway smooth muscle cells, we next investigated a direct, functional effect of GSK-3 inhibition on NF-κB driven gene transcription, by using a NF-κB reporter luciferase assay. Treatment with IL-1β (1 ng/mL) resulted in a significant 2.5 ± 1.0 fold increase in NF-κB reporter luciferase activity. GSK-3 inhibition did not have an effect on basal NF-κB driven gene-transcription, but fully inhibited IL-1β-induced luciferase activity (figure 7A). Similar results were obtained using 15% CSE (figure 7B). pRR-high EF1a vector transfected airway smooth muscle cells (control), did not respond to any of the treatments (data not shown). Collectively, these findings indicate that GSK-3 inhibition attenuates NF-κB-driven gene-transcription in airway smooth muscle, without affecting the degradation of the NF-κB inhibitory protein IκB-α, nuclear translocation or DNA binding capacity of the NF-κB subunit p65.

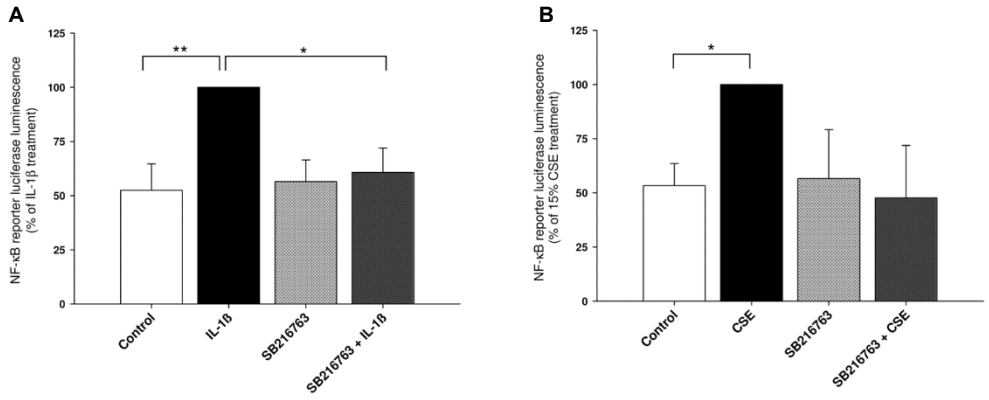

Figure 7: NF-κB-dependent gene-transcription is attenuated by GSK-3 inhibition. Airway smooth muscle cells were transfected with a pRR-High NF-κB-vector and (A) stimulated with IL-1β (1 ng/mL) and (B) stimulated with CSE (15%) in the presence or absence of the GSK-3 inhibitor SB216763 (10 μM). Responses shown represent means ± s.e.mean of 3-4 independent experiments, each performed in triplicate. *p< 0.05, **p<0.01.

Discussion

In this study, the contribution of GSK-3 to a number of CSE- and IL-1β-induced pro-inflammatory chemoattractant cytokine and growth factor secretion by airway smooth muscle cells was determined. We demonstrate that in airway smooth muscle these stimuli, both important factors in the development of COPD, concentration dependently induced the release of both VEGF-A and IL-8, whereas eotaxin release was induced by IL-1β only. Our results further show that neither of the stimuli activated β-catenin signalling and that selective inhibition of GSK-3 attenuated the secretion of IL-8, eotaxin and VEGF-A. Interestingly, the effects of GSK-3 inhibition were dependent on inhibition of NF-κB activity. Degradation of the NF-κB inhibitory protein IκB-α, nuclear translocation and DNA binding capacity of p65 NF-κB were, however, unaffected. Collectively, our study indicates that in airway smooth muscle GSK-3 signalling contributes to pro-inflammatory cytokine and growth factor secretion.

The expression of VEGF and IL-8 in the lung is raised in the airways of both asymptomatic and COPD smokers (47). Local production of VEGF, especially VEGF-A, in the airways has been implicated to drive angiogenesis, a predominant feature of structural tissue remodelling that occurs in COPD (54). Furthermore, VEGF is implicated in the pathogenesis of both chronic bronchitis and emphysema, although in the former it has been shown that VEGF levels are raised, the latter seems to be associated with a reduced expression of this growth factor (31). IL-8, an important chemotactic factor for neutrophils, is produced by various cell types in the airways, including airway smooth muscle, in response to cigarette smoke as well as inflammatory mediators implicated in the development of COPD (8; 21). Neutrophils are considered as important inflammatory cells in the pathogenesis of COPD (16). Moreover, eosinophilic airway inflammation, which is usually considered to be a characteristic feature of asthma, has also been demonstrated in a small percentage of COPD patients (48). Eotaxin might also play an important role in the progression of COPD, as this chemokine recruits eosinophils and lymphocytes to the airways during acute exacerbations (6). We demonstrate that the pro-inflammatory cytokine IL-1β was capable of potently inducing VEGF-A, IL-8 as well as eotaxin release from airway smooth muscle. Conversely, CSE induced the release of VEGF-A and IL-8, but failed to induce eotaxin release from airway smooth muscle. These findings are in agreement with a study showing that CSE did not induce eotaxin release and even attenuated TNF-α-induced eotaxin release from primary airway smooth muscle cells (40). This indicates that the release of cytokines can be differentially regulated by these pro-inflammatory stimuli. Consequently, insights into the mechanisms that regulate expression of these chemoattractant cytokines and VEGF-A are of great interest for the understanding

of the pathogenesis of chronic lung diseases, like COPD. Our results provide important new insights, as we demonstrate that the secretion of these cytokines and growth factors is partially dependent on GSK-3 signaling. The selective GSK-3 inhibitor SB216763 as well as CHIR/CT99021 dose-dependently inhibited IL-1β-induced eotaxin release by airway smooth muscle cells. The calculated EC_{50}-values for both SB216763 and CHIR/CT99021 are in agreement with the EC_{50}-values reported for both inhibitors on stimulating glycogen synthesis, indicating that the attenuation of cytokine release in airway smooth muscle cells by these inhibitors is primarily due to inhibition of GSK-3 (12; 46). Inhibition of GSK-3 may be strategy worth pursuing in reducing airway inflammation. Future studies are needed to investigate the potential beneficial effect of GSK-3 inhibition in animal models of chronic inflammatory lung disease.

Cigarette smoke (extract) is a complex mixture composed of over 4000 different organic and inorganic components, making it difficult to indicate which components are predominantly responsible for the induction of cytokine release by airway smooth muscle. Nonetheless, a study by Oltmanns *et al.*(40), demonstrated that human airway smooth muscle cells that were exposed to cigarette smoke showed increased expression of heme-oxygenase-1, an intracellular indicator of oxidative stress. Pretreatment with the anti-oxidant glutathione largely inhibited the CSE-induced IL-8 release by these cells. These findings indicate the oxidative stress induced by CSE is an important mechanism by which cytokine release is induced by airway smooth muscle cells (40). In addition, α,β-unsaturated aldehydes (i.e. acrolein and crotonaldehyde) present in CSE are capable of inducing the release of both VEGF and IL-8 from human lung fibroblasts and epithelial cells (37). Also endotoxins in cigarette smoke, in particular lipopolysaccharide (LPS), have been suggested to activate cells to produce inflammatory cytokines. LPS is, however, unlikely to contribute to the CSE-induced effects in our study, as LPS concentrations are below detection limit in the CSE we use for our studies (45). Collectively, although the exact chemical moiety in cigarette smoke that induces IL-8 and VEGF release by human ASM is not known, there is likely a major role for α,β-unsaturated aldehydes and components that induce oxidative stress.

β-Catenin-mediated gene transcription has been associated with the production of pro-inflammatory mediators, growth factors and cytokines (11; 36). For instance, in pulmonary artery smooth muscle cells (PASMC) the transforming growth factor (TGF)-β-induced release of VEGF-A is dependent on β-catenin signalling. In these cells, TGF-β inhibits GSK-3β activity resulting in increased β-catenin stability and subsequent TCF-dependent induction of VEGF-A (11). In addition, Clifford *et al.* (11) showed that inhibition of GSK-3 by SB216763 induced VEGF-A secretion, to a similar level as TGF-β. Likewise, IL-8 was identified as a transcriptional target of

Wnt/β-catenin signalling in endothelial cells. Thus, endothelial cells ectopically expressing Wnt1 or a degradation-resistant β-catenin (β-cateninS37A) produce IL-8 (36). Although we found that CSE induced a transient inhibitory ser9/21 phosphorylation of GSK-3, IL-1β did not (data not shown). Moreover, we show that neither CSE- nor IL-1β-induced β-catenin nuclear localization or the expression of the transcriptionally active, unphosphorylated form of β-catenin in airway smooth muscle cells, indicating that under these experimental conditions, β-catenin is not responsible for the CSE or IL-1β induced secretion of IL-8, eotaxin and VEGF-A from airway smooth muscle cells.

We demonstrate that 15% CSE-induced IL-8 release is partially inhibited by SB216763, whereas the IL-8 release induced by IL-1β is inhibited at the lowest concentration of IL-1β only. Therefore, an additional mechanism may be activated at higher concentrations of IL-1β which is independent of GSK-3. A possible mechanism could be the induction of cyclooxygenases (COX). Recently, it was demonstrated that IL-1β induces COX expression in airway smooth muscle cells (43). COX-1 and COX-2 dependent signaling has been shown to be relevant in the IL-8 release by airway smooth muscle cells (42). Therefore, COX-dependent signaling may play a more important role in IL-1β signaling than in cigarette smoke extract activated signaling in airway smooth muscle cells and explain the discrepancy observed in the role of GSK-3 in the IL-8 release induced by these stimuli.

A regulatory function for GSK-3 in NF-κB signalling has previously been demonstrated in the secretion of pro- and anti-inflammatory cytokines. The regulation of NF-κB by GSK-3 was shown for the first time in mice lacking the GSK-3β isoform, which caused apoptosis of hepatocytes and resulted in embryonic lethality. These mice displayed a phenotype which is consistent with mice having a dysfunction in the NF-κB pathway (26). This finding formed the basis for the hypothesis that GSK-3 might play a pivotal role in the regulation of the activation of NF-κB and, hence, inflammatory responses. In support, both *in vitro* and *in vivo* pro-inflammatory responses in different models of chronic diseases with an inflammatory component, are potently suppressed by administration of a GSK-3 inhibitor (2; 13; 18; 55). For instance, pro-inflammatory cytokine release induced by lipopolysaccharide (LPS), a component of the cell wall of gram negative bacteria, was effectively suppressed by administration of a GSK-3 inhibitor (35). GSK-3 has been demonstrated in a variety of cell types to intervene in the activation of NF-κB at different levels of the signalling cascade. Recently is has been demonstrated that activation of NF-κB pathway is essential for the upregulation of VEGF-A in the lung and for IL-8 and eotaxin secretion by airway smooth muscle (32; 53). Pro-inflammatory cytokines and CSE can activate the NF-κB signaling pathway in

airway smooth muscle cells (39). Indeed, we observed that inhibition of NF-κB by the selective IκB kinase-2 (IKK-2) inhibitor SC-514, attenuated the CSE- and IL-1β-induced eotaxin and IL-8 release in airway smooth muscle cells to a similar extent as observed with SB216763 (data not shown). The observed effects of the GSK-3 inhibitors SB216763 and CHIR/CT99021 on attenuating cytokine secretion can therefore likely be explained by reduction of NF-κB signaling. This was confirmed in our subsequent studies that indicated SB216763 fully repressed NF-κB dependent gene transcription.

In a variety of cell types it has been demonstrated that GSK-3 is involved in the regulation of NF-κB signaling by controlling the activation or transcriptional responses of this pathway. However, despite the numerous studies in various cell types and tissues no consensus exists on the underlying mechanisms. One of the first steps in the NF-κB pathway is the activation of IκB-α kinase (IKK) and subsequent degradation of IK-Bα. In fibroblasts derived from GSK-3β gene-deleted mice, activation of IKK and subsequent degradation of IκB-α in response to TNF-α were abolished. Accordingly, NF-κB activation by TNF-α, LPS, IL-1β and cigarette smoke condensate was completely suppressed in these GSK-3$\beta^{-/-}$ fibroblasts (52). However, and consistent with our results in airway smooth muscle, Hoeflich *et al.* (26) demonstrated that inhibition of the NF-κB pathway in these GSK-3$\beta^{-/-}$ fibroblasts is not via these early steps of NF-κB activation, but rather via the regulation of NF-κB nuclear activity (26). In hepatocytes and monocytes it was also found that the presence of the GSK-3 inhibitor SB216763 did not alter the rate or extent of LPS- and TNF-α-induced activation of IKK or the degradation of the NF-κB inhibitory protein IκB-α (35; 49). The next steps in the activation of NF-κB signalling are nuclear translocation and subsequent DNA binding of the p65/p50 NF-κB subunits (24), processes that are possibly dependent on GSK-3 signalling (18). We demonstrate that nuclear p65 NF-κB abundance is unaffected by SB216763, which is in agreement with several other studies (35; 49; 51). The role of GSK-3 in NF-κB phosphorylation and DNA binding, however, is controversial and indefinite. The GSK-3 inhibitor 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) was demonstrated to attenuate pro-inflammatory cytokine production in TNF-α-stimulated human bronchial epithelial cells via inhibition of the NF-κB pathway. In agreement with our findings, the binding capacity of NF-κB to DNA was not affected by TDZD-8 in these cells (2). Further, in hepatocytes and monocytes, GSK-3 inhibition attenuates NF-kB driven reporter gene transcription without any effect on NF-κB binding activity (35; 49). On the other hand, NF-κB DNA binding activity was reduced in GSK-3 null murine embryonic fibroblasts and it was established that GSK-3β was required for efficient expression of specific NF-κB-induced genes (51). The discrepancy between binding and transcriptional activity of NF-κB can be explained by several mechanisms. It was suggested that

GSK-3 plays a role in the phosphorylation of the NF-κB subunits. GSK-3 signaling may regulate phosphorylation of p65 at different locations, for instance serine 536 (e.g. ser 536) or the COOH terminus site of p65 NF-κB (2; 49). However, in GSK-3 null murine embryonic fibroblasts the cytokine-induced phosphorylation of the ser536-p65 and the phosphorylation of p105 NF-κB subunit were not altered compared to fibroblasts of wild type mice (51). Moreover, in the monocytes GSK-3 inhibition did not affect the amount nor the duration of ser276 or ser536-p65 phosphorylation (35). The effect of GSK-3 inhibition on NF-κB signaling could possibly be explained by a reduction in the recruitment of transcriptional co-activators required for functional NF-κB signaling (35). Clearly, future investigations into the specific mechanisms that regulate the reduced NF-κB-dependent gene transcription by GSK-3 inhibition are required. In view of the results from our current work, and the available literature, the nuclear events that follow NF-κB binding and regulate NF-κB transcriptional activity will need to be investigated in more detail.

In conclusion, the results from our study indicate that airway smooth muscle cells release IL-8, eotaxin and VEGF in response to CSE and/or IL-1β, two important factors in the development of COPD. Pharmacological inhibition of GSK-3 greatly attenuated the release of these chemoattractant cytokines and VEGF-A by reducing NF-κB-dependent gene transcription. These results imply a role for GSK-3 in cigarette smoke-induced inflammation, which is of clear interest to the pathogenesis of airway diseases. Future studies are needed to investigate the potential beneficial effect of GSK-3 inhibition in animal models of chronic inflammatory airway diseases.

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References

1. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR and Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408: 297-315, 2007.
2. Bao Z, Lim S, Liao W, Lin Y, Thiemermann C, Leung BP and Wong WS. Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. *Am J Respir Crit Care Med* 176: 431-438, 2007.
3. Barnes PJ, Shapiro SD and Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J* 22: 672-688, 2003.
4. Bentley JK, Deng H, Linn MJ, Lei J, Dokshin GA, Fingar DC, Bitar KN, Henderson WR, Jr. and Hershenson MB. Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3(beta) phosphorylation in a mouse model of asthma. *Am J Physiol Lung Cell Mol Physiol* 296: L176-L184, 2009.
5. Bhalla DK, Hirata F, Rishi AK and Gairola CG. Cigarette smoke, inflammation, and lung injury: a mechanistic perspective. *J Toxicol Environ Health B Crit Rev* 12: 45-64, 2009.
6. Bocchino V, Bertorelli G, Bertrand CP, Ponath PD, Newman W, Franco C, Marruchella A, Merlini S, Del DM, Zhuo X and Olivieri D. Eotaxin and CCR3 are up-regulated in exacerbations of chronic bronchitis. *Allergy* 57: 17-22, 2002.
7. Chung KF. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *Eur Respir J* 15: 961-968, 2000.
8. Chung KF. The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2: 347-354, 2005.
9. Chung KF, Patel HJ, Fadlon EJ, Rousell J, Haddad EB, Jose PJ, Mitchell J and Belvisi M. Induction of eotaxin expression and release from human airway smooth muscle cells by IL-1beta and TNFalpha: effects of IL-10 and corticosteroids. *Br J Pharmacol* 127: 1145-1150, 1999.
10. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell* 127: 469-480, 2006.
11. Clifford RL, Deacon K and Knox AJ. Novel regulation of vascular endothelial growth factor-A (VEGF-A) by transforming growth factor (beta)1: requirement for Smads, (beta)-CATENIN, AND GSK3(beta). *J Biol Chem* 283: 35337-35353, 2008.
12. Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee CL, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD and Holder JC. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 7: 793-803, 2000.
13. Cuzzocrea S, Mazzon E, Di PR, Muia C, Crisafulli C, Dugo L, Collin M, Britti D, Caputi AP and Thiemermann C. Glycogen synthase kinase-3beta inhibition attenuates the degree of arthritis caused by type II collagen in the mouse. *Clin Immunol* 120: 57-67, 2006.
14. Deng H, Dokshin GA, Lei J, Goldsmith AM, Bitar KN, Fingar DC, Hershenson MB and Bentley JK. Inhibition of glycogen synthase kinase-3beta is sufficient for airway smooth muscle hypertrophy. *J Biol Chem* 283: 10198-10207, 2008.
15. Deng J, Xia W, Miller SA, Wen Y, Wang HY and Hung MC. Crossregulation of NF-kappaB by the APC/GSK-3beta/beta-catenin pathway. *Mol Carcinog* 39: 139-146, 2004.
16. Di SA, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF and Saetta M. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 158: 1277-1285, 1998.
17. Doble BW and Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116: 1175-1186, 2003.
18. Dugo L, Collin M and Thiemermann C. Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. *Shock* 27: 113-123, 2007.
19. Edwards MR, Bartlett NW, Clarke D, Birrell M, Belvisi M and Johnston SL. Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol Ther* 121: 1-13, 2009.
20. Gosens R, Dueck G, Rector E, Nunes RO, Gerthoffer WT, Unruh H, Zaagsma J, Meurs H and Halayko AJ. Cooperative regulation of GSK-3 by muscarinic and PDGF receptors is associated with airway myocyte proliferation. *Am J Physiol Lung Cell Mol Physiol* 293: L1348-L1358, 2007.

21. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J, Kolahian S, Halayko AJ, Hiemstra PS and Zuyderduyn S. Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. *Eur Respir J* 2009.
22. Gosens R, Stelmack GL, Dueck G, McNeill KD, Yamasaki A, Gerthoffer WT, Unruh H, Gounni AS, Zaagsma J and Halayko AJ. Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 291: L523-L534, 2006.
23. Hardt SE and Sadoshima J. Glycogen synthase kinase-3beta: a novel regulator of cardiac hypertrophy and development. *Circ Res* 90: 1055-1063, 2002.
24. Hayden MS and Ghosh S. Shared principles in NF-kappaB signaling. *Cell* 132: 344-362, 2008.
25. Hinoi T, Yamamoto H, Kishida M, Takada S, Kishida S and Kikuchi A. Complex formation of adenomatous polyposis coli gene product and axin facilitates glycogen synthase kinase-3 beta-dependent phosphorylation of beta-catenin and down-regulates beta-catenin. *J Biol Chem* 275: 34399-34406, 2000.
26. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O and Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406: 86-90, 2000.
27. Howarth PH, Knox AJ, Amrani Y, Tliba O, Panettieri RA, Jr. and Johnson M. Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 114: S32-S50, 2004.
28. Howe LR, Subbaramaiah K, Chung WJ, Dannenberg AJ and Brown AM. Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. *Cancer Res* 59: 1572-1577, 1999.
29. Jansen SR, Van Ziel AM, Baarsma HA and Gosens R. {beta}-Catenin regulates airway smooth muscle contraction. *Am J Physiol Lung Cell Mol Physiol* 299: L204-L214, 2010.
30. Jope RS, Yuskaitis CJ and Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res* 32: 577-595, 2007.
31. Kanazawa H, Asai K, Hirata K and Yoshikawa J. Possible effects of vascular endothelial growth factor in the pathogenesis of chronic obstructive pulmonary disease. *Am J Med* 114: 354-358, 2003.
32. Keslacy S, Tliba O, Baidouri H and Amrani Y. Inhibition of tumor necrosis factor-alpha-inducible inflammatory genes by interferon-gamma is associated with altered nuclear factor-kappaB transactivation and enhanced histone deacetylase activity. *Mol Pharmacol* 71: 609-618, 2007.
33. Lazaar AL and Panettieri RA, Jr. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin Immunol* 116: 488-495, 2005.
34. Lee YH, Suzuki YJ, Griffin AJ and Day RM. Hepatocyte growth factor regulates cyclooxygenase-2 expression via beta-catenin, Akt, and p42/p44 MAPK in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 294: L778-L786, 2008.
35. Martin M, Rehani K, Jope RS and Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol* 6: 777-784, 2005.
36. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM and Kitajewski J. Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. *Angiogenesis* 8: 43-51, 2005.
37. Moretto N, Facchinetti F, Southworth T, Civelli M, Singh D and Patacchini R. alpha,beta-Unsaturated aldehydes contained in cigarette smoke elicit IL-8 release in pulmonary cells through mitogen-activated protein kinases. *Am J Physiol Lung Cell Mol Physiol* 296: L839-L848, 2009.
38. Nunes RO, Schmidt M, Dueck G, Baarsma H, Halayko AJ, Kerstjens HA, Meurs H and Gosens R. GSK-3/beta-catenin signaling axis in airway smooth muscle: role in mitogenic signaling. *Am J Physiol Lung Cell Mol Physiol* 294: L1110-L1118, 2008.
39. Oenema TA, Kolahian S, Nanninga JE, Rieks D, Hiemstra PS, Zuyderduyn S, Halayko AJ, Meurs H and Gosens R. Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle. *Respir Res* 11: 130, 2010.
40. Oltmanns U, Chung KF, Walters M, John M and Mitchell JA. Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle. *Respir Res* 6: 74, 2005.
41. Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA and Billadeau DD. Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. *Cancer Res* 65: 2076-2081, 2005.

42. Pang L and Knox AJ. Bradykinin stimulates IL-8 production in cultured human airway smooth muscle cells: role of cyclooxygenase products. *J Immunol* 161: 2509-2515, 1998.
43. Pascual RM, Carr EM, Seeds MC, Guo M, Panettieri RA, Jr., Peters SP and Penn RB. Regulatory features of interleukin-1beta-mediated prostaglandin E2 synthesis in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 290: L501-L508, 2006.
44. Pauwels RA, Buist AS, Ma P, Jenkins CR and Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. *Respir Care* 46: 798-825, 2001.
45. Pera T, Gosens R, Lesterhuis AH, Sami R, Toorn M, Zaagsma J and Meurs H. Cigarette smoke and lipopolysaccharide induce a proliferative airway smooth muscle phenotype. *Respir Res* 11: 48, 2010.
46. Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond ME and Harrison SD. Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes* 52: 588-595, 2003.
47. Rovina N, Papapetropoulos A, Kollintza A, Michailidou M, Simoes DC, Roussos C and Gratziou C. Vascular endothelial growth factor: an angiogenic factor reflecting airway inflammation in healthy smokers and in patients with bronchitis type of chronic obstructive pulmonary disease? *Respir Res* 8: 53, 2007.
48. Saha S and Brightling CE. Eosinophilic airway inflammation in COPD. *Int J Chron Obstruct Pulmon Dis* 1: 39-47, 2006.
49. Schwabe RF and Brenner DA. Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 283: G204-G211, 2002.
50. Skurk C, Maatz H, Rocnik E, Bialik A, Force T and Walsh K. Glycogen-Synthase Kinase3beta/beta-catenin axis promotes angiogenesis through activation of vascular endothelial growth factor signaling in endothelial cells. *Circ Res* 96: 308-318, 2005.
51. Steinbrecher KA, Wilson W, III, Cogswell PC and Baldwin AS. Glycogen synthase kinase 3beta functions to specify gene-specific, NF-kappaB-dependent transcription. *Mol Cell Biol* 25: 8444-8455, 2005.
52. Takada Y, Fang X, Jamaluddin MS, Boyd DD and Aggarwal BB. Genetic deletion of glycogen synthase kinase-3beta abrogates activation of IkappaBalpha kinase, JNK, Akt, and p44/p42 MAPK but potentiates apoptosis induced by tumor necrosis factor. *J Biol Chem* 279: 39541-39554, 2004.
53. Tong Q, Zheng L, Lin L, Li B, Wang D, Huang C and Li D. VEGF is upregulated by hypoxia-induced mitogenic factor via the PI-3K/Akt-NF-kappaB signaling pathway. *Respir Res* 7: 37, 2006.
54. Walters EH, Reid D, Soltani A and Ward C. Angiogenesis: a potentially critical part of remodelling in chronic airway diseases? *Pharmacol Ther* 118: 128-137, 2008.
55. Whittle BJ, Varga C, Posa A, Molnar A, Collin M and Thiemermann C. Reduction of experimental colitis in the rat by inhibitors of glycogen synthase kinase-3beta. *Br J Pharmacol* 147: 575-582, 2006.
56. Zhang X, Gaspard JP and Chung DC. Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. *Cancer Res* 61: 6050-6054, 2001.

Chapter 6

Glycogen synthase kinase-3 (GSK-3) inhibition prevents pulmonary and extrapulmonary sequelae of LPS-induced chronic obstructive pulmonary disease in guinea pigs

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Abstract

Glycogen synthase kinase-3 (GSK-3) is a constitutively active kinase that regulates multiple signaling proteins and transcription factors involved in a myriad of cellular processes. The kinase acts as a negative regulator in WNT/β-catenin signaling and is critically involved in the smad pathway. Activation of both pathways may contribute to the fibroproliferative features of chronic obstructive pulmonary disease (COPD). In the present study, we investigated the effect of the selective GSK-3 inhibitor SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione) on pulmonary remodelling and extrapulmonary pathology in an established guinea pig model of lipopolysaccharide (LPS)-induced COPD. Guinea pigs were instilled intranasally with LPS or saline twice weekly for 12 weeks and pretreated with either intranasally instilled SB216763 or corresponding vehicle prior to each LPS/saline challenge. Repeated LPS exposure activated β-catenin signaling, primarily in the airway epithelium and submucosa. LPS also induced tissue remodelling as indicated by increased pulmonary fibronectin expression and small airway collagen content. Inhibition of GSK-3 by SB216763 prevented the small airway remodelling and, unexpectedly, inhibited the activation of β-catenin *in vivo*. *In vitro* experiments supported this observation and indicated that SB216763 inhibited TGF-β induced β-catenin activation and matrix protein expression in pulmonary fibroblasts. LPS or SB216763 treatment had no effect on the airway smooth muscle content and alveolar airspace size. However, GSK-3 inhibition prevented the LPS-induced extrapulmonary pathological features, including right ventricle hypertrophy and the tendency in skeletal muscle atrophy. In conclusion, our findings suggest that GSK-3 inhibition is beneficial in attenuating pulmonary remodelling as well as extrapulmonary pathology in a guinea pig model of COPD, and that locally reduced LPS-induced β-catenin activation appears in part to underlie this effect. Collectively, this indicates that GSK-3 may be a novel drug target for the treatment of COPD.

Introduction

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine/threonine kinase, occurring in the two closely related isoforms GSK-3α and GSK-3β which share high homology in their kinase domains [1-3]. Originally, GSK-3 was discovered for its role in glucose metabolism by regulating glycogen synthase activity [3, 4]. Over the years, interest in GSK-3 signaling increased as it became apparent that this kinase regulates various physiological pathways involved a wide array of processes, including protein synthesis, cell differentiation, apoptosis and cell survival [5, 6]. Currently, over fifty putative substrates have been identified including structural proteins, various intracellular signaling intermediates and transcription factors [6]. For instance, GSK-3 is critically involved as a negative regulator in the canonical WNT/β-catenin signaling pathway and it has been linked to the regulation of smad-dependent signaling [5, 6]. Both these pathways which are important in developmental processes and may be activated during pathological conditions in the lungs [7-10] .

In the WNT signaling pathway, GSK-3 is the primary kinase that regulates cellular expression of the transcriptional co-activator β-catenin by phosphorylation, thereby targeting it for proteosomal degradation [11]. In pulmonary fibroblasts, we recently demonstrated that the pro-fibrotic mediator transforming growth factor-β (TGF-β) induces an inhibitory phosphorylation of GSK-3 and activates β-catenin signaling, which in turn contributed to myofibroblast differentiation and extracellular matrix deposition by these cells [12]. Interestingly, β-catenin activation and extracellular matrix deposition was enhanced in fibroblasts of individuals with chronic obstructive pulmonary disease (COPD). In addition, the fibroblast from individuals with COPD showed increased gene expression of WNT ligands, FZD receptors and β-catenin in response to TGF-β [12]. Aberrant activation of canonical WNT signaling is also observed in idiopathic pulmonary fibrosis (IPF), which is characterized by interstitial fibrosis in the lungs due to increased myofibroblast activation with nuclear expression of β-catenin [7-9].

Despite its inhibitory role in pro-fibrogenic β-catenin signaling, GSK-3 has been shown to be required for bleomycin-induced lung fibrosis in mice [13]. In line with this, we have shown in human pulmonary fibroblasts that GSK-3 is required for myofibroblast differentiation and matrix protein expression (Chapter 4). Mechanistically, this is explained by activation of CREB signalling in response to GSK-3 inhibition, which can attenuate smad-dependent transcriptional responses. It appears therefore that GSK-3 inhibition plays a bipartite role in pathological tissue remodelling, although this dual role is still controversial. On the one hand, GSK-3 is the main negative regulator of β-catenin of which increased activation is

associated with fibroproliferative diseases, while on the other hand GSK-3 inhibition may attenuate smad-dependent gene transcription and attenuate fibrotic responses.

Therefore, in the present study, we investigated the effect of GSK-3 inhibition on β-catenin activation, matrix protein expression and extrapulmonary pathology in an established guinea pig model of lipopolysaccharide (LPS)-induced COPD and in human lung fibroblasts, using the selective inhibitor 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763) [14]. LPS is an endotoxin in the outer membrane of gram negative bacteria, that is present as a contaminant in environmental pollution, organic dusts and cigarette smoke, factors that have been associated with COPD development [15, 16]. Furthermore, bacterial endotoxins may contribute to COPD exacerbations, which are episodes of acute worsening of symptoms at times accompanied by an impairment of lung function [17]. Accordingly, we and others have previously demonstrated that LPS can induce pulmonary and extrapulmonary pathological features resembling COPD pathophysiology in various animal models [14, 18, 19].

Materials and methods

Animals
Outbred, male, specified pathogen-free Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom) were used. All protocols describes in this study were approved by the University of Groningen Committee for Animal Experimentation.

Experimental protocol
Thirty-six guinea pigs were randomly assigned to four experimental groups, composed of vehicle treated, saline challenged (n=9); vehicle treated; LPS-challenged (n=9), SB216763 treated, saline challenged (n=9) and SB216763 treated; LPS-challenged (n=9). Guinea pigs were treated twice weekly for 12 consecutive weeks by intranasal instillation of 100 µL SB216763 (2 mM in 10% v/v DMSO in sterile saline) or vehicle (10% DMSO v/v in sterile saline). After the intranasally instilled solution was aspirated, the animals were kept in an upright position for an additional 2 minutes, to allow sufficient spreading of the fluid throughout the lungs. Thirty minutes after the instillations of SB216763 or vehicle, the animals were intranasally instilled with 100 µL LPS (10 mg/ml in sterile saline) or sterile saline. Twenty four hours after the last instillation, the guinea pigs were killed by experimental concussion, followed by rapid exsanguination. The heart, lungs and specific skeletal muscles of each animal were resected and kept on ice for immediate further processing.

Tissue processing and histological analyses
The left lung lobe was inflated and fixed with formalin at a constant pressure of 25 cm H_2O for 24 hours. The formalin fixed lungs were embedded in paraffin and subsequently cut in tissue-sections of 4 µm. The mean linear intercept (LMI), a measure for alveolar airspace size, was determined in tissue-sections stained with haematoxylin and eosin. The LMI was determined as described previously [14], by using 20-25 photo-microscopic images (magnification 200x) per animal. The LMI analysis was performed twice by two individuals in a blinded manner. For evaluation of airway collagen, the tissue-sections (4 µm) were stained with Sirius Red and counterstained with haematoxylin. Airways were digitally photographed (100-200 X magnification) and using ImageJ software, each image was split into RGB channels. The green channel images were used for further analysis and converted to binary images using the threshold function setting the threshold value identical for all images. The positively stained area in the airway wall, from adventitial border to the basement membrane, was digitally quantified in at least 2 airways per animal. The airway collagen area was then normalized to the squared basement membrane length.

The upper right lung lobe was immediately frozen in liquid nitrogen after resection. Transverse frozen sections (4 µM) of the right lung lobe were used for immunohistochemical analysis. The smooth muscle area was identified using immunohistochemical staining for smooth-muscle-specific myosin heavy chain (sm-MHC; dilution 1:100, Neomarkers, Fremont, CA, USA). For identification of activated β-catenin, sections were stained for non-phosphorylated β-catenin (dilution 1:50; Active β-catenin; ABC clone 8E7, Millipore, Amsterdam, The Netherlands). The specific primary antibodies were visualized by using horseradish peroxidase (HRP)-linked secondary antibodies, followed by a 3-amino-9-ethylcarbazole (AEC) staining (for active β-catenin) according to the manufacturer's instructions (AEC chromogen kit, Sigma, St. Louis, MO, USA) or a diaminobenzidine staining (0.1 mg/ml for sm-MHC). Sections were counter stained with haematoxylin. The airways within sections were digitally photographed (200 X magnification) and classified as cartilaginous or non-cartilaginous. All immunohistochemical analyses were performed using ImageJ software. Per animal, at least 2 lung sections were prepared per staining, each section containing 2-5 airways. The sm-MHC positively stained area was digitally quantified and normalized to the squared basement membrane length.

Immunoblotting

The lower right lung lobe was used for protein analysis by immunoblotting. Lung homogenates were prepared by pulverizing the frozen tissue under liquid nitrogen, after which 300 mg tissue was sonicated in 1 ml of ice-cold radio-immunoprecipation (RIPA) buffer supplemented with protease and phosphatase inhibitors (composition: Tris-HCl 50.0 mM, NaCl 150.0 mM, EDTA 1.0 mM, $Na3VO_4$ 1.0 mM, NaF 1.0 mM, Na-deoxycholate 0.25 % and 1% Igepal (NP-40), supplemented with 5 mM β-glycerophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 10 µg/ml pepstatin; at pH 7.4).

Equal amounts of protein (50 µg/lane) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analyzed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisistion software (Syngene; Cambridge; UK). Band intensities were quantified by densitometry using GeneTools analysis software (Syngene; Cambridge; UK).

Measurement of skeletal muscle atrophy
Skeletal muscles from the hind limbs were prepared free of surrounding tissue The plantaris, extensor digitorum longus (EDL), tibialis, soleus and gastrocnemius muscles were collected by standardized dissection methods and subsequently weighed [20].

Cell culture
MRC-5 lung fibroblasts [21] (ATCC CCL 171) were cultured in Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM L-glutamine, 100 mg/l streptomycin and 100 U/ml penicillin. Unless otherwise specified, for each experiment cells were grown to confluence and subsequently culture medium was substituted with Ham's F12 medium supplemented with 0.5% (v/v) FBS, 2 mM L-glutamine, 100 mg/l streptomycin and 100 U/ml penicillin for a period of 24 hours. Cells were stimulated for different time-points with TGF-β_1 in Ham's F12 medium supplemented with 0.5% FBS, L-glutamine and antibiotics. When applied, the pharmacological inhibitor SB216763 was added 30 minutes before the addition of TGF-β_1. To obtain whole cell lysates from MRC human lung fibroblasts, cells were first washed after stimulation with ice-cold Hank's Balanced Salt Solution (HBSS; composition [mg/l]: KCl 400, KH_2PO_4 60, NaCl 8000, $NaHCO_3$ 350, $Na_2HPO_4.1H_2O$ 50, glucose 1000, pH: 7.4). The cells were lysed in ice-cold sodiumdodecylsulphate (SDS) buffer (composition: 62.5 mM Tris, 2% w/v SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 7 mg/ml pepstatin A, pH 6.8). Protein concentration was determined by Pierce protein determination according to the manufacturer's instructions. Lung homogenates were stored at -80 ^oC till further use.

siRNA transfection
MRC-5 fibroblasts were grown to 90% confluence in 6-well cluster plates and transiently transfected with double stranded siRNA targeted against the GSK-3 transcript, which targets both GSK-3α and GSK-3β (Santa Cruz biotechnology, CA, USA). Cells were transfected in serum-free Ham's F12 without any supplements using 200 pmol of siRNA in combination with lipofectamine 2000 transfection reagent. Control transfections were performed using a non-silencing control siRNA (Qiagen, Venlo, The Netherlands). After 6 hours of transfection, cells were washed once with warm (37^oC) HBSS followed by a period of 24 hours in Ham's F12 supplemented with 0.5% FBS, L-glutamine and antibiotics. Subsequently, medium was refreshed and cells were stimulated with TGF-β_1 (2 ng/ml) for 48 hours. The cells were lysed in ice-cold SDS buffer. Protein concentration was determined by Pierce protein determination according to the manufacturer's instructions.

Antibodies and reagents

The mouse anti-smooth-muscle-specific myosin heavy chain (sm-MHC) antibody was from Neomarkers (Fremont, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody, HRP-conjugated goat anti-rabbit antibody, HRP-conjugated rabbit anti-goat antibody and lipopolysaccharides (LPS) from *Escherichia coli* (055:B5) were purchased from Sigma (St. Louis, MO, USA). Mouse anti-GSK-3 antibody, goat anti-fibronectin (C20) antibody and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-phospho-Ser9/21-GSK-3 antibody was from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-total β-catenin antibody was from BD Biosciences (San Jose, CA, USA). Mouse anti-non-phosphorylated-β-catenin antibody (clone 8E7) was from Millipore (Amsterdam, the Netherlands). The selective GSK-3 inhibitor 3-(2,4-Dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763) was obtained from Tocris Cookson (Bristol, UK). Recombinant human TGF-β_1 was from R&D systems (Abingdon, UK). All other chemicals were of analytical grade.

Statistical analysis

Data represent means ± S.E.M, from *n* separate experiments. Statistical significance of differences was evaluated by one-way ANOVA followed by a Newman-Keuls multiple comparison test. Differences were considered to be statistically significant when $p<0.05$.

Results

Effect of repeated LPS-instillation and pharmacological inhibition of GSK-3 on β-catenin activation

First, we investigated the activation of β-catenin signaling in whole lung homogenates in response to repeated intranasal instillation of LPS. The endotoxin LPS clearly induced the expression of active β-catenin in whole lung homogenates compared to the saline treated animals (figure 1A and B). Immunohistological analysis revealed that LPS activated β-catenin signaling in both the large (cartilaginous) and smaller (non-cartilaginous) airways, particularly in the epithelial cells (figure 1C). The selective GSK-3 inhibitor SB216763 did not significantly affect the expression of the active form of β-catenin compared to that in saline treated animals (figure 1A and B). Unexpectedly however, selective inhibition of GSK-3 attenuated the LPS-induced expression of β-catenin to levels comparable to those in saline treated animals (figure 1A and B).

GSK-3 is considered a constitutively active kinase, which is inhibited upon serine phosphorylation (i.e. ser9 on GSK-3β and ser21 on GSK-3α). The phospho-serines act as a pseudo-substrate for the kinase itself, thereby competitively preventing the accessibility of other substrates to the active site of the kinase [6]. LPS did not induce the inhibitory serine phosphorylation of GSK-3 in whole lung homogenates. Treatment with SB216763 had no effect on GSK-3 phosphorylation either in saline or LPS-exposed animals (figure 1A and 1D).

Effect of GSK-3 inhibition on β-catenin expression in lung fibroblasts

In view of the unexpected observation that LPS-induced β-catenin was attenuated by GSK-3 inhibition, we aimed to confirm this effect in *in vitro* experiments. We investigated the effect of the selective GSK-3 inhibitor SB216763 on TGF-β-induced β-catenin expression in MRC-5 human lung fibroblasts. Cells were stimulated with TGF-β_1 resulting in a time-dependent induction of both total and non-phosphorylated (active) β-catenin expression (figure 2A-D). Non-phosphorylated β-catenin was already significantly induced after 24 hours of TGF-β_1 stimulation (figure 2A), whereas 48 hours of stimulation resulted in a profound increase in total β-catenin expression (figure 2C and 2D). Pharmacological inhibition of GSK-3 by SB216763 (10 μM) largely attenuated the induction of active and total β-catenin induced by TGF-β_1 in MRC-5 fibroblasts (figure 2A-D). Moreover, silencing of GSK-3 expression by specific siRNA also attenuated the TGF-β-induced expression of active β-catenin in these cells (figure 2E). These findings in human lung fibroblasts corroborate the *in vivo* findings.

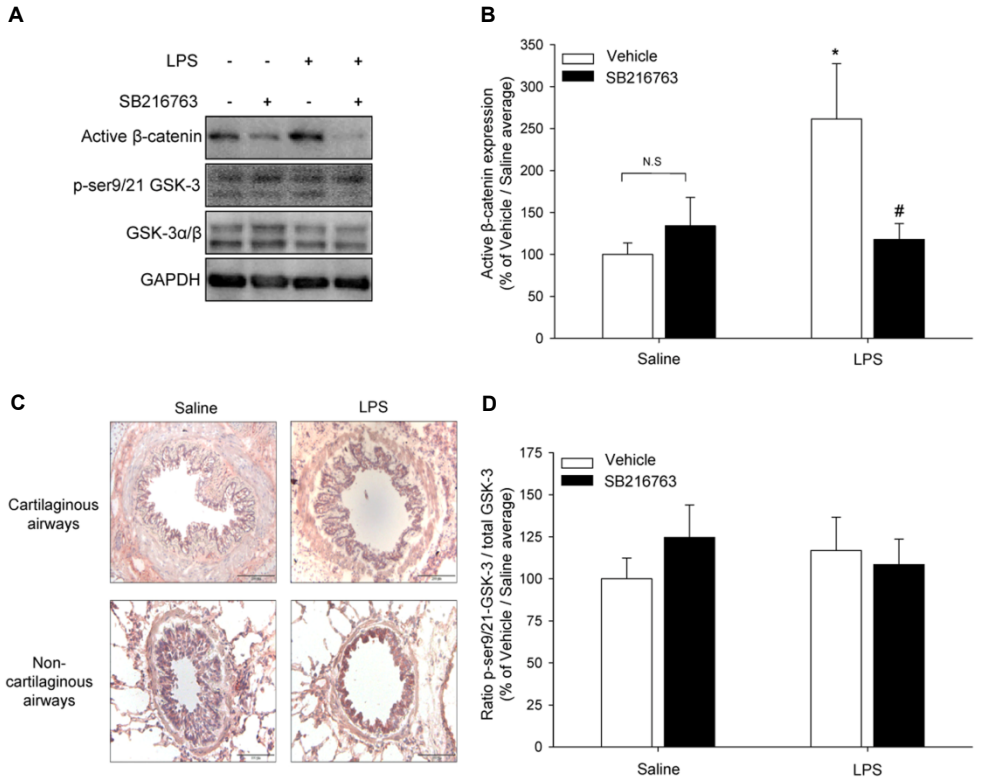
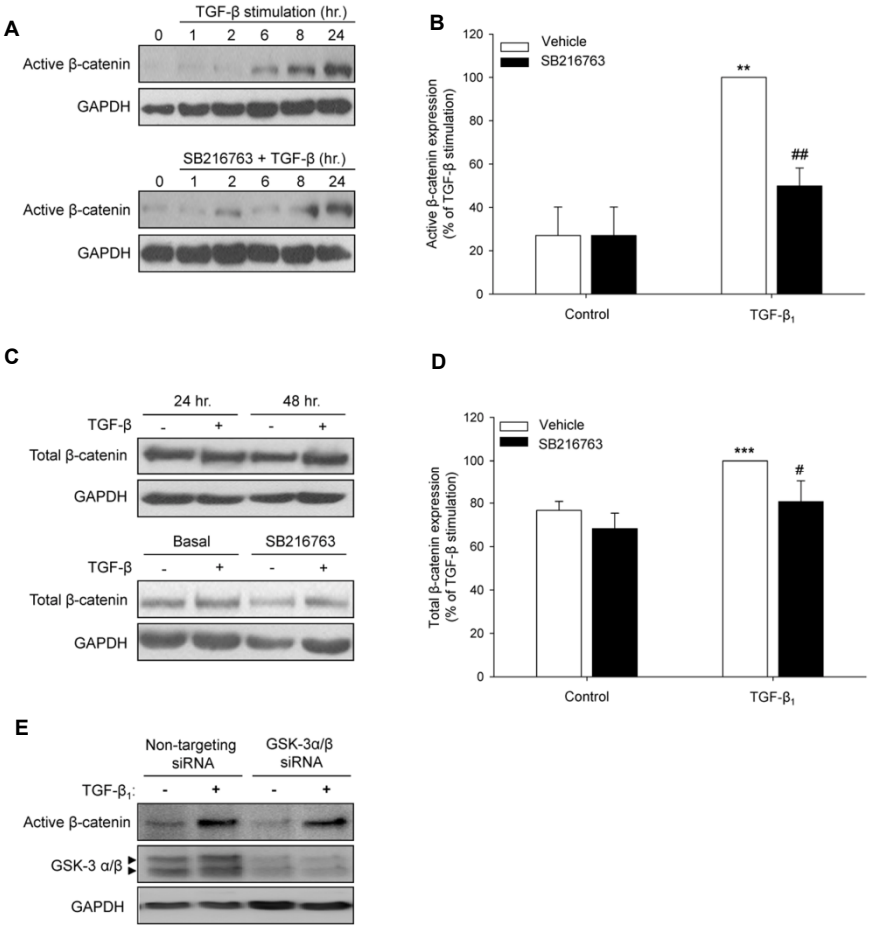


Figure 1: Activation of β-catenin in response to repeated intranasal LPS challenge is prevented by treatment with the selective GSK-3 inhibitor SB216763. (A) Expression of active β-catenin, phosphorylated GSK-3 (ser9/21 GSK-3) and total GSK-3 was evaluated in whole lung homogenates 24 hours after the last challenge by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. (B) Responses of repeated LPS challenge and SB216763 treatment on active β-catenin expression were quantified by densitometry, representing mean ± s.e.m. of 9 animals per group. (C) Immunohistological analysis of active β-catenin (red) in large (cartilaginous) and small (non-cartilaginous) airways of saline and LPS treated animals. (D) The effect of repeated LPS challenge and SB216763 treatment on GSK-3 phosphorylation was quantified by densitometry, representing mean ± s.e.m. of 9 animals per group. *p<0.05 compared to vehicle/saline treated animals (control group) and $^{\#}$p<0.05 compared to vehicle/LPS treated animals (LPS group).



▲ ***Figure 2: Activation of β-catenin in pulmonary fibroblasts in response to TGF-β stimulation is prevented by treatment with the selective GSK-3 inhibitor SB216763.*** MRC5 human lung fibroblasts were stimulated for various time-points with TGF-β (2 ng/ml) in the presence or absence of the selective GSK-3 inhibitor SB216763 (10 μM). (A-B) Expression of active β-catenin was evaluated in whole cell lysates by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. Responses of 24 hours (active β-catenin) and 48 hours (total β-catenin) were quantified by densitometry, representing mean ± s.e.m. of 3-4 independent experiments. (C) *Upper panel:* Expression of total β-catenin was evaluated in whole cell lysates after 24 and 48 hours of TGF-β stimulation by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. *Lower panel:* Expression of total β-catenin was evaluated in whole cell lysates after 48 hours of TGF-β stimulation in the presence or absence of SB216763 (10 μM). Equal protein loading was verified by the analysis of GAPDH. (D) Responses of 48 hour stimulation were quantified by densitometry, representing mean ± s.e.m. of 7-11 independent experiments. (E) Subconfluent MRC-5 lung fibroblast cultures were transfected with a siRNA against the GSK-3α/β transcript. Control cultures were transfected with a non-targeting control siRNA. Transfected cells were treated with TGF-β_1 (2 ng/ml) for 48 hours. Expression of active β-catenin and GSK-3 was evaluated in whole cell lysates and equal protein loading was verified by the analysis of GAPDH. **p<0.01 compared to vehicle treated cells (control) and $^{\#\#}$p<0.01 compared to vehicle/LPS treated animals (LPS group).

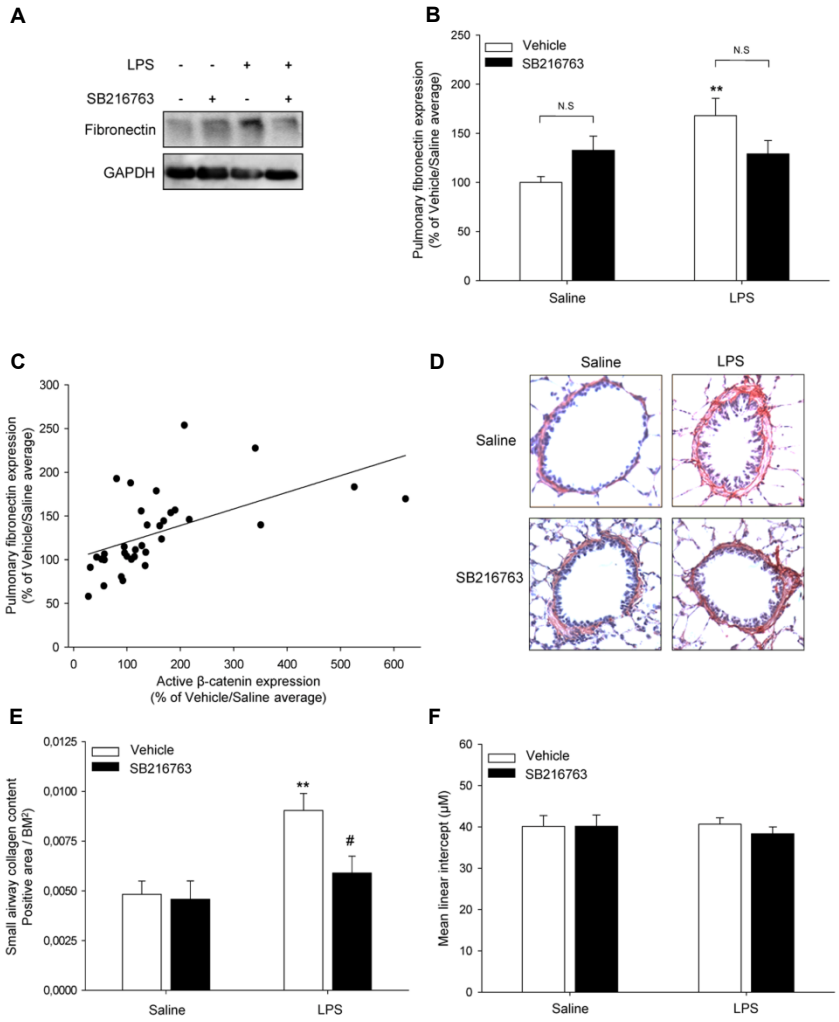
Effect of repeated LPS instillation and GSK-3 inhibition on extracellular matrix turnover in the lungs

To evaluate the effects of repeated LPS instillation on airway fibrosis, the lungs of the guinea pigs were analyzed for the expression of the extracellular matrix proteins fibronectin and collagen. Repeated LPS instillation causes a significant upregulation of fibronectin expression in whole lung homogenates (figure 3A and B). Pulmonary fibronectin expression appeared to be upregulated by GSK-3 inhibition; however, this was not statistically significant. Interestingly, fibronectin expression after repeated LPS instillation and treatment with SB216763 was similar to the effect of treatment with just SB216763. Statistical analysis revealed a trend towards a negative interaction between the effect of SB216763 and of LPS ($p=0.052$, determined by two-way ANOVA).

Fibrotic changes in the lungs may be due to activation of β-catenin signaling [8, 12, 22]. Therefore, we analyzed the correlation between active β-catenin expression and the amount of fibronectin in whole lung homogenate (figure 3C). A significant linear correlation ($R= 0.552$; $p<0.001$) exists between the presence of active β-catenin and pulmonary fibronectin expression (for both parameters the average of vehicle/saline treated animals was set to 100%).

Next, we determined the expression of collagen in non-cartilaginous airways by quantitative analysis of Sirius Red staining in these airways. Similar to the increase in pulmonary fibronectin expression, repeated LPS instillation increased small airway collagen content by 1.88 ± 0.18 fold compared to the average of the saline treated animals (figure 3D and E). Topical treatment of the airways with intranasally instilled SB216763 fully inhibited the LPS-induced increase in collagen deposition in the walls of the small airways, whereas the selective GSK-3 inhibitor did not affect the collagen content in saline treated animals (figure 3D and E).

Emphysema, a pathological feature defined by the loss of the alveolar structure and increased parenchymal airspaces may be caused by tissue destruction in combination with an impaired repair process within the parenchyma [16]. To evaluate the effect of GSK-3 inhibition on the size of the alveolar airspaces, the mean linear intercept (MLI) was determined in paraffin-embedded lung sections. Repeated LPS instillation for 12 weeks did not significantly affect the MLI and, more importantly, inhibition of GSK-3 by SB216763 did not affect the size of the alveolar airspaces in either saline- or LPS-instilled animals (figure 3F). Collectively, this indicates that repeated instillation of LPS induces alterations in pulmonary extracellular matrix expression and that inhibition of GSK-3 is beneficial in attenuating small airway fibrosis without affecting alveolar airspace size.



▲ ***Figure 3: Effect of repeated intranasal LPS challenge and treatment with the selective GSK-3 inhibitor SB216763 on extracellular matrix deposition in the lung.*** (A) Expression of fibronectin was evaluated in whole lung homogenates 24 hours after the last challenge by immunoblotting using specific antibodies. Equal protein loading was verified by the analysis of GAPDH. (B) Effects of repeated LPS challenge and SB216763 treatment on fibronectin expression were quantified by densitometry, representing mean ± s.e.m. of 9 animals per group. (C) Correlation between pulmonary fibronectin and active β-catenin expression. For both parameters the average of saline/saline treated animals is set to 100%. (D-E) Histological staining of the extracellular matrix protein collagen using sirius red. The non-cartilaginous airways were digitally photographed (100-200 X magnification) and analyzed by using ImageJ software. Effects of repeated LPS challenge and SB216763 treatment on airway collagen expression were quantified, representing mean ± s.e.m. of 9 animals per group. (E) The mean linear intercept (LMI), a measure for alveolar airspace size, was determined by staining the tissue-sections with haematoxylin and eosin. Data represent means ± s.e.m. of 9 animals per group. **p<0.01 compared to vehicle/saline treated animals (control group) and $^{\#}$p<0.05 compared to vehicle/LPS treated animals (LPS group).

Repeated LPS instillation and GSK-3 inhibition do not affect smooth muscle content *in vivo*

The airway smooth muscle content in cartilaginous and non-cartilaginous airways was determined by staining transverse frozen lung sections for the specific marker smooth-muscle myosin heavy chain (sm-MHC). Representative photomicrographs of serial lung sections containing the larger (cartilaginous) and small (non-cartilaginous) airways are shown in figure 4A and B. Morphometric analysis revealed that neither repeated LPS instillation nor SB216763 treatment affected the smooth muscle content in either the cartilaginous or the non-cartilaginous airways (figure 4C and D).

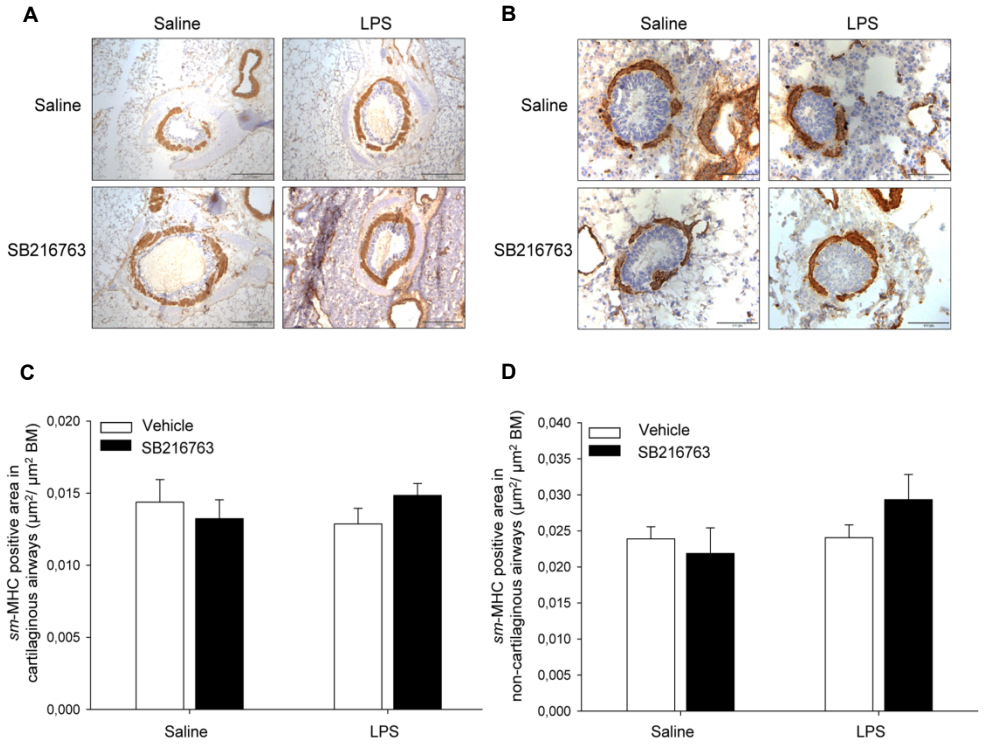


Figure 4: Repeated LPS instillation and pharmacological inhibition of GSK-3 by SB216763 does not affect airway smooth muscle content. Immunohistological analysis of sm-MHC positive area in (A) large (cartilaginous) and (B) small (non-cartilaginous) airways. (C-D) Effects of repeated LPS challenge and SB216763 treatment on airway sm-MHC expression were quantified, representing mean ± s.e.m. of 9 animals per group.

Extrapulmonary effects of repeated LPS instillation

By definition, COPD is a disease with significant extrapulmonary pathology that contributes to disease severity [16]. Two well-known co-morbidities in COPD are the occurrence of pulmonary hypertension resulting in alterations in structure and function of the right ventricle of the heart and skeletal muscle weakness [23-25].

Repeated LPS challenge induced right ventricle hypertrophy in the guinea pigs as indicated by a significant 1.48 ± 0.13-fold increase in the ratio of right ventricle weight over total heart weight compared to saline treated animals (figure 5A). SB216763 fully prevented the LPS-induced right ventricle hypertrophy; whereas the selective GSK-3 inhibitor did not have an effect in saline treated animals (figure 5A). Neither LPS nor SB216763 influenced the increase in body weight of the guinea pigs during the experimental procedures (figure 5B)

In addition to the cardiovascular alterations, we investigated if LPS induced changes in skeletal muscle weight. The plantaris, extensor digitorum longus (EDL), tibialis, soleus and gastrocnemius muscles from the hind limbs of the animals were collected and independently weighed. Repeated instillation of LPS resulted in a modest decrease in the weight of the extensor digitorum longus (EDL), tibialis and the gastrocnemius muscle, although this decrease was not significant (figure 5C-G). Pulmonary administration of the selective GSK-3 inhibitor SB216763 appeared to prevent the LPS-induced alterations in these skeletal muscles.

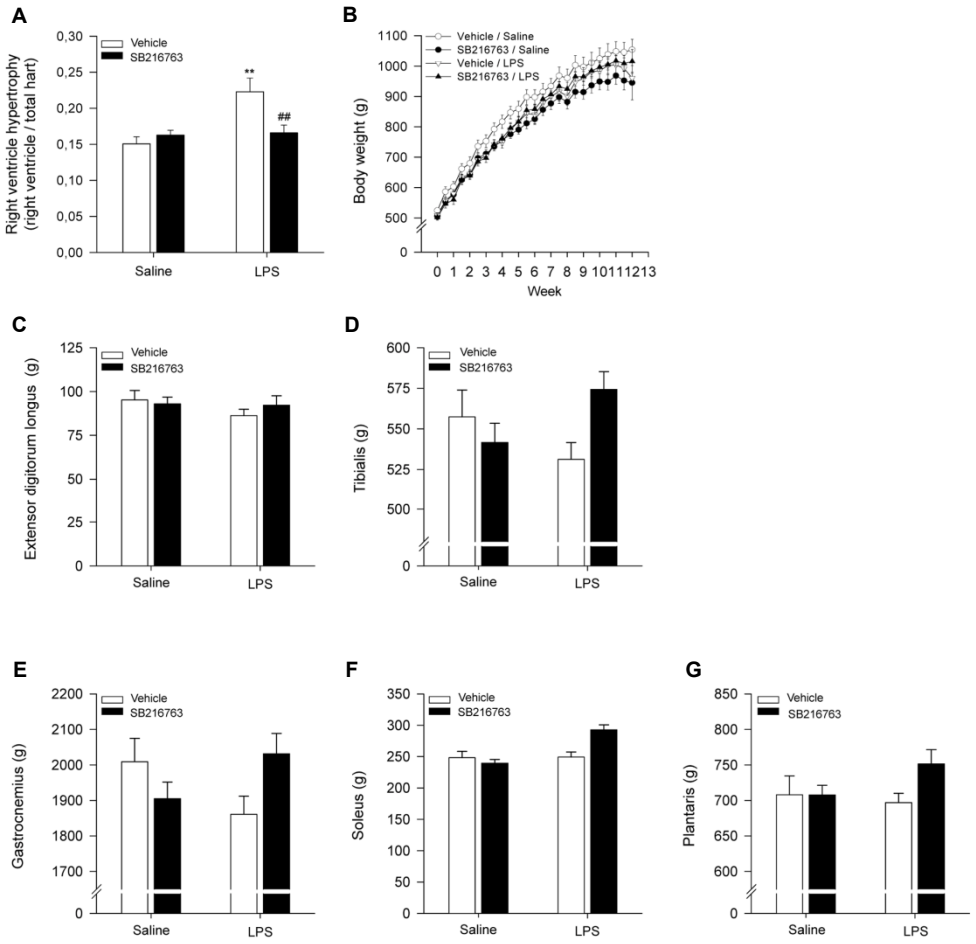


Figure 5: Extrapulmonary effects of repeated intranasal LPS instillation are attenuated by GSK-3 inhibition. (A) Effect of repeated LPS instillation and GSK-3 inhibition by SB216763 on right ventricle hypertrophy. Effects of repeated LPS challenge and SB216763 treatment on size of right ventricle were quantified, representing mean ± s.e.m. of 9 animals per group. (B) Body weight of guinea pigs during the experimental procedures, representing mean ± s.e.m. of 9 animals per group. (C-G) Effects of repeated LPS instillation and GSK-3 inhibition (SB216763) on skeletal muscle weight; (C) extensor digitorum longus (EDL), (D) tibialis, (E) gastrocnemius, (F) soleus and (G) plantaris muscles. Results represent mean ± s.e.m. of 9 animals per group. **p<0.01 compared to vehicle/saline treated animals (control group) and $^{\#\#}$p<0.01 compared to vehicle/LPS treated animals (LPS group).

Discussion

In this study, we demonstrate that glycogen synthase kinase-3 (GSK-3) signaling significantly contributes to the development of pathological features in an animal model of COPD. In this animal model, repeated intranasal LPS instillation induced the activation of β-catenin signaling and remodelling with an increase in pulmonary fibronectin expression and enhanced collagen content in the smaller, non-cartilaginous airways. Furthermore, we demonstrate that the intranasal instillation of LPS has extrapulmonary effects as it induces right ventricle hypertrophy and tends to reduce the weight of specific skeletal muscles. Unexpectedly, pharmacological inhibition of GSK-3 by topical administration of the small molecule inhibitor SB216763 prevented the LPS-induced activation of β-catenin signaling. *In vitro* studies confirmed this finding and indicated that SB216763 prevented TGF-β-induced β-catenin signaling in pulmonary fibroblasts. Further, *in vivo* treatment with SB216763 prevented the small airway remodelling, right ventricle hypertrophy and skeletal muscle atrophy, and had no detrimental effect on alveolar airspace size or airway smooth muscle content. Collectively, these data indicate that GSK-3 plays a paradoxical dual role in β-catenin signaling and may be a beneficial therapeutic target in chronic obstructive airway diseases like COPD.

Airway fibrosis is a characteristic feature of COPD, which contributes to airway wall thickening and airflow limitation [26]. We demonstrate that repeated LPS instillation resulted in increased expression of the extracellular matrix proteins fibronectin and collagen. The pulmonary expression of fibronectin significantly correlated to the protein level of activated β-catenin, which was predominantly present in the epithelial cells lining the airways and the submucosa. We and others have previously shown that pulmonary fibronectin expression is regulated by canonical WNT/β-catenin signaling [22, 27, 28]. Activation of β-catenin is important in normal wound healing, however aberrant activation of this transcriptional co-activator has been associated with various fibroproliferative diseases, including chronic lung diseases [7, 8, 29]. β-Catenin may directly be responsible for the transcription of *fibronectin*, via its interaction with T-cell-factor/lymphoid enhancer factor (TCF/LEF) transcription factors [30]. Furthermore, β-catenin may also increase fibronectin expression in an indirect manner by up regulating TGF-β expression and subsequent activation of smad signaling [31]. Further analysis revealed that LPS also induces small airway fibrosis as determined by collagen content in the non-cartilaginous airways. Pharmacological inhibition of GSK-3 by topical administration of SB216763 prevented the LPS-induced collagen expression. These findings are consistent with those of Kneidinger and colleagues, who showed that intraperitoneal administration of the GSK-3 inhibitor LiCl was capable of decreasing pulmonary collagen expression in a murine model of elastase-induced emphysema

[32]. Furthermore, the selective GSK-3 inhibitor SB216763 has been demonstrated to attenuate pulmonary fibrosis induced by bleomycin [13]. In the same study it was shown that attenuation of the fibrogenic processes upon GSK-3 inhibition occurred independently of the inflammatory response, suggesting a direct effect of GSK-3 on cells regulating the fibrotic response [13, 32]. In line with this contention, we have previously shown that GSK-3 inhibition or silencing of the kinase by siRNA attenuates specific cellular responses in pulmonary fibroblasts (chapter 4). Pharmacological inhibition of GSK-3 by SB216763 resulted in an increase in ser133 cyclic adenosine 3'5' monophosphate (cAMP) response element binding protein (CREB) phosphorylation in pulmonary fibroblasts, which was associated with inhibition of functional TGF-β signaling (chapter 4). In various cells it has been demonstrated that smad-dependent signaling can be functionally antagonized by activation of CREB, which provides an explanation for the inhibitory effects of SB216763 on TGF-β signaling and airway fibrosis [33-36] (chapter 4). Unfortunately, due to lack of availability of phospho-serine133 specific antibodies against guinea pig CREB, it was not possible to determine the phosphorylation status of CREB in our studies. Nonetheless, GSK-3 mediated regulation of CREB and smad-dependent signaling appears a plausible explanation for the paradoxical inhibition of LPS and TGF-β induced β-catenin expression and subsequent matrix protein production by SB216763. Growth factors, including TGF-β, regulate cellular β-catenin expression by smad mediated gene-transcription in addition to GSK-3 dependent posttranslational effects on β-catenin protein stability [37, 38]. In support, TGF-β induced β-catenin expression could be inhibited by smad 3 inhibition in pulmonary fibroblasts (data not shown). Collectively, the currently presented data indicate that *in vivo* activation of β-catenin signaling is associated with an increase in the pulmonary extracellular matrix deposition, whereas selective inhibition of GSK-3 prevents this LPS-induced process. The inhibition of β-catenin activation may occur through increased CREB signaling and subsequent functional antagonism of smad mediated β-catenin gene transcription, but this hypothesis needs further investigation in future studies.

In addition to fibrosis, increased smooth muscle content in the airways may be part of the airway remodelling, contributing to COPD pathophysiology [39]. It is important to note, that alterations in airway smooth muscle content are observed in individuals with very severe COPD only [40]. In our guinea pig model of LPS-induced COPD, we did not observe alterations in smooth muscle content, as determined by sm-MHC positive area, in either the large (cartilaginous) or smaller (non-cartilaginous) airways, which is in agreement with previously published findings in this model [14]. Of interest is that smooth muscle mass did not change in response to GSK-3 inhibition either. Published findings indicate that growth factor induced inhibition of GSK-3 promotes airway smooth muscle cell proliferation

and hypertrophy [41-43]. Further, airway smooth muscle growth in response to allergen exposure correlates with GSK-3 inactivation in airway smooth muscle in mice [44]. The observation that pharmacological inhibition of GSK-3 using SB216763 is not sufficient to promote airway smooth muscle growth is therefore reassuring and provides further support for the suitability of GSK-3 as a drug target for the treatment of COPD.

COPD is a disease with significant extrapulmonary effects that contribute to disease severity [16]. Pulmonary hypertension causing alterations in structure and function of the right ventricle of the heart, and skeletal muscle weakness are well-known co-morbidities in COPD [23-25]. Therefore, we investigated right ventricle size and skeletal muscle weight in response to repeated LPS instillation. LPS induced right ventricle hypertrophy, which was fully prevented by SB216763. This indicates that GSK-3 contributes to this pathological feature and therefore possibly to the development of pulmonary hypertension. Although investigations on the underlying mechanisms were not part of the design of the current study, it is well known that both vascular remodelling and functional changes in the vessel wall may lead to increased resistance in the pulmonary vasculature, causing pulmonary hypertension [23]. In addition, we demonstrate that LPS instillation may induce loss of functional skeletal muscle a process which may involve systemic inflammation [45]. The modest non-significant alterations in skeletal muscle weight due to LPS treatment were apparently prevented by SB216763 (non-significant). This may be due to attenuated inflammatory responses in response to GSK-3 inhibition [46-49]. In addition, GSK-3 signaling has been demonstrated to be directly involved in both basal and stimulus induced atrophy of skeletal muscle [50], suggesting that prevention of muscle atrophy may have been a direct effect or due to systemic effects of the inhibitor. Collectively, we show that GSK-3 inhibition prevents extrapulmonary effects induced by repeated LPS instillation.

Taken together, this study demonstrates that topical application of the selective GSK-3 inhibitor SB216763 is capable of preventing both pulmonary remodelling and extrapulmonary effects in a guinea pig model of COPD. Although the exact mechanism(s) underlying these effects remains to be established, we provided evidence that the anti-remodelling properties of the drug may be related to attenuation of β-catenin activation. In conclusion, our findings suggest that inhibition of GSK-3 may provide a novel means for the treatment of chronic airway diseases, such as COPD.

Acknowledgements
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References

1. Woodgett JR (1990) Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 9: 2431-2438.
2. Woodgett JR (1991) cDNA cloning and properties of glycogen synthase kinase-3. Methods Enzymol 200: 564-577.
3. Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A (2009) Glycogen synthase kinase 3: more than a namesake. Br J Pharmacol 156: 885-898.
4. Embi N, Rylatt DB, Cohen P (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem 107: 519-527.
5. Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359: 1-16.
6. Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29: 95-102.
7. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, et al. (2003) Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. Am J Pathol 162: 1495-1502.
8. Konigshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, et al. (2008) Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One 3: e2142.
9. Konigshoff M, Kramer M, Balsara N, Wilhelm J, Amarie OV, et al. (2009) WNT1-inducible signaling protein-1 mediates pulmonary fibrosis in mice and is upregulated in humans with idiopathic pulmonary fibrosis. J Clin Invest 119: 772-787.
10. Postma DS, Timens W (2006) Remodeling in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 3: 434-439.
11. Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65: 391-426.
12. Baarsma HA, Spanjer AI, Haitsma G, Engelbertink LH, Meurs H, et al. (2011) Activation of WNT / beta-Catenin Signaling in Pulmonary Fibroblasts by TGF-beta(1) Is Increased in Chronic Obstructive Pulmonary Disease. PLoS One 6: e25450.
13. Gurrieri C, Piazza F, Gnoato M, Montini B, Biasutto L, et al. (2010) 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), a glycogen synthase kinase-3 inhibitor, displays therapeutic properties in a mouse model of pulmonary inflammation and fibrosis. J Pharmacol Exp Ther 332: 785-794.
14. Pera T, Zuidhof A, Valadas J, Smit M, Schoemaker RG, et al. (2011) Tiotropium inhibits pulmonary inflammation and remodelling in a guinea pig model of COPD. Eur Respir J 38: 789-796.
15. Eduard W, Pearce N, Douwes J (2009) Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 136: 716-725.
16. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, et al. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 176: 532-555.
17. Veeramachaneni SB, Sethi S (2006) Pathogenesis of bacterial exacerbations of COPD. COPD 3: 109-115.
18. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, Wouters EF (2002) Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. Am J Respir Cell Mol Biol 26: 152-159.
19. Toward TJ, Broadley KJ (2002) Goblet cell hyperplasia, airway function, and leukocyte infiltration after chronic lipopolysaccharide exposure in conscious guinea pigs: effects of rolipram and dexamethasone. J Pharmacol Exp Ther 302: 814-821.
20. Gosker HR, Langen RC, Bracke KR, Joos GF, Brusselle GG, et al. (2009) Extrapulmonary manifestations of chronic obstructive pulmonary disease in a mouse model of chronic cigarette smoke exposure. Am J Respir Cell Mol Biol 40: 710-716.
21. Jacobs JP, Jones CM, Baille JP (1970) Characteristics of a human diploid cell designated MRC-5. Nature 227: 168-170.
22. Baarsma HA, Menzen MH, Halayko AJ, Meurs H, Kerstjens HA, et al. (2011) β-Catenin signaling is required for TGF-β1-induced extracellular matrix production by airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol .

23. MacNee W (2010) Right heart function in COPD. Semin Respir Crit Care Med 31: 295-312.
24. Reid WD, Rurak J, Harris RL (2009) Skeletal muscle response to inflammation--lessons for chronic obstructive pulmonary disease. Crit Care Med 37: S372-S383.
25. Agusti AG, Sauleda J, Miralles C, Gomez C, Togores B, et al. (2002) Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 166: 485-489.
26. Barnes PJ, Shapiro SD, Pauwels RA (2003) Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur Respir J 22: 672-688.
27. Baarsma HA, Engelbertink LHJM, Haitsma G, Postma DS, Timens W, et al. (2010) Wnt / β-catenin signaling regulates TGF-β-induced differentiation of lung fibroblasts. Am J Respir Crit Care Med 181: A2080.
28. De Langhe SP, Sala FG, Del Moral PM, Fairbanks TJ, Yamada KM, et al. (2005) Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung. Dev Biol 277: 316-331.
29. Bowley E, O'Gorman DB, Gan BS (2007) Beta-catenin signaling in fibroproliferative disease. J Surg Res 138: 141-150.
30. Gradl D, Kuhl M, Wedlich D (1999) The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. Mol Cell Biol 19: 5576-5587.
31. Carthy JM, Garmaroudi FS, Luo Z, McManus BM (2011) Wnt3a Induces Myofibroblast Differentiation by Upregulating TGF-beta Signaling Through SMAD2 in a beta-Catenin-Dependent Manner. PLoS One 6: e19809.
32. Kneidinger N, Yildirim AO, Callegari J, Takenaka S, Stein MM, et al. (2011) Activation of the WNT/beta-catenin pathway attenuates experimental emphysema. Am J Respir Crit Care Med 183: 723-733.
33. Gotschel F, Kern C, Lang S, Sparna T, Markmann C, et al. (2008) Inhibition of GSK3 differentially modulates NF-kappaB, CREB, AP-1 and beta-catenin signaling in hepatocytes, but fails to promote TNF-alpha-induced apoptosis. Exp Cell Res 314: 1351-1366.
34. Grimes CA, Jope RS (2001) CREB DNA binding activity is inhibited by glycogen synthase kinase-3 beta and facilitated by lithium. J Neurochem 78: 1219-1232.
35. Schiller M, Dennler S, Anderegg U, Kokot A, Simon JC, et al. (2010) Increased cAMP levels modulate transforming growth factor-beta/Smad-induced expression of extracellular matrix components and other key fibroblast effector functions. J Biol Chem 285: 409-421.
36. Tullai JW, Chen J, Schaffer ME, Kamenetsky E, Kasif S, et al. (2007) Glycogen synthase kinase-3 represses cyclic AMP response element-binding protein (CREB)-targeted immediate early genes in quiescent cells. J Biol Chem 282: 9482-9491.
37. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, et al. (2006) Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. FASEB J 20: 692-701.
38. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, et al. (2010) De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. FASEB J 24: 757-768.
39. Hogg JC (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 364: 709-721.
40. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, et al. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 350: 2645-2653.
41. Deng H, Dokshin GA, Lei J, Goldsmith AM, Bitar KN, et al. (2008) Inhibition of glycogen synthase kinase-3beta is sufficient for airway smooth muscle hypertrophy. J Biol Chem 283: 10198-10207.
42. Deng H, Hershenson MB, Lei J, Anyanwu AC, Pinsky DJ, et al. (2010) Pulmonary artery smooth muscle hypertrophy: roles of glycogen synthase kinase-3beta and p70 ribosomal S6 kinase. Am J Physiol Lung Cell Mol Physiol 298: L793-L803.
43. Nunes RO, Schmidt M, Dueck G, Baarsma H, Halayko AJ, et al. (2008) GSK-3/beta-catenin signaling axis in airway smooth muscle: role in mitogenic signaling. Am J Physiol Lung Cell Mol Physiol 294: L1110-L1118.
44. Bentley JK, Deng H, Linn MJ, Lei J, Dokshin GA, et al. (2009) Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3(beta) phosphorylation in a mouse model of asthma. Am J Physiol Lung Cell Mol Physiol 296: L176-L184.

45. Haegens A, Schols AM, Gorissen SH, van Essen AL, Snepvangers F, et al. (2012) NF-kappaB activation and polyubiquitin conjugation are required for pulmonary inflammation-induced diaphragm atrophy. Am J Physiol Lung Cell Mol Physiol 302: L103-L110.
46. Dugo L, Collin M, Thiemermann C (2007) Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. Shock 27: 113-123.
47. Jope RS, Yuskaitis CJ, Beurel E (2007) Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. Neurochem Res 32: 577-595.
48. Bao Z, Lim S, Liao W, Lin Y, Thiemermann C, et al. (2007) Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. Am J Respir Crit Care Med 176: 431-438.
49. Cuzzocrea S, Genovese T, Mazzon E, Esposito E, Muia C, et al. (2007) Glycogen synthase kinase-3beta inhibition attenuates the development of bleomycin-induced lung injury. Int J Immunopathol Pharmacol 20: 619-630.
50. Verhees KJ, Schols AM, Kelders MC, Op den Kamp CM, van d, V, et al. (2011) Glycogen synthase kinase-3beta is required for the induction of skeletal muscle atrophy. Am J Physiol Cell Physiol 301: C995-C1007.

Chapter 7

General discussion and summary

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is defined and characterized by persistent airflow limitation that is usually progressive and associated with an enhanced inflammatory response in the airways and the lung to noxious particles and gases [1]. The persistent airflow limitation is the resultant of small airway disease together with parenchymal tissue destruction (emphysema), two pathological features in COPD of which the relative severity can vary from individual to individual [2, 3]. During the course of COPD development, inflammation is an eminent pathological feature that is evident in mild disease and increases with disease severity. Cigarette smoke is the main risk factor associated with disease development, although environmental and genetic factors contribute to the disease susceptibility as well [2]. The molecular mechanisms contributing to the pathophysiology of COPD have not yet been fully elucidated.

WNT-dependent and independent cellular signaling by β-catenin and glycogen synthase kinase-3 (GSK-3)

The primary objective of this thesis was to establish the functional roles of β-catenin and glycogen synthase kinase-3 (GSK-3) in the pathological processes that underpin COPD. Both β-catenin and GSK-3 are critically involved in the complex canonical WNT signaling pathway (**Chapter 1**). In short, activation of this specific pathway is initiated by a WNT ligand (extracellular glycoprotein), which binds to two distinct families of cell surface receptors, namely the frizzled (FZD) receptors and low-density lipoprotein receptor related protein (LRP) receptors. In the absence of an extracellular WNT ligand, cytosolic β-catenin is targeted for degradation by a cytosolic multiprotein complex called the destruction complex. This complex consists of GSK-3, axin, adenomatous polyposis coli (APC) and casein kinase-1 (CK-1). GSK-3 in cooperation with CK-1 is responsible for the phosphorylation of β-catenin, which targets this specific transcriptional co-activator for proteosomal degradation [4-6]. Binding of a WNT ligand to the cell-surface receptors activates a downstream signaling cascade resulting in a disengaged destruction complex and consequently increased stability of cytosolic β-catenin (**Chapter 1**). Subsequently, β-catenin can translocate to the nucleus and associate with T-cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors to induce gene-transcription. Thus, GSK-3 acts in the canonical WNT signaling pathway as a negative regulator of β-catenin [4-6].

In addition to their role in canonical WNT signaling, both proteins are involved in a variety of other cellular functions and signaling pathways. For instance, β-catenin plays an important role in stabilizing cell-cell contacts, being a component of the cadherens-based adherens junctions [7]. This cytoskeletal function of β-catenin is of physiological relevance, as it contributes for example to force generation by

airway smooth muscle cells as well as for the barrier function of the airway epithelium [7, 8]. On the other hand, GSK-3 has been demonstrated to be involved in a myriad of cellular processes independently of its effect on β-catenin signaling. The GSK-3 mediated cellular processes include insulin signaling, microtubule dynamics and cytoskeletal rearrangement, cell differentiation, protein synthesis, cell motility, intracellular vesicular transport, as well as cell proliferation and cell survival [9]. The kinase is an unconventional enzyme as it is considered to be a constitutively active kinase, which means high kinase activity at basal conditions, which is altered in response to a variety of endogenous and exogenous signals [9, 10]. In addition to WNT mediated inhibition of GSK-3, phosphorylation of specific serines (ser9 on GSK-3β and ser21 on GSK-3α) attenuates the kinase activity [10, 11]. GSK-3 regulates over 50 putative substrates, including structural proteins, signaling intermediates and transcription factors. Although both β-catenin and GSK-3 are implicated in various cellular responses, their contribution to chronic airway diseases particular in relation to COPD development and progression required further exploration.

Involvement of β-catenin and GSK-3 signaling in airway remodeling: focus on the airway smooth muscle

One of the two main pathological features in COPD is airway disease, which is predominantly characterized by remodeling of the wall of the peripheral airways, especially the small airways [12, 13]. The airway remodeling in chronic obstructive lung diseases, includes increased airway smooth muscle mass and altered extracellular matrix profile in the airways, which may contribute cooperatively to airway hyperresponsiveness and the airflow obstruction [14-16]. Increased airway smooth muscle mass is associated with decreased lung function in severe asthma and may contribute to COPD pathogenesis, particularly in more severe states of disease [12, 17]. Cigarette smoke, the main risk factor for developing COPD, can have direct effects on the airway wall and cause activation of remodeling processes. For instance, pulsatile exposure of airway smooth muscle cells with soluble components of cigarette smoke results in enhanced proliferation, which may underlie the increase in smooth muscle mass in individuals with COPD [18]. Furthermore, both *in vitro* and *in vivo* experiments have demonstrated that exposure to cigarette smoke results in increased expression of fibrogenic growth factors, including transforming growth factor-β (TGF-β), which very frequently appears to be independent of an inflammatory response [19-22]. Accordingly, the expression of fibrogenic growth factors, like TGF-β, is upregulated in the lung of individuals with COPD [23-27]. An emerging interest in the role of TGF-β in COPD pathogenesis has evolved, as single nucleotide polymorphisms (SNPs) in the *TGF-β* gene are associated with the development of COPD [23-27]. The mechanisms leading to the development and progression of airway remodeling are not well

understood, but airway smooth muscle cells may contribute to the process through cell proliferation and in addition by producing and releasing various inflammatory mediators, growth factors, and extracellular matrix proteins in response to pathogenetic factors [28-32].

Extracellular matrix deposition by the airway smooth muscle

In several chronic lung diseases an altered expression and composition of extracellular matrix proteins within and surrounding the airway smooth muscle bundle has also been observed, which contributes to disease pathogenesis [31, 33]. In COPD, changes in airway smooth muscle mass and altered extracellular matrix deposition are less pronounced compared to asthma, but may become of major importance in more severe stages of disease [12]. The expression of fibronectin, hyaluronan, versican, biglycan, lumican, and specific collagens are increased within as well as surrounding the airway smooth muscle has been observed in chronic airway diseases [33-35]. Accordingly, airway smooth muscle cells show enhanced production of extracellular matrix proteins in response to TGF-β stimulation, which we used as a model in our studies in which we investigated the role of β-catenin signaling in this process (**Chapter 2**). In particular, the expression of collagen III, fibronectin and versican were upregulated in airway smooth muscle cells in response to TGF-β. It was observed that the upregulation of these extracellular matrix proteins was preceded by the activation of β-catenin signaling and accompanied by a synchronous, endogenous inhibition of GSK-3. Pharmacological inhibition of the interaction of β-catenin with the transcription factor TCF-4 by the small molecule PKF115-584 prevented the TGF-β-induced upregulation of the extracellular matrix proteins. Additional evidence that reinforced the involvement of β-catenin in regulating TGF-β-induced extracellular matrix protein expression came from studies using specific small interference RNA (siRNA) to selectively silence β-catenin, the results of which were in full agreement with the findings using PKF115-584. It appears that β-catenin directs TGF-β signaling to specific intracellular pathways, as the expression of the conventional smad-dependent gene *plasminogen activator inhibitor-1* (PAI-1) is not affected by either PKF115-584 or the specific β-catenin siRNA treatment. Moreover, the studies described in **Chapter 2** show that activation of β-catenin signaling is sufficient to activate the transcription of the extracellular matrix proteins by airway smooth muscle cells. This was established by expression of the transcriptionally active, non-degradable β-catenin mutant (S33Y-β-catenin), which induced the protein expression of fibronectin. Collectively, our data indicate that β-catenin signaling is activated in response to the fibrogenic growth factor TGF-β in airway smooth muscle cells. In these cells activation of β-catenin is required and sufficient for activation extracellular matrix production, which could be pharmacologically targeted by using the small molecule PKF115-584. This study contributes to the

understanding of molecular mechanisms that may underlie airway remodeling and in particular the excess of extracellular matrix proteins within and surrounding the smooth muscle layer. The extracellular matrix proteins do not only physically contribute to narrowing of the airway lumen size, but also influences cellular responses of structural cells lining the airway wall, including the airway smooth muscle layer, fibroblasts and epithelial cells [31]. These modified cellular responses due to alterations in extracellular matrix composition may significantly contribute to disease pathogenesis. Furthermore, activation of β-catenin contributes to airway smooth muscle cell proliferation in response to mitogens, like platelet derived growth factor (PDGF) and serum [11]. Because of this, β-catenin contributes once more to airway remodeling by increasing the airway smooth muscle mass. However, the *in vivo* contribution of β-catenin signaling to airway remodeling in individuals with COPD needs still to be elucidated. Nevertheless, β-catenin-mediated extracellular matrix production by airway smooth muscle cells can be pharmacologically targeted and may have potential therapeutic relevance for the treatment of airway remodeling in COPD.

The airway smooth muscle as potential source of cytokines and growth factors
In addition to the contributing to airway remodeling by regulating the ECM composition, the airway smooth muscle may significantly contribute to the persistent inflammatory process, which is an eminent characteristic of COPD. The airway smooth muscle is increasingly recognized as an important cellular source of (pro-inflammatory) cytokines, chemokines, growth factors, enzymes and other mediators in response to various stimuli [32, 36-38]. As mentioned, the main risk factor for development of COPD is chronic pulsatile exposure of structural cells lining the airway and alveoli to cigarette smoke. Components of cigarette smoke, as well as a variety of pro-inflammatory cytokines (e.g. IL-1β) are capable of activating the synthetic function of airway smooth muscle cells (**Chapter 5** and [28, 32, 36-38]). Cultured airway smooth muscle cells produce and release eotaxin, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) in response to soluble components of cigarette smoke (i.e. cigarette smoke extract: CSE) and/or IL-1β stimulation (**Chapter 5**). The secreted proteins potentially contribute to COPD pathogenesis, as the cytokines play an important role in recruiting and activating inflammatory cells, whereas VEGF may contribute to angiogenesis and airway remodeling [32, 39-41]. The exact chemical moiety of cigarette smoke extract responsible for the induction of cytokine and growth factor release by smooth muscle cells is not yet elucidated. Similarly, the molecular pathways underlying this process have also not completely been established. Activation of both the nuclear factor-kappa B (NF-κB) signaling pathway and of β-catenin-dependent gene transcription has been associated with the production of these pro-inflammatory cytokines and growth factor [37, 42-45]. Nevertheless, cigarette

smoke and IL-1β did not activate β-catenin signaling on their own in the cultured airway smooth muscle cells (**Chapter 5**). Since GSK-3 differentially regulates NF-κB pathway activation and β-catenin signaling, it could act as an important regulator in cigarette smoke extract- and cytokine-induced pro-inflammatory signaling in these cells [4, 46, 47]. Pharmacological inhibition of GSK-3 attenuated the production and release of the eotaxin, VEGF and IL-8 by airway smooth muscle cells, although cellular β-catenin expression was increased. Cigarette smoke extract and IL-1β stimulation caused the activation of the NF-κB pathway, as monitored by a decrease in the NF-κB inhibitory protein Iκ-Bα and nuclear translocation of the p65 subunit of NF-κB. Inhibition of GSK-3 did not affect either the degradation of Iκ-Bα or the nuclear translocation, but fully prevented the transcriptional activity of the pathway (**Chapter 5**). Collectively, this indicates that in airway smooth muscle cells GSK-3 signaling significantly contributes to the inflammatory response initiated by (soluble components of) cigarette smoke and cytokines by regulating NF-κB-mediated transcriptional activity. Therefore, inhibition of GSK-3 may be a strategy worth pursuing in reducing airway inflammation in chronic obstructive lung diseases.

Remarkably, inactivation of GSK-3 caused a dose-dependent increase in β-catenin expression in the airway smooth muscle cells, which was not associated with the production and secretion of pro-inflammatory cytokines. However, upregulation of β-catenin expression is closely linked to the production of extracellular matrix proteins by these cells (**Chapter 2**). Therefore, the activation of β-catenin signaling and its corresponding consequences in airway smooth muscle cells may be a possible drawback for the potential therapeutic use of GSK-3 inhibitors. Moreover, airway smooth muscle hypertrophy and hyperplasia, two processes which may be responsible for the increased smooth muscle mass observed in chronic obstructive lung diseases, both correlate to endogenous inactivation of GSK-3 by serine phosphorylation in a murine model of asthma [48]. Yet, *in vivo* studies in animal models of chronic obstructive lung diseases, including COPD, in which GSK-3 is pharmacologically targeted by small molecule inhibitors do not mention or show changes in smooth muscle content and/or enhanced extracellular matrix deposition in the airways (**Chapter 6** and [49, 50]). Rather, some of these studies demonstrated decreased expression of specific extracellular matrix components in the lungs, particularly in the airways. This can possibly be explained by the direct effect of attenuating GSK-3 activity in cells involved in fibrogenic responses or be due to the attenuation to the inflammation in response to GSK-3 inhibition (**Chapters 4, 5** and [47, 49-53]), which will be discussed in more detail further on.

A new paradigm for tissue repair in the COPD affected lung: canonical WNT/β-catenin pathway activation and GSK-3 signaling in pulmonary fibroblasts

Emphysema is another important pathological feature of COPD, which in contrast to airway remodeling is characterized by progressive loss of functional lung tissue. Emphysema is defined as a process resulting in the loss of functional lung tissue due to chronic inflammation and tissue destruction. Current therapeutic options for the treatment of smoking-related emphysema is sparse and only moderately effective [54]. Several mechanisms focusing on the chronic injury induced by cigarette smoke have been postulated to contribute to emphysema, including an imbalance in the expression of proteases and anti-proteases, increased apoptosis of epithelial cells and attenuation of the immune response in the lung [54]. Most studies focused on the processes that induce the chronic injury, whereas the repair processes in the lung are less extensively investigated, although they may not be of less importance. Emphysema could be considered a deficient repair process after initial tissue damage as a resultant of prolonged exposure to cigarette smoke. Pulmonary fibroblasts are important for the regeneration of tissue after it has been injured and although the emphysematous lung is predominantly characterized by destruction of functional lung tissue, there are indications that fibroblasts are actually active in the alveolar walls of these emphysematous lung regions [55, 56].

The molecular mechanisms contributing to parenchymal tissue repair are not well defined, but restoring functional lung tissue may constitute a feasible target for therapeutic intervention in COPD. Re-activation of pathways involved in lung development may form a rational approach to achieve this aim. In this respect, the canonical WNT signaling pathway is of potential interest as it is required for proper normal lung development during the embryonic stages [57-59]. Several studies have demonstrated that activation of canonical WNT signaling may contribute to tissue repair and this pathway may cooperatively act with TGF-β signaling [60-63]. A screen for components of the WNT signaling pathway in human pulmonary fibroblasts showed that the majority of genes required for functional WNT signaling are expressed, although considerable differences in the degree of expression are present (**Chapter 3**). Particularly, the WNT ligands WNT-5A, WNT-5B and WNT-16, the FZD receptors FZD_2, FZD_6 and FZD_8 as well as the intracellular signaling protein DVL3 and the key-effector of canonical WNT signaling, β-catenin, were abundantly expressed. Conversely, WNT-3A, WNT-6, WNT-9A, FZD_3, FZD_9 and FZD_{10} were barely expressed. Stimulation of these cells with TGF-β induced the expression of specific WNT components, whereas others were not altered in response to this stimulus. Especially the expression of WNT-5B, FZD_6, FZD_8 and β-catenin was sensitive to TGF-β. Remarkably, the expression of the induced WNT components was enhanced in pulmonary fibroblasts of individuals with COPD. Of

interest is that this intrinsic difference in WNT pathway expression was seen for pulmonary fibroblasts from both GOLD stage II and IV COPD patients. On the protein level, TGF-β induced a time-dependent increase in the expression of the transcriptional co-activator β-catenin. More specifically, the cytosolic pool and the nuclear pool of β-catenin were enhanced by this growth factor. By using both molecular biological and pharmacological approaches, we demonstrated that activation of β-catenin signaling was required for full transdifferentiation of fibroblasts in the more active myofibroblasts in response to TGF-β. In line with enhanced upregulation of genes required for functional WNT signaling in fibroblasts from individuals with COPD, the TGF-β-induced activation of β-catenin on the protein level was also enhanced in these cells. Accordingly, the extracellular matrix protein fibronectin (a canonical WNT/β-catenin target) was significantly more deposited by fibroblasts from individuals with COPD compared to fibroblasts from individuals without COPD. Collectively, the studies described in **Chapter 3** show that β-catenin signaling contributes to myofibroblast differentiation and that pulmonary fibroblasts from individuals with COPD show enhanced WNT pathway expression, activation of β-catenin signaling and deposition of fibronectin.

These findings suggest that activation of canonical WNT signaling contributes to tissue repair and may be potentially beneficial to arrest emphysema progression. In line with this contention, a recent *in vivo* study demonstrated that activation of WNT/β-catenin signaling in experimental emphysema indeed attenuated parenchymal tissue destruction by increasing pulmonary repair [64]. This study demonstrated that lung tissue from COPD patients in general did not show major alterations in expression of canonical WNT pathways components or activation status of the pathway. However, emphysematous lung regions were characterized by decreased expression of β-catenin, particularly in the alveolar type II (ATII) epithelial cells. In the two *in vivo* models of elastin- or cigarette smoke-induced emphysema described in that study, intraperitoneal administration of LiCl (a relatively non-selective inhibitor that also targets GSK-3) activated β-catenin signaling in ATII epithelial cells, improved the lung architecture, decreased airspace enlargement and enhanced the dynamic lung compliance [64].

In the lung, epithelial cells and pulmonary fibroblasts are in close proximity and capable of influencing each other's responses. In COPD, however, communication between these two cell types may be dysfunctional and may contribute to disease development and progression. In this context the functional interaction of canonical and non-canonical WNT signaling may be of specific interest, as activation of canonical WNT signaling can be (functionally) antagonized by WNT ligands that activate non-canonical WNT signaling. For instance, canonical WNT signaling activated by WNT-3A can be dose-dependently attenuated by the non-canonical

WNT-5A [65]. It appears that in pulmonary fibroblasts the expression of WNT components that have been primarily linked to the activation of non-canonical WNT signaling are induced in response to TGF-β (**Chapter 3**). This TGF-β-induced response on the expression of WNT components is enhanced in pulmonary fibroblasts of individuals with moderate and very severe COPD (GOLD stage II and IV, respectively). As the alveolar epithelium requires canonical WNT signalling for proper regeneration and repair [66], it can be hypothesized that the balance in canonical and non-canonical WNT signaling between the alveolar epithelium and the pulmonary fibroblasts is disrupted in COPD pathogenesis via such a mechanism. The increased release of non-canonical WNT ligands by pulmonary fibroblasts might inhibit the canonical WNT signaling in epithelial cells, thereby attenuating their proliferative response and repair mechanisms. This imbalance in WNT signaling may possibly contribute to the development of emphysema, which is an attractive hypothesis for further studies. Also, this suggests that restoration of the normal balance between canonical and non-canonical WNT signalling in the epithelial mesenchymal trophic unit is a therapeutic strategy worth investigating.

GSK-3 as a potential therapeutic target for COPD

A note of caution is warranted for using either activators or inhibitors of the WNT pathway as their effects are very context dependent, and chronic activation could lead to a deviant repair potential and may promote a potent fibrotic response [66]. However, pharmacological inhibition of GSK-3 in the pulmonary fibroblasts did not result in enhanced myofibroblast differentiation or fibrotic responses (**Chapter 4**). Rather, inhibition or siRNA silencing of the kinase prevented TGF-β-induced myofibroblast differentiation. In pulmonary fibroblasts, attenuation of GSK-3 activity was associated with the activation of cAMP responsive element binding protein (CREB), which functionally antagonizes TGF-β-induced smad signaling without affecting the phosphorylation status of these intracellular signaling proteins. Worth mentioning is that myofibroblast differentiation of pulmonary fibroblasts from individuals with COPD is also dependent on GSK-3 signaling. This suggests that this kinase mediates important cellular functions in peripheral lung fibroblasts that are still operative in COPD. An unexpected finding was that loss of GSK-3 actually results in decreased expression of active β-catenin in these cells (**Chapter 6**). Growth factors, including TGF-β, regulate cellular β-catenin expression by smad mediated gene-transcription in addition to GSK-3 dependent posttranslational effects on β-catenin protein stability [67, 68]. Therefore, a plausible explanation for the unanticipated finding may be that in pulmonary fibroblasts the expression of β-catenin is predominantly determined by the rate of *de novo* synthesis, which is attenuated upon GSK-3 inhibition. Collectively, this indicates that various cellular responses of pulmonary fibroblasts like extracellular matrix deposition and the

differentiation process are dependent on GSK-3 signaling, by its suppressive action on CREB activation.

To corroborate these *in vitro* findings and to evaluate the therapeutic potential of GKS-3 as molecular target *in vivo*, the effect of topical administration of the selective GSK-3 inhibitor SB216763 on pulmonary extrapulmonary pathological features in an established guinea pig model of LPS-induced COPD was investigated (**Chapter 6** and [69]). LPS is an endotoxin in the outer membrane of gram negative bacteria, that is present as contaminant in environmental pollution, organic dusts and cigarette smoke, factors that have been causally associated with COPD development [3, 70]. Furthermore, bacterial endotoxins may contribute to COPD exacerbations, which are episodes of acute worsening of symptoms at times accompanied by an impairment of lung function [71]. Accordingly, LPS has been demonstrated in various animal models to induce pulmonary and extrapulmonary pathological features resembling COPD pathophysiology [69, 72, 73]. In the guinea pig, repeated LPS exposure activated β-catenin signaling and induced tissue remodelling indicated by increased pulmonary fibronectin expression and small airway collagen content; whereas the extrapulmonary pathology was characterized by right ventricle hypertrophy and a tendency to skeletal muscle atrophy (**Chapter 6**). Selective inhibition of GSK-3 by intranasal instillation of SB216763 largely prevented the LPS-induced pulmonary and extrapulmonary pathological features. In line with the findings in pulmonary fibroblasts, GSK-3 inhibition attenuated the LPS-induced activation of β-catenin signaling *in vivo*, which may in part explain the decrease of pulmonary pathological features due to pretreatment with SB216763 in this guinea pig model of COPD. In addition to the direct effect in cells that regulate fibrogenic responses, GSK-3 inhibition may also attenuate inflammatory responses by regulating NF-κB signaling [47]. As mentioned, airway smooth muscle cells may contribute to the pulmonary inflammation by secreting several cytokines and growth factors, which can be attenuated by GSK-3 inhibition (**Chapter 5**). Accordingly, several *in vitro* and *in vivo* studies investigating other chronic inflammatory diseases have demonstrated that GSK-3 can be beneficial in decreasing inflammatory responses [47, 51, 74-76]. Furthermore, the observations that pharmacological inhibition of GSK-3 does not promote an increase in airway smooth muscle content, or affect the alveolar airspace size, or induce (major) adverse side effects *in vivo* is reassuring. Moreover, small molecule inhibitors of GSK-3 are currently being investigated clinically for the treatment of Alzheimer's disease and diabetes mellitus, while LiCl (a relative non-selective inhibitor that also targets GSK-3) is used clinically for over 50 years in the treatment of dipolar disorder [53, 77, 78]. This once more indicates that GSK-3 is may be a feasible therapeutic target for

chronic (inflammatory) diseases in human and provides further support for the suitability of GSK-3 as a drug target for the treatment of COPD.

Main conclusions

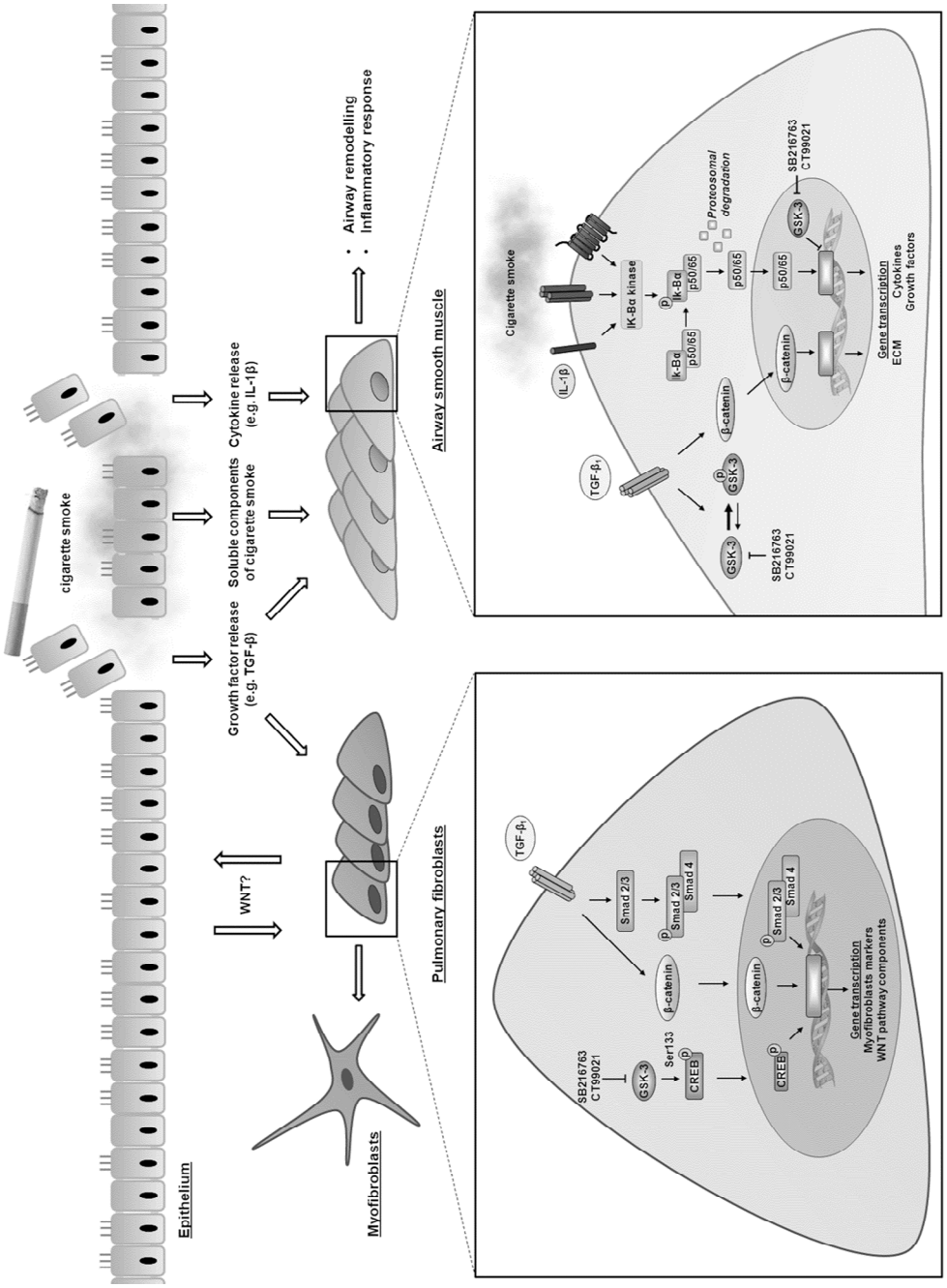
In conclusion, the studies described in this thesis revealed that:

- In both airway smooth muscle cells and pulmonary fibroblasts, β-catenin signaling is activated in response to TGF-β (**Chapters 2 and 3**).
- In airway smooth muscle cells the activation of β-catenin signaling is required and sufficient for increasing the extracellular matrix deposition. In particular, the expression of fibronectin, collagen III and versican is regulated by β-catenin signaling in these cells (**Chapter 2**).
- Pulmonary fibroblasts express the majority of components required for functional WNT signaling. TGF-β induces gene expression of specific WNT pathway components, which is enhanced in pulmonary fibroblasts from individuals with moderate to very severe COPD (GOLD stage II and IV, respectively) (**Chapter 3**).
- Activation of β-catenin signaling by TGF-β contributes to the full differentiation of pulmonary fibroblasts into myofibroblasts. The activation of β-catenin is enhanced in fibroblasts from individuals with moderate to very severe COPD (GOLD stage II and IV, respectively), which may underlie the enhanced fibronectin deposition by these cells in response to TGF-β (**Chapter 3**).
- The β-catenin-mediated extracellular matrix deposition by smooth muscle cells or pulmonary fibroblasts can be pharmacologically inhibited by disruption of the interaction of β-catenin with the transcription factor TCF-4 using the small molecule PKF115-584 or quercetin (**Chapters 2 and 3**).
- GSK-3 signaling actively contributes to myofibroblast differentiation by suppressing CREB signaling. Inhibition or silencing of GSK-3 in pulmonary fibroblasts results in increased ser133-CREB phosphorylation and attenuated TGF-β-induced β-catenin expression. The activated CREB can prevent myofibroblast differentiation by functionally antagonizing TGF-β-dependent smad signaling (**Chapter 4**).
- Cigarette smoke extract (CSE) and the pro-inflammatory cytokine IL-1β activate the secretion of eotaxin, IL-8 and VEGF from airway smooth muscle cells. Pharmacological inhibition of GSK-3 attenuates the CSE- and IL-1β-induced cytokine and growth factor secretion by inhibiting NF-κB-dependent gene transcription (**Chapter 5**).
- GSK-3 contributes to the pulmonary and extrapulmonary pathological features in a guinea pig model of LPS-induced COPD. Repeated LPS instillation activated β-catenin signaling and induced tissue remodelling indicated by increased pulmonary fibronectin expression and small airway

collagen content. The extrapulmonary pathology consists of right ventricle hypertrophy and modest alterations in skeletal muscle weight. The pathology induced by LPS could to a large extent be prevented by topical administration of the selective GSK-3 inhibitor SB216763 (**Chapter 6)**.

Taken together, the described *in vitro* studies in airway smooth muscle cells and pulmonary fibroblasts demonstrate that both β-catenin and GSK-3 signaling are important in specific cellular processes that may contribute to COPD pathogenesis (figure 1). Furthermore, the *in vivo* study shows that topical application of the pharmacological GSK-3 inhibitor SB216763 is capable of preventing both pulmonary and extrapulmonary pathological features in a guinea pig model of COPD. This suggests that therapeutic intervention of GSK-3 and/or β-catenin signaling may provide a novel means for the treatment of chronic airway diseases, such as COPD.

► *Figure 1:* **Insight in β-catenin and GSK-3 signaling in cellular processes which potentially contribute to COPD pathogenesis.** Chronic obstructive pulmonary disease (COPD) is primarily caused by smoking of cigarettes. Chronic pulsatile exposure to cigarette smoke damages the epithelium lining the airways and alveoli. In response to this damage, the epithelium secretes various pro-inflammatory cytokines (e.g. IL-1β) or growth factors (.e.g. TGF-β). Moreover, soluble components of cigarette smoke can diffuse across the epithelial layer. In turn, these mediators activate the pulmonary fibroblasts and the airway smooth muscle. In the pulmonary fibroblasts, TGF-β increases cellular β-catenin expression, which translocates to the nucleus to induce transcription of genes that contribute to myofibroblast differentiation. Furthermore, the gene expression of WNT pathway components is increased by TGF-β. In pulmonary fibroblasts from individuals with COPD, the gene expression of specific WNT components, the activation of β-catenin, and subsequent fibronectin deposition are enhanced in response to TGF-β (**Chapter 3**). In addition, TGF-β activates the smad signaling pathway in these cells which contributes to myofibroblast differentiation. Pharmacological inhibition of GSK-3 by SB216763 or CT99021 attenuates smad-dependent signaling via the activation of cAMP responsive element binding protein (CREB) (**Chapter 4**). In airway smooth muscle cells, TGF-β activates β-catenin signaling via inactivation of GSK-3 via ser9/21 phosphorylation and *de novo* synthesis. Nuclear β-catenin induces gene transcription of extracellular matrix genes (**Chapter 2**). The soluble components of cigarette smoke and IL-1β activate the NF-κB pathway, which is required for the transcription and secretion of eotaxin, VEGF and IL-8 by airway smooth muscle cells. Pharmacological inhibition of GSK-3 by attenuates the NF-κB-mediated transcription, without affecting Iκ-Bα degradation or p65 NF-κB nuclear translocation or DNA-binding (**Chapter 5**)


Epithelium
cigarette smoke
WNT?
Growth factor release (e.g. TGF-β)
Soluble components of cigarette smoke
Cytokine release (e.g. IL-1β)
Airway remodelling
Inflammatory response
Myofibroblasts
Pulmonary fibroblasts
Airway smooth muscle
TGF-β1
SB216763 CT99021
GSK-3
Ser133
CREB
β-catenin
Smad 2/3
Smad 4
Gene transcription
Myofibroblasts markers
WNT pathway components
Cigarette smoke
IL-1β
IK-Bα kinase
IK-Bα
p50/65
Proteosomal degradation
Gene transcription
ECM
Cytokines
Growth factors

Future perspectives:
The studies described in this thesis show that β-catenin and GSK-3 signaling play an important role in human airway smooth muscle and fibroblast biology. Furthermore, we demonstrate that WNT signaling may be enhanced in the pulmonary fibroblasts of individuals with COPD. To date it is unclear if enhanced WNT signaling by these cells may be beneficial or detrimental in the pathophysiology of COPD and this requires further investigation. Therefore, future studies will need to investigate the functional consequences of canonical and non-canonical WNT signaling in the pulmonary fibroblasts to provide more insight in the specific cellular responses regulated by these pathways in relation to COPD pathogenesis. Particularly interesting would be to study the function of both WNT-5B and FZD_8 and their possible interconnectivity, as this specific WNT ligand and receptor are the main WNT pathway components induced by TGF-β in the pulmonary fibroblast. In addition, the secreted (non-canonical) WNT ligands may extend their effects in the lungs beyond the pulmonary fibroblasts. In this respect, a specific cell type that may come in focus is the alveolar type II epithelial cell, which shows decreased nuclear β-catenin expression in emphysematous lung regions. As mentioned, alveolar epithelial cells highly depend on canonical WNT signaling for proliferation and to repair tissue after injury. Several studies suggest that non-canonical WNT ligands can functionally antagonize canonical WNT signaling. Therefore, it can be postulated that increased secretion of non-canonical WNT ligands by pulmonary fibroblasts disrupts the proliferative response and repair mechanisms by the alveolar epithelium thereby contributing to the development of emphysema, which is an attractive hypothesis for further studies. Furthermore, restoration of the normal balance between canonical and non-canonical WNT signalling in the epithelial mesenchymal trophic unit is a therapeutic strategy worth investigating.

A variety of intracellular signaling pathways that use GSK-3 as a molecular regulator in cellular processes, have links to several human diseases. Accordingly, GSK-3 inhibitors arise as promising drugs for pharmacotherapy of several pathologies including Alzheimer's disease, diabetes mellitus cancer, stroke, mood disorders and inflammatory diseases with promising results [53]. Our *in vitro* and *in vivo* studies show that GSK-3 inhibition is capable of preventing several pathological features of COPD. The current small molecule inhibitors of GSK-3 do not discriminate between the GSK-3α and GSK-3β isoform. The potential beneficial effect(s) of selectivity for one of the isoforms for treatment needs to be explored. In addition, for the potential treatment of patients with COPD it would be preferable to have a pulmonary formulation of the GSK-3 inhibitor to have a locally therapeutically effective concentration of the compound and to circumvent possible systemic adverse effects. Besides these technical issues, the clinical applicability

of GSK-3 inhibitors in the treatment of COPD needs additional justification. Most pre-clinical data concerning the effectiveness of GSK-3 inhibitors has been focused on preventing pathological features of diseases, rather than therapeutic intervention in established disease. Up to date two *in vivo* studies have touched upon the potential therapeutic relevance of GSK-3 inhibitors in established lung disease [50, 64]. Although GSK-3 inhibition is beneficial in preventing pathological features of lung disease, the small number of studies suggesting therapeutic benefits of inhibiting the kinase in established disease is not yet completely reassuring. Additional, thorough and comprehensive pre-clinical studies dealing with this specific issue may therefore be advisable, before testing GSK-3 inhibitors for the treatment of COPD in human in a clinical setting.

Reference List

1. J.Vestbo, A.G.Augusti, A.Anzueto, P.J.Barnes, L.M.Fabbri, P.Jones, et al. Global initiative for Chronic Obstructive Lung Diseases (GOLD); Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease (revised 2011). 2011. Ref Type: Internet Communication
2. Molfino NA, Jeffery PK (2007) Chronic obstructive pulmonary disease: histopathology, inflammation and potential therapies. Pulm Pharmacol Ther 20: 462-472.
3. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, et al. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 176: 532-555.
4. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480.
5. Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20: 781-810.
6. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell 17: 9-26.
7. Baum B, Georgiou M (2011) Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. J Cell Biol 192: 907-917.
8. Jansen SR, Van Ziel AM, Baarsma HA, Gosens R (2010) {beta}-Catenin regulates airway smooth muscle contraction. Am J Physiol Lung Cell Mol Physiol 299: L204-L214.
9. Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359: 1-16.
10. Cohen P, Frame S (2001) The renaissance of GSK3. Nat Rev Mol Cell Biol 2: 769-776.
11. Nunes RO, Schmidt M, Dueck G, Baarsma H, Halayko AJ, et al. (2008) GSK-3/beta-catenin signaling axis in airway smooth muscle: role in mitogenic signaling. Am J Physiol Lung Cell Mol Physiol 294: L1110-L1118.
12. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, et al. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 350: 2645-2653.
13. Hogg JC (2004) Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet 364: 709-721.
14. Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P (2010) Pathophysiology of bronchial smooth muscle remodelling in asthma. Eur Respir J 36: 1174-1184.
15. Johnson PR (2001) Role of human airway smooth muscle in altered extracellular matrix production in asthma. Clin Exp Pharmacol Physiol 28: 233-236.
16. Postma DS, Timens W (2006) Remodeling in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 3: 434-439.
17. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, et al. (2005) Differences in airway remodeling between subjects with severe and moderate asthma. J Allergy Clin Immunol 116: 544-549.
18. Pera T, Gosens R, Lesterhuis AH, Sami R, Toorn M, et al. (2010) Cigarette smoke and lipopolysaccharide induce a proliferative airway smooth muscle phenotype. Respir Res 11: 48.
19. Churg A, Tai H, Coulthard T, Wang R, Wright JL (2006) Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. Am J Respir Crit Care Med 174: 1327-1334.
20. Churg A, Zhou S, Preobrazhenska O, Tai H, Wang R, et al. (2009) Expression of profibrotic mediators in small airways versus parenchyma after cigarette smoke exposure. Am J Respir Cell Mol Biol 40: 268-276.
21. Wang RD, Tai H, Xie C, Wang X, Wright JL, et al. (2003) Cigarette smoke produces airway wall remodeling in rat tracheal explants. Am J Respir Crit Care Med 168: 1232-1236.

22. Wang RD, Wright JL, Churg A (2005) Transforming growth factor-beta1 drives airway remodeling in cigarette smoke-exposed tracheal explants. Am J Respir Cell Mol Biol 33: 387-393.
23. van Diemen CC, Postma DS, Vonk JM, Bruinenberg M, Nolte IM, et al. (2006) Decorin and TGF-beta1 polymorphisms and development of COPD in a general population. Respir Res 7: 89.
24. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl 34: 50s-59s.
25. de Boer WI, van SA, Sont JK, Sharma HS, Stolk J, et al. (1998) Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 158: 1951-1957.
26. Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, et al. (2001) Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). Am J Respir Crit Care Med 163: 1476-1483.
27. Konigshoff M, Kneidinger N, Eickelberg O (2009) TGF-beta signaling in COPD: deciphering genetic and cellular susceptibilities for future therapeutic regimen. Swiss Med Wkly 139: 554-563.
28. Chung KF (2000) Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? Eur Respir J 15: 961-968.
29. Black JL, Burgess JK, Johnson PR (2003) Airway smooth muscle--its relationship to the extracellular matrix. Respir Physiol Neurobiol 137: 339-346.
30. Lazaar AL, Panettieri RA, Jr. (2005) Airway smooth muscle: a modulator of airway remodeling in asthma. J Allergy Clin Immunol 116: 488-495.
31. Dekkers BG, Maarsingh H, Meurs H, Gosens R (2009) Airway structural components drive airway smooth muscle remodeling in asthma. Proc Am Thorac Soc 6: 683-692.
32. Chung KF (2005) The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. Proc Am Thorac Soc 2: 347-354.
33. Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, et al. (2008) Extracellular matrix components and regulators in the airway smooth muscle in asthma. Eur Respir J 32: 61-69.
34. Pini L, Hamid Q, Shannon J, Lemelin L, Olivenstein R, et al. (2007) Differences in proteoglycan deposition in the airways of moderate and severe asthmatics. Eur Respir J 29: 71-77.
35. Roberts CR, Burke AK (1998) Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. Can Respir J 5: 48-50.
36. Oltmanns U, Chung KF, Walters M, John M, Mitchell JA (2005) Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle. Respir Res 6: 74.
37. Oenema TA, Kolahian S, Nanninga JE, Rieks D, Hiemstra PS, et al. (2010) Pro-inflammatory mechanisms of muscarinic receptor stimulation in airway smooth muscle. Respir Res 11: 130.
38. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, et al. (2009) Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. Eur Respir J .
39. Di SA, Capelli A, Lusuardi M, Balbo P, Vecchio C, et al. (1998) Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am J Respir Crit Care Med 158: 1277-1285.
40. Saha S, Brightling CE (2006) Eosinophilic airway inflammation in COPD. Int J Chron Obstruct Pulmon Dis 1: 39-47.
41. Walters EH, Reid D, Soltani A, Ward C (2008) Angiogenesis: a potentially critical part of remodelling in chronic airway diseases? Pharmacol Ther 118: 128-137.
42. Zhang X, Gaspard JP, Chung DC (2001) Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res 61: 6050-6054.

43. Masckauchan TN, Shawber CJ, Funahashi Y, Li CM, Kitajewski J (2005) Wnt/beta-catenin signaling induces proliferation, survival and interleukin-8 in human endothelial cells. Angiogenesis 8: 43-51.
44. Clifford RL, Deacon K, Knox AJ (2008) Novel regulation of vascular endothelial growth factor-A (VEGF-A) by transforming growth factor (beta)1: requirement for Smads, (beta)-CATENIN, AND GSK3(beta). J Biol Chem 283: 35337-35353.
45. Baker RG, Hayden MS, Ghosh S (2011) NF-kappaB, inflammation, and metabolic disease. Cell Metab 13: 11-22.
46. Doble BW, Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci 116: 1175-1186.
47. Dugo L, Collin M, Thiemermann C (2007) Glycogen synthase kinase 3beta as a target for the therapy of shock and inflammation. Shock 27: 113-123.
48. Bentley JK, Deng H, Linn MJ, Lei J, Dokshin GA, et al. (2009) Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3(beta) phosphorylation in a mouse model of asthma. Am J Physiol Lung Cell Mol Physiol 296: L176-L184.
49. Bao Z, Lim S, Liao W, Lin Y, Thiemermann C, et al. (2007) Glycogen synthase kinase-3beta inhibition attenuates asthma in mice. Am J Respir Crit Care Med 176: 431-438.
50. Gurrieri C, Piazza F, Gnoato M, Montini B, Biasutto L, et al. (2010) 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), a glycogen synthase kinase-3 inhibitor, displays therapeutic properties in a mouse model of pulmonary inflammation and fibrosis. J Pharmacol Exp Ther 332: 785-794.
51. Dugo L, Collin M, Allen DA, Patel NS, Bauer I, et al. (2005) GSK-3beta inhibitors attenuate the organ injury/dysfunction caused by endotoxemia in the rat. Crit Care Med 33: 1903-1912.
52. Martin M, Rehani K, Jope RS, Michalek SM (2005) Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 6: 777-784.
53. Martinez A (2008) Preclinical efficacy on GSK-3 inhibitors: towards a future generation of powerful drugs. Med Res Rev 28: 773-796.
54. Chung KF, Adcock IM (2008) Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. Eur Respir J 31: 1334-1356.
55. Vlahovic G, Russell ML, Mercer RR, Crapo JD (1999) Cellular and connective tissue changes in alveolar septal walls in emphysema. Am J Respir Crit Care Med 160: 2086-2092.
56. Lang MR, Fiaux GW, Gillooly M, Stewart JA, Hulmes DJ, et al. (1994) Collagen content of alveolar wall tissue in emphysematous and non-emphysematous lungs. Thorax 49: 319-326.
57. Goss AM, Tian Y, Tsukiyama T, Cohen ED, Zhou D, et al. (2009) Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. Dev Cell 17: 290-298.
58. Goss AM, Morrisey EE (2010) Wnt signaling and specification of the respiratory endoderm. Cell Cycle 9: 10-11.
59. Goss AM, Tian Y, Cheng L, Yang J, Zhou D, et al. (2011) Wnt2 signaling is necessary and sufficient to activate the airway smooth muscle program in the lung by regulating myocardin/Mrtf-B and Fgf10 expression. Dev Biol .
60. Attisano L, Labbe E (2004) TGFbeta and Wnt pathway cross-talk. Cancer Metastasis Rev 23: 53-61.
61. Labbe E, Letamendia A, Attisano L (2000) Association of Smads with lymphoid enhancer binding factor 1/T cell-specific factor mediates cooperative signaling by the transforming growth factor-beta and wnt pathways. Proc Natl Acad Sci U S A 97: 8358-8363.
62. Labbe E, Lock L, Letamendia A, Gorska AE, Gryfe R, et al. (2007) Transcriptional cooperation between the transforming growth factor-beta and Wnt pathways in mammary and intestinal tumorigenesis. Cancer Res 67: 75-84.
63. Letamendia A, Labbe E, Attisano L (2001) Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. J Bone Joint Surg Am 83-A Suppl 1: S31-S39.

64. Kneidinger N, Yildirim AO, Callegari J, Takenaka S, Stein MM, et al. (2011) Activation of the WNT/beta-catenin pathway attenuates experimental emphysema. Am J Respir Crit Care Med 183: 723-733.
65. Nemeth MJ, Topol L, Anderson SM, Yang Y, Bodine DM (2007) Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. Proc Natl Acad Sci U S A 104: 15436-15441.
66. Beers MF, Morrisey EE (2011) The three R's of lung health and disease: repair, remodeling, and regeneration. J Clin Invest 121: 2065-2073.
67. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, et al. (2006) Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. FASEB J 20: 692-701.
68. Gosens R, Baarsma HA, Heijink IH, Oenema TA, Halayko AJ, et al. (2010) De novo synthesis of {beta}-catenin via H-Ras and MEK regulates airway smooth muscle growth. FASEB J 24: 757-768.
69. Pera T, Zuidhof A, Valadas J, Smit M, Schoemaker RG, et al. (2011) Tiotropium inhibits pulmonary inflammation and remodelling in a guinea pig model of COPD. Eur Respir J 38: 789-796.
70. Eduard W, Pearce N, Douwes J (2009) Chronic bronchitis, COPD, and lung function in farmers: the role of biological agents. Chest 136: 716-725.
71. Veeramachaneni SB, Sethi S (2006) Pathogenesis of bacterial exacerbations of COPD. COPD 3: 109-115.
72. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, Wouters EF (2002) Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. Am J Respir Cell Mol Biol 26: 152-159.
73. Toward TJ, Broadley KJ (2002) Goblet cell hyperplasia, airway function, and leukocyte infiltration after chronic lipopolysaccharide exposure in conscious Guinea pigs: effects of rolipram and dexamethasone. J Pharmacol Exp Ther 302: 814-821.
74. Jope RS, Yuskaitis CJ, Beurel E (2007) Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. Neurochem Res 32: 577-595.
75. Ougolkov AV, Fernandez-Zapico ME, Savoy DN, Urrutia RA, Billadeau DD (2005) Glycogen synthase kinase-3beta participates in nuclear factor kappaB-mediated gene transcription and cell survival in pancreatic cancer cells. Cancer Res 65: 2076-2081.
76. Schwabe RF, Brenner DA (2002) Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. Am J Physiol Gastrointest Liver Physiol 283: G204-G211.
77. Rey JP, Ellies DL (2010) Wnt modulators in the biotech pipeline. Dev Dyn 239: 102-114.
78. Martinez A, Gil C, Perez DI (2011) Glycogen synthase kinase 3 inhibitors in the next horizon for Alzheimer's disease treatment. Int J Alzheimers Dis 2011: 280502.

Nederlandse samenvatting

Nederlandse samenvatting

Chronisch obstructief longlijden

Chronisch obstructief longlijden of in het Engels *chronic obstructive pulmonary disease* (COPD) is een verzamelnaam voor chronische bronchitis (ontsteking van de luchtwegen) en longemfyseem. COPD is een chronische aandoening aan de longen die ademhaling bemoeilijkt en voornamelijk veroorzaakt wordt door het roken van tabak. De ziekte is gekarakteriseerd door een progressieve afname van de longfunctie als gevolg van de aanhoudende ontsteking en structurele veranderingen in de longen. Het ziekteproces speelt zich voornamelijk af in de kleine luchtwegen en het longweefsel rond en aan het einde van de luchtwegen, het zogenoemde parenchym. De chronische ontsteking zorgt voor verdikking van de luchtwegen en een toegenomen slijmproductie, wat gezamenlijk leidt tot een vernauwing van de luchtwegen. Bij emfyseem treedt er in het parenchym afbraak op van de longblaasjes, welke noodzakelijk zijn voor de gasuitwisseling die plaatsvindt in de longen. Bovendien speelt het parenchym een belangrijke rol in het open houden van de kleine luchtwegen bij het uitademen als de druk in de thoraxholte toeneemt. De symptomen die een patiënt met COPD ondervindt zijn onder andere chronisch hoesten, slijm opgeven, kortademigheid en benauwdheid. De diagnose COPD kan worden gesteld door het bepalen van longfunctie, waarbij gekeken wordt naar de totale hoeveelheid lucht die uitgeademd kan worden (FVC; forced vital capacity) en de hoeveelheid lucht die in 1 seconde kan worden uitgeblazen, ofwel de FEV_1 (forced expiratory volume in 1 second). De gemeten FEV_1 waarde is afhankelijk van verschillende factoren zoals leeftijd, sekse, lengte en etniciteit van de persoon en daarom wordt FEV_1 meestal weergegeven als percentage van de voorspelde waarde, waarbij er gecorrigeerd wordt voor deze factoren. Voor de algemene populatie geldt dat FEV_1/FVC tussen de 0,7 en 0,8 ligt, waarbij een waarde kleiner dan 0,7 kan duiden op luchtwegobstructie en de mogelijkheid dat de persoon aan COPD lijdt. De gemeten longfunctieparameters geven de ernst van de ziekte weer, welke wordt ingedeeld in 4 stadia volgens de *Global Initiative for Chronic Obstructive Lung Diseases* (GOLD) criteria (tabel 1).

Tabel 1: Indeling van de ernst van COPD volgens de GOLD-criteria en frequentieverdeling van ernststadia bij patiënten met COPD in de Nederlandse populatie[*]

GOLD stadium		FEV_1/FVC	FEV_1	Frequentieverdeling
I	Licht	< 0,7	>80	28%
II	Matig ernstig	< 0,7	50-80	54%
III	Ernstig	< 0,7	30-50	15%
IV	Zeer ernstig	< 0,7	<30	3%

** Bron: GOLD report 2010 update, www.goldcopd.org.*

Patiënten met licht COPD (stadium 1) hebben weinig klachten en vaak zijn deze personen zich er niet van bewust dat hun longfunctie verminderd is. Daarentegen

hebben met name patiënten met zeer ernstig COPD (stadium 4) een dusdanig sterk verminderde longfunctie, dat medicamenteuze behandeling eigenlijk altijd nodig is en soms dagelijks toediening van extra zuurstof noodzakelijk is. Bij deze groep patiënten kunnen episodes van plotselinge verergering van de ziekte, zogenoemde exacerbaties, levensbedreigend zijn. Naast de pathologische veranderingen in de longen die zorgen voor een verminderde longfunctie, kunnen bij COPD ook andere organen aangetast worden. Deze extrapulmonale of systemische effecten zijn vaak het gevolg van de chronische ontsteking en enkele voorbeelden van aandoeningen die optreden als gevolg van COPD zijn een afname in gewicht en functie van skeletspieren en het ontstaan van atherosclerotische hart- en vaatziekten. Over het algemeen leidt COPD tot vroegtijdige sterfte en momenteel is COPD de vijfde doodsoorzaak door ziekte wereldwijd. Volgens inschattingen van de Wereldgezondheidsorganisatie (WHO) zal het sterftecijfer als gevolg van roken en daaraan gerelateerde ziekten de aankomende jaren nog sterk toenemen.

Stoppen met roken is de eerste en belangrijkste stap in de behandeling van COPD. Door te stoppen met roken kan de progressieve afname in longfunctie vertraagd worden, zelfs bij zeer ernstig COPD (stadium 4). Momenteel is er geen medicatie beschikbaar die de ziekte kan voorkomen of genezen. De huidige (inhalatie)medicatie bestaat uit luchtwegverwijdende en ontstekingsremmende middelen die de luchtwegklachten kunnen verminderen en/of de verergering van de symptomen kunnen tegengaan. Voor de ontwikkeling van nieuwe geneesmiddelen is onderzoek nodig naar de processen die kunnen bijdragen aan de ontwikkeling van deze chronische ziekte. De moleculaire mechanismen die bijdragen aan de ontwikkeling en voortgang van COPD zijn vooralsnog grotendeels nog onduidelijk. De studies beschreven in dit proefschrift richten zich op de eiwitten β-catenine en glycogeen synthase kinase-3 (GSK-3) en hun rol in de processen die mogelijk ten grondslag liggen aan de pathofysiologie van COPD.

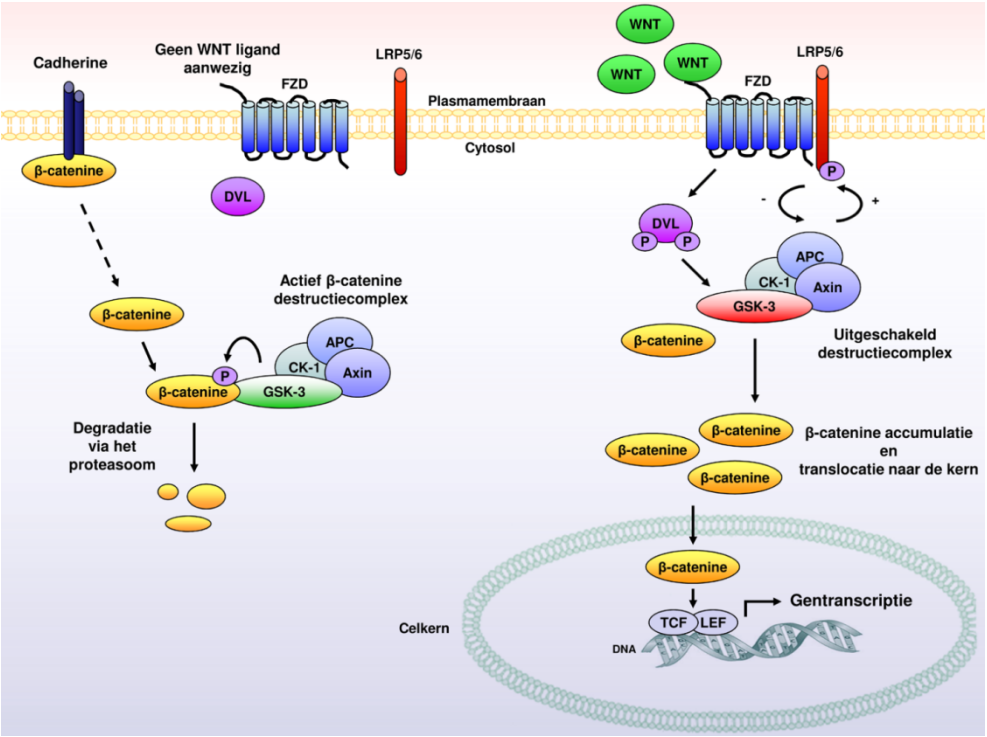
WNT-afhankelijke en onafhankelijke signaaltransductie door β-catenine en glycogeen synthase kinase-3 (GSK-3)

Zowel β-catenine als glycogeen synthase kinase-3 (GSK-3) maken deel uit van de complexe *Wingless/integrase-1* (WNT) signaaltransductiecascade (figuur 1 en **hoofdstuk 1**). De WNT signaaltransductieroute vervult een belangrijke rol in cellulaire processen die cruciaal zijn tijdens embryonale ontwikkeling en tijdens het handhaven van de homeostase in volgroeide weefsels en organen. Deze signaalroute wordt geactiveerd doordat een WNT ligand (eiwit dat zich buiten de cel bevindt) bindt aan Frizzled (FZD) of de low-density lipoprotein receptor related proteins (LRP5/6)-receptoren die zich op het celmembraan bevinden (figuur 1). Binding van WNT ligand aan deze receptoren leidt tot de activatie van de

intracellulaire WNT signaaltransductie, wat uiteindelijk resulteert in veranderingen in gentranscriptie. De WNT route bestaat uit een β-catenine afhankelijke en onafhankelijke signaaltransductie. De rol van β-catenine in de cel is tweeledig. Ten eerste speelt het eiwit een belangrijke rol in cel-cel contact door zijn interactie met cadherines. Het maakt daarbij deel uit van zogenoemde “adherens juncties” die zich tussen cellen bevinden. Ten tweede functioneert β-catenine als transcriptionele co-activator in de WNT signaaltransductie. In de afwezigheid van een extracellulair WNT ligand is β-catenine gelokaliseerd aan het plasmamembraan door zijn interactie met cadherines. Vrij β-catenine, dat wil zeggen ongebonden β-catenine dat zich in het cytosol bevindt, wordt herkend en gefosforyleerd door het “β-catenine destructiecomplex”. Dit complex bestaat uit de eiwitten adenomatous polyposis coli (APC), axin en de twee kinases caseïne-kinase-1 (CK-1) en GSK-3. Beide kinases zijn verantwoordelijk voor sequentiële fosforylering van β-catenine, waardoor dit eiwit gemarkeerd wordt voor afbraak door het proteasoom (figuur 1). Actief GSK-3 heeft dus een negatieve werking op vrij β-catenine. Echter, binding van een WNT ligand aan de FZD en LRP receptoren leidt tot signaaltransductie die resulteert in de inactivering van het destructiecomplex. Inactivering van het destructiecomplex is gecompliceerd en nog niet volledig opgehelderd, maar het proces gaat gepaard met de rekrutering en fosforylering van zogenoemde dishevelled (DVL) eiwitten en de fosforylering van de LRP5/6 receptoren. Interessant is dat de fosforylering van de LRP5/6 receptoren gemedieerd wordt door GSK-3, waardoor er een negatieve feedback ontstaat (figuur 1). De inactivering van het destructiecomplex leidt tot accumulatie van vrij β-catenine en de daaropvolgende translocatie van deze transcriptionele co-activator naar de celkern. Aldaar gaat β-catenine een interactie aan met de transcriptiefactoren T-cell factor (TCF) / Lymphoid enhancer factor (LEF) en induceert het gentranscriptie. Momenteel zijn meer dan 100 genen bekend die gereguleerd kunnen worden door de activatie van WNT/β-catenin signaaltransductie, waaronder diverse transcriptiefactoren (bijv. cMYC), eiwitten betrokken bij de regulatie van de celcyclus (bijv. cycline D1), diverse groeifactoren, extracellulaire matrixeiwitten, maar ook ontstekingsbevorderende cytokinen en enzymen (o.a. interleukine-8 en cyclo-oxygenase-2).

Naast hun rol in de WNT signaaltransductieroute hebben β-catenine en GSK-3 nog additionele functies in de cel. Zoals reeds aangegeven is β-catenine belangrijk voor de stabilisatie van cel-cel contacten. Deze functie van β-catenine is ook van fysiologisch belang, omdat het bijvoorbeeld bijdraagt aan de krachtontwikkeling door luchtweg gladde-spiercellen zoals recent aangetoond is in ons laboratorium. Evenzo is van GSK-3 bekend dat het bij een breed scala aan cellulaire processen betrokken is, onafhankelijk van het effect van dit kinase op β-catenine. GSK-3 komt voor in twee specifieke isovormen, namelijk GSK-3α (molecuulgewicht: 51

kDa) en GSK-3β (47 kDa). Enkele cellulaire processen waarbij GSK-3 betrokken is, zijn insuline signaaltransductie, differentiatie van cellen, eiwitsynthese, celmotiliteit en celgroei. GSK-3 modificeert deze cellulaire processen door het reguleren van meer dan 50 specifieke substraten, waaronder diverse enzymen, structurele eiwitten en transcriptiefactoren. GSK-3 is een onconventioneel enzym, omdat het een hoge activiteit heeft onder basale condities. De activiteit van het kinase kan echter beïnvloed worden door diverse exogene en endogene stimuli. Zoals genoemd heeft WNT signaaltransductie een negatieve invloed op de GSK-3 activiteit, maar ook fosforylering van specifieke aminozuren (serine 9 in GSK-3α en serine 21 in GSK-3β) heeft een remmende werking op het kinase. Zowel β-catenine als GSK-3 zijn betrokken bij diverse cellulaire processen, echter hun bijdrage aan de pathologische processen die ten grondslag liggen aan de ontwikkeling van chronische luchtwegziekten, met name COPD, is grotendeels onbekend en daarom werd mede dit onderzoek opgezet.



Figuur 1: Schematische weergave van β-catenine-afhankelijke WNT signalering. *Afwezigheid van een extracellulair WNT ligand (links)*: vrij β-catenine wordt herkend door het actieve destructiecomplex, bestaande uit axin, adenomatous polyposis C (APC), caseïne kinase-1 (CK-1) and glycogeen synthase kinase-3 (GSK-3). Het destructiecomplex is verantwoordelijk voor de fosforylering van β-catenine, waardoor dit eiwit gemarkeerd wordt voor afbraak door het proteasoom. *Aanwezigheid van WNT liganden (rechts):* een WNT ligand bindt aan de FZD en LRP5/6 receptoren op het plasmamembraan. Binding van WNT aan deze receptoren leidt tot activatie van de intracellulaire WNT signaaltransductiecascade via rekrutering van dishevelled (DVL) eiwitten. Activatie van DVL in combinatie met fosforylering van LPR5/6 leidt tot inactivatie van het destructiecomplex. Hierdoor kan β-catenine accumuleren in het cytosol en vindt er translocatie plaats van β-catenine naar de celkern. In de celkern gaat de transcriptionele co-activator β-catenine een interactie aan met de transcriptiefactoren T-cell factor (TCF)/Lymphoid enhancer factor (LEF) en induceert het gentranscriptie.

Bijdrage van β-catenine en GSK-3 signaaltransductie aan luchtweg-remodelling: specifieke focus op de luchtweg-gladde spier

Een belangrijk pathologisch verschijnsel bij chronische obstructieve longziekten is het optreden van structurele veranderingen in de luchtwegen, ofwel luchtwegremodelling. Deze structurele veranderingen manifesteren zich voornamelijk in de kleine luchtwegen (<2 mm diameter in doorsnee) en worden gekarakteriseerd door een toename van de luchtweg-gladde spiermassa en veranderingen in de samenstelling en hoeveelheid van extracellulaire matrixeiwitten. Gezamenlijk dragen deze veranderingen in belangrijke mate bij aan zowel de luchtweghyperreactiviteit als de luchtwegobstructie. Een toegenomen luchtweg-gladde spiermassa is bijvoorbeeld geassocieerd met een verminderde longfunctie in ernstig astma. Ook bij COPD kan een toegenomen luchtweg-gladde spiermassa worden waargenomen, voornamelijk in ernstig en zeer ernstig COPD (stadium III en IV). Tabaksrook, de belangrijkste risicofactor voor het ontstaan van COPD, kan een direct effect hebben op de cellen die de wand van luchtwegen bekleden. Hierdoor kunnen processen die betrokken zijn bij luchtwegremodelling geactiveerd worden. Zo kan bijvoorbeeld blootstelling van luchtweg-gladde spiercellen aan sigarettenrook de groei van deze cellen activeren en dit zou mogelijk kunnen verklaren waarom er een toegenomen luchtweg-gladde spiermassa wordt waargenomen bij COPD. Daarnaast hebben diverse experimenten in cellen (*in vitro* experimenten*)* en proefdieren (*in vivo* experimenten) aangetoond dat blootstelling van diverse organen, inclusief de longen, aan sigarettenrook resulteert in een verhoogde expressie van de groeifactor transforming growth factor-β (TGF-β). Overeenkomstig is een verhoogde pulmonale expressie van TGF-β waargenomen in patiënten met COPD. Hierbij is er tevens sprake van een sterke associatie tussen de pathofysiologie van COPD en bepaalde varianten (polymorfismen) van het TGF-β gen. De mechanismen die leiden tot de ontwikkeling en het (progressieve) verloop van luchtwegremodelling zijn slechts ten dele bekend, maar luchtweg-gladde spiercellen zouden in belangrijke mate aan dit proces kunnen bijdragen door toename in celgroei (hyperplasie) en/of celgrootte (hypertrofie), maar ook door middel van het synthetiseren en uitscheiden van diverse ontstekingsmediatoren, groeifactoren en extracellulair matrixeiwitten.

Afzetting van extracellulaire matrix door luchtweg-gladde spiercellen

In de luchtwegen van patiënten met chronische luchtwegziekten kan een toegenomen expressie van extracellulaire matrixeiwitten worden waargenomen rond de gladde spiercellen. Voornamelijk de expressie van fibronectine, hyaluronan, versican, biglycan, lumican en specifieke subtypen collageen is hierbij verhoogd. De veranderingen in luchtweg-gladde spiermassa en extracellulaire matrixafzetting zijn bij COPD in het algemeen minder uitgesproken dan bij astma,

doch deze veranderingen kunnen van groot belang zijn in de pathofysiologie van COPD en dan met name de vergevorderde stadia van de ziekte. De studies beschreven in **hoofdstuk 2**, waarin de bijdrage van β-catenine signaaltransductie aan de extracellulaire matrixeiwit productie onderzocht werd, tonen aan dat stimulatie van luchtweg-gladde spiercellen met TGF-β leidt tot een toegenomen expressie van de extracellulaire matrixeiwitten collageen Iαl, fibronectine en versican. De toename in extracellulaire matrixeiwit expressie geïnduceerd door TGF-β werd vooraf gegaan door activering van β-catenine signaaltransductie en een endogene remming van GSK-3 ten gevolge van van serine fosforylering. Farmacologische inhibitie van de interactie tussen β-catenine en de transcriptiefactor TCF-4 door de verbinding PKF115-584 verhinderde de TGF-β geïnduceerde inductie van de extracellulaire matrixeiwitten in luchtweg-gladde spiercellen. De bijdrage van β-catenine aan dit proces werd nogmaals bevestigd door experimenten, waarbij de expressie van β-catenine verlaagd werd door middel van specifiek siRNA. De resultaten van deze experimenten waren volledig in overeenstemming met de resultaten die verkregen waren met PKF115-584. Opmerkelijk was dat de TGF-β geïnduceerde expressie van het conventionele smad-afhankelijke gen *plasminogen activator inhibitor-1* (PAI-1) niet beïnvloed werd door PKF115-584 of het siRNA specifiek gericht tegen β-catenine. Dit impliceert dat niet alle TGF-β geactiveerde gentranscriptie afhankelijk is van β-catenine en dat activatie van deze transcriptionele co-activator bijdraagt aan de transcriptie van specifieke genen. Bovendien tonen de studies beschreven in **hoofdstuk 2** aan dat transcriptioneel actief β-catenine niet alleen een bijdrage levert aan TGF-β geïnduceerde extracellulaire matrixafzetting door luchtweg-gladde spiercellen, maar ook dat het zelfstandig in staat is om transcriptie van deze eiwitten te induceren. Dit kon worden aangetoond door de luchtweg-gladde spiercellen te transfecteren met een transcriptioneel-actieve en degradatie-ongevoelige β-catenine mutant (S33Y-β-catenine). Het S33Y-β-catenine heeft een mutatie van het aminozuur op positie 33 waarbij een serine (S) is vervangen door een tyrosine (Y), waardoor GSK-3 niet meer in staat is om dit β-catenine te fosforyleren en te markeren voor afbraak. Transfectie met S33Y-β-catenine zorgde ervoor dat de luchtweg-gladde spiercellen meer fibronectine aanmaakten. Samengevat laten de studies beschreven in **hoofdstuk 2** zien dat in luchtweg-gladde spiercellen β-catenine signaaltransductie geactiveerd wordt door de pro-fibrogene groeifactor TGF-β. Activatie van β-catenine als transcriptionele co-activator leidt tot een toename in aanmaak en afzetting van extracellulaire matrixeiwitten in deze cellen, die geremd kon worden door farmacologische interventie met PKF115-584. Deze studie geeft inzicht in een moleculair mechanisme dat zou kunnen bijdragen aan luchtwegremodelling. De uitgescheiden extracellulaire matrixeiwitten dragen niet alleen fysiek bij aan vernauwing van het lumen van de luchtwegen, maar beïnvloeden ook in sterke mate de cellulaire

processen van structurele cellen in de luchtwegwand zoals de gladde spiercellen, fibroblasten en epitheelcellen. Veranderingen in de samenstelling en hoeveelheid extracellulaire matrix in de luchtwegen zijn van belang bij het ziekteproces van chronische obstructieve longziekten, zoals astma en COPD. Activatie van β-catenine leidt niet alleen tot een toename in extracellulaire matrix afzetting, maar het draagt ook bij aan de proliferatie (het proces dat leidt tot een toename in celaantal) van luchtweg-gladde spiercellen na stimulatie met groeifactoren, zoals PDGF en serum. Ergo, activatie van β-catenine signaaltransductie zou kunnen bijdragen aan luchtwegremodelling via een toename in extracellulaire matrixafzetting, maar ook door bij te dragen aan een toename in luchtweg gladde-spiermassa. Echter, in hoeverre β-catenine signaaltransductie verantwoordelijk is voor de luchtwegremodelling in patiënten met COPD is tot op heden nog niet duidelijk. Desalniettemin tonen wij aan dat de β-catenine-gemedieerde aanmaak en afzetting van extracellulaire matrixeiwitten door luchtweg-gladde spiercellen farmacologisch geremd kan worden, wat van therapeutische waarde zou kunnen zijn voor de behandeling van luchtwegremodelling bij COPD.

De luchtweg-gladde spiercel als potentiële bron van cytokinen en groeifactoren
De luchtweg-gladde spiercel wordt in toenemende mate herkend als een potentiële bron van (ontstekingsbevorderende) cytokinen, chemokinen, groeifactoren, enzymen en andere mediatoren die een rol kunnen spelen in chronische obstructieve longziekten. Zoals reeds genoemd, chronische en herhaaldelijke blootstelling van de luchtwegen en het parenchym aan sigarettenrook is de belangrijkste risicofactor voor het ontwikkelen van COPD. Componenten van sigarettenrook alsmede ontstekingsbevorderende cytokinen, bijvoorbeeld interleukine-1β (IL-1β) kunnen de gladde spiercellen activeren om cytokinen en groeifactoren uit te scheiden (**hoofdstuk 5**). Om blootstelling van de luchtweg gladde-spiercellen aan sigarettenrook werd er in de studies beschreven in **hoofdstuk 5** gebruik gemaakt van sigarettenrookextract. Dit sigarettenrookextract is een mengsel van allerlei verschillende chemische componenten die zich in sigarettenrook bevinden en die oplosbaar zijn in het medium, waarin de cellen gekweekt worden. Luchtweg-gladde spiercellen produceren als reactie op sigarettenrookextract en/of IL-1β de cytokinen eotaxine, IL-8 en de groeifactor vascular endothelial growth factor (VEGF) (**hoofdstuk 5**). Deze uitgescheiden eiwitten kunnen in belangrijke mate bijdragen aan de pathogenese van COPD. Zo kunnen de uitgescheiden cytokinen diverse ontstekingsbevorderende cellen aantrekken en activeren, terwijl VEGF een belangrijke rol speelt in angiogenese (vorming van nieuwe bloedvaten) en luchtwegremodelling. De exacte chemische componenten in sigarettenrookextract die verantwoordelijk zijn voor de inductie van cytokine- en groeifactorafgifte door de luchtweg-gladde spiercellen zijn nog niet opgehelderd. De onderliggende moleculaire mechanismen die betrokken zijn bij dit

proces zijn eveneens nog niet volledig in kaart gebracht. Activatie van zowel de zogenoemde nuclear factor-kappa B (NF-κB) signaaltransductieroute als van β-catenine-afhankelijke gentranscriptie is in verband gebracht met de productie van de reeds genoemde cytokinen en VEGF. Echter, sigarettenrookextract en IL-1β zijn op zichzelf niet in staat om β-catenine signaaltransductie te activeren in luchtweg-gladde spiercellen (**hoofdstuk 5**). Vanuit de literatuur is bekend dat GSK-3 potentieel betrokken is bij de regulatie van de NF-κB signaaltransductieroute en dat het kinase fungeert als negatieve regulator van β-catenine. Daarom was onze hypothese dat het kinase een belangrijke rol zou kunnen vervullen in de door sigarettenrookextract en IL-1β geïnduceerde ontstekingsreactie in luchtweg-gladde spiercellen. Farmacologische remming van GSK-3 deed de afgifte van eotaxine, IL-8 en VEGF door luchtweg gladde-spiercellen als reactie op sigarettenrookextract en IL-1β afnemen, terwijl de cellulaire expressie van β-catenine toenam als gevolg van de GSK-3 remming. Zowel sigarettenrookextract als IL-1β zorgde voor de afbraak van Iκ-Bα (een endogene remmer van de NF-κB signaaltransductieroute) en voor de translocatie van de p65-subunit van NF-κB naar de kern. Deze twee cellulaire processen tonen aan dat de NF-κB signaaltransductieroute geactiveerd werd door beide stimuli. Farmacologische remming van GSK-3 met SB216763 had geen effect op de afbraak van Iκ-Bα of de translocatie van p65 NF-κB, maar remde volledig de transcriptionele activiteit van de NF-κB signaaltransductieroute (**hoofdstuk 5**). Samengevat betekent dit dat GSK-3 signaaltransductie in luchtweg-gladde spiercellen bijdraagt aan de ontstekingsreactie geïnitieerd door (oplosbare componenten van) sigarettenrook en het cytokine IL-1β, via NF-κB gemedieerde gentranscriptie. Hieruit kan geconcludeerd worden dat remming van GSK-3 een nieuwe strategie zou kunnen zijn voor het verminderen van ontstekingsprocessen in de luchtwegen bij chronische obstructieve longziekten. Interessant is dat vermindering van de GSK-3 activiteit gepaard gaat met een toename van β-catenine, welke niet geassocieerd is met de aanmaak en vrijzetting van cytokinen en groeifactoren. Een toename in β-catenine expressie in luchtweg-gladde spiercellen kan echter leiden tot een toegenomen aanmaak van extracellulaire matrixeiwitten, zoals de studies beschreven in **hoofdstuk 2** aantonen. Een potentiële beperking van het therapeutisch gebruik van GSK-3 remmers zou in theorie dus kunnen zijn dat de extracellulaire matrixafzetting in de luchtwegen toeneemt, waardoor luchtwegremodelling versterkt wordt. Tevens is aangetoond in muizen dat hypertrofie (toename in celgrootte) en hyperplasie (toename in celaantal) van de luchtweg-gladde spier correleert met endogene inactivatie van GSK-3 (d.w.z. serine fosforylatie van beide GSK-3 isovormen). Desalniettemin vermeldt geen enkele *in vivo* studie, waarin het GSK-3 als mogelijk farmacologisch target onderzocht is, significante veranderingen in de hoeveelheid en grootte van de luchtweg-gladde spier en/of toegenomen extracellular matrixafzetting in de

luchtwegen (zie ook **hoofdstuk 6**). Deze studies tonen zelfs het tegendeel aan, waarbij een afname gezien wordt van specifieke extracellulaire matrixeiwitten in de longen, en dan voornamelijk in de luchtwegen. Deze schijnbare tegenstelling kan mogelijk verklaard worden door de directe effecten van GSK-3 remming in cellen die betrokken zijn bij fibrotische reacties, of door een verminderde ontstekingsreactie *in vivo* als gevolg van GSK-3 inhibitie (**hoofdstuk 4 en 5**).

Een nieuw paradigma voor weefselherstel in de door COPD aangetaste long: WNT/β-catenine en GSK-3 signaaltransductie in longfibroblasten

Een belangrijk pathologisch verschijnsel dat optreedt bij COPD is emfyseem, dat in tegenstelling tot luchtwegremodelling gekarakteriseerd wordt door progressief verlies van longweefsel als gevolg van de chronische ontsteking en weefselschade. De huidige therapeutische mogelijkheden voor de behandeling van aan roken gerelateerd emfyseem zijn schaars en slechts beperkt effectief. Diverse mechanismen dragen mogelijk bij aan de ontwikkeling van de chronische weefselschade, waaronder een verstoring van de balans in expressie van proteasen en anti-proteasen in de long, een toegenomen apoptose (geprogrammeerde celdood) van alveolaire epitheelcellen, alsmede een verminderde afweerreactie van het immuunsysteem in de longen. De meeste studies richten zich op de processen die betrokken zijn bij het veroorzaken van chronische weefselschade, terwijl de processen die betrokken bij weefselherstel minder onderzocht zijn. De processen die betrokken zijn bij weefselherstel zijn echter niet van ondergeschikt belang. Emfyseem zou kunnen worden beschouwd als een verminderd herstel van parenchymweefsel, als gevolg van beschadiging door chronische blootstelling aan sigarettenrook. Longfibroblasten zijn cellen die primair van belang zijn voor het herstellen van weefselschade. Alhoewel de emfysemateuze long primair gekarakteriseerd wordt door afname van longweefsel, zijn er indicaties dat fibroblasten in de longblaasjes (alveoli) nog steeds actief zijn.

De moleculaire mechanismen die kunnen bijdragen aan weefselschade en -herstel van het longparenchym zijn nog niet goed opgehelderd. Het herstel van longweefsel of het tegengaan van verdere beschadiging zou een reëel doel kunnen zijn voor therapeutische interventie bij de behandeling van emfyseem. Activatie van signaaltransductieroutes die betrokken zijn bij de ontwikkeling van de longen vormen een rationele benadering om dit doel te bereiken. In dit opzicht is de WNT signaaltransductieroute van potentieel belang, omdat deze in belangrijke mate bijdraagt aan de longontwikkeling tijdens de verschillende embryonale fasen. Diverse studies hebben aangetoond dat activatie van de WNT signaaltransductieroute ook kan bijdragen aan weefselherstel en dat deze route daarin samenwerkt met de TGF-β signaaltransductie. Een screening van genen die noodzakelijk zijn voor functionele WNT signalering toonde aan dat het merendeel

van deze genen tot expressie wordt gebracht in primaire longfibroblasten. Er waren echter duidelijke verschillen waarneembaar in de mate waarin deze specifieke genen tot expressie worden gebracht (**hoofdstuk 3**). Vooral de WNT liganden WNT-5A, WNT-5B en WNT16, de FZD receptoren FZD_2, FZD_6 en FZD_8, alsmede DVL3 en β-catenine werden hoog tot expressie gebracht. Daarentegen werden WNT-3A, WNT-6, WNT-9A, FZD_3, FZD_9 en FZD_{10} in geringe mate tot expressie gebracht.

Stimulatie van de longfibroblasten met de groeifactor TGF-β induceerde genexpressie van specifieke WNT liganden en receptoren, terwijl de expressie van andere WNT genen niet beïnvloed werd. Met name de expressie van WNT-5B, FZD_6, FZD_8 en β-catenine was gevoelig voor stimulatie met TGF-β. Opmerkelijk was dat de expressie van enkele van deze genen aanzienlijk hoger was in longfibroblasten van patiënten met matig of zeer ernstig COPD (respectievelijk stadium II en stadium IV). Naast een toegenomen genexpressie leidde stimulatie met TGF-β ook tot een toename in de eiwitexpressie van β-catenine. De TGF-β stimulatie resulteerde eveneens in een toegenomen cytosolaire en nucleaire expressie van transcriptioneel actief β-catenine (**hoofdstuk 3**). Door gebruik te maken van verschillende moleculair biologische en farmacologische technieken, toonden we aan dat activatie van β-catenine signaaltransductie bijdraagt aan het differentiatieproces van fibroblasten tot meer actieve myofibroblasten. TGF-β stimulatie leidde tot een versterkte activatie van β-catenine signaaltransductie in longfibroblasten van COPD patiënten in vergelijking tot fibroblasten van de controle groep. Hiermee overeenkomstig, resulteerde de toegenomen activatie van β-catenine in versterkte afzetting van het extracellulaire matrixeiwit fibronectine (een β-catenine gereguleerd gen) door fibroblasten van COPD patiënten in vergelijking tot de controle groep (**hoofdstuk 3**). Samengevat, de studies beschreven in **hoofdstuk 3** tonen aan dat longfibroblasten van COPD patiënten een verhoogde genexpressie van WNT liganden en receptoren hebben, dat activatie van β-catenine signaaltransductie bijdraagt aan myofibroblast differentiatie en dat longfibroblasten van COPD patiënten een toegenomen activering van β-catenine signaaltransductie en dientengevolge fibronectine afzetting vertonen. De verhoogde WNT expressie kan aldus als een poging tot weefselherstel gezien worden bij patiënten met COPD.

Deze bevindingen suggereren dat activatie van WNT signaaltransductie bijdraagt aan weefselherstel, wat implicaties heeft zou kunnen hebben voor het verminderen van de progressie van emfyseem. Inderdaad, recent heeft een *in vivo* studie aangetoond dat therapeutische activatie van de WNT/β-catenine signaaltransductie leidt tot een vermindering van emfyseem in muizen. Deze studie toonde verder aan dat genexpressie van specifieke componenten van de WNT

signaaltransductieroute, alsmede de activiteit van deze route, nagenoeg identiek was in longweefsel van COPD patiënten en in de controle groep. Opmerkelijk was echter, dat de nucleaire expressie van β-catenine verminderd was in alveolaire type II (AT II) epitheelcellen in gebieden die beschadigd waren als gevolg van emfyseem. Door gebruik te maken van twee verschillende *in vivo* modellen van sigarettenrook en elastine geïnduceerd emfyseem, toonden de auteurs van deze studie aan dat intraperitoneale toediening van LiCl (een relatief aselectieve remmer die ook GSK-3 activiteit remt) leidt tot activatie van β-catenine signaaltransductie in AT II cellen. Activatie van β-catenine in deze cellen resulteerde vervolgens in herstel van het parenchymweefsel en tot een verbeterde longfunctie.

In de longen bevinden de epitheelcellen en de longfibroblasten zich in elkaars nabijheid en zijn ze in staat elkaars cellulaire responsen te beïnvloeden. De communicatie tussen deze cellen zou mogelijk verstoord kunnen zijn bij COPD en zodoende kunnen bijdragen aan het ziekteproces. Ook in deze context zou signaaltransductie via WNT erg belangrijk kunnen zijn. Sommige WNT liganden leiden tot cellulaire processen zonder dat ze β-catenine signaaltransductie activeren en worden daarom niet-canonieke WNT liganden genoemd. Deze specifieke WNT liganden zijn in staat om signaaltransductie aangestuurd door canonieke WNT liganden, die wel leiden tot stabilisatie en activatie van β-catenine, te antagoneren. De door WNT-3A geïnduceerde stabilisatie en activatie van β-catenine kan bijvoorbeeld concentratie-afhankelijk worden voorkomen door cellen te stimuleren met WNT-5A (niet-canoniek WNT ligand). Belangrijk hierbij is dat AT II cellen afhankelijk zijn van de canonieke WNT signaaltransductie om te prolifereren en om weefsel te herstellen. Het lijkt er echter op dat longfibroblasten als reactie op TGF-β stimulatie met name componenten van de niet-canonieke WNT signaaltransductie tot expressie brengen en dat dit versterkt is in fibroblasten van COPD patiënten (**hoofdstuk 3**). Daarom zou de balans in de wederzijdse communicatie tussen longfibroblasten en AT II cellen via canonieke en niet-canonieke WNT signaaltransductie verstoord kunnen zijn bij de pathofysiologie van COPD. De verhoogde afgifte van niet-canonieke WNT liganden door de longfibroblasten zorgt er met andere woorden voor dat canonieke WNT signaaltransductie in ATII onderdrukt wordt, wat weer tot gevolg heeft dat de proliferatie van ATII cellen en de capaciteit om weefsel te repareren verminderd. De onbalans in WNT-gemedieerde cellulaire communicatie tussen deze twee celtypen kan mogelijk de ontwikkeling en progressie van emfyseem verklaren en daarom is deze hypothese bijzonder aantrekkelijk om in de toekomst verder te bestuderen. Tevens suggereert dit dat het herstellen van het normale evenwicht in canonieke en niet-canonieke WNT signaaltransductie tussen het epitheel en het onderliggende mesenchymale weefsel een therapeutische strategie zou kunnen zijn voor de behandeling van COPD.

GSK-3 als potentieel therapeutisch doelwit voor de behandeling van COPD

Uit het voorgaande blijkt dat activatie van canonieke WNT signaaltransductie zou kunnen bijdragen aan weefselherstel en dat GSK-3 functioneert als een negatieve regulator van deze signaaltransductieroute. De effecten van de WNT signaaltransductieroute zijn echter context-afhankelijk en langdurige activatie van deze specifieke route zou nadelige gevolgen kunnen hebben voor weefselfibrose. Remming van GSK-3 in longfibroblasten leidt echter niet tot een toegenomen myofibroblastdifferentiatie (**hoofdstuk 4**). Integendeel, farmacologische remming van GSK-3 of downregulatie van het kinase door middel van siRNA voorkomt de TGF-β geïnduceerde myofibroblastdifferentiatie. In longfibroblasten leidt een verminderde activiteit van GSK-3 tot een toegenomen fosforylatie en zodoende activatie van het zogenoemde eiwit cAMP-responsive element binding protein (CREB). Gefosforyleerd CREB functioneert als competitieve antagonist van TGF-β-geïnduceerde Smad signaaltransductie, zonder dat de fosforyleringsstatus van de Smad-eiwitten wordt beïnvloed. Interessant is dat GSK-3 in dezelfde mate betrokken is bij myofibroblastdifferentiatie in primaire longfibroblasten afkomstig van COPD patiënten. Dit suggereert dat GSK-3 belangrijke cellulaire functies van longfibroblasten reguleert en dat die nog intact zijn in patiënten met COPD. Een onverwachte bevinding was dat vermindering van de GSK-3 activiteit in longfibroblasten resulteert in een afname van TGF-β geïnduceerde β-catenine expressie in plaats van een toename, zoals dit uit de schematische weergave in figuur 1 verwacht zou worden (**hoofdstuk 6**). De cellulaire expressie van β-catenine wordt bepaald door de mate waarin het eiwit door *de novo* synthese wordt aangemaakt en de mate waarin β-catenine afgebroken wordt door posttranslationele processen. In longfibroblasten leidt stimulatie met verscheidene groeifactoren, waaronder TGF-β, tot een toegenomen smad-afhankelijke gentranscriptie van β-catenine, waardoor de cellulaire expressie van het eiwit toeneemt. Zoals genoemd, resulteert verminderde activiteit van GSK-3 tot een toegenomen CREB activatie, hetgeen de smad-afhankelijke signaaltransductie onderdrukt. Een plausibele verklaring voor verminderde TGF-β-geïnduceerde β-catenine expressie na inhibitie van GSK-3 is daarom dat dit een gevolg kan zijn van de onderdrukking van smad-afhankelijke *de novo* synthese van β-catenine. Kortom, verscheidene cellulaire responsen van longfibroblasten, waaronder de afzetting van extracellulaire matrixeiwitten en myofibroblast differentiatie, zijn afhankelijk van GSK-3 activiteit via de onderdrukking van CREB-gemedieerde signaaltransductie.

Om onze *in vitro* bevindingen te bevestigen en het therapeutische potentieel van GSK-3 remming verder te onderzoeken, zijn de effecten van de GSK-3 remmer SB216763 in een caviamodel van lipopolysaccharide (LPS)-geïnduceerd COPD onderzocht (**hoofdstuk 6**). LPS is een endotoxine dat deel uitmaakt van het

buitenmembraan van gram-negatieve bacteriën en is tevens onderdeel van sigarettenrook en andere pathogene stimuli die geassocieerd zijn met de ontwikkeling van COPD. In diverse diermodellen is aangetoond dat herhaaldelijke LPS blootstelling ziekteverschijnselen induceert die overeenkomen met de pathofysiologie van COPD. Dit geldt ook voor het gebruikte caviamodel, waarbij herhaaldelijke intranasale toediening van LPS (2x per week gedurende12 weken) leidde tot pulmonale en extrapulmonale ziekteverschijnselen. De pathologische veranderingen in de longen waren geassocieerd met activatie van β-catenine signaaltransductie en werden gekarakteriseerd door een toegenomen fibronectine expressie en een toename in collageen afzetting in de kleine luchtwegen. Tevens induceerde LPS cardiovasculaire remodelling, waaronder hypertrofie van het rechter ventrikel en er was een tendens tot skeletspieratrofie (**hoofdstuk 6**). Selectieve remming van GSK-3 door intranasale instillatie van SB216763 was in staat om de LPS-geïnduceerde pulmonale en extrapulmonale pathologische kenmerken grotendeels te voorkomen in de cavia. In overeenstemming met de bevindingen in de longfibroblasten, leidde GSK-3 remming in het caviamodel van LPS geïnduceerd COPD ook tot een verminderde activatie van β-catenine. De afname in actief β-catenine zou een verklaring kunnen zijn voor de afname in pathologische verschijnselen na farmacologische remming van GSK-3 in dit *in vivo* model van COPD. Naast een direct effect van GSK-3 remming in cellen die betrokken zijn bij het fibrotische proces, kan de afname in pathologische verschijnselen ook het gevolg zijn van een verminderde ontstekingsreactie ten gevolge van GSK-3 remming. Zoals aangetoond in **hoofdstuk 5,** leidt GSK-3 remming in luchtweg-gladde spiercellen tot een verminderde productie van ontstekingsbevorderende cytokinen en groeifactoren. Tevens hebben diverse *in vitro* en *in vivo* studies, die model stonden voor een breed scala aan andere chronische aandoeningen dan COPD, aangetoond dat GSK-3 inhibitie een gunstig effect heeft op de ontstekingsreactie. Geruststellend is dat onze studie aantoont dat farmacologische remming van GSK-3 niet leidt tot luchtwegremodelling of andere ernstige bijwerkingen. Bovendien wordt de toepasbaarheid van GSK-3 remmers klinisch onderzocht voor de behandeling van de ziekte van Alzheimer en diabetes mellitus, terwijl LiCl al meer dan 50 jaar klinisch gebruikt wordt in de psychiatrie voor de behandeling van manische depressiviteit. Gezamenlijk tonen deze bevindingen aan dat GSK-3 een therapeutisch target kan zijn voor de behandeling van chronische ontstekingsziekten en dat GSK-3 remmers mogelijk in de toekomst toepasbaar zijn voor de behandeling van COPD.

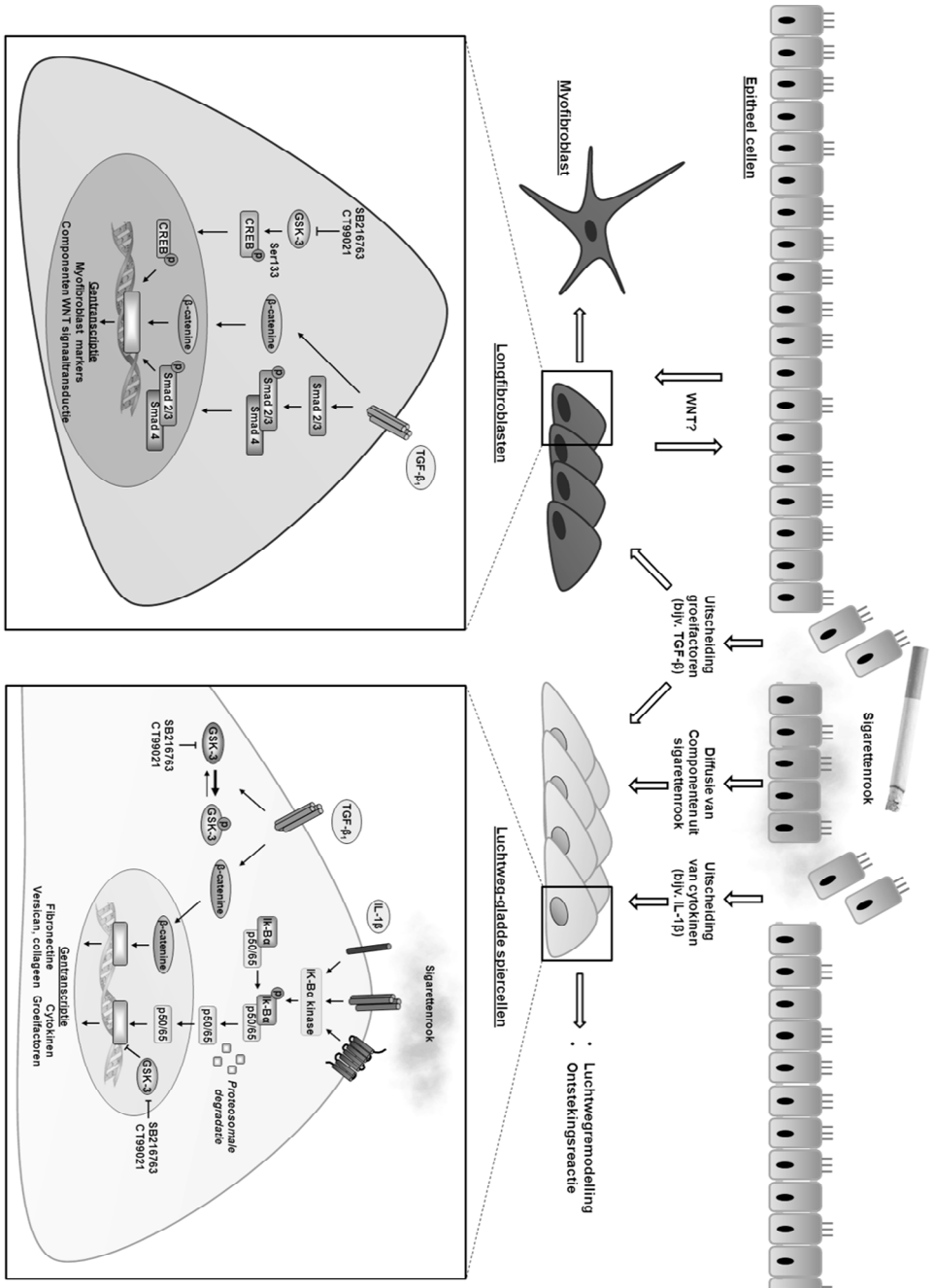
Conclusies

De studies beschreven in dit proefschrift hebben het volgende aangetoond:

- Stimulatie met TGF-β leidt in zowel luchtweg-gladde spiercellen als in longfibroblasten tot de activatie van β-catenine signaaltransductie (**hoofdstukken 2 en 3**)
- Activatie van β-catenine signaaltransductie in luchtweg-gladde spiercellen leidt tot aanmaak en afzetting van de extracellulaire matrixeiwitten fibronectine, collageen Iαl en versican (**hoofdstuk 2**)
- Longfibroblasten brengen de genen tot expressie die noodzakelijk zijn voor functionele WNT signaaltransductie. Stimulatie met TGF-β leidt tot een toename in genexpressie van specifieke componenten van de WNT signaaltransductieroute en deze inductie is sterker in fibroblasten van patiënten met matig en zeer ernstig COPD in vergelijking tot fibroblasten van de controle groep (**hoofdstuk 3**).
- Activatie van β-catenine signaaltransductie door TGF-β draagt bij aan het differentiatieproces van fibroblasten naar meer actieve myofibroblasten. In fibroblasten van COPD patiënten (stadium II en IV) is deze activatie van β-catenine toegenomen en leidt dit tot een versterkte afzetting van het extracellulaire matrixeiwit fibronectine (**hoofdstuk 3**).
- β-catenine-gemedieerde extracellulaire matrixafzetting door zowel luchtweg-gladde spiercellen als door longfibroblasten kan farmacologisch geremd worden door de interactie tussen β-catenine en de transcriptiefactor TCF-4 te verstoren met behulp van de farmacologische remmers PKF115-584 en quercetin (**hoofdstukken 2 en 3**).
- GSK-3 signaaltransductie draagt bij aan het differentiatieproces van fibroblasten naar myofibroblasten geïnduceerd door TGF-β stimulatie. Farmacologische remming van GSK-3 of downregulatie van het kinase door middel van specifiek siRNA leidt tot een toegenomen fosforylering van CREB. Gefosforyleerd CREB voorkomt myofibroblast differentiatie door de smad signaaltransductie te antagoneren (**hoofdstuk 4**).
- Stimulatie van luchtweg-gladde spiercellen met sigarettenrookextract of het ontstekingsbevorderende cytokine IL-1β resulteert in vrijzetting van eotaxine, IL-8 en VEGF door deze cellen. Farmacologische remming van GSK-3 vermindert de vrijzetting van deze cytokinen en groeifactor-vrijzetting geïnduceerd door sigarettenrookextract en IL-1β door de NF-κB gemedieerde gentranscriptie te onderdrukken (**hoofdstuk 5**).
- GSK-3 signaaltransductie draagt bij aan de pulmonale en extrapulmonale pathologische verschijnselen in een caviamodel van LPS geïnduceerd COPD. Herhaaldelijke intranasale instillatie van LPS leidt in de cavia tot activatie van β-catenine en remodellering van de luchtwegen. Dit laatste wordt gekarakteriseerd door een toename in fibronectine expressie en een

toegenomen collageen expressie in de kleine luchtwegen. Tevens leidt intranasale LPS toediening tot hypertrofie van het rechter ventrikel en er is een tendens waarneembaar voor atrofie van de skeletspieren. Deze pathologische verschijnselen geïnduceerd door LPS kunnen grotendeels voorkomen worden door intranasale toediening van de GSK-3 remmer SB216763 (**hoofdstuk 6**).

De diverse *in vitro* studies beschreven in dit proefschrift tonen aan dat zowel β-catenine als GSK-3 een belangrijke rol spelen in luchtweg-gladde spiercellen en longfibroblasten bij specifieke cellulaire processen die kunnen bijdragen aan de pathofysiologie van COPD. Een schematisch overzicht van de resultaten uit de *in vitro* studies is weergegeven in figuur 2. De *in vivo* studie beschreven in dit proefschrift toont aan dat pulmonale toediening van de GSK-3 remmer SB216763 in staat is om de pulmonale en extrapulmonale ziekteverschijnselen geïnduceerd door intranasale LPS instillatie te voorkomen. Gezamenlijk suggereren de resultaten van de *in vitro* en *in vivo* studies dat farmacologische interventie in GSK-3 en β-catenine gemedieerde signaaltransductie de pathologische processen die ten grondslag liggen aan de ontwikkeling en progressie van COPD kan voorkomen. Deze bevindingen openen nieuwe mogelijkheden voor de (toekomstige) ontwikkeling van nieuwe therapieën voor de behandeling van chronisch obstructieve longziekten, zoals COPD.


Epitheel cellen
Sigaretterook
WNT?
Uitscheiding groeifactoren (bijv. TGF-β)
Diffusie van Componenten uit sigaretterook
Uitscheiding van cytokinen (bijv. IL-1β)
Myofibroblast
Longfibroblasten
Luchtwegremodelling
Ontstekingsreactie
Luchtwegs-gladde spiercellen
SB216763
CT99021
GSK-3
Ser133
CREB
β-catenine
Smad 2/3
Smad 4
TGF-β1
Gentranscripte
Myofibroblast markers
Componenten WNT signaaltransductie
Sigaretterook
IL-1β
IK-Bα kinase
IK-Bα
p50/65
Proteosomale degradatie
Gentranscripte
Fibronectine
Versican, collageen
Cytokinen
Groeifactoren

◄ *Figuur 2:* **Rol van β-catenine en GSK-3 signaaltransductie in cellulaire processen die bijdragen aan de pathofysiologie van COPD.** Chronic obstructive pulmonary disease (COPD; chronisch obstructief longlijden) wordt primair veroorzaakt door het roken van sigaretten of andere tabaksproducten. Chronische blootstelling van cellen in de luchtwegen kan leiden tot weefselschade en veranderingen in de structuur van de longen. Blootstelling van epitheelcellen aan sigarettenrook leidt tot de uitscheiding van diverse ontstekingsbevorderende cytokinen (bijv. IL-1β) en groeifactoren (bijv. TGF-β). De uitgescheiden mediatoren kunnen de luchtweg-gladde spiercellen of de longfibroblasten activeren. Tevens kunnen diverse componenten van sigarettenrook diffunderen naar deze cellen die zich onder het epitheel bevinden. Activatie van de longfibroblasten door TGF-β leidt tot een toename in cellulaire β-catenine expressie. Vervolgens vindt translocatie van β-catenine naar de kern plaats, waar het bijdraagt aan gentranscriptie van genen die geassocieerd zijn met de differentiatie van fibroblasten naar myofibroblasten (**hoofdstuk 3**). Tevens leidt TGF-β stimulatie in deze cellen tot een verhoging van genen van de WNT signaaltransductieroute. In longfibroblasten van COPD patiënten is de expressie van genen voor de WNT signaaltransductieroute, de activatie van β-catenine alsmede de afzetting van fibronectine door TGF-β stimulatie toegenomen in vergelijking tot longfibroblasten van personen zonder COPD (**hoofdstuk 3**). Stimulatie van deze cellen met TGF-β leidt eveneens tot de activatie van de smad signaaltransductieroute dat bijdraagt aan het differentiatieproces van de fibroblasten. Farmacologische remming van GSK-3 door SB216763 of CT/CHIR99021 leidt tot een vermindering van smad gemedieerde gentranscriptie, doordat de CREB signaaltransductie wordt geactiveerd. Actief CREB kan smad-afhankelijke gentranscriptie antagoneren, zonder dat de fosforylering van de smad eiwitten beïnvloed wordt (**hoofdstuk 4**). In de luchtweg gladde-spiercellen leidt TGF-β stimulatie tot activatie van β-catenine signaaltransductie door inactivatie van GSK-3 via serine fosforylatie en *de novo* synthese van β-catenine. In deze cellen leidt de activatie van β-catenine tot gentranscriptie van extracellulaire matrixeiwitten (**hoofdstuk 2**). Behandeling van de luchtweg-gladde spiercellen met sigarettenrookextract of IL-1β induceert de activatie van de NF-κB signaaltransductieroute, wat noodzakelijk is voor de transcriptie en het vrijzetten van eotaxine, VEGF en IL-8 door deze cellen. Farmacologische remming van GSK-3 vermindert de NF-κB gemedieerde gentranscriptie, zonder dat de afbraak van Iκ-Bα of de nucleaire translocatie en DNA binding van p65 NF-κB beïnvloed wordt (**hoofdstuk 5**).

Dankwoord

Dankwoord / Acknowledgements

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Curriculum Vitae

The author of this thesis was born on the 12th of March 1983 in Boornbergum, municipality Smallingerland, The Netherlands. After finishing his pre-university education (Atheneum, Groningen) in 2001, he started his Pharmacy study at the University of Groningen. He obtained his Bachelor degree (BSc.) in 2004 and his Master's degree as well as his pharmacist's degree (MSc. and PharmD) in 2007. His Master's thesis on the effects of insulin on airway smooth muscle phenotype was completed at the Department of Molecular Pharmacology, University of Groningen. After his graduation in July 2007, he was appointed as instructor of the practical pharmacology course for Pharmacy and Pharmaceutical Sciences students at the University of Groningen. In March 2008 he initiated his PhD-study at the Department of Molecular Pharmacology, where he worked on a research project funded by the Netherlands Asthma Foundation (NAF grant: 3.2.07.023) entitled: 'The β-catenin/GSK-3 signaling axis: a central transducer of chronic airway remodelling and emphysema in COPD', the results of which are presented in this thesis.

List of publications

Publications

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Full articles

Baarsma HA, Engelbertink HJM, van Hees LJ, Menzen MH, Meurs H, Timens W, Postma DS, Kerstjens HAM, Gosens R. *Glycogen synthase kinase-3 (GSK-3) regulates TGF-β_1-induced differentiation of pulmonary fibroblasts via suppression of CREB signaling.* Submitted 2012.

Baarsma HA, Spanjer AI, Haitsma GH, Engelbertink LH, Meurs H, Jonker MR, Timens W, Postma DS, Kerstjens HA, Gosens R. *Activation of WNT / β-catenin signaling in pulmonary fibroblasts by TGF-β_1 is increased in chronic obstructive pulmonary disease*. Plos One, 2011; 6(9):e25450.

Baarsma HA, Menzen MH, Halayko AJ, Meurs H, Kerstjens HA, Gosens R. *β-catenin is required for TGF-β_1-induced extracellular matrix production by airway smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2011; 301(6):L956-65

Baarsma HA, Meurs H, Halayko AJ, Menzen MH, Schmidt M, Kerstjens HA, Gosens R. *Glycogen synthase kinase-3 regulates cigarette smoke extract- and IL-1β-induced cytokine secretion by airway smooth muscle.* Am J Physiol Lung Cell Mol Physiol, 2011; 300(6):L910-9

Jansen SR, Van Ziel AM, **Baarsma HA**, Gosens R. *β-catenin regulates airway smooth muscle contraction.* Am J Physiol Lung Cell Mol Physiol, 2010; 299(2): L20-14

Gosens R, **Baarsma HA**, Heijink IH, Oenema TA, Halayko AJ, Meurs H, Schmidt M. *De novo synthesis of β-catenin via H-RAS and MEK regulates airway smooth muscle growth.* FASEB J. 2010 (3):757-68

Nunes RO, Schmidt M, Dueck G, **Baarsma H**, Halayko AJ, Kerstjens HA, Meurs H, Gosens R. *GSK-3/β-catenin signaling axis in airway smooth muscle: role in mitogenic signaling.* Am J Physiol Lung Cell Mol Physiol, 2008; 294(6):L1110-8

Schaafsma D, McNeill KD, Stelmack GL, Gosens R, **Baarsma HA**, Dekkers BG, Frohwerk E, Penninks JM, Sharma P, Ens KM, Nelemans SA, Zaagsma J, Halayko AJ, Meurs H. *Insulin increases the expression of contractile phenotypic markers in airway smooth muscle.* Am J Physiol Cell Physiol, 2007; 293(1):C429-39

Abstracts

Baarsma HA, Engelbertink LHJM, van Hees LJ, Rodewald M, Postma DS, Timens W, Meurs H, Kerstjens HAM, Gosens R. *Myofibroblast Differentiation by TGF-β1 requires Glycogen Synthase Kinase-3 (GSK-3) signaling*. Respir Crit Care Med 183;2011:A5336

Baarsma HA, Menzen MH, Halayko AJ, Meurs H, Kerstjens HAM, Gosens R. *β-Catenin signaling is required for TGF-β_1-induced ECM production by airway smooth muscle.* Am J Respir Crit Care Med 183;2011:A4060

Kumawat K, Bos IST, Borger P, Roth M, Tamm M, Postma DS, Koppelman GH, Siedlinski M, Halayko AJ, Menzen MH, **Baarsma HA**, Schmidt M, Gosens R. *Autocrine Wnt5a Signaling Is Increased In Asthma And Regulates TGF-Beta1 Induced ECM Production By Airway Smooth Muscle Cells.* Am J Respir Crit Care Med 183;2011:A4058

Baarsma HA, Engelbertink LHJM, Postma DS, Timens W, Meurs H, Kerstjens HAM, Gosens R. *Glycogen synthase kinase-3 is required for transforming growth factor-β-induced myofibroblast differentiation*. Am J Respir Crit Care Med 181;2010:A2081

Baarsma HA, Engelbertink LJHM, Haitsma GH, Postma DS, Timens W, Kerstjens HAM, Meurs H, Gosens R. *Wnt / ß-catenin signaling regulates transforming growth factor-β-induced differentiation of lung fibroblast.* Am J Respir Crit Care Med 181;2010:A2080

Baarsma HA, Haitsma GH, Engelbertink LHJM, Postma DS, Timens W, Kerstjens HA, Meurs H, Gosens R. W*NT/β-catenin signaling regulates TGF-β_1-induced differentiation of lung fibroblasts*. Naunyn-Schmiedebergs archives of pharmacology 2010, 381(1)56-56.

Bos IST, **Baarsma HA**, Halayko AJ, Gosens R. *Functional Wnt signaling in airway smooth muscle.* Am J Respir Crit Care Med 181;2010:A2126

Jansen SR, van Ziel M, **Baarsma HA**, Gosens R. *β-catenin regulates airway smooth muscle contraction.* Am J Respir Crit Care Med 181;2010:A5292

Baarsma HA, Meurs H, Halayko AJ, Gosens R. *Wnt pathway gene expression in airway smooth muscle and bronchial epithelial cells.* Am J Respir Crit Care Med 179;2009:A3895

Baarsma HA, Meurs H, Halayko AJ, Kerstjens HAM, Gosens R. *GSK-3 inhibition attenuates cigarette smoke and IL-1β-induced pro-inflammatory cytokine secretion by airway smooth muscle cells.* Am J Respir Crit Care Med 179;2009:A3901

Baarsma HA, Meurs H, Halayko AJ, Kerstjens HA, Gosens R. *GSK-3 inhibition attenuates cigarette smoke extract- and IL-1β-induced pro-inflammatory cytokine secretion by airway smooth muscle.* Naunyn-Schmiedebergs archives of pharmacology 2009, 380(3)263-263.

Gosens R, **Baarsma HA**, Heijink IH, Halayko AJ, Meurs H, Schmidt M. *A MEK/β-catenin signaling pathway regulates airway smooth muscle (ASM) growth.* Am J Respir Crit Care Med 179;2009:A3916

Gosens R, **Baarsma HA**, Heijink IH, Oenema TA, Halayko AJ, Meurs H, Schmidt M. *A MEK/β-catenin signaling pathway regulates airway smooth muscle growth.* Naunyn-Schmiedebergs archives of pharmacology 2009, 379, 11-11.

Schaafsma D, McNeill KD, Gosens R, **Baarsma HA**, Penninks JM, Zaagsma J, Nelemans SA, Halayko AJ, Meurs H. *Insulin increases the expression of contractile phenotypic markers in airway smooth muscle (ASM).* Naunyn-Schmiedebergs archives of pharmacology 2007, 375(2), 153-154.

Abbreviations

List of abbreviations

Abbreviations

18S rRNA:	18S ribosomal RNA
ABC:	Active (non-phosphorylated) β-catenin
α-sm-actin:	Alpha-smooth muscle-actin
ANOVA:	Analysis of variance
AP-1:	Activator protein-1
APC:	Adenomatous polyposis coli
ASM:	Airway smooth muscle
BMI:	Body mass index
cAMP:	Adenosine 3'5' cyclic monophosphate
CBP:	CREB binding protein
Cby:	Chibby
CK-1:	Casein kinase-1
COPD:	Chronic obstructive pulmonary disease
CREB:	cAMP responsive element binding protein
CSE:	Cigarette smoke extract
CTGF:	Connective tissue growth factor
DKK:	Dickkopf
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DVL:	Dishevelled
EC_{50}:	Concentration of the stimulus eliciting half-maximum response
ECM:	Extracellular matrix
eGFP:	Enhanced green fluorescent protein
ERK-1/2:	Extracellular signal-regulated kinase 1 and 2 (MAPK)
FBS:	Foetal bovine serum
FEV_1:	Forced expiratory volume in 1 second
FZD:	Frizzled receptor
FVC:	Forced vital capacity
GAG:	Glycosaminoglycan
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GSK-3:	Glycogen synthase kinase-3
HBSS:	Hank's balanced salt solution
HMG:	High mobility group
hTERT:	Human telomerase reverse transcriptase
ICAT:	Inhibitor of β-catenin and TCF4
IKK:	Iκ-Bα kinase
IL:	Interleukin

IL-1β:	Interleukin-1beta
IL-8:	Interleukin-8 (CXCL-8)
IPF:	Idiopathic pulmonary fibrosis
ITS:	Insulin, transferrin and selenium
Iκ-Bα:	NF-κB inhibitory protein I kappa-B alpha
JNK:	c-Jun N-terminal kinase
LEF-1:	Lymphoid enhancer factor-1
Lgs:	Legless
LPS:	Lipopolysaccharide
LRP:	Low-density lipoprotein receptor related proteins
MAPK:	Mitogen activated protein kinase
MEK1:	Mitogen activated protein kinase kinase (MAPKK)
MMP:	Matrix metalloproteinase
(m)RNA:	(messenger) Ribonucleic acid
NAF:	Netherlands asthma foundation
NF-κB:	Nuclear factor kappa B
NLK:	Nemo-like kinase
Pack-year:	20 manufactured cigarettes (one pack) smoked per day for one year
PAI-1:	Plasminogen activator inhibitor-1
PASMC:	Pulmonary artery smooth muscle cells
PBS:	Phosphate-buffered saline
PDE:	Phosphodiesterase
PKA:	Protein kinase A
PKB:	Protein kinase B (AKT)
PKC:	Protein kinase C
PLC:	Phospholipase C
PP:	Protein phosphatase
Pygo:	Pygopus
RIPA:	Radio-immunoprecipation assay buffer
R-smad:	Receptor regulated smad
RT:	Room temperature
RT-PCR:	Real time polymerase chain reaction
S33Y β-catenin:	Degradation resistant β-catenin mutant
SDS PAGE:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sFRP:	Secreted Frizzled related protein
siRNA:	Small interference ribonucleic acid
Smad:	Small phenotype and mothers against decapentaplegic related protein
sm-MHC:	Smooth muscle myosin heavy chain
TBST:	Tris-buffered saline tween

TCF:	T-cell factor
TGF-β:	Transforming growth factor-beta
TLE:	Transducin-like enhancer of split
TOP/FOP flash:	TCF reporter assay
VASP:	Vasodilator-stimulated phoshoprotein
VEGF:	Vascular endothelial growth factor
Wg:	Wingless
WIF-1:	WNT inhibitory factor-1
Wls/Evi:	Wntless / evenness-interrupted
WNT:	Wingless / integrase-1