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RESEARCH PAPER

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

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BACKGROUND
Chronic lung diseases such as asthma, COPD and pulmonary fibrosis are characterized by abnormal extracellular matrix (ECM) turnover. TGF-β is a key mediator stimulating ECM production by recruiting and activating lung fibroblasts and initiating their differentiation process into more active myofibroblasts. Glycogen synthase kinase-3 (GSK-3) regulates various intracellular signalling pathways; its role in TGF-β_1-induced myofibroblast differentiation is currently largely unknown.

PURPOSE
To determine the contribution of GSK-3 signalling in 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 time- and dose-dependent increases of α-sm-actin 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 CT/CHIR99021 fully inhibited the 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 signalling.

CONCLUSION AND IMPLICATION
We demonstrate that GSK-3 signalling regulates TGF-β_1-induced myofibroblast differentiation by regulating CREB phosphorylation. GSK-3 may constitute a useful target for treatment of chronic lung diseases.

Abbreviations
AP-1, activator protein-1; CBP, CREB-binding protein; COPD, chronic obstructive pulmonary disease; CREB, cAMP response element-binding protein; ECM, extracellular matrix; GSK-3, glycogen synthase kinase-3; IKK, Iκ-Bα kinase; IL-8, interleukin-8 (CXCL-8); Iκ-Bα, NF-κB inhibitory protein Iκ-Bα; R-smad, receptor-regulated smad; smad, small phenotype and mothers against decapentaplegic-related protein; TCF, T-cell factor; VASP, vasodilator-stimulated phosphoprotein; α-sm-actin, α -smooth muscle-actin

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 and Woodgett, 2003; Frame and Cohen, 2001; Jope and Johnson, 2004). However, over the past few decades, GSK-3 has been shown to contribute to various other signalling pathways that are involved in a wide variety of cellular functions including gene transcription, protein translation, apoptosis and cell cycle progression (Frame and Cohen, 2001; Jope and Johnson, 2004; Gosens *et al.*, 2007). GSK-3α and GSK-3β exert their cellular functions by regulating a variety of signalling proteins and transcription factors, including NF-κB, activator protein-1 (AP-1), cAMP 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 and Johnson, 2004; Liang and Chuang, 2006; Baarsma *et al.*, 2011a). 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 (Ding *et al.*, 2000; Götschel *et al.*, 2008). However, the role of GSK-3 signalling 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, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis (Noordhoek *et al.*, 2003; 2005; Kranenburg *et al.*, 2006; Togo *et al.*, 2008; Lofdahl *et al.*, 2011). Growth factors released during the persistent chronic inflammation, in particular TGF-β, attract and activate pulmonary fibroblasts (Morty *et al.*, 2009). In addition, locally up-regulated 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 (α-sm-actin)-containing stress fibres (Scotton and Chambers, 2007). 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). 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) (Massagué, 2000; Schiller *et al.*, 2004). In addition to smad-dependent signalling, smad-independent signalling cascades like MAPKs and NF-κB are activated in response to TGF-β_1, which collectively define the cellular responses (Massagué, 2000).

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

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'; http://www.federa.org). Clinical characteristics of the groups are presented in Table 1.

Table 1

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 1 year. FEV_1 % predicted = forced expiratory volume in 1 s 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.

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}) FBS, 2 mM L-glutamine, 100 μg 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 μg L^{-1} streptomycin and 100 U mL^{-1} penicillin for a period of 24 h. 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 h. 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, CT/CHIR99021, SIS3, U0126, SC-514, PS1145) or forskolin were added 30 min 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 six-well cluster plates and transiently transfect with double-stranded siRNA targeted against the GSK-3 transcript, which targets both GSK-3α and GSK-3β (Santa Cruz Biotechnology, Santa Cruz, 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 (Invitrogen, Carlsbad, CA, USA). Control transfections were performed using a non-silencing control siRNA (Qiagen, Venlo, The Netherlands). After 6 h of transfection, cells were washed once with warm (37°C) Hank's balanced salt solution [HBSS; composition (mg L^{-1}): 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 h 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}) for 48 h. The cells were lysed in ice-cold SDS buffer. Protein concentration was determined by Pierce protein determination according to the manufacturer's instructions.

Preparation of cell lysates

To obtain whole cell lysates, cells were washed once with ice-cold (4°C) HBSS then lysed in ice-cold SDS buffer (composition: 62.5 mM Tris, 2% w.v^{-1} SDS, 1 mM NaF, 1 mM Na_3VO_4, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, 7 μg 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°C till further use.

Western blot analysis

Equal amounts of protein (10–50 μg lane^{-1}) were subjected to electrophoresis on polyacrylamide gels, transferred to nitrocellulose membranes and analysed for the proteins of interest using specific primary and HRP-conjugated secondary antibodies. By using enhanced chemiluminescence reagents, bands were subsequently recorded in the G:BOX iChemi gel documentation system equipped with GeneSnap image acquisition software (Syngene, Cambridge, UK) for the data shown in Figures 1, 4, 5 and 6 with the exception of the data shown in Figure 2, which was recorded using film. Band intensities were quantified by densitometry using GeneTools analysis software (Syngene) for the data shown in Figures 1, 4, 5 and 6 with the exception of the data shown in Figure 2, which was analysed using Totallab quantification software (Nonlinear Dynamics, Newcastle, UK).

Cytochemistry

MRC5 lung fibroblasts were plated onto Lab-Tek™ borosilicate chamber slides (Thermo Scientific, Etten-Leur, The Netherlands) and treated with TGF-β_1 (2 ng mL^{-1}) for 48 h, fixed for 15 min at 4°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°C in CB buffer containing 3% PFA and 0.3% Triton X-100. Filamentous actin was stained with Alexa Fluor 488 phalloidin (15 min at room temperature) and nuclei with Hoechst 33342. After staining, coverslips were mounted using ProLong Gold antifade reagent (Invitrogen) and analysed by using an Olympus AX70 microscope equipped with digital image capture system (ColorView Soft System with Olympus U CMAD2 lens; Olympus, Hamburg, Germany).

mRNA isolation and real-time PCR analysis

Total mRNA was extracted using the RNeasy mini kit (Qiagen). 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, DE, 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 Table 2. Real-time PCR data were analysed using the comparative cycle threshold (Cq: amplification cycle number) method. Cycle parameters were: denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for 40 cycles followed by 5 min at 72°C. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA ($Cq_{\text{gene of interest}} - Cq_{\text{18S rRNA}}$; 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

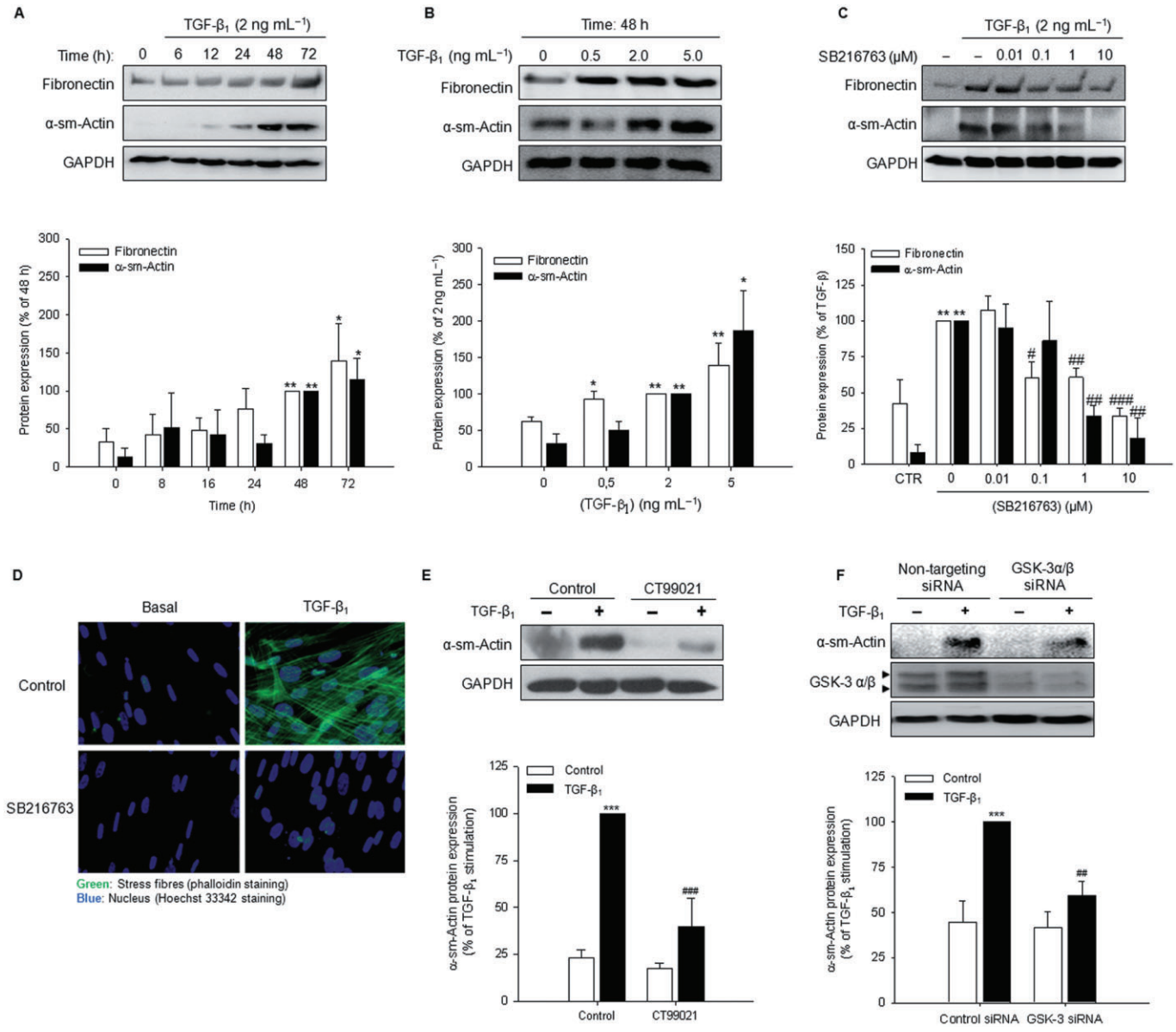

Figure 1

TGF-β_1-induced myofibroblast differentiation of lung fibroblasts is attenuated by inhibition of GSK-3. (A-B) 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 several time-points (A) or with several concentrations of TGF-β_1 for 48 h (B). 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. Data shown are the means ± SEM of four to five independent experiments. (C) 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 h in the presence or absence of the selective GSK-3 inhibitor SB216763 (0.01–10 μM). Expression of fibronectin and α-sm-actin was evaluated in whole cell lysates by immunoblotting using specific antibodies. Data shown are the means ± SEM of five independent experiments. (D) Cytochemical evaluation of stress fibre 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 33342; blue). Pictures were taken at 400 × magnification. (E) MRC5 human lung fibroblasts were stimulated for 48 h with TGF-β_1 (2 ng mL^{-1}) in the presence or absence of the selective GSK-3 inhibitor CT/CHIR99021 (1 μM). Expression of α-sm-actin was evaluated in whole cell lysates by immunoblotting using specific antibodies and responses were quantified by densitometry representing mean ± SEM of four independent experiments. (F) Silencing of GSK-3 by specific siRNA attenuates TGF-β_1-induced myofibroblast differentiation. MRC-5 lung fibroblasts were transfected with a siRNA against GSK-3α and GSK-3β whereas control cultures were transfected with a non-targeting control siRNA. Transfected cells were treated with TGF-β_1 (2 ng mL^{-1}) for 48 h. Expression of α-sm-actin and total GSK-3 (i.e. GSK-3α and GSK-3β) 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.

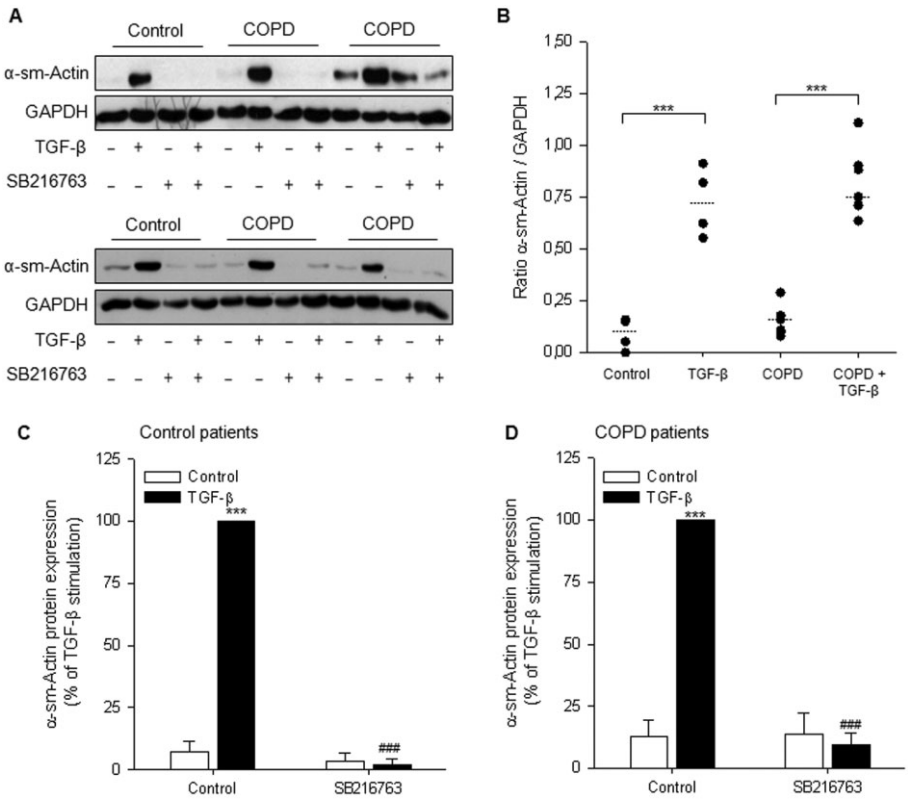

Figure 2

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 without (C) and with COPD (D). ***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.

Table 2

Primers used for the determination of myofibroblast markers and housekeeping genes by qRT-PCR analysis

Gene	NCBI accession number			Primer sequence	
Fibronectin	NM_212482	Forward	5′	TCG AGG AGG AAA TTC CAA TG	3′
		Reverse	5′	ACA CAC GTG CAC CTC AT CAT	3′
α-sm-actin	NM_001141945	Forward	5′	GAC CCT GAA GTA CCC GAT AGA AC	3′
		Reverse	5′	GGG CAA CAC GAA GCT CAT TG	3′
18S rRNA	NR_003286.2	Forward	5′	CGC CGC TAG AGG TGA AAT TC	3′
		Reverse	5′	TTG GCA AAT GCT TTC GCT C	3′
PAI-1	NG_013213.1	Forward	5′	CGC CAG AGC AGG ACG AA	3′
		Reverse	5′	GGA CAC ATC TGC ATC CTG AAG TT	3′
CTGF	NM_001901	Forward	5′	CCG TAC TCC CAA AAT CTC CA	3′
		Reverse	5′	GTA ATG GCA CGC ACA GGT CT	3′

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 $2^{-(\Delta\Delta Cq)}$.

Cytokine ELISA

Confluent MRC5 human lung fibroblasts were washed twice with warm (37°C) HBSS followed by a period of 24 h 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 h (Bain *et al.*, 2007). Cell supernatants were harvested 24 h after stimulation and stored at −20°C until assayed for IL-8 (CXCL8). Cytokine levels were determined by specific ELISA according to the manufacturers' instructions (IL-8 kit Sanquin, Amsterdam, The Netherlands).

SBE4-luciferase assay

For the smad binding element (SBE)4-luciferase assay, cells were grown to ~95% confluence in six-well cluster plates and then transfected with 0.5 μg plasmid DNA encoding SBE4-firefly luciferase and with 0.12 μg plasmid DNA encoding CMV-renilla luciferase using Lipofectamine 2000™ in serum- and antibiotics-free DMEM. After 6 h, medium was changed to DMEM supplemented with antibiotics and 10% FBS, in which cells were grown for another 18 h. Cells were then serum-deprived for 24 h, after which cells were subjected to TGF-β_1 (2 ng mL^{-1}) stimulation in the absence or presence of SB216763 (10 μM) for another 24 h. Luciferase activity was then assayed using a dual firefly/renilla luciferase assay system (Promega, Madison, WI, USA). SBE4-luciferase activity was normalized to CMV-renilla activity.

Antibodies and reagents

Mouse anti-α-sm-actin, 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- GAPDH antibody were obtained from Santa Cruz Biotechnology. 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 h 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). Myofibroblast differentiation was evident after 48 h of treatment (Figure 1A). 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 that showed submaximal induction at 2 ng mL^{-1} (Figure 1B). Based on these initial findings, we selected the submaximal concentration of 2 ng mL^{-1} TGF-β_1 and 48 h of treatment for further experiments.

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

Next, we determined the contribution of GSK-3 signalling 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 with an EC_{50} of 0.47 ± 0.19 μM and 0.17 ± 0.11 μM respectively (Figure 1C). These EC_{50} values for SB216763 are very similar to the EC_{50} values of this compound on GSK-3 activity, β-catenin expression and cytokine expression, as previously reported by us and others (Bain *et al.*, 2007; Baarsma *et al.*, 2011a). DMSO did not affect myofibroblast differentiation (data not shown). The role of GSK-3 in fibronectin and α-sm-actin expression in response to TGF-β_1 stimulation was next investigated in more detail. 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 fibres (filamentous actin; green) in these cells, another indication of myofibroblast differentiation (Figure 1D). Similar to the effect on α-sm-actin and fibronectin expression, SB216763 (10 μM) prevented the formation of stress fibres in these cells. To confirm the requirement of GSK-3 in TGF-β_1-induced α-sm-actin expression, we used a distinct and structurally unrelated GSK-3 inhibitor, namely CT/CHIR99021 (1 μM). Of note, these small molecules used for inhibition of GSK-3 have dissimilar off-target effects (Bain *et al.*, 2007). In agreement with SB216763, CT/CHIR99021 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 1E). Moreover, silencing of GSK-3 expression by specific siRNA attenuated the TGF-β_1-induced expression of α-sm-actin (Figure 1F). The non-targeting control siRNA does not interfere with TGF-β induced α-sm-actin and fibronectin expression (Baarsma *et al.*, 2011b). Taken together, these data indicate that GSK-3 inhibition attenuates TGF-β_1-induced responses in human lung fibroblasts.

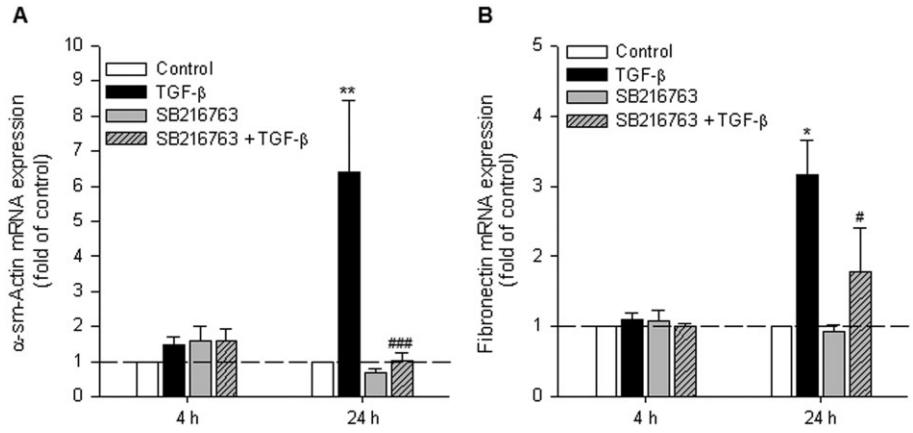

Figure 3

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 4 h and 24 h with TGF-β_1 (2 ng mL^{-1}) in the presence or absence of the selective GSK-3 inhibitor 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 ± SEM of four 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.

We next used primary human lung fibroblasts, both from individuals with and without COPD (the clinical characteristics are presented in Table 1). 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).

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 h 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 h 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).

Activation of NF-κB signalling 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 signalling 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 signalling by controlling the activation or transcriptional responses of this pathway (Hoeflich *et al.*, 2000; Baarsma *et al.*, 2011a). Therefore, we studied the activation of NF-κB signalling in response to TGF-β_1 and its contribution to myofibroblast differentiation. Activation of the NF-κB signalling pathway was determined by measuring the expression of the NF-κB inhibitory protein Iκ-Bα. We found a small 19.3 ± 3.8% decrease of Iκ-Bα expression after 1 h of TGF-β_1 stimulation, indicating that the pathway is only minimally activated in response to this growth factor (Figure 4A). In line with this, the DNA binding activity of p65 NF-κB was only very modestly enhanced to 1.2-fold of basal at this time-point (data not shown). Next, we determined the contribution of NF-κB signalling 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,C). These findings indicate that TGF-β_1-induced IL-8 secretion is completely inhibited by SC-514 or PS-1145 (Figure 4C), suggesting a significant contribution of NF-κB activation in IL-8 secretion. Activation of the NF-κB signalling pathway is not required for myofibroblast differentiation, indicating that the effects of GSK-3 inhibition require distinct signalling intermediates.

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 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 (Guo *et al.*, 2008; Sebe *et al.*, 2008). The involvement of the smad and ERK-1/2 signalling pathway in the differentiation process was investigated by using the specific inhibitor of smad3 (SIS3: 3 μM) and the selective MEK1/2

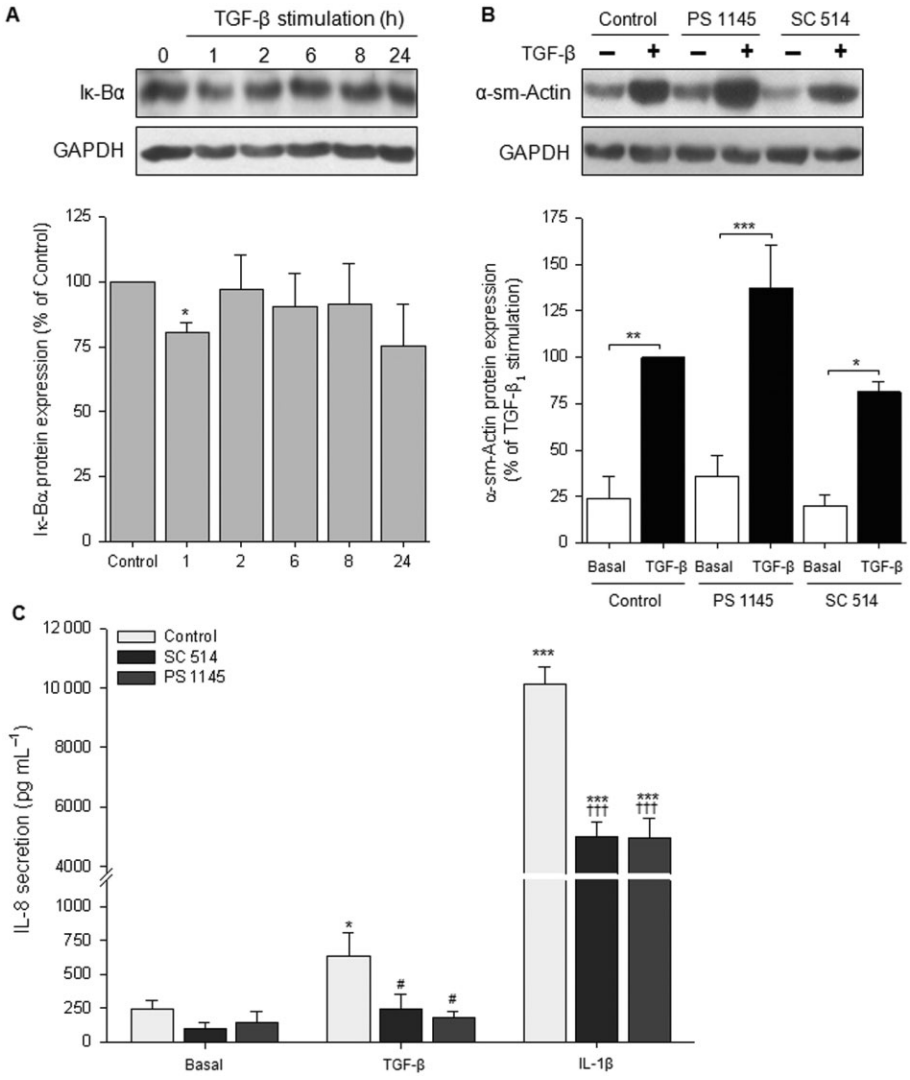

Figure 4

Activation of NF-κB signalling by TGF-β_1 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 ± SEM of three 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 signalling to TGF-β_1-induced myofibroblast differentiation. MRC5 human lung fibroblasts were stimulated for 48 h with TGF-β1 in the presence or absence of either PS-1145 (10 μM) or SC-514 (50 μM), two distinct inhibitors of 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 ± SEM of three 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 h 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 ± SEM of three 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 $^{\dagger\dagger\dagger}P$ < 0.001 compared to IL-1β stimulation; determined by a one-way ANOVA followed by a Newman–Keuls multiple comparison test.

inhibitor U0126 (3 μM) respectively (Jinnin *et al.*, 2006; Bain *et al.*, 2007). Attenuation of smad signalling decreased the TGF-β_1-induced expression of α-sm-actin, indicating the requirement of smad signalling in the differentiation process, whereas pharmacological inhibition of ERK1/2 enhanced basal and TGF-β_1-induced expression of α-sm-actin, indicative of negative regulation by this pathway (Figure 5A).

Although TGF-β_1 activates ERK-1/2 by phosphorylation (Figure 5B), inhibition of GSK-3 by SB216763 had no effect on the basal or TGF-β_1-induced phosphorylation of ERK-1/2 (Figure 5B). Stimulation with TGF-β_1 also 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). However, inhibition of

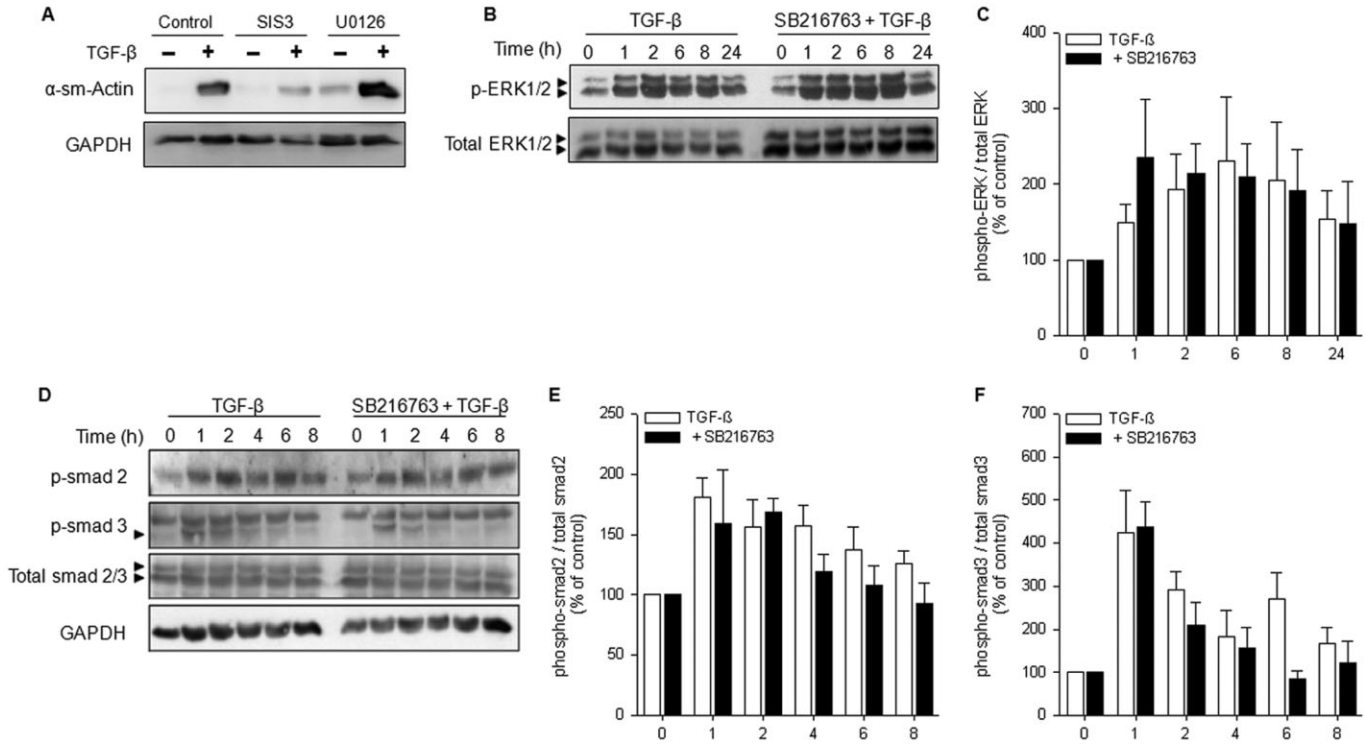

Figure 5

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 signalling to myofibroblast differentiation. 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 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 three independent experiments are shown. (B-F) MRC5 human lung 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 inhibitor SB216763 (10 μM). (B) Time-dependent phosphorylation of ERK1/2 in response to TGF-β_1 (2 ng mL^{-1}). (C) Quantification of phospho-Thr202/Tyr204 ERK-1/2 expression evaluated by immunoblotting using specific antibodies. (D) Time-dependent phosphorylation of smad2/3 in response to TGF-β_1 (2 ng mL^{-1}). (E) Quantification of phospho-ser465/467 smad2 and (F) quantification of phospho-ser423/425 smad3 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 ± SEM of three to six 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 by SB216763 had no major effect on basal or TGF-β_1-induced phosphorylation or cellular expression of smad2 or smad3 (Figure 5B).

These data suggest that smad and ERK1/2 signalling are activated by TGF-β and that these signalling pathways have opposite effects on myofibroblast differentiation. GSK-3 signalling 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 signalling intermediates.

GSK-3 inhibition promotes CREB activation in human lung fibroblasts

GSK-3 signalling has been demonstrated to have a modulatory effect on CREB signalling in various cell types (Grimes and Jope, 2001a,b; Tullai *et al.*, 2007; Götschel *et al.*, 2008). Interestingly, we observed that inhibition of GSK-3 by SB216763 (10 μM, 1.5 h) 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 h) did not result in increased ser133-CREB phosphorylation (Figure 6A). Phosphorylated CREB can act as a functional antagonist of smad signalling via competition for the common transcriptional co-activator CBP (CREB-binding partner) (Schiller *et al.*, 2010). In agreement with these findings, the expression of the canonical smad target genes PAI-1 and CTGF in response to TGF-β_1 was repressed by GSK-3 inhibition (Figure 6B,C). Also, TGF-β_1-activated smad-dependent promoter regulation, assayed using the SBE4-luciferase reporter assay, was repressed by SB216763 (Figure 6D).

In view of these data, we further investigated the effect of CREB activation on myofibroblast differentiation. In order to activate CREB signalling in MRC5 human lung fibroblasts, we used forskolin, an agent that elevates cellular cAMP levels by

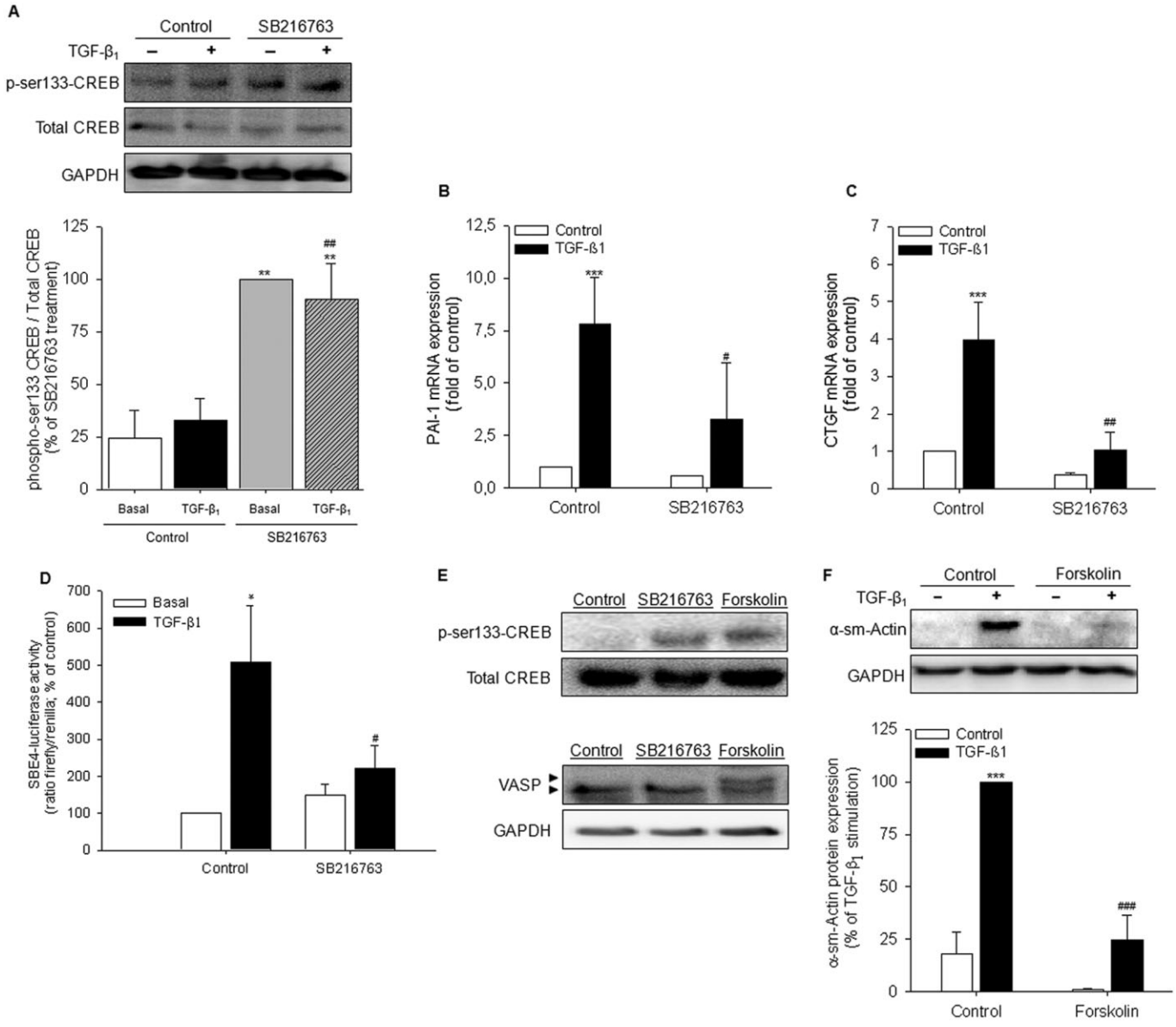

Figure 6

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 h with TGF-β_1 (2 ng mL^{-1}) in the presence or absence of the selective GSK-3 inhibitor SB216763 (10 μM). 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 ± SEM of three to four independent experiments. $^{**}P < 0.01$ compared to basal control, $^{\#}P < 0.05$ compared to TGF-β_1 in the absence of SB216763; determined by a one-way ANOVA followed by a Newman–Keuls multiple comparison test. (B-C) mRNA expression of canonical smad-dependent genes (B) PAI-1 and (C) CTGF in response to TGF-β (2 ng mL^{-1}, 24 h) in the presence or absence of SB216763 (10 μM). Data represent mean ± SEM of three to eight independent experiments. $^{***}P < 0.001$ compared to basal control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to TGF-β_1 stimulation; determined by a one-way ANOVA followed by a Newman–Keuls multiple comparison test. (D) Smad-dependent promoter regulation in response to TGF-β_1 and SB216763. MRC5 cells were transfected with the SBE4-firefly luciferase and the CMV-renilla luciferase reporter. Data shown represent mean normalized SBE4-luciferase activity ± SEM of six independent experiments. $^{*}P < 0.05$ compared to basal control, $^{\#}P < 0.05$ compared to TGF-β1 stimulation; determined by a one-way ANOVA followed by a Newman–Keuls multiple comparison test. (E) Evaluation of CREB phosphorylation (top panel) and VASP phosphorylation (lower panel) in response to SB216763 (10 μM, 1.5 h) or forskolin (2 μM, 1.5 h). (F) 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 ± SEM of three 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.

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 6E). By using a vasodilator-stimulated phosphoprotein (VASP)-specific antibody that recognizes both phospho-VASP (upper band) and total VASP (upper and lower band), we show that forskolin induced the ser157 phosphorylation of VASP, 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 6E). To reinforce that CREB signalling 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 6F). These findings imply that CREB signalling 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.

Discussion

We determined the contribution of GSK-3 signalling 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 and fibronectin. 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 signalling 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 (adult) lung fibroblasts. Activation of NF-κB was not required for myofibroblast differentiation and GSK-3 was not involved in the phosphorylation of R-smads or ERK-1/2 by TGF-β_1. Rather, inhibition of GSK-3 resulted in increased levels of phosphorylated ser133-CREB, which we propose acts as a functional antagonist of smad signalling and thereby prevents myofibroblast differentiation. Collectively, these data indicate that the GSK-3 regulates myofibroblast differentiation presumably by repressing CREB signalling.

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. In COPD, a variable degree of airway wall fibrosis is present and additionally insufficient parenchymal tissue repair, both contributing to disease development (Hogg *et al.*, 2004; Coward *et al.*, 2010). 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 patients often develop emphysema, in which the fibroblast function may be inadequate or insufficient to restore tissue damage (Noordhoek *et al.*, 2003; Rennard *et al.*, 2006; Zandvoort *et al.*, 2008). 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 (Kotaru *et al.*, 2006; Zhou *et al.*, 2011).

TGF-β_1 is a multifunctional cytokine that regulates various cellular functions by acting on several signalling pathways. Stimulation of pulmonary fibroblasts with TGF-β_1 resulted in only a modest decrease of the NF-κB-inhibitory protein Iκ-Bα, which indicates that NF-κB signalling is not activated to a significant extent. Two distinct IKK inhibitors (i.e. PS-1145 and SC-514) did not at all affect TGF-β_1-induced α-sm-actin expression, indicating that TGF-β_1-activated NF-κB signalling 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 signalling by GSK-3 (Takada *et al.*, 2004; Wang *et al.*, 2006). Smad phosphorylation is critical in canonical TGF-β_1 signalling 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 (Massagué, 2000; Schiller *et al.*, 2004). 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). 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 (Ghosh *et al.*, 2000; Goodman and Smolik, 2000). Phosphorylation of CREB on serine 133 results in increased activity as this phosphorylation is required for recruitment of the transcriptional co-activators (Goodman and 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 (Fiol *et al.*, 1994; Johannessen and Moens, 2007; Tullai *et al.*, 2007; Liang *et al.*, 2008). We show that inhibition of constitutive GSK-3 activity 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. In human dermal fibroblasts, activation of CREB signalling also attenuated smad-dependent signalling and expression of type 1 collagen and plasminogen activator inhibitor 1 (PAI-1) (Ghosh *et al.*, 2000; Schiller *et al.*, 2010). 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 (Vardouli *et al.*, 2005; Akhmetshina *et al.*, 2008). 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 and Moens, 2007). Remarkably, SB216763 induced ser133 phosphorylation of CREB, but did not alter the phosphorylation status of VASP. This suggests that PKA is not involved and implies 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 signalling is required for the activation of phosphatases, which are involved in the dephosphorylation of CREB (Johannessen *et al.*, 2004; Johannessen and Moens, 2007). In support of the hypothesis that GSK-3-mediated CREB signalling is capable of functionally antagonizing fibrotic responses by reducing the transcriptional complexes of activated smads with CBP/p300, SB216763 reduced the expression of the canonical smad target genes CTGF and PAI-1 in response to TGF-β_1 and attenuated smad-dependent promoter regulation, assayed using the SBE4-luciferase reporter. Attempts to directly address the role of GSK-3-mediated CREB activation using siRNA knockdown of CREB were unsuccessful, as knockdown of CREB interferes with fibronectin and α-sm-actin expression at baseline (data not shown). Nonetheless, our current data and the published data summarized earlier provide a solid and plausible rationale for a mechanism in which inhibition of GSK-3 in pulmonary fibroblasts results in increased phosphorylation of ser133-CREB, which attenuates TGF-β_1-induced myofibroblast differentiation.

GSK-3 signalling contributes to many cellular responses by regulating a variety of transcription factors and transcriptional co-activators (Doble and Woodgett, 2003). 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 signalling by phosphorylating cytosolic β-catenin, thereby targeting it for proteosomal degradation (Doble and Woodgett, 2003). Consequently, pharmacological inhibition of GSK-3 would be expected to result in increased β-catenin signalling and enhanced myofibroblast differentiation in response to TGF-β_1. Indeed, in airway smooth muscle cells, GSK-3 inhibition promotes α-sm-actin expression and differentiation (Deng *et al.*, 2008). 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 and Woodgett, 2003; Jope and Johnson, 2004). Compartmentalization of GSK-3 resulting in differential downstream signalling 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 signalling 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 prevents inflammatory and fibrotic responses in the lung *in vivo* and it was suggested that these effects of GSK-3 inhibition were due to suppression of NF-κB activation and subsequent inhibition of the recruitment and activation of inflammatory cells (Cuzzocrea *et al.*, 2006; 2007; Bao *et al.*, 2007). 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 signalling may regulate the onset of fibrosis in the lung by directly acting on the deposition of 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 signalling 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, presumably via activation of CREB-dependent signalling. 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 signalling pathways.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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