Inhibition of glycogen synthase kinase 3 induces dermal fibrosis by activation of the canonical Wnt pathway

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Abstract: OBJECTIVE: Glycogen synthase kinase 3 (GSK-3) regulates the phosphorylation and subsequent degradation of β-catenin, thereby preventing aberrant activation of the canonical Wnt pathway. A study was undertaken to define the role of GSK-3 in fibroblast activation and in experimental models of systemic sclerosis (SSc). METHODS: siRNA and specific inhibitors were used to inhibit GSK-3 in cultured fibroblasts and in mice. Activation of the canonical Wnt signalling was analysed by determining the levels of nuclear β-catenin and by measuring the mRNA levels of the Wnt target gene Axin2. The effects of GSK-3 on the release of collagen were evaluated in human dermal fibroblasts and in the mouse model of bleomycin-induced skin fibrosis in tight-skin-1 (tsk-1) mice. RESULTS: Targeting GSK-3 potently activated the canonical Wnt pathway in fibroblasts in vitro and in vivo. Inactivation of GSK-3 dose-dependently stimulated the release of collagen from cultured fibroblasts in a β-catenin-dependent manner and further resulted in progressive accumulation of collagen and dermal thickening in mice. Inhibition of GSK-3 aggravated experimental fibrosis in bleomycin-challenged mice and in tsk-1 mice. CONCLUSION: Inhibition of GSK-3 activates the canonical Wnt pathway in fibroblasts, stimulates the release of collagen from fibroblasts, exacerbates experimental fibrosis and is sufficient to induce fibrosis. GSK-3 is therefore a key regulator of the canonical Wnt signalling in fibroblasts and inhibition of GSK-3 results in fibroblast activation and increased release of collagen.

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Inhibition of glycogen synthase kinase 3β induces dermal fibrosis by activation of the canonical Wnt pathway

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ABSTRACT

Objective: Glykogen Synthase Kinase 3β (GSK-3) regulates the phosphorylation and subsequent degradation of β-catenin, thereby preventing aberrant activation of the canonical Wnt pathway. The aim of the present study was to define the role of GSK-3 for fibroblast activation and in experimental models of SSc.

Methods: We used siRNA and specific inhibitors to inhibit GSK-3 in cultured fibroblasts and in mice. The activation of the canonical Wnt-signaling was analyzed by determining the levels of nuclear β-catenin and by measuring the mRNA levels of the Wnt target gene Axin2. The effects of GSK-3 on the release of collagen were evaluated in human dermal fibroblasts and in the mouse model of bleomycin induced skin fibrosis in the tight-skin-1 (tsk-1) mice.

Results: Targeting GSK-3 potently activated the canonical Wnt pathway in fibroblasts in vitro and in vivo. Inactivation of GSK-3 dose-dependently stimulated the release of collagen from cultured fibroblasts in a β-catenin dependent manner and it further resulted in progressive accumulation of collagen and dermal thickening in mice. Finally, inhibition of GSK-3 aggravated experimental fibrosis in bleomycin challenged mice and in tsk-1 mice.

Conclusion: We demonstrated that inhibition of GSK-3 activates the canonical Wnt pathway in fibroblasts, stimulates the release of collagen from fibroblasts, exacerbates experimental fibrosis and is sufficient to induce fibrosis. Thus, GSK-3 is a key regulator of the canonical Wnt signaling in fibroblasts and inhibition of GSK-3 results in fibroblast activation and increased release of collagen.
Systemic sclerosis (SSc) is a chronic fibrosing disease with unknown etiology. The most obvious histopathological finding of involved tissues is the excessive accumulation of extracellular matrix components.\cite{1} The resulting tissue fibrosis often disrupts the physiological tissue structure and results in dysfunction of affected organs. Activated fibroblasts are key players in SSc that produce and release the extracellular matrix components. The molecular mechanisms for the uncontrolled activation of SSc fibroblasts are only partially known.\cite{1}

Wnt signaling profoundly affects developmental processes during embryogenesis and tissue homeostasis in adults.\cite{2,3} The canonical Wnt signaling pathway is best characterized among the different Wnt cascades. Aberrant Wnt signaling has been implicated in a variety of different diseases and might also play a role in fibrotic disorders such as SSc. First evidence suggested that several Wnt proteins might be overexpressed in tight-skin-1 mice, a common animal model for studies in SSc.\cite{4} Moreover, overexpression of Wnt 10b in the skin resulted in dermal fibrosis (J. Varga, unpublished results). Thus, the canonical Wnt signaling might be a key player in the pathogenesis of SSc.

GSK-3 is a ubiquitously expressed serine/threonine kinase that plays a pivotal role for the regulation the canonical Wnt pathway.\cite{2,5} In the absence of Wnt proteins, GSK-3 forms a complex together with Axin, Adenomatous Polyposis Coli (APC) protein and Casein Kinase Iα (CKI α). This catalytically active complex phosphorylates cytosolic β-catenin, which in turn induces ubiquitinylation and subsequent proteasomal degradation of β-catenin.\cite{5} Mutations that inhibit the phosphorylation of β-catenin by GSK-3 highlight the importance of GSK-3 for the regulation of the canonical Wnt pathway: In various tumors such as hepatocellular carcinoma, carcinomas of the ovaries and endometrium, these mutations result in ligand independent accumulation of β-catenin with uncontrolled transcription of Wnt target genes.\cite{6,7} We analyzed in the present study the consequences of GSK-3 inhibition for fibroblast activation and the development of tissue fibrosis.
MATERIAL AND METHODS (A more detailed description of the material and methods used for this study can be found in the supplementary material)

Patients and fibroblast cultures

All patients fulfilled the criteria for SSc as suggested by LeRoy et al.[8] Biopsies from SSc patients (n = 9) were taken from involved skin (supplementary table 1). Fibroblasts from healthy controls (n = 6) were obtained from skin biopsies of age- and sex-matched volunteers. Fibroblasts were prepared as described previously.[9] All SSc patients and healthy volunteers signed an informed consent form approved by the local institutional review boards.

Incubation with small molecule inhibitors of GSK-3

Stimulation experiments were performed in DMEM/0.1% FCS. Dermal fibroblasts were incubated with two small molecule inhibitors of GSK-3, N-(4-Methoxybenzyl)-N’-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418, Calbiochem, Darmstadt, Germany) and 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763, Tocris, Bristol, UK) in concentrations from 10 nM to 10 μM for 24 h. Fibroblasts incubated with the same volumes of the solvent DMSO were used as controls. Both inhibitors are highly specific for GSK-3. AR-A014418 inhibits GSK-3 with an IC\textsubscript{50} value of 104 nM and does not inhibit related kinases such as CDK2 or CDK5 (IC\textsubscript{50} for CDK2 and CDK5 > 100 μmol/L).[10] Similarly, SB216763 inhibits GSK-3 with an IC\textsubscript{50} value of 34 nM with an IC\textsubscript{50} for related protein kinases, which is above 10 μM.[11]

Knockdown of GSK-3 in dermal fibroblasts by siRNA

In addition to pharmacological inhibition, GSK-3 was targeted by siRNA mediated knock down using a pre-designed siRNA and the nucleofection technique as described previously.[12]
Quantitative Real time PCR

Gene expression was quantified by TaqMan or by SYBR Green real-time PCR using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described.[13]

Collagen measurements

The collagen content was quantified by the hydroxyproline assay.[14]

Western blot analysis

Western blot analyses were performed as described using polyclonal antibodies against human β-catenin (R&D Systems, Minneapolis, United States) at a dilution of 1:1000 and anti-human α-tubulin antibodies (Sigma; dilution 1:1000) as primary antibodies.

Microtiter tetrazolium (MTT) assay

The colorimetric MTT Assay is an established method of analyzing the cell viability in cytotoxicity investigations.[15] Briefly, the number of viable cells directly correlates with the release of formazan upon MTT [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] exposure.[15, 16] Formazan is detected colorimetrically. Cultured SSc fibroblasts were incubated with SB216763 and AR-A014418 at concentrations of 1μM and 10 μM, respectively. The MTT-Assay was performed as described previously[19]. Untreated fibroblasts were used as negative controls. Fibroblasts incubated with 50 % DMSO served as positive controls.

Immunhistochemistry for β-catenin and α-smooth muscle actin
β-catenin was detected incubating skin sections with polyclonal goat-anti-mouse β-catenin antibodies (R&D Systems) at a dilution of 1:300 at 4 °C overnight. To identify nuclear accumulation of β-catenin, nuclei were counterstained with 4’,6-Diamidino-2-phenylindole (DAPI Santa Cruz Biotechnology, California, USA) at a dilution of 1:1000 for 10 min. In each section, cells positive for nuclear β-catenin were counted in 20 randomly chosen high-power fields. To confirm that Wnt signaling is active in fibroblasts, skin sections were triple stained for β-catenin, DAPI, and the selective fibroblast marker prolyl-4-hydroxylase-β (Acris Antibodies, Herfold, Germany) at a dilution of 1:50 at 4°C overnight. Myofibroblasts were identified by staining for α-smooth muscle actin as described.[17] In each section, α-SMA–positive cells were counted in 6 randomly chosen high-power fields.

Histological analysis

Dermal thickness at the injection sites was analyzed as described previously.[18]

Effects of GSK-3 inhibition on the release of extracellular matrix in vivo

Six week old, female DBA/2 mice (Janvier, Le Genest St Isle, France) were treated with intraperitoneal injections of the GSK-3 inhibitor SB216763 at concentrations of 0.6 mg/kg every other day. To analyze potential changes caused by the activation of GSK-3 over time, one group of mice were treated for 4 weeks and another group of mice for 8 weeks. DBA/2 mice with intraperitoneal injections of the solvent 20 % DMSO dissolved 0.9 % NaCl were used as controls.

Bleomycin induced dermal fibrosis

Skin fibrosis was induced in 6 week old, female DBA/2 mice by local injections of bleomycin for 21 days.[19, 20] AR-A014418 was applied in final concentrations of 4 mg/kg/bid, SB21676 at concentrations of 0.6 mg/kg/bid by intraperitoneal injections every
other day. Mice from the control group and the bleomycin group were injected the solvent of the GSK-3 inhibitors (20 % DMSO dissolved 0.9 % NaCl) to control for the intraperitoneal injections in the treatment groups. After 21 d, mice were sacrificed by cervical dislocation. In total, 32 mice were analyzed.

**Inhibition of GSK-3 in the tight-skin-1 mouse model**

Three groups of mice were analyzed. The first group was a control group consisting of pa/pa mice not bearing the tsk-1 mutation. The second group consisted of mock-treated tsk-1 mice. The third group consisted of tsk-1 mice receiving intraperitoneal injections of SB216763 at a concentration of 0.6 mg/kg every other day. The treatment was started at the age of five weeks. All mice were sacrificed at an age of 10 weeks. In total, 21 mice were analyzed.

**Statistics**

Data are expressed as mean ± standard error of the mean. The Wilcoxon signed rank test for related samples and the Mann-Whitney-U-test for non-related samples were used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.
RESULTS

Inhibition of GSK-3 stimulates the release of collagen from SSc fibroblasts

Incubation of SSc fibroblasts with SB216763, a highly specific inhibitor of GSK-3, increased the mRNA levels of col1a1 by 178 ± 28 % and 210 ± 28 % at concentrations of 0.1μM and 1 μM, respectively. The stimulatory effects of SB216763 on the mRNA levels of col1a1 peaked at a concentration of 10 μM with a mean increase of 260 ± 49 % (p < 0.05) (fig 1a). Consistent with the induction of mRNA for col1a1, incubation with SB216763 increased the release of collagen with a maximal induction of 161 ± 2 % (p < 0.05) (fig 1b). We obtained similar results with AR-A014418, another structurally unrelated inhibitor of GSK-3 (data not shown). Comparable increases in col1a1 mRNA and collagen protein upon incubation with inhibitors of GSK-3 were observed healthy dermal fibroblast, suggesting an ubiquitous response pattern rather than an SSc-specific phenomenon (data not shown).

We then targeted the expression of GSK-3 with siRNA to confirm the effects of the pharmacological inhibition by an independent approach. Transfection with siRNA against GSK-3 reduced the mRNA levels of GSK-3 by 91 ± 2 %. Consistent with pharmacologic inhibition, siRNA against GSK-3 increased the mRNA levels of col1a1 by 50 ± 11 % compared to fibroblasts transfected with scrambled siRNA (fig 1c).

To exclude that the increased release of collagen upon pharmacological inhibition of GSK-3 resulted from toxic effects we determined the effects of SB216763 and AR-A014418 on the cell viability using the MTT assay. Treatment with SB216763 and AR-A014418 in concentrations up to 10 μM did not alter the cell viability of SSc fibroblasts (supplementary figure 1). In contrast, we observed a reduction of viable cells to 11 ± 24 % in control fibroblasts treated with DMSO.

Inhibition of GSK-3 activates the canonical Wnt signaling cascade in dermal fibroblasts
We next investigated whether pharmacological inhibition of GSK-3 activates canonical Wnt signaling. In this context, we assessed nuclear β-catenin and the mRNA levels of Axin2, an established target gene of the canonical Wnt cascade.[2] Incubation with SB216763 significantly increased the nuclear accumulation of β-catenin in cultured dermal fibroblasts of SSc patients by 296 ± 41 % compared to controls (supplementary fig 2a). Moreover, incubation with SB216763 increased the mRNA levels of Axin2 by 930 ± 360 % (p < 0.05) (supplementary fig 2b). Similar results were also observed with fibroblasts isolated from healthy volunteers (data not shown). These data demonstrate that GSK-3 is a crucial regulator of the canonical Wnt pathway in dermal fibroblasts.

**SB216763 stimulates the collagen synthesis in a β-catenin dependent manner**

To investigate, whether the canonical Wnt pathway mediates the pro-fibrotic effects observed upon inactivation of GSK-3, we inhibited the canonical Wnt pathway by knockdown of β-catenin in healthy dermal fibroblasts. siRNA against β-catenin decreased the mRNA levels of β-catenin by 87 ± 5 %, confirming an effective inactivation of this central mediator of canonical Wnt signaling. In cells treated with non-targeting siRNA, SB216763 increased the expression of col1a1 mRNA by 187 ± 30 % (p < 0.05) compared to controls. By contrast, incubation with SB216763 did not alter the levels of col1a1 mRNA in cells transfected with siRNA against β-catenin compared to mock treated controls (fig 2a). Consistently, siRNA against β-catenin also abolished the stimulatory effects of SB216763 on the release of collagen protein (fig 2b). Thus, inhibition of GSK-3 induces the collagen synthesis by activating canonical Wnt signaling.

**Inhibition of GSK-3 is sufficient to induce fibrosis**

To study the effects of GSK-3 in vivo, we treated DBA/2 mice with SB216763. We did not observe any signs of toxicity related to the treatment with SB216761. The body
weight, the texture of the fur and the activity did not differ between mice treated with SB216761 and sham treated controls. Gross examination of the colon also did not reveal tumour formation. However, no in-depth histological or molecular examination was performed. In addition, the treatment period with inhibitors of GSK3 may have been too short to induce tumour formation.

SB216763 potently activated the canonical Wnt pathway in murine skin. The number of fibroblasts with nuclear staining for β-catenin increased from 11 ± 4 % in mock-treated mice to 50 ± 5 % upon treatment with SB216763 for eight weeks (p = 0.02). Consistently, the mRNA levels of the Wnt target gene Axin2 were induced by 598 ± 139 % (p = 0.003) in mice treated with SB216763 (fig 3a and 3b and supplementary fig 3).

Activation of the canonical Wnt cascade by SB216763 resulted in progressive dermal fibrosis (fig 3c, supplementary fig 4a). The dermal thickness increased by 35 ± 6 % compared to mock-treated controls after four weeks of treatment with SB216763 (p = 0.04). This effect increased further to 65 ± 9 % after eight weeks of treatment (p = 0.001) (fig 3d). Consistently, mice treated with SB216763 exhibited increased hydroxyproline content in the skin (129 ± 22 % as compared to controls; p = 0.04) (fig 3e). Treatment with SB216763 also enhanced the differentiation of resting fibroblasts into myofibroblasts with increases of 264 ± 12 % after eight weeks (p = 0.002) (fig 3f, supplementary figure 4b).

**Inhibition of GSK-3 enhances the activation of the canonical Wnt cascade and exacerbates bleomycin induced dermal fibrosis**

We next investigated whether inhibition of GSK-3 may also exacerbate fibrosis in different experimental models of SSc. First, we investigated the effects of GSK-3 in the mouse model of bleomycin induced dermal fibrosis, an established model for early, inflammatory stages of SSc.
Challenge with bleomycin or SB216763 activated the canonical Wnt pathway. Inhibition of GSK-3 in bleomycin treated mice resulted in a further activation of the canonical Wnt pathway. The number of fibroblasts positive for nuclear β-catenin increased from 57 ± 6% in bleomycin challenged, mock-treated mice and 45 ± 4% in SB216763 treated mice to 79 ± 4% bleomycin challenged, SB216763 treated mice (p = 0.05 compared to bleomycin alone) (fig 4a). Moreover, the mRNA levels of Axin2 were upregulated by 277 ± 168% in bleomycin challenged, SB216763 treated mice compared to bleomycin challenged, mock-treated mice (p = 0.01) (fig 4b).

We further analyzed, whether inhibition of GSK-3 and subsequent activation of the Wnt pathway may affect the outcome of bleomycin induced dermal fibrosis. Bleomycin and SB216763 were both sufficient to induce fibrosis alone (fig3, fig4). However, inhibition of GSK-3 exacerbated bleomycin induced dermal fibrosis. Treatment of bleomycin challenged mice with SB216763 increased dermal thickening by further 84 ± 15% compared to bleomycin alone (p < 0.001) (fig 4c and 4d). SB216763 also enhanced the hydroxyproline content (65 ± 11%; p = 0.04) (fig 4e) and myofibroblast counts (84% ± 23%, p = 0.005) (figure 4f) compared to bleomycin or SB216763 alone.

**Inhibition of GSK-3 exacerbates the tight skin phenotype**

We next investigated the effects of the GSK-3 inhibition in tsk-1 mice, which serve as a model of later, less inflammatory stages of SSc.[21] Inhibition of GSK-3 further enhanced the activation of the canonical Wnt pathway in this model and increased the number of fibroblasts positive for nuclear β-catenin from 57 ± 4% in mock-treated tsk-1 mice to 84 ± 5% (p = 0.03). The number of fibroblasts positive for nuclear β-catenin in pa/pa control mice treated with SB216763 was 37 ± 5% (p = 0.04) (fig 5a). Treatment of pa/pa mice with SB216763 modestly increased the hypodermal thickness and the numbers of myofibroblasts compared to sham-treated pa/pa mice. Inhibition of GSK-3 by SB216763 exacerbated the tsk-
1 phenotype and increased hypodermal thickness by 59 ± 5 % as compared to mock-treated tsk-1 mice (p = 0.05) (fig 5b and fig 5c). The differentiation of resting fibroblasts into myofibroblasts also increased upon GSK-3 inhibition by 63 ± 15 % (p = 0.05) (fig 5d).
DISCUSSION

Herein, we demonstrated that GSK-3 is a central regulator of fibroblast activation and collagen synthesis. Inhibition of GSK-3 by specific inhibitors or siRNA prevented the degradation of β-catenin, resulted in nuclear accumulation of β-catenin and in the transcription of Wnt target genes in dermal fibroblasts in vitro as well as in vivo. Inhibition of GSK-3 activated the canonical Wnt pathway in resting fibroblasts and healthy mice. In addition, inhibition of GSK3 further stimulated the Wnt pathway in different models of experimental fibrosis, in which the canonical Wnt pathway was already activated. We found nuclear accumulation of β-catenin and elevated levels of Axin2 mRNA, both common markers of activated canonical Wnt signaling,[2] in dermal fibroblasts of mice challenged with bleomycin and in tsk-1 mice. Of note, both markers further increased upon inhibition of GSK-3 in both mouse models. Thus, GSK-3 controls the canonical Wnt signaling pathway not only under physiologic conditions, but also in the context of fibrosis.

Our study highlights the importance of a proper function of GSK-3 and a tight regulation of the canonical Wnt pathway in fibroblasts. Inactivation of GSK-3 resulted in increased release of collagen from cultured fibroblasts in vitro. Moreover, GSK-3 inhibition induced dermal fibrosis in vivo with accumulation of collagen, dermal thickening, and differentiation of resting fibroblasts into myofibroblasts. Treatment with the small molecule inhibitor SB216763 also enhanced dermal fibrosis in the mouse model of bleomycin induced fibrosis and in tsk-1 mice. However, as a limitation, the effect of the inhibition of GSK-3 on other organs than the skin has not been analyzed in our study. Inhibition of the canonical Wnt pathway by siRNA mediated knockdown of β-catenin completely prevented the pro-fibrotic effects of the inactivation of GSK-3 and abrogated the induction of collagen by SB216763. Our data indicate that inhibition or loss of GSK-3 potently stimulates the release of collagen in fibroblasts and that this stimulation is mediated by the canonical Wnt pathway. However, GSK-3 does not only regulate the canonical Wnt pathway, but also integrates signals from
other cascades such as the Notch- and the Hedgehog pathway [22, 23]. Interestingly, Notch- and Hedgehog signaling both have recently been suggested to contribute to tissue fibrosis in SSc [24-27]. Although inhibition of canonical Wnt signaling by knockdown of β-catenin alone was sufficient to fully abrogate the pro-fibrotic effects of the inhibition of GSK-3 in cultured fibroblasts, we cannot exclude that dysregulation of the Notch- and the Hedgehog pathway might have contributed to the pro-fibrotic effects of SB216763 in vivo.

Inhibition of GSK-3 by SB216763 has also been described to reduce the inflammatory response upon the activation of Toll-like-receptor 2 (TLR2) signaling in human monocytes by shifting the balance between pro- and anti-inflammatory cytokines [28]. Interestingly, activation of TLR-2 signaling also contributes to bleomycin induced dermal fibrosis [29]. Thus, effects of SB216763 on TLR2 might have influenced the results obtained with SB216763 in vitro and in vivo. However, considering the pro-fibrotic effects of TLR2 and the inhibitory effects of SB216763 on TLR2 signaling, the effects of SB216763 on TLR2 would have ameliorated rather than exacerbated the pro-fibrotic effects of SB216763, Moreover, AR-A014418, another, structurally not closely related inhibitor of GSK-3 that has not been reported to alter TLR2 signaling and specific siRNA mediated knockdown of GSK-3 stimulated the release of collagen in cultured fibroblasts to a similar degree as SB216763.

In addition to fibrosis, GSK-3 might also play a crucial role in wound healing. Mice harboring a fibroblast specific knockout of GSK-3 exhibited accelerated wound closure and excessive scaring in a dermal punch wound model.[30] Consistent with our results, fibroblasts deficient for GSK-3 were activated and showed enhanced adhesion, migration, and gel contraction.[30] Furthermore, GSK-3 might regulate the release of extracellular matrix not only in fibroblasts, but also osteoblasts. A recent study demonstrated that locally increased levels of vascular endothelial growth factor (VEGF) increased bone mass.[31] Overexpression of VEGF resulted in aberrant activation of osteoblasts with uncontrolled release of bone matrix. VEGF induced the osteoblast activation by inhibited GSK-3 activity
and stimulated the canonical Wnt signaling pathway [31]. In turn, Wnt 3a mediated inhibition of GSK-3 also enhances the release of VEGF in osteoblasts [32]. This effect is also mimicked by inhibition of GSK-3 with SB216763 [32]. Thus, GSK-3 mediated inhibition of the canonical Wnt pathway is not only important for proper regulation of fibroblast functions in fibrotic diseases, but also plays a central role for tissue homeostasis in other cell types.

In summary, we showed that inhibition of GSK-3 stimulates the canonical Wnt pathway in fibroblasts and induces fibrosis in a Wnt dependent manner. Moreover, inhibition of GSK-3 exacerbates fibrosis in different experimental models of SSc. Our data demonstrate that GSK-3 is a crucial regulator of the canonical Wnt pathway that prevents the uncontrolled activation of fibroblasts and development of tissue fibrosis.
ACKNOWLEDGMENTS

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COMPETING INTERESTS

The authors declare no conflicts of interest.

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REFERENCES

FIGURE LEGENDS

**Figure 1** Inhibition of GSK-3 stimulates the release of collagen in SSc fibroblasts. Cultured dermal fibroblasts from SSc patients (n = 6) were incubated with SB216763 at concentrations of 0.1 μM, 1.0 μM and 10 μM. Fibroblasts incubated with the same volumes of the solvent DMSO were used as controls. After 24h of incubation treated fibroblasts and control fibroblasts were lysed for further analysis. **(A)** Incubation with SB216763 increased the mRNA levels of col1a1 in SSc fibroblasts in a dose dependent manner. **(B)** SB21673 increased the release of collagen protein. The release of collagen from SSc fibroblasts was determined using the hydroxyproline assay (n = 4). **(C)** To confirm the effects of pharmacological inhibition, GSK-3 was knocked down in SSc fibroblasts by siRNA (n = 4). Knockdown of GSK-3 increased the expression of col1a1 mRNA in SSc fibroblasts. * p < 0.05 compared to controls.

**Figure 2** Activation of the canonical Wnt pathway mediates the stimulatory effects of SB216763 on collagen synthesis. To assess, whether the activation of the canonical Wnt signaling mediates the pro-fibrotic effects of SB216763, healthy dermal fibroblasts (n = 4) were transfected with β-catenin targeting siRNA or non-targeting mock siRNA. Knockdown of β-catenin completely abrogated the stimulatory effects of SB216763 on col1a1 mRNA **(A)** and collagen protein as measured by the hydroxyproline content in the supernatant **(B)** after 24h, demonstrating that SB216763 stimulated the collagen synthesis in fibroblasts by activating canonical Wnt signaling. * p < 0.05 compared to controls.

**Figure 3** Inhibition of GSK-3 activates the canonical Wnt signaling cascade and induces dermal fibrosis. To investigate, whether inhibition of GSK-3 is sufficient to induce fibrosis, DBA/2 mice were treated with SB216763 alone. A subgroup of mice received injections of
SB216763 for four weeks (n = 4), another subgroup of mice was treated for a period of 8 weeks (n = 5). The control group consisted of eight mice.

(A) The number of fibroblasts stained positive for nuclear β-catenin is significantly increased compared to mock treated controls after 8 weeks of treatment. (IHC) (B) Increased mRNA levels of the Wnt target gene Axin2 upon 8 weeks of treatment with SB216763. (C) Treatment with SB216763 was sufficient to induce dermal fibrosis in mice. Representative sections are shown at 100 fold magnification. (D) Progressive increase in dermal thickness in SB216763 treated mice after 4 and after 8 weeks. (E) Inhibition of GSK-3 increased the hydroxyproline content in the skin. (F) Treatment with SB216763 stimulated the differentiation of resting fibroblasts into myofibroblasts. * p < 0.05 compared to controls.

**Figure 4** Inhibition of GSK-3 exacerbates bleomycin induced dermal fibrosis. To investigate, whether inhibition of GSK-3 exacerbates bleomycin induced experimental fibrosis, mice were treated with SB216763 and challenged with bleomycin (n = 8). Control groups consisted of sham treated, bleomycin challenged mice (n = 8), sham treated mice injected with NaCl (n = 8) and sham treated mice injected with SB216763 (n = 4). (A) Treatment with SB216763 further augments the bleomycin induced accumulation of nuclear β-catenin IHC (B) and the mRNA levels of Axin2. (C) Representative sections of mice injected with NaCl, bleomycin, SB216763 and bleomycin plus SB216763 at 200 fold magnification. (D) Increased dermal thickening in bleomycin challenged mice upon treatment with SB216763. (E) Enhanced hydroxyproline content (F) and increased myofibroblast counts in the skin of mice injected with SB216763 and bleomycin. * p < 0.05 as compared to bleomycin challenged, mock-treated mice.
**Figure 5** Inhibition of GSK-3 enhances the activation of canonical Wnt signaling in tsk-1 mice and exacerbates the tsk-1 phenotype. To assess the effects of the inhibition of GSK-3 in the tsk-1 mouse model, tsk-1 mice were treated with SB216763 (n = 6). Control groups consisted of sham-treated tsk-1 mice (n = 5), mice without the tsk-1 mutation (pa/pa) treated with SB216763 (n = 6) and sham treated pa/pa mice (n = 8). Treatment with SB216763 resulted in increased fibrosis. (A) Activation of the canonical Wnt pathway as analyzed by staining for nuclear β-catenin. (B) Treatment with SB216763 enhanced skin fibrosis in tsk-1 mice. Representative sections are shown at 40 fold magnification. (C) Inhibition of GSK-3 increases hypodermal thickening as compared to mock treated tsk-1 mice. (D) Treatment with SB216763 also enhanced myofibroblast differentiation compared to mock-treated tsk-1 mice. White bars indicate the hypodermal thickness. * p < 0.05 as compared to mock-treated tsk-1 mice.
Inhibition of glycogen synthase kinase 3β induces dermal fibrosis by activation of the canonical Wnt pathway

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SUPPLEMENTS

MATERIAL AND METHODS

Patients and fibroblast cultures

Fibroblast cultures were obtained from skin biopsies of SSc patients. All patients fulfilled the criteria for SSc as suggested by LeRoy et al.[1] Biopsies from SSc patients (n = 9) were taken from involved skin. Healthy fibroblasts (n = 6) were obtained from skin biopsies of healthy age- and sex-matched volunteers (table 1). Fibroblasts were prepared by outgrowth of skin biopsies and cultured in DMEM containing 10 % heat inactivated fetal calf serum (FCS), 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2.5 μg/ml amphotericin B (all Invitrogen, Karlsruhe, Germany) as described previously.[2] All patients and healthy volunteers signed an informed consent form approved by the local institutional review boards.
Incubation with small molecule inhibitors of GSK-3

Stimulation experiments were performed in DMEM/0.1% FCS. Dermal fibroblasts were incubated with two small molecule inhibitors of GSK-3, N-(4-Methoxybenzyl)-N´-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418, Calbiochem, Darmstadt, Germany) and 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763, Tocris, Bristol, UK) in concentrations from 10 nM to 10 μM for 24 h. Fibroblasts incubated with the same volumes of the solvent DMSO were used as controls. Both inhibitors are highly specific for GSK-3. AR-A014418 inhibits GSK-3 with an IC$_{50}$ value of 104 nM and does not inhibit related kinases such as CDK2 or CDK5 (IC$_{50}$ for CDK2 and CDK5 > 100 μmol/L).[3] Similarly, SB216763 inhibits GSK-3 with an IC$_{50}$ value of 34 nM with an IC$_{50}$ for related protein kinases, which is above 10 μM.[4]
Knockdown of GSK-3 in dermal fibroblasts by siRNA

In addition to pharmacological inhibition, GSK-3 was targeted by siRNA mediated knock down using a pre-designed siRNA and the nucleofection technique as described previously.[5] siRNA duplexes with the following sequences were used: sense 5’-CACCUGCACUCUUCACUU-3’, antisense 5’-AAGUUGAAGAGUGCGAGGUG-3’. Fibroblasts transfected with non-targeting control siRNA (Ambion, Darmstadt, Germany) served as controls. Cells were resuspended in Nucleofector solution (Amaza, Cologne, Germany), and 1.5 μg of siRNA against GSK-3β or control siRNA were added. Six hours after nucleofection, the medium was changed to eliminate the nucleofector solution. Fibroblasts were lysed for further analyses after 30 h.

Quantitative Real time PCR

Total RNA was isolated with the NucleoSpin RNA II extraction system (Machery-Nagel, Düren, Germany). Reverse transcription into cDNA was performed using random hexamers.[6, 7] Gene expression was quantified by TaqMan or by SYBR Green real-time PCR using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Specific primer pairs for each gene were designed with the Primer 3 software. The following primer pairs were used for SYBR Green real time PCR: for human α1(I) procollagen, 5’-TCAAGAGAAGGCTCACGGATGG-3’ (forward), 5’-TCACGGTCACGAAACCACATT-3’ (reverse); for human Axin2 5’-CATGACGGACACGAGCTGATAGA-3’ (forward), 5’-ACTGCCCACACGATAAGGAG-3’ (reverse); for murine Axin2, 5’GCCACCAAGACCTACATACGA-3’ (forward), 5’-GAGCGATCTGGTTGCTTCTT-3’ (reverse). To normalize for the amounts of loaded cDNA a pre-developed β-actin assay (Applied Biosystems) was used. Differences were calculated with the threshold cycle (Ct) and the comparative Ct method for relative quantification. Genomic contamination and formation of primer dimers were excluded with samples without enzyme in the reverse transcription
(non-RT-controls), samples without cDNA (no template controls) and dissociation curve analysis.

Collagen measurements

The collagen content was quantified by the hydroxyproline assay.[8] Confluent cells in 25 cm$^2$ culture dishes were incubated for 24 hours with 1 ml DMEM/0.1 % FCS prior to stimulation. For quantification of the hydroxyproline content in murine skin, tissue samples were digested in 500 µl 0.5 M hydrochloric acid at 120° C for 3 h. After adjusting the pH to 6-7, chloramine T (0.06 M) was added to 250µl of each sample. Samples were mixed and incubated for 20 min at room temperature. Afterwards, 3.15 M perchloric acid and 20 % p-dimethylaminobenzaldehyde were added and samples were incubated for 20 min at 60 °C. The absorbance was determined at 557 nm with a Spectra MAX 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

For Western Blot analysis, cells were harvested in PBS and lysed with RIPA buffer containing 1 % nonidet P-40, 0.5 % sodium desoxycholate (Sigma, Deisenhofen, Germany), 0.1 % SDS, 2 mM sodium orthovanadate (Sigma), 0.1 % sodium dodecylsulfate (SDS, Sigma-Aldrich), 0.1 % 1,4-Dithiothreitol (Merck, Darmstadt, Germany). Cell lysates were incubated on ice for 10 min and centrifuged at 12,700 g for 20 min. The protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois,USA). Six micrograms of protein from each sample were separated by 10 % SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Roth, Karlsruhe, Germany) according to standard protocols.[9] After blocking with 5 % nonfat milk powder for 1 h, immunoblots were incubated with polyclonal antibodies against human β-catenin (R&D Systems, Minneapolis, United States) at a dilution of 1:1000 at 4 °C overnight.
Horseradish peroxidase-conjugated rabbit anti-goat antibodies (Dako, Hamburg, Germany) at a dilution of 1:1000 were used as secondary antibodies. Signals were detected with ECL Western Blotting Detection Reagents (Amersham Bioscience, Freiburg, Germany) and exposed to high performance chemiluminescence film (Amersham Biosciences). To confirm the equal loading of proteins, the amount of α-tubulin was visualized using mouse anti-human α-tubulin antibodies (Sigma; dilution 1:1000) and horseradish peroxidase-conjugated goat anti-mouse antibodies (Dako, dilution 1:10000). The intensity of the bands on Western Blots was quantified using the AlphaImager 1220 (Version 5.5) Software.

**Microtiter tetrazolium (MTT) assay**

The colorimetric MTT Assay is an established method to analyzing cell viability in response to pharmacologic treatments. [10] The number of viable cells and their metabolic activity directly correlates with the release of the colorimetrically detectable formazan upon MTT [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] exposure. [10],[11] Cultured SSc fibroblasts were incubated with SB216763 and AR-A014418 at concentrations of 1 μM and 10 μM, respectively. MTT was added at final concentrations of 1 mg/ml and the cells were incubated at 37 °C for 4 h. After dilution with 300 μl 0.04 M HCl in isopropanol, the metabolic activity was analyzed using the ELISA-Reader at a test wavelength of 570 nm with a control wavelength of 630 nm. Untreated fibroblasts were used as negative controls. Fibroblasts incubated with 50 % DMSO served as positive controls.

**Immunohistochemistry for β-catenin and α-smooth muscle actin**

To analyze the effects of GSK-3 inhibition as a negative regulator of canonical Wnt-signaling, the nuclear accumulation of β-catenin was determined by immunohistochemistry. Skin sections were deparaffinized and incubated with 10 % horse serum / 5 % bovine serum albumine for 1 h. β-catenin was detected incubating skin sections with polyclonal goat-anti-
mouse β-catenin antibodies (R&D Systems) at a dilution of 1:300 at 4 °C overnight. To identify nuclear accumulation of β-catenin, nuclei were counterstained with 4’,6-Diamidino-2-phenylindole (DAPI Santa Cruz Biotechnology, California, USA) at a dilution of 1:1000 for 10 min. β-catenin was visualized with DAB peroxidase substrate solution (Sigma-Aldrich). In each patient, cells positive for nuclear β-catenin were counted in 20 randomly chosen high-power fields.

Myofibroblasts were identified by staining for α-smooth muscle actin.[16] After counterstaining with hematoxylin, the number of myofibroblasts was counted from 6 different sections of lesional skin at 200 fold magnification for each mouse by an examiner blinded to the treatment of the mice.

**Histological analysis**

Lesional skin was fixed in 4 % formalin and embedded in paraffin. For the determination of dermal thickness, five-micrometer-thick sections were stained with hematoxylin and eosin. Dermal thickness at the injection sites was analyzed with a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands) measuring the largest distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction as described previously.[12] The analysis was performed by an experienced examiner blinded to the treatment of the mice.

Trichrome staining was performed to directly visualize collagen fibers. After deparaffinization and rehydration, skin sections were incubated in Bouin’s Solution at 56 °C for 15 min and then stained with Weigert’s iron hematoxylin and Biebrich Scarlet-Acid Fuchsin. After incubation with phosphotungstic and phosphomolybdic acid, sections were counterstained with aniline blue for five minutes at room temperature. All reagents for histologic evaluation were obtained from Sigma-Aldrich.
Effects of GSK-3 inhibition on the release of extracellular matrix in vivo

Six week old, female DBA/2 mice (Janvier, Le Genest St Isle, France) were treated with intraperitoneal injections of the GSK-3 inhibitor SB216763 at concentrations of 0.6 mg/kg every other day. To analyze potential changes caused by the activation of GSK-3 over time, one group of mice were treated for 4 weeks and another group of mice for 8 weeks. DBA/2 mice with intraperitoneal injections of the solvent 20 % DMSO dissolved in 0.9 % NaCl were used as controls. 15 mice were analyzed in these experiments.

Bleomycin induced dermal fibrosis

Skin fibrosis was induced in 6 week old, female DBA/2 mice by local injections of bleomycin for 21 days.[13, 14] One hundred microliters of bleomycin dissolved in 0.9 % natrium chloride (NaCl) at a concentration of 0.5 mg/ml were administered every other day by subcutaneous injections in defined areas of the upper back. Subcutaneous injections of 0.9% NaCl at the same volume of 100 µl served as controls. To investigate whether inhibition of GSK-3 aggravates experimental fibrosis, two subgroups were additionally treated with different inhibitors of GSK-3, AR-A014418 and SB216763. AR-A014418 was applied in final concentrations of 4 mg/kg/bid, SB21676 at concentrations of 0.6 mg/kg/bid by intraperitoneal injections every other day. Mice from the control group and the bleomycin group were injected the solvent of the GSK-3 inhibitors (20 % DMSO dissolved in 0.9 % NaCl) to control for the intraperitoneal injections in the treatment groups. After 21 d, mice were sacrificed by cervical dislocation. In total, 32 mice were analyzed.

Inhibition of GSK-3 in the tight-skin 1 mouse model

In addition to the mouse model of bleomycin induced dermal fibrosis, the tight-skin (tsk-1) mouse model of SSc was used to evaluate the pro-fibrotic potential of the selective inhibition of GSK-3. Due to a dominant mutation in fibrillin-1, the phenotype of tsk-1 is
characterized by an increased hypodermal thickness.[15-17] We analyzed three groups of mice. The first group was a control group consisting of pa/pa mice not bearing the tsk-1 mutation. The second group consisted of mock-treated tsk-1 mice. The third group consisted of tsk-1 mice receiving intraperitoneal injections of SB216763 at a concentration of 0.6 mg/kg every other day. The treatment was started at the age of five weeks. All mice were sacrificed at an age of 10 weeks. In total, 21 mice were analyzed.

Statistics

Data are expressed as mean ± standard error of the mean. The Wilcoxon signed rank tests for related samples and the Mann-Whitney-U-test for non-related samples were used for statistical analyses. A p-value of less than 0.05 was considered statistically significant.
REFERENCES

**Supplementary Figure legends**

**Supplementary figure 1:** Inhibition of GSK-3 does not reduce the viability of dermal fibroblasts. SSc fibroblasts were incubated with SB216763 and AR-A014418 at concentrations of 1 µM and 10 µM, respectively, for 20 h. Mock fibroblasts were used as negative controls. Fibroblasts incubated with 50 % DMSO served as positive controls. In each group, three lines of SSc fibroblasts were analysed.

**Supplementary figure 2:** Incubation with the selective GSK-3 inhibitor SB216763 activated the canonical Wnt cascade in cultured dermal fibroblasts from SSc patients. 2A: SB216763 induced nuclear accumulation of β-catenin in cultured dermal fibroblasts of SSc patients as determined by western blot. SSc fibroblasts were incubated with SB216763 for 36 h (n = 4). SSc fibroblasts in the control group (n = 4) were incubated with the same volume of the solvent DMSO. Cells were harvested for further analysis after 24 h. 2B: Consistent with the increase of nuclear β-catenin, SB216763 dose-dependently increased the expression of mRNA of Axin 2. SSc fibroblasts in the treatment group (n = 6) and in the control group (n = 6) were lysed after 24 h. * p < 0.05 compared to controls.

**Supplementary figure 3:** Treatment with SB216763 activated the canonical Wnt cascade in the dermal fibroblasts in vivo. Nuclear staining for of β-catenin in fibroblasts was identified by triple staining for prolyl-4-hydroxylase-β (red), β-catenin (green) and DAPI (blue). Grey arrowheads mark fibroblasts with nuclear staining for β-catenin. White arrows indicate fibroblasts with cytoplasmatic, but not nuclear staining of β-catenin. Fibroblasts, in which staining for β-catenin was restricted to the nucleus were counted as negative as were fibroblasts with absent or very weak staining for β-catenin. Fibroblasts, for which the nucleus
was not present on the section, were not counted. Two representative images of sham treated mice and mice treated with SB216761 are shown at 1000-fold magnification.

**Supplementary figure 4:** Inhibition of GSK-3 induces dermal fibrosis. To investigate, whether inhibition of GSK-3 is sufficient to induce fibrosis, DBA/2 mice were treated with SB216763 alone. A subgroup of mice received injections of SB216763 for four weeks (n = 4), another subgroup of mice was treated for a period of 8 weeks (n = 5). The control group received intraperitoneal injections of NaCl for 8 weeks (n = 8). Representative sections are shown at 200 and 400 fold magnification in **4A**.

**4B:** Treatment with SB216763 induces the differentiation myofibroblasts. A subgroup of mice received injections of SB216763 for four weeks, another subgroup of mice was treated for a period of 8 weeks. The control group received intraperitoneal injections of NaCl for 8 weeks. Skin sections were stained for αSMA and counterstained with hematoxylin. Representative sections are shown at 400 fold magnification.