ARTHRITIS & RHEUMATISM
Vol. 64, No. 9, September 2012, pp 3006–3015
DOI 10.1002/art.34500

JAK-2 as a Novel Mediator of the Profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis

Clara Dees,[1] Michal Tomcik,[2] Katrin Palumbo-Zerr,[1] Alfiya Distler,[1] Christian Beyer,[1] Veronika Lang,[1] Angelika Horn,[1] Pawel Zerr,[1] Jochen Zwerina,[1] Kolja Gelse,[1] Oliver Distler,[3] Georg Schett,[1] and Jörg H. W. Distler[1]

Objective. **To investigate whether JAK-2 contributes to the pathologic activation of fibroblasts in patients with systemic sclerosis (SSc) and to evaluate the antifibrotic potential of JAK-2 inhibition for the treatment of SSc.**

Methods. **Activation of JAK-2 in human skin and in experimental fibrosis was determined by immunohistochemical analysis. JAK-2 signaling was inhibited by the selective JAK-2 inhibitor TG101209 or by small interfering RNA. Bleomycin-induced dermal fibrosis in mice and TSK-1 mice were used to evaluate the antifibrotic potential of specific JAK-2 inhibition in vivo.**

Results. **Increased activation of JAK-2 was detected in the skin of patients with SSc, particularly in fibroblasts. The activation of JAK-2 was dependent on transforming growth factor β (TGFβ) and persisted in cultured SSc fibroblasts. Inhibition of JAK-2 reduced basal collagen synthesis selectively in SSc fibroblasts but not in resting healthy dermal fibroblasts. Moreover, inhibition of JAK-2 prevented the stimulatory effects of TGFβ on fibroblasts. Treatment with TG101209 not only prevented bleomycin-induced fibrosis but also effectively reduced skin fibrosis in TSK-1 mice.**

Conclusion. **We demonstrated that JAK-2 is activated in a TGFβ-dependent manner in SSc. Considering the potent antifibrotic effects of JAK-2 inhibition, our study might have direct translational implications, because inhibitors of JAK-2 are currently being evaluated in clinical trials for myeloproliferative disorders and would also be available for evaluation in patients with SSc.**

Fibrotic diseases such as systemic sclerosis (SSc) are characterized by uncontrolled activation of fibroblasts that release excessive amounts of extracellular matrix (ECM) components such as collagen, glycosaminoglycan, and fibronectin (1). The accumulation of ECM proteins and the resulting fibrosis destroy tissue architecture and contribute significantly to the high morbidity and mortality associated with SSc (2). Several cytokines and growth factors such as transforming growth factor β (TGFβ), platelet-derived growth factor, and different interleukins have been shown to activate resting fibroblasts and to enhance the production of ECM proteins (3). However, the precise molecular mechanisms for the persistent activation of fibroblasts in SSc are still incompletely understood. Thus, molecularly guided translational therapies targeting the activation of fibroblasts are currently not available.

Janus kinases (JAKs) are receptor-associated tyrosine kinases with central roles in cytokine and growth

Supported by the Deutsche Forschungsgesellschaft and the Interdisciplinary Center of Clinical Research in Erlangen (IZKF grant A20). Dr. Tomcik's work was supported by CMH Research Projects no. 00000023728. Dr. J. H. W. Distler is recipient of the Ernst Jung Foundation Career Support Award of Medicine.

[1]Clara Dees, Dipl Biol, Katrin Palumbo-Zerr, MSc, Alfiya Distler, PhD, Christian Beyer, MD, Veronika Lang, MD, Angelika Horn, MD, Pawel Zerr, MSc, Jochen Zwerina, MD, Kolja Gelse, MD, Georg Schett, MD, Jörg H. W. Distler, MD: University of Erlangen–Nuremberg, Erlangen, Germany; [2]Michal Tomcik, MD: University of Erlangen–Nuremberg, Erlangen, Germany, and Charles University Prague, Prague, Czech Republic; [3]Oliver Distler, MD: Zurich Center of Integrative Human Physiology and University Hospital Zurich, Zurich, Switzerland.

Dr. O. Distler has consultancy relationships and/or has received research funding from Actelion, Pfizer, Ergonex, Bristol-Myers Squibb, Sanofi Aventis, United BioSource, Medac, Swedish Orphan Biovitrum, Novartis, 4D Science, and Active Biotec in the area of potential treatments of scleroderma. Dr. J. H. W. Distler has consultancy relationships and/or has received research funding from Celgene, Bayer Pharma, JB Therapeutics, Anaphore, Sanofi Aventis, Novartis, Array Biopharma, Boehringer Ingelheim, Actelion, Pfizer, Ergonex, Bristol-Myers Squibb, and Active Biotec in the area of potential treatments of scleroderma and owns stock in 4D Science.

Address correspondence to Jörg H. W. Distler, MD, Department of Internal Medicine 3 and Institute for Clinical Immunology, University of Erlangen–Nuremberg, Universitätstrasse 29, 91054 Erlangen, Germany. E-mail: joerg.distler@uk-erlangen.de.

Submitted for publication July 6, 2011; accepted in revised form April 5, 2012.

factor signaling. The JAK proteins have 7 JAK homology (JH) domains (4). The kinase activity is located in the JH1 domain, whereas the pseudokinase domain JH2 has essential regulatory function (4,5). The remaining domains (JH3 to JH7) are necessary for protein–protein interactions with cytokine receptors and STAT proteins (4). Upon cytokine binding to the receptor, JAK kinases become activated and phosphorylate tyrosine residues in the cytoplasmic region of the receptor (6). STAT proteins are recruited to these phosphorylation sites and are themselves phosphorylated and activated by JAKs. Activated STATs dimerize and translocate into the nucleus, where they activate transcription of several target genes (6). JAK-2 is a key regulator of cytokine signaling, and alterations in JAK-2 signaling cause profound changes in response to cytokine stimulation. Point mutations in the JAK-2 gene that result in constitutive activation of JAK-2 have been identified as key events in the molecular pathogenesis of myeloproliferative diseases (5,6). The crucial role of JAK-2 in myeloproliferative diseases stimulated the development of inhibitors of JAK-2, several of which are currently being evaluated in clinical trials, with the first results being promising (7).

In the present study, we evaluated the role of JAK-2 in the pathogenesis of SSc and analyzed the antifibrotic potential of JAK-2 inhibition as a novel approach to treatment. We demonstrate that JAK-2 is activated in SSc in a TGFβ-dependent manner and mediates the stimulatory effects of TGFβ on fibroblasts. Selective inhibition of JAK-2 prevented fibroblast activation and experimental fibrosis in different models. Considering the availability of JAK-2 inhibitors, our findings might have direct translational implications and stimulate clinical trials with JAK-2 inhibitors in patients with SSc or other fibrotic diseases.

PATIENTS AND METHODS

Patients and fibroblast cultures. Fibroblast cultures were generated from skin biopsy specimens obtained from the lesional skin of 20 patients with SSc (15 women and 5 men). Control fibroblasts were generated from skin biopsy samples obtained from 17 healthy age- and sex-matched volunteers, as previously described (8). All of the patients fulfilled the criteria for SSc suggested by LeRoy et al (9). At the time of biopsy for the generation of dermal fibroblast cultures, the median age of the patients was 50 years (range 20–67 years). Of the 20 patients with SSc, 10 had limited SSc, and 10 had diffuse disease. The median disease duration (measured from the onset of the first non-Raynaud's symptom attributable to SSc) was 5 years (range 1–16 years). None of the patients were receiving disease-modifying antirheumatic drugs, corticosteroids, or nonsteroidal antiinflammatory drugs. All patients and control subjects signed a consent form approved by the local institutional review boards.

Pharmacologic inhibition of JAK-2 signaling. For pharmacologic blockade of JAK-2 signaling, we used the highly selective JAK-2 inhibitor TG101209 (50% inhibition concentration value of 6 n*M*), which has 30-fold higher selectivity for JAK-2 compared with other JAK kinases at the concentrations used in this study (10). Dermal fibroblasts were incubated with TG101209 (TargeGen) in concentrations ranging from 50 n*M* to 1,000 n*M*. This range covers the plasma concentrations of TG101209 that were obtained after therapeutic doses in the first clinical studies. In a subset of experiments, recombinant human TGFβ1 (10 ng/ml) (R&D Systems) was added 60 minutes after the inhibitor.

Nucleofection with small interfering RNA (siRNA). Dermal fibroblasts were transfected with 1.5 μg of an siRNA duplex against JAK-2, using a human dermal fibroblast Nucleofector kit (Amaxa), as previously described (11). The sequences of the siRNAs were as follows: sense 5′-GAACAGGAUUUACAGUUAU-3′ and antisense 5′-AUAACUGUAAAUCCUGUUC-3′. Fibroblasts transfected with nontargeting siRNAs (Ambion) served as controls. The medium was changed after 6 hours to remove the Nucleofector solution. After 12 hours, cells were stimulated with TGFβ1. Thirty-six hours after siRNA transfection, fibroblasts were harvested for further analysis.

Real-time quantitative polymerase chain reaction (PCR). Total RNA was isolated with a NucleoSpin RNA II extraction system (Machery-Nagel) and reverse transcribed into complementary DNA (cDNA), as previously described (12). Gene expression was quantified by real-time PCR using an ABI Prism 7300 Sequence Detection System (Applied Biosystems). Specific primer pairs for each gene were designed with Primer3 software. The sequences of the human JAK-2 primers were as follows: forward 5′-TTTGGCAACAGACAAATGGA-3′ and reverse 5′-TGCAGATTTCCCACAAAGTG-3′. A predeveloped β-actin assay (Applied Biosystems) was used to normalize for the amounts of loaded cDNA. Dissociation curve analysis, samples without enzyme in reverse transcription (non-RT controls), and no-template controls were used as negative controls to exclude genomic DNA contamination and formation of primer dimers. Differences were calculated with the threshold cycle (C_t) and the comparative C_t method for relative quantification.

Collagen measurements. Total soluble collagen in cell culture supernatants was quantified using a Sircol collagen assay (Biocolor) as previously described (13). Briefly, cell culture supernatant was mixed with sirius red dye for 30 minutes at room temperature. After centrifugation, the pellet was dissolved in alkali reagent. Measurement was performed using a SpectraMax 190 microplate spectrophotometer (Molecular Devices) at a wavelength of 540 nm. To analyze the collagen content in skin samples, a hydroxyproline assay was performed using punch biopsy specimens of lesional skin (diameter 3 mm), as described previously (14,15).

Western blot analysis. PVDF membranes were incubated with anti-human JAK-2 antibodies (Abcam) or anti–pSTAT-3 (Cell Signaling Technology) overnight at 4°C. Horseradish peroxidase–conjugated antibodies (Dako) were used as secondary antibodies. Equal loading of proteins was confirmed by visualization of β-actin (Sigma).

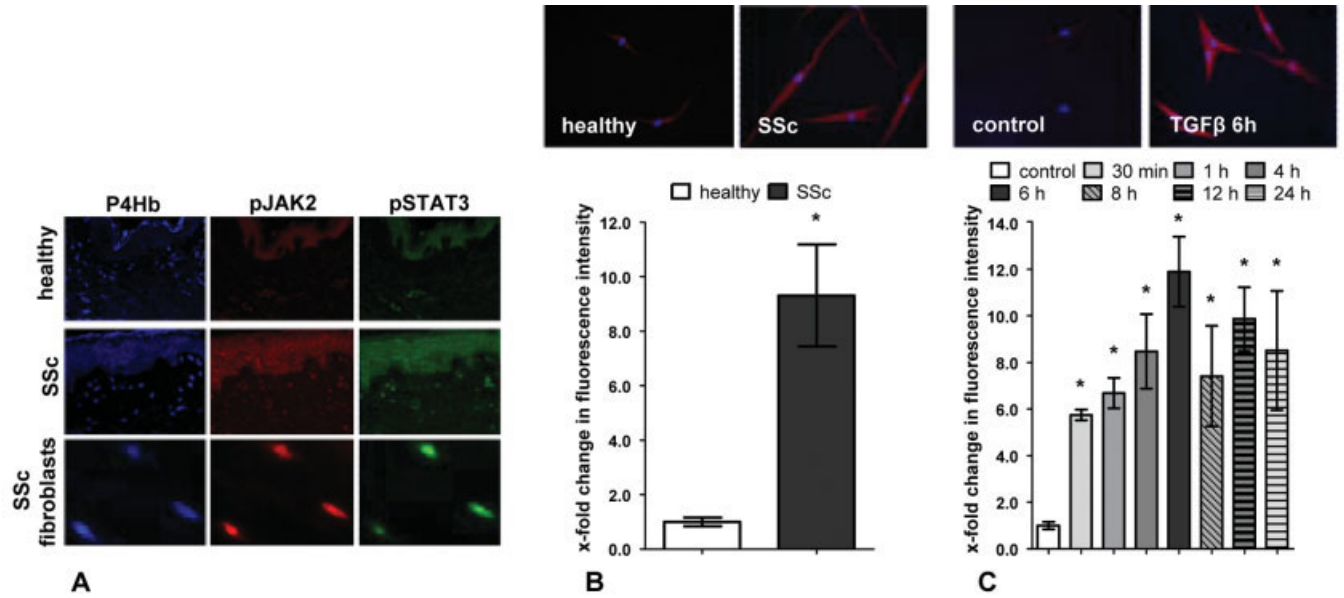

Figure 1. JAK-2 signaling activation in systemic sclerosis (SSc). **A,** Intense staining for pJAK-2 and pSTAT-3 was observed in fibroblasts in skin sections from patients with SSc (n = 10) as analyzed by costaining for the fibroblast marker prolyl 4-hydroxylase β (P4Hb), whereas skin sections from healthy individuals (n = 11) showed only weak staining. Representative images are shown. Original magnification $\times$ 200 (top and middle rows); $\times$ 1,000 (bottom row). **B,** Activation of JAK-2 persisted in cultured SSc fibroblasts. Elevated levels of pJAK-2 (Tyr1007/1008) were detected in SSc fibroblasts compared with cultured fibroblasts obtained from healthy volunteers (n = 4 each). **C,** Stimulation with transforming growth factor β (TGFβ) induced JAK-2 activation and increased the levels of pJAK-2 in a time-dependent manner (n = 4). **B** and **C,** Representative images of pJAK-2–stained fibroblasts are shown. Original magnification $\times$ 200. Bars show the mean $\pm$ SEM. $* = P < 0.05$ versus unstimulated healthy dermal fibroblasts.

Immunocytochemical analysis. Dermal fibroblasts were serum-starved in Dulbecco's modified Eagle's medium–Ham's F-12 containing 0.1% fetal calf serum for 24 hours before the experiments. For time response experiments, serum-starved cells were stimulated with TGFβ1 (10 ng/ml) for time periods ranging from 30 minutes to 24 hours. After fixation with 4% paraformaldehyde, permeabilization with 0.25% Triton X-100, and a blocking step with 5% horse serum, fibroblasts were incubated with rabbit anti–pJAK-2 (Tyr1007/1008) (Epitomics) or mouse anti–α-smooth muscle actin (anti–α-SMA) monoclonal antibodies (clone 1A4; Sigma-Aldrich) at 4°C overnight. Fibroblasts incubated with irrelevant isotype antibodies were used as controls. Alexa Fluor 594–conjugated goat anti-rabbit or Alexa Fluor 488–conjugated goat anti-mouse antibodies (Invitrogen) served as secondary antibodies. Stress fibers were stained with 5 units/ml rhodamine-conjugated phalloidin (Invitrogen) for 20 minutes at room temperature. Counterstaining of nuclei was performed with DAPI (Santa Cruz Biotechnology) for 10 minutes at room temperature. The fluorescence intensity was quantified using ImageJ software version 1.44.

Immunohistochemical analysis. Immunohistochemical analysis of paraffin-embedded sections was performed as previously described (16). Cells positive for α-SMA in mouse sections were detected by incubation with anti–α-SMA monoclonal antibodies (clone 1A4; Sigma-Aldrich). The levels of pJAK-2 and pSTAT-3 in patients with SSc and controls were assessed by staining with anti–pJAK-2 monoclonal antibodies (Tyr1007/1008) (Epitomics) and anti–pSTAT-3 monoclonal antibodies (Cell Signaling Technology) at 4°C overnight. Fibroblast-specific staining was confirmed by staining with anti–prolyl 4-hydroxylase β monoclonal antibodies (Acris Antibodies). Irrelevant isotype antibodies in the same concentration were used as controls. Antibodies labeled with horseradish peroxidase (Dako), Alexa Fluor 350, Alexa Fluor 488, and Alexa Fluor 594 (all from Invitrogen) were used as secondary antibodies. The expression of α-SMA and pJAK-2 in mouse sections was visualized with diaminobenzidine peroxidase substrate solution (Sigma-Aldrich).

Immunohistochemical staining for pJAK-2 was analyzed in a semiquantitative manner. The intensity of staining in fibroblasts, endothelial cells, and keratinocytes was quantified using a scale of 0 to 3, where 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = intense staining. Six high-power fields were evaluated in each slide (n = 2 slides per patient). The reading was performed in a blinded manner by 2 independent, experienced reviewers.

Bleomycin-induced experimental fibrosis. Skin fibrosis was induced in 6-week-old, pathogen-free, female C57BL/6 mice (Charles River) by injection of bleomycin, as previously described (17,18). Subcutaneous injections of 100 μl 0.9% NaCl, the solvent for bleomycin, were used as controls. To investigate whether inhibition of JAK-2 signaling exerts antifibrotic effects in vivo, 2 groups of mice were treated with TG101209 at concentrations of 30 mg/kg and 100 mg/kg twice daily by oral gavage (10,19). One group of mice injected with bleomycin received sham treatment with the carrier solution of TG101209. All groups consisted of 8 mice each. After 21 days,

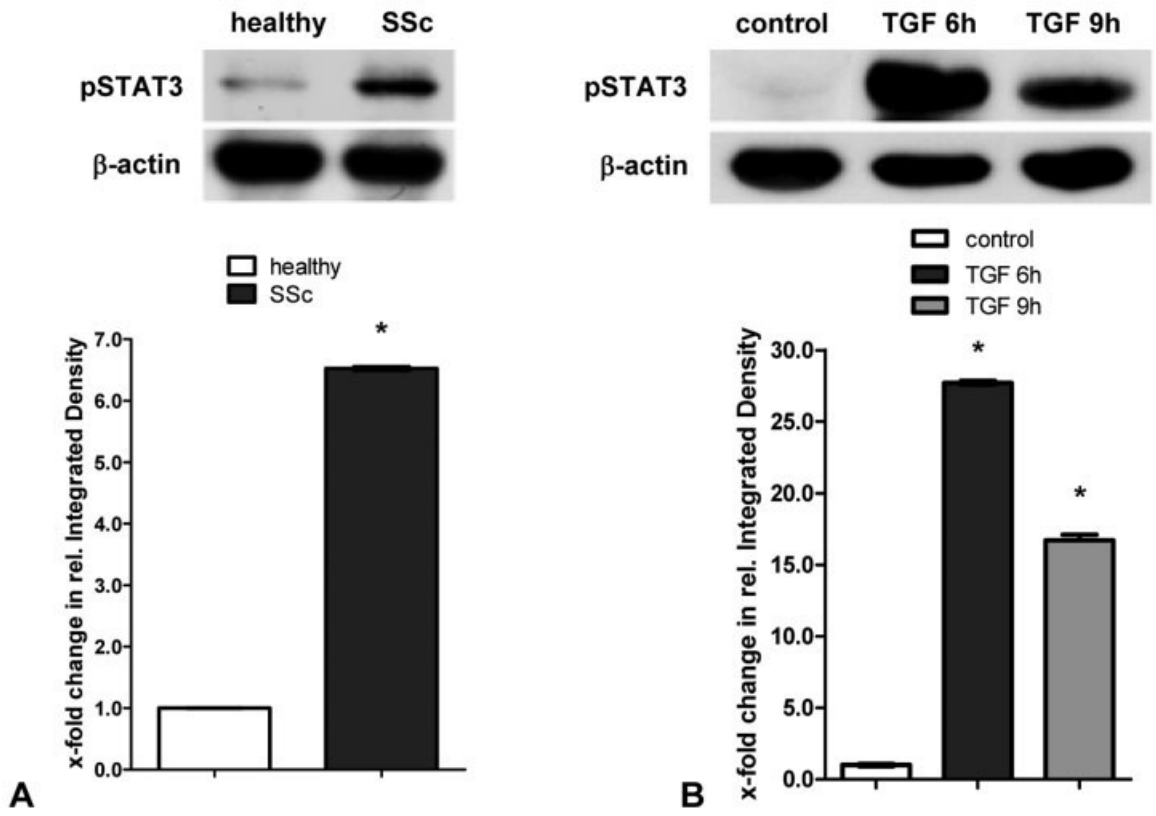

Figure 2. STAT-3 activation in SSc. **A,** The levels of phosphorylated and activated STAT-3 were strongly increased in cultured fibroblasts from patients with SSc compared with fibroblasts from healthy individuals (n = 3 each). **B,** Stimulation of healthy dermal fibroblasts with TGFβ induced phosphorylation of STAT-3, with maximum induction observed after 6 hours of stimulation (n = 3). Bars show the mean ± SEM. $* = P < 0.05$ versus healthy dermal fibroblasts or unstimulated control fibroblasts. Representative blots are shown. rel. = relative (see Figure 1 for other definitions).

the mice were killed by cervical dislocation. All mouse experiments were approved by the local ethics committee.

TSK-1 mouse model. In addition to the mouse model of bleomycin-induced dermal fibrosis, the TSK-1 mouse model of SSc was used to evaluate the antifibrotic potential of specific inhibition of JAK-2 signaling (20). Three groups of mice were analyzed. One group of TSK-1 mice was treated with TG101209 at a concentration of 100 mg/kg twice daily by oral gavage, and another TSK-1 mouse group received mock treatment with the carrier solution. The third group, composed of control littermates not carrying the TSK-1 mutation (*pa*/*pa*), also received mock treatment. The control groups consisted of 10 mice each, and the treatment group comprised 5 mice. Treatment was started at age 5 weeks. After 5 weeks of treatment, the mice were killed by cervical dislocation.

Evaluation of toxicity. Because inhibition of JAK-2 may be accompanied by toxic side effects, the mice treated with TG101209 were monitored daily with analyses of body weight, activity, and fur texture. In addition, the numbers of leukocytes, erythrocytes, and thrombocytes in the peripheral blood were assessed.

Histologic analysis. The injected skin areas of all mice were fixed in 4% formalin and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin for the determination of dermal thickness. Dermal thickness at the injection sites was analyzed using a Nikon Eclipse 80i microscope, as previously described (17). The measurements were performed by an examiner who was blinded to the treatment of the mice.

Statistical analysis. Data are expressed as the mean ± SEM. Wilcoxon's signed rank tests for related samples and the Mann-Whitney U test for nonrelated samples were used for the statistical analyses. *P* values less than 0.05 were considered significant.

RESULTS

Activation of JAK-2 in SSc. First, we analyzed the activation status of JAK-2 in patients with SSc. Active JAK-2 can be identified by phosphorylation on Tyr1007/1008 (pJAK-2) (21,22). Intense staining for pJAK-2 was detected in skin sections and fibroblasts from all patients with SSc (Figure 1A). In contrast to what was observed in patients with SSc, staining for pJAK-2 was reduced in healthy individuals. In particular, only a minority of fibroblasts from healthy individuals stained positive for pJAK-2. Semiquantitative analysis of staining demonstrated that the levels of pJAK-2 were significantly

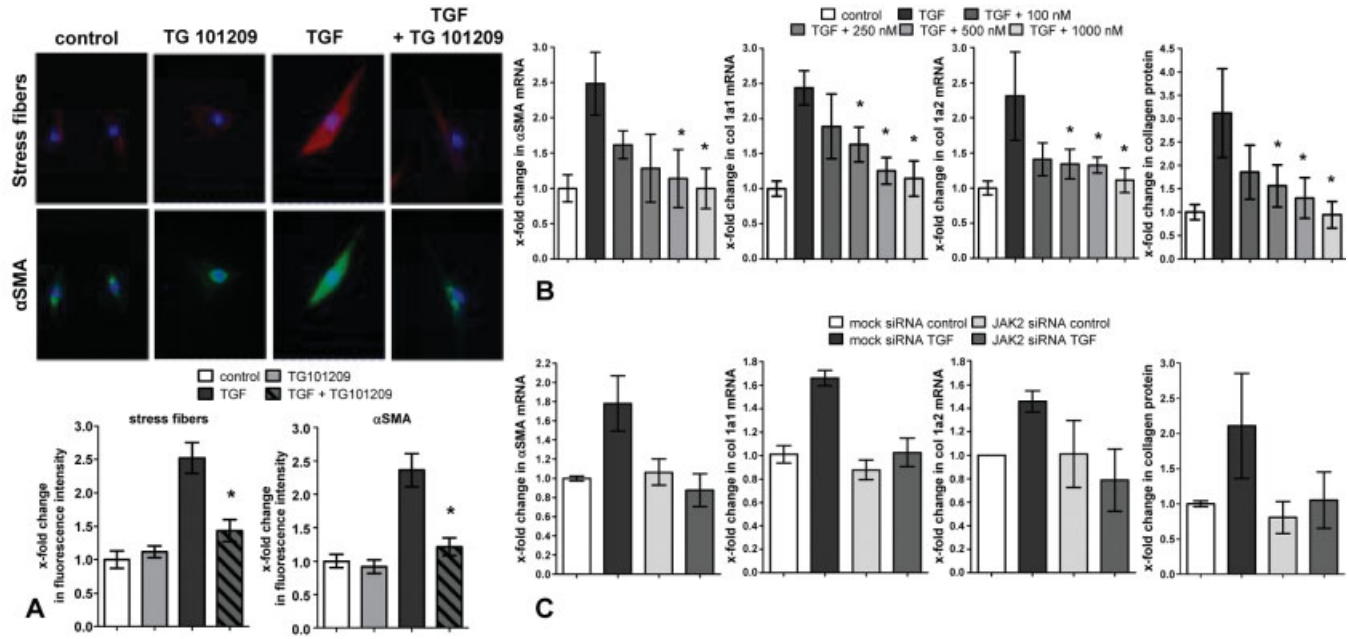

Figure 3. Inhibition of JAK-2 prevents activation of healthy dermal fibroblasts by TGFβ. **A,** Incubation with TG101209 reduced the induction of stress fibers and α-smooth muscle actin (α-SMA) protein production by TGFβ as determined by immunofluorescence analysis (n = 4). Original magnification $\times$ 400. **B,** Incubation with TG101209 dose-dependently reduced the mRNA levels of α-SMA, COL1A1, and COL1A2 and completely prevented the release of collagen protein from TGFβ-stimulated healthy dermal fibroblasts, as analyzed by Sircol assay (n = 8 each). **C,** Knockdown of JAK-2 by small interfering RNA (siRNA) reduced TGFβ-stimulated induction of α-SMA, COL1A1, and COL1A2 mRNA expression and decreased the accumulation of collagen protein in cell culture supernatants, as assessed by Sircol assay (n = 4 each). Bars show the mean $\pm$ SEM. $*$ = $P < 0.05$ versus TGFβ-stimulated fibroblasts without TG101209. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

up-regulated in fibroblasts from SSc skin sections, by a mean $\pm$ SEM of 114 $\pm$ 24% compared with fibroblasts in skin sections from healthy subjects ($P = 0.008$). In addition, the levels of pJAK-2 in endothelial cells from SSc skin sections were also increased, by 112 $\pm$ 29% ($P = 0.027$). Quantitative analysis showed that the levels of pJAK-2 in SSc keratinocytes were up-regulated by 220 $\pm$ 41%, although statistical significance was not reached ($P = 0.323$).

Staining for pSTAT-3, the major downstream mediator of canonical JAK-2 signaling, was also performed. Consistent with the increased levels of JAK-2, staining for pSTAT-3 was increased in SSc and was particularly prominent in SSc fibroblasts (Figure 1A). The staining patterns of pJAK-2 and pSTAT-3 were overlapping.

Of note, the activation of JAK-2 persisted in SSc fibroblasts in vitro. Immunofluorescence analysis showed prominent and intense staining for pJAK-2 in cultured fibroblasts from SSc patients, with a mean $\pm$ SEM 9.3 $\pm$ 1.9-fold increase in fluorescence intensity compared with that in fibroblasts from healthy volunteers ($P < 0.05$) (Figure 1B). In addition, activation of STAT-3, which is the major mediator of JAK-2 signaling, was also increased in SSc fibroblasts compared with fibroblasts from healthy individuals ($P < 0.05$) (Figure 2A).

To investigate whether TGFβ stimulates JAK-2 signaling, we incubated dermal fibroblasts from healthy individuals with TGFβ. TGFβ increased the levels of pJAK-2 in a time-dependent manner, with maximal effects observed 6 hours after TGFβ stimulation (Figure 1C). Furthermore, the levels of pSTAT-3 increased in parallel to those of pJAK-2 upon TGFβ stimulation (Figure 2B). Taken together, these results suggest that JAK-2 is activated in a TGFβ-dependent manner in SSc.

Inhibition of JAK-2 in TGFβ-stimulated fibroblasts. We next evaluated whether JAK-2 mediates the profibrotic effects of TGFβ. Activated fibroblasts and myofibroblasts can be identified by increased formation of stress fibers and expression of α-SMA protein. Indeed, the formation of stress fibers and α-SMA protein levels decreased upon pharmacologic inhibition of JAK-2 using 1 μM TG101209 (mean $\pm$ SEM 74 $\pm$ 12% and 84 $\pm$ 11%, respectively; $P < 0.05$ for both) (Figure 3A). Preincubation with TG101209 potently reduced

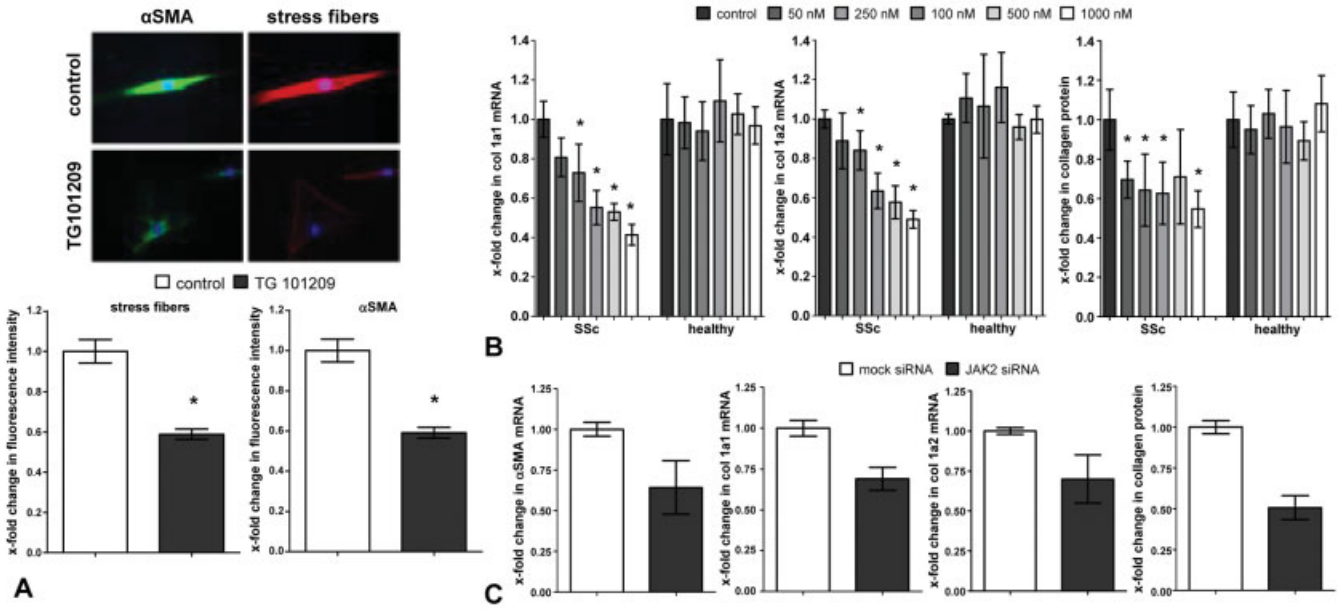

Figure 4. JAK-2 inhibition prevents the active phenotype of systemic sclerosis (SSc) fibroblasts. **A,** Inhibition of JAK-2 decreased the formation of stress fibers and the synthesis of α-smooth muscle actin (α-SMA) protein. Original magnification $\times$ 400. **B,** Incubation with TG101209 dose-dependently reduced basal mRNA expression of COL1A1 and COL1A2 (as assessed by real-time quantitative polymerase chain reaction) and decreased the release of collagen protein from SSc fibroblasts, as assessed using a Sircol collagen assay (n = 8 each). No reduction in basal collagen synthesis was observed in resting healthy dermal fibroblasts upon incubation with TG101209 (n = 6). **C,** Nucleofection of SSc fibroblasts with 1.5 μg of small interfering RNA (siRNA) against JAK-2 efficiently reduced the mRNA levels of α-SMA, COL1A1, and COL1A2 and reduced the release of collagen, as assessed using a Sircol collagen assay (n = 4 each). Bars show the mean $\pm$ SEM. $* = P < 0.05$ versus untreated or mock-transfected fibroblasts. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

messenger RNA (mRNA) expression of the TGFβ target genes plasminogen activator inhibitor 1 (*PAI1*), connective tissue growth factor (*CTGF*), and *ACTA* in healthy dermal fibroblasts stimulated with TGFβ (additional information is available from the corresponding author). Furthermore, the stimulatory effects of TGFβ on the release of collagen in healthy dermal fibroblasts were efficiently reduced in a dose-dependent manner (Figure 3B). The mRNA levels of *COL1A1* and *COL1A2* decreased by up to 90 $\pm$ 21% and 92 $\pm$ 14%, respectively, in TG101209-treated cells compared with mock-treated fibroblasts stimulated with TGFβ ($P <$ 0.05 for both) (Figure 3B). In addition, the TGFβ-induced increase in secreted collagen protein was completely prevented by treatment with TG101209 ($P <$ 0.05) (Figure 3B).

To exclude the possibility that the observed effects of TG101209 were mediated by off-target effects, JAK-2 signaling was inhibited by transfection with siRNA against JAK-2. Transfection of fibroblasts from healthy volunteers with siRNA effectively inhibited the expression of both JAK-2 mRNA and JAK-2 protein, by a mean $\pm$ SEM of 74 $\pm$ 3% and 90 $\pm$ 2%, respectively, compared with fibroblasts transfected with nontargeting control siRNA (additional information is available from the corresponding author). Similar to the results obtained with TG101209, siRNA against JAK-2 prevented the stimulatory effects of TGFβ on *ACTA*, *COL1A1*, and *COL1A2* mRNA (Figure 3C). The release of collagen protein in supernatants of transfected and TGFβ-stimulated fibroblasts also decreased by 69 $\pm$ 16% (Figure 3C).

JAK-2 inhibition in nonstimulated fibroblasts. TGFβ signaling is persistently active in cultured fibroblasts from patients with SSc for several passages and is thought to play a central role in maintenance of the activated phenotype of SSc fibroblasts. After demonstrating that JAK-2 mediated the stimulatory effects of TGFβ on fibroblasts and that JAK-2 was persistently activated in SSc fibroblasts, we hypothesized that inhibition of JAK-2 might reverse the activated phenotype of cultured SSc fibroblasts. Indeed, incubation of SSc fibroblasts with the JAK-2 inhibitor TG101209 dose-dependently decreased the mRNA expression of the TGFβ target genes *PAI1*, *CTGF*, and *ACTA* (additional information is available from the corresponding author).

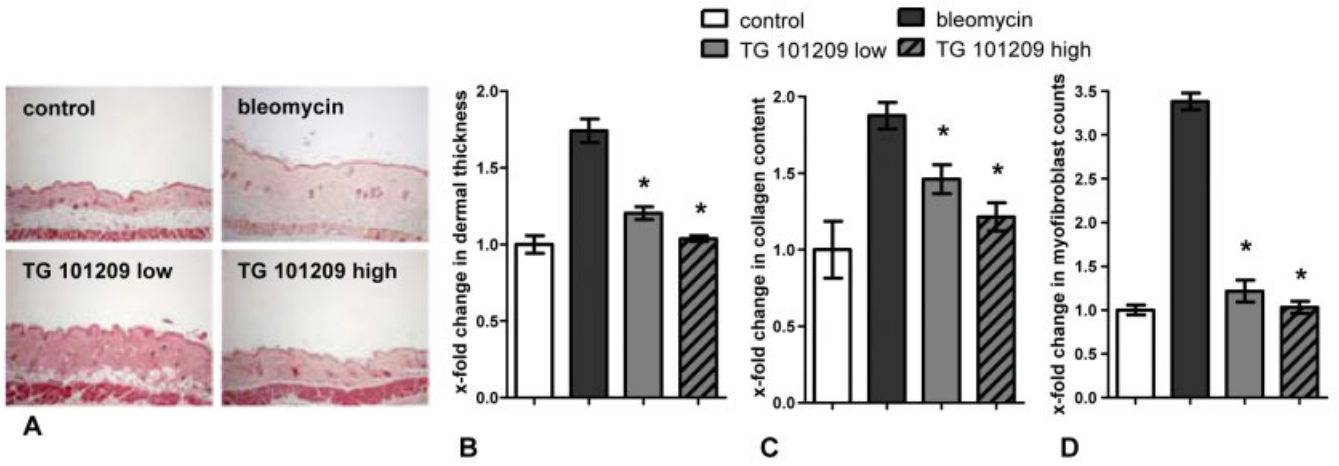

Figure 5. Experimental fibrosis is prevented upon inhibition of JAK-2 signaling. **A,** Representative hematoxylin and eosin–stained skin sections from control mice injected with NaCl (n = 7), bleomycin-challenged mice receiving mock treatment (n = 8), mice injected with bleomycin and treated with TG101209 at a dosage of 30 mg/kg twice daily (n = 8), and bleomycin-challenged mice receiving TG101209 at a dosage of 100 mg/kg twice daily (n = 8) are shown. Original magnification × 100. **B–D,** Treatment with TG101209 reduced bleomycin-induced dermal thickening (**B**), collagen accumulation as analyzed by hydroxyproline assay (**C**), and myofibroblast counts (**D**). Bars show the mean ± SEM. ∗ = $P < 0.05$ versus bleomycin-challenged mice without antifibrotic treatment. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

In addition, inhibition of JAK-2 reduced the formation of stress fibers and the production of α-SMA protein by a mean ± SEM of 41 ± 5% and 41 ± 6%, respectively ($P < 0.05$ for both) (Figure 4A). Incubation with TG101209 also effectively reduced collagen synthesis in SSc fibroblasts (Figure 4B). In the absence of exogenous stimulation, 1 μM TG101209 reduced the mRNA levels of *COL1A1* and *COL1A2* by 59 ± 4% and 51 ± 3%, respectively ($P < 0.05$ for both) (Figure 4B). The basal release of collagen protein from SSc fibroblasts decreased by 50 ± 12% ($P < 0.05$) (Figure 4B). Small interfering RNA–mediated knockdown of JAK-2 also significantly reduced basal collagen mRNA and protein levels as well as the expression of α-SMA in SSc fibroblasts (Figure 4C).

Consistent with the persistent activation of JAK-2 in cultured SSc fibroblasts, but not in fibroblasts from healthy individuals, TG101209 did not alter the basal levels of collagen mRNA or protein in normal dermal fibroblasts (Figure 4B).

Prevention of bleomycin-induced skin fibrosis by JAK-2 inhibition. The mouse model of bleomycin-induced dermal fibrosis was used first to evaluate the antifibrotic potential of JAK-2 inhibition in vivo. Immunohistochemical analysis showed increased staining for activated JAK-2 in fibroblasts from bleomycin-injected mice compared with control mice injected with NaCl (additional information is available from the corresponding author), demonstrating that the model of bleomycin-induced fibrosis mimics activation of JAK-2 in patients with SSc.

A massive accumulation of thickened collagen bundles was observed in mice injected with bleomycin (Figures 5A and B). Treatment with TG101209 at a dosage of 30 mg/kg twice daily significantly reduced dermal thickening, by a mean ± SEM of 72 ± 8% ($P = 0.02$ compared with sham-treated, bleomycin-challenged mice) (Figure 5B). At higher dosages of 100 mg/kg twice daily, dermal thickening was completely prevented ($P = 0.007$) (Figure 5B). The hydroxyproline content and the number of myofibroblasts in lesional skin were also efficiently reduced upon inhibition of JAK-2. The hydroxyproline content decreased dose-dependently by up to 76 ± 7% ($P < 0.001$) (Figure 5C). The differentiation of resting fibroblasts into myofibroblasts was completely abrogated by TG101209 at both concentrations ($P = 0.001$ compared with sham-treated, bleomycin-challenged mice) (Figure 5D).

Protection of TSK-1 mice from fibrosis by inhibition of JAK-2. To evaluate the antifibrotic effects of JAK-2 inhibition in a less inflammatory model that resembles later, noninflammatory stages of SSc (23), we treated TSK-1 mice with TG101209. Increased levels of phosphorylated and thereby activated JAK-2 were observed in TSK-1 mice compared with *pa*/*pa* control mice (additional information is available from the corresponding author).

Fibrotic changes in TSK-1 mice were efficiently decreased by treatment with the JAK-2 inhibitor TG101209 (Figure 6). Following treatment with TG101209, hypodermal thickening in these mice was reduced by a mean ± SEM of 82 ± 10% ($P = 0.002$ versus sham-treated TSK-1 mice) (Figures 6A and B), and the

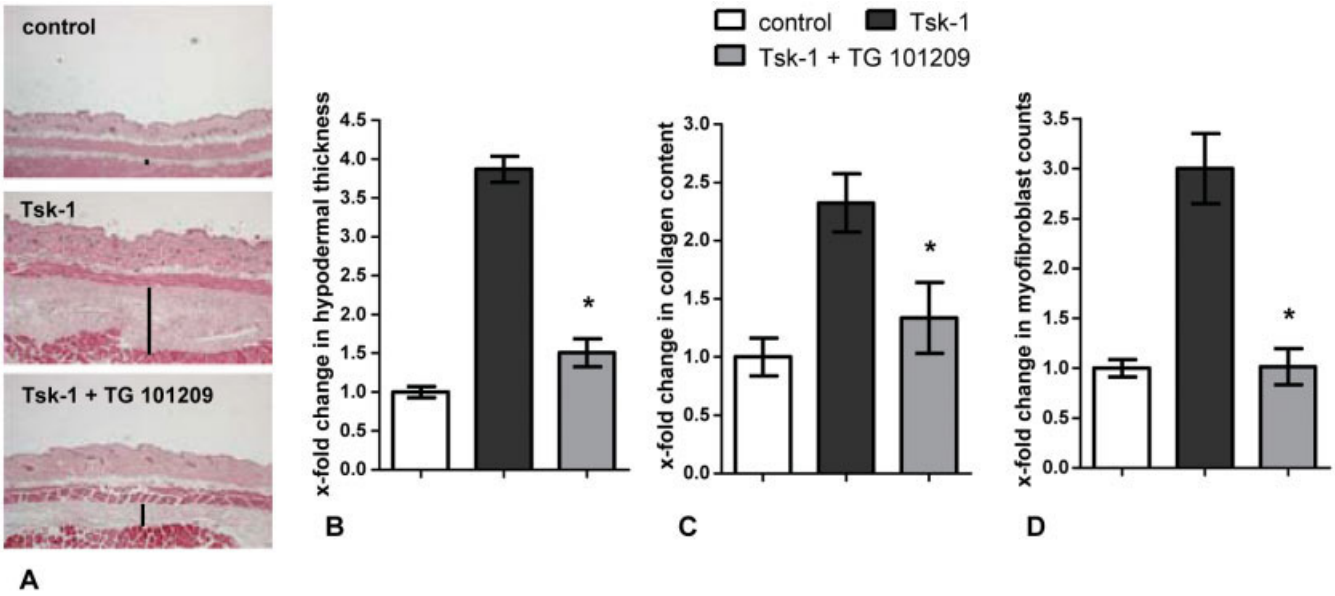

Figure 6. Amelioration of the TSK-1 phenotype by treatment with TG101209. **A,** Representative images of skin sections obtained from control mice without the TSK-1 allele (n = 18), mock-treated TSK-1 mice (n = 11), and TSK-1 mice treated with TG101209 (100 mg/kg twice daily) for 5 weeks (n = 5) are shown. Vertical bars indicate hypodermal thickness. Original magnification × 40. **B–D,** Treatment with TG101209 reduced hypodermal thickening (**B**), hydroxyproline content (**C**), and the differentiation of resting fibroblasts into myofibroblasts (**D**). Bars show the mean ± SEM. ∗ = $P < 0.05$ versus TSK-1 mice without antifibrotic treatment. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

hydroxyproline content in the skin decreased by 75 ± 25% ($P = 0.03$) (Figure 6C). In addition, the numbers of myofibroblasts in TSK-1 mice treated with TG101209 were reduced to the levels of nonfibrotic control mice ($P = 0.01$ versus sham-treated TSK-1 mice) (Figure 6D).

Absence of side effects of JAK-2 inhibition in experimental fibrosis. To exclude the possibility that the antifibrotic effects of TG101209 are accompanied by major toxic side effects, the mice treated with TG101209 were closely monitored. No clinical signs of toxicity were observed, and the mean body weight, the activity, and the fur texture were not altered in mice treated with TG101209 at dosages of 30 mg/kg twice daily and 100 mg/kg twice daily. In addition, regular blood cell counts were performed to exclude bone marrow toxicity. We did not observe any signs of bone marrow toxicity in mice treated with TG101209, and the numbers of leukocytes, erythrocytes, or thrombocytes in the peripheral blood of these mice did not differ from those of sham-treated controls. Thus, TG101209 at antifibrotic doses did not cause major adverse events in experimental models of SSc.

DISCUSSION

A typical feature of SSc fibroblasts is endogenous activation with persistently increased expression of myofibroblast markers and excessive release of collagen, which persists even in the absence of exogenous stimuli for several passages in vitro (1,3). This autonomous activation of SSc fibroblasts is thought to mediate progression of fibrosis in the later stages of SSc. Aberrant TGFβ signaling has been identified as a major molecular mechanism underlying the pathologic activation of SSc fibroblasts. Indeed, the expression of TGFβ and its receptors is increased in cultured SSc fibroblasts, whereas the expression of inhibitory components such as Smad7 is decreased (3,24). The persistent autocrine stimulation of TGFβ signaling induces an activated phenotype in SSc fibroblasts, with myofibroblast differentiation and increased release of collagen.

In the current study, we demonstrated that inhibition of JAK-2 is sufficient to reverse the characteristic phenotype of cultured SSc fibroblasts. Persistent phosphorylation and thereby activation of JAK-2 was observed in cultured SSc fibroblasts but not in fibroblasts from age- and sex-matched healthy individuals. Targeting of JAK-2 in SSc fibroblasts abrogated the pathologic activation of TGFβ signaling, prevented myofibroblast differentiation, and normalized the release of collagen. The inhibitory effects in the absence of exogenous stimulation were specific for SSc fibroblasts and were not observed in fibroblasts from healthy individuals.

Of interest, JAK-2 not only may act as a down-

stream mediator of TGFβ in fibroblasts but also may amplify TGFβ signaling by stimulating the expression of TGFβ (25,26). Both inhibition of STAT-3 and overexpression of suppressor of cytokine signaling 1 reduced the expression of TGFβ in different studies (25,26). Consistent with these findings, incubation with TG101209 dose-dependently decreased the levels of TGFβ mRNA in SSc fibroblasts. Considering the selective activation in SSc fibroblasts and the important role of JAK-2 for fibroblast activation, inhibition of JAK-2 might be a novel approach to prevent aberrant TGFβ signaling and to control the pathologic activation of SSc fibroblasts. By demonstrating that pJAK-2 and pSTAT-3 colocalize in vitro and in vivo, we provide the first evidence that TGFβ activates canonical JAK/STAT signaling to regulate the release of collagen. However, further studies are required to dissect the molecular mechanisms by which JAK-2 regulates the profibrotic effects of TGFβ.

In the current study, we demonstrated that doses of TG101209 that have potent antifibrotic effects in preclinical models are well tolerated. We did not observe any obvious signs of toxicity such as weight loss, reduced activity, changes in fur texture, or cytopenia in mice treated with TG101209 in dosages of 30 mg/kg twice daily and 100 mg/kg twice daily. Consistent with our findings in murine models, JAK-2 inhibitors were rather well tolerated in clinical studies in patients with myeloproliferative diseases. In the largest study available to date, 12 serious adverse events possibly related to treatment occurred in 143 patients who were treated for a mean duration of 14.7 months with the nonselective JAK-1/2 inhibitor INCB018424 (27). Moreover, some of the adverse events observed in these clinical trials may not be directly related to the inhibition of JAK-2. Diarrhea was reported in association with only some of the JAK-2 inhibitors, indicating that diarrhea may not be a class effect. Consistent with this hypothesis, we did not observe any signs of gastrointestinal toxicity such as diarrhea or weight loss associated with TG101209 treatment in our preclinical model. We also did not observe any clinical signs of bone marrow toxicity in mice treated with TG101209, but thrombocytopenia and anemia accounted for the majority of grade 3 or grade 4 adverse events in studies in patients with myelodysplastic diseases (7,28,29). However, cytopenia might be the consequence of the expansion of malignant cells in the bone marrow and splenomegaly during the natural course of myelodysplastic syndromes. Although some JAK-2 inhibitors may suppress bone marrow, improvement of anemia related to myelodysplasia was observed for other inhibitors such as CYT387 (28). Taken together, these data suggest that targeting JAK-2 in SSc and other fibrotic diseases may not be limited by toxicity.

Inhibition of JAK-2 signaling by a small molecule inhibitor or siRNA significantly reduced the release of collagen in vitro and prevented the differentiation of resting fibroblasts into myofibroblasts. Moreover, TG101209 completely prevented bleomycin-induced dermal fibrosis, which serves as a model for the early stages of SSc, with inflammation-dependent activation of resident fibroblasts (23,30). Consistent with its inhibitory effects on cultured fibroblasts, TG101209 also significantly reduced skin fibrosis in the TSK-1 mouse model, which is characterized by endogenous activation of fibroblasts and mimics the later, noninflammatory stages of SSc (23). The mechanism of action with direct targeting of fibroblast activation and the efficacy in inflammation-driven models and in noninflammatory models of fibrosis suggest that targeting of JAK-2 might be of interest in terms of treatment of patients with different stages of SSc and also as treatment of other fibrotic diseases.

These results may have direct translational implications. Currently, 8 different inhibitors of JAK-2 are being evaluated in clinical trials (www.clinicaltrials.gov). Seven of these inhibitors are being tested in patients with oncologic diseases, primarily myelodysplastic syndromes, but also acute leukemias and solid tumors. However, because of the promising effects and the good tolerability observed in earlier studies, inhibition of JAK-2 also became interesting for other, less fatal diseases. Indeed, the nonselective JAK-1/2 inhibitor LY3009104 is currently being evaluated in a phase IIb study, using 4 different doses on a background of methotrexate in patients with rheumatoid arthritis. Thus, a plethora of different pharmacologic inhibitors of JAK-2 would be available for clinical trials in SSc and other fibrotic diseases. This is particularly important because organ failure due to excessive accumulation of ECM is a major reason for the high morbidity and mortality associated with SSc, and efficient antifibrotic therapies are not yet available for clinical use (31–33).

In summary, we demonstrated that JAK-2 is activated in a TGFβ-dependent manner in SSc. Inhibition of JAK-2 decreased the basal release of collagen from SSc fibroblasts and prevented the stimulatory effects of TGFβ on healthy dermal fibroblasts. Inhibition of JAK-2 exerts potent antifibrotic effects in different preclinical models. Considering that several pharmacologic inhibitors of JAK-2 are available and are well tolerated, the targeting of JAK-2 might be an interesting molecular approach to the treatment of SSc and other fibrotic diseases.

ACKNOWLEDGMENTS

We would like to thank Maria Halter and Anna-Maria Herrmann for excellent technical support.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. J. H. W. Distler had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Dees, O. Distler, J. H. W. Distler.

Acquisition of data. Dees, Tomcik, Palumbo-Zerr, A. Distler, Lang, Horn, Zerr, J. H. W. Distler.

Analysis and interpretation of data. Dees, Tomcik, Beyer, Zwerina, Gelse, O. Distler, Schett, J. H. W. Distler.

REFERENCES

1. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med 2009;360:1989–2003.
2. Tyndall AJ, Bannert B, Vonk M, Airo P, Cozzi F, Carreira PE, et al. Causes and risk factors for death in systemic sclerosis: a study from the EULAR Scleroderma Trials and Research (EUSTAR) database. Ann Rheum Dis 2010;69:1809–15.
3. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007;117:557–67.
4. Yamaoka K, Saharinen P, Pesu M, Holt VE III, Silvennoinen O, O'Shea JJ. The Janus kinases (Jaks). Genome Biol 2004;5:253.
5. Baker SJ, Rane SG, Reddy EP. Hematopoietic cytokine receptor signaling. Oncogene 2007;26:6724–37.
6. Valentino L, Pierre J. JAK/STAT signal transduction: regulators and implication in hematological malignancies. Biochem Pharmacol 2006;71:713–21.
7. Verstovsek S. Therapeutic potential of JAK2 inhibitors. Hematology Am Soc Hematol Educ Program 2009;2009:636–42.
8. Akhmetshina A, Dees C, Pileckyte M, Szucs G, Spriewald BM, Zwerina J, et al. Rho-associated kinases are crucial for myofibroblast differentiation and production of extracellular matrix in scleroderma fibroblasts. Arthritis Rheum 2008;58:2553–64.
9. LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol 2001;28:1573–6.
10. Pardanani A, Hood J, Lasho T, Levine RL, Martin MB, Noronha G, et al. TG101209, a small molecule JAK2-selective kinase inhibitor potently inhibits myeloproliferative disorder-associated JAK2V617F and MPLW515L/K mutations. Leukemia 2007;21:1658–68.
11. Distler JH, Jungel A, Pileckyte M, Zwerina J, Michel BA, Gay RE, et al. Hypoxia-induced increase in the production of extracellular matrix proteins in systemic sclerosis. Arthritis Rheum 2007;56:4203–15.
12. Dees C, Tomcik M, Zerr P, Akhmetshina A, Horn A, Palumbo K, et al. Notch signalling regulates fibroblast activation and collagen release in systemic sclerosis. Ann Rheum Dis 2011;70:1304–10.
13. Palumbo K, Zerr P, Tomcik M, Vollath S, Dees C, Akhmetshina A, et al. The transcription factor JunD mediates transforming growth factor β-induced fibroblast activation and fibrosis in systemic sclerosis. Ann Rheum Dis 2011;70:1320–6.
14. Dees C, Zerr P, Tomcik M, Beyer C, Horn A, Akhmetshina A, et al. Inhibition of Notch signaling prevents experimental fibrosis and induces regression of established fibrosis. Arthritis Rheum 2011;63:1396–404.
15. Woessner JF Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440–7.
16. Dees C, Akhmetshina A, Zerr P, Reich N, Palumbo K, Horn A, et al. Platelet-derived serotonin links vascular disease and tissue fibrosis. J Exp Med 2011;208:961–72.
17. Distler JH, Jungel A, Huber LC, Schulze-Horsel U, Zwerina J, Gay RE, et al. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. Arthritis Rheum 2007;56:311–22.
18. Marquart S, Zerr P, Akhmetshina A, Palumbo K, Reich N, Tomcik M, et al. Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. Arthritis Rheum 2010;62:3467–76.
19. Russell RC, Sufan RI, Zhou B, Heir P, Bunda S, Sybingco SS, et al. Loss of JAK2 regulation via a heterodimeric VHL-SOCS1 E3 ubiquitin ligase underlies Chuvash polycythemia. Nat Med 2011;17:845–53.
20. Akhmetshina A, Venalis P, Dees C, Busch N, Zwerina J, Schett G, et al. Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis. Arthritis Rheum 2009;60:219–24.
21. Lasho T, Tefferi A, Pardanani A. Inhibition of JAK-STAT signaling by TG101348: a novel mechanism for inhibition of KITD816V-dependent growth in mast cell leukemia cells. Leukemia 2010;24:1378–80.
22. Wang Y, Fiskus W, Chong DG, Buckley KM, Natarajan K, Rao R, et al. Cotreatment with panobinostat and JAK2 inhibitor TG101209 attenuates JAK2V617F levels and signaling and exerts synergistic cytotoxic effects against human myeloproliferative neoplastic cells. Blood 2009;114:5024–33.
23. Beyer C, Schett G, Distler O, Distler JH. Animal models of systemic sclerosis: prospects and limitations [review]. Arthritis Rheum 2010;62:2831–44.
24. Kawakami T, Ihn H, Xu W, Smith E, LeRoy C, Trojanowska M. Increased expression of TGF-β receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-β signaling to scleroderma phenotype. J Invest Dermatol 1998;110:47–51.
25. Pang M, Ma L, Gong R, Tolbert E, Mao H, Ponnusamy M, et al. A novel STAT3 inhibitor, S3I-201, attenuates renal interstitial fibroblast activation and interstitial fibrosis in obstructive nephropathy. Kidney Int 2010;78:257–68.
26. Shi Y, Zhang Y, Wang C, Du C, Zhao S, Qi Z, et al. Suppressor of cytokine signaling-1 reduces high glucose-induced TGF-β1 and fibronectin synthesis in human mesangial cells. FEBS Lett 2008;582:3484–8.
27. Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA, et al. Safety and efficacy of INCB018424, a JAK1 and JAK2 inhibitor, in myelofibrosis. N Engl J Med 2010;363:1117–27.
28. Pardanani A, Vannucchi AM, Passamonti F, Cervantes F, Barbui T, Tefferi A. JAK inhibitor therapy for myelofibrosis: critical assessment of value and limitations. Leukemia 2011;25:218–25.
29. Santos FP, Verstovsek S. JAK2 inhibitors: what's the true therapeutic potential? Blood Rev 2011;25:53–63.
30. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, et al. Animal model of sclerotic skin. I: local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 1999;112:456–62.
31. Charles C, Clements P, Furst DE. Systemic sclerosis: hypothesis-driven treatment strategies. Lancet 2006;367:1683–91.
32. Kim R, Meyer KC. Therapies for interstitial lung disease: past, present and future. Ther Adv Respir Dis 2008;2:319–38.
33. Swigris JJ, Brown KK, Make BJ, Wamboldt FS. Pulmonary rehabilitation in idiopathic pulmonary fibrosis: a call for continued investigation. Respir Med 2008;102:1675–80.