The histone demethylase Jumonji domain-containing protein 3 (JMJD3) regulates fibroblast activation in systemic sclerosis

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Abstract:

Objectives Systemic sclerosis (SSc) fibroblasts remain activated even in the absence of exogenous stimuli. Epigenetic alterations are thought to play a role for this endogenous activation. Trimethylation of histone H3 on lysine 27 (H3K27me3) is regulated by Jumonji domain-containing protein 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat on chromosome X (UTX) in a therapeutically targetable manner. The aim of this study was to explore H3K27me3 demethylases as potential targets for the treatment of fibrosis.

Methods JMJD3 was inactivated by small interfering RNA-mediated knockdown and by pharmacological inhibition with GSKJ4. The effects of targeted inactivation of JMJD3 were analysed in cultured fibroblasts and in the murine models of bleomycin-induced and topoisomerase-I (topoI)-induced fibrosis. H3K27me3 at the *FRA2* promoter was analysed by ChIP.

Results The expression of JMJD3, but not of UTX, was increased in fibroblasts in SSc skin and in experimental fibrosis in a transforming growth factor beta (TGFβ)-dependent manner. Inactivation of JMJD3 reversed the activated fibroblast phenotype in SSc fibroblasts and prevented the activation of healthy dermal fibroblasts by TGFβ. Pharmacological inhibition of JMJD3 ameliorated bleomycin-induced and topoI-induced fibrosis in well-tolerated doses. JMJD3 regulated fibroblast activation in a FRA2-dependent manner: Inactivation of JMJD3 reduced the expression of FRA2 by inducing accumulation of H3K27me3 at the *FRA2*promoter. Moreover, the antifibrotic effects of JMJD3 inhibition were reduced on knockdown of *FRA2*.

Conclusion We present first evidence for a deregulation of JMJD3 in SSc. JMJD3 modulates fibroblast activation by regulating the levels of H3K27me3 at the promoter of *FRA2*. Targeted inhibition of JMJD3 limits the aberrant activation of SSc fibroblasts and exerts antifibrotic effects in two murine models.

Introduction

Systemic sclerosis (SSc) is a chronic fibrosing connective tissue disease that affects the skin and various internal organs. A central hallmark of SSc is the uncontrolled and persistent activation of fibroblasts, which release excessive amounts of extracellular matrix.[1] The accumulation of extracellular matrix proteins perturbs the physiological architecture of affected tissues, thus leading to progressive dysfunction.[2] The chronically activated fibroblast phenotype in SSc relies at least in part on endogenous mechanisms. This is highlighted by the persistently activated phenotype of SSc fibroblasts even after several passages in culture.[3]In the absence of exogenous stimuli, SSc fibroblasts continue to express myofibroblast markers and release increased amounts of extracellular matrix. Although several core factors of fibroblast activation such as transforming growth factor beta (TGFβ) have been identified, the molecular mechanisms leading to the persistent endogenous fibroblast activation in SSc are incompletely understood and targeted therapies are still not available for clinical use.

Accumulating evidence suggests that epigenetic alterations may play a role in the pathogenesis of SSc.[4–23] Different epigenetic modifications such as DNA methylation,[4–7] histone acetylation,[8] histone deacetylation,[5 9–11] histone methylation,[12]microRNAs[13–22] and long non-coding RNAs[23] have been shown to be deregulated in SSc fibroblasts and have been suggested to perpetuate fibroblast activation. Trimethylation of histone H3 on lysine 27 (H3K27me3) is a common histone modification that represses the transcription of target genes.[24] H3K27me3 might inhibit gene expression by different mechanisms: H3K27me3 can provide a binding surface for chromatin remodelling enzymes with specific methyl–lysine binding sites, it can interfere with the binding of proteins that interact selectively with unmethylated histones or it can affect the accessibility of neighbouring histone residues for other epigenetic modifications. H3K27me3 is regulated by a specific set of histone–methyltransferases and histone–demethylases in a therapeutically targetable manner.[24] H3K27me3 is executed by polycomb repressive complexes including enhancer of zeste 2 (EZH2) and suppressor of zeste 12 (SUZ12) as central components. Two highly specific histone–demethylases, Jumonji domain-containing protein 3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat on chromosome X (UTX), are capable to reverse the H3K27me3 modification. The activity of H3K27 methyltransferases and H3K27me3 demethylases is tightly controlled under physiological conditions. Deregulation of this delicate balance has been implicated in the pathogenesis of several tumours,[25] but more recently also in fibroblast activation.[12 26] Inhibition of H3K27 trimethylation by 3-deazaneplanocin A (DZNep), a combined inhibitor of the synthesis of S-adenosylhomocysteine and of the H3K27 methyltransferase EZH2, promoted fibroblast activation and induced fibrosis.[12]

In the present study, we tested the hypothesis that targeted inhibition of H3K27me3 demethylases may be a novel approach to interfere with the aberrant activation of SSc fibroblasts. We aimed to study the expression and regulation of H3K27me3 demethylases in SSc, to investigate their role in fibroblast activation and to assess the antifibrotic effects and the tolerability of targeting them in preclinical models of fibrosis.

Materials and methods can be found in the online supplement.

Results

JMJD3 is overexpressed in SSc fibroblasts

We first analysed the expression of the H3K27me3 demethylases in SSc. We observed increased expression of JMJD3 in the skin of patients with SSc compared with matched healthy individuals. Co-staining with the fibroblast marker prolyl-4-hydroxylase-β (P4Hβ) demonstrated particular high levels of JMJD3 in SSc skin fibroblasts (figure 1A). We also observed overexpression of JMJD3 in murine models of SSc such as bleomycin-induced skin fibrosis, the B10.D2 (H-2^d) → BALB/c (H-2^d) model of sclerodermatous chronic Graft versus Host Disease (cGvHD), and topoisomerase-I (topoI)-induced dermal fibrosis (online supplementary figure 1). Of interest, the expression of JMJD3 remained elevated in SSc fibroblasts even after several passages with increased mRNA and protein levels of JMJD3 in cultured dermal SSc fibroblasts compared with dermal fibroblasts from healthy individuals

(figure 1A). The expression levels of UTX were much lower than the expression of JMJD3 and no significant differences were observed between patients with SSc and controls (online supplementary figure 2). Consistent with previous findings,[12] the overall H3K27me3 content in SSc skin was rather upregulated despite the upregulation of the JMJD3 (online upplementary figure 3). These findings suggest that the increased activity of JMJD3 may be counterbalanced by enhanced activity of H3K27-histone methyltransferase activity, which is consistent with reports on increased EZH2 expression in fibrotic diseases.[27 28]

Given the consistent upregulation of JMJD3 in SSc and murine models of skin fibrosis as well as in cultured fibroblasts, we hypothesised that a core pathway of fibrosis may drive the overexpression of JMJD3 in fibrosis. Incubation of human dermal fibroblasts with recombinant TGFβ increased the mRNA as well as the protein levels of JMJD3 (figure 1B). Consistent with the upregulation of the histone demethylase JMJD3 on stimulation with TGFβ, the levels of H3K27me3 decreased in fibroblasts stimulated with TGFβ (figure 1C). To decipher the intracellular signalling cascade that mediates the induction of JMJD3 by TGFβ, we targeted SMAD signalling by siRNA-mediated knockdown of SMAD3 in fibroblasts. Knockdown of SMAD3 prevented the induction of JMJD3 by TGFβ (figure 1D), demonstrating a SMAD-dependent regulation.

Consistent with the results in vitro, we observed increased expression of JMJD3 in skin fibroblasts of mice with fibroblast-specific overexpression of a constitutively active TGFβ receptor type 1 (TBR^{act}) compared with control mice (figure 1E). Moreover, treatment of bleomycin-challenged mice with SD208, a specific inhibitor of the TGFβ receptor I-kinase activity, prevented the bleomycin-induced upregulation of JMJD3 (online supplementary figure 4). Together, these data demonstrate that TGFβ signalling is sufficient and required to induce JMJD3 in fibrosis in a SMAD3-dependent manner.

Targeted inactivation of JMJD3 inhibits fibroblast activation

We next aimed to investigate the role of JMJD3 in fibroblast activation. GSKJ4 has recently been described as a specific inhibitor of H3K27me3 demethylases.[29] We therefore used GSKJ4 as a first approach to target JMJD3 in fibroblasts. Incubation of SSc fibroblasts with GSKJ4 dose-dependently increased H3K27me3 in SSc fibroblasts (figure 2A). GSKJ4 also induced accumulation of H3K27me3 in healthy fibroblasts (online supplementary figure 5A), confirming that GSKJ4 is a potent tool to modulate the activity of JMJD3. Moreover, GSKJ4 ameliorated the TGFβ-induced downregulation of H3K27me3 in healthy fibroblasts (online supplementary figure 5B). The inhibition of JMJD3 by GSKJ4 was associated with impaired collagen release. Incubation with GSKJ4 decreased the mRNA levels of *COL1A1*, reduced the levels of type I collagen protein and decreased the hydroxyproline levels in SSc fibroblasts in a dose-dependent manner (figure 2B). Incubation with GSKJ4 also delayed closure of the artificial wound in scratch assays (figure 2D). Of note, those antifibrotic effects were observed in non-toxic concentrations as shown by lack of effects on the metabolic activity in MTT assays (online supplementary figure 6).

JMJD3 modulates fibroblast activity in a FRA2-dependent manner

We and others demonstrated previously that the AP1 transcription factor FRA2 is overexpressed in dermal fibroblasts of patients with SSc and plays a role in fibroblast activation in SSc.[30 31] We therefore aimed to investigate whether the inhibitory effects of targeting JMJD3 depend on the regulation of FRA2. First we analysed the levels of H3K27me3 at the *FRA2* promoter in fibroblasts isolated from patients with SSc and healthy individuals by ChIP. Consistent with the overexpression of FRA2 in SSc fibroblasts and the inhibitory effects of H3K27me3 on gene expression, H3K27me3 at the *FRA2* promoter was reduced in SSc fibroblasts compared with control fibroblasts (figure 4A). The reduced H3K27me3 at the *FRA2* promoter in SSc fibroblasts was resembled in healthy fibroblasts by stimulation with TGFβ. Incubation of normal dermal fibroblasts with TGFβ strongly reduced H3K27me3 at the *FRA2* promoter (figure 4B). Consistent with a JMJD3-mediated effect, treatment with GSKJ4 prevented the inhibitory effects of TGFβ on H3K27me3 at the *FRA2* promoter (figure 4B). Treatment with GSKJ4 also prevented the TGFβ-induced upregulation of the FRA2 protein in fibroblasts (figure 4C). The role of JMJD3 for the regulation of FRA2 expression in fibroblasts was

further confirmed by the finding that siRNA-mediated knockdown of JMJD3 also inhibited the induction of FRA2 by TGFβ (figure 4D). In line with these *in vitro*results, treatment with GSKJ4 reduced the expression of FRA2 in topoI-induced fibrosis (figure 4E). To provide functional evidence for FRA2 as target for the antifibrotic effects of targeting JMJD3 in fibroblasts, we assessed the effects of GSKJ4 in human fibroblasts on knockdown of *FRA2*. Consistent with a central role of FRA2 as a mediator of the regulatory effects of JMJD3 in fibroblasts, knockdown of *FRA2* strongly reduced the inhibitory effects of GSKJ4 on TGFβ-induced collagen synthesis (figure 4F).

Treatment with GSKJ4 ameliorates experimental fibrosis

We next analysed whether the inhibitory effects of GSKJ4 on fibroblast activation *in vitro* translate into antifibrotic effects in murine SSc models. We employed bleomycin-induced skin fibrosis as a model for inflammation-driven stages of SSc. Treatment of bleomycin-challenged mice with GSKJ4 increased H3K27me3 levels as compared with vehicle-treated controls and non-fibrotic mice (figure 5A). The elevated levels of H3K27me3 were associated with potent antifibrotic effects and treatment with GSKJ4-reduced bleomycin-induced dermal thickening, accumulation of myofibroblasts and the hydroxyproline content (figure 5B). In addition to prevention of bleomycin-induced dermal fibrosis, we demonstrate that treatment with GSKJ4 also induces regression of pre-established bleomycin-induced dermal fibrosis (figure 5C).

Treatment with GSKJ4 also ameliorated topoI-induced fibrosis as a model that emphasises the autoimmune component of SSc. TopoI-challenged mice treated with GSKJ4 showed increased H3K27me3 levels and reduced dermal thickness, myofibroblast counts and hydroxyproline levels compared with vehicle-treated, topoI-challenged mice (figure 6). Treatment with GSKJ4 also ameliorated topoI-induced pulmonary fibrosis (figure 6).

Of note, treatment with GSKJ4 in antifibrotic doses was well tolerated and no signs of toxicity were observed by clinical monitoring or on necropsy. GSKJ4-treated mice showed no differences in activity, body weight, texture of the fur or consistency of the stool compared with vehicle-treated controls.

Previous studies suggest that JMJD3 is implicated in alternative activation of macrophages in response to helminth infection.[32]Given the central role of alternatively activated macrophages in the pathogenesis of fibrotic diseases, we analysed the effects of GSKJ4 treatment on macrophage influx and M2 polarisation in bleomycin-induced and topoI-induced fibrosis. Treatment with GSKJ4 did not reduce the total number of macrophages or the number of alternatively activated macrophages (online supplementary figures 8 and 9). We also investigated changes in T-cell and B cell numbers on treatment with GSKJ4 and observed that treatment with GSKJ4 significantly reduced T-cell counts in bleomycin-induced as well as topoI-induced skin fibrosis (online supplementary figures 8 and 9). We further analysed whether treatment with GSKJ4 may interfere with the immune response in topoI-challenged mice, but did not observe decreases in the levels of antitopoI antibodies in GSKJ4 treatment mice as compared with vehicle-treated controls (online supplementary figure 10).

Discussion

Accumulating evidence suggests a key role of epigenetic alterations as central drivers in the pathogenesis of fibrotic diseases such as SSc. Among those epigenetic modifications, histone methylation may play a central role. We demonstrated recently that inhibition of the H3K27 methyltransferase EZH2 by the adenosine analogue DZNep promotes fibroblast activation. In the present study, we extend those findings and demonstrate that targeted inhibition of H3K27me3-demethylases may be a novel antifibrotic treatment. Pharmacological or genetic inactivation of JMJD3 ameliorated the endogenous activation of SSc fibroblasts with decreased expression of myofibroblast markers and reduced collagen release. Targeted inhibition of JMJD3 also prevented the TGFβ-induced differentiation of resting fibroblasts derived from healthy donors into myofibroblasts. Together, these findings provide evidence that JMJD3 is an important regulator of fibroblast activation in SSc.

Although novel therapeutic options for patients with inflammatory SSc such as tocilizumab may soon become available, the medical need for effective antifibrotic therapies remains very high. This therapeutic gap may be filled by targeting epigenetic alterations as exemplified by the potent antifibrotic effects of inhibition of JMJD3 in our preclinical models. However, SSc is a heterogeneous disease and it is thus of crucial importance to address heterogeneity by analysing samples from different subpopulations of SSc. We included fibroblasts derived from patients with limited and diffuse cutaneous SSc, from patients with early and longstanding disease, from patients with clinically stable and progressive disease, from patients with active inflammation and from patients without clinical evidence of overt inflammation. We also analysed the effects of JMJD3 inhibition in two different mouse models, one of early inflammatory stages of SSc and one that emphasises the autoimmune nature of SSc and mimics patients with antitopoI antibodies and high risk of progression and major internal organ involvement to address the heterogeneity as far as possible. We observed potent antifibrotic effects of JMJD3 inhibition in fibroblasts from all patient subgroups as well as in both mouse models, demonstrating that the therapeutic effects of JMJD3 inhibition may not be restricted to certain subgroups only. However, further studies in additional models are advised to demonstrate effects in other subpopulations and to study the effects on other manifestations of SSc than fibrosis. In particular, additional studies of the role of JMJD3 in SSc vasculopathy would be of interest, given the strong expression of JMJD3 in vessels of patients with SSc observed in our study.

Mechanistically, our in vitro studies point to a direct inhibition of fibroblast activation for the antifibrotic effects of JMJD3. However, we also observed significantly reduced T-cell counts in the skin of mice challenged with bleomycin or topo and JMJD3 has been implicated in alternative macrophage polarisation in response to helminth infection[32] and in the differentiation of TH17 cells.[33]Anti-inflammatory effects with inhibition of T-cell responses may thus have contributed to the potent antifibrotic effects of GSKJ4 and the broad-spectrum efficacy across different preclinical models.

Despite the increased expression of JMJD3, the total levels of H3K27me3 are modestly upregulated in SSc skin, in SSc fibroblasts and in murine models of SSc. These findings suggest that the elevated levels of H3K27me3 in SSc fibroblasts are due to a pronounced increase in H3K27-histone methyltransferase activity that overcomes the upregulation of JMJD3. This concept is supported by previous studies demonstrating increased expression of the H3K27 methyltransferase EZH2 in fibrotic conditions.[27 28] In this model, the upregulation of total H3K27me3 in SSc would be a compensatory attempt trying to counteract the aberrant activation of SSc fibroblasts. The increase in the levels of H3K27me3 achieved by this endogenous counter-regulation limits fibroblast activation to a certain extent as shown by even more pronounced activation of SSc fibroblasts on inhibition of H3K27 methyltransferases.[12] However, this compensatory increase alone is not sufficient to prevent the aberrant fibroblast activation in SSc. We demonstrate in the present study that the pharmacological inhibition of JMJD3 enhances this endogenous antifibrotic mechanism and induces further accumulation of H3K27me3 in SSc fibroblasts and in experimental fibrosis. This increase in H3K27me3 translated into potent antifibrotic effects with reduced fibroblast activation and ameliorated skin fibrosis.

Although we show that specific targeting of JMJD3 can reverse the activated phenotype of SSc fibroblasts, the epigenetic changes in SSc fibroblasts are not restricted to alterations of H3K27 methylation and other epigenetic modifications have also been shown to be key drivers of the persistent activation of SSc fibroblasts.[4–23] Different epigenetic modifications are known to interact and cross-regulate each other. As we are just beginning to understand the complex interactions between epigenetic marks, further studies are required to characterise the network of epigenetic modifications in SSc and to identify central upstream regulators that coordinate the epigenetic dysregulation in SSc.

We present evidence that the antifibrotic effects of JMJD3-inhibition are mediated to a significant extent by downregulation of FRA2. FRA2 is a member of the AP1 family of transcription factors that has previously been shown to play a central role in the pathogenesis of SSc. Overexpression of FRA2 in SSc is directly linked to fibrotic tissue remodelling.[30] Despite a modest increase in the overall

accumulation of H3K27 trimethylation, H3K27 trimethylation at the *FRA2* promoter is reduced in SSc fibroblasts, demonstrating an impaired epigenetic silencing of FRA2 expression in SSc fibroblasts. Inactivation of JMJD3 promotes accumulation of inhibitory H3K27me3 marks at the promoter of *FRA2*, thereby reducing the expression of the profibrotic mediator FRA2 in cultured fibroblasts and in murine models of fibrosis. The crucial role of impaired FRA2 expression for the antifibrotic effects of targeting JMJD3 is highlighted by our studies in FRA2-deficient fibroblasts. The antifibrotic effects of JMJD3 inhibition are strongly reduced on knockdown of FRA2 in fibroblasts. However, inhibition of JMJD3 will not selectively affect the FRA2 expression, but will also modulate the expression of other target genes. Despite the prominent role of FRA2, the altered expression of those genes may also contribute to the antifibrotic effects of JMJD3 inhibition.

Given the regulatory effects of JMJD3 on FRA2 and the central role of FRA2 for the vascular pathogenesis of SSc[31 34] one might speculate that targeting JMJD3 may also improve vascular features of SSc. However, further studies are required to confirm a JMJD3-dependent regulation of FRA2 in endothelial cells and to investigate the effects of JMJD3 inhibition on vascular manifestations of SSc.

In summary, we provide first evidence for a deregulation of the H3K27me3 demethylase JMJD3 in SSc and demonstrate that JMJD3 promotes fibroblast activation by loosening the epigenetic breaks on FRA2 expression. Targeted inhibition of JMJD3 limits the aberrant activation of SSc fibroblasts and exerts potent antifibrotic effects in murine models. JMJD3 may therefore be a novel target for antifibrotic therapies.

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Figures:

Figure 1
The expression of JMJD3 is upregulated in SSc in a TGFβ-dependent manner. (A) Expression of JMJD3 in SSc and healthy skin analysed by immunofluorescence by triple staining for JMJD3 (green), the fibroblast marker P4Hβ (red) and DAPI. Representative sections are shown at 200-fold and 400-fold magnification (n=7). (B and C) Effects of TGFβ on the mRNA and protein levels of JMJD3 (B) and on the levels of H3K27me3 (C) in cultured human dermal fibroblasts (n=5). (D) Effects of siRNA-mediated knockdown of SMAD3 on TGFβ-induced JMJD3 expression in fibroblasts (n=4). (E) Expression of JMJD3 in TBR^{act} mice compared with wild-type mice as analysed by immunofluorescence with triple staining for JMJD3 (green), vimentin (red) and DAPI. Data are presented as median with IQR (n=5, *p<0.05; **p<0.01; ***p<0.001). DAPI, 4',6-diamidino-2-phenylindole; H3K27me3, trimethylation of histone H3 on lysine 27; JMJD3, Jumonji domain-containing protein 3; P4Hβ, prolyl-4-hydroxylase-β; siRNA, small interfering RNA; SMAD3, SMAD family member 3; SSc, systemic sclerosis; TGFβ, transforming growth factor beta; TBR, TGF beta receptor 1.

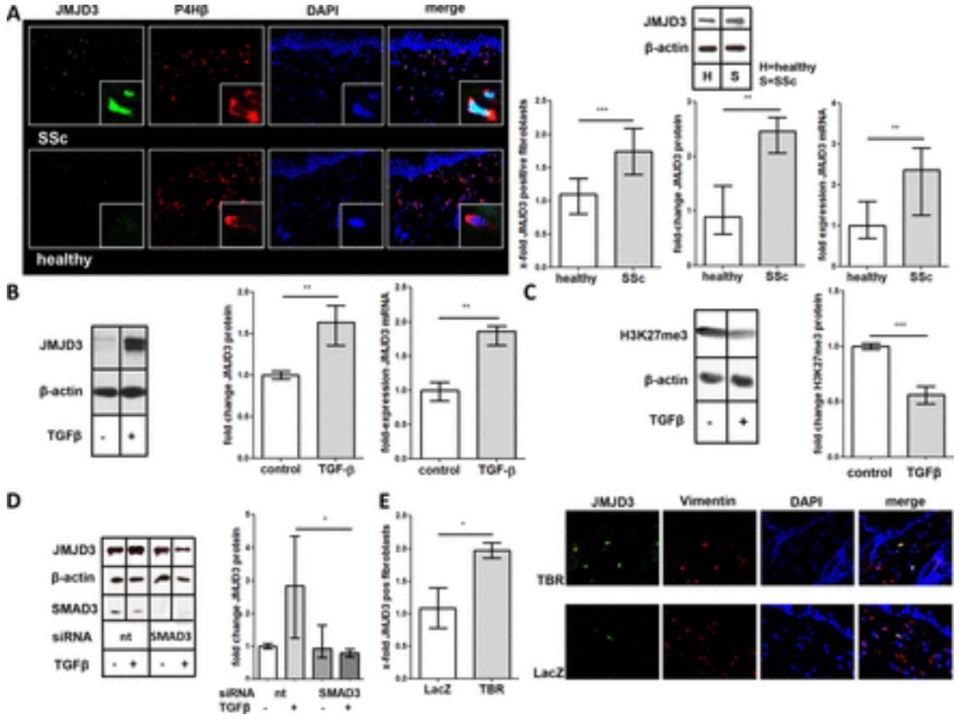

Figure 2

Pharmacological inhibition of JMJD3 inhibits fibroblast activation, collagen release and migration in vitro. (A) Levels of H3K27me3 in cultured fibroblasts on incubation with GSKJ4 (n=6). (B) Effects of GSKJ4 on the levels of *COL1A1* mRNA and type I collagen protein in SSc fibroblasts in the absence of exogenous stimuli (n=8). (C) Collagen release of TGFβ-stimulated fibroblasts from healthy donors incubated with GSKJ4 (n=5). (D) Effects of GSKJ4 on the PDGF-induced closure of an artificial wound (n=5). Data are presented as median with IQR (*p<0.05; **p<0.01; ***p<0.001). H3K27me3, trimethylation of histone H3 on lysine 27; JMJD3, Jumonji domain-containing protein 3; PDGF, platelet-derived growth factor; SSc, systemic sclerosis; TGFβ, transforming growth factor beta.

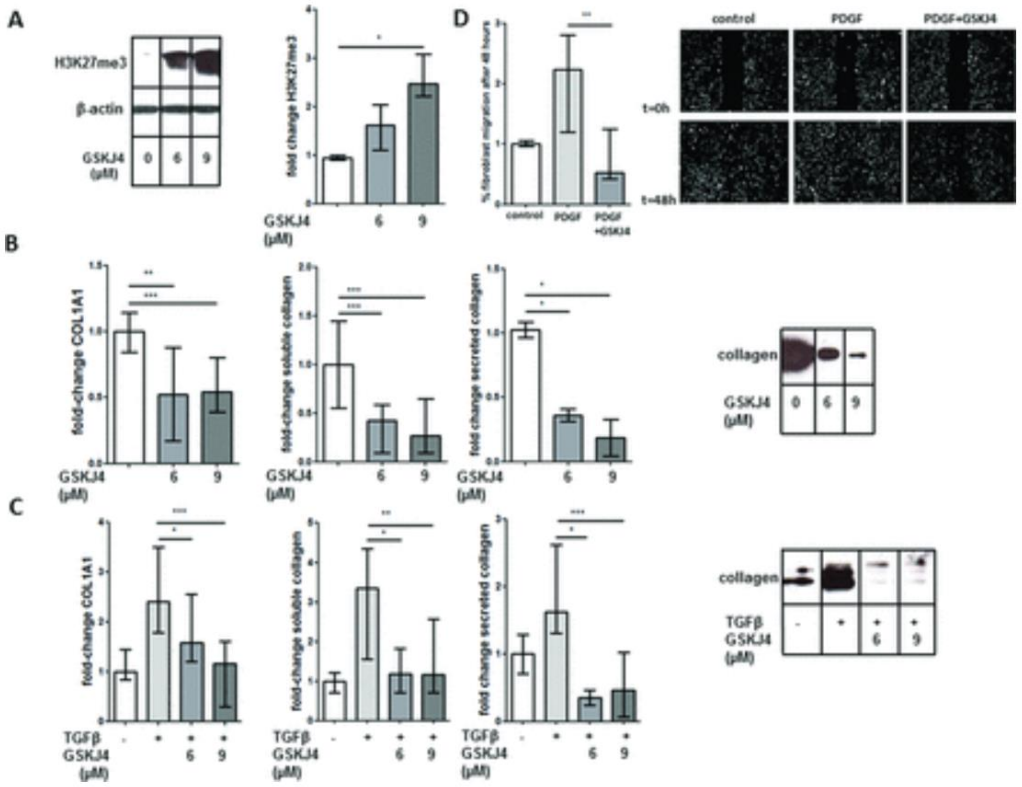

Figure 3

siRNA-mediated knockdown of JMJD3 inhibits fibroblast activation. (A) mRNA and protein levels of JMJD3 on siRNA-mediated knockdown (n=4). (B and C) Effects of GSKJ4 on the levels of *COL1A1* mRNA and type I collagen protein (B) and on the expression level of the myofibroblast marker αSMA and on the formation of stress fibres (C) in TGFβ-stimulated fibroblasts from healthy donors (n=4). Data are presented as median with IQR (*p<0.05; **p<0.01; ***p<0.001). αSMA, α-smooth muscle actin; H3K27me3, trimethylation of histone H3 on lysine 27; JMJD3, Jumonji domain-containing protein 3; siRNA, small interfering RNA; TGFβ, transforming growth factor beta.

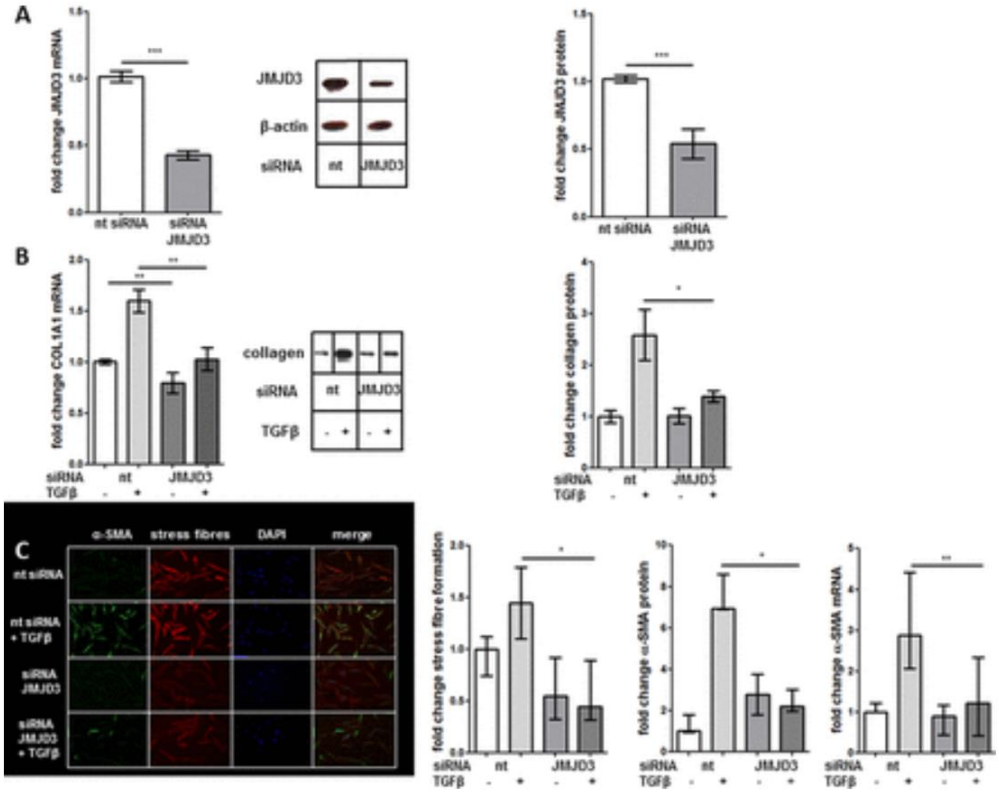

Figure 4

JMJD3 regulates fibroblast activation in a FRA2-dependent manner. (A) Levels of H3K27me3 at the *FRA2* promoter in SSc fibroblasts compared with fibroblasts from matched healthy individuals (n=6) as analysed by ChIP assays. (B) Effects of TGFβ and GSKJ4 at a concentration of 6 µM on H3K27me3 at the *FRA*2 promoter in dermal fibroblasts from healthy individuals (n=6). The results of qRT-PCR were normalised to sample-related input values. (C) Expression levels of FRA2 protein in fibroblasts incubated with TGFβ and GSKJ4 (6 µM). One representative western blot and quantification of four independent experiments are shown. (D) Effects of the siRNA-mediated knockdown of JMJD3 on the mRNA and protein levels of FRA2 in TGFβ-stimulated fibroblasts (n=4 for RNA and protein). (E) FRA2 expression in topoI-induced dermal fibrosis on treatment with GSKJ4 (n=5 mice per group). (F) Blunted effects of GSKJ4 on TGFβ-induced collagen release on siRNA-mediated knockdown of FRA2. One representative western blot and quantification of four independent experiments are shown. Data are presented as median with IQR (*p<0.05; **p<0.01; ***p<0.001). H3K27me3, trimethylation of histone H3 on lysine 27; JMJD3, Jumonji domain-containing protein 3; siRNA, small interfering RNA; SSc, systemic sclerosis; TGFβ, transforming growth factor beta; topoI, topoisomerase-I.

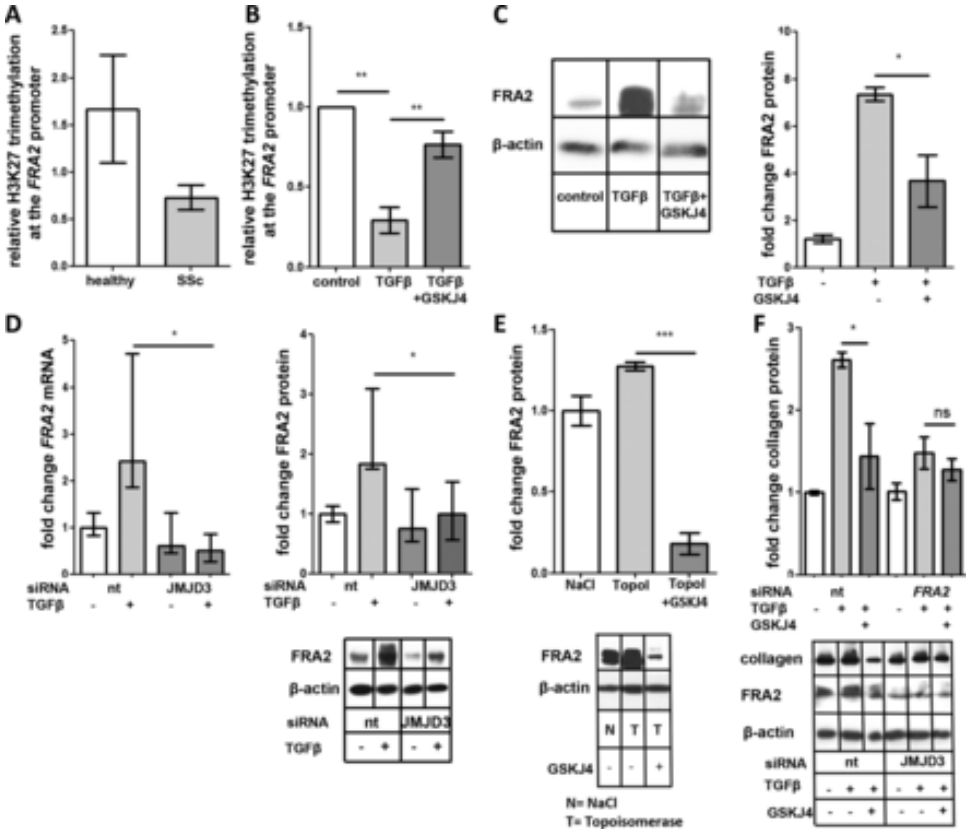

Figure 5

Treatment with GSKJ4 does not only prevent bleomycin-induced dermal fibrosis, but also promotes its regression. (A) Upregulation of H3K27me3: levels of H3K27me3 in bleomycin-challenged mice on treatment with GSKJ4 as analysed by immunofluorescence with triple-staining for H3K27me3 (red), vimentin (green) and DAPI and quantification of the staining intensity in murine skin sections and western blot of murine skin (n=5 for both). (B) GSKJ4 ameliorates bleomycin-induced fibrosis: representative images of trichrome-stained sections shown at 100-fold magnification and quantification of dermal thickness, myofibroblast counts and hydroxyproline content (n=5 for all groups). (C) GSKJ4 induces regression of pre-established fibrosis: representative images of trichrome-stained sections shown at 100-fold magnification and quantification of dermal thickness, myofibroblast counts and hydroxyproline content (data are presented as median with IQR, n=5 for all groups *p<0.05; **p<0.01; ***p<0.001). DAPI, 4',6-diamidino-2-phenylindole; H3K27me3, trimethylation of histone H3 on lysine 27.

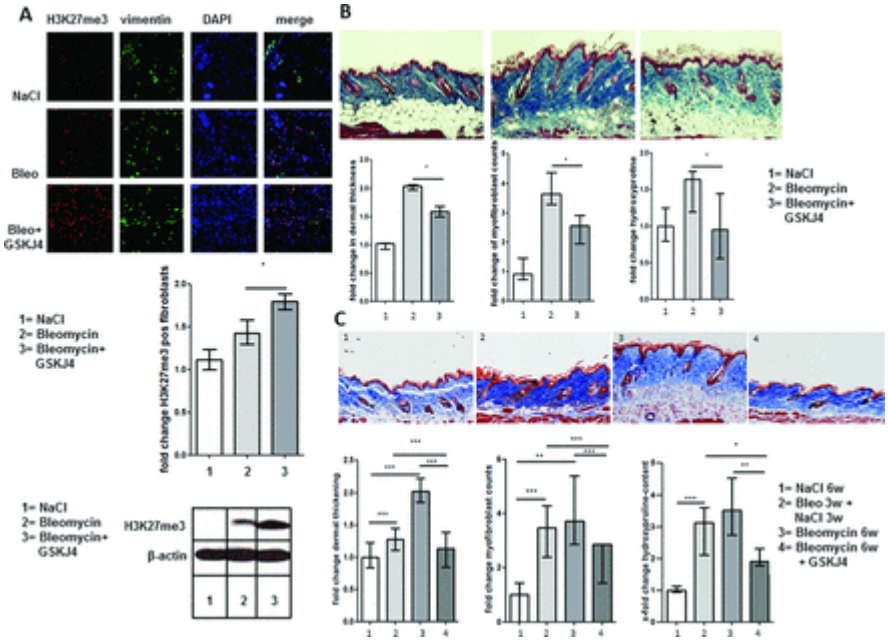

Figure 6

Treatment with GSKJ4 ameliorates topoI-induced fibrosis. (A) Upregulation of H3K27me3: levels of H3K27me3 in topoI-challenged mice on treatment with GSKJ4 as analysed by immunofluorescence with triple-staining for H3K27me3 (green), the fibroblast marker vimentin (red) and DAPI and quantification of the staining intensity in murine skin sections and by western blot of murine skin tissue (n=5). (B) GSKJ4 ameliorates topoI-induced skin fibrosis: representative images of trichrome-stained sections shown at 100-fold magnification and quantification of dermal thickness (n=5), myofibroblast counts and hydroxyproline content. (C) GSKJ4 ameliorates topoI-induced pulmonary fibrosis: representative images of Sirius red staining at 200-fold magnification and quantification of the fibrotic area (n=6), hydroxyproline content (n=6), Ashcroft score (n=6). Data are presented as median with IQR (*p<0.05; **p<0.01; ***p<0.001). DAPI, 4',6-diamidino-2-phenylindole; H3K27me3, trimethylation of histone H3 on lysine 27; topoI, topoisomerase-I.