Neue Einsichten in die anomale Aktivierung von Fibroblasten in der Systemischen Sklerose – Casein Kinase 2-, Poly(ADP-ribose)-Polymerase 1- und Janus Kinase 1-vermittelte Transaktivierung von Janus Kinase 2 als neue Mechanismen

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Novel insights into the aberrant activation of fibroblasts in systemic sclerosis - Casein kinase 2, Poly(ADP-ribose)-Polymerase 1, and Janus kinase1 mediated transactivation of Janus kinase2 as novel players

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1 SUMMARY

A major hallmark of Systemic Sclerosis (SSc) is the uncontrolled and persistent activation of fibroblasts, which release excessive amounts of extracellular matrix. The accumulation of extracellular matrix proteins disrupts the tissue architecture leading to organ dysfunction and causing high morbidity and mortality in patients. Transforming growth factor-β (TGFβ) has been characterized as a key-mediator of fibroblast activation in SSc and other fibrotic diseases. Numerous studies demonstrate increased TGFβ signaling in SSc and in other fibrotic diseases and highlight that persistent activation of TGFβ is sufficient to induce fibrosis. However, the precise molecular mechanisms and the intracellular signaling cascades that mediate the TGFβ-induced activation of fibroblasts are still incompletely understood.

The purpose of the first part of the study was to characterize whether Casein kinase II (CK2) contributes to the pathologic activation of fibroblasts in patients with SSc and to evaluate the anti-fibrotic potential of CK2 inhibition. We found that expression of CK2 was increased in skin fibroblasts of SSc patients. Inhibition of CK2 by selective CK2 inhibitor 4, 5, 6, 7-Tetrabromobenzotriazole (TBB) abrogated the TGFβ induced activation of JAK2/STAT3 signaling and prevented the stimulatory effects of TGFβ on collagen release and myofibroblasts differentiation in cultured fibroblasts. Treatment with TBB also ameliorated bleomycin-induced dermal fibrosis as well as skin fibrosis induced by adenoviral overexpression of a constitutively active TGFβ receptor I (AdTBR). The anti-fibrotic effects of TBB in both mouse models were accompanied by reduced activation of Janus kinase 2 (JAK2) / signal transducer and activator of transcription 3 (STAT3) signaling.

In the second part of the study, the role of the enzyme poly(ADP-ribose) polymerase-1 (PARP-1) in the pathogenesis of SSc was investigated. Decreased expression of PARP-1 was detected in skin sections of SSc patients, particularly in fibroblasts. Upon stimulation of fibroblasts with TGFβ, PARP-1 binds to Smad3, which inhibited Smad-dependent gene
expression via poly(ADP-ribosyl)ation (PARylation). Inactivation of PARP-1 promoted accumulation of phosphorylated Smad3, enhanced Smad-dependent transcription and upregulated the expression of TGFβ/Smad target genes. Treatment with 3-Aminobenzamide (3AB) enhanced the effect of TGFβ on collagen release and myofibroblast differentiation in vitro. Inhibition of PARP-1 also exacerbated experimental fibrosis in vivo. PARP-1 deficiency induced a more severe fibrotic response to bleomycin with increased dermal thickening, hydroxyproline contents and myofibroblast counts. Additionally, inhibition of PARylation strongly exacerbated fibrosis in Tsk-1 mice. We demonstrate that PARP-1 negatively regulates canonical TGFβ signaling in experimental skin fibrosis. The downregulation of PARP-1 in SSc fibroblasts may thus directly contribute to hyperactive TGFβ signaling and to persistent fibroblast activation in SSc.

The third part of the study evaluates strategies to overcome transactivation of Janus kinase 2 (JAK2) by JAK1 induced by long-term inhibition of JAK2. We observed that the inhibitory effect of the selective inhibitor TG101209 on JAK2 decreased upon long-time incubation, resulting in ineffective inhibition of TGFβ-induced fibroblast activation and collagen release. In bleomycin-induced pulmonary fibrosis and AdTBR-induced skin fibrosis, combined inhibition of JAK2 and heat-shock protein 90 (HSP90) by cotreatment with TG101209 and 17-DMAG had more potent anti-fibrotic effects as compared to monotherapy with either of the two compounds. Moreover, combined inhibition of JAK1 and JAK2 by Ruxolitinib showed also more pronounced anti-fibrotic effects than the selective JAK2 inhibitor TG101209.

The results of the thesis might have clinical implications. Small molecules to target CK2, JAKs or HSP90 are either in advanced clinical development or already approved for clinical use. Targeting of CK2 and JAK2 may thus be novel therapeutic approaches for SSc and other fibrotic diseases. My thesis also identifies PARP-1 as a central regulator of skin fibrosis by
interacting with TGFβ signaling, and implicates that therapies re-activating PARP-1 may be of interest for clinical evaluation.
2 ZUSAMMENFASSUNG


Im ersten Teil dieser Studie sollte der Einfluss von Casein Kinase II (CK2) auf die pathologische Aktivierung von Fibroblasten in SSc-Patienten und das antifibrotische Potential von CK2 Inhibitoren evaluiert werden. Es konnte gezeigt werden, dass die Expression von CK2 in Hautfibroblasten von SSc-Patienten erhöht war. Inhibition von CK2 durch den selektiven CK2-Inhibitor 4, 5, 6, 7-Tetrabromobenzotriazole (TBB) setzte die TGFβ induzierte Aktivierung der JAK2/STAT3-Signalkaskade außer Kraft und verhinderte sowohl die TGFβ induzierte Freisetzung von Kollagen als auch die Differenzierung von Myofibroblasten in kultivierten Fibroblasten. Eine Behandlung mit TBB führte darüber hinaus zu einer Verbesserung der Fibrose sowohl im Modell der Bleomycin-induzierten dermalen Fibrose als auch bei Hautfibrose, die durch adenovirale Überexpression eines konstitutiv aktiven TGFβ Rezeptor I (AdTBR) induziert wurde. Die antifibrotischen Eigenschaften von
TBB in beiden Mausmodellen gingen mit einer reduzierten Aktivierung des JAK2 (Janus kinase 2) / STAT3 (signal transducer and activator of transcription 3)-Signalweges einher.


Im dritten Teil der Studie werden Strategien zur Umgehung einer Transaktivierung der Janus Kinase 2 (JAK2) durch JAK1, welche durch Langzeitinhibtion von JAK2 hervorgerufen wird. Wir konnten beobachten, dass der inhibierende Effekt des selektiven Inhibitors TG101209 auf JAK2 bei länger andauernder Inkubation abnimmt. Dies führt zu einer ineffektiven Inhibierung der TGFβ-induzierten Aktivierung von Fibroblasten und Kollagenfreisetzung. In den Modellen der Bleomycin-induzierten Lungenfibrose und der
3 INTRODUCTION

3.1 Systemic sclerosis

Scleroderma (systemic sclerosis) is a complex disease in which extensive fibrosis, vascular alterations, inflammation and autoantibodies against various cellular antigens are the principal features. Scleroderma can lead to severe dysfunction and failure of almost any internal organ. However, the mechanisms of scleroderma are unclear [1]. The term “scleroderma” consists of the Greek words “skleros” (hard) and “derma” (skin) and refers to the fibrosing and hardening of the skin, which is the most obvious sign of the disease. The more common term “systemic sclerosis (SSc)” is not limited to the skin but also includes systemic manifestations such as autoimmunity and organ fibrosis.

Depending on the skin involvement, there are two major subgroups in the commonly accepted classification of scleroderma: limited cutaneous scleroderma and diffuse cutaneous scleroderma [2]. Fibrosis is mainly restricted to the hands, arms, and face in limited cutaneous scleroderma, which dominated by vascular manifestations, skin and organ fibrosis is generally limited and slow to progress. Diffuse cutaneous scleroderma is a rapidly progressing disorder that affects a large area of the skin and compromises one or more internal organs and is dominated by rapidly progressive fibrosis of the skin, lungs, and other internal organs [3].

SSc has a worldwide distribution and is 3 - 5 times more frequent in women than in men and the age at diagnosis ranges from 30 to 50 years, though the disease can also occur in children and elderly people [4]. Fibrosis in SSc is not restricted to a single organ, but rather affects many organs and accounts for much of the morbidity and mortality associated with this disease [5]. The fibrotic process is most frequently observed in the skin, lungs, gastrointestinal tract, heart, tendons and ligaments; widespread perivascular fibrosis also occurs [6]. Raynaud’s phenomenon is present for several years before fibrosis appears,
pulmonary hypertension is frequent, and anti-centromere antibodies occur in 50 to 90% of patients [7].

### 3.2 Fibrosis

Fibrosis is the most characteristic pathological hallmark of SSc, and it is especially prominent in the diffuse cutaneous SSc [8]. Tissue architecture is progressively replaced by collagen-rich extracellular matrix (ECM), which leads to functional impairment of affected organs. The ECM is composed of a cellular compartment of resident and infiltrating cells and a connective tissue compartment of collagens, proteoglycans, fibrillins, and adhesion molecules [6]. The most abundant ECM components are collagens, a family of proteins that play a critical role in organ development, growth, and differentiation.

The ECM also serves as a reservoir for TGFβ, CTGF, and other growth factors and matricellular proteins that control mesenchymal cell differentiation, function, and survival. The expression of ECM genes is normally regulated by paracrine and/or autocrine actions of soluble mediators as well as by cell-cell contact, hypoxia, and contact with the surrounding ECM [9].

Overproduction by fibroblasts and related mesenchymal cells activated by soluble factors in an autocrine and/or paracrine manner results in excessive connective tissue accumulation. Impaired ECM degradation and turnover and expansion of the pool of mesenchymal cells in affected tissues further contribute to ECM accumulation [6].

Fibroblasts, the key effectors of the fibrotic process, are induced by soluble mediators generated in the local cellular microenvironment by platelets, endothelial cells, epithelial cells, and inflammatory cells to secrete collagens and other ECM macromolecules. Fibroblasts are contracted, organized, and remodeled by ECM molecules, secrete and activate growth factors
and cytokines, and transdifferentiate into contractile myofibroblasts [10]. In the normal circumstances, fibroblast repair program is self-limited, but pathological fibrotic responses are sustained and amplified fibroblast activation. The inappropriate fibroblast activation is the fundamental pathogenetic alteration underlying fibrosis in SSc. (Figure 3.1)

![Diagram of fibroblast activation]

**Figure 3.1:** Mechanism of fibroblasts activation. Vascular injury, autoimmunity and inflammation cytokine induce fibroblasts to differentiate into myofibroblasts. Myofibroblasts synthesize extra ECM protein leading to fibrosis. Adapted and modified from [5].

Of the multiple cytokines implicated in SSc, TGFβ is considered to be the most important regulator of both physiological and pathological fibrogenesis [11]. In addition to TGFβ, many cytokines, growth factors, and chemokines that regulate mesenchymal cell function has been shown to be overexpressed or abnormally regulated in patients with SSc [12-14].

Fibrosis is the cause of the main symptoms of the disease. It replaces the vascular inflammatory phase of SSc gradually and disrupts the architecture of the affected tissue ultimately. Fibrosis in the skin begins from the lower dermis and upper subcutaneous layer and occurs together with microvasculature loss, appendages reduction, and reticular structure and the rete ridges loss. In the early stages, the typical character is mixture of different
collagen types, proteoglycans, and elastic fibers including fibrillin, whereas in later stage type I collagen accumulates [15, 16].

3.2.1 Animal models of systemic sclerosis

So far, the complex interplay of vasculopathy, the cellular immune system, and fibrosis in SSc remains enigmatic. To develop effective therapies targeting a broad spectrum of pathologic processes in SSc, several murine and avian models are available to study different aspects of the disease [17]. Because none of these models can encompass all features of human SSc, a critical selection of animal models is the basis for successful in vivo studies (Figure 3.2).

<table>
<thead>
<tr>
<th>Vasculopathy</th>
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<td>Bleomycin-induced skin fibrosis</td>
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Figure 3.2: Key pathophysiologic features (Vasculopathy, fibrosis, inflammation, and autoimmunity) in systemic sclerosis (SSc) and their representation by different animal models. Adapted and modified from [17].

3.2.1.1 The mouse model of bleomycin-induced dermal fibrosis
The model of bleomycin-induced subcutaneous fibrosis is widely used in SSc research. Bleomycin, originally isolated from Streptomyces verticillus, has strong anti-tumor effects and being used in many types of cancer [18, 19].

Bleomycin-induced skin fibrosis model mimics the early stage of inflammatory changes in SSc. Bleomycin treatment induces the production of reactive oxygen species, causes damage to endothelial cells and other cell types, and leads to the expression of adhesion molecules [20]. This attracts leukocytes to infiltrate into lesional skin with upregulated TGFβ and chemokine expression, followed by dermal fibrosis with accumulation of α-SMA–expressing myofibroblasts, which are the activated resident fibroblasts [21-23]. Activated fibroblasts then release large amounts of ECM, which result in skin fibrosis at the site of bleomycin injection and leading to dermal fibrosis with thickened collagen bundles and increased dermal thickness similar to that seen in human SSc [21]. Meanwhile, as in SSc, the presence of anti-nuclear autoantibodies, such as anti-Scl-70, anti-U1 RNP, and anti-histone antibodies, may also suggest systemic disease involvement in this model [24, 25].

Injections of bleomycin in a defined area of the upper back for at least three weeks induce local SSc-like fibrotic changes of the skin, which last for up to six weeks after treatment [21]. Many mouse strains respond to subcutaneous treatment with bleomycin, but the severity of the skin fibrosis varies between different strains [26]. Limitations of bleomycin induced fibrosis are: fibrosis is limited to the site of injection, typical vascular phenomena that precede fibrosis in human SSc are usually absent, and bleomycin induced fibrosis model may respond to treatment with anti-inflammatory therapies that are not effective in the human disease [17].

### 3.2.1.2 The mouse model of AdTBR (TGFβ receptor 1)-induced dermal fibrosis

TGFβ is a critical pro-fibrotic mediator in human SSc that activates fibroblasts and induces the production of ECM. Incubation of normal fibroblasts with TGFβ induces an SSc-like pro-
fibrotic phenotype. Thus, fibroblast-specific stimulation of TGFβ signaling in vivo may induce several features of fibrosis observed in human SSc [27-29].

Dermal fibrosis induced by adenoviral overexpression of a constitutively active TGFβ receptor I (AdTBR) serves as an approach to reflect fibrotic pathologies of human SSc and to study TGFβ signaling in fibroblast activation [30]. 6.67x10^7 infectious units of replication deficient type V adenoviruses overexpressing TBR were injected intradermally into defined areas of 1 cm^2 at the upper back. Mice injected with adenoviruses carrying a LacZ reporter gene served as controls. The first injection was performed at an age of 4 weeks and repeated every 2 weeks. The mice were sacrificed 8 weeks after the first injection.

But there are some limitations: it does not recapitulate the activation of several other profibrotic pathways as observed in human SSc; autoimmune and inflammatory phenomena might be absent in this model; the vasculopathy is also different substantially from that in human SSc; adenoviral activation of TGFβ signaling is not restricted to fibroblasts but may also affect other cell types [17].

3.2.1.3 The tight-skin 1 (Tsk-1) mouse model

The mutation of Tsk-1 mice in the gene encoding fibrillin-1 results in skin tethering and subcutaneous hyperplasia [31]. There is no phenotype of lung or renal fibrosis in Tsk-1 mice [32]. Mice homozygous for the Tsk-1 allele die in utero during gestation [33]. Heterozygous Tsk-1 mice spontaneously develop SSc-like features, with skin fibrosis and autoimmunity [34, 35]. The mechanism of fibrosis developing in Tsk-1 is still not clear. Evidences suggest that abnormal fibrillin might activate TGFβ signaling which may further lead to ECM production in fibroblasts and progressive fibrosis [36].

Increased autoantibodies against SSc-specific antigens (e.g., topoisomerase I) and against other autoantigens (e.g., RNA polymerase I, single-stranded, and double-stranded DNA
(dsDNA)) [37-43] are found in Tsk-1 mice. Meanwhile, overexpression of CD19 in Tsk-1 mice leads to chronic B cell activation, suggesting that B cells might play an important role in fibrosis development. The increased production of IL-6 might contribute to the progression of fibrosis. By contrast, a deficiency in CD19 can ameliorate fibrosis in Tsk-1 mice [40].

The Tsk-1 mice showed thickening of the hypodermis develops and it does not fully mimic the histologic changes of human SSc because of lack typical features of vasculopathy such as endothelial cell apoptosis and lack of prominent inflammatory infiltrates [32, 44]. Therefore Tsk-1 mouse is commonly used to study less inflammatory stages of SSc.

3.2.1.4 The mouse model of bleomycin-induced pulmonary fibrosis

Pulmonary fibrosis is a common respiratory problem of end-stage lung disease caused by environmental toxins, radiation, or chemotherapy treatments for cancer or other chronic inflammatory diseases [45-47]. Fibrosis results in reduced lung function and has a high mortality rate [48]. For instance, Bleomycin is the chemotherapeutic drug for testicular cancer, and it leads to lung fibrosis as a side effect in 10% of patients [46].

Bleomycin-induced lung fibrosis is a standard mice model for pulmonary fibrosis. The inflammatory and fibrotic events are similar to those in human pulmonary fibrosis [49]. The intratracheal instillation (IT) of bleomycin into mice causes the accumulation of leukocytes, the activation of fibroblasts and fibroblast-like cells and the deposition of collagen [50-52]. Another key feature of this experimental model is uncontrolled tissue repair that are driven by pro-fibrotic cytokines such as TGFβ, PDGF, CTGF, hypoxia and so on [5, 53-56].

Bleomycin-induced pulmonary fibrosis model is served to study inflammatory and fibrotic changes in the lung, the generic processes that occur when inflamed tissues are unable to effectively repair themselves and are replaced by collagenous scars [57].
3.2.2 TGFβ signaling

Transforming growth factor-β (TGFβ) family is a group of structurally related growth factors. The TGFβ superfamily comprises TGFβ, activin, nodal, bone morphogenetic proteins (BMPs), and others. These growth factors play critical roles in regulating a wide range of biological processes during embryonic development and adult tissue homeostasis, and deregulation of the signal transduction has been associated with many human diseases, including cancer and tissue fibrosis [58, 59].

3.2.2.1 Receptors and intracellular signaling

The functional complex of TGFβ family receptors at the cell surface consists of two ‘type II’ and two ‘type I’ transmembrane serine/threonine kinase receptors [60, 61]. Ligand binding triggers the formation of the receptor heterocomplex, type II receptor phosphorylates and activate type I receptor [62]. The activated type I receptor recruits and phosphorylates the receptor-activated Smads (R-Smad, such as Smad1, Smad2, Smad3, Smad5, and Smad8) proteins, which then form a heterocomplex with the co-Smad Smad4. The Smad complexes then translocate into the nucleus, where they regulate transcription of the target genes [63, 64]. Different member of the TGFβ family have different combination of type I and type II receptors and R-Smads. For TGFβ signaling, the type I receptor TβRI/ALK5 and the type II receptor TβRII are involved to activate Smad2/3.

Upon activation, heterocomplex of Smad2, Smad3 and Smad4 translocate from the cytoplasm into the nucleus, where they recognize and bind to a cis-acting conserved DNA sequence (CAGAC) that defines the consensus SMAD-binding element (SBE), recruit coactivators such as p300/CBP to the DNA and result in transcription of target gene [11, 64].
Activation of R-Smads by type I receptor kinases is inhibited by structurally divergent Smad6 or Smad7. Smad7 interacts with Smurf1 or Smurf2, the E3 ubiquitin ligases, which mediate ubiquitination and consequent degradation of R-Smads and the type I receptors [65, 66].

### 3.2.2.2 TGFβ and fibrosis

Transforming growth factor-β (TGFβ) has been characterized as a key-mediator of fibroblast activation in SSc and other fibrotic diseases [67]. Numerous studies demonstrate increased TGFβ signaling in SSc and in other fibrotic diseases and highlight that persistent activation of TGFβ is sufficient to induce fibrosis. However, the underlying molecular mechanisms and the intracellular signaling cascades that mediate the TGFβ-induced activation of fibroblasts that lead to the uncontrolled activation of TGFβ signaling remain incompletely understood.

TGFβ is a potent pro-fibrotic cytokine [68]. TGFβ-dependent genes are overexpressed in biopsy from skin lesions in patients with scleroderma [69]. TGFβ is also the strongest inducer of myofibroblast transdifferentiation from normal fibroblasts, and it modulates the expression of various receptors, such as receptors for TGFβ and PDGF [70, 71].

In scleroderma fibroblasts, TGFβ upregulates connective-tissue growth factor (CTGF) [72], which has biologic activities similar to TGFβ. Increased TGFβ and CTGF expression has been found in scleroderma lesions, and enhanced TGFβ signaling in fibroblasts causes skin fibrosis in mouse model that shows the clinical and histologic features of scleroderma [28].

The extracellular matrix (ECM) serves as a reservoir for an inactive form TGFβ, the latent TGFβ, by latent TGFβ binding proteins (LTBPs) [6]. Upon activation, TGFβ binds to receptors and induces SMAD-mediated signal transduction. Activated SMAD proteins transfer into the nucleus, bind to SBE elements, and trigger mRNA synthesis of target gene [10]. Smad-dependent or Smad-independent signaling downstream of TGFβ has been extensively described in scleroderma cells [5].
3.2.3 Casein Kinase 2 (CK2)

3.2.3.1 Structure and function of CK2

Casein kinase II (CK2) is a constitutively active serine/threonine protein kinase [73]. CK2 consists of a tetrameric complex with two regulatory CK2β subunits and two catalytic subunits (α and/or α') in a homozygous or heterozygous composition (Figure 3.4) [74, 75]. CK2 is crucial for cell and tissue homeostasis by regulating proliferation, differentiation, apoptosis and senescence [76]. There is mounting evidence indicating that deregulation of CK2 is involved in cellular transformation and cancer. Abnormally high expression of CK2 was detected in different tumors, including mammary- [77], prostate- [78], lung- [79], head and neck- [80] and kidney cancer [75, 81].

![Figure 3.4: Tetrameric complex of CK2. Adapted from Mol Cell Biochem (2008) 316:57–62 [74]](image)

3.2.3.2 CK2 inhibition

Inhibition of CK2 demonstrated potent anti-tumor effects in several preclinical models [82, 83]. These results stimulated development of novel CK2 inhibitors with 4,5,6,7-tetrabromobenzotriazole (TBB), which has been shown to display a striking selectivity toward CK2 either Ser/Thr or Tyr specific [84], as a lead compound for novel CK2 inhibitors [74].
One of those novel CK2 inhibitors has recently entered first clinical trials (http://clinicaltrials.gov; NCT00891280 and NCT01199718).

Of particular interest, CK2 has recently been discovered as a novel interaction partner of JAK proteins [85]. Based on the recent identification of JAK2 as a crucial downstream target of TGFβ signaling in fibroblasts [86], we hypothesized that CK2 may be a novel regulator of fibroblast activation in SSc (Figure 3.5). Here, we aimed to analyze CK2 activation in SSc, to investigate its role in fibroblast activation and to characterize the anti-fibrotic potential of CK2 inhibitors in preclinical models of SSc.

Figure 3.5: Mechanism hypothesis that CK2 regulate JAK2/STAT3 pathway [87].
3.2.4 The JAK/STAT pathway

The Janus kinase (JAK) / signal transducer and activator of transcription (STAT) signaling pathway is an evolutionarily conserved signaling pathway mediates cellular response to various cytokines, interferons and growth factors [88]. These responses include proliferation, differentiation, migration, apoptosis, and cell survival. JAK/STAT signaling also play an essential role of numerous developmental and homeostatic processes, such as hematopoiesis, differentiation of immune cell, stem cell maintenance, organismal growth, and mammary gland development [89].

3.2.4.1 Pathway components

The mammalian JAK family has four members: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2) [90]. Each contains a conserved kinase domain and a related, but catalytically inactivate, pseudo-kinase domain at the carboxyl terminus and selectively bind different receptor chains.

There are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [91], which are highly homologous in several regions.

3.2.4.2 Signal transduction and regulation

Upon engagement of ligand, receptor-associated Janus kinases (JAKs) are activated and phosphorylate both each other and the intracellular part of their receptors, creating docking sites for cytoplasmic signal transducers and activators of transcription (STATs). JAK-mediated phosphorylation activates STATs, which bind DNA and regulate gene expression [92].

Signaling through the JAK/STAT pathway is initiated when a cytokine ligand binds to its related receptor (Fig. 3.6). This leads to conformational changes in the cytoplasmic portion of the receptor, initiating activation of receptor associated members of the JAK family of kinases.
The JAKs mediate phosphorylation at the specific receptor tyrosine residues, which then serve as docking sites for STATs and other signaling molecules. Once recruited to the receptor, STATs are also phosphorylated by JAKs, on a single tyrosine residue and get activated [93]. Once activated, STATs dissociated from the receptor and then dimerize through a reciprocal phosphotyrosine–SH2 domain interaction (“classical dimerization”) (Figure 3.6) [94, 95]. Only activated and dimerized STATs are competent to translocate to the nucleus and bind to members of the GAS (gamma activated site) family of STATs. Studies have showed various details about how STAT dimers bind DNA, but the structural motifs which regulate nuclear import are not well characterized yet [94-96].

Figure 3.6: The JAK/STAT signaling pathway. Adapted and modified from [96].
3.2.4.3 Comparison of different JAK inhibitors

The dysfunction of JAK2 has been implicated in myeloproliferative disorders (MPDs) and leukemias. JAK2 is essential in erythropoiesis [97], primary myelofibrosis (PMF) [98], and systemic sclerosis [86]. Several JAK inhibitors have been developed and recent studies have described the therapeutic effect of JAK2 inhibitors in myeloproliferative disorders [99], skin fibrosis [100] and graft-versus-host-disease [101].

However, chronic inhibition by selective JAK2 inhibitors leads to reactivating JAK/STAT signalling and with heterodimerization between activated JAK2 and JAK1 or TYK2 in myeloproliferative neoplasm cells [102]. Therefore other strategies to target JAK are necessary to overcome the loss of efficacy of JAK2 inhibition with prolonged treatment.

TG101209

TG101209 is a selective small molecule inhibitor of JAK2, it has been found to be potent inhibitors of JAK2V617F and MPLW515L/K mutations associated with polycythemia vera (PV) and primary myelofibrosis (PMF), respectively [99, 103, 104].

Recent studies showed the effects of TG101209 are inhibiting proliferation and inducing cytotoxicity in myeloma patient cells [105], selectively inhibiting JAK2 versus JAK3 kinase-dependent cell proliferation in hematopoietic disease model [99], and reducing collagen synthesis in SSc fibroblasts, preventing skin fibrosis in fibrotic model [86].

17DMAG

HSP90 is a ubiquitously expressed protein chaperone, which has been shown to stabilize a number of client proteins, including tyrosine kinases such as EGFR [106] and BCR-ABL [107, 108]. As a result, ATP-competitive HSP90 inhibitors, including the benzoquinone ansamycin 17-AAG and its derivates 17-DMAG and IPI-504, have been developed and investigated for the treatment of different malignancies [109, 110].
HSP90 inhibition leads to degradation of JAK2, demonstrating that JAK2 is an HSP90 chaperone client. Thus it represents an alternative approach to JAK2 inhibition of potential benefit for the treatment of patients with JAK2-dependent malignancies [111]. Recent studies showed that HSP90 inhibition abrogates proliferation of MPL mutation cell line, and blocked the signal transduction of JAK2 in MPL mutation cell line [102].

**Ruxolitinib**

Ruxolitinib (INCB018424, Jakafi, Incyte), the first-in-class oral small molecule inhibitor, is a potent for inhibiting both JAK1 and JAK2, and it has received FDA approval in 2011 for the treatment of polycythemia vera and myelofibrosis [112]. Ruxolitinib is a cyclopentylpropionitrile derivative. Based on preclinical studies, ruxolitinib treatment in myelofibrosis leads to attenuation of inflammatory cytokine signaling by inhibiting JAK1 and JAK2, results in anti-proliferative and pro-apoptotic effects [113]. Ruxolitinib is also an effective therapy in improving symptoms in patients with myelofibrosis irrespective of JAK2 mutational status [114].

### 3.2.5 Poly ADP-polymerase-1 (PARP-1)

**3.2.5.1 PARP Family**

Poly (ADP-ribose) polymerases (PARPs) are enzymes that transfer ADP-ribose groups onto various substrate proteins either as mono- or oligomeric moieties or as linear or branched poly-ADP-ribose (PAR) chains [115, 116]. Seventeen PARP family members have been identified based on the homology to PARP-1 [116, 117]. PARP family members compromises four subfamilies, which are grouped by their domain structures: DNA-dependent PARPs, namely PARP-1, PARP-2 and PARP-3 [118-120]; the tankyrases, including tankyrase 1 (also known as PARP-5A) and tankyrase 2 (also
known as PARP-5B); the CCCH (Cys-Cys-Cys-His) PARPs, including TIPARP (also known as PARP-7), PARP-12, PARP-13.1 and PARP-13.2; and the macroPARPs, PARP-9, PARP-14 and PARP-15. PARP family members that do not fit into these four subfamilies have special domain structures. These PARP subfamilies have different enzymatic activities and some are non-enzymatic: PARP-1, PARP-2, PARP-4, tankyrase 1 and tankyrase 2 have clear catalytic activity (poly(ADP-ribosyl)ation), whereas PARP-3, PARP-10, PARP-14 and PARP-15 catalyse mono(ADP-ribosyl)ation; the remaining PARP family members are either catalytically inactive or require specific and, as yet undetermined, cofactors to trigger ADP-ribosylation [121, 122]

3.2.5.2 Function of PARP-1

As the attachment of PAR chains, a process also known as poly(ADP-ribosyl)ation (PARylation), modulates protein half-life or subcellular localization, PARP-1 has profound regulatory effects on various nuclear and cytoplasmic processes [123]. Of the 18 members of the PARP family in humans, PARP-1 is by far most well characterized [115]. PARP-1 is involved in multiple aspects of cellular metabolism, such as transcription, chromatin remodeling, apoptosis, and DNA repair [124]. PARP-1 has a highly conserved structure including an N-terminal double zinc finger DNA-binding domain (DBD), a nuclear localization signal, a central automodification domain, and a C-terminal catalytic domain [125, 126]. PARP-1 catalyzes the polymerization of ADP-ribose units from donor NAD+ molecules on target proteins, resulting in the attachment of linear or branched polymers. Poly ADP-ribose (PAR) is a branched polymer of repeating ADP-ribose units. (Figure 3.7) PAR is heterogeneous with respect to length and extent of branching. There could be as many as 200
ADP-ribose units per line *in vitro* or about 20–50 ADP-ribose units for one branch [125]. The consequences of linking a long negatively charged polymer to a protein are potentially profound, as numerous *in vitro* and *in vivo* studies have now demonstrated for PAR [125-127].

![Chemical structures of NAD+, nicotinamide (NAm), and PAR. Adapted from Genes & development, 2005 Sep 1; 19(17):1951-1967 [115]](image)

Figure 3.7: Chemical structures of NAD+, nicotinamide (NAm), and PAR. Adapted from Genes & development, 2005 Sep 1; 19(17):1951-1967 [115]

Other covalent protein modifications, poly(ADP-ribosyl)ation is reversible [125]. PAR catabolism is promoted primarily by poly(ADP-ribose) glycohydrolase (PARG), an enzyme that catalyzes the hydrolysis of glycosidic linkages between the ADP-ribose units of PAR [125, 128] (Figure 3.8).
3.2.5.3 Interaction of PARP-1 and TGFβ pathway

Of particular interest for fibrotic diseases, PARP-1 has recently been shown to PARylate Smad3 and to either positively or negatively regulate Smad-mediated transcription, depending on the cellular context [129, 130] (Figure 3.9).

Figure 3.9: Scheme of the role of PARP-1 as a negative feedback regulator of TGFβ signaling [129].

3.2.5.4 Inhibition of PARP-1

Moreover, PARP-1 can contribute to carcinogenesis by promoting cancer cell survival in response to genotoxic insults, which may allow cells to survive and accumulate DNA damage. The involvement of PARP proteins in the cellular response to DNA damage or cellular stress responses suggests that PARPs are attractive candidates for novel therapies for the treatment of cancer and for autoimmune diseases [123, 131, 132]. Indeed, numerous PARP inhibitors have been developed, some of which have recently entered clinical trials for stroke or cancer (http://clinicaltrials.gov; NCT01983358 and NCT01351909).
4 AIMS OF THE STUDY

4.1 Aims of the study

Thus, overall aim of the current thesis was to improve our understanding of the molecular pathogenesis of aberrant fibroblast activation in SSc and to define novel potential targets for the treatment of fibrosis. Specifically, we had three aims. In the first part, we aimed to investigate the role of CK2 in SSc and the contribution to fibroblast activation by regulating JAK2/STAT3 signaling and to evaluate its potential as molecular targets for the treatment of fibrosis in SSc. In the second part, we aimed to demonstrate the regulatory effect of PARP-1 on TGFβ signaling in experimental skin fibrosis. In the third part, we aimed to investigate potential loss of anti-fibrotic activity of selective JAK2 inhibitors upon long-term treatment and potential strategies to prevent that loss of efficacy.
4.2 Declaration


The necessary permissions to re-publish this work were acquired and have been awarded from the responsible publishers.

I declare that the thesis work is done by me independently under the supervision of my supervisors and some of the lab members showed me techniques for some experiments.
4.3 Statement of contribution for the results in the thesis

Some results of the first part of the thesis have been published as: Inhibition of casein kinase II reduces TGFβ induced fibroblast activation and ameliorates experimental fibrosis.


The contribution of the individual coauthors is listed below.

Yun Zhang: performed all experiments for this publication by her own, in part supported and supervised by coauthors.

Clara Dees: contributed to acquisition and interpretation of data: supervised Yun Zhang during her first time in the laboratory and introduced her in novel techniques.

Christian Beyer: contributed to acquisition and interpretation of data: provided skin samples.

Neng-yu Lin: contributed to the acquisition of data by trouble shooting with microscopes.

Alfiya Distler: contributed to the acquisition of data by providing skin samples.

Pawel Zerr: contributed to the acquisition of data and manuscript preparation.

Katrin Palumbo-Zerr: contributed to the acquisition of data.

Laura Susok: contributed to the manuscript preparation and provided patient samples.

Alexander Kreuter: contributed to the manuscript preparation and provided patient samples.

Oliver Distler: contributed to the preparation of the manuscript and interpretation of data.

Georg Schett: contributed to the preparation of the manuscript and interpretation of data.

Jörg H. W. Distler: interpretation of results, design of the study and preparation of the manuscript.
AIMS OF THE STUDY

We are aiming to publish the results of the second part and are currently preparing a manuscript entitled: Poly(ADP-ribose) polymerase-1 regulates the pro-fibrotic Effects of Transforming Growth Factor-β signaling in Systemic Sclerosis.

The contribution of the individual coauthors is listed below.

Yun Zhang: performed all experiments for this publication, in part supported and supervised by coauthors.

Clara Dees: contributed to acquisition and interpretation of data: supervised Yun Zhang during her first time in the laboratory and introduced her in novel techniques.

Sebastian Pötter: contributed to acquisition part of the data.

Katrin Palumbo-Zerr: contributed to acquisition of data by trouble shooting with co-immunoprecipitation.

Christian Beyer: provided skin samples.

Pawel Zerr: contributed to acquisition of data by trouble shooting with reporter assay.

Ruifang Liang: contributed to acquisition part of the data.

Kolja Gelse: provided human skin biopsies.

Oliver Distler: contributed to the preparation of the manuscript.

Georg Schett: contributed to the preparation of the manuscript.

Jörg H. W. Distler: interpretation of results, design of the study and preparation of the manuscript.

We are also aiming to publish the results of the third part. We are currently planning with the following authors. The contribution of the individual coauthors is listed below.

Yun Zhang: performed all experiments for this publication, in part supported by coauthors.

Clara Dees: provided inhibitors.

Alina Soare: helped with blinded quantification of Ashcroft score.
Oliver Distler: contributed to the preparation of the manuscript.

Georg Schett: contributed to the preparation of the manuscript.

Jörg H. W. Distler: interpretation of results, design of the study and preparation of the manuscript.

Herein, I confirm that the statements above are correctly reflecting Yun Zhang’s contribution.

Prof. Dr. Jörg Distler
5 MATERIAL AND METHODS

5.1 Material

5.1.1 Chemicals and consumables

Chemicals, as not indicated otherwise, were obtained from Sigma-Aldrich (Steinheim, Germany), Roth (Karlsruhe, Germany), and Merck (Darmstadt, Germany). Bleomycin (0.5 mg/ml in 0.9% NaCl) was obtained from the pharmacy of the University Hospital Erlangen. Consumables were purchased from Eppendorf (Hamburg, Germany), Biozym (Hessisch Oldendorf, Germany), Applied Biosystems (Darmstadt, Germany), Sarstedt (Nümbrecht, Germany), Greiner Bio-One (Solingen, Germany), Millipore (Schwalbach, Germany), Nunc (Hanau, Germany), BD Biosciences (Heidelberg, Germany), Pfim medical (Cologne, Germany), Macherey-Nagel (Düren, Germany) and Nerbe plus (Winsen, Germany).

5.1.2 Patients

Skin biopsies were obtained from affected skin of the forearm from SSc patients. Biopsies from healthy age- and sex-matched volunteers served as controls. All patients fulfilled the 2013 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for SSc. 20 patients had diffuse cutaneous SSc, 21 had limited cutaneous SSc. The median age of SSc patients was 46 years (range: 19 - 68 years) and their median disease duration was 6 years (range: 0.5 - 12 years). All patients were positive for anti-nuclear antibodies; 7 patients were positive for anti-topoisoromerase-1 antibodies, 15 patients were positive for anti-centromere antibodies. Skin fibrosis was progressive in 10 patients with diffuse cutaneous SSc at the time of the biopsy, whereas the other 10 patients had stable skin fibrosis. Fibrosis was stable in all patients with limited cutaneous SSc. None of the patients was treated with immunosuppressive or other potentially disease modifying drugs at the time of biopsy.

Scar tissue was obtained from 4 patients with keloids and 5 patients with hypertrophic scars. Two of the patients with keloids were males, two were females. The age ranged from 28 years to 45 years (median 37.5 years). Of the patients with hypertrophic scars, one was male and the other four were females. The age ranged from 35 years to 86 years (median 45 years).

All patients and controls signed a consent form approved by the local institutional review boards. Skin samples of 12 SSc patients and 10 controls were applied in the experiments of this study.

5.1.3 Mice

FVB, C57Bl/6 and B6.Cg-Fbn1<sup>tsk</sup>+/-Pldn<sup>pa</sup>/J (Tsk-1) mice with age-matched pa/pa (wildtype) mice were obtained from Janvier (Le Genest-Saint-Isle, France). PARP-1<sup>-/-</sup> mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). All mice were kept in wire-top cages with wooden bedding (Abedd, Vienna, Austria) in a 12 hour light-dark cycle. Pellet food (Ssniff, Soest, Germany) and water were given ad libitum. All mouse experiments were approved by the local
ethical committee.

5.1.4 Stimulants, inhibitors and antibodies

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
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<td>R&amp;D Systems</td>
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Table 1: Stimulatory agent and concentration used for stimulation experiments in cell culture

<table>
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<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Specificity</th>
<th>Concentration</th>
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</thead>
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<tr>
<td>TBB 4,5,6,7-tetrabromobenzotriazole</td>
<td>CK2</td>
<td><strong>IC₅₀</strong>: 0.9 and 1.6 μM</td>
<td>1.0 μM or 5.0 μM, 2.5 mg/kg/d or 10.0 mg/kg/d</td>
<td>Tocris</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Ki</strong>: for different CK2 subunits, from 80 nM to 210 nM</td>
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<td></td>
</tr>
<tr>
<td>SD208 2,4-disubstituted pteridine</td>
<td>TGFβ receptor</td>
<td><strong>IC₅₀</strong>: 49 nM</td>
<td>1.0 μM, 20 mg/kg/bid</td>
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<tr>
<td>3AB 3-Aminobenzamide</td>
<td>PARP-1</td>
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<td>Tocris</td>
</tr>
<tr>
<td>PJ34 hydrochloride</td>
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<td>TG101209</td>
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</tr>
<tr>
<td>Ruxolitinib</td>
<td>JAK1/JAK2</td>
<td><strong>IC₅₀</strong>: 3.3 nM/2.8 nM</td>
<td>5 μM, 20 mg/kg/bid</td>
<td>LC Laboratories</td>
</tr>
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</table>

Table 2: Inhibitors used in the study with Ki and IC₅₀ values, respectively. For *in vitro* use, the concentration is indicated in mM, μM or nM, for *in vivo* use, the concentration is given in mg/kg body weight.
<table>
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<td>1:200 (WB)</td>
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### MATERIAL AND METHODS

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**Table 3:** Antibodies used for neutralization, immunohistochemistry and Western Blot. IHC = Immunohistochemistry; WB = Western Blot; AF = Alexa Fluor, HRP = horseradish peroxidase, AP = Alkaline phosphatase.

### 5.1.5 PCR Primers and siRNA

All primers were obtained from Metabion (Martinsried, Germany); siRNA was purchased from Eurogentec (Cologne, Germany).

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<thead>
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<td>---------------</td>
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<td>human Smad3 forward</td>
<td>TGG GCT GAA GCG CAC TGA CC</td>
</tr>
<tr>
<td>human Smad3 reverse</td>
<td>CCG CGG CTC TTG CCC ACA T</td>
</tr>
<tr>
<td>human PAI-1 forward</td>
<td>TCA TTG CTG CCC CTT ATG A</td>
</tr>
<tr>
<td>human PAI-1 reverse</td>
<td>GTT GGT GAG GGC AGA GAG AG</td>
</tr>
<tr>
<td>human SMAD7 forward</td>
<td>TAC TCC AGA TAC CCG ATG GAT T</td>
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### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
</tr>
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<tbody>
<tr>
<td>human SMAD7 reverse</td>
<td>TCT GGA CAG TCT GCA GTT GG</td>
</tr>
<tr>
<td>human CTGF forward</td>
<td>AAC TCA CAC AAC AAC TCT TCC CCG C</td>
</tr>
<tr>
<td>human CTGF reverse</td>
<td>GAG TCG CAC TGG CTG TCT CCT CT</td>
</tr>
<tr>
<td>SBE on human α-SMA promoter forward</td>
<td>TAG AGC GAA TGA GCG AGC AG</td>
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<td>SBE on human α-SMA promoter reverse</td>
<td>GAG TGG CCT GTT AGG AAC CC</td>
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<td>mouse PARP-1 forward</td>
<td>ACA CTT GGC CTG GTG GAC ATT GTG</td>
</tr>
<tr>
<td>mouse PARP-1 reverse</td>
<td>AGG ACC GGA AGA TCC AGT ACC TGC</td>
</tr>
<tr>
<td>mouse PAI-1 forward</td>
<td>ACG TTG TGG AAC TGC CCT AC</td>
</tr>
<tr>
<td>mouse PAI-1 reverse</td>
<td>AGC GAT GAA CAT GCT GAG G</td>
</tr>
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<td>GCT CAA TTC GGA CAA CAA GAG</td>
</tr>
<tr>
<td>mouse SMAD7 reverse</td>
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</tr>
<tr>
<td>mouse CTGF forward</td>
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</tr>
<tr>
<td>mouse CTGF reverse</td>
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<td>mouse collagen 1a1 forward</td>
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</tr>
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<td>mouse collagen 1a1 reverse</td>
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</tr>
<tr>
<td>mouse collagen 1a2 forward</td>
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<tr>
<td>mouse collagen 1a2 reverse</td>
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</tr>
<tr>
<td>mouse fibronectin forward</td>
<td>AAG ACC ATA CCT GCC GAA TG</td>
</tr>
<tr>
<td>mouse fibronectin reverse</td>
<td>GAA CAT GAC CGA TTT GGA CC</td>
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**Table 4:** PCR primers used for SYBR Green real-time PCR in human samples

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<thead>
<tr>
<th>Primer</th>
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<tr>
<td>PARP-1/- Wild type Reverse</td>
<td>CCA GCG CAG CTC AGA GAA GCC A</td>
</tr>
<tr>
<td>PARP-1/- Common</td>
<td>CAT GTT CGA TGG GAA AGT CCC</td>
</tr>
<tr>
<td>PARP-1/- Mutant reverse</td>
<td>AGG TGA GAT GAC AGG AGA TC</td>
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**Table 5:** PCR primers used for genotyping of mice.
### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence (5’ → 3’)</th>
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<tr>
<td>human Smad3 sense</td>
<td>GCC UGG UCA AGA AAC UCA A55</td>
</tr>
<tr>
<td>human Smad3 antisense</td>
<td>UUG AGU UUC UUG ACC AGG C55</td>
</tr>
</tbody>
</table>

Table 6: siRNA sequences used for nucleofection experiments.

#### 5.1.6 Buffers and solutions

**Western Blot**

1x Phosphate buffered saline (PBS) / Tween (PBST)

- 1.5 M NaCl
- 60 mM Na₂HPO₄ · 2 H₂O
- 20.5 mM NaH₂PO₄ · 2 H₂O
- 0.1 % Tween-20
- Dilute in ddH₂O

NP-40 Lysis buffer

- 150 mM NaCl
- 1.0 % (v/v) NP-40
- 50mM Tris-Cl pH 8.0
- 1 % (v/v) Nonidet P-40

Lysis Buffer A (cytoplasmic fraction)

- 10 mM HEPES
- 1.5 mM MgCl₂
- 10 mM KCl
- 0.5 mM DTT
- 0.05% NP-40
- Add 1mM Na-orthovanadate
- 5mM Na-Fluoride before use

Lysis Buffer B (nuclear fraction)

- 5 mM HEPES
- 1.5 mM MgCl₂
- 0.2 mM EDTA
- 0.5 mM DTT
- 26% glycerol
- 300mM NaCl
- Add 1mM Na-orthovanadate
- 5mM Na-Fluoride before use
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Running buffer (10x)

- 250 mM Tris
- 2 M Glycin
- 1 % (w/v) SDS

Dilute 1:10 with ddH2O

Transfer buffer (10x)

- Tris 58g
- Glycin 29g
- SDS 3.76g
- pH 8.6 for 1L

Dilute 1:10 and add 20 % (v/v) methanol

Loading buffer (5x)

- 300mM Tris HCl pH 6.8
- 10% (w/v) SDS
- 50% (v/v) Glycin
- 0.05% (w/v) Bromphenol blue
- 5% (w/v) β-Mercaptoethanol

Dilute 1:5 with protein sample

Blocking buffer

- 5% (w/v) BSA in PBST

Reporter assay

Luciferase assay buffer

- 25mM Glycyl glycine
- 15 mM HKPO4
- 15 mM MgSO4
- 4 mM EGTA pH 7.8
- 2 mM ATP (add before use)
- 2 mM DTT (add before use)

in ddH2O

Luciferin

- 0.3 mg/mL in ddH2O

MTT assay
### MATERIAL AND METHODS

**MTT reagent**

0.5 % (w/v) in 1x PBS

**MTT lysis buffer**

99.4 % (v/v) DMSO
0.6 % (v/v) HCl (37 %)
10 % (w/v) SDS

**Hydroxyproline assay**

**Assay Buffer (pH 6.0)**

- 240 mM citric acid monohydrate
- 880 mM sodium acetate trihydrate
- 850 mM NaOH
- 0.2 mM acetic acid in ddH₂O

**Hydroxyproline stock solution**

0.01 % *trans*-hydroxyproline in assay buffer

**Chloramine T solution**

62 mM Chloramine T in ddH₂O

**Benzaldehyde / Perchloric Acid**

- 1 M p-(Dimethylamino)benzaldehyde
- 60 % (v/v) 2-Methoxyethanol
- 26 % Perchloric Acid (70 %) in ddH₂O

**Immunohistochemistry**

**1x PBS (pH 7.4)**

- 1.5 M NaCl
- 60 mM Na₂HPO₄ · 2 H₂O
- 20.5 mM NaH₂PO₄ · 2 H₂O in ddH₂O

**Citrate buffer (pH 6.0)**

10 mM Trisodium citrate dihydrate

**Tris-EDTA (pH 9.0)**

10 mM Tris
1 mM EDTA
### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td><strong>DAB stock solution</strong></td>
<td>1 % (w/v) DAB in ddH₂O</td>
</tr>
<tr>
<td></td>
<td>3-5 drops of 10 M HCl</td>
</tr>
<tr>
<td><strong>DAB working solution</strong></td>
<td>5 % (v/v) DAB stock solution</td>
</tr>
<tr>
<td></td>
<td>5 % (v/v) of 0.3 % H₂O₂ in 1x PBS</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
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</tr>
<tr>
<td>Hematoxylin</td>
<td>0.1 % (w/v) Hematoxylin</td>
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<tr>
<td></td>
<td>0.02 % (w/v) NaJO³</td>
</tr>
<tr>
<td></td>
<td>105 mM Potassium alum dodecahydrate</td>
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<tr>
<td></td>
<td>300 mM Chloral hydrate</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v) crystallized citric acid in ddH₂O</td>
</tr>
<tr>
<td>Eosin</td>
<td>0.3 % (w/v) Eosin</td>
</tr>
<tr>
<td></td>
<td>0.01 % (v/v) acetic acid in ddH₂O</td>
</tr>
<tr>
<td>Weigerts Iron Hematoxylin Solution</td>
<td>1 volume of Solution Part A</td>
</tr>
<tr>
<td></td>
<td>1 volume of Solution Part B</td>
</tr>
<tr>
<td></td>
<td>Stable for 10 days</td>
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<tr>
<td>Biebrich Scarlet-Acid Fuchsin</td>
<td>0.9 % (w/v) Biebrich Scarlet</td>
</tr>
<tr>
<td></td>
<td>0.1 % (w/v) Acid Fuchsin</td>
</tr>
<tr>
<td></td>
<td>1 % Acetic Acid in ddH₂O</td>
</tr>
<tr>
<td>Phosphotungstic / Phosphomolybdic acid</td>
<td>2.5 % (w/v) Phosphotungstic Acid</td>
</tr>
<tr>
<td></td>
<td>2.5 % (w/v) Phosphomolybdic Acid in ddH₂O</td>
</tr>
<tr>
<td>Aniline Blue</td>
<td>2.4 % (w/v) Aniline Blue</td>
</tr>
<tr>
<td></td>
<td>2 % Acetic Acid in ddH₂O</td>
</tr>
<tr>
<td>Sirius red</td>
<td>0.1 % (w/v) Direct red 90 in picric acid</td>
</tr>
</tbody>
</table>
5.2 Methods

5.2.1 Fibroblast culture

Fibroblast cultures were obtained from skin biopsies from SSc patients and healthy volunteers. All patients fulfilled the 2013 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for SSc. Biopsies from SSc patients were taken from involved skin of the forearm. Control fibroblasts were obtained from skin biopsies of healthy age- and sex-matched volunteers. After enzymatic digestion of the skin biopsies with dispase II (Invitrogen, Karlsruhe, Germany), fibroblasts were cultured in DMEM-F12 containing 10 % heat-inactivated (30 min at 56°C) 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). Cultured fibroblasts were kept in 75 cm² culture flasks at 37°C with 5 % CO₂ and 90 % relative humidity. Fibroblasts from passages 4-8 were used for the experiments.

5.2.2 Stimulation of cultured fibroblasts

For stimulation experiments, confluent dermal fibroblasts were cultured in DMEM-F12 containing 0.1 % FCS for 24 h before the experiments. Dermal fibroblasts were stimulated with recombinant TGFβ at the concentrations of 10ng/ml for different time point.

5.2.3 Inhibition of different pathways

5.2.3.1 Selective inhibition of TGFβ signaling

SSc fibroblasts were incubated with SD208 (2, 4-disubstituted pteridine) at a concentration of 1.0 μM as described [30]. The Ki values of all chemical inhibitors according to the NIMH Ki Database are given in Table 2 (Chapter 5.1.4). TGFβ was added one hour after the inhibitors at a concentration of 10.0 ng/ml.

5.2.3.2 Inhibition of CK2

Cells were incubated with the selective inhibitors TBB (4,5,6,7-tetramobenzotriazole) at concentrations of 1.0 and 5.0 μM [84]. TGFβ was added one hour after the inhibitors at a concentration of 10.0 ng/ml.

5.2.3.3 Blockade of the PARP-1
To assess the effects of PARP-1 inhibition, dermal fibroblasts were incubated with the PARP-1 inhibitor 3AB (3-Aminobenzamide) at concentrations of 1.0 mM. In a subset of experiments, TGFβ was added one hour after at a concentration of 10.0 ng/ml.

**5.2.3.4 Selective inhibition of JAKs**

For JAK2 inhibition, fibroblasts were incubated with TG101209 at a concentration of 500 nM as described [86]. 500 nM 17-DMAG was applied to inhibit HSP90 in fibroblasts culture [100]. To inhibit JAK1 and JAK2, fibroblasts were incubated with pan-inhibitor Ruxolitinib at a concentration of 5.0 μM [113, 133]. In a subset of experiments, TGFβ was added one hour after at a concentration of 10.0 ng/ml.

**5.2.3.5 Nucleofection with siRNA**

Fibroblasts were transfected with 1.5 μg siRNA duplexes against Smad3 using the human dermal fibroblast Nucleofector Kit (Amaxa GmbH, Cologne, Germany) [134]. The siRNA sequences are given in Table 7 (chapter 5.1.5). Fibroblasts transfected with non-targeting control siRNAs were used as controls. Medium was changed after 6 h to remove the nucleofector solution. Cells were harvested 48 h after transfection.

**5.2.4 Overexpression of target protein**

**5.2.4.1 Nucleofection with pcDNA3.1-PARP-1 plasmid**

pcDNA3.1 plasmid was applied as vector to overexpress PARP-1 and transfected into fibroblasts using the human dermal fibroblast Nucleofector Kit (Amaxa GmbH, Cologne, Germany) [135]. Fibroblasts transfected with pcDNA3.1 plasmid backbone were used as controls.

**5.2.5 Luciferase reporter assay**

Human fibroblasts were cultured in 12-well-plates. 1000 ifu CAGA viruses per fibroblast were added in 1% serum supernatant for 48 hours [129, 136]. Afterwards, CAGA virus infected cells were serum-starved in DMEM-F12 with 0.1% FBS for 24 hours, then stimulated with TGFβ (10 ng/ml) and treated with 3AB (1mM) for up to 72 hours. The infections were conducted in triplicate for different treatments of each cell line. Fibroblasts were harvest in passive lysis buffer (Promega, Madison, WI, USA). The luciferase activities of fibroblast lysates were evaluated using Microwin software.
5.2.6 Microtiter tetrazolium (MTT) assay

The metabolic activity of dermal fibroblasts incubated with 17-DMAG was measured using the MTT [3, 4, 5-dimethylthiazol-2-yl] 2, 5-diphenyl-tetrazolium bromide] method [137]. SSc and healthy dermal fibroblasts were cultured in 96 well plates (1000 cells / well) for 72h with 500nM 17-DMAG in 80µl medium. Untreated fibroblasts were used as negative controls and fibroblasts incubated with 50 % DMSO served as positive controls. 20µl MTT (5mg/ml) was added 4 h before the end of incubation time. After remove the medium dilution, fibroblasts were lysed in 100µl MTT lysis buffer; the wells were analyzed using an ELISA plate reader (Bioteck, Bad Friedrichshall, Germany) at a test wavelength of 570 nm and 630nm. The metabolic activities were calculated as OD$_{570}$-OD$_{630}$.

5.2.7 Quantitative real-time PCR

Total RNA from cells was isolated with a NucleoSpin RNA II extraction system (Machery-Nagel, Dueren, Germany) and tissues were homogenized before isolation. The RNA samples were reverse transcribed into complementary DNA (cDNA) using random hexamers, as previously described [138]. Gene expression was quantified by real-time PCR using the MxPro 3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA). Specific primer pairs for each gene were designed with Primer6 software. The sequences of the human and mouse primers are summarized in Table 4. β-actin primer was used to normalize for the amounts of loaded cDNA. All samples were measured in duplicates. 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 (Ct) and the comparative Ct method for relative quantification.

5.2.8 Quantification of collagen protein

5.2.8.1 SirCol assay from cell culture supernatants

Total soluble collagen in cell culture supernatants was measured by the SirCol collagen assay (Biocolor, Belfast, Northern Ireland) [139]. For these experiments, 400 µl of Sirius Red Dye were mixed with 200 µl of cell culture supernatant for 30 min at room temperature. After centrifugation at 12000 g for 10 min, the supernatants were discarded and the pellet was washed with acid-salt-wash reagent. Then centrifuged at 12000 g for 10 min again and dissolved in Alkali Reagent. The samples were mixed and 100 µl of each sample were measured in an ELISA reader (Bioteck, Bad Friedrichshall, Germany) at a wavelength of 555 nm.

5.2.8.2 Hydroxyproline assay from skin and lung biopsies

To detect highly crosslinked collagen in mouse tissue, the collagen content in skin samples was
analyzed by determination of the hydroxyproline content [140]. Two punch biopsies (3 mm in diameter) per mouse were digested in 6M HCl for three hours at 120°C and the pH values were adjusted to 6-7. Samples were incubated for 20 min at room temperature after chloramine T (0.06 M) was added. 3.15 M perchloric acid, 20% p-dimethylaminobenzaldehyde and 60% Methoxyethanol were added into samples followed by incubation for additional 20 min at 60 °C. The absorbance was determined with an ELISA reader (Bioteck, Bad Friedrichshall, Germany) at a wavelength of 557 nm.

Lung lopes were dried in eppi tubes at 80°C for 3-4 hours and the weight of each dry biopsy were measured for hydroxyproline assay. After digestion at 120 °C overnight in 300μL 6M HCl, samples were analyzed as skin and the absorbance values were divided by the weight.

5.2.9 Co-Immunoprecipitation (co-IP)

TGFβ stimulated fibroblasts were harvested and lysed in IP lysis/wash buffer (Thermo scientific, IL, USA). Lysates were incubated at 4°C overnight with anti-Smad3 antibodies (Santa Cruz Biotechnology, TX, USA) or anti-PAR antibodies (Trevigen), protein A/G plus-agarose beads (Thermo scientific) and proteinase inhibitors. Precipitated immune-complexes were released by boiling with SDS electrophoresis sample buffer and prepared for Western Blot analysis.

5.2.10 Western Blot

Nuclear and cytoplasmic extracts or whole cell lysates were prepared as previously described [141, 142]. Whole cell lysates were obtained by adding RIPA buffer to the cell pellet. For separation of the nuclear and cytoplasmic fraction, cell pellets were first incubated with lysis buffer A. After centrifugation, the supernatant containing cytoplasmic proteins was collected and pellets were homogenized and re-dissolved in lysis buffer B to obtain nuclear extracts. Protein concentration was detected by protein assay dye reagent (Bio Rad, Hercules, CA, USA). After blocking with 5 % BSA in PBST, PVDF membranes were incubated with different primary antibody listed in Table 3 at 4°C overnight. Horseradish peroxidase-conjugated antibodies (Dako, Hamburg, Germany) were used as secondary antibodies. Equal loading of proteins was confirmed by visualization of cytoplasmic protein β-actin (Sigma-Aldrich), nuclear protein lamin A/C (Cell Signaling Technology) or cytoplasmic protein GAPDH (Abcam).

5.2.11 Chromatin-immunoprecipitation (ChIP)

Cultured fibroblasts were fixed and sheared by sonication using ChIP-IT Express kit (Active motif, La Hulpe, Belgium). The sheared chromatin, protein G magnetic beads, Smad3 antibody (cell signaling technology), proteinase inhibitors and ChIP buffer were mixed in siliconized tube and gently shaked at 4°C overnight. On the second day, the protein G magnetic beads were washed and the chromatin was eluted for quantitative real-time PCR. Sheared chromatin frozen before precipitation was used to normalize the loading of cDNA. Differences were calculated with
the comparative Ct method for relative quantification.

5.2.12 Immunohisto- and Immunocytochemistry

5.2.12.1 Immunohistochemical stainings

Immunohistochemical analysis of paraffin-embedded sections was performed as previously described [143]. Sections were deparaffinized and epitope retrieval was performed with boiling Tris-EDTA buffer and citrate buffer (pH 6) for ten minutes. After blocking of endogenous peroxidase with 3 % H₂O₂ and serum blocking with 5 % horse serum in 2 % bovine serum albumin, The levels of CK2α and CK2β in patients with SSc and controls were assessed by staining with anti-CK2α (Epitomics, Burlingame, CA, USA) and CK2β antibodies (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. Fibroblasts were identified by staining with anti-prolyl-4-hydroxylase-β monoclonal antibodies (Acris Antibodies, Herford, Germany) [144]. Irrelevant isotype antibodies in the same concentration were used as controls. Antibodies labeled with horseradish peroxidase (Dako, Hamburg, Germany), Alexa Fluor 488, and Alexa Fluor 594 (both Invitrogen, Carlsbad, California, USA) were used as secondary antibodies. The expression of pJAK2 (Tyr1007/1008) antibodies (Epitomics, Burlingame, CA, USA) and pSTAT3 monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA) was visualized with diaminobenzidine peroxidase substrate solution (Sigma-Aldrich). Semiquantitative scoring of the staining was performed as follows: The epidermis, fibroblasts (spindle-shaped single cells in the dermis) and vessels were scored separately. No staining was scored as 0, faint staining or staining in very few cells was scored as 1, moderate staining was scored as 2 and very intense staining in the majority of cells was scored as 3.

Cells positive for anti-α-smooth muscle actin (α-SMA) in murine sections were detected by incubation with anti-α-SMA monoclonal antibodies (clone 1A4; Sigma-Aldrich, Steinheim, Germany). Rabbit anti-mouse antibodies labeled with horseradish peroxidase were used as secondary antibodies. The expression of α-SMA was visualized with diaminobenzidine peroxidase substrate solution (Sigma-Aldrich). The number of myofibroblasts was counted from 6 different sections of lesional skin for each mouse by an examiner blinded to the treatment of the mice.

The levels of PARP-1 were assessed by staining with anti-PARP-1 antibodies (Abcam, Cambridge, MA, USA) at 4°C overnight. Fibroblasts were identified by staining with anti-prolyl-4-hydroxylase-β monoclonal antibodies (Acris Antibodies, Herford, Germany). Irrelevant isotype antibodies in the same concentration were used as controls. Antibodies labeled with horseradish peroxidase (Dako, Hamburg, Germany), Alexa Fluor 488 (Invitrogen) were used as secondary antibodies. The expression of PARP-1 was visualized with 3,3'-diaminobenzidine peroxidase substrate peroxidase substrate solution (Sigma-Aldrich). Semiquantitative scoring of the staining was performed as follows: The epidermis, fibroblasts (spindle-shaped single cells in the dermis) and vessels were scored separately in the following manner: No staining was scored as 0, faint staining or staining in very few cells was scored as 1, moderate staining was scored as 2 and very intense staining in the majority of cells was scored as 3.
All immunohistochemical stainings were analyzed using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands).

5.2.12.2 Immunocytochemistry and visualization of stress fibers

Serum-starved cells were stimulated with TGFβ (10ng/ml) for 24 hours. After fixation with 4 % methanol-free paraformaldehyde (PFA), permeabilization in 0.25 % Triton X-100 in PBS and blocking with 10 % horse serum, fibroblasts were incubated with mouse anti-α-SMA monoclonal antibodies (clone 1A4; Sigma-Aldrich). Fibroblasts incubated with irrelevant isotype antibodies were used as controls. Alexa Fluor 488 goat anti-mouse antibodies (Invitrogen, Carlsbad, California, USA) served as secondary antibodies. Stress fibers were stained with rhodamine-conjugated phalloidin (Invitrogen) for 20 minutes at room temperature [86]. Counterstaining of nuclei was performed with 4',6-diamidino-2-phenylindole DAPI (Santa Cruz Biotechnology) for 10 minutes at room temperature.

PARylation and accumulation of phosphorylated Smad3 in cultured fibroblasts were visualized by incubation with mouse monoclonal antibodies against PAR (Trevigen, Gaithersburg, MD, USA) and rabbit monoclonal antibodies against pSmad3 (Cell signaling technology). Alexa Fluor 350 goat anti-mouse antibodies (Invitrogen) and Alexa Fluor 488 goat anti-rabbit antibodies (Invitrogen) served as secondary antibodies.

The fluorescence intensity was analyzed using a Nikon Eclipse 80i microscope (Nikon) and quantified with ImageJ software version 1.44.

5.2.13 Animal models

5.2.13.1 Prevention of bleomycin-induced dermal fibrosis

Bleomycin-induced dermal fibrosis was induced in 6-week-old female FVB or C57Bl/6 mice (Charles River) by local injection of bleomycin for 28 days. Control mice were injected with 0.9% NaCl, the solvent for bleomycin [145]. 6-weeks-old mice were anaesthetized with 0.9 % NaCl : ketaminchloride 100 mg/ml : xylazinchloride 2 % (2 : 1 : 1) mixture. Shave larger area of about 2 cm x 2 cm between the shoulders at the back of the mouse. Mark an area of 1 cm x 1 cm between the shoulders at the back of the mouse. 100µL 0.5 mg/ml bleomycin/ 0.9 % NaCl was injected every other day at one of five different positions. Injections should be performed subcutaneously. As soon as the opening of the needle has completely invaded the skin, bleomycin/NaCl should be injected. To evaluate the effects of CK2 inhibition, mice were treated with TBB in doses of 2.5 mg/kg/day or 10 mg/kg/day by i.p. injection. To evaluate the effect of PARP-1 inhibition, 10mg/kg 3AB were injected every other day intraperitoneally, or 30mg/kg/d PJ34 were injected intraperitoneally. PAPR-1 knockout mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) [146]. To evaluate the effects of genetic inactivation of PARP-1 using PARP-1 knockout mice, the following groups were examined: (1) wild type mice injected with 0.9% NaCl; (2) PARP-1 knockout mice injected with 0.9% NaCl; (3) wild type mice challenged with
bleomycin; (4) PARP-1 knockout mice challenged with bleomycin.

### 5.2.13.2 Treatment of established bleomycin-induced dermal fibrosis

For established bleomycin-induced fibrosis, mice were injected with bleomycin for up to six weeks [147, 148] and treatment with TBB was initiated only after three weeks of pre-challenge with bleomycin. To assess the extent of fibrosis before the onset of treatment, one group of mice was injected with 0.9% NaCl for 3 weeks, followed by injections of 0.9% NaCl for another 3 weeks. One group of mice was injected 3 weeks of bleomycin and 3 weeks of 0.9% NaCl for spontaneous regression of fibrosis. The third group received bleomycin injections for 6 weeks. The last group received bleomycin injections for 3 weeks followed by bleomycin injections for the next 3 weeks with TBB treatment.

### 5.2.13.3 Prevention of TBR-induced dermal fibrosis

To induce dermal fibrosis by selective activation of TGFβ signaling, attenuated adenoviruses encoding for a constitutively active TGFβ receptor type I (adTBR) were diluted to a final concentration of 6.67x10⁷ ifu in 250μl sterile PBS. 4-weeks-old C57Bl/6 mice were anesthetized with Rompun: Ketavet: PBS (1:1:2) and injected with 250μl diluted virus at five positions in a defined 1cmx1cm area on the upper back. Attenuated adenoviruses encoding for LacZ at the same concentration were used as controls. AdTBR and adLacZ injection were repeated every two weeks until age 12 weeks. CK2 inhibitor TBB (10.0mg/kg/d), HSP90 inhibitor 17-DMAG (0.35mg every third day) was injected intraperitoneally, JAK2 inhibitor TG101209 (30mg/kg/d), JAKs pan-inhibitor Ruxolitinib (20mg/kg/bid) were applied to mice by oral gavage for 8 weeks from the first adTBR and adLacZ injection until the end of the model. The mice were sacrifice at age 12 weeks. The skin samples were harvested and analyzed by quantification of dermal thickening, myofibroblast counts and hydroxyproline content.

### 5.2.13.4 Tight-skin 1 mouse model

B6.Cg-Fbn1tsk+/+Pldnja/J (Tsk-1) mice and age-matched pa/pa (wildtype) controls were analyzed for the treatment of later, non-inflammatory stages of SSc. Treatment of Tsk-1 as well as pa/pa control mice started at the age of 5 weeks and were continued for 5 weeks. 3AB were injected 10 mg/kg every other day by injection. The following groups were examined: (1) pa/pa and sham treatment; (2) Tsk-1 and sham treatment; (3) Tsk-1 treated with 3AB 10 mg/kg every other day by intraperitoneal injection. Mice were sacrificed after five weeks of treatment by cervical dislocation. The skin fibrosis was analyzed by quantification of hypodermal thickening, myofibroblast counts and hydroxyproline content.
5.2.13.5 Bleomycin-induced pulmonary fibrosis

8-weeks-old male C57Bl/6 mice were anesthetized by isoforene inhalation and hanged vertically on their teeth under stereo microscope(Nickon). The tongues were gently grabbed using a blunted forceps, 50μl bleomycin or NaCl were injected using high pressure syringe with microsprayer (Penn century) through the voice cord into the lung of the mice. HSP90 inhibitor 17-DMAG (0.35mg every third day) was injected intraperitoneally, JAK2 inhibitor TG101209 (30mg/kg/d), JAKs pan-inhibitor Ruxolitinib (20mg/kg/bid) were applied to mice by oral gavage for 4 week from the bleomycin injection until sacrificing at age 12 weeks. Lungs were harvest for collagen content analysis and myofibroblasts number counting.

5.2.14 Histological analyses

5.2.14.1 Hematoxylin / Eosin staining

Paraffin-embedded histological sections were stained with hematoxylin and eosin for the determination of dermal or hypodermal thickness [149]. The injected skin areas were fixed in 4 % formalin and embedded in paraffin. After deparaffinization and rehydration by xylene and ethanol, five-micrometer histological sections were stained with hematoxylin and eosin, and then covered with mounting medium and cover slips. Pictures of the stained section were taken with a Nikon Eclipse 80i microscope (Nikon) at 100 fold magnification, the dermal or hypodermal thickness was analyzed at 100 fold or 40 fold magnification, respectively. The dermal thickness was determined by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction as described [150]. The hypodermal thickness was measured as the distance from the bottom of muscle layer beneath subcutaneous fat to next muscle layer. The measurements were done by an examiner blinded to the treatment of the mice. Dermal thickness was calculated as the fold increase compared with controls in each series.

5.2.14.2 Trichrome staining

For visualization of collagen, trichrome staining was performed using Masson’s Trichrome Staining kit (Sigma-Aldrich). After deparaffinization and rehydration, skin sections were incubated in Bouin’s Solution for 15 min at 56°C and then with Weigert’s iron hematoxylin and Biebrich Scarlet-Acid Fuchsin. Then the sections were incubated with phosphotungstic and phosphomolybdic acid and followed by with aniline blue for two minutes at room temperature. Finally, sections were dehydrated and covered with permanent mounting medium.

Ashcroft score has been widely used in bleomycin-induced pulmonary fibrosis in animals [151, 152]. It was assigned by a numerical scale, with grades from 0 to 8, of the amount of fibrotic tissue in histological samples. Normal lung was scored as 0, minimal fibrous thickening of alveolar or bronchiolar vessels was scored as 1; moderate thickening of walls without obvious damage to lung architecture was scored as 3; increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses was scored as 5; severe
distortion of structure and large fibrous areas; “honeycomb lung” is placed in this category was scored as 7; total fibrous obliteration of the field was scored as 8.

5.2.14.3 Sirius red staining

Sirius red staining was used for collagen staining and lung fibrotic area measurement. Paraffin sections were de-waxed and hydrated as HE staining. Nuclei were stained with Weigert’s haematoxylin for 8 minutes. One hour Picro-sirius red (Sigma-Aldrich) incubation was followed by wash in acidified water twice. Dehydrated sections in isopropanol and xylene then covered by resinous medium. Fibrotic area in lung section were measured by Image J and calculated as the fold change compare with controls.

5.2.15 Statistics

All results were tested for normal distribution using the D’Agostino & Pearson omnibus normality test. In case of normal distribution, results were further evaluated using the student’s t test. For not normally distributed samples, a Mann-Whitney U test was performed. Data are represented as column bar graph with median and IQR using GraphPad Prism V.4.0 Software (GraphPad Software, San Diego, California, USA). A p-value of less than 0.05 was considered statistically significant. P-values are expressed as follows: 0.05 >p> 0.01 as *; 0.01 >p> 0.001 as **; p< 0.001 as ***.
6 RESULTS

6.1 Evaluation of the JAK/STAT signaling pathway after CK2 inhibition in the development of skin fibrosis

6.1.1 Upregulation of CK2 in SSc

6.1.1.1 Expression of CK2α and CK2β are increased in SSc

To analyze the expression of CK2 in skin sections of SSc patients and matched healthy volunteers, immunochemical staining for CK2 subunits CK2α and CK2β were applied. CK2α and CK2β were detectable in fibrotic skin of SSc patients as well as in normal skin of healthy individuals. The results showed that both CK2α and CK2β (Figure 6.1 A) were more intensely stained in SSc skin compared to healthy individuals with prominent expression in spindle-shaped fibroblasts in the dermis. Semiquantitative scoring of CK2α and CK2β staining in fibroblasts, epidermis and vessels were performed separately (Figure 6.1 B).

![Image](image_url)

**Figure 6.1:** The expression of CK2 is increased in SSc patient skin. (A) Expression of CK2α (upper row) and CK2β (lower row) were detected by immunohistochemistry in the skin biopsies of healthy volunteers and SSc patients. Representative examples of SSc patients and healthy individuals are shown at 400-fold magnification (n=8 each). (B) Semiquantitative analysis of staining confirmed that the levels of CK2 were significantly
upregulated in fibroblasts in SSc skin compared to fibroblasts in the skin of healthy individuals.

Double staining with the fibroblast marker prolyl-4-hydroxylase β (P4H β) confirmed that all SSc fibroblasts express high levels of CK2α and CK2β, whereas only a minority of fibroblasts stained positive for CK2 in skin sections from healthy individuals (Figure 6.2 A). Analysis of the stainings confirmed that the levels of CK2α and CK2β were significantly up-regulated in fibroblasts in SSc patients (Figure 6.2 B).

![Figure 6.2: The expression of CK2 is increased in SSc fibroblasts. (A) Double staining of CK2α or CK2β with the fibroblast marker prolyl-4-hydroxylase β (P4H β). Representative images of SSc patients and healthy individuals are shown at 1000-fold magnification (n=8 each). (B) Semiquantitative analysis of CK2 expression in P4H β positive fibroblasts in SSc patients and controls.](image)

**6.1.1.2 CK2α and CK2β are upregulated in a TGFβ-dependent manner**

Aberrant TGFβ signaling is a major hallmark of SSc and other fibrotic diseases. To investigate whether TGFβ stimulates CK2 signaling, healthy human dermal fibroblasts were incubated with recombinant TGFβ. The mRNA levels of CK2α, CK2α’ and CK2β increased significantly after TGFβ stimulation (Figure 6.3 A). Stimulation with TGFβ also upregulated the protein levels of CK2α and CK2β by 58% (p=0.0001) and 48% (p=0.0139), respectively.
(Figure 6.3 B). Thus, CK2 is overexpressed in a TGFβ-dependent manner in cultured fibroblasts.

**Figure 6.3:** Upregulation of CK2 by TGFβ. (A) Stimulation with TGFβ upregulated the mRNA levels of CK2α, CK2α’ and CK2β in cultured fibroblasts from healthy individuals (n = 4). (B) Stimulation of healthy dermal fibroblasts with TGFβ increased the levels CK2α and CK2β protein (n = 4).

In murine skin, the mRNA levels of CK2α, CK2α’ and CK2β significantly increased upon standardized challenge with the pro-fibrotic agent bleomycin compared with non-fibrotic control (Figure 6.4). However, treatment with the selective TGFβRI inhibitor SD208 prevented the induction of CK2α, CK2α’ and CK2β mRNA by bleomycin (Figure 6.4). Taken together, these results suggested that CK2 is overexpressed in a TGFβ-dependent manner in SSc.

**Figure 6.4:** Treatment with the selective TGFβRI inhibitor SD208 prevents the increases of CK2α, CK2α’ and CK2β mRNA by bleomycin in murine skin (n = 6).
6.1.2 CK2 regulates fibroblast activation *in vitro*

6.1.2.1 Inhibition of CK2 prevents myofibroblast differentiation and decreases the release of collagen in fibroblasts

To detect whether CK2 might be required for the pro-fibrotic effects of TGFβ, we firstly investigated the effects of CK2 inhibition by the selective CK2 inhibitor 4,5,6,7-Tetrabromobenzotriazole (TBB) on myofibroblast differentiation. Myofibroblasts can be detected by the increased expression of α-SMA and formation of stress fibers. CK2 inhibition prevented TGFβ induced myofibroblast differentiation, but had little effects on the basal levels of α-SMA and stress fibers. Incubation with TBB reduced the TGFβ-induced upregulation of α-SMA mRNA by 68% (p=0.0284), the induction of α-SMA protein by 38% (p=0.0280) and the formation of stress fibers by 55% compared to TGFβ stimulated, mock-treated cells (p=0.0064) (Figure 6.5 A and B).

![Figure 6.5: Inhibition of CK2 prevents myofibroblast differentiation. (A) Incubation with the CK2 inhibitor TBB reduced the stimulatory effects of TGFβ on α-SMA protein production and prevented the formation of stress fibers.](image)

*indicates p<0.05, **indicates p<0.01.*
fibers (n = 4 each). Representative images of fibroblasts are shown at 200-fold magnification. (B) α-SMA mRNA level and quantification analysis of α-SMA expression and stress fibers formation (n = 4 each).

We next analyzed the effect of CK2 inhibition on the release of collagen. Incubation with TBB reduced the stimulatory effects of TGFβ in a dose-dependent manner. The mRNA levels of col1a1 and col1a2 decreased by 88% (p=0.0083) and 84% (p=0.0219), respectively, compared with mock-treated fibroblasts stimulated with TGFβ (Figure 6.6). Consistently, TBB reduced the TGFβ-induced release of collagen protein by 82% (p=0.0042) (Figure 6.6).

6.1.3 Effect of CK2 inhibition on JAK/STAT pathway

6.1.3.1 Inhibition of CK2 suppresses TGFβ induced JAK2/STAT3 activation

JAK2 and its downstream mediator STAT3 have recently been identified as intracellular mediators of the pro-fibrotic effects of TGFβ in fibroblasts. To investigate whether CK2 regulates JAK2/STAT3 activation in fibroblasts, we analyzed the phosphorylation and subcellular localization of JAK2 and STAT3 in response to TGFβ and TBB. (Figure 6.7 A and B)
Figure 6.7: Inhibition of CK2 suppresses TGFβ induced JAK2/STAT3 signaling. Stimulation of fibroblasts with TGFβ increased the levels of pJAK2 in the cytoplasm (A) and pSTAT3 in nucleus (B) without altering the total levels of JAK2 and STAT3. Inhibition of CK2 by TBB prevented the TGFβ induced phosphorylation of JAK2 and STAT3 (A and B). (n = 3 each).

Stimulation with TGFβ induced phosphorylation of JAK2 in the cytoplasmic fraction, but did not alter the total levels of JAK2 or induce its nuclear translocation. TGFβ also increased the nuclear levels of phosphorylated and thereby activated STAT3. Co-incubation with TBB completely prevented the TGFβ induced accumulation of phosphorylated JAK2 (Figure 6.7 A). In addition, TBB also abrogated the nuclear accumulation of phosphorylated STAT3 (Figure 6.7 B). These data suggested that CK2 is essentially required for TGFβ-induced activation of JAK2/STAT3 in SSc fibroblasts. In contrast, inhibition of CK2 by TBB did not prevent the TGFβ induced phosphorylation of Smad3 in cultured healthy human fibroblasts.
(Figures 6.8 A and B). These results indicated that CK2 may not regulate canonical TGFβ / Smad signaling.

![Western blot and graph showing changes in pSmad3, Smad3, and β-actin levels](image)

**Figure 6.8:** Inhibition of CK2 does not effect TGFβ signaling. (A) Western blot shows the levels of pSmad3 after stimulation of fibroblasts with TGFβ. Inhibition of CK2 by TBB does not effect the level of TGFβ induced phosphorylation of Smad3 (n = 3 each). (B) Quantitative analysis confirmed there is no significant change of pSmad3 expression after TBB treatment in TGFβ stimulated fibroblasts.

### 6.1.4 Anti-fibrotic effect of CK2 inhibition in experimental fibrosis

#### 6.1.4.1 Inhibition of CK2 prevents bleomycin-induced skin fibrosis

We next investigated whether inhibition of CK2 can prevent fibrosis in murine models of SSc. First, we examined the effects of CK2 in the mouse model of bleomycin-induced skin fibrosis. Injection of bleomycin induces prominent skin fibrosis resembling early, inflammatory stages of SSc [17]. Consistent with the findings in human SSc, the levels of CK2α and CK2β were increased in bleomycin-challenged mice fibroblasts, epidermis and vessels compared to NaCl.
treated mice (Figures 6.9 A and B), demonstrating that bleomycin-induced skin fibrosis is a suitable model to study CK2 signaling in SSc.

![Image](image1)

**Figure 6.9:** The expression of CK2 is increased in bleomycin-induced fibrosis. (A) Immunohistochemistry staining of CK2α and CK2β in bleomycin-challenged mice compared to NaCl treated mice. (B) Semiquantification analysis showed levels of CK2 were significantly upregulated in fibroblasts of bleomycin-challenged mice skin compared to fibroblasts of NaCl treated mice skin.

Next, we investigated whether pharmacological targeting of CK2 pathway with TBB can prevent bleomycin-induced fibrosis. In bleomycin-injected mice, a massive accumulation of collagen bundles with prominent dermal thickening by 121 % was observed (p = 0.0043 vs. NaCl injected control mice) In treatment group, bleomycin-challenged mice were treated with TBB intraperitoneally at a dose of 2.5 mg/kg/d or 10 mg/kg/d, mice were well tolerated in TBB treatment group and no difference were observed compare with vehicle-treated group. 2.5 mg/kg/d of TBB significantly reduced dermal thickening (p=0.0362) compared with
RESULTS

vehicle-treated, bleomycin-challenged mice (Figures 6.10 A and B). TBB treatment reduced
dermal thickening in a dose-dependent manner. At higher dosages of 10 mg/kg/d, dermal
thickening was further decreased (p< 0.0001) (Figure 6.10 A and B). Consistent with reduced
dermal thickening, the hydroxyproline content decreased dose-dependently by up to 47%
(p=0.0002) at 10mg/kg (Figure 6.10 B). The differentiation of resting fibroblasts into
myofibroblasts was also significantly reduced by TBB (p=0.0002) (Figure 6.10 B).

![Figure 6.10: CK2 inhibition prevents bleomycin induced fibrosis.](image)

(A) Representative hematoxylin and eosin–stained skin sections from control mice injected with 0.9% NaCl (n = 6), bleomycin-challenged mice receiving vehicle treatment (n = 6), mice injected with bleomycin and treated with TBB at doses of 2.5 mg/kg/d or 10 mg/kg/d (n = 6 each) are shown at 100-fold magnification. (B) Treatment with TBB reduced bleomycin-induced dermal thickening and decreased the hydroxyproline content and the myofibroblast counts.

To test if the anti-fibrotic effects of CK2 inhibition are consistent with our findings on the
molecular mechanism in vitro, pJAK2 and pSTAT3 immunohistochemical staining were

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applied in bleomycin-induced fibrotic mice skin. Both pJAK2 and pSTAT3 were strongly stained in bleomycin-challenged mice compare with control mice treated with NaCl. In TBB treatment group, the anti-fibrotic effects of CK2 inhibition were paralleled by impaired JAK2/STAT3 activation. Treatment with TBB prevented the accumulation of pJAK2 and pSTAT3 in bleomycin-challenged mice and reduced the levels of pJAK2 and pSTAT3 to that of non-fibrotic control mice (Figure 6.11 A and B).

![Figure 6.11: CK2 inhibition suppresses JAK2/STAT3 signaling in bleomycin induced fibrosis. (A) Inhibition of CK2 prevented the bleomycin-induced activation of JAK2/STAT3 signaling and reduced the levels of pJAK2 and pSTAT3 in fibrotic skin. Representative images are shown at 400-fold magnification. (B) Semiquantitative analysis of staining confirmed that the levels of pJAK2 and pSTAT3 were significantly down-regulated in fibroblasts upon treatment with TBB.]

6.1.4.2 CK2 inhibition ameliorates TBR-induced fibrosis

We further evaluated the anti-fibrotic effects of CK2 inhibition in the mouse model of TBR-induced fibrosis. In the immunochemical staining of CK2α and CK2β, according to semiquantitative scoring, TBR-induced fibrosis increased the expression of CK2α in
fibroblasts, epidermis and vessels in mice skin. Overexpression of TBR also increased the expression of CK2β in fibroblasts and vessels significantly (Figure 6.12 A and B).

**Figure 6.12:** The expression of CK2 is increased in TBR-induced fibrosis. (A) Immunohistochemistry staining of CK2α and CK2β in TBR-challenged mice compared to LacZ control mice. Representative pictures are shown at 400-fold magnification. (B) Semiquantitative analysis showed levels of CK2 were significantly upregulated in fibroblasts of bleomycin-challenged mice skin compared to fibroblasts of LacZ control mice skin (n = 6 for all groups).

Overexpression of TBR induced prominent dermal thickening and accumulation of collagen compared to LacZ control mice. Similarly to the effect of CK2 inhibition in bleomycin model of SSc, treatment with TBB reduced dermal thickening by 38% in TBR-induced fibrosis compared to vehicle-treated TBR-challenged mice (p<0.0001) (Figure 6.13 A and B). The hydroxyproline content and the number of myofibroblasts were also both significantly reduced upon TBB treatment in TBR-induced skin fibrosis (Figure 6.13 B).
RESULTS

Figure 6.13: CK2 inhibition prevents TBR-induced fibrosis. (A) Representative hematoxylin and eosin–stained skin sections from control mice injected with LacZ-AAV, mice injected with TBR-AVV receiving vehicle treatment and mice injected with TBR-AVV and treated with TBB are shown at 100-fold magnification (n = 6 for all groups). (B) Treatment with TBB reduced TBR-induced dermal thickening, collagen accumulation and myofibroblast differentiation versus vehicle-treated TBR-AAV mice.

Similarly to bleomycin-challenged mice, pJAK2 and pSTAT3 expression in the dermis increased in TBR-challenged fibrotic mice skin compared with LacZ control mice. Consistent with the anti-fibrotic effects, immunochemical staining of pJAK2 and pSTAT3 in the murine skin section showed that TBB treatment completely prevented activation of JAK2/STAT3 signaling induced by overexpression of TBR (Figure 6.14 A and B).
Figure 6.14: CK2 inhibition suppresses JAK2/STAT3 signaling in TBR-induced fibrosis. (A) Immunohistochemistry demonstrated an upregulation of pJAK2 and pSTAT3 in the skin of TBR-AAV mice, which was completely prevented by TBB treatment. Representative images are shown at 400-fold magnification. (B) Semiquantitative analysis of staining demonstrated decreased levels of pJAK2 and pSTAT3 in fibroblasts of TBR mice treated with TBB compared to vehicle-treated TBR-AAV mice.

6.1.4.3 Inhibition of CK2 induces regression of pre-established bleomycin-induced skin fibrosis

We next analyzed whether pharmacologic inhibition of CK2 is also effective in therapeutic settings, when treatment is initiated after fibrosis has already been established. Prolonged injections of bleomycin to six weeks induced progressive skin fibrosis. Treatment with TBB for the last three weeks did not only prevent progression of bleomycin-induced skin fibrosis, but also reduced the dermal thickness, the hydroxyproline content and the myofibroblast counts (p<0.0001 for all outcomes) to below the levels of mice injected with bleomycin for only three weeks (pre-treatment levels), demonstrating that TBB does not only prevent experimental fibrosis, but can also induce regression of pre-established bleomycin-induced fibrosis (Figures 6.15 A and B).
Figure 6.15: Inhibition of CK2 by TBB cures established bleomycin-induced fibrosis. (A) Representative hematoxylin and eosin–stained skin sections from control mice injected with 0.9% NaCl (n = 6) for 6 weeks, bleomycin-challenged mice for 3 weeks then 0.9% NaCl for 3 weeks (n = 6), mice injected with bleomycin for 6 weeks (n=6), mice injected with bleomycin for 6 weeks and treated with TBB for last 3 weeks at doses of 10 mg/kg/d (n = 6) are shown at 100-fold magnification. (B) Treatment with TBB reduced bleomycin-induced dermal thickening and decreased the hydroxyproline content and the myofibroblast counts of established fibrosis.
6.2 PARP-1 regulates the pro-fibrotic Effects of Transforming Growth Factor-β signaling in Systemic Sclerosis

6.2.1 Reduction of the PARP-1 expression in fibrotic disease

6.2.1.1 The expression of PARP-1 is decreased in SSc

We firstly analyzed the expression of PARP-1 in the skin of SSc patients and matched healthy volunteers by immunofluorescence and immunohistochemistry. SSc skin showed less intensive staining of PARP-1 compared to healthy skin (Figure 6.16 A and Figure 6.17 A). Co-staining of PARP-1 and the fibroblast marker prolyl-4-hydroxylase-β (P4Hβ) demonstrated reduced expression of PARP-1 in SSc fibroblasts (Figure 6.16 A, B and Figure 6.17 A). Semiquantitative analysis confirmed decreased expression of PARP-1 in SSc fibroblasts, less PARP-1 positive fibroblasts in SSc patient skin as compared to healthy skin (Figure 6.17 B). It also showed trends towards more pronounced decreases in patients with diffuse cutaneous SSc and active disease as compared to limited cutaneous SSc and stable disease.

Figure 6.16: The expression of PARP-1 is decreased in SSc. (A) PARP-1 immunofluorescence staining in the skin of SSc patients and matched healthy volunteers skin samples with co-staining for the fibroblast marker prolyl-4-hydroxylase-β (P4H) and DAPI as well as overlays of all three stainings are shown at 400-fold magnification. (B) Semiquantitative analysis of PARP-1 staining in fibroblasts in the skin of SSc patients and healthy volunteers (n=12 SSc patients and 10 controls).
Figure 6.17: The expression of PARP-1 is decreased in SSc fibroblasts. (A) PARP-1 immunohistochemistry staining in the skin of SSc patients and matched healthy volunteers skin samples with co-staining for the fibroblast marker prolyl-4-hydroxylase-β (P4Hβ) and DAPI as well as overlays of all three stainings are shown at 200-fold and 1000-fold magnification (n=12 SSc patients and 10 controls). (B) Relative number of PARP-1 positive fibroblasts and the mean intensity per fibroblasts in SSc and healthy human skin (n=12 SSc patients and 10 controls).

Compare with healthy human fibroblasts, PARP-1 mRNA levels were also significantly decreased in cultured fibroblasts from SSc patients even after several passages in vitro (Figure 6.18 A). Similarly, the nuclear levels of PARP-1 protein were reduced significantly in cultured SSc fibroblasts compare with healthy fibroblasts (Figure 6.18 B).
Figure 6.18: The expression of PARP-1 in cultured SSc fibroblasts: (A) mRNA levels of PARP-1 in fibroblasts of healthy individuals and SSc patients. (B) protein levels of PARP-1 in fibroblasts of healthy individuals and SSc patients.

The immunofluorescence and immunochemical staining of PARP-1 and fibroblasts marker vimentin were performed in bleomycin-induced fibrotic and control mice skin. (Figure 6.19 A) Semiquantitative analysis results of PARP-1 staining showed that the findings in human SSc skin were mimicked by murine models of SSc with reduced PARP-1 levels in fibroblasts in bleomycin-induced fibrosis compared with control mice. (Figure 6.19 B)
RESULTS

For another experimental fibrosis model, in Tsk-1 mice, which spontaneously develop fibrosis in the skin, co-staining of PARP-1 and fibroblast marker vimentin together with the semiquantification analysis showed that compared to non-fibrotic control mice, PARP-1 expression level decreased in the fibroblasts of Tsk-1 mice skin (Figure 6.20 A and B).

Figure 6.19: The expression of PARP-1 is decreased in Bleomycin-induced skin fibrosis. (A) Immunohistochemistry staining for PARP-1 with costaining for vimentin and DAPI at 400-fold magnification. (B) Semiquantitative analyses of PARP-1 expression in the skin of mice challenged with bleomycin and non-fibrotic control mice (n=6).
6.2.1.2 The levels of PARP-1 are reduced by TGFβ stimulation

Given the central role of TGFβ in fibrotic diseases, we wondered whether the expression of PARP-1 might be regulated by TGFβ in the context of fibrosis. Indeed, we observed a time-dependent decrease of PARP-1 mRNA upon stimulation with TGFβ in cultured human healthy fibroblasts. (Figure 6.21 A) Moreover, a time-dependent downregulation of PARP-1 protein level by TGFβ were observed and analyzed by western blot. (Figure 6.21 B)
RESULTS

Figure 6.21: TGFβ downregulates the expression of PARP-1 in healthy fibroblasts. (A) mRNA levels of PARP-1 in human fibroblasts stimulated with TGFβ (n=4 biological replicates with ≥ 2 technical replicates). (B) Western blot for the protein levels of PARP-1 in human fibroblasts at different time points after stimulation with TGFβ (n=4 biological replicates with ≥ technical replicates).

To investigate if the inhibitory effects of TGFβ on PARP-1 were dependent on canonical Smad signaling, siRNA of SMAD3 were applied to knockdown SMAD3, which abrogated the TGFβ-induced downregulation of PARP-1 at mRNA levels and protein levels. (Figure 6.22 A) Meanwhile, the transfection efficiency of siRNA of Smad3 was confirmed by significantly decreased mRNA levels and protein levels. (Figure 6.22 B)
Moreover, selective inhibition of TGFβ signaling in bleomycin-induced fibrosis by treatment of mice with the TGFβ receptor type I kinase inhibitor SD208 prevented the downregulation of PARP-1 in vivo (Figure 6.23 A). Consistently, western blot analysis showed that inhibition of TGFβ signaling by SD208 incubation in SSc fibroblasts upregulated PARP-1 expression (Figure 6.23 B). These results further highlighted the central role of TGFβ in regulating PARP-1 expression in fibrosis.

Figure 6.22: siRNA-mediated knockdown of Smad3 abrogated TGFβ induced downregulation of PARP-1 in healthy fibroblasts. (A) Effects of siRNA-mediated knockdown of Smad3 on the mRNA and protein levels of PARP-1 (n=4 biological replicates with ≥ 2 technical replicates). (B) Efficiency of siRNA-mediated knockdown of Smad3 on the mRNA (left) or protein (right) levels of Smad3 in TGFβ stimulated human fibroblasts (n=4 biological replicates with ≥ 2 technical replicates).

RESULTS
RESULTS

6.2.2 Inhibition of PARP-1

6.2.2.1 Inhibition of PARP-1 enhances fibroblast activation and collagen release

We next investigated whether PARP-1 in turn might regulate TGFβ-induced fibroblast activation. Inhibition of PARP-1 by 3AB enhanced the stimulatory effects of TGFβ on myofibroblast differentiation. Incubation with 3AB further increased the mRNA level of α-SMA (Figure 6.24 A), the expression of α-SMA protein and stress fiber formation (Figure 6.24 B) as compared to fibroblasts stimulated with TGFβ alone.
**RESULTS**

**Figure 6.24:** Inhibition of PARP-1 promotes fibroblasts differentiation. (A) mRNA levels of α-SMA in TGFβ stimulated human fibroblasts with or without PARP-1 inhibition. (B) Immunofluorescence staining and quantification for α-SMA and stress fibers in fibroblasts incubated with TGFβ and 3AB at 200-fold magnification (n=4 biological replicates with ≥ 2 technical replicates).

Inhibition of PARP-1 also fostered TGFβ-induced collagen release and increased the mRNA levels of col1a1 and col1a2 as well as the release of collagen protein into the supernatant (Figure 6.25 A). In addition, the levels of type I collagen protein also increased after PARP-1 inhibition by 3AB in TGFβ stimulated fibroblast lysate (Figure 6.25 B).
Figure 6.25: Inhibition of PARP-1 promotes fibroblasts collagen release. (A) mRNA and protein levels of collagen in TGFβ stimulated healthy human fibroblasts after inhibition of PARP-1 by 3AB. (B) Protein levels of type I collagen in TGFβ stimulated healthy human fibroblasts increased after inhibition of PARP-1 by 3AB.

In contrast, overexpression of PARP-1 inhibited TGFβ-induced fibroblast activation and reduced the mRNA levels of α-SMA, col1a1 and col1a2 (Figure 6.26 A). Type I collagen protein in fibroblast lysate and the release of collagen protein were also inhibited by PARP-1 overexpression (Figure 6.26 A and B).
RESULTS

6.2.2.2 PARP-1 binds to Smad3 to enhance TGFβ signaling

To further characterize the effect of PARP-1 on TGFβ signaling, we analyzed the levels of pSmad3 after inhibition of PARP-1 in TGFβ stimulated fibroblasts. Treatment with 3AB further enhanced the accumulation of pSmad3 in TGFβ stimulated fibroblasts as shown by immunofluorescence and western blot (Figure 6.27 A and B).
Figure 6.27: Inhibition of PARP-1 enhances TGFβ induced pSmad3 accumulation. (A) Immunofluorescence staining pictures for pSmad3 in TGFβ stimulated fibroblasts incubated with 3AB at 200-fold magnification and quantification of the relative fluorescence intensity of pSmad3. (B) Western blot for pSmad3 in TGFβ stimulated fibroblasts incubated with 3AB and quantification of the relative integrated density of pSmad3 (n=3 biological replicates with 2 technical replicates).

Smad-dependent reporter assays showed increased Smad-dependent transcription upon
inhibition of PARP-1 (Figure 6.28 A). Consistently, the mRNA levels of the classical TGFβ / Smad target genes PAI-1, SMAD7 and CTGF were further increased by inhibition of PARP-1 in TGFβ stimulated cells (Figure 6.28 A). ChIP assays further demonstrated that inhibition of PARP1 enhances TGFβ-dependent binding of Smad3 to SBE in the promoter of the α-SMA gene (Figure 6.28 B).

Figure 6.28: Inhibition of PARP-1 enhances TGFβ signaling. (A) Transcriptional activity of Smad in Smad-binding elements (SBEs) reporter assays in fibroblasts stimulated with TGFβ and co-incubated with 3AB; mRNA levels of the TGFβ target genes PAI-1, SMAD7 and CTGF after inhibition of PARP-1 (n=4 biological replicates with 2 technical replicates). (B) PARP-1 inhibition enhanced the effects of TGFβ stimulation on binding of Smad3 to SBEs of the human α-SMA promoter in ChIP assays (n=4 biological replicates with 2 technical replicates).

Co-staining of fibroblasts for PARylation, pSmad2/3 and Smad2/3 demonstrated that the staining for PARylation colocalizes with the staining for pSmad2/3 and Smad2/3 in fibroblasts
stimulated with TGFβ (Figure 6.29 A) and that this PARylation is effectively blocked by 3AB (Figure 6.29 B).

**Figure 6.29:** PARylation is blocked by 3AB. (A) Representative immunofluorescence pictures of PAR, pSmad2/3 and Smad2/3 at 1000-fold magnification. (B) Quantification of related integrated density of PARylated protein. (n=4 biological replicates with ≥ 2 technical replicates).

Furthermore, western blot of PARylated protein upon TGFβ stimulation and 3AB incubation at different time points confirmed that PARylation is inhibited by 3AB (Figure 6.30).
To further investigate a potential direct binding of Smad3 by PARP-1 and subsequent PARylation of Smad3, we performed co-immunoprecipitation assays. Precipitation with antibodies against Smad3 demonstrated that stimulation with TGFβ promoted binding of PARP-1 to Smad3 and PARylation of Smad3 (Figure 6.31 A). TGFβ-induced PARylation of Smad3 was inhibited by co-incubation with 3AB. We additionally performed co-immunoprecipitation using antibodies against PARylation (Figure 6.31 B). The results confirmed the TGFβ-induced PARylation of Smad3 and of phosphorylated Smad3.
6.2.3 Targeting PARP-1 in experimental fibrosis

6.2.3.1 Genetic and pharmacologic inactivation of PARP-1 exacerbates bleomycin-induced dermal fibrosis

We next aimed to analyze the role of PARP-1 in experimental fibrosis. We firstly evaluated the outcome of PARP-1-deficient (PARP-1$^{-/-}$) mice in bleomycin-induced fibrosis. In the absence of bleomycin, PARP-1$^{-/-}$ mice demonstrated a normal skin phenotype (Figure 6.32 A). However, PARP-1$^{-/-}$ mice developed more severe fibrosis upon injection of bleomycin as compared to PARP-1$^{+/+}$ littermates (Figure 6.32 A). Skin thickening, hydroxyproline content and differentiation of resting fibroblasts into metabolically active myofibroblasts were more pronounced in PARP-1$^{-/-}$ mice injected with bleomycin as compared to PARP-1$^{+/+}$ mice (Figures 6.32 B-D).
RESULTS

Figure 6.32: Deficiency in PARP-1 exacerbates bleomycin-induced skin fibrosis. (A) Representative trichrome-stained and hematoxylin and eosin–stained skin sections at 100-fold magnification. (B-D) Effects of PARP-1-deficiency on bleomycin-induced dermal thickening (B), the hydroxyproline content (C) and the myofibroblast counts (D) (n = 6 mice for all groups and outcomes).

We also observed increased mRNA levels of the classical TGFβ / Smad target genes PAI-1, SMAD7 and CTGF compared to PARP-1+/+ mice (Figure 6.33 A-C).

Figure 6.33: Increased transcription of TGFβ / Smad target genes in PARP-1 deficient mice challenged with bleomycin as compared to wildtype littermates. (A) mRNA levels of PAI-1 (B) SMAD7 (C) CTGF (n = 6).

Consistent with the findings in PARP-1−/− mice, pharmacological inactivation of PARP-1 by
RESULTS

two structurally non-related inhibitors, 3AB and PJ34, also exacerbated bleomycin-induced skin fibrosis (Figure 6.34) with enhanced skin thickening (Figure 6.34 B), collagen accumulation (Figure 6.34 C), and myofibroblast differentiation (Figure 6.34 D) as compared to vehicle-treated, bleomycin-challenged mice.

![Trichrom-stained and hematoxylin and eosin–stained skin sections](image)

**Figure 6.34**: Pharmacologic inhibition of PARP-1 by 3AB or PJ34 exacerbates bleomycin-induced skin fibrosis. (A) Representative trichrom-stained and hematoxylin and eosin–stained skin sections are shown at 100-fold magnification. (B-D) Inhibition of PARP-1 enhanced bleomycin-induced dermal thickening (B), increased the hydroxyproline content (C) and stimulated myofibroblast differentiation (D) (n = 6 for each group in all readouts).

### 6.2.3.2 Inhibition of PARP-1 promotes fibrosis in Tsk-1 mice

Bleomycin-induced dermal fibrosis serves as a model for early, inflammatory stages of SSc, but is less appropriate for less inflammatory subgroups of SSc patients. To overcome this
RESULTS

limitation, we aimed to study the effects of PARP-1 inactivation in the Tsk-1 model that resembles non-inflammatory stages of SSc. Inhibition of PARP-1 by 3AB enhanced the Tsk-1 phenotype (Figure 6.35 A). Hypodermal thickening, accumulation of collagen and myofibroblast counts were all increased in Tsk-1 mice treated with 3AB as compared to vehicle treated Tsk-1 mice (Figure 6.35 B-D).

**Figure 6.35:** Inhibition of PARP-1 by 3AB promotes fibrosis in Tsk-1 mice. **(A)** Representative trichrome-stained and hematoxylin and eosin–stained skin sections at 100-fold magnification. **(B-D)** Hypodermal thickening **(B)**, hydroxyproline content **(C)** and myofibroblast counts **(D)** in control mice, Tsk-1 mice and Tsk-1 mice treated with 3AB. (n = 6 mice for all groups and outcomes).
6.3 Cross activation of JAK2 by JAK1 and preventive implication for the treatment of fibrosis

6.3.1 Inhibition of JAKs in vitro

6.3.1.1 Inhibition of JAKs decreases fibroblast activation and collagen release

To evaluate the effect of the selective JAK2 inhibitor TG101209 on TGFβ stimulation in healthy human fibroblasts, TGFβ stimulated fibroblasts were incubated with TG101209 for 1 day, 3 days, 7 days or 13 days. The stimulatory effects of TGFβ on fibroblasts with upregulation of the mRNA levels of col1a1, col1a2 and of collagen protein were reduced after 1 day TG101209 incubation compared with sham-treated fibroblasts (Figure 6.36). However, after 3 days, 7 days and 13 days, the inhibitory effect of TG101209 on collagen release decreased and TG101209 became progressively ineffective (Figure 6.36).

6.3.1.2 Inhibition of JAKs decreases pJAK1/pJAK2 expression

To analyze the levels of pJAK1 and pJAK2 in TGFβ stimulated human fibroblasts, double staining of pJAK1 and pJAK2 was performed (Figure 6.37). Immunofluorescence staining showed that pJAK1 and pJAK2 accumulated upon stimulation with TGFβ. Short-term incubation of fibroblasts with TG101209 for one day decreased the levels of pJAK2 without affecting the levels of pJAK1 or the total levels of JAK1 and JAK2. In contrast, the inhibitory
effect of TG101209 on pJAK2 decreased over time and long-time incubation with TG101209 did not significantly reduce the TGFβ-induced accumulation of pJAK2 after 3 days, 7 days or 13 days (Figure 6.37 A and B).

Figure 6.37: Long-time incubation of TGFβ stimulated fibroblasts with TG101209. (A) pJAK1 and pJAK2 expression after stimulation with TGFβ and incubation with TG101209 for 1 day, 3 days, 7 days or 13 days. Representative immunofluorescence pictures are shown at 1000-fold magnification (B) Quantitative analysis of pJAK1 and pJAK2. (n=3)

Inhibition of HSP90 has recently been shown to promote JAK2 degradation [111]. We therefore analyzed whether co-incubation with an inhibitor of HSP90, 17-DMAG, could prevent the loss of efficacy of selective JAK2 inhibition with prolonged incubation. The levels of pJAK1 and pJAK2 were determined by immunofluorescence staining (Figure 6.38 A). Quantitative analysis showed consistent with previous results that inhibition of JAK2 by TG101209 modulated the levels of pJAK2 without affecting the total levels of JAK2 and that the effects of TG101209 decreased upon long-term incubation. Incubation with 17-DMAG
reduced the levels of pJAK2 as well as the total levels of JAK2 to a comparable extent. In contrast to TG101209, 17-DMAG was also effective at long-term incubation and repressed the TGFβ-induced activation of JAK2 throughout the observation period (Figure 6.38 B). Co-incubation with TG101209 and 17-DMAG was particularly effective in inhibiting the TGFβ-induced accumulation of pJAK2 (Figure 6.38 B).
Consistent with the persistent downregulation of pJAK2, 17-DMAG also inhibited the profibrotic effects of TGFβ on fibroblasts after 1 day, 3 days and 7 days with decreased collagen release and reduced myofibroblast differentiation (Figure 6.39). Most pronounced anti-fibrotic effects were observed by co-incubation with TG101209 and 17-DMAG.

![Figure 6.39: Collagen release after Long-time incubation of JAK2 and HSP90 inhibitors in TGFβ stimulated fibroblasts. (n=3 with ≥ 3 technical replicates)](image)

The results obtained by immunofluorescence staining were confirmed by western blot. Analyses of protein lysates from fibroblasts stimulated with TGFβ for 3 days and coinubated with 17-DMAG, TG101209 or Ruxolitinib demonstrated an increase of pJAK2 upon stimulation with TGFβ and decreased in fibroblasts incubated with TG101209, 17-DMAG or the combined inhibitor of JAK1 and JAK2, Ruxolitinib. Only incubation with 17-DMAG, but not with TG101209 or Ruxolitinib decreased the total levels of JAK2 (Figure 6.40 A and B).
6.3.2 Inhibition of JAKs in experimental fibrosis

6.3.2.1 Pharmacologic inactivation of JAKs by different pharmacologic approaches in bleomycin-induced lung fibrosis

To analyze the anti-fibrotic effect of different pharmaceutical strategies to target JAK2 in vivo, we firstly employed the mouse model of bleomycin-induced pulmonary fibrosis. TG101209, 17-DMAG or Ruxolitinib treatment were initiated in mice model. Sirius red staining and subsequent quantification of the stained area demonstrated that treatment of
bleomycin-challenged mice with TG101209, 17-DMAG or Ruxolitinib all significantly reduced the fibrotic area as compared with vehicle-treated, bleomycin-challenged mice (Figure 6.41 A and B). Treatment with TG101209, 17-DMAG or Ruxolitinib also reduced the numbers of myofibroblasts and the hydroxyproline content. (Figure 6.41 B). Of note, combination therapy with TG101209 and 17-DMAG more effectively reduced the fibrotic area and the myofibroblast counts as compared to monotherapy with either TG101209 or 17-DMAG. In addition, Ruxolitinib decreased fibrotic area and the myofibroblast counts more prominently compared with TG101209 treatment.

Figure 6.41: Bleomycin-induced pulmonary fibrosis. (A) Representative Sirius red stainings of lungs from mice challenged with bleomycin and treated with TG101209, 17-DMAG or Ruxolitinib either alone or in combinations
are shown at 100-fold magnification. (B) Effects of TG101209, 17-DMAG or Ruxolitinib on the fibrotic area, hydroxyproline content and myofibroblast counts (n=6).

Ashcroft scores as an outcome of severe distortion of structure, large fibrous areas, and honeycomb lesions [151-153] that is also used for histological grading of patients with idiopathic pulmonary fibrosis, demonstrated a significant amelioration of bleomycin-induced pulmonary damage in mice treated with TG101209, 17-DMAG and Ruloxitinib (Figure 6.42 A). Combined inhibition of JAK2 and HSP90 by cotreatment with TG101209 and 17-DMAG was more effective than each inhibitor alone. Moreover, the anti-fibrotic effects of combined inhibition of JAK1 and JAK2 by Ruxolitinib were also more pronounced than with the selective JAK2 inhibitor TG101209 (Figure 6.42 B).
Figure 6.42: Ashcroft scores in the model of bleomycin-induced pulmonary fibrosis. (A) Representative Trichrome staining pictures of bleomycin induced lung fibrosis treated with TG101209, 17-DMAG or Ruxolitinib are shown at 100-fold magnification. (B) Treatment with TG101209, 17-DMAG or Ruxolitinib reduced bleomycin-induced lung Ashcroft score as compared to vehicle-treated mice challenged with bleomycin (n=6).

Furthermore, the mRNA levels of type I collagen and of fibronectin were analyzed in vehicle-treated fibrotic and non-fibrotic control groups and in the four different treatment groups. Treatment with TG101209, 17-DMAG and Ruxolitinib reduced the mRNA levels of col1a1, col1a2 and fibronectin in the lungs of bleomycin-challenged mice (Figure 6.43). The mRNA level of col1a1 further decreased in TG101209 and 17-DMAG combined treatment group compared with single treatment. In addition, Ruxolitinib more effectively inhibited col1a1 mRNA expression than TG101209 (Figure 6.43).

Figure 6.43: mRNA levels of major proteins of the ECM in the lungs of mice challenged with bleomycin and treated with TG101209, 17-DMAG or Ruxolitinib (n=6).

6.3.2.2 Pharmacologic targeting of JAK2 by different strategies in TBR-induced skin fibrosis

Next, we compared the effects of the different strategies to target JAK2 signaling in the mouse model of TBR-induced skin fibrosis model. Treatment with TG101209, 17-DMAG and
Ruxolitinib reduced TBR-induced dermal thickening, prevented myofibroblast differentiation and reduced the hydroxyproline content (Figure 6.44 A and B). Co-treatment with TG101209 and 17-DMAG was more effective as compared to monotherapy with TG101209 or 17-DMAG (Figure 6.44 B). Furthermore, the anti-fibrotic effects of combined inhibition of JAK1 and JAK2 by Ruxolitinib were also more pronounced than with the selective JAK2 inhibitor TG101209.

Figure 6.44: TBR-induced dermal fibrosis. (A) Representative H&E staining pictures of TBR-induced skin fibrosis treated with TG101209, 17-DMAG or Ruxolitinib are shown at 100-fold magnification. (B) Effects of the treatment with TG101209, 17-DMAG or Ruxolitinib on dermal thickening, myofibroblast differentiation and hydroxyproline content (n=6)
7 DISCUSSION

7.1 Inhibition of CK2 prevents activation of JAK2/STAT3 and ameliorates fibrotic disease

Aberrant TGFβ signaling is a hallmark of SSc and other fibrotic diseases. However, the molecular mechanisms underlying the persistent activation of TGFβ signaling are incompletely understood. Here, we demonstrated that TGFβ stimulates the expression of CK2α and CK2β. TGFβ upregulates mRNA and protein levels of CK2α and CK2β in fibroblasts, whereas inhibition of TGFβ signaling prevents the induction of both CK2 subunits in fibrotic skin. The activation of CK2 in turn is required for the pro-fibrotic effects of TGFβ on fibroblasts. Inhibition of CK2 ameliorates the pro-fibrotic effects of TGFβ and reduces the release of extracellular matrix in vitro and in vivo. The induction of CK2 by TGFβ may thus enhance the pro-fibrotic effects of TGFβ thereby creating a vicious cycle that contributes to aberrant TGFβ signaling and persistent fibroblast activation in SSc and in other fibrotic diseases.

We demonstrate that CK2 does not modify canonical, Smad-dependent TGFβ signaling, but rather regulates TGFβ induced activation of JAK2. JAK2 has recently been identified as novel intracellular mediator of TGFβ signaling. JAK2 is activated in SSc in a TGFβ-dependent manner and pharmacologic or genetic inactivation of JAK2 reduces the pro-fibrotic effects of TGFβ. Although the precise mechanisms are unknown, we demonstrate here that inhibition of CK2 prevents phosphorylation of JAK2, thereby reducing activation and nuclear translocation of its downstream mediator STAT3 in cultured human fibroblasts and in two mouse models of SSc. Consistent with these findings, CK2 inhibitors have recently been shown to suppress the constitutive autophosphorylation of mutated JAK2V617F and induce apoptosis in cells from patients with polycythemia vera [85].

The potent anti-fibrotic effects of CK2 inhibitors in vitro and in different mouse models in vivo indicated that CK2 may also positively regulate other pro-fibrotic pathways in addition to
JAK2 signaling. Indeed, accumulating evidence from the cancer field demonstrates that CK2 can enhance the stability of β-catenin, thus facilitating canonical Wnt signaling and tumorigenesis [154]. However, further studies are required to investigate the effect of CK2 inhibitors on Wnt signaling in fibrotic diseases and to analyze the relative contribution of targeting JAK2 and Wnt signaling to the anti-fibrotic effects of CK2 inhibitors.

We demonstrated that CK2 inhibitors exert potent anti-fibrotic effects in two different mouse models in pharmacologically relevant doses. The CK2 inhibitor TBB effectively reduced the histological features of fibrosis, decreased collagen content and prevented the differentiation of resting fibroblasts into myofibroblasts in the mouse models of bleomycin- and TBR induced fibrosis. Of note, TBB was well tolerated and toxic effects were neither observed by clinically nor on necroscopy. Both mouse models mimic different aspects and different stages of SSc. The mouse model of bleomycin-induced skin fibrosis resembles early, inflammatory stages of SSc with leukocyte infiltration and subsequent activation of fibroblasts by leukocyte-derived mediators. In contrast, the TBR model resembles later, non-inflammatory stages of SSc with endogenous activation of SSc fibroblasts and persistently activated TGFβ signaling. The potent anti-fibrotic effects of TBB in those models indicate that CK2 inhibition may be effective in the inflammatory subset of SSc patients as well as in SSc patients in later, less inflammatory stages. These findings could have translational implications, because first CK2 inhibitors recently entered clinical trials in patients with different malignancies (http://clinicaltrials.gov; NCT00891280 and NCT01199718). In addition to the more advanced programs with ATP-competitive compounds, several new, non ATP-competitive inhibitors are currently in clinical development [155]. However, further preclinical evaluation of CK2 in fibrotic diseases is warranted. This should include analyses of the anti-fibrotic effects of CK2 inhibitors in other organ systems,
evaluation of the effects on established fibrosis and investigation of the role of CK2 on endothelial cells and on SSc-associated microvascular disease.

In summary, we demonstrated that TGFβ induces CK2α and CK2β, which are in turn required for the pro-fibrotic effects of TGFβ. Inactivation of CK2 prevents the TGFβ induced activation of JAK2 / STAT3 and significantly ameliorates fibrosis in different mouse models of SSc. These findings may have translational implications, as CK2 inhibitors were well tolerated in anti-fibrotic doses and potent CK2 inhibitors are currently in clinical trials.

7.2 PARP-1 plays a central role in fibroblast activation and tissue fibrosis

We demonstrate in the present study that the expression of PARP-1 is decreased in SSc patients and in murine models of skin fibrosis. The downregulation of PARP-1 persisted in vitro and resulted in reduced PARylation in cultured SSc fibroblasts even after several passages in culture. We provide evidence that the downregulation of PARP-1 is due to endogenous activation of TGFβ signaling in SSc fibroblasts. The impaired PARylation in SSc has profound effects on canonical TGFβ signaling. Under physiologic conditions, PARylation induces a negative feedback-loop to limit canonical TGFβ signaling. TGFβ induces binding of PARP-1 to Smad3 with subsequent PARylation of Smad3. The PARylation of Smad3 has been shown to induce dissociation of Smad3 from DNA, thereby abrogating the Smad-dependent transcription of TGFβ target genes [129]. However, our data demonstrate that chronically activated TGFβ signaling as in SSc interferes with PARP-1-induced inhibition of TGFβ / Smad signaling. Persistently high levels of TGFβ decrease the expression of PARP-1 in fibroblasts, leading to decreased association of PARP-1 with Smad3 and thus impaired PARylation-induced inactivation of Smad3. Besides regulation by TGFβ, enhanced oxidative stress may also contribute to the deregulation of PARP-1 expression in SSc, given that PARP-1 is a DNA nick sensor enzyme that is activated by DNA breaks [124, 156].
The reduced PARP-1 activity in turn further promotes canonical TGFβ/Smad signaling in SSc. Inhibition of PARylation, either by knockdown of PARP-1 or by PARP-1 inhibitors stimulated Smad-dependent transcription with enhanced Smad reporter activity and increased levels of classical Smad target genes. Consistent with the central role of TGFβ signaling in fibrogenesis [157], the enhanced TGFβ/Smad signaling in skin fibrosis translates directly into hyper-activation of fibroblasts. Inactivation of PARP-1 enhanced the stimulatory effects of TGFβ on myofibroblast differentiation and on the release of collagen in vitro. Moreover, inhibition of PAPR1 also exacerbates skin fibrosis in bleomycin-challenged mice and in Tsk-1 mice with increased differentiation of resting fibroblasts into myofibroblasts and increased accumulation of collagen in fibrotic skin. Of note, the role of PARP-1 in fibrosis may differ depending on the affected organ systems. In internal organs such as liver and kidneys, PARP-1 promotes epithelial-to-mesenchymal differentiation (EMT) and fibrosis [158, 159]. Consistent with the different outcomes in fibrosis, inhibitory as well as stimulatory effects of PARP-1 on Smad signaling have been reported in vitro[129, 130]. Those different outcomes are likely explained by cell- and context specific effects of PARP-1. In the context of fibrosis, PARP-1 fosters the transdifferentiation of epithelial cells into fibroblasts and may thus promote fibrosis in organs where myofibroblasts primarily descend from transdifferentiated epithelial cells. However, PARP-1 negatively regulates TGFβ/Smad signaling in resident fibroblasts and thus promotes fibrosis in tissues such as the skin where myofibroblasts are predominantly derived from resident mesenchymal cells independently of EMT.

Our data further also provide evidence that different members of the PARP family have opposing effects on fibroblast activation and tissue fibrosis. PARP-5a and PARP-5b, which are also known as tankyrases, form a subfamily with in the PARP family [160]. Unlike other PARPs, PARP-5a and PARP-5b can PARylate axin, a central component of the β-catenin destruction complex. PARylation of axin increases its stability, thereby fostering the
degradation of β-catenin, which inhibits canonical Wnt-signaling [160, 161]. Consistent with the potent stimulatory effects of Wnt signaling on fibroblast activation and collagen synthesis [30, 144, 162], selective inactivation of PARP-5a and PARP-5b demonstrated potent anti-fibrotic effects in preclinical models of fibrosis. Indeed, tankyrase inhibitors are currently considered as candidates for clinical trials in fibrotic diseases [145, 163]. In contrast to the anti-fibrotic effects of tankyrases, inactivation of PARP-1 exerts pro-fibrotic effects by enhancing canonical TGF-β signaling. Moreover, with non-selective PARP-1 inhibitors such as 3AB and PJ34, the anti-fibrotic effects of targeting PARP-5a and PARP-5b are outweighed by the pro-fibrotic effects of inactivating PARP-1. Those findings have direct implications on the developmental program with tankyrase inhibitors in fibrotic diseases, because they warrant the use of highly selective tankyrase inhibitors without effects on PARP-1.

In summary, our study identifies PARP-1 as a central regulator of skin fibrosis. Stimulation with TGFβ recruits PARP-1 to Smad3, resulting in PARylation-induced inactivation of Smad3. However, in SSc, this regulatory feedback loop is inactivated by downregulation of PARP-1. The inactivation of PARP-1 fosters canonical TGFβ signaling, stimulates fibroblast activation and exacerbates experimental fibrosis and may thus contribute to persistent fibroblast activation and progression of fibrosis in SSc.

7.3 Cross activation of JAK2 by JAK1 and preventive implication for the treatment of fibrosis

TGFβ activates JAK2 with accumulation of pJAK2 and the activation of JAK2 has been shown to contribute to the pro-fibrotic effects of TGFβ [138]. Short-term incubation with selective JAK2 inhibitors effectively blocked TGFβ-induced fibroblast activation and fibrosis. We now demonstrate that the efficacy of JAK2 inhibitors decreases over time. After an initial suppression, pJAK2 re-accumulates and markers of fibroblast activation re-increase with
prolonged incubation with JAK2 inhibitors. Based on recent finding in myeloproliferative neoplasm cancer cells [102], we hypothesized that the re-activation of JAK2 might be mediated by trans-phosphorylation of JAK2 by JAK1, which remains active upon incubation with modern, highly selective JAK2 inhibitors such as TG101209. Consistent with this hypothesis, we demonstrate that TGFβ activates JAK1 and promotes dimerization of JAK1 with JAK2 in fibroblasts to induce trans-phosphorylation of JAK2. As SSc and other fibrotic disease would require chronic treatment, the decreasing efficacy of JAK2 inhibition upon long-time application would be a major limitation. We thus tested potential strategies to overcome the loss of efficacy of JAK2 inhibition with prolonged treatment: (I) to prevent the transactivation of JAK2 by JAK1 with pan-JAK inhibitors, such as ruxolitinib, which is well tolerated and approved for clinical use; (II) to simultaneously treat with HSP90 inhibitors, which target JAK2 signaling by destabilizing JAK2 protein. This approach is one of clinical relevance as HSP90 inhibitors themselves exert anti-fibrotic effects [100, 164] and several HSP90 inhibitors are in advanced clinical testing (clinicaltrial.gov).

Both approaches effectively prevented JAK1-induced trans-activation of JAK2. The persistent inhibition of JAK2 translated into effective downregulation of TGFβ signaling and potent anti-fibrotic effects in two different mouse models. Of note, treatment with the pan-JAK inhibition ruxolitinib or with TG101209 in combination with the HSP90 inhibitor 17-DMAG reduced histological fibrosis scores and myofibroblast counts more effectively than selective JAK2 inhibitor TG101209, thus providing proof for the importance to target JAK1-dependent trans-activation of JAK2 in fibrotic condition.

7.4 Conclusion
This study elucidates different mechanisms of fibroblast activation and identifies novel
potential targets for the treatment of fibrosis. First of all, we demonstrated a link between CK2 inhibition and JAK2/STAT3 signaling. Inactivation of CK2 by TBB prevents the TGFβ induced activation of JAK2/STAT3 and significantly ameliorates fibrosis in different mouse models of SSc. Secondly, we demonstrated that PARP-1 is a central regulator of skin fibrosis by interacting with TGFβ signaling. PARylation induces inactivation of Smad3, which demonstrated that the regulatory feedback loop is in activated by downregulation of PARP-1 in SSc. Finally, we establish two strategies to overcome the loss of efficacy of JAK2 inhibition with prolonged treatment. Combined treatment with selective JAK2 inhibitors in combination with HSP90 inhibitors or combined inhibition of JAK1 and JAK2 can prevent the reactivation of JAK2 signaling and is more effective than monotherapies in experimental models of skin and lung fibrosis. These findings might have translational implications. Since inhibitors for CK2, JAK2 and HSP90 are already approved or are currently evaluated in clinical trials for other diseases, these findings might stimulate the initiation of clinical trials in patients with SSc and other fibrotic diseases.
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ABBREVIATIONS

3AB 3-Aminobenzamide
ADP adenosine di-phosphate
AF Alexa Fluor
AP Alkaline Phosphatase
ATP adenosine tri-phosphate
bid *lat.: bis in die* (twice daily)
BSA bovine serum albumin
CK2 Casein Kinase 2
cDNA complementary desoxyribonucleic acid
COL collagen
Ct Threshold cycle
CTGF connective tissue growth factor
DAB 3,3-Diaminobenzidine
DAPI 4',6-diamidino-2-phenylindole
ddH2O double distilled water
DMEM Dulbecco’s modified Eagle medium
DMSO Dimethyl sulfoxide
DNA desoxyribonucleic acid
DTT Dithiothreitol
ECM extracellular matrix
EDTA Ethylenediamine tetraacetic acid
e.g. *lat.: exempli gratia* (for example)
EGTA ethylene glycol tetraacetic acid
ELISA Enzyme-linked Immunosorbent Assay
FCS fetal calf serum
g gravitational force
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<th>Abbreviation</th>
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<tr>
<td>HE</td>
<td>hematoxylin-eosin staining</td>
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<tr>
<td>HRP</td>
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<td>IC50</td>
<td>half maximal inhibitory concentration</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>SMAD</td>
<td>mothers against decapentaplegic homolog</td>
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**Units**

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Greek letters

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$\beta$  
$\delta / \Delta$  
$\mu$

alpha
beta
delta
mu

Unit prefixes

k  
m  
$\mu$  
n
kilo ($10^3$)  
milli ($10^{-3}$)  
micro ($10^{-6}$)  
nano ($10^{-9}$)
10 CURRICULUM VITAE

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Department of Internal Medicine 3 and Institute for
Clinical Immunology at the University of Erlangen-
Nuremberg: “Novel insights into the aberrant
activation of fibroblasts in systemic sclerosis -
Casein kinase 2, Poly(ADP-ribose)-Polymerase 1,
and Janus kinase1mediated transactivation of Janus
kinase2 as novel players”
Scientific abstracts:

2013 EULAR Annual European Congress of Rheumatology
“Inhibition of Casein Kinase-2 alleviates the profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis.”
(Oral presentation)

2013 ACR/ARHP Annual Scientific Meeting
“Inhibition of Casein kinase II reduces TGFβ induced fibroblast activation and ameliorates experimental fibrosis.”
(Oral presentation)

2014 EULAR Annual European Congress of Rheumatology
“Poly(ADP-ribose) polymerase-1 (PARP-1) suppresses the profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis.”
(Poster presentation)

2014 ACR/ARHP Annual Scientific Meeting
“Poly(ADP-ribose) polymerase-1 (PARP-1) suppresses the profibrotic Effects of Transforming Growth Factor β in Systemic Sclerosis.”
(Poster presentation)

2015 14th International Workshop on Scleroderma Research
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