EXTENDED REPORT

S100A4 amplifies TGF-β-induced fibroblast activation in systemic sclerosis

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ABSTRACT

Objectives S100A4 is a calcium binding protein with regulatory functions in cell homeostasis, proliferation and differentiation that has been shown to promote cancer progression and metastasis. In the present study, we evaluated the role of S100A4 in fibroblast activation in systemic sclerosis (SSc).

Methods The expression of S100A4 was analysed in human samples, murine models of SSc and in cultured fibroblasts by real-time PCR, immunohistochemistry and western blot. The functional role of S100A4 was evaluated using siRNA, overexpression, recombinant protein and S100A4 knockout (S100A4−/−) mice. Transforming growth factor β (TGF-β) signalling was assessed by reporter assays, staining for phosphorylated Smad2/3 and analyses of target genes.

Results The expression of S100A4 was increased in SSc skin and in experimental fibrosis in a TGF-β/Smad-dependent manner. Overexpression of S100A4 or stimulation with recombinant S100A4 induced an activated phenotype in resting normal fibroblasts. In contrast, knockdown of S100A4 reduced the pro-fibrotic effects of TGF-β and decreased the release of collagen. S100A4−/− mice were protected from bleomycin-induced skin fibrosis with reduced dermal thickening, decreased hydroxyproline content and lower myofibroblast counts. Deficiency of S100A4 also ameliorated fibrosis in the tight-skin-1 (Tsk-1) mouse model.

Conclusions We characterised S100A4 as a downstream mediator of the stimulatory effects of TGF-β on fibroblasts in SSc. TGF-β induces the expression of S100A4 to stimulate the release of collagen in fibroblasts and induce fibrosis. Since S100A4 is essentially required for the pro-fibrotic effects of TGF-β and neutralising antibodies against S100A4 are currently evaluated, S100A4 might be a candidate for novel antifibrotic therapies.

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disease characterised by pathological remodelling of connective tissue. The hallmark of SSc is an excessive accumulation of extracellular matrix (ECM) caused by an increased release of collagen and other ECM components by pathologically activated fibroblasts.1 Considerable evidence demonstrates a fundamental role of transforming growth factor β (TGF-β) in the pathogenesis of SSc and other fibrotic diseases. TGF-β signalling is persistently activated in fibrotic diseases, upregulates the synthesis of collagen in fibroblasts and induces fibrosis in vivo.2 Moreover, TGF-β induces an expression profile in resting normal fibroblasts that is reminiscent of SSc fibroblasts. However, the knowledge of the central role of TGF-β in the aberrant activation of SSc has not yet been successfully translated into antifibrotic therapies.

S100A4 (also known as metastasin, calvasculin, pEL98, 18A2, FSP1, p9Ka and 42A) belongs to the family of EF-hand calcium binding S100 proteins.3 S100A4 has intracellular as well as extracellular functions. Intracellular S100A4 regulates several processes that are fundamental for cell homeostasis and differentiation such as gene transcription, cytoskeletal rearrangement and cell proliferation.4 When secreted into extracellular space, S100A4 exerts cytokine-like effects and regulates remodelling of ECM,5 angiogenesis6 and cell survival.7 S100A4 has been extensively studied in tumours and described to promote tumour progression and metastasis.1, 8 However, more recently, it became apparent that S100A4 is also implicated in pathogenesis of non-malignant human disorders. Of particular interest, S100A4 has been described to be involved in the pathogenesis of rheumatoid arthritis (RA)9, 9 and inflammatory myopathies.10 In RA, increased expression of S100A4 might contribute to the aggressive phenotype of RA synovial fibroblasts.11 Increased expression of S100A4 in inflammatory myopathies may act as a cytokine-like factor and promote muscle fibre damage by stimulating the release of pro-inflammatory cytokines from mononuclear cells.10

In the present study, we evaluated the role of S100A4 in the pathogenesis of SSc. We demonstrate that TGF-β stimulates the expression of S100A4 in SSc and that the induction of S100A4 is required for the pro-fibrotic effects of TGF-β.

MATERIAL AND METHODS

Patients and fibroblast cultures

Dermal fibroblasts were isolated from skin biopsies of SSc patients (n=20) and matched healthy volunteers (n=18) as described.12 13 All biopsies were taken from involved skin of the radial aspect of the forearm, 15±2 cm proximal of the styloid process of the ulna. Prior to RNA isolation, biopsies were stored in RNAlater (Invitrogen, Karlsruhe, Germany) and biopsies for histological analysis.
were stored in 50% ethanol after fixation. Fibroblasts from passages 4–8 were used for the experiments. All SSc patients fulfilled the criteria for SSc as suggested by LeRoy and Medsger. Sixteen patients were female, four were male. Twelve had limited cutaneous SSc, and eight had diffuse cutaneous SSc. Seven patients were in an active phase, and 13 in a stable phase according to the EUSTAR activity score. The median age of SSc patients was 47 years (range 19–66 years), and their median disease duration was 5 years (range 0.5–11 years). None of the patients received disease-modifying anti-rheumatic drugs or glucocorticoids at the time of biopsy. All patients and control subjects signed a consent form approved by the local institutional review boards.

**Stimulation with S100A4 and TGF-β and treatment with anti-S100A4 antibodies**

In selective experiments, cells were incubated with recombinant TGF-β (10 ng/mL) (PeproTech, Hamburg, Germany), or recombinant S100A4 protein at 0.01, 0.1, 1 or 10 μg/mL (R&D Systems, Abingdon, UK), or treated with anti-S100A4 antibodies (5 μg/mL). The sequences of the primer pairs used for the amplification of the human S100A4 gene are shown in online supplementary table S1. The PCR products were ligated into pcDNA3.1(+) expression vectors (Life Technologies, Darmstadt, Germany). After sequencing, plasmids were amplified in *Escherichia coli* Dh5α (Novagen, Darmstadt, Germany) and selected by ampicillin. Single colonies were picked and tested by PCR. After amplification in LB medium, plasmids were isolated using a commercial isolation kit (Promega, Madison, USA).

**Cloning of human S100A4**

The sequences of the primer pairs used for the amplification of the human S100A4 gene are shown in online supplementary table S1. The primer pairs used for the amplification of the human S100A4 gene are shown in online supplementary table S4. The sequences of all primers are summarised in online supplementary table S4. Gene expression was quantified by the ΔΔCt method. After amplification, plasmids were transfected into dermal fibroblasts using the 4D-Nucleofector (Lonza, Cologne, Germany). Gene silencing was achieved by transfection of 3 μg predesigned siRNA duplexes against S100A4 or Smad3 (both Thermo Scientific, Bonn, Germany) as described. Empty pcDNA3.1(+) vectors and non-targeting siRNAs (Life Technologies) served as controls. The sequences of the used siRNAs are shown in online supplementary table S2.

**Reporter assays**

Cells were transfected with the -353 COL1A2-Luc construct or a common pSv-β-Galactosidase control vector (β-gal). For selective experiments, cells were transfected with 1000 MOI of Ad-CAGA-Luc construct or Ad-LacZ as a control for transduction efficiency. Luciferase activity was determined using a microplate luminometer (Berthold Technologies, Bad Herrenalb, Germany).

**Quantitative real-time PCR**

Gene expression was quantified by SYBR Green real-time PCR using an ABI Prism 7300 System (Life Technologies). The sequences of all primers are summarised in online supplementary table S3. β-Actin was used to normalise for the amounts of loaded cDNA.

**Western blot analysis**

Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and incubated with anti-S100A4, anti-p-Smad3 or anti-Smad3 antibodies (both from Santa Cruz Biotechnology, Heidelberg, Germany), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Glostrup, Denmark). β-Actin served as loading control.

**Quantification of collagen protein**

The amount of soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, Northern Ireland) as previously described.

**Immunohistochemistry and immunofluorescence staining**

Skin sections and dermal fibroblasts were stained with rabbit anti-S100A4, anti-α-smooth muscle actin (α-SMA) (Sigma-Aldrich, Steinheim, Germany), anti-pro-ß1-hydroxylase-ß (P4H) (Acris Antibodies, Herford, Germany), anti-vimentin (Abcam, Cambridge, UK) or anti-p-Smad2/3 antibodies (Santa Cruz). Irrelevant isotype matched immunoglobulins were used as controls. The staining intensity was quantified using ImageJ software V1.44.

**Mouse models of fibrosis**

Mice deficient in S100A4 have previously been described. The mouse model of bleomycin-induced skin fibrosis was performed according to established protocols. S100A4-deficient mice ([S100A4<sup>−/−</sup>]) were crossed with tight-skin-1 (Tsk-1) mice. Genotyping of Tsk-1 mice and S100A4<sup>−/−</sup> mice was performed by PCR with the primers shown in online supplementary table S4.

**Analysis of murine skin**

Dermal thickness, myofibroblasts counts and hydroxyproline content were analysed as described previously.

**Statistics**

All data are presented as median with IQR, and differences between the groups were analysed using the Mann–Whitney U test. p Values are expressed as follows: 0.05 > p > 0.01 as *; 0.01 > p > 0.001 as **; p < 0.001 as ***.

**RESULTS**

**S100A4 expression is increased in SSc fibroblasts**

S100A4 protein was upregulated in the lesional skin of SSc patients as compared with healthy individuals both on the mRNA and the protein level (figure 1A,B). In healthy skin, S100A4 was expressed at low levels in a limited number of fibroblasts, but also in vascular cells and in the basal layer of epidermis (figure 1B1,B3–5). In contrast, in SSc skin, a prominent staining for S100A4 was detected in fibroblasts, with additional staining in vessels, perivascular inflammatory infiltrates and in the basal layer of epidermis (figure 1B2,B6–8). Vessels showed similar staining pattern in both healthy and SSc skin (figure 1B4,B7). Of particular interest, P4H-positive fibroblasts as well as α-SMA-positive myofibroblasts stained particularly intense for S100A4. S100A4 expression was detected in both cytoplasm and nucleus (see online supplementary figure S1A,B). Consistent with the findings in SSc, increased S100A4 mRNA and protein levels were detected in mice challenged with bleomycin (figure 1C,D) and in Tsk-1 mice (figure 1E,F). In fibrotic murine skin, S100A4 expression was prominent in the fibroblasts and perivascular inflammatory infiltrates (figure 1D1–4, F1–8). The specificity of the S100A4 staining was proven by the absence of any staining in S100A4 knockout mice (see online supplementary figure S1C).

**TGF-β induces S100A4 in a Smad3-dependent manner**

The overexpression of S100A4 persisted in cultured SSc fibroblasts, which maintained increased mRNA and protein levels of...
S100A4 compared with control fibroblasts even after several passages in culture. S100A4 expression in dermal fibroblasts was both nuclear and cytoplasmic (figure 2A,B). Based on the central role of TGF-β in fibrosis, we speculated that TGF-β signalling may drive the expression of S100A4. Indeed, S100A4-positive fibroblasts in the lesional SSc skin uniformly co-stained for p-Smad2/3, demonstrating activation of canonical TGF-β signalling in S100A4 expressing fibroblasts (figure 2C). Incubation of healthy fibroblasts with TGF-β increased the mRNA and protein levels of S100A4 (figure 2D,E). The induction of S100A4 by TGF-β was dependent on canonical TGF-β/Smad signalling, as knockdown of Smad3 (see online supplementary figure 2A,B) abrogated the stimulatory effects of TGF-β on S100A4 expression (figure 2D,E).

**S100A4 induces fibroblast activation and collagen synthesis**

To analyse the functional consequences of the upregulation of S100A4 in SSc, we overexpressed S100A4 in fibroblasts. Transfection with pcDNA3.1_S100A4 expression vectors resulted in strongly increased mRNA and protein levels of S100A4 compared with controls (see online supplementary figure 2C,D). Overexpression of S100A4 activated canonical TGF-β/Smad signalling in fibroblasts with enhanced activity in SBE reporter assays (figure 3A), increased accumulation of p-Smad3 (figure 3B) and upregulation of classical TGF-β/Smad target genes such as PAI-1 and Smad7 (figure 3C). Consistent with activated TGF-β signalling, the release of collagen was also increased. Overexpression of S100A4 stimulated the activity of COL1A2 reporter assays (figure 3D), induced the mRNA levels of COL1A1 and COL1A2 (figure 3E) and increased the release of collagen protein into the supernatants (figure 3F).

We next analysed whether extracellular S100A4 can also activate the release of collagen in fibroblasts. Indeed, recombinant S100A4 increased the expression of aSMA (see online supplementary figure S3A), induced accumulation of p-Smad3 (see online supplementary figure S3A) and upregulated the mRNA expression of PAI-1 and Smad7 (see online supplementary figure S3C). In line with activated TGF-β signalling, recombinant S100A4 induced the mRNA levels of COL1A1 and COL1A2 (see online supplementary figure S3D) and increased the release of collagen protein into the supernatants in a dose-dependent manner (see online supplementary figure S3E). Stimulation with S100A4 did not alter the expression of matrix metalloproteinase-1 (MMP-1) (see online supplementary figure S3F), MMP-3 or tissue inhibitors of MMP (TIMP)-1, 2, 3 (data not shown). The stimulatory effects of TGF-β on the release of type I collagen were reduced upon treatment with anti-S100A4 antibody (see online supplementary figure S4A,B), thus supporting the theory that S100A4 protein secreted from dermal fibroblasts acts on its own in an autocrine or paracrine manner.
Knockdown of S100A4 abrogates the stimulatory effects of TGF-β on fibroblasts

Based on the findings that TGF-β induces the expression of S100A4 and that S100A4 potently activates resting fibroblasts, we speculated that S100A4 may be a downstream mediator of the pro-fibrotic effects of TGF-β. To test this hypothesis, we assessed the effects of TGF-β in S100A4 knockdown fibroblasts. Transfection of cultured fibroblasts with S100A4 siRNA effectively reduced the expression of S100A4 (see online supplementary figure S5A,B). Knockdown of S100A4 did not affect the expression of TGF-β target genes in resting cells or reduce the basal collagen synthesis (figure 4A–F), but reduced SBE reporter activity (figure 4A), Smad3 phosphorylation (figure 4B) and impaired the induction of PAI-1 and Smad7 in TGF-β-stimulated cells (figure 4C). Knockdown of S100A4 also inhibited the stimulatory effects of TGF-β on collagen synthesis with reduced COL1A2 reporter activation (figure 4D), decreased levels of COL1A1 and COL1A2 mRNA (figure 4E) and impaired release of collagen protein (figure 4F).

Similar to the results with siRNA-mediated knockdown of S100A4 in human fibroblasts, the pro-fibrotic effects of TGF-β were also blunted in murine fibroblasts isolated from S100A4−/− mice compared with fibroblasts from S100A4+/+ mice (see online supplementary figure S6A–D).

S100A4 knockout mice are protected from bleomycin-induced fibrosis

We next aimed to analyse the role of S100A4 on fibroblast activation in vivo. We first analysed the number of fibroblasts in unchallenged S100A4−/− mice and wildtype littermates. Fibroblast counts did not differ in these groups (see online supplementary figure S7A). We next evaluated the outcome of S100A4−/− in bleomycin-induced skin fibrosis.39 S100A4−/− mice were protected from bleomycin-induced fibrosis with a 74% reduction in dermal thickening compared with S100A4+/+ mice (p<0.01) (figure 5A, B). Furthermore, the hydroxyproline content in lesional skin and differentiation of resting fibroblasts into myofibroblasts were also significantly reduced in S100A4−/− mice (figure 5C,D). Consistent with the in vitro findings, activation of canonical TGF-β/Smad signalling was impaired in S100A4−/− dermal fibroblasts upon bleomycin challenge compared with S100A4+/+ littermates (figure 5E, see online supplementary figure S7B).

Deficiency in S100A4 ameliorates fibrosis in Tsk-1 mice

In the Tsk-1 model, hypodermal thickening was reduced by 66% in S100A4−/− mice carrying the Tsk-1 allele compared with Tsk-1 mice expressing normal levels of S100A4 (p<0.001) (figure 6A,B). The hydroxyproline content and the myofibroblast counts were also reduced in S100A4−/− Tsk-1 mice.
We also observed significantly lower levels of p-Smad2/3 in the dermal fibroblasts of S100A4−/− Tsk-1 mice compared with S100A4+/+ Tsk-1 littermates (figure 6E, see online supplementary figure S7C).

DISCUSSION

We demonstrate that S100A4 is upregulated in SSc and in different models of experimental fibrosis. The expression of S100A4 was strongly increased in fibrotic skin, particularly in fibroblasts, perivascular inflammatory infiltrates and in the basal layer of epidermis. Our data are in line with recent findings in other autoimmune inflammatory diseases, such as RA, inflammatory myopathies, where S100A4 was found to be expressed in macrophages, granulocytes, mast cells, fibroblasts, pericytes, endothelial cells, dendritic cells and T cells, but not B cells or stem cells. We did not observe any differences in S100A4 expression patients with limited versus diffuse cutaneous SSc, or correlations with disease duration or activity. However, the number of patients in our study was too limited for subgroup analyses and further studies in larger cohorts may be needed for final conclusions.

Our data show on multiple experimental levels that the overexpression of S100A4 in SSc is mediated by canonical TGF-β signalling: (1) The expression pattern of S100A4 colocalised with staining for p-Smad3. (2) TGF-β upregulated the mRNA and protein levels of S100A4. (3) Knockdown of Smad3 abrogated the stimulatory effects of TGF-β on S100A4 expression. (4) The expression of S100A4 was also upregulated in cultured SSc fibroblasts, which is in line with endogenous activation of TGF-β signalling in cultured SSc fibroblasts. The stimulatory effects of TGF-β on S100A4 are not restricted to fibroblasts, but TGF-β also induces S100A4 in endometrial cancer cells and in multipotent corneal progenitor cells. These findings also identify TGF-β as an important regulator of S100A4. However, other factors such as hypoxia may also contribute to the expression of S100A4 in SSc. Indeed, the oxygen levels are severely decreased in SSc and hypoxia has recently been shown to induce the expression of S100A4.

S100A4 amplifies canonical TGF-β signalling in SSc. S100A4 is not only induced by TGF-β, but also actively promotes canonical TGF-β signalling in fibroblasts. Overexpression of S100A4 or stimulation with recombinant S100A4 activated TGF-β signalling and induced Smad-dependent transcription. In contrast, inactivation of S100A4 abrogated the stimulatory effects of TGF-β on fibroblasts. Although the molecular mechanisms by which S100A4 regulates Smad-dependent transcription in SSc fibroblasts remain to be elucidated, recent findings in HEK-293T demonstrate that S100A4 can physically interact with Smad proteins in a calcium-dependent manner, suggesting that S100A4 may modulate DNA binding or cofactor recruitment of Smad proteins. Together, these findings highlight the regulatory effects of S100A4 on canonical TGF-β signalling and suggest that overexpression of S100A4 may contribute to aberrant TGF-β signalling in SSc.

Our results also demonstrate that inactivation of S100A4 exerts potent antifibrotic effects. Knockdown of S100A4 inhibits

Figure 3  Overexpression of S100A4 activates transforming growth factor β (TGF-β) signalling and stimulates the release of collagen. (A–C) Overexpression of S100A4 stimulates the activity of SBE reporter constructs (A) (n=9), induces an accumulation of p-Smad3 (B) and upregulates the mRNAs levels of the prototypical Smad target genes PAI-1 and Smad7 (C for both) (n=6 for both). (D–F) Overexpression of S100A4 stimulates the activity of COL1A2 reporter assays (D) (n=9), increases the mRNA levels of COL1A1 and COL1A2 (E for both) (n=6 for both) and upregulates the release of collagen protein (F) (n=4).

Figure 4  Inactivation of S100A4 inhibits canonical transforming growth factor β (TGF-β) signalling in fibroblasts. (A–C) siRNA-mediated knockdown of S100A4 (S100A4 siRNA) reduces the stimulatory effects of TGF-β on SBE reporter constructs (A) (n=9), inhibits Smad3 phosphorylation (p-Smad3) (B) and prevents the induction of PAI-1 and Smad7 by TGF-β (C for both) (n=6 for both). (D–F) Furthermore, knockdown of S100A4 abrogates the stimulatory effects of TGF-β on collagen synthesis with reduced activity of COL1A2 reporter assays (D) (n=9), decreased mRNA levels of COL1A1 and COL1A2 (E for both) (n=6 for both) and reduced collagen secretion (F) (n=4).

Figure 5  S100A4−/− mice are protected from bleomycin-induced skin fibrosis. The pro-fibrotic effects of bleomycin are ameliorated in S100A4−/− mice (A) with reduced dermal thickening (B), decreased hydroxyproline content (C), impaired myofibroblast differentiation (D) and reduced accumulation of p-Smad3 in fibroblasts (E). Representative images of trichrome-stained sections are shown at 200-fold magnification in (A) (n=9 for S100A4+/+ NaCl, n=6 for S100A4+/+ bleomycin, n=7 for S100A4−/− NaCl, n=6 for S100A4−/− bleomycin).
TGF-β/Smad-dependent transcription and prevents aberrant activation of canonical TGF-β signalling in murine models of SSc. The reduction of canonical TGF-β signalling translates directly into inhibitory effects on fibroblast activation. Knockdown of S100A4 prevented the TGF-β-induced upregulation of type I collagen in cultured fibroblasts. Moreover, S100A4−/− mice were protected from experimental fibrosis in two experimental models. The murine models used herein mimic different aspects of SSc.39 47 The potent antifibrotic effects of the silencing of S100A4 in both models indicate that S100A4 inhibition may be effective in inflammatory as well as in non-inflammatory subsets of SSc patients. Moreover, inactivation of S100A4 may not only be effective in models of skin fibrosis, but also in other fibrotic conditions such as pulmonary or cardiac fibrosis,48 49 highlighting that targeting of S100A4 may be an interesting antifibrotic approach. However, due to our genetic approach, we were only able to assess prevention of fibrosis. For pharmacological approaches, for example, with antibodies against S100A4, it will be important to also assess the antifibrotic effects of targeting S100A4 in therapeutic settings. Inactivation of S100A4 seems not to affect the physiologic collagen synthesis and targeting of S100A4 may thus theoretically not perturb the normal collagen homeostasis. Knockdown of S100A4 did not decrease the basal release of type I collagen from resting fibroblasts, and S100A4−/− mice display a normal architecture of the skin and of internal organs with comparable levels of collagen as in wild-type littermates. However, further studies are needed to assess the effects of targeting S100A4 on tissue homeostasis, particularly upon chronic inhibition as required for fibrotic diseases.

In summary, we demonstrate that S100A4 is a key regulator of fibroblast activation in SSc. S100A4 is induced by TGF-β and in turn amplifies the pro-fibrotic effects of canonical TGF-β/Smad signalling. Inactivation of S100A4 severely impairs TGF-β-induced fibroblast activation and ameliorates fibrosis in different mouse models. These findings have direct translational implications because different approaches to target S100A4 are currently in clinical development with first promising results. Recently, blocking antibodies against S100A4 have been shown to ameliorate psoriasis in a human psoriasis xenograft SCID mouse model.16 Furthermore, a transcriptional inhibitor of S100A4, calcimycin, inhibited colon cancer metastasis in a xenograft model.50

**Figure 6** Deficiency in S100A4 ameliorates the Tsk-1 phenotype. Fibrosis is reduced in S100A4−/− Tsk-1 mice compared with S100A4+/+ Tsk-1 mice (A) with reduced hypodermal thickening (B), decreased hydroxyproline content (C), lower myofibroblast counts (D) and decreased accumulation of p-Smad2/3 in fibroblasts (E). Representative images of trichrome-stained sections are shown at 200-fold magnification in (A). (n=10 for S100A4+/+ pa/pa, n=8 for S100A4+/+ Tsk-1, n=10 for S100A4−/− pa/pa, n=11 for S100A4−/− Tsk-1).

**Contributors** Design of the study: MT, LS and JHWD. Acquisition of data: MT, KP-Z, PZ, JA, CD, BS, AD, CB and LAC. Interpretation of data: MT, KP-Z, PZ, RB, OD, MG, GS, LS and JHWD. Manuscript preparation: MT, GS, LS and JHWD.

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