# Clinical and pathogenic significance of S100A4 overexpression in systemic sclerosis

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**Abstract**

Objectives

We have studied the Damage Associated Molecular Pattern (DAMP) protein S100A4 as a driver of fibroblast activation in systemic sclerosis (SSc).

Methods

S100A4 protein concentration was measured by ELISA in serum of SSc (n=94) and healthy controls (n=15). Protein expression in skin fibroblast cultures from diffuse cutaneous SSc (SScF, n=6) and healthy controls (NF, n=6) was assessed. Recombinant S100A4 and a high affinity anti-S100A4 neutralising monoclonal antibody (AX-202) were tested on SScF and NF.

Results

Median [range] S100A4 (ng/ml) was higher in serum of SSc (89.9 [15.0-240.0]) than healthy controls (71.4 [7.9-131.8]; p=0.027). There was association with SSc-ILD (p=0.025, n=55), scleroderma renal crisis (p=0.026, n=4). Median [range] S100A4 (ng/ml) was higher in culture supernatants of SScF (4.19 [0.52-8.42]) than NF controls (0.28 [0.02-3.29]; p<0.0001). AX-202 reduced the constitutive profibrotic gene and protein expression phenotype of SScF. Genome-wide RNAseq analysis identified an S100A4 activated signature in NF overlapping the hallmark gene expression signature of SScF. Thus, 464 differentially expressed genes (FDR<0.001 and FC>1.5) induced in NF by S100A4, were also constitutively overexpressed, and downregulated by AX-202, in SScF. Pathway mapping of these S100A4 dependent genes in SSc showed the most significant enriched Kegg® pathways (FDR<0.001) were regulation of stem cell pluripotency (4.6-fold) and metabolic pathways (1.9-fold).

Conclusion

Our findings provide compelling evidence for a profibrotic role for S100A4 in SSc and suggest that serum level may be a biomarker of major organ manifestations and disease severity. This study supports examining the therapeutic potential of targeting S100A4 in SSc.

**Introduction**

Systemic sclerosis (SSc) is characterised by fibrosis in the skin and internal organs and structural vasculopathy [1]. The aetiopathogenesis of SSc is incompletely understood although activation of fibroblasts to produce excessive extracellular matrix is a hallmark of disease and factors that regulate or determine fibroblast activation are considered central to SSc pathobiology [2], including recently identified epigenetic mechanisms [3].

S100A4 is one of a family of proteins originally defined by solubility of the prototypic members in 100% saturated ammonium sulphate [4]. In earlier differential gene expression studies examining epithelial-mesenchymal trans-differentiation it was identified and named fibroblast specific protein-1 (FSP1) [5]. However, it has subsequently been demonstrated to have a broader expression by multiple cell types including cells within the innate and adaptive immune system. S100A4 has intracellular and extracellular functions. Within the cell, the presence of S100A4 is related to apoptosis, migration, and mesenchymal cell fibrogenesis [6]. Specifically, S100A4 nuclear complex interacts with members of the proteasome complex to degrade p53, stimulating mesenchymal progenitor cell proliferation and expansion in idiopathic lung fibrosis [7].

Extracellular S100A4 has been characterised as a Damage Associated Molecular Pattern (DAMP) protein. DAMPs are alarm signals which are upregulated and released from cells in response to stress or injury that alerts the surrounding tissue to danger by triggering downstream pro-inflammatory pathways through interaction with Pattern Recognition Receptors (PRRs). S100A4 has been shown to signal via TLR4 and RAGE (receptor for advanced glycation end-products) [8,9,10] Consistent with activation of RAGE and TLR4, S100A4 induces expression and secretion of inflammatory cytokines, growth factors and MMPs to stimulate proinflammatory pathways and extracellular matrix remodelling [11, 12, 13]. Reports show that S100A4 modulates immune cell function and may impact in tumour biology by promoting tumour metastasis [14].

Previous reports highlight the potential of an antibody directed against S100A4 as a possible anti-fibrotic treatment, suggesting S100A4 trapping could have clinical and biological benefit in systemic sclerosis [15, 16]. We have studied expression and function of S100A4 in systemic sclerosis (SSc) to further explore its relevance as a driver of fibroblast activation.

**Methods**

***Patients and biological samples***

Clinical manifestations were recorded based on the assessment of the latest clinic visit. We used consensus definitions for major organ-based complications of SSc defined previously [17]. All patients met the 2013 ACR/EULAR criteria for SSc. Serum samples were also collected from age- and sex-matched healthy controls (HC). All samples were stored at -80 C until further analysis. Further details are in Suppelmentary Methods.

***Fibroblast culture***

Fibroblasts (6 cell line normal (NF) and diffuse cutaneous SSc (SScF) each) were grown to 80% confluence in DMEM with 10% foetal bovine serum and then serum starved in DMEM containing 0.5% bovine serum albumin (BSA) for overnight (16 hours). These were then treated with either increasing doses of S100A4 (0.01-10 μg/ml) or TGFβ (10 ng/ml) for an additional 24 hours for RNA and protein extraction. For inhibition experiments, cells were pre-incubated in the presence of AX-202 (1-10ug/ml) or selective ALK5 inhibitor SB431524 (10 μM/ml) for an hour prior to stimulation with either recombinant S100A4 or TGFβ. Recombinant S100A4 used in these experiments has been confirmed to be free of any significant endotoxin contamination. Isotype matched IgG1 and IgG4 antibodies (Invitrogen) were used in control experiments for anti-TLR4 and AX-202 respectively.

***S100A4 ELISA measurement***

We measured serum S100A4 and compared across the disease subsets and evaluated for any major association with ANA or skin or lung fibrosis. In addition, levels were measured in tissue culture supernatants from fibroblast monolayer cultures from SSc and healthy control skin biopsies. Human Protein S100A4 (S100A4) ELISA Kit. Catalog Number. CSB EL020632HU (Cusabio, USA). Data were expressed as ng/ml based upon the standard curve readings.

***Western blot analysis***

To investigate the effect of recombinant S100A4 protein on ECM production western blot analysis was performed as described previously [18]. The recombinant S100A4 protein was provided by Arxx Therapeutics, Oslo. Recombinant Human TGF-β1 Protein from R&D Systems (Catalog Number: 240-B). Evaluation of Col1, αSMA and CTGF in normal fibroblasts was initially performed including a range of concentrations of endotoxin-free S100A4 at 0.01, 0.1, 1 and 10 μg/ml. To investigate whether TLR4 receptor mediated signalling might mediate some of the effect of S100A4 on normal dermal fibroblasts, additional experiments using a neutralising antibody against TLR4 (Invitrogen)

was undertaken. Later experiments compared control (NDF) and SSc fibroblasts and explored the effect of AX-202, and anti-S100A4 neutralising antibody, and the pharmacological ALK5 inhibitor SB431524. Representative blots were also probed for total ERK and phospho-ERK expression to examine non-canonical TGFbeta and potential TLR4 signalling using p44/42 MAPK (Erk1/2) Antibody #9102 and Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101 (Cell Signaling Technology). Molecular weight was confirmed using standard markers and is indicated in representative blots.

Following treatment with either S100A4 or TGFβ for 24 hours, fibroblasts were then lysed in RIPA buffer and quantified. Western blot analysis was performed as previously described [17]. Protein antigens were detected by ECL (Amersham, Buckinghamshire, UK) and quantified using a camera imager (Biospectrum AC Imaging; UVP, Cambridge, UK). Arbitrary integrated density values were recorded.

***Collagen gel contraction and scratch wound migration assay***

These assays were performed as described previously and detailed in the Supplementary Methods file.

***RNA extraction and quantitative reverse PCR***

RNA was subjected to qPCR analysis using a QuantiFast SYBR Green PCR kit (Qiagen). αSMA, Col1,CTGF and S100A4 gene expression was normalized against the expression of the β-actin gene or the TATA box binding protein. Treatment of fibroblasts with the ALK5 inhibitor SB431524 was used to assess dependence of S100A4 expression and activity on canonical TGFβ signalling.

***RNAseq analysis of fibroblast cultures***

Approximately $1 \times 10^6$ fibroblast cells were collected for each sample and pelleted into RLT Plus Buffer (Qiagen). RNA extraction was performed using the RNeasy kit (Qiagen) according to the manufacturer's protocol, including on-column DNase digestion. The concentration of total RNA samples was measured using NanoDrop 8000 (Thermo Scientific, Waltham, MA, USA), and the integrity of RNA samples was determined with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Approximately 500 ng total RNA was used as an input for library generation using TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). The size of the libraries was confirmed using 2200 TapeStation and High Sensitivity D1K screen tape (Agilent Technologies), and their concentration was measured by a quantitative polymerase chain reaction–based method

(KAPA Biosystems, Wilmington, MA, USA). Libraries were sequenced on HiSeq4000 (Illumina, San Diego, CA, USA) to generate 30 × 106 single-end 50-base pair reads.

***Statistical analysis***

For serum S100A4 levels and supernatant ELISA results simple non-parametric descriptive and inferential analysis was performed using Mann Whitney test and computing absolute P values taking account of ties among values. For correlation analysis of serum S100A4 and mRSS Spearman rho (r) was calculated with two-tailed P-value. A similar approach was used for comparison of the quantitative protein expression, migration and gel contraction data.

For analysis of individual RNAseq, individual datasets were compared using the R studio statistical package. Overall PCA plot showed clustering of each of the fibroblast groups according to disease status (SSc or healthy control) and experimental treatment. Pairwise comparison was undertaken to identify differential gene expression (DGE). These data were used for genome wide comparison by volcano plot and then gene lists generated and ranked by FDR. For further analysis the threshold of FDR<0.001 and FC at least 1.5 (Log2 FC >0.58). Overlapping gene set that was significantly induced in NDF by S100A4, significantly different between SSc and control cultures and that were attenuated in SSc fibroblasts by AX-202 was defined for Kegg® pathway enrichment for further analysis and interpretation as outlined below.

**Results**

***Elevated serum levels of S100A4 in SSc associate with major organ involvement***

To evaluate if circulating S100A4 is upregulated in SSc, we measured the levels in 93 patients with SSc and 15 healthy controls. Cohort characteristics are summarised in **Table 1**. In summary, SSc samples had significantly higher S100A4 than healthy controls (HC) (median 89.92, range 15-255 and 71.41, 7.9-139.7 respectively, p=0.027) and the levels were not affected by skin subset (**Table 2, Figure 1A and B**).

**Table 1** **Summary of clinical and demographic features for study cohort**

| | n | % | |
|---|---|---|---|
| **Total** | 93 | 100 | |
| **female** | 77 | 83 | |
| **diffuse** | 49 | 53 | |
| | | | |
| **Autoantibody** | | | |
| **ANA** | 91 | 98 | |
| **ATA** | 22 | 24 | |
| **ARA** | 30 | 32 | |
| **ACA** | 27 | 29 | |
| **other** | 13 | 14 | |
| | | | |
| **Organ-based complications** | | | |
| **Lung fibrosis (ILD)** | 37 | 40 | |
| **Cardiac involvement** | 4 | 4 | |
| **PH** | 7 | 8 | |
| **SRC** | 4 | 4 | |
| **Severe GIT** | 44 | 47 | |
| | | | |
| | **mean** | **SD** | **median** |
| **Duration SSc (yr)** | 13.4 | 10.6 | 9.6 |
| **Current mRSS** | 8.9 | 6.2 | 7 |
| **Peak mRSS** | 16.1 | 9.5 | 12 |

**Table 2** **Summary of S100A4 levels (ng/ml) in serum for SSc and controls**

| | | n | mean | median | sd | range | |
|---|---|---|---|---|---|---|---|
| | | | | | | lowest | highest |
| **Diagnosis** | Controls | 15 | 71.9 | 71.4 | 32.4 | 7.9 | 139.7 |
| | SSc | 93 | 99.8 | 89.9 | 47.2 | 15.0 | 255.0 |
| **Subset** | dcSSc | 49 | 104.7 | 95.7 | 48.5 | 25.9 | 255.0 |
| | lcSSc | 43 | 94.6 | 88.7 | 43.0 | 15.0 | 243.4 |
| **Complication** | | | | | | | |
| ILD | yes | 37 | 111.1 | 96.8 | 41.3 | 51.0 | 239.8 |
| | no | 55 | 92.6 | 86.8 | 47.9 | 15.0 | 255.0 |
| Cardiac | yes | 4 | 117.3 | 117.2 | 16.9 | 95.6 | 139.2 |
| | no | 88 | 99.2 | 88.5 | 47.0 | 15.0 | 255.0 |
| SRC | yes | 4 | 160.3 | 144.5 | 58.6 | 97.1 | 255.0 |
| | no | 88 | 97.3 | 88.5 | 43.7 | 15.0 | 243.4 |
| PH | yes | 7 | 129.2 | 138.6 | 45.1 | 67.4 | 190.2 |
| | no | 85 | 97.6 | 88.7 | 45.5 | 15.0 | 255.0 |

It is known that S100A4 is implicated in development of various organ fibrosis including lung fibrosis with activation of fibroblasts. We explore if upregulated S100A4 levels associates with potential clinical manifestations. There was also statistically significant association with SSc-ILD (median 96.79 range, 51.0-239.8 compared with 86.83, 15-255 non-ILD, p=0.024), SSc renal crisis (median 144.5, range 97.1-255.0 compared with 88.5, 15.0-243.4, p=0.02, but only 4 SRC cases) and trend of increase in pulmonary hypertension (138.6, 67.4-190.2 PH compared with 88.73, 15.0-255.0 no-PH, p=0.07). No association with stratified GI disease was observed assessed by UCLA-SCTC GIT2.0 overall score (data not shown). There was a tendency for S100A4 to fall in longer disease duration (r=-0.18, p=0.09) (but no association with patient age) (data not shown) and a weak (r=0.28, p=0.07) correlation with current mRSS in dcSSc but not with peak mRSS (**Figure 1G and I**).

***SSc fibroblasts demonstrate elevated expression of S100A4***

To determine whether S100A4 is involved in the fibrotic response in SSc, Western blot analysis was performed using fibroblast lysates from early dcSSc and control forearm dermal fibroblasts. The

summary data are representative of a series of technical replicates and assays that were optimised to give clear protein results. Housekeeping protein was used as a loading and western technical control. Data are shown in **Figure 2A** with quantitation using densitometry image unit confirming that SSc cell layer had increased S100A4 expression compared with NF (n=6 for each) by Western blot analysis (median [range] RDU NF=0.0.14 [0.11-2.37]; SScF 9.09 [3.11-9.58]; p=0.0022). (**Figure 2B**).

Additional experiments examined tissue culture supernatants and showed significant elevation for SSc compared with healthy control dermal fibroblast culture medium (**Figure 2C**). Median [range] S100A4 (ng/ml) was higher in culture supernatants of SScF (4.19 [0.52-8.42]) than NF controls (0.28 [0.02-3.29]; $p<0.0001$) suggesting that in addition to increased cell layer expression there was also enhanced release of soluble S100A4 protein into SSc culture medium. Upregulation of S100A4 protein appears to be at least partly determined at the transcriptional level with elevated mRNA using qPCR and this effect appears at least partially due to canonical TGFbeta signalling as the level in SSc fibroblasts is reduced after using an ALK5 inhibitor (**Figure 2D**)

***Recombinant S100A4 promotes a fibrotic phenotype in control fibroblasts***

Previous studies have demonstrated that S100A4 may have a profibrotic effect but its role in extracellular matrix (ECM) synthesis is unclear. As collagen was significantly upregulated in SSc fibroblasts, we explored whether S100A4 may promote ECM production in dermal fibroblasts. As shown in **Figure 3A**, incubation of isolated dermal fibroblasts from healthy controls with variable concentrations of recombinant S100A4 resulted in significant concentration-dependent increase in all three ECM proteins: pro(I)collagen, $\alpha$SMA and CTGF with maximal induction at 1 ug/ml S100A4 comparable to TGF$\beta$1 (10 ng/ml) (**Figure 3**; $p<0.05$). ERK phosphorylation in response to S100A4 is also shown in **Figure 3D** and this is attenuated by blocking TLR4, confirming that TLR4 engagement may be important in the effect of S100A4 on normal dermal fibroblasts. This is further demonstrated for the profibrotic proteins with partial attenuation of the effect of S100A4 in presence of a neutralising antibody specific for TLR4 (**Figure 3D**).

Induction of a profibrotic phenotype in control fibroblasts is further shown by promotion of 3-D collagen(I) lattice contraction over 24 hours. This assay reflects migration and contraction of fibroblasts as well as fibroblast-collagen binding. For each treatment condition, we show representative experiments including data from the first experiments and biological replicates with

additional HC fibroblasts strain are shown below. The effect of recombinant S100A4 is compared with recombinant TGFβ1 (**Figure 3B**).

Dermal fibroblast migration was explored more directly using the scratch wound assay in replicate experiments there is concentration-dependent promotion of fibroblast migration leaning to smaller residual scratch wound at 48 hours approaching the effect of recombinant TGFβ1 at higher concentration (**Figure 3C**).

***The S100A4 neutralising monoclonal antibody AX-202 attenuates fibroblast activation***

Having demonstrated that S100A4 protein induces a profibrotic phenotype in control dermal fibroblast, we next explored the effect of the neutralising anti-S100A4 antibody AX-202 on fibroblasts activated with a fixed concentration (0.5 ug/ml) of S100A4. Data in **Figure 4** summarise a series of experiments using independent control fibroblasts. Preincubation with AX-202 prior to addition of recombinant S100A4 abrogated the induction of the ECM production in normal dermal fibroblasts. Representative blots and quantitation from a series of independent experiments are shown in the upper panel (**Figure 4A**). These effects were not observed using an IgG4 isotype matched control monoclonal antibody as shown in **Figure 4B**. Attenuation of S100A4 promoted contraction of fibroblasts populated collagen(I) lattices is shown with a representative experiment and quantitation for replicate studies over an incubation period of 24 hours (**Figure 4C**). It is noted that the blocking antibody appears to have peak effect on gel contraction at a concentration of 5μg/ml and less effect at higher concentration, consistent with trends also observed for fibrotic protein expression. Promotion of gel contraction together with upregulation of αSMA protein suggest that at least some of the cells are transformed into myofibroblast phenotype. Consistent with previous reports of S100A4 dependent intestinal fibroblast contractile activity in response to TGFβ1, normal dermal fibroblast migratory capacity is attenuated in a concentration dependent manner (**Figure 4D**).

***Blocking antibody against S100A4 attenuates the SSc fibroblast fibrotic phenotype***

SSc fibroblasts have a constitutive pro-fibrotic phenotype which is confirmed in a series of independent experiment summarised in **Figure 5**. Western blot analysis compared the effect of S100A4 and AX-202 with or without recombinant TGFβ1. AX-202 had no effect on unstimulated normal fibroblasts but reduced levels of all there profibrotic proteins in NDF activated by TGFβ1. Conversely, the constitutive overexpression of proteins by SScF was attenuated by AX-202 and effect of TGFβ1 on SScF was also attenuated (**Figure 5A**). To further explore the effect of recombinant

S100A4 and AX-202 on fibroblasts from healthy controls and SSc, we analysed mRNA from fibroblast monolayers by qPCR. Data in **Figure 5B** summarise a representative experiment from a series of studies. Using a specific TGFβ type1 receptor (SB431524), there was reduction in stimulatory effect of S100A4 suggesting that S100A4 may be a downstream mediator of the profibrotic effect of TGFβ in SSc (**Figure 5B**).

***Gene expression and pathway analysis for in SSc and control fibroblasts by AX-202 demonstrate a consistent anti-fibrotic effect***

Genome-wide transcriptomic analysis by RNAseq confirmed and extended our findings from RT-PCR and protein expression. Data are summarised in **Figure 6**. Biological replicates for 5 skin biopsies (SScF) taken from involved skin of early stage dcSSc were compared with 5 fibroblast strains grown from healthy donor biopsies (NDF). The effect of S100A4 on NDF was determined in the presence or absence of SB431524, a pharmacological inhibitor of ALK5, the type 1 TGFβ receptor kinase. The effect of AX-202 on SScF was assessed and the data were integrated to determine a set of genes that are differentially expressed between NDF and SScF, induced in NDF by recombinant S100A4 and significantly attenuated in SScF by AX-202.

Principal component analysis (PCA) was used for dimensional reduction to compare overall gene expression differences between the 5 different fibroblasts culture groups (**Figure 6A**). The results demonstrate that untreated NDF and SScF have the greatest separation. Treatment of NDF with S100A4 moves the cluster towards that of untreated SScF and the ALK5i largely reverses this change. When SScF are treated with AX-202 the cluster moves towards that of the untreated NDF.
We first explored the effect of recombinant S100A4 on normal dermal fibroblasts, with 1804 significantly different transcripts using FDR threshold of 0.01. Pre-treatment with the ALK5 inhibitor SB431524 substantially attenuated the gene expression signature of S100A4 on normal fibroblasts, with significant differential expression (FDR <0.01) of 1213 mRNA transcripts and with only 5 genes remaining significantly different from untreated normal fibroblasts at the same threshold (<0.01) and 8 with an FDR threshold of below 0.05. As expected, there was a typical differential gene expression profile between SSc dermal fibroblasts and normal fibroblasts that included 2351 genes (FDR<0.01). There was substantial overlap between these genes and those that were significantly modulated in the same direction by treating normal fibroblasts with S100A4. Treatment of SSc fibroblasts with AX-202 significantly attenuated the SSc gene expression with 1254 transcripts identified at the FDR threshold of 0.01. Differential gene expression is summarised in **Table 3**.

**Table 3 Summary of differential gene expression (DGE) with FDR thresholds (<0.01 and <0.05) for pairwise comparison of fibroblast RNAseq data**

| Differential gene expression comparison | Total genes | DGE FDR (n) | |
|---|---|---|---|
| | | <0.01 | <0.05 |
| NDF versus NDF-S100 | 10703 | 1804 | 2739 |
| NDF-S100 versus ALK5i-NDF-S100 | 10579 | 1213 | 1961 |
| S100-NDF-ALK5i versus NDF | 10895 | 5 | 8 |
| NDF versus SScF | 10718 | 2351 | 3423 |
| SScF versus SScF-AX-202 | 10520 | 1254 | 2144 |
| S100-NDF versus SScF | 10216 | 215 | 314 |

There was substantial overlap between the altered gene expression induced by S100A4 and the down regulation of genes in SSc by AX-202 and many of these were also shared with the differential expression signature of untreated SSc compared with control fibroblasts. To derive a set of SScF differentially expressed genes that may represent those regulated by S100A4 we interrogated the three differentially expressed gene sets using a combined threshold of FC>1.5 and FDR<0.001. In this way we defined 646 overlapping genes between those that differentiate NDF and NDF-S100A4 (n=1168), SScF and NDF (n=1503) and AX-202 treated SScF (n=685). These overlapping gene sets are summarised in **Figure 6B**.

Further analyses examining the differentially expressed genes regulated by S100A4 in normal dermal fibroblasts are shown in **Figure 6C**, including annotation of the top 20 genes by FDR. Attenuation of the gene expression signature induced in NDF by S100A4 in the presence of SB431524 is shown in **Figure 6D**. The top 20 differentially expressed genes for untreated SScF compared with NDF define a profibrotic signature aligned with previous published datasets. This is partially attenuated by treating SScF with AX-202, confirming at a genome wide dataset the results of qPCR experiments summaries in **Figure 5B**. A full list of relevant genes from this figure is included as a supplementary results file.

Having defined the 464 overlapping genes that could reflect S100A4 activation in SScF, gene set enrichment analysis was used to map pathways that were significantly enriched in this overlapping gene set. Kegg® Pathway analysis of enrichment scores for these overlapping genes is summarised in **Figure 6G**. Key pathways that were enriched significantly included glycosaminoglycan biosynthesis, pluripotency of stem cells, TGFβ signalling, Hippo signalling and metabolic pathways.

**Discussion**

Taken together the results of this study provide robust evidence supporting a potential role for S100A4 as a direct determinant of fibroblast activation in SSc and a plausible therapeutic target in this intractable disease. We show that levels of S100A4 protein are increased in SSc and that there is a correlation with key clinical traits and that levels are increased in association with important complications such as lung fibrosis. Recombinant S100A4 protein activates normal fibroblasts and promotes a fibrotic phenotype characterised by increased collagen gel contraction, enhance migration and elevated production of key proteins including collagen type I, CTGF and αSMA. Examination of mRNA levels for the relevant target genes extended these findings.

The biology of S100A4 has been elucidated from studies over the past two decades [19-22]. It was first linked to fibroblast biology when cloned and labelled FSP1 [5]. This led to studies of fibroblast activation in cancer biology and other spheres. It was used to drive gene expression in transgenic mice and for fibroblast specific genetic recombination [23]. Subsequently expression in broader range of cell types has limited use as a fibroblast marker compared with genetic regulatory elements subcloned from α2(I)collagen [24]. Previous reports have suggested a close link between TGFβ effect and S100A4 [14] and our findings also support this, based upon the overlap in gene signature with pathways know to be altered by TGFβ and most compellingly by the substantial attenuation of S100A4 gene modulation by co-incubation with an ALK5 inhibitor. Several mechanisms for this interplay between S100A4 and TGFβ have been proposed with physical interaction of S100A4 with Smad3 in renal fibroblasts. In addition, S100A4 may function as a bridge for the engagement of Smad3 and Smad4 thus regulating the TGFβ/Smad3 signalling pathway [20]. Tomcik et al. [14] reported that TGF-β1 induced S100A4 expression and enhanced fibroblast activation in systemic sclerosis at least partly through induction of the canonical TGFβ/Smad pathway. In addition, non-canonical TGFbeta signalling via MAP kinase pathways, such as ERK, are also activated by TLR4 engagement. Data from the present study suggest that TLR4 receptor activation at least partly induces a profibrotic phenotype in normal fibroblasts that is associated with ERK phosphorylation. This is consistent with published reports on S100A4 induced ERK responses via TLR4 in peripheral blood mononuclear cells from patients with rheumatoid arthritis [25]. In another experimental model of lung fibrosis, both S100A4 and TGFβ are critical factors downstream of VEGFR1- tyrosine kinase signalling, and this may underlie the potential utility of targeting this pathway in ILD [26]. Wen recently reported that niclosamide reduced S100A4 expression and fibrotic response in experimental model of chronic kidney disease [27]. However, niclosamide is not specific for S100A4 and may influence Wnt/β-catenin, mTORC1 and STAT3 signalling pathways [28].

It has been shown that S100A4 may be released from cells in culture and our studies support this by showing elevated levels in the supernatant of cultured SSc fibroblasts [29] including micro-vesicles released from cells [30]. Elevated serum levels observed in SSc together with clinical associations are consistent with an increase in the extracellular space that may directly link to intravascular levels. Recent studies of interstitial dermal blister fluid and plasma are supportive of the present dataset [31]

There are many other relevant aspects of S100A4 biology that relate to tissue repair, fibroblasts biology and relevant aspects of adaptive and innate immune function. In addition, its role intracellularly and as an extracellular mediator or modulator of other cytokines or growth factors is compelling. This broad impact is supported by our RNAseq data suggesting that additional to being a potential marker of disease severity and candidate mediator, S100A4 may be targeted therapeutically. The effects of TGFβ activation of normal fibroblasts are abrogated in vitro and the intrinsic fibrotic phenotype of SSc is attenuated. Our results provide robust support for the antifibrotic effect of a human monoclonal antibody targeting S100A4.

Degradation of the endothelial barrier in response to perivascular inflammation is critical to the altered SSc microvasculature affecting various vascular beds linking to key clinical vascular phenotypes including digital vasculopathy, PH and SRC. Paracrine interaction between extracellular S100A4 and endothelial RAGE mediating downregulation of VE-cadherin and occludin expression is reported to facilitate transmigration of melanoma cells through endothelial cell monolayer [32]. This observation supports previous report that S100A13-regulated NFκB activation in human endothelial cells that can be abrogated by sRAGE [33]. Recent studies showed that S100A4 and αSMA do not overlap in the cardiac tissues following myocardial infarct [34] indicating that both markers denote discrete cell types. In contrast, depleting αSMA [35] or S100A4 fibroblasts [36] resulted in attenuation of renal fibrosis. This supports the current knowledge that the molecular and functional divergence of myofibroblasts in a tissue and disease-specific context.

Based upon the promising data from functional studies and protein or RNA expression analysis we undertook additional experiments using explant dermal fibroblasts from healthy donors or systemic sclerosis patients. Our results confirm overlap between the genes that were differentially expressed after S100A4 treated of NDF and those that depend on ALK5 activity. Finally, we defined an overlapping cohort of genes that are upregulated by S100A4, attenuated by AX-202 in SScFB and are included in the hallmark signature of SSc-FB. These genes allow pathway mapping to give granular

insight into the pathways that are altered in SScF that may be S100A4 regulated and provide a valuable candidate gene expression signature that could be refined and validated in human clinical studies exploring potential therapeutic benefit of anti-S100A4 in human disease such as SSc.

Strengths of our study include well matched samples from of SSc patients and healthy volunteer subjects, the multilevel confirmation of findings using complementary functional assays or fibrotic phenotype, gene and protein expression including high dimensional analysis by RNAseq. However, there are also some limitations that include the relatively small number of biological replicates that cannot be expected to reflect the diversity of systemic sclerosis and the potential limitation of explant fibroblasts culture in assessing only some subpopulations of dermal fibroblasts. Nevertheless, the consistency of our findings across the different experimental platforms and the high degree of statistical significance suggests that the results are reproducible and robust. Specificity of the ALK5i and limitations of recombinant protein may also be considered unavoidable technical limitations.

In conclusion, our findings highlight that S100A4 may be a molecular marker, pathogenic mediator and potential therapeutic target in systemic sclerosis and support future trials targeting S100A4 protein in vivo as an antifibrotic therapy. A clinical trial in SSc would give insight into efficacy in a disease with high unmet need and be a relevant platform for defining safety or possible toxicity.

**Competing interests:** CPD has received research grants to the institution from Servier, Horizon, Arxx Therapeutics, and GlaxoSmithKline, consulting fees from Arxx Therapeutics, Roche, Janssen, GlaxoSmithKline, Bayer, Sanofi, Galapagos, Boehringer Ingelheim, CSL Behring, and Acceleron, and honoraria from Janssen, Boehringer Ingelheim, and Corbus.

SB, RH, JK and JH are employees of Arxx Therapeutics.

Other authors have no relevant disclosures.

**Contributorship:** CD, JK and JH conceived the study. CD, XS, RM, KC, VH obtained the data. CD, VO, JH and XS drafted the manuscript. All authors interpreted the data, edited the manuscript and approved the final submitted version.

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**Ethical approval:** This study was approved by London-Hampstead (IRAS Ref. 270295) and London-Fulham (IRAS Ref. 279682) NHS National Research Ethics Committees.

**Data sharing:** Study data and protocols will be made available for the purposes of academic research upon reasonable request to the corresponding author.

**Patient and public involvement:** Patient and Public Involvement is included in all our emerging clinical studies related to assess priorities and disseminate findings but not for the design of laboratory experiments included in this manuscript.

**References**


1. Denton, C. P., & Khanna, D. (2017). Systemic sclerosis. Lancet (London, England), 390(10103), 1685–1699. https://doi.org/10.1016/S0140-6736(17)30933-9
2. Allanore, Y., Simms, R., Distler, O., Trojanowska, M., Pope, J., Denton, C. P., & Varga, J. (2015). Systemic sclerosis. Nature reviews. Disease primers, 1, 15002. https://doi.org/10.1038/nrdp.2015.2
3. Tsou PS, Varga J, O'Reilly S. Advances in epigenetics in systemic sclerosis: molecular mechanisms and therapeutic potential. Nat Rev Rheumatol. 2021 Oct;17(10):596-607. doi: 10.1038/s41584-021-00683-2. Epub 2021 Sep 3. PMID: 34480165.
4. Bresnick, A. R., Weber, D. J., & Zimmer, D. B. (2015). S100 proteins in cancer. Nature reviews. Cancer, 15(2), 96–109. https://doi.org/10.1038/nrc3893
5. Strutz, F., Okada, H., Lo, C. W., Danoff, T., Carone, R. L., Tomaszewski, J. E., & Neilson, E. G. (1995). Identification and characterization of a fibroblast marker: FSP1. The Journal of cell biology, 130(2), 393–405. https://doi.org/10.1083/jcb.130.2.393
6. Xia, H., Herrera, J., Smith, K., Yang, L., Gilbertsen, A., Benyumov, A., Racila, E., Bitterman, P. B., & Henke, C. A. (2021). Hyaluronan/CD44 axis regulates S100A4-mediated mesenchymal progenitor cell fibrogenicity in idiopathic pulmonary fibrosis. American journal of physiology. Lung cellular and molecular physiology, 320(5), L926–L941. https://doi.org/10.1152/ajplung.00456.2020
7. Xia, H., Gilbertsen, A., Herrera, J., Racila, E., Smith, K., Peterson, M., Griffin, T., Benyumov, A., Yang, L., Bitterman, P. B., & Henke, C. A. (2017). Calcium-binding protein S100A4 confers mesenchymal progenitor cell fibrogenicity in idiopathic pulmonary fibrosis. The Journal of clinical investigation, 127(7), 2586–2597. https://doi.org/10.1172/JCI90832
8. Roh, J. S., & Sohn, D. H. (2018). Damage-Associated Molecular Patterns in Inflammatory Diseases. Immune network, 18(4), e27. https://doi.org/10.4110/in.2018.18.e27
9. Dahlmann M, Okhrimenko A, Marcinkowski P, Osterland M, Herrmann P, Smith J, et al. RAGE mediates S100A4-induced cell motility via MAPK/ERK and hypoxia signaling and is a prognostic biomarker for human colorectal cancer metastasis. Oncotarget. 2014;5(10):3220-33.
10. Cerezo LA, Remakova M, Tomcik M, Gay S, Neidhart M, Lukanidin E, et al. The metastasis-associated protein S100A4 promotes the inflammatory response of mononuclear cells via the TLR4 signalling pathway in rheumatoid arthritis. Rheumatology (Oxford). 2014;53(8):1520-6.
11. Yuan, Q., Hou, S., Zhai, J., Tian, T., Wu, Y., Wu, Z., He, J., Chen, Z., & Zhang, J. (2019). S100A4 promotes inflammation but suppresses lipid accumulation via the STAT3 pathway in chronic ethanol-induced fatty liver. Journal of molecular medicine (Berlin, Germany), 97(10), 1399–1412. https://doi.org/10.1007/s00109-019-01808-7
12. Cerezo LA, Remakova M, Tomcik M, Gay S, Neidhart M, Lukanidin E, et al. The metastasis-associated protein S100A4 promotes the inflammatory response of mononuclear cells via the TLR4 signalling pathway in rheumatoid arthritis. Rheumatology (Oxford). 2014;53(8):1520-6.
13. Li Z, Li Y, Liu S, Qin Z. Extracellular S100A4 as a key player in fibrotic diseases. J Cell Mol Med. 2020 Jun;24(11):5973-5983.
14. Ambartsumian, N., Klingelhöfer, J., & Grigorian, M. (2019). The Multifaceted S100A4 Protein in Cancer and Inflammation. Methods in molecular biology (Clifton, N.J.), 1929, 339–365. https://doi.org/10.1007/978-1-4939-9030-6_22

15. Tomcik M, Palumbo-Zerr K, Zerr P, Avouac J, Dees C, Sumova B, Distler A, Beyer C, Cerezo LA, Becvar R, Distler O, Grigorian M, Schett G, Senolt L, Distler JH. S100A4 amplifies TGF-β-induced fibroblast activation in systemic sclerosis. Ann Rheum Dis. 2015 Sep;74(9):1748-55. doi: 10.1136/annrheumdis-2013-204516. Epub 2014 Apr 7. PMID: 24709861.
16. Li Y, Bao J, Bian Y, Erben U, Wang P, Song K, Liu S, Li Z, Gao Z, Qin Z. S100A4+ Macrophages Are Necessary for Pulmonary Fibrosis by Activating Lung Fibroblasts. Front Immunol. 2018 Aug 6;9:1776.
17. Nihtyanova, S. I., Sari, A., Harvey, J. C., Leslie, A., Derrett-Smith, E. C., Fonseca, C., Ong, V. H., & Denton, C. P. (2020). Using Autoantibodies and Cutaneous Subset to Develop Outcome-Based Disease Classification in Systemic Sclerosis. Arthritis & rheumatology (Hoboken, N.J.), 72(3), 465–476. https://doi.org/10.1002/art.41153
18. Denton, C. P., Ong, V. H., Xu, S., Chen-Harris, H., Modrusan, Z., Lafyatis, R., Khanna, D., Jahreis, A., Siegel, J., & Sornasse, T. (2018). Therapeutic interleukin-6 blockade reverses transforming growth factor-beta pathway activation in dermal fibroblasts: insights from the faSScinate clinical trial in systemic sclerosis. Annals of the rheumatic diseases, 77(9), 1362–1371. https://doi.org/10.1136/annrheumdis-2018-213031
19. Abdelfattah N, Kumar P, Wang C, Leu JS, Flynn WF, Gao R, Baskin DS, Pichumani K, Ijare OB, Wood SL, Powell SZ, Haviland DL, Parker Kerrigan BC, Lang FF, Prabhu SS, Huntoon KM, Jiang W, Kim BYS, George J, Yun K. Single-cell analysis of human glioma and immune cells identifies S100A4 as an immunotherapy target. Nat Commun. 2022 Feb 9;13(1):767. doi: 10.1038/s41467-022-28372-y. PMID: 35140215; PMCID: PMC8828877.
20. Ackerman, J. E.; Nichols, A. E.; Studentsova, V.; Best, K. T.; Knapp, E.; Loiselle, A. E. Cell Non-Autonomous Functions of S100a4 Drive Fibrotic Tendon Healing. Elife 2019, 8. https://doi.org/10.7554/eLife.45342.
21. Bresnick AR, Weber DJ, Zimmer DB. S100 proteins in cancer. Nat Rev Cancer. 2015 Feb;15(2):96-109. doi: 10.1038/nrc3893. PMID: 25614008; PMCID: PMC4369764.
22. Bresnick AR. S100 proteins as therapeutic targets. Biophys Rev. 2018 Dec;10(6):1617-1629. doi: 10.1007/s12551-018-0471-y. Epub 2018 Oct 31. PMID: 30382555; PMCID: PMC6297089.
23. Cardot-Ruffino, V., Chauvet, V., Caligaris, C., Bertrand-Chapel, A., Chuvin, N., Pommier, R. M., Valcourt, U., Vincent, D., Martel, S., Aires, S., Kaniewski, B., Dubus, P., Cassier, P., Sentis, S., & Bartholin, L. (2020). Generation of an Fsp1 (fibroblast-specific protein 1)-Flpo transgenic mouse strain. Genesis, 58(5), e23359. https://doi.org/10.1002/dvg.23359
24. Zheng, B., Zhang, Z., Black, C. M., de Crombrugghe, B., & Denton, C. P. (2002). Ligand-dependent genetic recombination in fibroblasts: a potentially powerful technique for investigating gene function in fibrosis. The American journal of pathology, 160(5), 1609–1617. https://doi.org/10.1016/S0002-9440(10)61108-X
25. Cerezo LA, Remáková M, Tomčik M, Gay S, Neidhart M, Lukanidin E, Pavelka K, Grigorian M, Vencovský J, Šenolt L. The metastasis-associated protein S100A4 promotes the inflammatory response of mononuclear cells via the TLR4 signalling pathway in rheumatoid arthritis. Rheumatology, 2014 Aug;53(8):1520-6. doi: 10.1093/rheumatology/keu031. Epub 2014 Mar 18. PMID: 24643522.
26. Lee, J.-U.; Chang, H. S.; Shim, E.-Y.; Park, J.-S.; Koh, E.-S.; Shin, H.-K.; Park, J.-S.; Park, C.-S. The S100 Calcium-Binding Protein A4 Level Is Elevated in the Lungs of Patients with Idiopathic Pulmonary Fibrosis. Respir Med 2020, 171, 105945. https://doi.org/10.1016/j.rmed.2020.105945.

27. Wen J, Jiao B, Tran M, Wang Y. Pharmacological Inhibition of S100A4 Attenuates Fibroblast Activation and Renal Fibrosis. Cells. 2022 Sep 5;11(17):2762.
28. Kortüm, B., Radhakrishnan, H., Zincke, F., Sachse, C., Burock, S., Keilholz, U., Dahlmann, M., Walther, W., Dittmar, G., Kobelt, D., & Stein, U. (2022). Combinatorial treatment with statins and niclosamide prevents CRC dissemination by unhinging the MACC1-β-catenin-S100A4 axis of metastasis. Oncogene, 41(39), 4446–4458. https://doi.org/10.1038/s41388-022-02407-6
29. Kim H, Kim B, Il Kim S, Kim HJ, Ryu BY, Chung J, Lee ZH, Kim HH. S100A4 released from highly bone-metastatic breast cancer cells plays a critical role in osteolysis. Bone Res. 2019 Sep 23;7:30. doi: 10.1038/s41413-019-0068-5. PMID: 31667000; PMCID: PMC6804941.
30. Forst B, Hansen MT, Klingelhöfer J, Møller HD, Nielsen GH, Grum-Schwensen B, Ambartsumian N, Lukanidin E, Grigorian M. Metastasis-inducing S100A4 and RANTES cooperate in promoting tumor progression in mice. PLoS One. 2010 Apr 28;5(4):e10374. doi: 10.1371/journal.pone.0010374. PMID: 20442771; PMCID: PMC2860983.
31. Clark, K. E., Csomor, E., Campochiaro, C., Galwey, N., Nevin, K., Morse, M. A., ... & Denton, C. P. (2022). Integrated analysis of dermal blister fluid proteomics and genome-wide skin gene expression in systemic sclerosis: an observational study. The Lancet Rheumatology, 4(7), e507-e516.
32. Herwig, N., Belter, B., & Pietzsch, J. (2016). Extracellular S100A4 affects endothelial cell integrity and stimulates transmigration of A375 melanoma cells. Biochemical and biophysical research communications, 477(4), 963–969. https://doi.org/10.1016/j.bbrc.2016.07.009
33. Hsieh H.L., B.W. Schafer, J.A. Cox, et al., S100A13 and S100A6 exhibit distinct translocation pathways in endothelial cells, J. Cell Sci. 115 (2002) 3149e3158.
34. Saraswati S, Marrow SMW, Watch LA, Young PP. Identification of a pro-angiogenic functional role for FSP1-positive fibroblast subtype in wound healing. Nat Commun. 2019 Jul 9;10(1):3027.
35. LeBleu VS, Teng Y, O'Connell JT, Charytan D, Müller GA, Müller CA, Sugimoto H, Kalluri R. Identification of human epididymis protein-4 as a fibroblast-derived mediator of fibrosis. Nat Med. 2013 Feb;19(2):227-31.
36. Iwano M, Fischer A, Okada H, Plieth D, Xue C, Danoff TM, Neilson EG. Conditional abatement of tissue fibrosis using nucleoside analogs to selectively corrupt DNA replication in transgenic fibroblasts. Mol Ther. 2001 Feb;3(2):149-59.

**Key messages**

**What is already known about this subject?**

- The Damage Associated Molecular Pattern (DAMP) protein S100A4 is profibrotic in preclinical mouse models. It has potential intra- and extracellular effects that may be relevant to cancer metastasis.

**What does this study add?**

- This study shows that recombinant S100A4 protein promotes a fibrotic phenotype in normal fibroblasts resembling that of unstimulated systemic sclerosis fibroblasts.
- We demonstrate that a monoclonal antibody (AX-202) targeting S100A4 reverses the fibrotic phenotype of SSc fibroblasts.
- Using integrated high dimensional RNA sequencing we define a signature of S100A4 regulated genes in SSc fibroblasts.

**How might this impact on clinical practice or future developments?**

- By implicating S100A4 as a molecular marker, mediator, and potential treatment target in SSc our results strongly support clinical development of AX-202 as a potential antifibrotic therapy.

**Figures and Legends**

**Fig. 1 Clinical associations of circulating S100A4protein in systemic sclerosis**

Plasma levels of circulating S100A4 were elevated in SSc patients compared with healthy control subjects (A) and the average level was numerically higher in dcSSc (B). There was significant increase in serum S100A4 in cases with interstitial lung disease (SSc-ILD) (C), a trend for higher levels in cardiac SSc (D) and pulmonary hypertension (PH) (E). Levels were higher in patients with a history of scleroderma renal crisis (SRC) (F). There was a weak, non-significant, correlation between serum S100A4 and current skin score (mRSS) in dcSSc (G) but not in lcSSc (H). Nor was there any association in either disease subset between peak mRSS and S100A4 (I, J).

**Fig. 2 Elevated expression and release of S100A4 by systemic sclerosis fibroblasts**

Fibroblast cell layer lysates had increased S100A4 protein demonstrated by Western blot (A) and quantified (B). Tissue culture supernatant confirmed elevated levels of S100A4 in a series of biological replicate samples taken from dcSSc or healthy control fibroblast explant cultures (C). Quantitative PCR confirms increased mRNA in dcSSc that is partially inhibited by the ALK5 inhibitor SB431524 (D).

**Fig. 3 Recombinant S100A4 protein induces a profibrotic phenotype in normal dermal fibroblasts**

Recombinant S100A4 induced concentration-dependent upregulation profibrotic protein markers ($\alpha$SMA, CTGF, Pro(I)collagen) in fibroblasts from healthy control skin biopsies (A) and promoted 3-dimensional type I collagen gel contraction over 24 hours (B). There was also promotion of fibroblasts migration in a 48 hour scratch wound assay (C). Phosphorylation of ERK is induced by S100A4 and partially attenuated by anti-TLR4 which also partially inhibits upregulation of profibrotic proteins consistent with binding of S100A4 to TLR4 (D). Data shown are representative of a series of 3 independent experiments and summary data from biological and technical replicates are shown graphically for each panel.

**Fig. 4 Effect of anti-S1004A neutralising antibody (AX-202) on normal dermal fibroblasts**

Anti-S100A4 antibody showed concentration dependent attenuation of S100A4 (0.5ug/ml) treated normal dermal fibroblasts using the specific anti-S100A4 neutralising antibody AX-202. There was attenuation of profibrotic protein expression (aSMA, CTGF, Pro(I)collagen) (A). These effects were not observed using an IgG4 isotype matched control monoclonal antibody (B). There was also inhibition of S100A4 induced 3-dimenesional collagen gel contraction at 24 hours (C) and fibroblasts

migration (D) in a 48 hour scratch wound assay. Representative data are shown and summary results for a series of biological replicate experiments.

**Fig. 5 Effect of anti-S100A4 neutralising antibody (AX-202) on systemic sclerosis fibroblasts**

The upper panel (A) shows a series of three independent experiments testing health control (n=3) or systemic sclerosis (n=6) fibroblast explant cultures from punch skin biopsies. The effect of AX-202 on fibroblasts with or without TGFβ1 is shown, and histograms summarise data for each of the profibrotic proteins in cell layer lysates. In the lower panel (B) quantitative RT-PCR shows effect in normal fibroblasts and AX-202 on SSc fibroblasts treated with recombinant S100A4, TGFβ1, AX-202 and the selective ALK5 inhibitor SB431524.

**Fig. 6 Genome wide transcriptomic analysis of S100A4 regulated genes in systemic sclerosis and control fibroblasts**

RNA sequencing was used to examine genome-wide transcription in healthy donor control fibroblasts treated with recombinant S100A4 and systemic sclerosis fibroblasts treated with anti-S100A4 neutralising antibody (AX-202). Gene expression was also compared between SSc and control fibroblasts and for S100A4 activated normal fibroblasts treated with SB431524, a specific pharmacological inhibitor of ALK5 signalling. The cluster PCA plot shows overall differences between gene expression across groups (A). Pairwise comparison of significantly differentially expressed genes (DGE; FC>1.5; FDR<0.001) was performed for S100A4 activated normal dermal fibroblasts, for SSc compared with normal fibroblasts and for AX-202 treated SSc fibroblasts. The number of DGE is shown and a Venn diagram highlights 464 genes that overlap between these gene sets (B) and the overlapping genes are listed in **Supplementary Table 1**. Pairwise differential gene expression is shown in Volcano plots for S100A4 activated normal dermal fibroblasts (C), ALK5i treated S100A4 activated normal dermal fibroblasts (D), SSc compared with control fibroblasts (E) and AX-202 treated SSc fibroblast cultures. Data summarise 5 biological replicates for each pairwise comparison. Using the overlapping genes identified above, Kegg® pathway enrichment analysis was performed. The pathways highlighted represent a gene set that is significantly differentially expressed in SSc, modulated by S100A4 in normal fibroblasts and attenuated by AX-202 in SSc fibroblasts. Gene number, enrichment score and FDR are shown for each of the significantly enriched pathways.

**Supplementary material**

**Supplementary Methods file**

Prvides additional experimantal detail for protocols used in this study.

**Supplementary Table 1.**

Gene lists from Venn diagram in Panel B of Figure 6. Overlapping genes between pairwise genome wide comparison using FDR0.001 and FC1.5 with overlapping segments coded A to G.

Fig. 1 Clinical associations of circulating S100A4 protein in systemic sclerosis

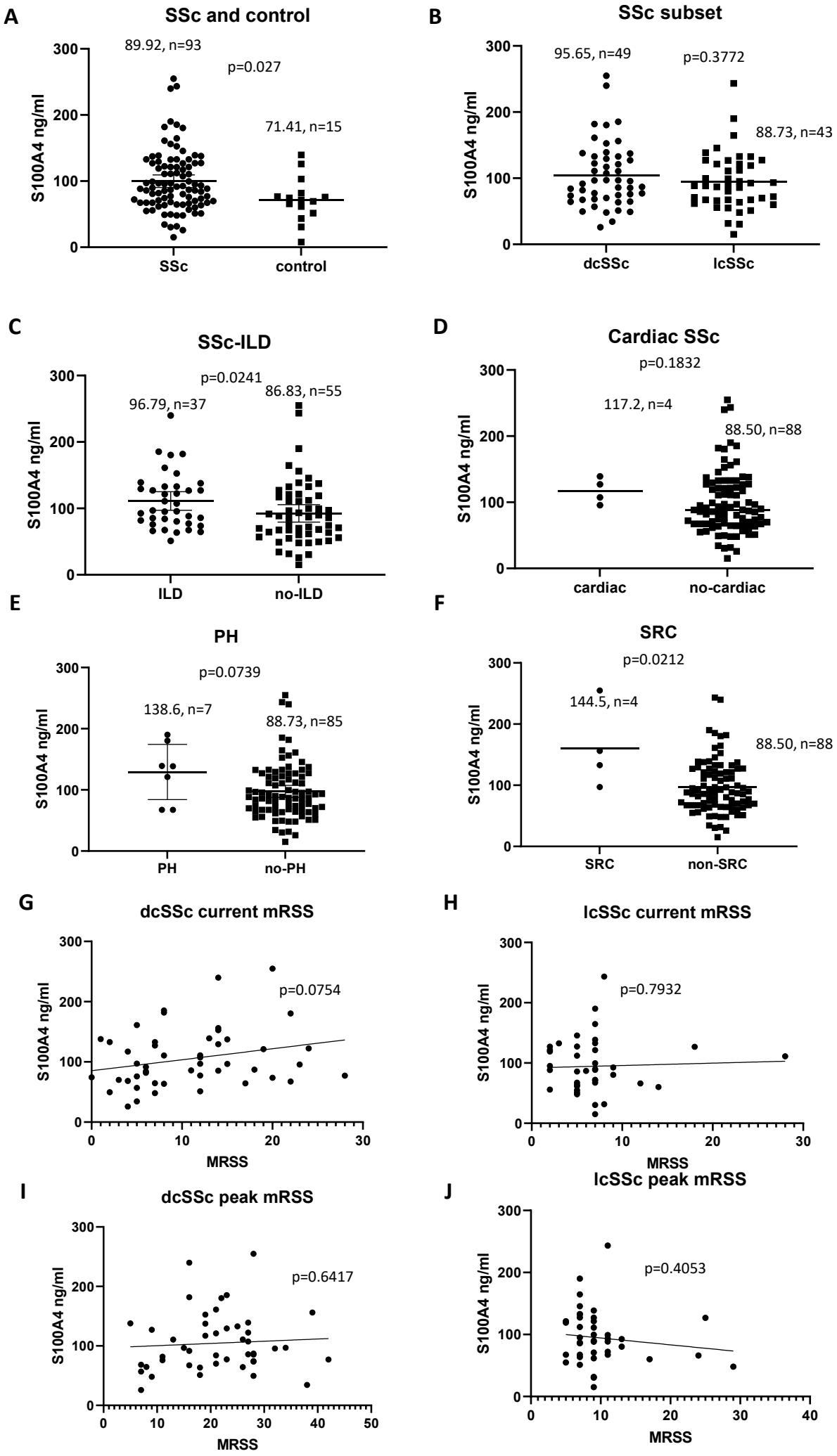
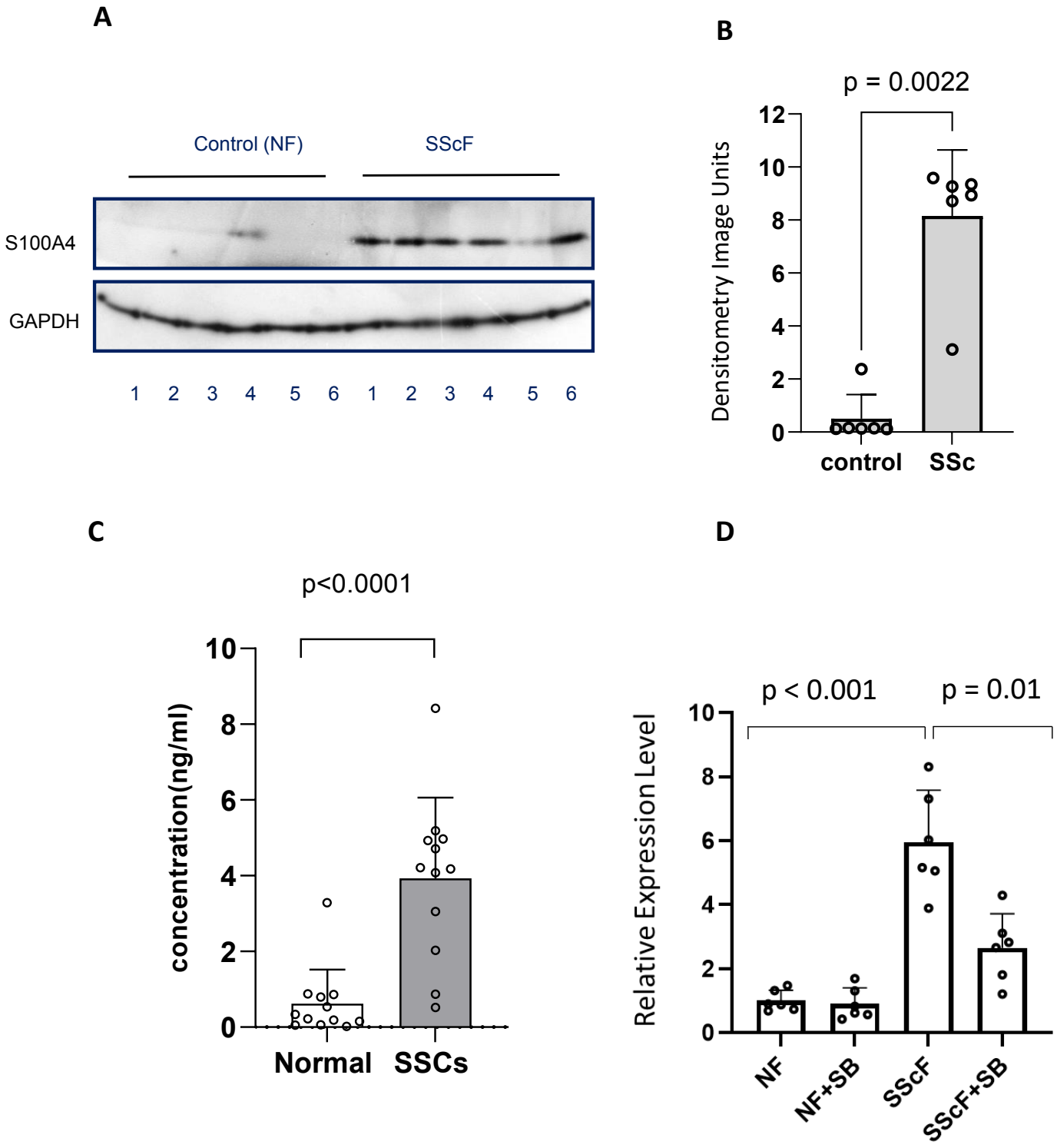


Fig. 2 Elevated expression and release of S100A4 by systemic sclerosis fibroblasts




SB = SB431524

Fig. 3 Recombinant S100A4 protein induces a profibrotic phenotype in normal dermal fibroblasts

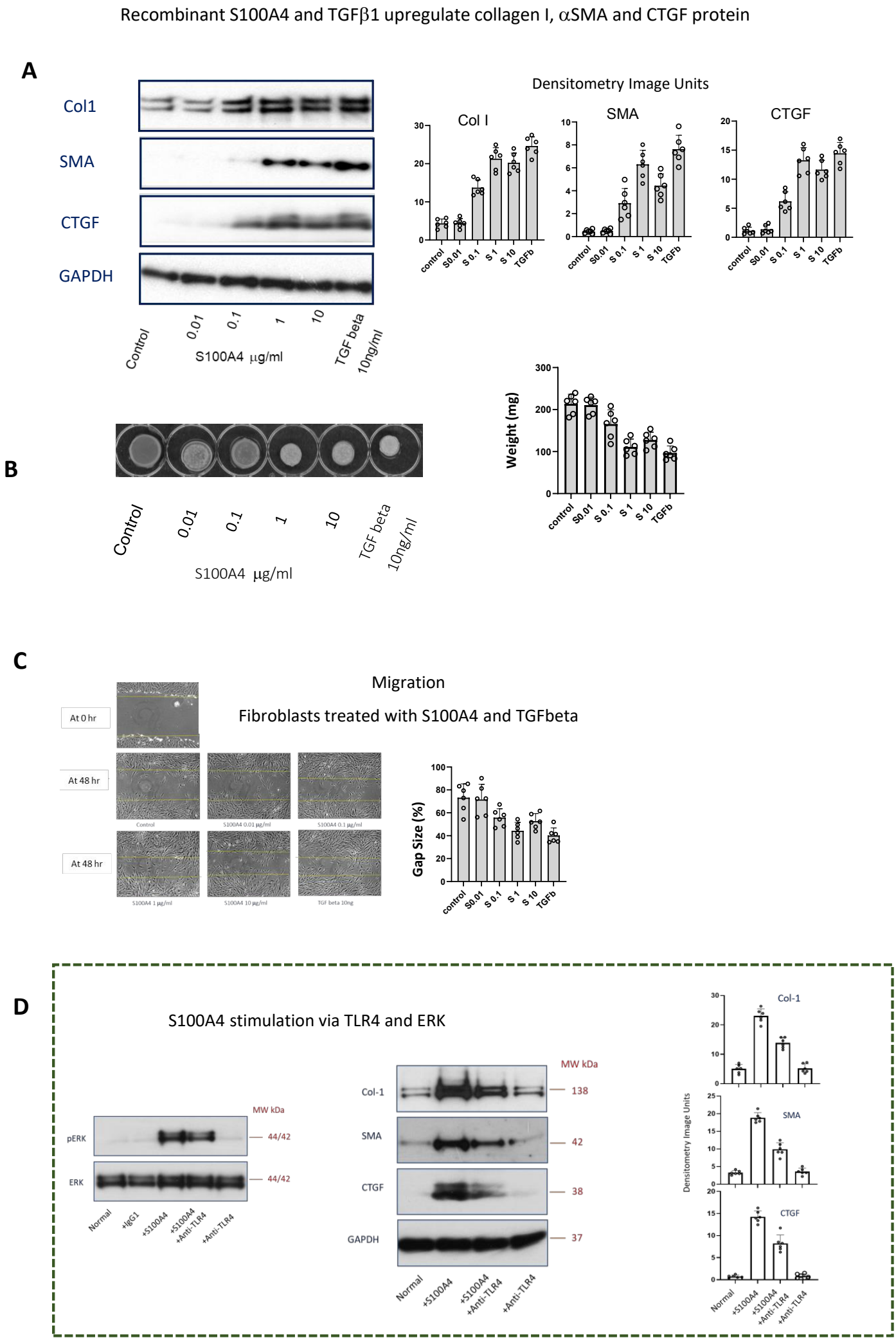
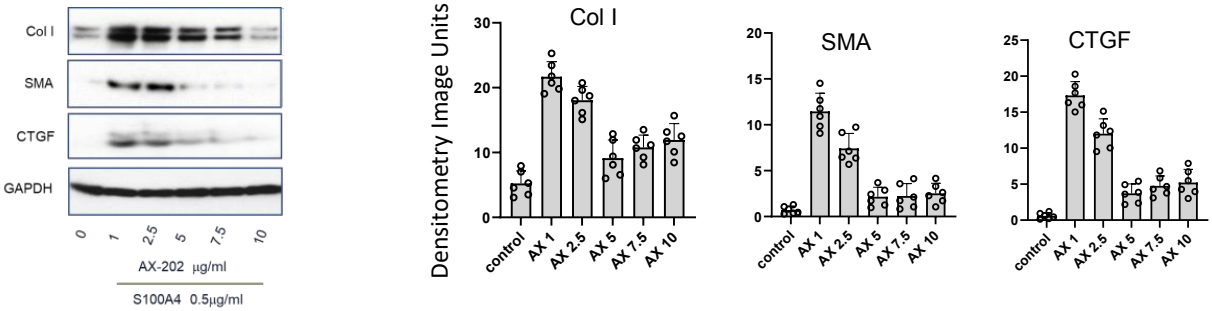


Fig. 4 Effect of anti-S1004A neutralising antibody (AX-202) on normal dermal fibroblasts

**A**

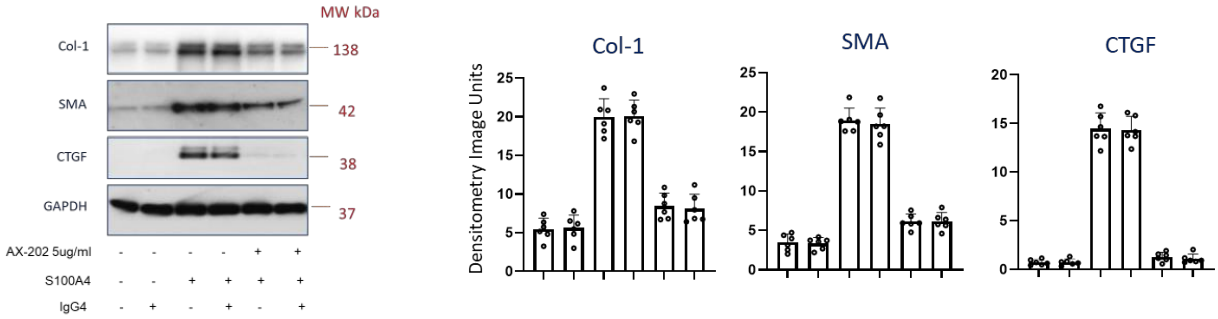
To inhibit S100A4 using neutralising anti-S100A4 antibody(AX-202)

- Dose Response in normal dermal fibroblasts (HC)



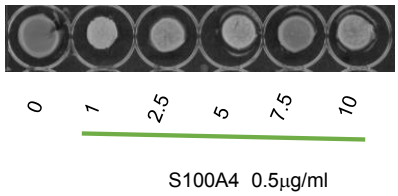

**B**

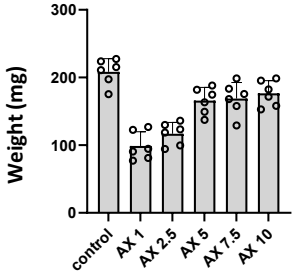
A concentration matched isotype control non S100A4 targeting antibody




**C**

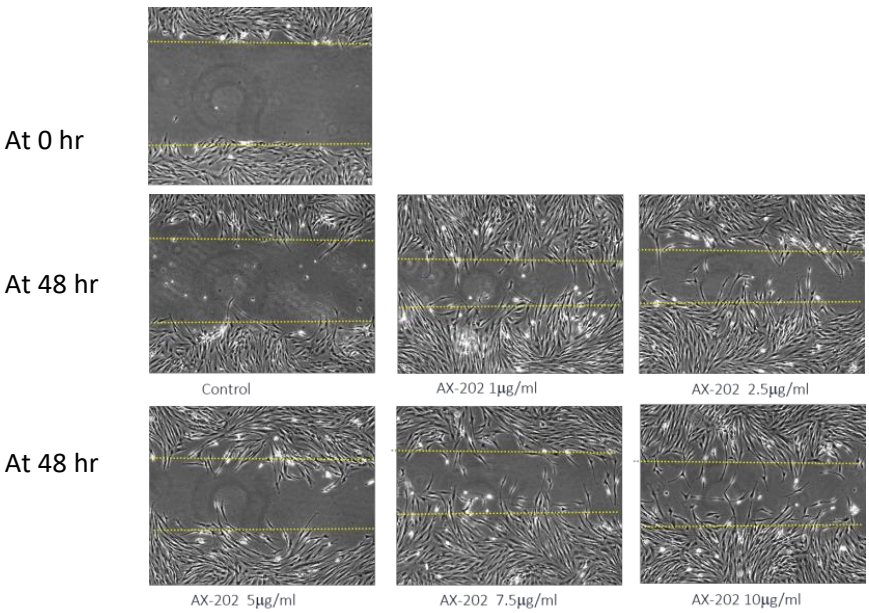
Inhibition of S100A4 using neutralizing anti-S100A4 antibody (AX-202)

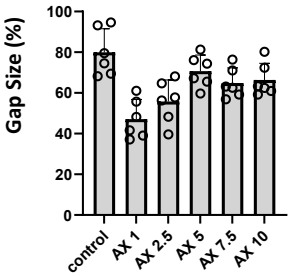



**D**

Inhibition of S100A4 (0.5µg/ml) using neutralising anti-S100A4 antibody (AX-202)



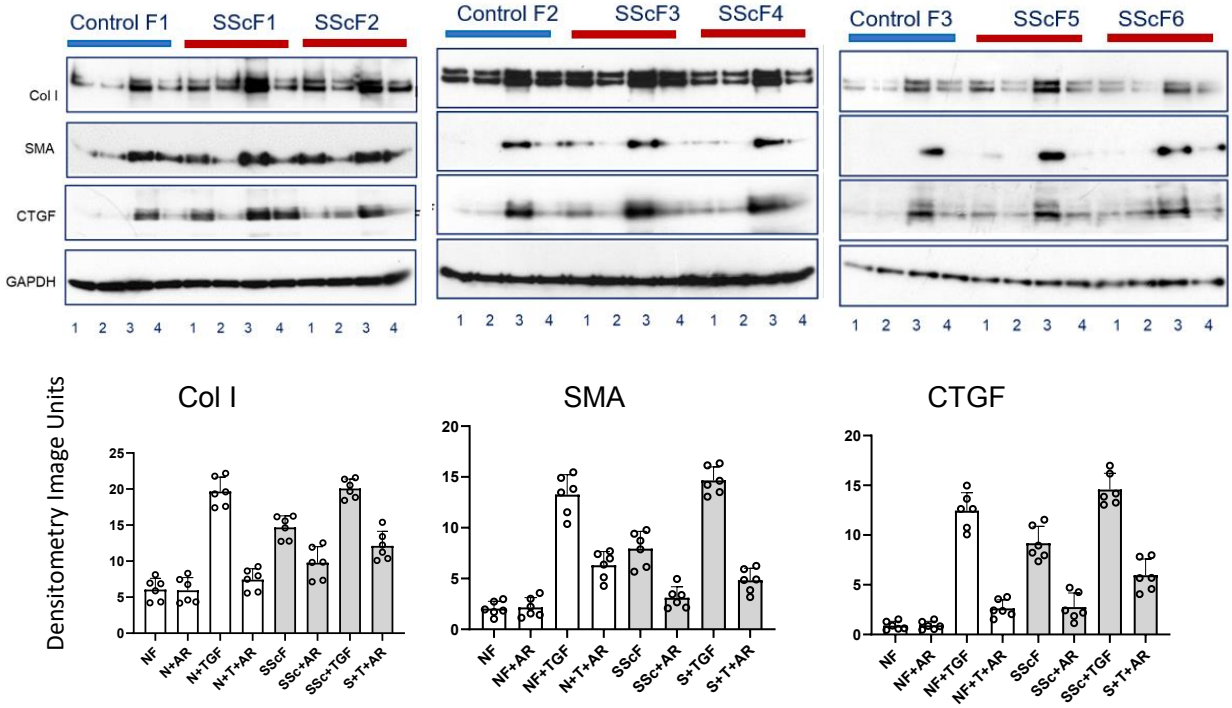




1. Control
2. AX-202 1µg/ml
3. AX-202 2.5µg/ml
4. AX-202 5µg/ml
5. AX-202 7.5µg/ml
6. AX-202 10µg/ml

Fig. 5 Effect of anti-S100A4 neutralising antibody (AX-202) on systemic sclerosis fibroblasts

**A**

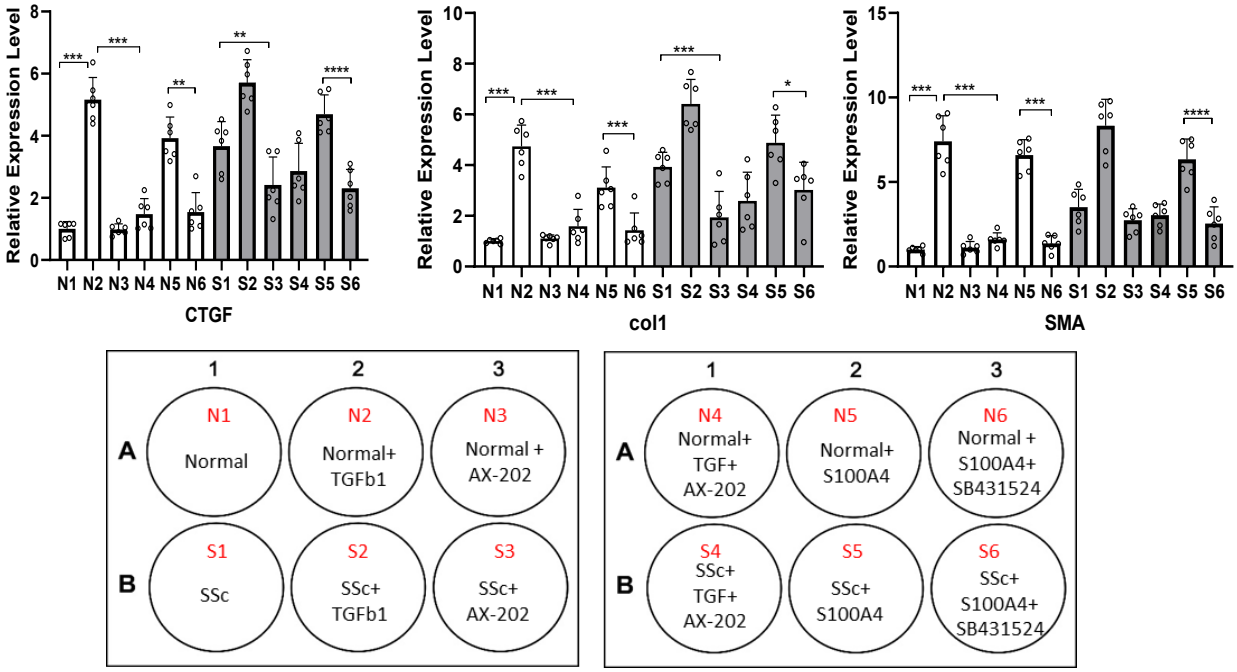
The effect of S100A4 neutralizing antibody (AX-202) on levels of Collagen 1, SMA and CTGF protein in normal and SSc fibroblasts




1. Control
2. S100A4 neutralizing antibody (AX-202)
3 TGFbeta1
4 TGFbeta1 + S100A4 neutralizing antibody (AX-202)

**B**

The change of RNA level in Scleroderma and normal fibroblasts with different treatments




S100A4 0.5µg/ml
Neutralizing anti-S100A4 antibody (AX-202) 5µg/ml
TGFbeta1 10 ng/ml
SB431524 : A selective inhibitor of TGF-β type I receptor/ALK5. 10 µM/ml

Fig. 6 Genome wide transcriptomic analysis of S100A4 regulated genes in systemic sclerosis and control fibroblasts