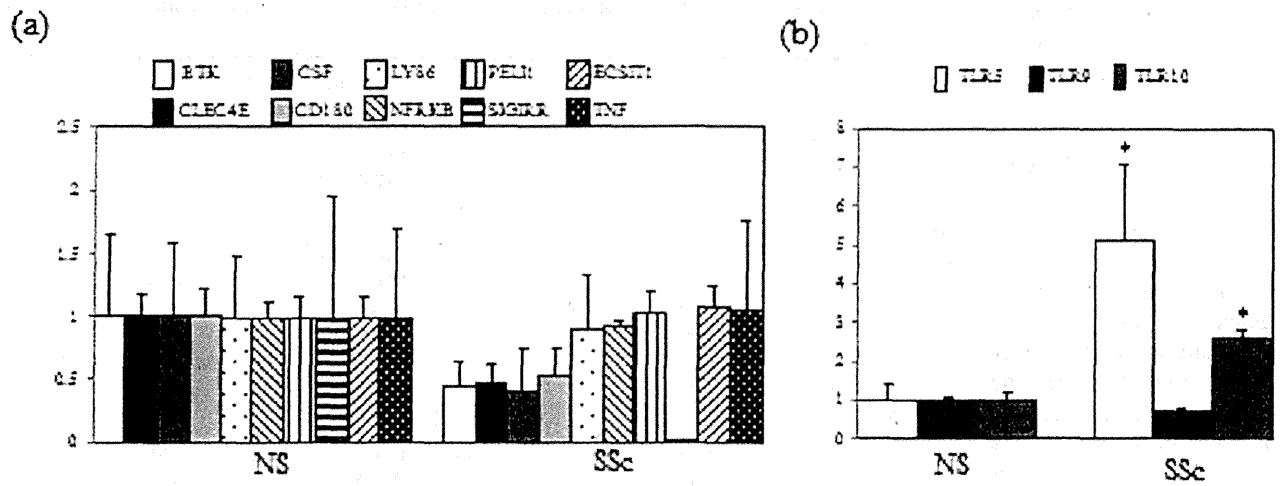


**Fig. 1.** Expression profiles of TLR-related genes. (**a**, **b**) The relative gene expression levels in 8 normal fibroblasts (NS) and 8 scleroderma fibroblasts (SSc) determined by real-time PCR. Error bars represent standard error. *$p<0.05$ compared with the values in NS (1.0).

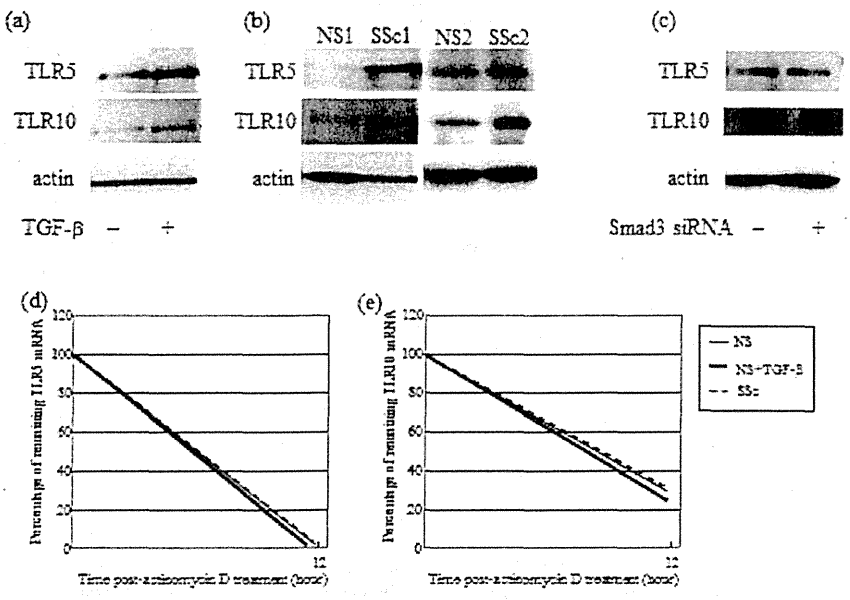


**Fig. 2.** TLR5/10 overexpression *in vitro*. (**a**) Normal fibroblasts were incubated in the presence or absence of TGF-β (2ng/ml) for 24 hours. Cell lysates were subjected to immunoblotting with antibody for TLR5 or TLR10. The same membrane was reprobed with anti-β-actin antibody as a loading control.
(**b**) Normal (NS) and scleroderma (SSc) fibroblasts were cultured independently under the same conditions until they were confluent, serum-starved for 24 hours, and harvested. Cell lysates were subjected to immunoblotting as described in Figure 1a.
(**c**) Scleroderma fibroblast at a density of $2\times10^4$ cells/well in twenty four-well culture plates were transfected with control siRNA or Smad3 siRNA for 96 hours. Cell lysates were subjected to immunoblotting as described in Figure 1a.
(**d**, **e**) Normal fibroblasts (NS, black lines), normal fibroblasts stimulated with TGF-β for 12 hours (NS+ TGF-β, bold lines) and scleroderma fibroblasts (SSc, dotted lines) were incubated with 5μg/ml of actinomycin D for 12 hours. mRNA expression of TLR5 (**d**) and TLR10 (**e**) was analysed by real-time PCR. The mRNA levels in cells before actinomycin D treatment were set at 100.

the up-regulation of TLR5 and TLR10 in SSc fibroblasts is due to the stimulation of endogenous TGF-β signalling, we determined the effect of the TGF-β blockade by Smad3 siRNA on TLR expression. The transfection of Smad3 siRNA into SSc fibroblasts resulted in the down-regulation of TLR proteins (Fig. 2c). Therefore, the up-regulated mRNA and protein expression of TLR5 and TLR10 in SSc fibroblasts is likely to be caused by the TGF-β activation in these cells.

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. To determine whether the increase of TLR5 and TLR10 mRNA in SSc fibroblasts takes place at the transcriptional or post-transcriptional level, de novo mRNA synthesis was blocked by actinomycin D, a RNA synthesis inhibitor, in normal and SSc fibroblasts in the presence or absence of TGF-β (Fig. 2d and e). There were no significant differences in mRNA decrease rate of TLR5 and TLR10 between normal and SSc fibroblasts in the presence or absence of TGF-β, indicating that the mRNA stability of TLR5 and TLR10 is not changed in SSc fibroblasts as well as TGF-β–treated normal fibroblasts. Taken together, the expression of TLR5 and TLR10 was likely to be increased at a transcriptional level in TGF-β-treated normal fibroblasts and SSc dermal fibroblasts.

Immunohistochemical staining using paraffin-embedded sections revealed that TLR5 and TLR10 expression was hardly detected in normal dermal fibroblasts, but was strongly detected in SSc fibroblasts between the thickened collagen bundles (Fig. 3). The result was consistent in 5 normal skin and 5 SSc skin. Thus, expression of TLR5 and TLR10 is increased in SSc both *in vivo* and *in vitro*.

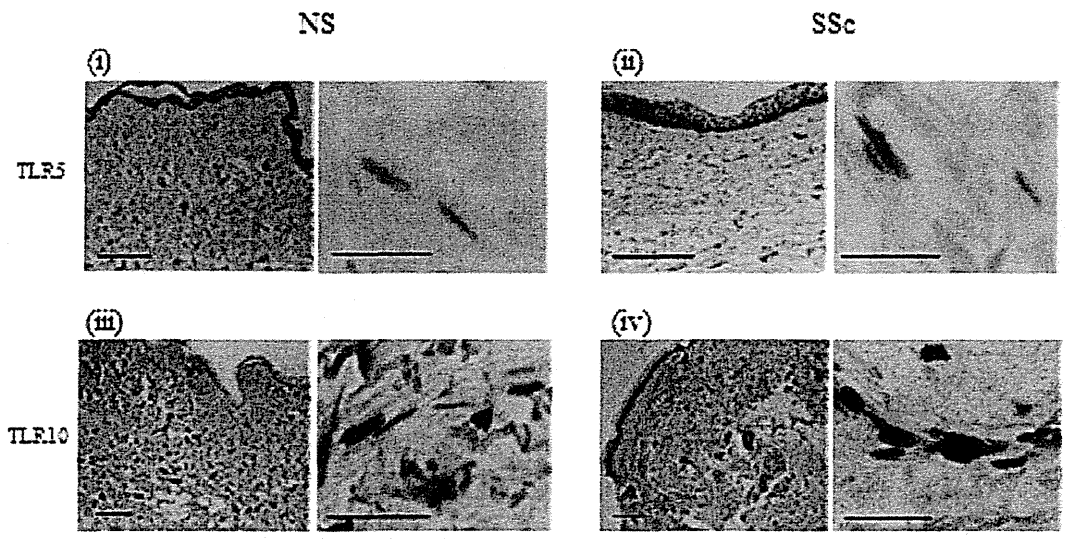


**Fig. 3.** *In vivo* expression of TLR5/10 in scleroderma.
Immunostaining of TLR5 and TLR10 in the normal skin (NS: i, iii) and scleroderma skin (SSc; ii, iv). Tissue sections were stained with antibodies against TLR5 or TLR10 as described in Materials and Methods. One experiment representative of five independent experiments is shown. Scale bar = 250μm (left), 20μm (right).

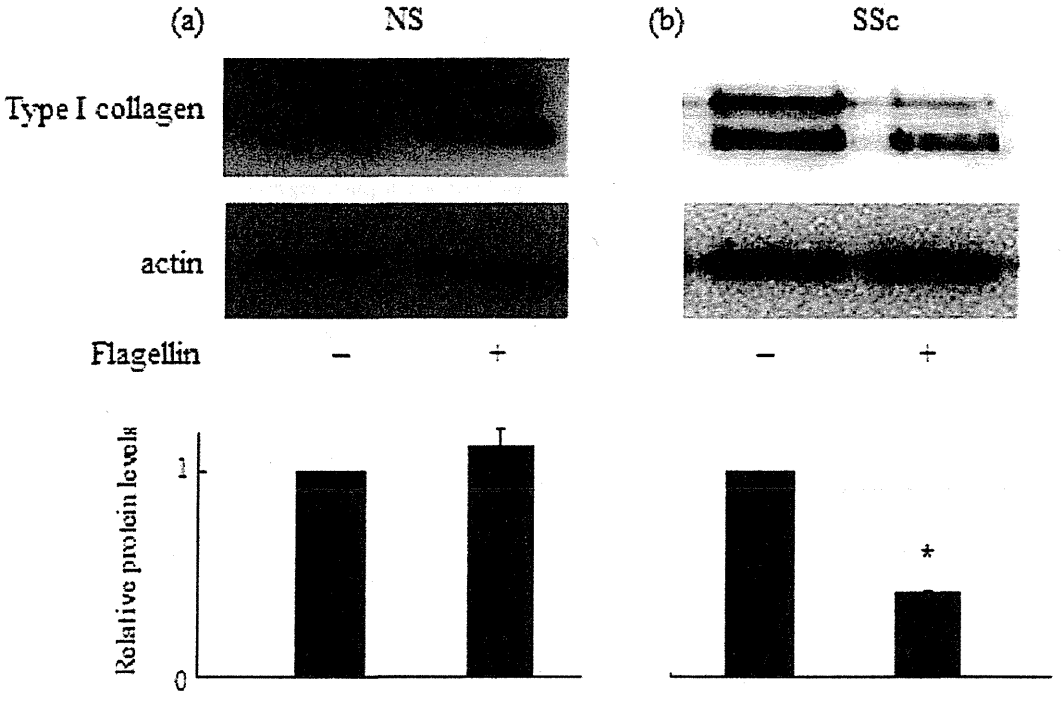


**Fig. 4.** TLR5 signalling regulates type I collagen expression *in vitro*.
Normal (NS, a) and scleroderma (SSc, b) fibroblasts at a density of $2\times10^4$ cells/well in twenty four-well culture plates were stimulated with control agonist or flagellin for 48 hours. Cell lysates were subjected to immunoblotting with antibody for type I collagen. The same membrane was reprobed with anti-β-actin antibody as a loading control. Levels of TLR5 protein quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the levels in control cells (1.0). *$p<0.05$.

*The function of TLR5 in SSc fibroblasts*

Next, we tried to examine the role of TLR5 in SSc fibroblasts using flagellin, the specific agonist of the TLR5. Treatment of normal fibroblasts with flagellin (0.1μg/ml, Enzo Life Science) for 48 hours did not affect the expression of type I collagen (Fig. 4a). On the other hand, the type I collagen expression was significantly reduced by the addition of flagellin in SSc fibroblasts (Fig. 4b). Accordingly, TLR5 activation may have negative effect on the collagen expression only in SSc fibroblasts.

## Discussion

This study demonstrated the expression and role of TLR5 and TLR10 in SSc by three major findings. First, we showed the expression profile of genes involved in human TLR signalling pathway in SSc fibroblasts, and identified that TLR5 and TLR10 is overexpressed specifically in SSc fibroblasts compared with normal fibroblasts. TLR5 recognises bacterial flagellin (20, 21). On the other hand, the exact function of TLR10 remains largely unexplored due to the fact that it is not present in mice. Although the expression of TLR5 and TLR10 has been found in breast and prostate cancer (22-24), our study is the first to demonstrate the expression of TLR5 and TLR10 in autoimmune diseases including SSc.

Next, we found that up-regulation of TLR5 and TLR10 in SSc fibroblasts was at a transcriptional level. In SSc

fibroblasts, various molecules including collagen, tenascin and MMP-1 are controlled by TGF-β/Smad3 signalling (25-27). Our study suggests the possibility that up-regulation of TLR5 or TLR10 in SSc fibroblasts is also induced by TGF-β/Smad3 activation.

Lastly, we investigated the effects of TLR signalling on the collagen expression in SSc fibroblasts. Since TLR5 activation inhibited the collagen expression only in SSc fibroblasts, TLR5 itself may have suppressive effects on collagen expression. Thus, TLR5 overexpression in SSc fibroblasts may be one of the negative feedback mechanisms for the collagen overexpression in SSc fibroblasts. The effect of flagellin was not seen in normal fibroblasts, probably because the expression level of TLR5 was low. On the other hand, at present, ligand of TLR10 is not identified and not commercially available, but the function of TLR10 in SSc needs to be clarified in the future.

In summary, TLR5 and TLR10 may play some roles in the pathogenesis of SSc. Investigation of the regulatory mechanisms of fibrosis by TLRs may lead to new therapeutic approaches of this disease.

**Acknowledgement**

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LETTER

# Platelet-rich plasma therapy is effective for the treatment of refractory skin ulcers in patients with systemic sclerosis

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**Keywords**

Skin ulcer, Systemic sclerosis, Platelet-rich plasma therapy

**History**

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**To the Editor,**

Systemic sclerosis (SSc) is a chronic connective tissue disease which includes of fibrosis, vasculopathy and autoimmunity [1]. Severe cutaneous manifestations caused by vasculopathy appear as skin ulcers, gangrene, and fistulas. They tend to be resistant to standard therapy, and patients suffer from pain due to both the lesions and troublesome treatment, which decrease the patients' quality of life. Therefore, developing new therapeutic approaches against the refractory skin ulcers in SSc patients is urgently needed [2]. Platelet-rich plasma (PRP) therapy has been reported to be effective for the treatment of chronic or refractory wounds, such as traumatic injuries, pressure ulcers, and diabetic ulcers [3]. PRP is defined as a plasma fraction whose concentration is above the peripheral blood level. It releases various growth factors that enhance wound-healing [4]. Although many reports have shown the potency of PRP therapy, no previous studies have identified the efficacy of PRP for the skin ulcers in SSc patients. We herein report our experiences with skin ulcers in two SSc patients who were successfully treated with PRP therapy, which could shorten the duration of treatments. Institutional review board approval and written informed consent were obtained before patients were entered into this study, according to the Declaration of Helsinki.

Case 1 was a 56-year-old male with a 10-year history of diffuse cutaneous SSc. He had suffered from an ulcer on the left-middle finger for 1 year (Figure 1a). Conservative therapies, such as intravenous prostaglandin and argatroban hydrate, were administered, but the wound was refractory to these treatments. Bosentan hydrate, one of the endothelin receptor antagonists, has been administered in the treatment of his pulmonary arterial hypertension. Phosphodiesterase type 5 (PDE5) inhibitors have never been administered. MRI showed no osteomyelitis. We tried PRP therapy to accelerate the wound-healing using a PRP kit (KYOCERA Medical Corporation, Osaka, Japan). The medical procedure was performed with reference to the manufacturer's protocol. We collected 20 mL of the patient's blood using a syringe containing sodium citrate. We then performed centrifugal separation of the blood at 600 g for 10 min. Next, we collected the platelets and leukocyte supernatant from the blood sampling syringe to a separation syringe. We then performed centrifugal separation at 2000 g for 5 min, removed the platelet-poor plasma supernatant from the separation syringe, and used the remaining 2 mL present in the separation syringe as the PRP. Just before using it, we added calcium to activate the platelets. Finally, we put the activated PRP on the surface of the ulcer. This patch of PRP was applied on the place of ulcer for a week. After the first PRP treatment, epithelialization was seen. Therefore, we completed the PRP treatment on the first attempt. The skin defect was almost diminished after 1 month (Figure 1b).

The second case was a 75-year-old female with a 14-year history of limited cutaneous SSc, positive for anti-centromere antibodies. Neither endothelin receptor antagonists nor PDE5 inhibitors have been administered before. She developed skin ulcers and gangrene on the right big, second, and little toes. MRI revealed osteomyelitis of the right big and little toes. Because of a lack of responsiveness to conservative therapies, we performed amputation below the right knee. However, cutaneous ulcers appeared on the surgical scar after 2 weeks (Figure 2a). We tried PRP therapy every week as described in Case 1. This resulted in good granulation tissue formation after a week. After 1 month (after the third PRP treatment), her ulcer showed good improvement (Figure 2b). After the fourth PRP treatment, the wound was treated with a full-thickness skin graft (FTSG) harvested from the right inguinal region. One week after the FTSG treatment, the ulcer lesion almost completely healed (Figure 2c).

Since the skin involvements in SSc, including ulcers and gangrene, tend to be unresponsive to standard therapies, their

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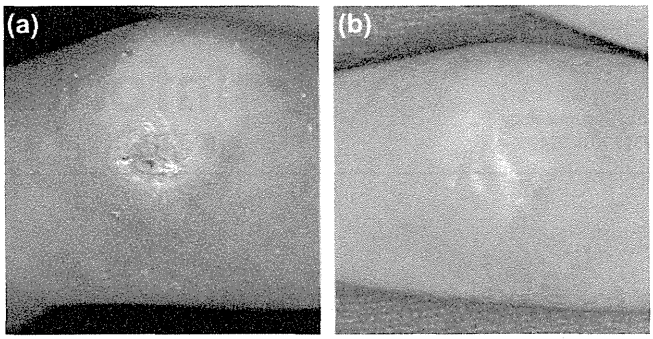


Figure 1. The ulcer of the left-middle finger in Case 1. (a) Before the PRP therapy; (b) 1 month after the first PRP treatment.

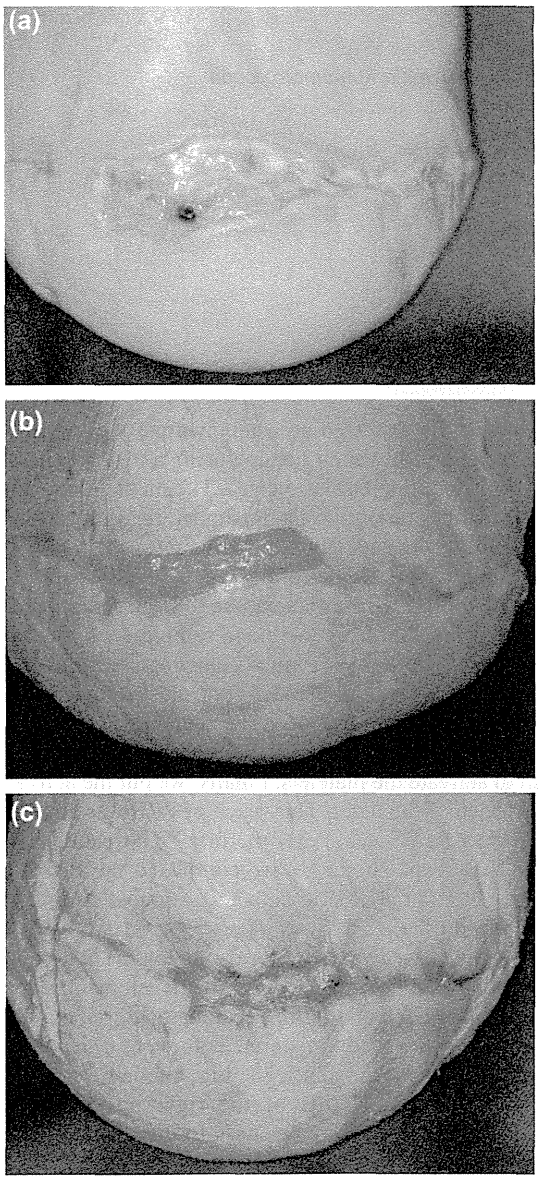


Figure 2. The ulcers of the right knee after the amputation in Case 2. (a) Before the PRP therapy; (b) after the third PRP treatment; (c) 1 week after the FTSG.

treatment is often difficult and expensive. However, we were able to achieve an almost complete response within a month using PRP therapy. These results indicate that PRP therapy is effective for the treatment of skin ulcers in SSc patients.

The reason why PRP therapy has such rapid effectiveness is mainly because of its high concentrations of growth factors released by platelets [4]. Platelets produce various growth factors, including PDGF, TGF-β, VEGF, and EGF, which stimulate the proliferation, migration, and differentiation of dermal fibroblasts, endothelial cells, and keratinocytes. Apart from these growth factors, PRP is thought to lead to granulation, vascularization, and epithelialization of the ulcer, which brings about rapid healing of the lesion [5]. To the best of our knowledge, this study is the first to report the successful treatment of skin ulcers using PRP therapy in patients with SSc. Although PRP therapy is not yet included in the EULAR recommendations [6], it can be an appropriate alternative therapy. In addition, PRP therapy may shorten the length of treatment, decreasing medical expenses and improving the patients' quality of life.

In conclusion, we herein demonstrated that PRP therapy has great effectiveness against refractory skin ulcers in SSc patients. To confirm the present findings, further studies will be needed in a large number of patients.

## Acknowledgements

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## Conflict of interest

None.

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CONNECTIVE TISSUE DISEASES

# New criteria improve recognition of early systemic sclerosis

*Yoshihide Asano and Shinichi Sato*

**In systemic sclerosis (SSc), new classification criteria highly sensitive to early and limited forms of the disease could facilitate not only the recognition of early SSc, but also the discovery of biomarkers of poor prognosis, with potential benefits for conducting clinical trials.**

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In rare and heterogeneous diseases, clinical trials to evaluate the efficacy of disease-modifying interventions are challenging. Systemic sclerosis (SSc) is no exception. SSc is a multisystem connective tissue disease of unknown aetiology that is characterized by immune abnormalities, vascular injury and resultant fibrosis of the skin and various internal organs. To enable selection of patients with definite disease for inclusion in clinical trials, the development of classification criteria is a critical task. The findings of a study by the Canadian Scleroderma Research Group (CSRG) demonstrate that the latest classification criteria for SSc serve as a new basis for identifying early disease.[1]

> "...the 1980 criteria fell short by excluding a considerable number of patients with SSc..."

The first widely accepted classification criteria for SSc, published in 1980 by the ACR as 'preliminary' criteria, were developed using patients with long-standing SSc.[2] The high sensitivity and specificity (97% and 98%, respectively) of these criteria in the derivation sample were externally validated in a separate large cohort. However, the ensuing widespread clinical use of nailfold capillaroscopy and SSc-specific autoantibody testing considerably improved the recognition of early disease and of limited cutaneous SSc (lcSSc), and subsequent research highlighted that the 1980 criteria fell short by excluding a considerable number of patients with SSc, in particular those with early disease and lcSSc.[3,4]

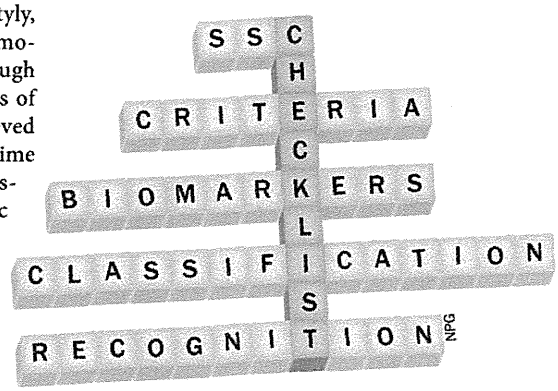
In 2013, following consideration of the definition of early SSc and several newly proposed criteria, a joint ACR–EULAR committee developed an updated criteria set for SSc. These new classification criteria had better sensitivity and specificity than the 1980 criteria even in the validation sample (91% versus 75% and 92% versus 72%, respectively).[5,6] An independent validation study by the CSRG has now also demonstrated the superior sensitivity of the 2013 criteria, as compared with the 1980 criteria, in a study of 724 patients with SSc (98.3% versus 88.3%). The new criteria were also more sensitive in subgroups of patients with lcSSc (98.8% versus 85.6%), anti-centromere antibodies (98.9% versus 79.8%), disease duration ≤3 years (98.7% versus 84.7%) or no skin involvement proximal to the metacarpophalangeal joints (97% versus 60%), findings that indicate the classification of more patients with early SSc and lcSSc as having definite disease.[1]

The 1980 criteria include skin thickening proximal to the metacarpophalangeal joints as the major criterion, with sclerodactyly, digital pitting scars and bibasilar pulmonary fibrosis as minor criteria.[2] Although the classification of SSc on the basis of one major or ≥2 minor criteria achieved high sensitivity and specificity at the time of publication, this formula put disproportionate emphasis on the fibrotic aspects of the disease. Therefore, diagnostic progress in vascular and immunological aspects of SSc reduced the sensitivity of the 1980 criteria. By contrast, the 2013 criteria assign almost equal importance to three major features of SSc: fibrosis, vasculopathy and immune abnormalities.

The 2013 criteria set uses a points system that classifies patients with a score ≥9 as having definite SSc. Skin thickening proximal to the metacarpophalangeal joints (9 points) is considered sufficient for classification, as in the 1980 criteria. Alternatively, seven other variously weighted items are included, namely skin thickening of the fingers (puffy fingers [2 points] or sclerodactyly [4 points], counting the higher score only), fingertip lesions (digital tip ulcers [2 points] or pitting scars [3 points], counting the higher score only), telangiectasia (2 points), abnormal nailfold capillaries (2 points), interstitial lung disease or pulmonary arterial hypertension (2 points), Raynaud phenomenon (3 points) and SSc-specific autoantibodies (3 points).[5,6]

The validation study by the CSRG found that the inclusion of Raynaud phenomenon as a criterion is the single most important contributor to the improved sensitivity of the 2013 criteria over the 1980 criteria; secondarily, puffy fingers, abnormal nailfold capillaries and anti-centromere antibodies also contributed to the improved sensitivity of the 2013 criteria, by facilitating the recognition of patients with early disease and lcSSc.[7] The CSRG's evaluation proves the importance of vascular and immunological aspects to the early diagnosis of SSc. This finding is plausible in terms of the pathophysiology of SSc because aberrant activation of immune and vascular systems occurs prior to the development of skin and organ fibrosis.

Another circumstance underlying the revision of the 1980 criteria seems to be



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the discussion about how to select patients with early SSc who have progressive disease for randomized controlled trials (RCTs). In 1995, the ACR published guidelines for clinical trials in SSc that recommended the inclusion of patients with early diffuse cutaneous SSc (dcSSc) in RCTs in which the main goal is 'prevention of worsening', with early dcSSc defined by both disease duration of <2 years and the presence of clinically apparent involvement of skin of the extremities proximal to the elbows or knees or of the trunk.[7] However, in at least three large RCTs (533 patients in total) of treatments for skin sclerosis (D-penicillamine, recombinant human relaxin and oral bovine type I collagen trials), the skin thickness score improved after the patients entered the clinical trials irrespective of disease duration,[8] suggesting that 'early disease' defined by the 1995 ACR guidelines fails to identify patients with progressive disease. In view of this situation, the criteria were amended in 2001, by incorporating microvascular changes and autoantibodies, to include an 'early SSc' classification.[9] A series of attempts to make new classification and diagnostic criteria especially for early SSc followed, including the VEDOSS (Very Early Diagnosis Of SSc) study.[10] Eventually, these activities established the essential basis for the development of the 2013 criteria, which markedly increased the classification of patients with early and limited cutaneous disease as having definite SSc.

Useful biomarkers are not available to estimate disease progression, except for SSc-related autoantibodies, but the establishment of a large registry of patients with early SSc by using the 2013 criteria would enable the identification of new biomarkers of progressive SSc. Such biomarkers would facilitate RCTs in patients with progressive disease, and thus the drawing of definitive conclusions regarding the potential disease-modifying effects of various existing and new therapies.

> **"Better recognition ... will help to trace the clinical course of early and progressive disease..."**

Overall, the major result of the GSRG's study is the validation of the high sensitivity of the 2013 criteria, not only in patients with SSc in general, but also in subgroups with early disease or lcSSc. Further validation of the sensitivity and specificity of the 2013 criteria in specific cohorts, particularly in certain ethnic groups, is awaited. Better recognition of early SSc, lcSSc and SSc sine scleroderma, which are missed by the 1980 criteria, will help to trace the clinical course of early and progressive disease; the identification of biomarkers would be quite useful in SSc RCTs focused on skin and lung involvement, for which no existing drugs have had definite benefits so far.

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**Competing Interests**
The authors declare no competing interests.

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**Title: Fli1 haploinsufficiency induces fibrosis, vascular activation and immune abnormalities resembling systemic sclerosis in bleomycin-treated mice**

**Running head: SSc phenotype induction by BLM in *Fli1*$^{+/-}$ mice**

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**Abstract**

**Objectives:** Friend leukemia virus integration 1 (Fli1) is a potential predisposing factor of systemic sclerosis (SSc), which is constitutively downregulated in the lesional skin of this disease by an epigenetic mechanism. To investigate the impact of Fli1 deficiency on the induction of an SSc phenotype in various cell types, we generated bleomycin-treated skin fibrosis in *Fli1*$^{+/-}$ mice and investigated the molecular mechanisms explaining its phenotypical alterations.

**Methods:** mRNA levels and protein expression of target molecules were examined by quantitative reverse transcription PCR and immunostaining. Transforming growth factor (TGF)-β bioassay was used to evaluate the activation of latent TGF-β. The occupancy of the target gene promoters with Fli1 was assessed with chromatin immunoprecipitation.

**Results:** Bleomycin induced greater dermal fibrosis in *Fli1*$^{+/-}$ mice than in wild type mice. Fli1 haploinsufficiency activated dermal fibroblasts via up-regulation of αVβ3 and αVβ5 integrins and activation of latent TGF-β. Dermal fibrosis in *Fli1*$^{+/-}$ mice was also attributable to endothelial-to-mesenchymal transition, which is directly induced by Fli1 deficiency and amplified by bleomycin. Th2/Th17 skewed inflammation and increased infiltration of mast cells and macrophages were seen partly due to the altered expression of cell adhesion molecules in endothelial cells as well as induction of the skin chemokines. *Fli1*$^{+/-}$ macrophages preferentially differentiated into an M2 phenotype by interleukin-4 or -13.

**Conclusions:** These results provide strong evidence for the fundamental role of Fli1 deficiency in inducing SSc-like phenotypic alterations in dermal fibroblasts, endothelial cells, and macrophages in a manner consistent with human disease.

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## Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy and fibrosis of the skin and certain internal organs (1). It is generally accepted that SSc is caused by the complex interplay between hereditary and environmental factors, leading to the accumulation of predisposing factors and the subsequent activation of endothelial cells, immune cells and fibroblasts (2). Various combinations of predisposing factors may explain disease heterogeneity and a variety of organ involvement in SSc.

Transcription factor Friend leukemia virus integration 1 (Fli1), a member of Ets transcription factor family, may represent such a predisposing factor of SSc, which is epigenetically suppressed in SSc skin and SSc dermal fibroblasts (3). Fli1 expression is decreased in non-lesional SSc skin in various cell types, including dermal fibroblasts, endothelial cells and perivascular inflammatory cells, suggesting that downregulation of Fli1 is an early event preceding the development of fibrosis (4). The factors that might be involved in downregulation of Fli1 include transforming growth factor (TGF)-β (4) and interferon-α (5) in addition to epigenetic mechanisms (3). Fli1 deficiency induces the SSc phenotype in dermal fibroblasts and dermal microvascular endothelial cells at the molecular levels (4-6). In dermal fibroblasts, Fli1 functions as a potent repressor of the *COL1A1* and *COL1A2* genes (4, 6-9) and its deficiency modulates the expression of other fibrosis-related genes, resulting in myofibroblastic differentiation (10-15). In endothelial cells, Fli1 deficiency suppresses the expression of genes regulating endothelial cell-endothelial cell and endothelial cell-pericyte interactions and up-regulates matrix metalloproteinase 9, thus promoting the remodeling of vascular basement membrane (16). Notably, endothelial cell-specific *Fli1* knockout (*Fli1* ECKO) mice reproduce the histological and functional abnormalities characteristic of SSc vasculopathy (16). However, *Fli1* ECKO or $Fli1^{+/-}$ mice do not spontaneously develop apparent clinical symptoms of SSc.

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Since the complexity of SSc cannot be entirely explained by genetic predisposition, epigenetic components such as Fli1 deficiency potentially plays a critical role in the development of this disease. The previous data regarding Fli1 deficient mice support this idea and further suggest that some additional factors may synergize with Fli1 deficiency to promote the development of clinically definite SSc. To assess this hypothesis, we generated a bleomycin (BLM)-induced murine model of SSc using *Fli1*$^{+/-}$ mice and investigated the role of Fli1 deficiency in the induction of SSc phenotype by focusing on the key cell types involved in the pathogenesis of SSc.

## Materials and methods

**Mice.** Eight-week-old C57BL/6 female mice were used. Breeding was from *Fli1*$^{+/+}$ × *Fli1*$^{+/-}$ parents. The derivation of *Fli1*$^{+/-}$ mice was described previously (17). All studies and procedures were approved by the Committee on Animal Experimentation of University of Tokyo Graduate School of Medicine.

**BLM-induced murine model of SSc.** BLM (200 μg) dissolved in phosphate buffered saline (PBS) or control PBS was injected subcutaneously into a single location on the back of the mice daily for 4 weeks (18).

**Histological assessment.** On the next day of the final injection, mice were sacrificed, and skin sections were taken. Six-μm thick sections were stained with H&E, Masson's trichrome, and toluidine blue. Dermal thickness was examined as previously described (19). Immunohistochemistry was performed using antibodies directed α-smooth muscle actin (mouse clone 1A4; Sigma-Aldrich, St. Louis, MO), connective tissue growth factor (CTGF/CCN2) (sc-14939; Santa Cruz Biotechnology, Santa Cruz, CA), integrin β3 (sc-6627; Santa Cruz Biotechnology), integrin β5 (sc-5401; Santa Cruz Biotechnology), CD4 (rat clone 2H9; Relia Tech Inc. Wobum, MA), CD8 (mouse clone 53-6.7; BD Pharmingen), F4/80 (rat clone A3-1; AbD Serotec, Eching, Germany), and arginase 1 (sc-18531; Santa Cruz Biotechnology). For immunofluorescence, goat

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anti-VE-cadherin antibody (sc-6458; Santa Cruz Biotechnology), rabbit anti-fibroblast specific protein 1 (FSP1) antibody (ab41532; abcam, Cambridge, U.K.), goat anti-integrin β3 antibody (sc-6627; Santa Cruz Biotechnology), and goat anti-integrin β5 antibody (sc-5401; Santa Cruz Biotechnology) were used as primary antibodies and FITC conjugated donkey anti-rabbit IgG antibody (sc-2090; Santa Cruz Biotechnology) and Alexa Fluor donkey 555 anti-goat IgG antibody (A21432; Invitrogen, Carlsbad, CA) were used as secondary antibodies. Coverslips were mounted by using Vectashield with DAPI (Vector Laboratories, Burlingame, CA), and staining was examined by using Bio Zero BZ-8000 (Keyence, Osaka, Japan) at 495 nm (green), 565 nm (red), and 400 nm (blue). Ten random grids were evaluated under high magnification by two independent researchers (T.T. and Y.A.) with blinded manners. All experiments included at least 4 mice per study group.

**Hydroxyproline measurement.** Following the instruction of QuickZyme Total Collagen Assay (QuickZyme Biosciences, Leiden, Netherlands), 6-mm punch biopsy skin samples were hydrolyzed with 6N HCl and collagen content was quantified. A hydroxyproline standard solution of 0–6 mg/ml was used to generate a standard curve.

**Cell culture.** Human dermal fibroblasts, human dermal microvascular endothelial cells (HDMECs), and peritoneal macrophages were prepared and maintained as described previously (8, 16, 20, 21). In some experiments, dermal fibroblasts were treated with 2 ng/ml of TGF-β1 (R & D Systems, Minneapolis, MN) and/or 20 μg/ml of RGD or RGE peptides (Takara Bio Inc., Shiga, Japan) for 24 hours and peritoneal macrophages were stimulated with 20 ng/ml of mouse interleukin (IL)-4 or 13 (R & D Systems) for 24 hours.

**siRNA.** Cells were transfected with 10 nM Fli1 siRNA or scrambled non-silencing RNA (SCR) (Santa Cruz Biotechnology) for 48 hours using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) shortly after seeding. Thereafter, cells were serum starved for 24 hours and subsequently harvested.

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**RNA isolation and quantitative reverse transcription PCR.** Total RNA was isolated from lower back skin of mice and distal one-third of the forearm of SSc patients and healthy controls with RNeasy spin columns (Qiagen). One μg of RNA was reverse transcribed using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA). Real-time quantitative PCR was carried out using SYBR Green PCR Master Mix (Life technologies, Gaithersburg, MD) on ABI prism 7000 (Life technologies) in triplicates. The mRNA levels were normalized to those of the GAPDH gene. The relative change in the levels of genes of interest was determined by the $2^{-\Delta\Delta CT}$ method. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification. The sequences of the primers used are shown in supplementary Table S1.

**TGF-β bioassay.** To determine the TGF-β activation, transformed mink lung reporter cells (TMLC) were co-cultured with either transfectant as described previously (22). TMLC and test cells were mixed at a ratio of 1:1 and suspended at $1 \times 10^6$ cells/ml in modified eagle medium containing 1% fetal bovine serum. These cells were plated at 200 μl/well in 12-well plates and cultured for 24 hours. Cell lysates were prepared using the Reporter Lysis buffer (Promega, Madison, WI), and the luciferase activity was determined using the Promega luciferase assay system. In experiments without cell-cell contact, similar co-cultures were performed in 24-well plates with inserts designed for attachment-dependent cell culture (Millicell-PCF 3 μm filter, Millipore, Bedford, MA), but $1.5 \times 10^5$ TMLC and test cells were added to lower and upper chambers, respectively. In some experiments, fibroblasts were treated with 2 ng/ml of TGF-β1 before co-culture with TMLC and co-cultures were conducted in the presence of 20 μg/ml of RGD or RGE peptides.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP assay was performed using EpiQuik ChIP kit (Epigentek, Farmingdale, NY). After reversal of crosslinking, the immunoprecipitated chromatin was amplified by PCR of specific regions of target genes. The amplified DNA products were resolved by agarose gel electrophoresis. Putative

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Fli1 binding site was predicted by Tfsitescan. Primer sequences are shown in supplementary Table S2.

**Extraction of collagen from skin by the acetic acid method.** The acetic acid extraction of collagen was performed as previously described (23, 24). Skin punches (8 mm) were taken from the dorsa of each mouse. Next, skin pieces were minced and incubated in 10 volumes of PBS overnight at 4°C with stirring. Tissue was harvested by centrifugation at 12,000 x g for 15 minutes and suspended in 10 volumes of cold 0.5 M acetic acid with or without the addition of pepsin (1:10 ratio of pepsin to tissue wet weight). Extraction was performed overnight at 4°C with stirring, and supernatant was dialyzed against 0.1 M acetic acid. Next, the dialysates with the addition of pepsin were treated with pepstatin A (Sigma-Aldrich), followed by lyophilization. Lyophilized proteins were resuspended in cold 0.1 M acetic acid and were tumbled for ~20 hours. Equal aliquots from each sample were neutralized with 1 M Tris base, boiled in sample buffer with the addition of 2-mercaptoethanol, and resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue. Collagen levels were quantified using public domain software ImageJ.

**Statistical analysis.** Statistical analysis was carried out using GraphPad Prism with one-way ANOVA with Bonferroni post-hoc tests for multiple group comparison and two-tailed unpaired t-test for two group comparison. For comparing two group values that did not follow Gaussian distribution, two-tailed Mann-Whitney U test was used. $P < 0.05$ was considered statistically significant. Within-group distributions are expressed as mean ± SEM.

## Results

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**BLM induces greater dermal thickness in *Fli1*$^{+/-}$ mice than in wild type mice.**

Four-week BLM injection induced greater dermal thickness in *Fli1*$^{+/-}$ mice than in wild type (WT) mice (2.4 fold versus 1.8 fold, P <0.05), while dermal thickness was comparable between PBS-treated *Fli1*$^{+/-}$ and WT mice (Figure 1A). In Masson's trichrome staining, an increase of signal intensity in response to BLM treatment was confirmed in both WT and *Fli1*$^{+/-}$ mice, but more importantly a slight to moderate increase of the signal intensity was detected not only in BLM-treated *Fli1*$^{+/-}$ mice compared with BLM-treated WT mice but also in PBS-treated *Fli1*$^{+/-}$ mice compared with PBS-treated WT mice (left panels of Figure 1B). This observation was also confirmed by hydroxyproline assay (a right panel of Figure 1B). Furthermore, the number of α-smooth muscle actin-positive cells showed the same trend, i.e. the significant elevation in both WT and *Fli1*$^{+/-}$ mice in response to BLM treatment and in *Fli1*$^{+/-}$ mice compared with WT mice after 4-week of PBS or BLM treatment (Figure 1C). Thus, Fli1 haploinsufficiency is sufficient to activate dermal fibroblasts *in vivo* and cooperatively establish tissue fibrosis together with additional factors induced by BLM. To elucidate the mechanism underlying this observation, we further investigated the impact of Fli1 haploinsufficiency on fibroblasts, endothelial cells and immune cells *in vivo* and *in vitro*. Since inflammation and dermal fibrosis reach the peak at day 7 and 28 after BLM injection, respectively (18, 25-27), skin samples collected at day 7 and 28 were used in the subsequent experiments.

**Fli1 haploinsufficiency cooperates with BLM in activation of latent TGF-β through the αVβ3 and αVβ5 integrin-dependent mechanism, leading to CTGF induction.**

To assess the impact of Fli1 haploinsufficiency on dermal fibroblasts, we first focused on TGF-β1 and CTGF. Fli1 haploinsufficiency significantly increased *Ctgf* mRNA levels while not affecting *Tgfb1* mRNA levels in murine skin after 4-week BLM injection (Figure 2A). Furthermore, CTGF was mainly expressed in dermal fibroblasts

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of BLM-treated mice as confirmed by colocalization of CTGF with FSP1, a marker of fibroblasts, in double immunofluorescence (Figure S1). Given that TGF-β strongly induces CTGF expression in dermal fibroblasts and that CTGF is indispensable for the TGF-β-dependent induction and maintenance of dermal fibrosis *in vivo* (28), we speculated that BLM may increase the sensitivity of *Fli1*$^{+/-}$ fibroblasts to TGF-β.

Since αVβ3 and αVβ5 integrins, receptors for latent form TGF-β, increase the sensitivity of SSc dermal fibroblasts to TGF-β by recruiting and activating latent TGF-β on cell surface (22, 29-31), the impact of Fli1 deficiency on the expression of these integrins was investigated *in vivo* and *in vitro*. mRNA levels of *Itgav*, *Itgb3* and *Itgb5* were modestly, but significantly increased at baseline in *Fli1*$^{+/-}$ mice compared with WT mice and BLM further potentiated expression of those genes, especially *Itgb3* and *Itgb5*, in *Fli1*$^{+/-}$ mice, while a smaller increase was observed in WT mice (Figure 2A). In immunostaining, the elevated expression of these integrins, especially in dermal fibroblasts, was confirmed at protein levels (Figure S2 and S3). Consistently, Fli1 siRNA, which achieved about 73% reduction of *Fli1* mRNA levels, significantly but moderately increased mRNA levels of *ITGAV*, *ITGB3*, and *ITGB5* compared to SCR in dermal fibroblasts (1.3, 1.6, and 1.3-fold increase, respectively; $P < 0.05$). Furthermore, TGF-β1 induced the mRNA expression of *ITGB3* and *ITGB5*, but not *ITGAV*, to a greater extent in Fli1 siRNA-treated fibroblasts compared to SCR-treated fibroblasts (2.3 and 1.9-fold increase, respectively; $P < 0.05$; Figure 2B).

To gain further insights into the Fli1-dependent activation of latent TGF-β, we employed a TGF-β bioassay by culturing Fli1 siRNA- or SCR-treated dermal fibroblasts with TMLC, a mink lung epithelial reporter cell line stably expressing a portion of the plasminogen activator inhibitor 1 promoter (32). When cultured as a mixture with fibroblasts, the luciferase activity was significantly higher in TMLC co-cultured with Fli1 siRNA-treated fibroblasts than in those co-cultured with SCR-treated fibroblasts (1.20-fold increase, $P < 0.01$; a left panel of Figure 2C). In

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contrast, when co-culture assays were conducted with inserts to separate TMLC and fibroblasts while allowing soluble molecules to pass, TMLC co-cultured with Fli1 siRNA-treated fibroblasts showed significantly lower induction of luciferase activity than those co-cultured with SCR-treated fibroblasts (0.78-fold decrease, $P < 0.05$; a middle panel of Figure 2C). These results, which are consistent with the previous reports (22, 30), indicate that a decrease of soluble activated TGF-β in cultured medium of Fli1 siRNA-treated fibroblasts is likely due to the activation of endogenous latent TGF-β and a subsequent consumption by fibroblasts. Given that Fli1 siRNA-dependent induction of luciferase activity was quite weak despite the high sensitivity of the TGF-β bioassay, the modest increase of αVβ3 and αVβ5 expression by Fli1 haploinsufficiency is likely to be insufficient for the induction of TGF-β-dependent genes, including CTGF. Since exogenous active TGF-β1 greatly increases these integrins in Fli1 siRNA-treated fibroblasts, TGF-β bioassay was also performed with Fli1 siRNA- or SCR-treated fibroblasts stimulated with active TGF-β1 before co-culture. Notably, pretreatment with active TGF-β1 further enhanced luciferase activity in TMLC co-cultured with Fli1 siRNA-treated fibroblasts compared to those co-cultured with SCR-treated fibroblasts (2.04-fold increase, $P < 0.01$; a left panel of Figure 2C). Furthermore, an RGD peptide that blocks the binding of αVβ3 and αVβ5 to latent TGF-β abolished elevated luciferase activity completely in TMLC co-cultured with Fli1 siRNA-treated fibroblasts and also greatly reduced this activity in TMLC co-cultured with Fli1 siRNA-treated fibroblasts pre-stimulated with active TGF-β1. RGD treatment, on the other hand, had a negligible effect on SCR-transfected fibroblasts pre-treated with active TGF-β1 (a right panel of Figure 2C), suggesting that the combination of Fli1 siRNA and exogenous TGF-β1 activates latent TGF-β in part through the integrin-mediated mechanism.

To elucidate the impact of integrin-dependent latent TGF-β activation on CTGF expression, we further evaluated *CTGF* mRNA levels in Fli1 siRNA- or SCR-treated fibroblasts stimulated with TGF-β1. TGF-β1 induced the expression of the

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*CTGF* gene to a greater extent in Fli1 siRNA-treated fibroblasts than in SCR-treated fibroblasts, while *CTGF* mRNA levels were comparable without TGF-β1 treatment (a left panel of Figure 2D). Furthermore, RGD peptide suppressed the expression of the *CTGF* gene in Fli1 siRNA-treated fibroblasts stimulated with TGF-β1 to the levels comparable with those in SCR-treated fibroblasts stimulated with TGF-β1 (a right panel of Figure 2D). These results indicate that gene silencing of Fli1 augments exogenous TGF-β-dependent CTGF induction through αVβ3 and αVβ5 integrin-dependent activation of latent TGF-β in dermal fibroblasts. Overall, these experiments strongly suggest that BLM in cooperation with Fli1 haploinsufficiency induces fibrogenic genes, including CTGF, at least partly through the integrin-mediated activation of latent TGF-β.

**Fli1 haploinsufficiency enhances endothelial-to-mesenchymal transition in response to BLM.**

Endothelial-to-mesenchymal transition (EndoMT) is a mechanism underlying pathological fibrosis, including BLM-induced pulmonary fibrosis (33-36). Therefore, we examined if Fli1 haploinsufficiency facilitates BLM-induced EndoMT by double immunofluorescence for FSP1 and VE-cadherin (Figure 3A and 3B). BLM-treated *Fli1*$^{+/-}$ mice showed a greater number of FSP1/VE-cadherin double positive cells distributed in the lower dermis than BLM-treated WT mice. Notably, FSP1/VE-cadherin double positive cells were detected in the dermis of PBS-treated *Fli1*$^{+/-}$ mice while absent in PBS-treated WT mice. In HDMECs, Fli1 siRNA decreased mRNA expression of an endothelial marker, *VE-cadherin*, and increased mRNA expression of mesenchymal markers, *FSP1* and *ACTA2*, and *SNAI1*, an essential transcription factor regulating EndoMT (Figure 3C). Furthermore, Fli1 occupied the promoter regions of the *FSP1* and *SNAI1* genes (Figure 3D) in addition to the previously reported *VE-cadherin* gene (16). Thus, Fli1 deficiency directly contributes to EndoMT and this

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process is further facilitated by BLM-induced factors.

**Fli1 haploinsufficiency modulates the expression of cell adhesion molecules related to a skewed T-helper cell response.**

We previously demonstrated that the increased infiltration of mast cells and macrophages and the preferential polarization toward T helper 2 (Th2) and T helper 17 (Th17) phenotypes contribute to the development of BLM-induced dermal fibrosis in mice (37). Furthermore, we demonstrated that Th2/Th17 polarization depends on the expression of intercellular adhesion molecule 1 (ICAM1) and glycosylation-dependent cell adhesion molecule 1 (GlyCAM1) acting as positive regulators and P-selectin and E-selectin as negative regulators of these processes (37). Therefore, we next investigated the impact of Fli1 on cell adhesion molecules *in vivo* and *in vitro*. mRNA levels of the *Icam1* and *Glycam1* genes were increased, while those of the *Selp* and *Sele* genes were decreased in BLM-treated *Fli1*$^{+/-}$ mice compared with BLM-treated WT mice at day 7 (Figure 4A). Furthermore, Fli1 siRNA, which achieved 89% reduction of *Fli1* mRNA levels, significantly increased mRNA levels of the *ICAM1* and *GlyCAM1* genes while decreasing those of the *SELP* and *SELE* genes in HDMECs (Figure 4B). Moreover, Fli1 occupied the promoter regions of the *ICAM1*, *SELP* and *SELE* genes (Figure 4C). Collectively, these results indicate that Fli1 haploinsufficiency directly modulates the expression of cell adhesion molecules, leading to the promotion of pro-fibrotic inflammatory cell infiltration and Th2/Th17 dominant immune polarization in BLM-treated mice.

**Fli1 haploinsufficiency reproduces the expression profiles of cytokines and chemokines characteristic of SSc.**

We next examined mRNA levels of various cytokines and chemokines implicated in the pathogenesis of SSc in the lesional skin of mice. The mRNA levels of *Il1b*, *Il4*, *Il6*, *Il10*,

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*Il17a*, *Ifng*, *Tnfa* and *Mcp1* were significantly higher and those of *Il12a* were significantly lower in BLM-treated *Fli1*$^{+/-}$ mice than in BLM-treated WT mice at day 7 (Figure 5A), while the levels were comparable between PBS-treated *Fli1*$^{+/-}$ and WT mice (Figure S3). Thus, Fli1 haploinsufficiency augments the expression levels of cytokines and chemokines characteristic of SSc in the lesional skin of BLM-treated mice.

**The number of macrophages and mast cells and the ratio of CD4$^+$/CD8$^+$ T cells are increased in the lesional skin of BLM-treated *Fli1*$^{+/-}$ mice.**

We next evaluated the phenotype of immune cells in the lesional skin of BLM-treated *Fli1*$^{+/-}$ mice. The number of macrophages and mast cells was markedly increased in BLM-treated *Fli1*$^{+/-}$ mice compared with BLM-treated WT mice at day 7 and 28 (Figure 5B and S4). In contrast, the number of CD4$^+$ and CD8$^+$ cells was decreased at day 7 and 28, while CD4$^+$/CD8$^+$ ratio was significantly increased at day 7 in BLM-treated *Fli1*$^{+/-}$ mice compared with BLM-treated WT mice (Figure 5C and S4). Given that the number of macrophages, especially alternatively activated macrophages (M2 macrophages), and mast cells and the ratio of CD4$^+$/CD8$^+$ T cells are increased in the lesional skin of early diffuse cutaneous SSc (38, 39), these data suggest that Fli1 haploinsufficiency supports the development of an SSc-like immune response in the lesional skin of BLM-treated mice.

**Fli1 haploinsufficiency promotes M2 macrophage infiltration in the lesional skin of BLM-treated mice and M2 differentiation of peritoneal macrophages by IL-4 or IL-13 stimulation.**

Since M2 differentiation of macrophages promotes tissue fibrosis in various pathological conditions (40-42), including SSc (39, 43, 44), we next investigated if Fli1 haploinsufficiency promotes M2 differentiation of macrophages. mRNA levels of M2