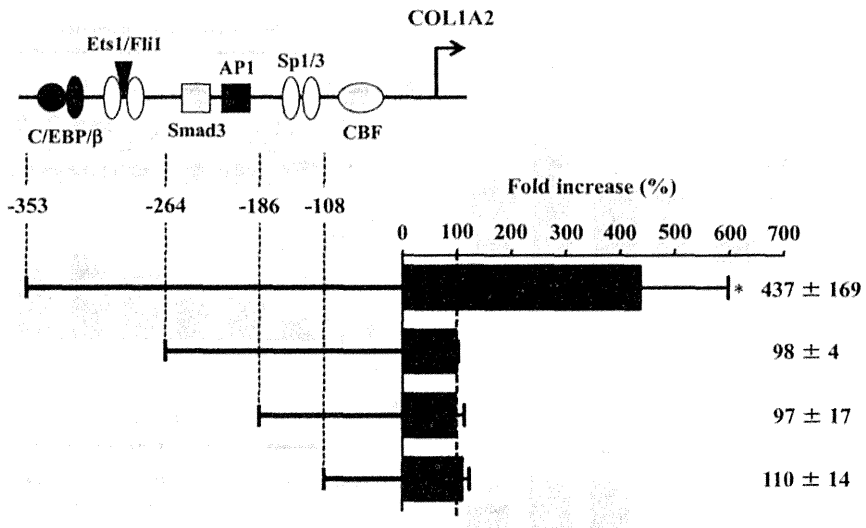


**Figure 2 Identification of the responsive element to endothelin-1 (ET-1) stimulation in the human α2 (I) collagen (COL1A2) promoter.** Normal dermal fibroblasts were transfected with 2 μg of the indicated 5′-deletion of the COL1A2/CAT construct and incubated for 48 hours. In some experiments, cells were treated with ET-1 (200 nM) for the last 24 hours. Values represent CAT mRNA levels relative to those of untreated cells transfected with the same COL1A2/CAT construct (100). The mean and SD from five separate experiments are shown. *$P$ <0.05 versus control cells untreated with ET-1.

threonine 312 in response to TGF-β stimulation [33]. Therefore, we next examined if ET-1 stimulation sequentially activates c-Abl and PKC-δ in normal dermal fibroblasts. As TGF-β1 increases the expression levels of c-Abl and PKC-δ in normal dermal fibroblasts [34,35], we looked at the effect of ET-1 on the expression levels of c-Abl and PKC-δ by immunoblotting. As shown in Figure 4A, the expression levels of c-Abl and PKC-δ were markedly increased as early as 15 minutes after the ET-1 stimulation in normal dermal fibroblasts. Given that the phosphorylation levels of c-Abl reflect its activation status and nuclear localization is required for PKC-δ to directly phosphorylate Fli1, we also evaluated the phosphorylation levels of c-Abl and nuclear localization of PKC-δ under the same condition. Consistently, ET-1 increased the phosphorylation levels of c-Abl (Figure 4A) and promoted the nuclear translocation of PKC-δ (Figure 4B) in normal dermal fibroblasts. Collectively, these results indicate that ET-1 stimulation activates the c-Abl/PKC-δ/Fli1 pathway and induces the expression of the *COL1A2* gene.

### *Bosentan, a dual endothelin receptor antagonist,* decreased the expression of the *COL1A2* gene by reversing the transcriptional activity of Fli1 in SSc dermal fibroblasts

Previous reports demonstrated that bosentan, a dual ET receptor antagonist, reverses a pro-fibrotic phenotype of SSc fibroblasts [8]. However, the detailed mechanism by which bosentan exerts its prominent anti-fibrotic effect on SSc fibroblasts has still remained unknown. We previously demonstrated that Fli1 deficiency contributes to the establishment of the pro-fibrotic phenotype in SSc fibroblasts and imatinib mesylate, which targets the c-Abl/PKC-δ/Fli1 pathway, reverses the pro-fibrotic phenotype of these cells [33,36]. Given that SSc fibroblasts are constitutively activated by autocrine stimulation of transforming growth factor- β (TGF-β), a potent inducer of ET-1, and produces an excessive amount of ET-1 [4,9,31,37,38], autocrine ET-1 appears to be involved in the self-activation system in SSc fibroblasts. The present observation that ET-1 inactivates the transcriptional activity of Fli1 suggests that the blockade of autocrine ET-1 by bosentan reverses the pro-fibrotic phenotype of SSc fibroblasts by reactivating the transcriptional repressor activity of Fli1. To address this issue, we performed a series of experiments using cultured SSc dermal fibroblasts.

Supporting the contribution of autocrine ET-1 to the activation of SSc dermal fibroblasts, exogenous ET-1 did not affect type I collagen expression (Figure 5A), whereas bosentan suppressed the expression of type I collagen in a dose-dependent manner without any effect on cell viability in SSc dermal fibroblasts (Figure 5B and Table 1). Furthermore, the total levels and the phosphorylation levels of c-Abl and the total levels and nuclear localization of PKC-δ were decreased in SSc dermal fibroblasts treated with bosentan (Figure 5C and 5D). Consistently, Fli1 phosphorylation at threonine 312 was reduced (Figure 5E) and the occupancy of Fli1 on COL1A2 promoter was increased in SSc dermal fibroblasts treated with bosentan (Figure 5F).

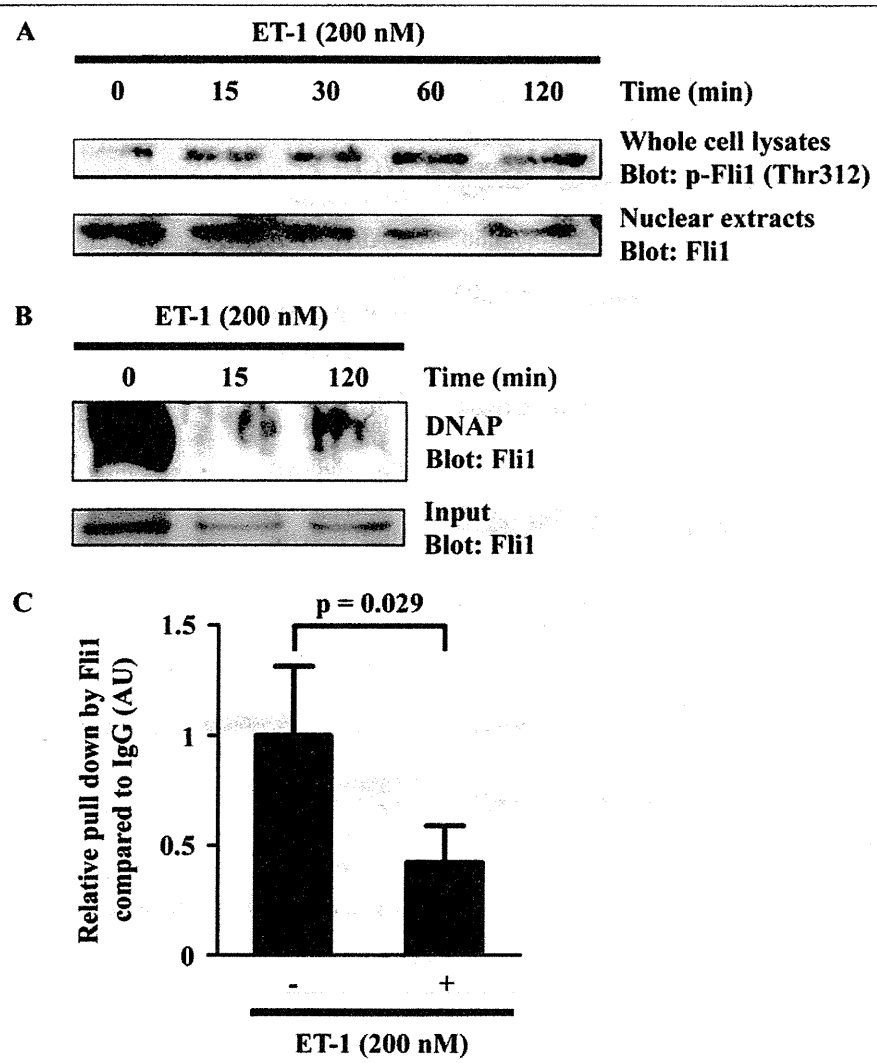


**Figure 3 Endothelin-1 (ET-1) decreased the binding of Fli1 to the human α2 (I) collagen (COL1A2) promoter via increasing its phosphorylation at threonine 312. (A)** Normal dermal fibroblasts were treated with ET-1 for the indicated period of time. Whole cell lysates were subjected to immunoblotting with anti-p-Fli1 antibody. To determine the total Fli1 levels, nuclear extracts were used for immunoblotting with anti-Fli1 antibody. **(B)** Nuclear extracts were incubated with biotin-labeled oligonucleotides. Proteins bound to these nucleotides were isolated with streptavidin-agarose beads, and Fli1 was detected by immunoblotting. Total Fli1 protein levels were determined by immunoblotting using the same nuclear extracts. **(C)** Chromatin was isolated from normal dermal fibroblasts and immunoprecipitated using rabbit anti-Fli1 antibody or rabbit IgG. After isolation of bound DNA, PCR amplification was carried out using COL1A2 promoter-specific primers. Input DNA was taken from each sample before addition of an antibody. The cycle threshold (Ct) values from IgG and Fli1 pull-down were subtracted by the number obtained from 10 × diluted input DNA. The relative pull-down of Fli1 was then normalized by the subtracted Ct value of IgG. The mean value of normal dermal fibroblasts without ET-1 stimulation was arbitrarily set at 1. All bands show one representative of three independent experiments. DNAP, DNA affinity precipitation; AU, arbitrary unit.

Importantly, bosentan did not affect the mRNA levels of the *FLI1* gene in SSc dermal fibroblasts (Figure 5G). Collectively, these results indicate that autocrine ET-1 contributes to the activation of SSc dermal fibroblasts and bosentan reverses a pro-fibrotic phenotype of SSc dermal fibroblasts by increasing the DNA binding ability of Fli1.

### Bosentan increased the expression of Fli1 protein in lesional dermal fibroblasts of the BLM-induced murine model of SSc

Finally, we investigated if bosentan increases the expression of Fli1 protein in lesional dermal fibroblasts of the BLM-induced SSc murine model because previous reports

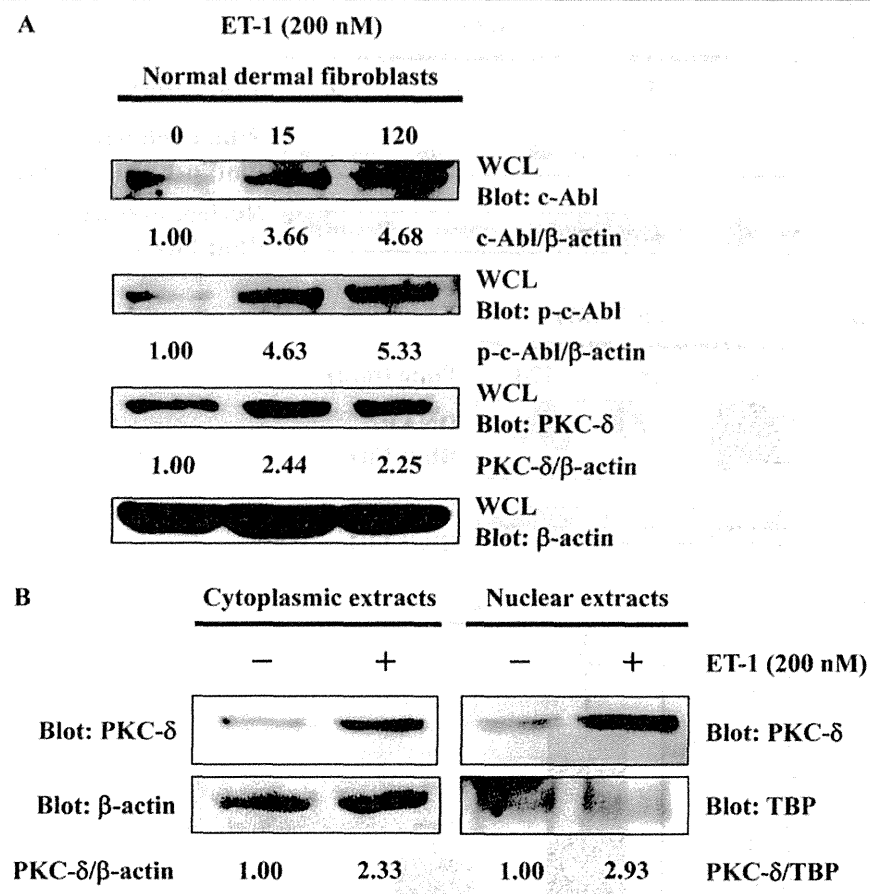


**Figure 4 Endothelin-1 (ET-1) promoted the nuclear localization of protein kinase C (PKC)-δ by activating c-Abl. (A)** Normal dermal fibroblasts were treated with 200 nM of ET-1 for the indicated period of time. The protein levels of c-Abl, PKC-δ, and β-actin and the phosphorylation levels of c-Abl were determined by immunoblotting using whole cell lysates (WCL). **(B)** Normal dermal fibroblasts were treated with ET-1 for 30 minutes and cytoplasmic and nuclear extracts were prepared. The protein levels of PKC-δ in the cytoplasm and nucleus were determined by immunoblotting. Equal amounts of loading were confirmed by immunoblotting for β-actin in cytoplasmic extracts and for TATA binding protein (TBP) in nuclear extracts. The values below each blot represent the relative levels of target molecules normalized by loading controls with densitometry.

demonstrated that bosentan prevents the development of dermal fibrosis in this model [12]. As we could reproduce the preventive effect of bosentan on dermal fibrosis in BLM-treated mice (Figure 6A), we carried out immunostaining for Fli1 in the skin samples taken from these mice. As shown in Figure 6B, in the absence of bosentan, the number of Fli1-positive dermal fibroblasts was much more decreased in dermal fibroblasts of BLM-treated mice than in those of PBS-treated mice. In contrast, when administered bosentan, the number of Fli1-positive dermal fibroblasts was comparable between BLM-treated mice and PBS-treated mice. Importantly, the signals of Fli1 and α-SMA, a marker of myofibroblasts, in double immunofluorescence were mutually exclusive in most of dermal fibroblasts (Figure 6C), indicating that Fli1 expression is closely related to the inactivation of dermal fibroblasts *in vivo*. Collectively, these results suggest that bosentan prevents the development of dermal fibrosis in the BLM-induced SSc murine model, at least partially, by increasing the expression of Fli1 protein in lesional dermal fibroblasts.

## Discussion

This study was undertaken to clarify the molecular mechanism underlying the pro-fibrotic effect of ET-1 on normal dermal fibroblasts and anti-fibrotic effect of bosentan on SSc dermal fibroblasts. A series of experiments demonstrated that ET-1 activates the c-Abl/PKC-δ/Fli1 pathway and reduces the DNA binding ability of Fli1, resulting in the induction of type I collagen expression. Given that a pro-fibrotic phenotype of SSc fibroblasts is largely due to stimulation by autocrine TGF-β, a potent inducer of ET-1, and those cells produce a much larger amount of ET-1 than normal dermal fibroblasts [1,7,38], it was speculated that the blockade of ET-1-dependent signaling by bosentan

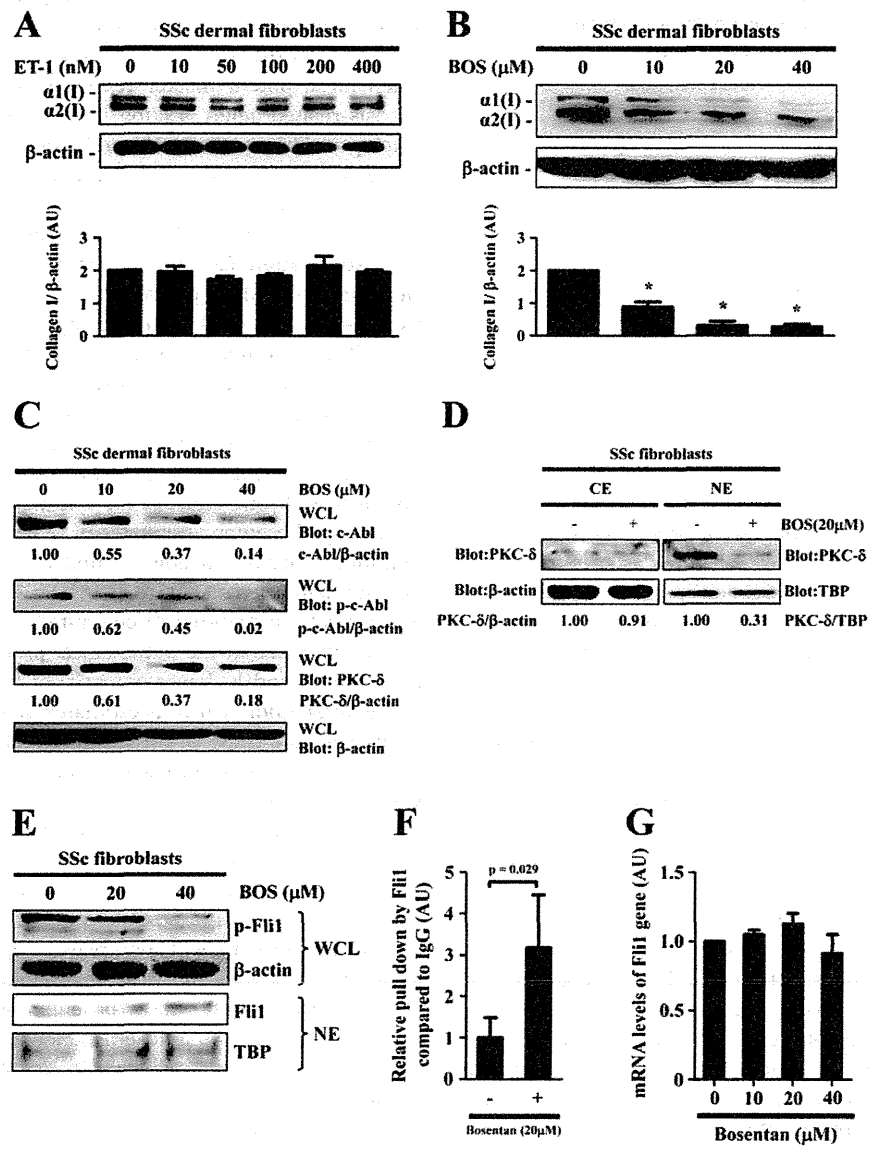


**Figure 5 Bosentan reversed the pro-fibrotic phenotype of systemic sclerosis (SSc) dermal fibroblasts via increasing the DNA binding of Fli1. (A-C)** Whole cell lysates (WCL) prepared from SSc fibroblasts treated with endothelin-1 (ET-1) for 24 hours **(A)** or bosentan (BOS) for 48 hours **(B, C)** were subjected to immunoblotting **(D)** Protein kinase C (PKC)-δ levels were examined by immunoblotting in cytoplasmic extracts (CE) and nuclear extracts (NE) prepared from SSc fibroblasts treated or untreated with bosentan for 48 hours. **(E-G)** SSc fibroblasts were treated or untreated with bosentan for 48 hours. Fli1 phosphorylation levels and total Fli1 levels were determined by immunoblotting **(E)**. Fli1 occupancy of the human α2 (I) collagen (COL1A2) promoter was determined by chromatin immunoprecipitation and reverse transcription (RT) real-time PCR **(F)**. Fli1 mRNA levels were determined by RT real-time PCR **(G)**. The graph represents fold change in type I collagen protein levels quantified by densitometry **(A, B)**, Fli1 occupancy of the COL1A2 promoter **(F)**, and Fli1 mRNA levels **(G)** in comparison to unstimulated controls, which were arbitrarily set at 1. Equal amounts of loading were confirmed by immunoblotting for β-actin in cytoplasmic extracts and for TATA binding protein (TBP) in nuclear extracts. The values below each blot represent the relative levels of target molecules normalized by loading controls with densitometry. α1(I), α1(I) procollagen; α2(I), α2(I) procollagen; AU, arbitrary unit.

reverses the pro-fibrotic phenotype of SSc dermal fibroblasts by increasing the DNA binding of Fli1 through the inhibition of the c-Abl /PKC-δ/Fli1 pathway, which is constitutively activated in those cells [33]. Supporting this hypothesis, exogenous ET-1 did not affect type I collagen expression, whereas bosentan suppressed the expression of

**Table 1 Viability of systemic sclerosis dermal fibroblasts in the presence or absence of bosentan, which was evaluated by trypan blue exclusion test**

| Duration of treatment (h) | Bosentan (μM) | | | |
|---|---|---|---|---|
| | 0 | 10 | 20 | 40 |
| 0 | 96.5 ± 0.05 | 98.8 ± 0.24 | 96.3 ± 0.70 | 97.3 ± 1.52 |
| 24 | 97.3 ± 1.13 | 97.8 ± 1.13 | 97.6 ± 1.68 | 96.3 ± 0.72 |
| 48 | 95.6 ± 0.64 | 96.2 ± 0.55 | 96.8 ± 0.25 | 95.1 ± 0.18 |

The impact of bosentan on cell viability was evaluated by trypan blue exclusion test. The percentage of cell viability was calculated according to the following formula: % cell viability = (viable cell count/total cell count) × 100. Data are expressed as the mean ± SD of results in three independent experiments.

c-Abl and PKC-δ, decreased the nuclear localization of PKC-δ and Fli1 phosphorylation at threonine 312, and eventually increased the DNA binding ability of Fli1 to the COL1A2 promoter resulting in the reduction of type I collagen expression in SSc dermal fibroblasts. Furthermore, bosentan increased the expression levels of Fli1 protein in lesional dermal fibroblasts of BLM-treated mice. As the protein stability of Fli1 increases when Fli1 binds to DNA, and is protected from degradation, these *in vivo* data suggest that bosentan prevents the development of dermal fibrosis in BLM-treated mice by increasing the DNA binding of Fli1 in lesional dermal fibroblasts. Collectively, these results indicate that the anti-fibrotic effect of bosentan on SSc dermal fibroblasts and BLM-induced SSc murine model is at least partly attributable to the increase in the DNA binding ability of Fli1.

ET-1 induces a pro-fibrotic phenotype in fibroblasts through increasing the expression of extracellular matrix (ECM) proteins, such as type I and III collagen and fibronectin, and decreasing the expression of matrix metalloproteinase 1 [39,40], therefore the blockade of ET-1 signaling has been thought to be a potential therapeutic strategy for fibrotic disorders, including SSc. The first study of the anti-fibrotic effect of bosentan on SSc fibroblasts was reported in 2004 by Shi-Wen *et al.* A series of studies from their group demonstrated that bosentan suppresses the expression of α-SMA, type I collagen, fibronectin, and CCN2 in SSc lung fibroblasts and ET-1 is a downstream mediator of pro-fibrotic responses to TGF-β in human lung fibroblasts [8,10,11]. Following these, Lagares *et al.* [12] revealed that these previous findings in lung fibroblasts are reproducible in dermal fibroblasts in *in vitro* experiments and *in vivo* animal models. Thus, ET-1 and its receptor antagonists have currently drawn much attention in the field of research on the mechanism and treatment of fibrotic disorders, but the detailed molecular mechanism explaining their effects has remained to be clarified. Since ET-1 induces a prominent fibrotic response even in the absence of Smad2/3 activation, which are important intracellular second messengers of TGF-β signaling especially in the regulation of the fibrotic gene program, ET-1 appears to inactivate a potent master repressor acting on a set of fibrosis-related genes. Taking into account that gene silencing of Fli1 results in the induction of *COL1A2* gene expression up to the level much greater than that achieved by TGF-β1 stimulation [23,25,29,32,33,41], we speculated that Fli1 may be inactivated in response to ET-1 stimulation. Consistent with this idea, a responsive element of COL1A2 promoter to ET-1 was located between −353 and −264 bp, including the Fli1 binding site, and ET-1 activated c-Abl and PKC-δ, resulting in Fli1 inactivation through its phosphorylation at threonine 312. Although other mechanisms may be involved in the pro-fibrotic effect of ET-1, the current data indicate that Fli1 largely contributes to the mechanism explaining the potent pro-fibrotic effect of ET-1 in dermal fibroblasts.

Although bosentan attenuates BLM-induced dermal and pulmonary fibrosis in animal models [12,42-44], its efficacy on skin sclerosis and ILD in SSc, to our best knowledge, has been reported to be limited [20,21]. In addition to the difference in the pathological process between SSc and animal models, lower potential of bosentan for good tissue distribution, which is estimated around 1% [22], has been cited as a cause of the unsuccessful outcome of oral bosentan therapy against skin sclerosis and ILD in SSc. Considering that the average peak plasma concentration after a single dose of 125 mg of bosentan, which is a maximal dose clinically used in humans, is 1,584 ng/ml (2.87 μM) in Caucasian and 1,922 ng/ml (3.48 μM) in Japanese subjects [45], 10 μM of bosentan, which has been used for *in vitro* experiments with fibroblasts, appears to be much higher than the concentration of bosentan in human skin tissue. In the present study, bosentan suppressed type I collagen expression in a dose-dependent manner up to 40 μM without affecting cell viability in SSc dermal fibroblasts, suggesting that endothelin receptor antagonists with better tissue distribution and tolerability may have potential as disease-modifying drugs targeting skin sclerosis and ILD in SSc by directly inactivating fibroblasts.

According to a previous report by Wang *et al.* [46], the expression of Fli1 is strongly suppressed at the transcription level by an epigenetic mechanism in SSc dermal fibroblasts. The authors demonstrated that the acetylation levels of histone H3 and H4 are decreased, while the methylation levels of CpG islands are increased, in the promoter region of the *FLI1* gene in SSc dermal fibroblasts compared with normal dermal fibroblasts, suggesting that the decrease in *FLI1* gene expression contributes to the developmental process of SSc as a potential predisposing factor. The most important observation in the present study was that bosentan reversed the decreased expression of Fli1 protein without

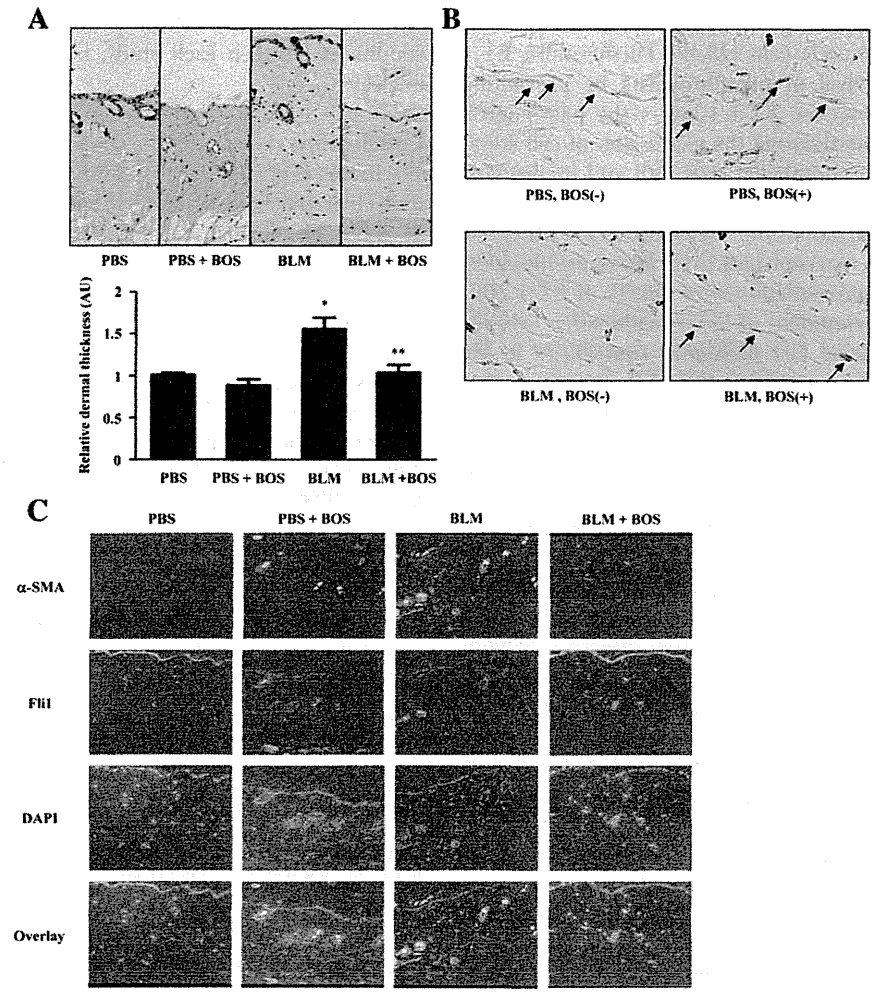


**Figure 6 Bosentan prevented the development of dermal fibrosis, at least partially, by increasing the expression of Fli1 protein in lesional dermal fibroblasts of bleomycin (BLM)-induced systemic sclerosis (SSc) murine model.** Wild-type C57BL/6 mice were injected with BLM (200 μg) or PBS into the skin of the back for 3 weeks. In some mice, bosentan (BOS) was administered intraperitoneally for 4 weeks from 1 week before BLM treatment. **(A)** The thickness of the skin on the back was evaluated by hematoxylin and eosin staining. The graph represents fold change in the dermal thickness in comparison to PBS-treated mice without bosentan administration, which was arbitrarily set at 1. **(B)** The expression levels of Fli1 protein were determined by immunohistochemistry. The arrows represent Fli1-positive dermal fibroblasts. **(C)** Double immunofluorescence for Fli1 (red) and α-SMA (green). Blue signals of nuclei were detected with 4′,6-diamidino-2-phenylindole (DAPI). The bottom panels represent the overlay of the three above. *$P$ <0.05 versus PBS-treated mice without bosentan. **$P$ <0.05 versus BLM-treated mice without bosentan. AU, arbitrary unit.

affecting its gene expression status in SSc dermal fibroblasts. These results suggest that bosentan can increase Fli1 protein levels regardless of the degree of epigenetic transcriptional suppression in those cells. Given that the reversion of epigenetically repressed disease-related genes by drugs is a general idea for the treatment of various diseases, bosentan may serve as a disease-modifying drug for SSc. Although the efficacy of bosentan on skin sclerosis and ILD is limited, recent papers demonstrated the potential of bosentan as a disease-modifying drug for SSc vasculopathy. In addition to the prevention of new digital ulcers by bosentan [15,16], Guiducci *et al.* [47] revealed that one-year treatment with bosentan significantly decreases the number of advanced nailfold capillary changes, such as capillary disorganization, ramified capillaries, and capillary loss, while increasing the number of relatively early changes, including enlarged capillaries, megacapillaries, and hemorrhage, in SSc patients. These clinical findings suggest that the effect of endothelin receptor antagonists on SSc vasculopathy goes beyond the reversal of the potent vasoconstrictive effects of endothelin. This notion has

been supported by some experimental data. For example, ET-1 has a potent mitogenic action on fibroblasts and vascular smooth muscle cells [48,49]. Furthermore, ET-1 prolongs the survival of myofibroblasts by preventing apoptosis [8]. Moreover, ET-1 triggers the pathological inflammation by modulating the expression of cell adhesion molecules on endothelial cells [50] and by promoting the production of free radical and fibrosis-inducing cytokines, such as monocyte chemoattractant protein-1 and TGF-β, from macrophages [51]. These proliferative, pro-fibrotic, and pro-inflammatory effects of ET-1 contribute to the development of SSc vasculopathy. Considering that endothelial Fli1 deficiency contributes to the development of SSc vasculopathy [52], bosentan may exert its disease-modifying effect on SSc vasculopathy by increasing endothelial Fli1 expression. The research regarding the effect of bosentan on endothelial Fli1 deficiency is currently underway in our laboratory.

Similar to bosentan, our latest paper demonstrated that imatinib reverses the decreased expression of Fli1 in SSc dermal fibroblasts by increasing its protein stability and without affecting its gene expression status [33], suggesting that imatinib is a potential disease-modifying drug for SSc patients. Consistent with this idea, imatinib moderately improved skin sclerosis of SSc patients in a couple of case reports [53,54] and case series [55-57]. Although imatinib was poorly tolerated and failed to show a significant effect on skin sclerosis of SSc patients in a couple of clinical trials [58], previous case reports suggest that some derivatives of imatinib with a better tolerability may be effective for skin sclerosis in a certain subset of SSc patients. Similarly, the clinical response of digital ulcers associated with SSc to bosentan varies from patients to patients [15,16]. Given that SSc is a multifactorial disease caused by a complex interaction between hereditary factors and environmental influences, these clinical data suggest that imatinib and bosentan may target some disease-associated factors, leading to the modification of the natural disease course in a certain subset of SSc patients. Given that both of c-Abl tyrosine kinase inhibitors and endothelin receptor antagonists target Fli1, a potential predisposing factor of SSc, further studies on the association between the degree of Fli1 suppression and the clinical efficacy of these drugs may provide us some clue to properly select good responders and effectively administer these treatments for SSc patients.

In the present study, 200 nM of ET-1 showed the maximal stimulatory effect on type I collagen expression in normal human dermal fibroblasts. However, previous reports demonstrated that 100 nM of ET-1 exhibits a significant stimulatory effect on the expression levels of type I collagen, fibronectin, fibrillin-1, and α-SMA and the gel contraction in murine embryonic fibroblasts and human normal lung and dermal fibroblasts [8,9,12,40,59]. This discrepancy may be attributable to the different sensitivity of fibroblasts to ET-1 and/or to the different experimental conditions between each study, but the actual reason is unclear.

## Conclusions

We herein reported the first study regarding the detailed molecular mechanism underlying the pro-fibrotic effect of ET-1 on normal dermal fibroblasts and the anti-fibrotic effect of bosentan on SSc dermal fibroblasts, in which transcription factor Fli1, a potential predisposing factor in SSc, is an important target. Although the efficacy of bosentan for dermal and pulmonary fibrotic conditions associated with SSc is limited, the present observation definitely provides us with a useful clue to further explore the potential of the upcoming new dual endothelin receptor antagonists with better clinical effects as disease-modifying drugs for SSc.

### Abbreviations

β-GAL: β-galactosidase; BLM: bleomycin; bp: base pairs; CAT: chloramphenicol acetyltransferase; ChIP: Chromatin immunoprecipitation; COL1A2: human α2 (I) collagen; ET-1: endothelin-1; EBS: Ets binding site; ILD: interstitial lung disease; PBS: phosphate buffered saline; PKC: protein kinase C; RT: reverse transcription; SSc: systemic sclerosis; TGF: transforming growth factor.

### Competing interests

Y Asano has received honoraria and research funding from Acterion.

### Authors' contributions

KA: data collection and analysis, drafting the manuscript and final approval of the manuscript. YA: conception and design, analysis and interpretation of data, manuscript writing, critical revision and final approval of the manuscript. NA: data collection and analysis, drafting the manuscript and final approval of the manuscript. SN: data collection and analysis, drafting the manuscript and final approval of the manuscript. T Taniguchi: data collection and analysis, drafting the manuscript and final approval of the manuscript. T Takahashi: data collection and analysis, drafting the manuscript and final approval of the manuscript. YI: data collection and analysis, drafting the manuscript and final approval of the manuscript. T Toyama: data collection and analysis, drafting the manuscript and final approval of the manuscript. SS: analysis and interpretation of data, critical revision and final approval of the manuscript. All authors read and approved the final manuscript.

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# Effects of the immunosuppressant rapamycin on the expression of human α2(I) collagen and matrix metalloproteinase 1 genes in scleroderma dermal fibroblasts

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ABSTRACT

*Background:* Rapamycin has been shown to exert an anti-fibrotic effect on skin fibrosis in a certain subset of patients with systemic sclerosis (SSc) and in bleomycin-treated animal models.
*Objectives:* To investigate the mechanism responsible for the anti-fibrotic effect of rapamycin especially by focusing on human α2(I) collagen (COL1A2) and matrix metalloproteinase 1 (MMP1) genes in normal and systemic sclerosis (SSc) dermal fibroblasts.
*Methods:* The expression levels of type I procollagen and MMP1 proteins were analyzed by immunoblotting and the mRNA levels of COL1A2 and MMP1 genes were evaluated by quantitative real-time RT-PCR. The activities of COL1A2 and MMP1 promoters were determined by reporter analysis.
*Results:* Rapamycin significantly decreased the levels of type I procollagen protein and COL1A2 mRNA, while significantly increasing the levels of MMP1 protein and mRNA in normal dermal fibroblasts. Similar effects of rapamycin were also observed in SSc dermal fibroblasts. Importantly, the inhibitory and stimulatory effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes, respectively, were significantly greater in SSc dermal fibroblasts than in normal dermal fibroblasts. In SSc dermal fibroblasts, rapamycin affected the expression of COL1A2 gene at the post-transcriptional level. In contrast, rapamycin altered the expression of MMP1 gene at the transcriptional level through the JNK/c-Jun signaling pathway in those cells.
*Conclusion:* Rapamycin has a potential to directly regulate the deposition of type I collagen in extracellular matrix through inhibiting type I collagen synthesis and promoting its degradation by MMP1, suggesting that this drug is useful for the treatment of SSc.


## 1. Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disorder characterized by initial vascular injuries and resultant fibrosis of the skin and certain internal organs. Although the pathogenesis of SSc still remains unknown, there has been a wealth of evidence suggesting that a number of humoral and cellular immune abnormal reactions, including B cells, T cells, and macrophages [1,2], in early SSc extensively damage and activate microvascular endothelial cells and fibroblasts in the dermis, eventually leading to the promotion of the excessive extracellular matrix (ECM) deposition [3–9]. Consistent with the notion that immune abnormality is a primary event in the pathogenesis of SSc, several studies have demonstrated that various immunosuppressive agents, such as cyclosporine A [10,11], tacrolimus [11], cyclophosphamide [9], methotrexate [12,13], and mycophenolate mofetil [14,15], are useful against the vascular and/or fibrotic lesions in this disorder.

Rapamycin, isolated from *Streptomyces hygroscopicus*, is macrolide antibiotic, which has also been known as a potent immunosuppressive drug with the property of calcineurin inhibitor as well as

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cyclosporine A and tacrolimus. Similarly to tacrolimus, rapamycin exerts a variety of effects on various cell types via binding to immunophilin FK506-binding protein (FKBP12). Following the binding, the complex inhibits mammalian target of rapamycin (mTOR), which regulates cell growth and proliferation as well as metabolic homeostasis [16,17]. In T cells, for instance, the complex inhibits cell growth and proliferation by impairing the response to growth-promoting cytokines including interleukin-2 and subsequently interfering with the downstream signaling pathways required for G1 progression [18,19]. Indeed, rapamycin has been widely used for the prevention of graft rejection in kidney transplant recipients due to its relatively lower nephrotoxicity than other calcineurin inhibitors [20–22]. Importantly, several experimental studies have shown that rapamycin also exerts an anti-fibrotic effect on liver and pulmonary fibrosis in animal models [23,24], supporting the conventional idea that the suppression of inflammation prior to fibrosis is efficacious for the treatment of fibrotic diseases. Alternatively, these experimental data may be attributable to the direct anti-fibrotic effect of rapamycin on dermal fibroblasts.

Previous clinical trials [9,12,13] demonstrated that a certain subset of immunosuppressants is useful for the treatment of skin fibrosis in SSc. Given that SSc fibroblasts keep producing excessive amount of ECM even in the in vitro culture system, where these cells are free from inflammatory attacks, these immunosuppressants appear to exert their anti-fibrotic effect via directly affecting homeostasis of the ECM, which is maintained by a balance between the production and the degradation of ECM proteins. Supporting this idea, we have previously shown that tacrolimus suppresses the pro-fibrotic property of SSc fibroblasts through decreasing the stability of α2(I) collagen (COL1A2) mRNA [25]. Based on these backgrounds with our recent data that rapamycin suppresses bleomycin-induced skin fibrosis in animal models [26], in this study we investigated if rapamycin affects the expression of COL1A2 and matrix metalloproteinase 1 (MMP1) genes using normal and SSc dermal fibroblasts. We also discussed the potential mechanism by which rapamycin exerts its anti-fibrotic effect in human diseases and animal models.

## 2. Materials and methods

### 2.1. Reagents

Rapamycin and SP600125 were purchased from Calbiochem (La Jolla, CA, USA). Actinomycin D and antibody for β-actin were purchased from Sigma (St. Louis, MO, USA). Antibody for type I collagen was purchased from Southern Biotechnology (Birmingham, AL, USA). Luciferase assay kit and β-galactosidase enzyme assay kit were purchased from Promega (Madison, WC, USA). Antibody for MMP1 was obtained from Chemicon (San Francisco, CA, USA). FuGENE 6 was obtained from Roche Diagnostics (Indianapolis, IN, USA).

### 2.2. Cell cultures

After getting institutional approval and informed consent, dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of seven patients with diffuse cutaneous SSc (dcSSc) and <2 years of skin thickening and from the corresponding areas of 5 healthy donors. Primary explant cultures were established and cells were maintained as described previously [27].

### 2.3. Immunoblotting

Subconfluent quiescent normal dermal fibroblasts were treated with rapamycin at the indicated concentrations or an equal amount of vehicle (dimethyl sulfoxide, DMSO). Then, cells were cultured for an additional 72 h. Subconfluent quiescent SSc dermal fibroblasts were treated in parallel with normal dermal fibroblasts. Cell lysates were prepared and subjected to immunoblotting as described previously [28].

### 2.4. RNA preparation and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using an RNeasy Protect Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instruction. First strand-cDNA synthesis was performed with oligo (dT) primer using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time RT-PCR was performed by PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primers for COL1A2, MMP1, COL3A1, COL5A1, fibronectin, tenascin C, tissue inhibitor of metalloproteinase 1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were purchased from Applied Biosystems.

### 2.5. Plasmid construction

The full-length clone of the human MMP1 promoter containing a fragment of promoter DNA linked to the luciferase (MMP1/Lux construct) reporter was kindly provided by Dr. Brinckerhoff [29]. A −772 COL1A2/Lux construct consisting of a COL1A2 gene fragment (+58 to −772 bp relative to the transcription start site) linked to the luciferase reporter gene was generated as previously described [30]. Plasmids used in transient transfection assays were purified twice on CsCl gradients. At least two different plasmid preparations were used for each experiment.

### 2.6. Transient transfection

Transfection of reporter constructs and the determination of promoter activity were performed as described previously [28].

### 2.7. Analysis of mRNA stabilities

To investigate the stability of COL1A2 and MMP1 mRNAs, subconfluent quiescent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1.0 μg/ml), and treated with rapamycin (1.0 nM) or an equal amount of vehicle (DMSO), for the indicated times prior to RNA extraction. The mRNA levels of COL1A2, MMP1, and GAPDH genes were analyzed by quantitative real-time RT-PCR.

### 2.8. Statistical analysis

Statistical analyses were carried out with the Mann–Whitney *U* test for comparison of means. *P* values less than 0.05 were considered significant.

## 3. Results

### 3.1. Cell viability of normal and SSc dermal fibroblasts treated with rapamycin

Before starting a series of substantial experiments, we investigated toxic effect of rapamycin on dermal fibroblasts. To this end, cell viability of normal and SSc dermal fibroblasts treated with indicated concentration of rapamycin was determined by trypan blue exclusion test. As shown in Table 1, in both of normal and SSc dermal fibroblasts, rapamycin failed to significantly affect the cell mortality at all of the concentrations tested up to 72 h.

**Table 1**
Cell viability of normal and SSc dermal fibroblasts treated with rapamycin.

| | 24 h | | | | |
|---|---|---|---|---|---|
| Rapamycin | 0 nM | 0.01 nM | 0.1 nM | 1.0 nM | 10 nM |
| Normal fibroblasts | 96.5 ± 2.7 | 96.6 ± 2.5 | 96.7 ± 3.1 | 96.6 ± 2.1 | 96.9 ± 1.1 |
| SSc fibroblasts | 97.6 ± 2.8 | 97.7 ± 1.2 | 97.6 ± 2.5 | 96.1 ± 2.7 | 97.2 ± 1.0 |
| | 48 h | | | | |
| Rapamycin | 0 nM | 0.01 nM | 0.1 nM | 1.0 nM | 10 nM |
| Normal fibroblasts | 98.3 ± 1.2 | 97.3 ± 1.2 | 98.0 ± 1.0 | 97.3 ± 1.2 | 97.0 ± 1.0 |
| SSc fibroblasts | 98.0 ± 1.0 | 97.7 ± 1.5 | 97.3 ± 1.5 | 97.3 ± 1.5 | 97.0 ± 1.7 |
| | 72 h | | | | |
| Rapamycin | 0 nM | 0.01 nM | 0.1 nM | 1.0 nM | 10 nM |
| Normal fibroblasts | 98.3 ± 0.6 | 97.7 ± 1.2 | 97.7 ± 1.2 | 96.3 ± 1.2 | 96.3 ± 1.2 |
| SSc fibroblasts | 96.7 ± 2.1 | 97.3 ± 1.5 | 97.3 ± 2.1 | 96.3 ± 2.5 | 96.6 ± 2.1 |

SSc, systemic sclerosis. The values represent mean ± standard deviation.

### 3.2. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in normal dermal fibroblasts

To determine the effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in normal dermal fibroblasts, cells were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) for 72 h. Each sample of cell lysate was analyzed by immunoblotting. As shown in Fig. 1A, the expression levels of type I procollagen proteins were reduced in a dose-dependent manner by the treatment with rapamycin. The inhibitory effect was statistically significant over the concentration of 1.0 nM. As shown in Fig. 1B, in contrast, the expression levels of MMP1 proteins were increased by the treatment with rapamycin. The stimulatory effect was statistically significant at the concentration of 0.1 nM and 1.0 nM.

### 3.3. Effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts

We next investigated the effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts. Under the same conditions described above, the mRNA levels of COL1A2 and MMP1 genes were determined by quantitative real-time RT-PCR. As shown in Fig. 1C, the mRNA levels of COL1A2 gene were decreased by the treatment with rapamycin in a dose-dependent manner, and the inhibitory effect was statistically significant over a concentration of 0.1 nM. In contrast, the mRNA levels of MMP1 gene were increased by the treatment with rapamycin in a dose-dependent manner and the stimulatory effect was statistically significant over a concentration of 0.1 nM (Fig. 1D). Taken together, these results suggest that rapamycin coordinately decreases the accumulation of type I collagen proteins in ECM through the suppression of type I collagen production and the acceleration of its degradation by MMP1.

### 3.4. Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins in SSc dermal fibroblasts

We further investigated if rapamycin affects the expression levels of type I procollagen and MMP1 proteins in SSc dermal fibroblasts. As shown in Fig. 2A, the expression levels of type I procollagen proteins were reduced in a dose-dependent manner by the treatment with rapamycin. The inhibitory effect on type I procollagen proteins was statistically significant over a concentration of 0.1 nM. In contrast, as shown in Fig. 2B, the expression levels of MMP1 proteins were increased in a dose-dependent manner by the treatment with rapamycin. The stimulatory effect on MMP1 proteins was statistically significant over a concentration of 1.0 nM. Importantly, the stimulatory effect of rapamycin on MMP1 proteins was much greater in SSc dermal fibroblasts than in normal dermal fibroblasts at a concentration of 10 nM (mean ± SD [fold increase]: 2.80 ± 1.29 vs. 1.22 ± 0.22, $P < 0.05$).

### 3.5. Effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts

We also determined the effects of rapamycin on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts by quantitative real-time RT-PCR. As shown in Fig. 2C, the mRNA levels of COL1A2 gene were decreased by the treatment with rapamycin in a dose-dependent manner, and the inhibitory effect was statistically significant over a concentration of 0.01 nM. In contrast, as shown in Fig. 2D, the mRNA levels of MMP1 gene were increased by the treatment with rapamycin in a dose-dependent manner, and the stimulatory effect was statistically significant over a concentration of 1.0 nM.

### 3.6. Rapamycin exerts greater inhibitory and stimulatory effects on the mRNA expression of COL1A2 and MMP1 genes, respectively, in SSc dermal fibroblasts than in normal dermal fibroblasts

To further assess if rapamycin exerts differential effects on the expression levels of COL1A2 and MMP1 genes between normal and SSc dermal fibroblasts, we focused on the magnitude of its inhibitory and stimulatory effects on the mRNA levels of COL1A2 and MMP1 genes, respectively, at the highest concentration (10 nM). As shown in Table 2, the basal expression levels of COL1A2 gene was significantly increased in SSc dermal fibroblasts compared with normal dermal fibroblasts (2.59 ± 1.44 arbitrary unit [AU] vs. 1.00 ± 0.18 AU, $P < 0.01$). After the treatment of rapamycin, the mRNA levels of COL1A2 gene in SSc dermal fibroblasts were decreased to the levels comparable to those in normal dermal fibroblasts (0.79 ± 0.33 AU vs. 0.71 ± 0.09 AU). Importantly, the magnitude of its inhibitory effect on the mRNA levels of COL1A2 gene was much greater in SSc dermal fibroblasts than in normal dermal fibroblasts (fold increase: 0.36 ± 0.18 vs. 0.71 ± 0.09, $P < 0.05$). On the other hand, the mRNA basal levels of MMP1 gene were comparable between SSc and normal dermal fibroblasts (0.86 ± 0.43 AU vs. 1.00 ± 0.25 AU). After the treatment of rapamycin, there was a trend toward the elevation of MMP1 mRNA levels in SSc dermal fibroblasts compared with normal dermal fibroblasts, but it did not reach a statistical difference (2.51 ± 1.11 AU vs. 144 ± 0.27 AU, $P = 0.073$). Similarly to COL1A2 gene, the magnitude of its stimulatory effect on the mRNA levels of MMP1 gene was much greater in SSc

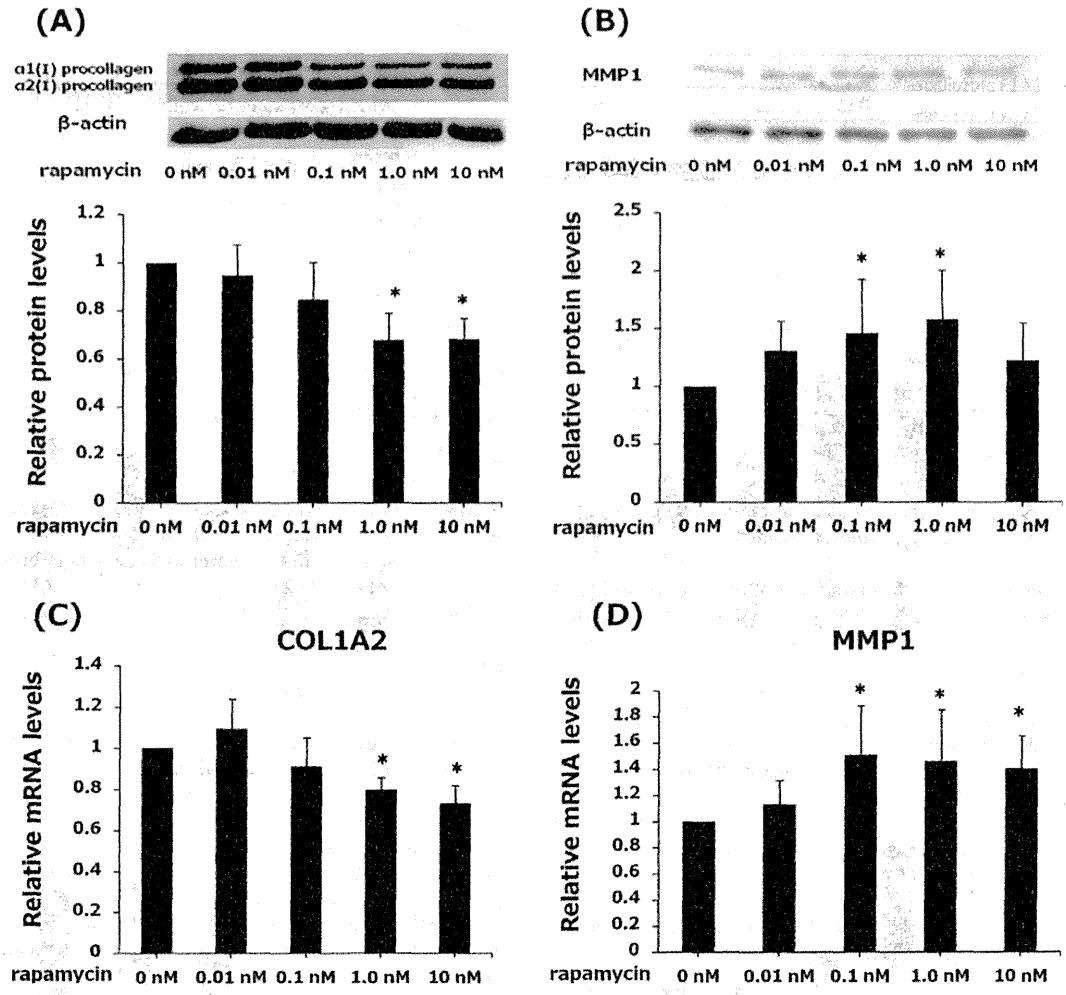


**Fig. 1.** Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins, and the mRNA levels of COL1A2 and MMP1 genes in normal dermal fibroblasts. (A) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) and incubated for 72 h. Each sample of cell lysate was analyzed by immunoblotting using anti-type I collagen antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated normal fibroblasts (bottom). Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (B) Subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 72 h. Each sample of cell lysate was analyzed by immunoblotting using anti-MMP1 antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (C) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of COL1A2 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of COL1A2 corrected for the mRNA levels of GAPDH gene in the same samples are shown relative to those in untreated normal dermal fibroblasts. Data are expressed as the mean and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts. (D) Subconfluent normal dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA for MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated normal dermal fibroblasts. Data are expressed as the mean and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated normal dermal fibroblasts.

dermal fibroblasts than in normal dermal fibroblasts (fold increase: $3.06 \pm 1.04$ vs. $1.46 \pm 0.14$, $P < 0.01$). Of note, there was a trend toward a negative correlation between basal levels and fold increase values in the mRNA levels of COL1A2 gene, but it did not reach a statistical significance probably due to the small number of samples ($r = -0.72$, $P = 0.088$). Collectively, these results indicate that rapamycin exerts greater effects on the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts with prominent fibrotic phenotype.

### *3.7. Rapamycin decreases the expression of COL1A2 gene at the post-transcriptional level in SSc dermal fibroblasts*

To further determine the mechanism responsible for the inhibitory effect of rapamycin on the expression of COL1A2 gene, we examined if rapamycin affects the expression levels of COL1A2 mRNA at the transcriptional level or at the post-transcriptional level. To this end, we investigated if rapamycin affects the stability of COL1A2 mRNA in SSc dermal fibroblasts using a transcriptional inhibitor, actinomycin D. One hour after the addition of actinomycin D, cells were treated with 1.0 nM rapamycin or the same amount of vehicle (DMSO) for the indicated period. The levels of remaining COL1A2 mRNA were determined by quantitative real-time RT-PCR. As shown in Fig. 3A, the stability of COL1A2 mRNA was significantly decreased in SSc dermal fibroblasts treated with 1.0 nM rapamycin compared with SSc dermal fibroblasts treated with DMSO at 24 h, indicating that rapamycin suppresses the expression of COL1A2 gene at the post-transcriptional level. To further examine if rapamycin affects the

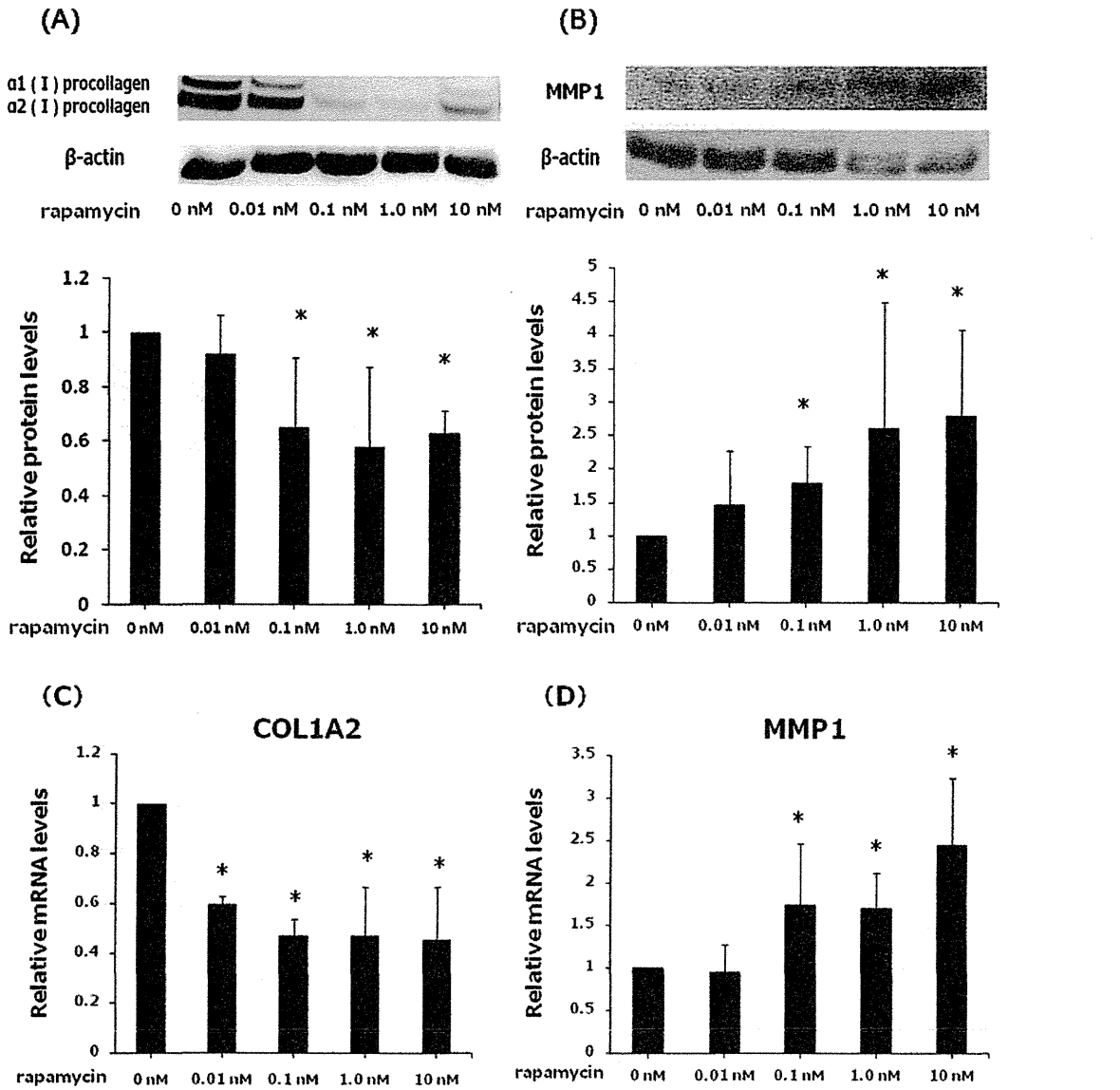


**Fig. 2.** Effects of rapamycin on the expression levels of type I procollagen and MMP1 proteins, and the mRNA levels of COL1A2 and MMP1 genes in SSc dermal fibroblasts. Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin or the same amount of vehicle (DMSO) and incubated for 72 h. (A) Each sample of cell lysate was analyzed by immunoblotting using anti-type I collagen antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of six independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of five or six independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) Each sample of cell lysate was analyzed by immunoblotting using anti-MMP1 antibody. The levels of β-actin proteins are shown as a loading control (top). Shown is a representative experiment of four independent experiments. The levels quantified by scanning densitometry are shown relative to the levels in untreated SSc dermal fibroblasts (bottom). Data are expressed as the mean and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (C) Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of COL1A2 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of COL1A2 corrected for the mRNA levels of GAPDH gene in the same samples are shown relative to those in SSc dermal fibroblasts treated with DMSO. Data are expressed as the mean and SD of four independent experiments. (D) Subconfluent SSc dermal fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The mRNA levels of MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 gene corrected for the levels of GAPDH gene in the same samples are shown relative to those in SSc dermal fibroblasts treated with DMSO. Data are expressed as the mean and SD of four or five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts.

transcriptional activity of COL1A2 gene, we performed transient transfection assay using the −772 COL1A2/Lux construct. As shown in Fig. 3B, the treatment with rapamycin for 48 h did not affect the promoter activity of COL1A2 gene in SSc dermal fibroblasts. These results indicate that rapamycin suppresses the expression of COL1A2 gene at the post-transcriptional level, but not at the transcriptional level.

### *3.8. Rapamycin increases the expression of MMP1 gene at the transcriptional level in SSc dermal fibroblasts*

We also carried out the same analyses to investigate the mechanism responsible for the stimulatory effect of rapamycin on the expression of MMP1 gene. As shown in Fig. 4A, the treatment with rapamycin did not affect the stability of mRNA in SSc dermal

**Table 2**
Comparison of the inhibitory effect of rapamycin on mRNA levels of COL1A2 gene and the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene between normal and SSc dermal fibroblasts.

| | COL1A2 mRNA | | | MMP1 mRNA | | |
|---|---|---|---|---|---|---|
| | Basal | Rapamycin | Fold increase | Basal | Rapamycin | Fold increase |
| Normal No. 1 | 0.88 | 0.65 | 0.74 | 0.70 | 1.12 | 1.59 |
| Normal No. 2 | 0.94 | 0.58 | 0.61 | 0.90 | 1.31 | 1.46 |
| Normal No. 3 | 0.91 | 0.75 | 0.82 | 1.13 | 1.71 | 1.52 |
| Normal No. 4 | 1.26 | 0.83 | 0.66 | 1.27 | 1.60 | 1.26 |
| Mean ± SD | 1.00 ± 0.18 | 0.70 ± 0.11 | 0.71 ± 0.09 | 1.00 ± 0.25 | 1.44 ± 0.27 | 1.46 ± 0.14 |
| SSc No. 1 | 1.31 | 0.54 | 0.41 | 0.39 | 0.81 | 2.07 |
| SSc No. 2 | 5.26 | 0.89 | 0.17 | 0.62 | 2.42 | 3.90 |
| SSc No. 3 | 1.59 | 0.65 | 0.41 | 0.64 | 3.00 | 4.69 |
| SSc No. 4 | 3.21 | 1.01 | 0.33 | 0.73 | 2.45 | 3.36 |
| SSc No. 5 | 3.35 | 0.70 | 0.21 | 0.83 | 2.54 | 3.06 |
| SSc No. 6 | 1.98 | 1.36 | 0.72 | 1.08 | 1.85 | 1.71 |
| SSc No. 7 | 1.40 | 0.36 | 0.26 | 1.70 | 4.48 | 2.63 |
| Mean ± SD | 2.59 ± 1.44** | 0.79 ± 0.33 | 0.36 ± 0.18* | 0.86 ± 0.43 | 2.51 ± 1.11 | 3.06 ± 1.04** |

* $P < 0.05$ vs. normal dermal fibroblasts.
** $P < 0.01$ vs. normal dermal fibroblasts.

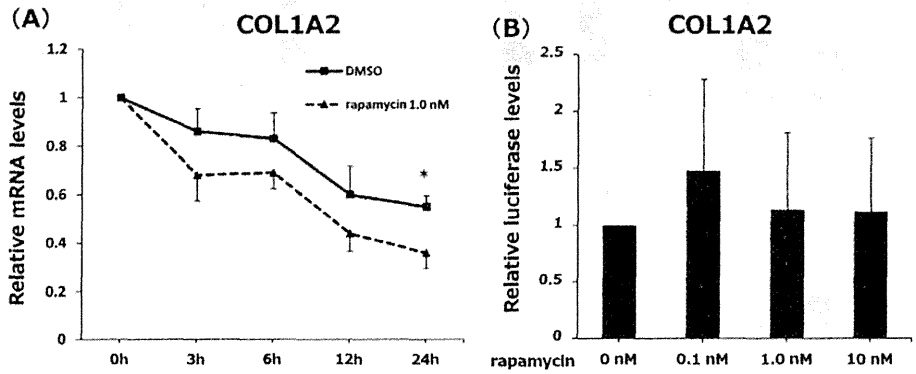


**Fig. 3.** Rapamycin decreases the expression of COL1A2 gene at the post-transcriptional level in SSc dermal fibroblasts. (A) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1 μg/ml) and treated with 1.0 nM rapamycin or vehicle for the indicated period prior to RNA extraction. The levels of remaining COL1A2 mRNA were analyzed by quantitative real-time RT-PCR. The stability of COL1A2 mRNA corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of four independent experiments. *$P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) SSc dermal fibroblasts were transiently transfected with the COL1A2/Lux construct. After incubation for 24 h, subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The promoter activities of COL1A2 and β-galactosidase genes were analyzed by luciferase assay. The promoter activity of COL1A2 corrected for the levels of β-galactosidase in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of five independent experiments.

fibroblasts, suggesting that rapamycin increases the expression of MMP1 gene at the transcriptional level. Consistently, as shown in Fig. 4B, the promoter activity of MMP1 gene was significantly increased after the exposure to rapamycin in a dose-dependent manner. Since a previous study showed that rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in human lung fibroblasts [31], we next determined whether rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in SSc dermal fibroblasts. As shown in Fig. 4C, SP600125, a specific JNK inhibitor, almost completely inhibited the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene in SSc dermal fibroblasts. These results indicate that rapamycin increases the expression of MMP1 gene through the activation of JNK/c-Jun signaling in SSc dermal fibroblasts as well as normal lung fibroblasts.

## 4. Discussion

This study was undertaken to clarify the effect of rapamycin on the expression levels of human COL1A2 and MMP-1 genes in normal and SSc dermal fibroblasts. A series of cell-based experimental data demonstrated that this reagent exerts anti-fibrotic effects on normal and SSc dermal fibroblasts through the suppression of COL1A2 gene expression and the up-regulated expression of MMP1 gene. Most importantly, the inhibitory effect of rapamycin on COL1A2 gene and the stimulatory effect of rapamycin on MMP1 gene were much greater in SSc dermal fibroblasts than in normal dermal fibroblasts. In addition to its immunosuppressive property, the present data provide another molecular basis for our previous finding that rapamycin inhibits the bleomycin-induced skin fibrosis in animal models [26]. These results indicate that this reagent possesses a therapeutic potential against skin fibrosis in SSc.

There are several different mechanisms regulating eukaryotic mRNA decay. A major mRNA decay pathway is initiated by shortening of poly(A) tail, followed by decapping of the 5′-end and subsequent 5′–3′ exonucleolytic degradation of the mRNA [32]. A number of studies have shown that the stabilization of type I collagen mRNA is deeply involved in fibrotic process due to excessive production of type I collagen proteins. The mechanism of mRNA stabilization has been well-studied in the mRNA of COL1A1 gene in human lung and dermal fibroblasts. Krupsky et al. [33]

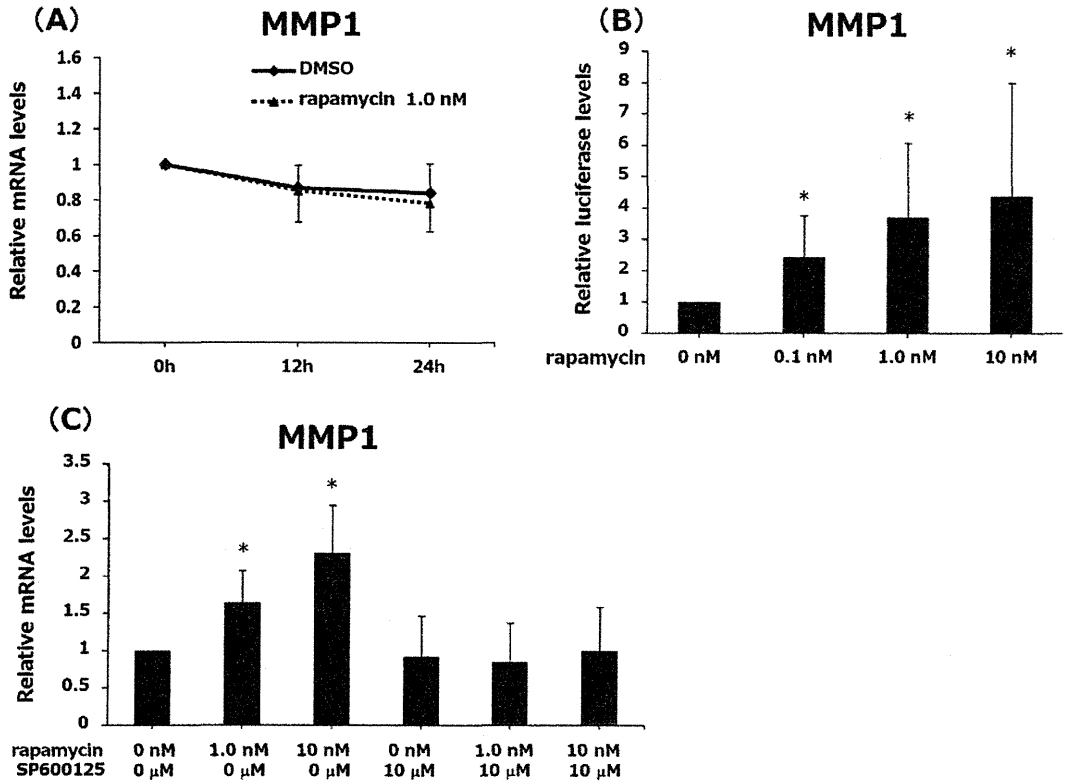


**Fig. 4.** Rapamycin increases the expression of MMP1 gene at the transcriptional level in SSc dermal fibroblasts. (A) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with actinomycin D (1 μg/ml) and treated with 1.0 nM rapamycin or vehicle for the indicated period prior to RNA extraction. The levels of remaining MMP1 mRNA were analyzed by quantitative real-time RT-PCR. The stability of MMP1 mRNA corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of four independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (B) SSc dermal fibroblasts were transiently transfected with the MMP1/Lux construct. After incubation for 24 h, subconfluent fibroblasts were treated with various concentrations of rapamycin and incubated for 48 h. The promoter activities of MMP1 and β-galactosidase genes were analyzed by luciferase assay. The promoter activity of MMP1 corrected for the levels of β-galactosidase in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of six independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts. (C) Subconfluent SSc dermal fibroblasts were pretreated for 1 h with the indicated concentration of rapamycin and incubated in the presence or absence of SP600125 (10 μM) for an additional 48 h. The mRNA levels of MMP1 and GAPDH genes were analyzed by quantitative real-time RT-PCR. The mRNA levels of MMP1 gene corrected for the levels of GAPDH gene in the same samples are shown relative to those in untreated SSc dermal fibroblasts. Values are the means and SD of five independent experiments. $^*P < 0.05$, as compared with the value in untreated SSc dermal fibroblasts.

demonstrated that the stability of COL1A1 mRNA is reduced by amino acid deprivation in human lung fibroblasts. Similarly, Varga et al. [34] disclosed that retinoic acid and prostaglandin $E_2$, which down-regulate the uptake of the transported amino acid, reduces the steady state mRNA levels of COL1A1 gene in human dermal fibroblasts. Importantly, another study revealed that rapamycin provokes a cellular stress response similar to amino acid decay [35]. Given that mTOR may act as a nutritional sensor, the inhibition of mTOR by rapamycin may reduce the stability of type I collagen mRNA through the similar signals caused by amino acid deprivation [31]. Although a stabilizing mechanism of human COL1A2 mRNA has not been well-studied, rapamycin may decrease the COL1A2 mRNA stability in SSc dermal fibroblasts through the mechanism similar to that regulating the stabilization of COL1A1 mRNA. These observations suggest that the treatment with rapamycin reduces the stability of COL1A2 mRNA through one or some of these pathways; however, further studies are required.

The post-transcriptional regulation of MMP1 gene expression still remains elusive, but a mechanism regulating the stability of MMP1 mRNA is the induction of rapid degradation through the binding of AU-rich element binding proteins to AU-rich sequences in 3′-untranslated region [36–38]. On the other hand, the transcriptional regulation of MMP1 gene has been well studied in various cell types [39]. Importantly, rapamycin increases the promoter activity of MMP1 gene through the activation of JNK/c-Jun/AP-1 signaling axis in human lung fibroblasts [31]. Consistently, in the present study, rapamycin increased the expression levels of MMP1 gene at the transcriptional level in SSc dermal fibroblasts. Furthermore, the specific JNK inhibitor, SP600125, almost completely prevented the stimulatory effect of rapamycin on the mRNA levels of MMP1 gene in SSc dermal fibroblasts. These results indicate that, similarly to lung fibroblasts, rapamycin increases the promoter activity of MMP1 gene through the activation of JNK/c-Jun cascade in SSc dermal fibroblasts.

Given that SSc dermal fibroblasts are characterized by the constitutive activation resulting in the excessive production of type I collagen proteins, the present observation that rapamycin reverses the mRNA levels of COL1A2 gene to normal levels by decreasing the mRNA stability strongly supports the potential of rapamycin to improve skin fibrosis in SSc. On the other hand, experimental data regarding the expression levels of MMP1 gene in SSc dermal fibroblasts are still controversial. Kuroda and Shinkai [40] reported that the mRNA levels of MMP1 gene in SSc dermal fibroblasts are altered in relation to disease duration. According to the report, although the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of <1 year are significantly higher than those in normal dermal fibroblasts, SSc dermal fibroblasts from patients with disease duration of 2–4 years

show low mRNA levels of MMP1 gene compared with normal dermal fibroblasts. Furthermore, the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of more than 6 years were comparable to those in normal dermal fibroblasts [40]. However, we could not reproduce the association of MMP1 mRNA levels with disease duration in SSc dermal fibroblasts in the present study as well as in a previous study [41]. In any case, one of the most important observations in this study was that rapamycin uniformly increased the mRNA levels of MMP1 gene in SSc dermal firboblasts irrespective of their basal expression levels. Collectively, rapamycin may achieve the reversal of skin sclerosis in early diffuse SSc by restoring altered homeostasis of the ECM as well as aberrant activation of immune system. These results support the previous clinical findings that rapamycin is efficacious for skin fibrosis equally to methotrexate in a certain subset of SSc patients [42].

Another important observation in this study was that rapamycin altered the expression levels of COL1A2 and MMP1 genes to a greater extent in SSc dermal fibroblasts than in normal dermal fibroblasts. This finding is plausible if mTOR is activated more greatly in SSc dermal fibroblasts than in normal dermal fibroblasts. Supporting this hypothesis, Pannu and Trojanowska reported that mTOR expression is elevated in SSc dermal fibroblasts in vitro [43]. Given that TGF-β stimulation activates mTOR in fibroblasts and that SSc dermal fibroblasts are constitutively activated by autocrine TGF-β stimulation [27,28,44,45], mTOR may be activated as a result of autocrine TGF-β stimulation in SSc dermal fibroblasts. Taken together with the evidence that rapamycin suppressed mRNA levels of COL3A1 and COL5A1 genes, while not those of fibronectin, tenascin C and tissue inhibitor of metalloproteinase 1 genes in SSc dermal fibroblasts (supplementary Fig. 1), it is likely that the activation of mTOR is largely involved in the regulation of fibrillar collagen (types I, III and V) and MMP1 and rapamycin reverses the altered expression of these genes in SSc dermal fibroblasts.

Although corticosteroid is likely to induce a couple of severe complications in SSc, including scleroderma renal crisis especially at the dose of >15 mg/day [46], it is widely used to suppress the inflammatory reactions prior to resultant fibrosis and to improve the skin fibrosis itself in early diffuse SSc [47]. This situation largely depends on the lack of established anti-fibrotic and anti-inflammatory treatments against this disorder. Since rapamycin has a relatively low-nephrotoxicity compared with other calcineurin inhibitors, such as cyclosporine A and tacrolimus [20–22], the combination therapy of rapamycin with corticosteroid may be potentially useful to decrease the dose of corticosteroid and to reduce the adverse events due to conventional calcineurin inhibitors, such as nephrotoxicity. Importantly, in case of renal transplantation, a suitable trough level of rapamycin to optimize efficacy and minimize adverse effects is thought to be between 5 and 15 ng/ml (5.5–16.5 nM) [48], where rapamycin achieves a maximal anti-fibrotic effect on SSc dermal fibroblasts in the present study. Therefore, rapamycin may be a safe and useful drug for the treatment of early diffuse SSc.

In summary, we elucidated that rapamycin has a potential to directly regulate the deposition of type I collagen in ECM through inhibiting type I collagen synthesis and promoting its degradation by MMP1. To the best of our knowledge, this is the first report demonstrating one of the potential mechanisms by which rapamycin exerts its anti-fibrotic effect in human diseases and animal models. Large clinical trials are required to assess the efficacy of rapamycin in patients with early diffuse SSc in the future.Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2014.02.002.

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ORIGINAL PAPER

# Fli1 deficiency contributes to the suppression of endothelial CXCL5 expression in systemic sclerosis

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**Abstract** CXCL5 is a member of CXC chemokines with neutrophilic chemoattractant and pro-angiogenic properties, which has been implicated in the pathological angiogenesis of rheumatoid arthritis and inflammatory bowel diseases. Since aberrant angiogenesis is also involved in the developmental process of systemic sclerosis (SSc), we herein measured serum CXCL5 levels in 63 SSc and 18 healthy subjects and investigated their clinical significance and the mechanism explaining altered expression of CXCL5 in SSc. Serum CXCL5 levels were significantly lower in SSc patients than in healthy subjects. In diffuse cutaneous SSc (dcSSc), serum CXCL5 levels were uniformly decreased in early stage (<1 year) and positively correlated with disease duration in patients with disease duration of <6 years. In non-early stage dcSSc (≥1 year), decreased serum CXCL5 levels were linked to the development of digital ulcers. Consistently, the expression levels of CXCL5 proteins were decreased in dermal blood vessels of early stage dcSSc. Importantly, Fli1 bound to the CXCL5 promoter and its gene silencing significantly suppressed the CXCL5 mRNA expression in human dermal microvascular endothelial cells. Furthermore, endothelial cell-specific Fli1 knockout mice, an animal model of SSc vasculopathy, exhibited decreased CXCL5 expression in dermal blood vessels. Collectively, these results indicate that CXCL5 is a member of angiogenesis-related genes, whose expression is suppressed at least partially due to Fli1 deficiency in SSc endothelial cells. Since Fli1 deficiency is deeply related to aberrant angiogenesis in SSc, it is plausible that serum CXCL5 levels inversely reflect the severity of SSc vasculopathy.

**Keywords** Systemic sclerosis · CXCL5 · Angiogenesis · Endothelial cells · Digital ulcer · Fli1

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## Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vascular injury, and fibrosis of skin and certain internal organs. Although the pathogenesis of SSc still remains unknown, aberrant angiogenesis potentially caused by autoimmunity and inflammation has been believed to precede and contribute to the development of fibrotic and vascular organ involvement [3].

CXCL5 is a member of CXC chemokines with glutamic acid-leucine-arginine (ELR) motif [19]. The properties of CXC chemokines generally depend on the presence or absence of the ELR motif in their N terminus; $ELR^+$ CXC chemokines are potent promoters of angiogenesis as well as potent activators and chemoattractants for neutrophils, while $ELR^-$ CXC chemokines are potent inhibitors of angiogenesis without neutrophil chemoattractant property [6]. Consistently, the altered local expression of CXCL5 has been implicated in the pathogenesis of diseases characterized by accelerated neovascularization accompanied with neutrophil infiltration, such as rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD). For example, CXCL5 levels are elevated in inflamed synovial tissue and

fluid in patients with RA as compared to those with osteoarthritis [16] and serum CXCL5 levels correlate with the activity of arthritis in RA [16] and its animal models [13]. Of note, the administration of anti-CXCL5 antibody improves the severity of joint involvement in animal models of arthritis induced by adjuvant or interleukin-17 injection [13, 26]. Similarly, CXCL5 expression is much more increased in intestinal epithelial cells of patients with Crohn's disease and ulcerative colitis than in those of healthy controls [33] and topical application of CXCL5 antisense oligonucleotide reduces murine colitis induced by the ingestion of dextran sodium sulfate [18]. In a certain subset of tumors, the expression levels of CXCL5 serve as a prognostic marker because the production of CXCL5 by tumor cells and/or normal cells surrounding them promotes their proliferation, migration, and invasion as well as angiogenesis in an autocrine or paracrine fashion. Importantly, a latest paper demonstrated that CXCL5 overexpression, alone or combined with intratumoral neutrophil infiltration, is a novel prognostic predictor for hepatocellular carcinoma [34]. Thus, CXCL5 plays a unique role under various pathological conditions, including tumorigenesis, metastasis, and autoimmune inflammatory diseases, by coordinately regulating angiogenesis and neutrophil infiltration.

Although another $ELR^+$ CXCL chemokine, interleukin-8/CXCL8, has been shown to be potentially involved in the pathogenesis of interstitial lung disease associated with SSc [7, 10, 12, 27, 28], the roles of other $ELR^+$ CXCL chemokines have not been well studied in this disorder. Since aberrant angiogenesis is the central pathological event leading to fibrotic and vascular organ involvement in SSc and CXCL5 is implicated in pathological angiogenesis associated with RA [13, 16, 26] and IBD [18, 33], we interested in the role of CXCL5 during the developmental process of SSc. To address this issue, we herein investigated the clinical significance of serum CXCL5 levels and the mechanism explaining altered expression of CXCL5 in SSc.

## Materials and methods

### Patients

Serum samples, frozen at −80 °C until assayed, were obtained from 63 SSc patients [59 women, 4 men; age, median (25–75 percentiles) 59 years (51–68.5); disease duration, 6 years (2–13.5)] and 19 healthy individuals [17 women, 2 men; age, 55 years (47.5–61.8)] after getting written informed consent. Patients treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by the LeRoy's classification system: 31 diffuse cutaneous SSc (dcSSc) patients [age, 57 years (47.5–62); disease duration, 5 years (1.5–15)] and 32 limited cutaneous SSc (lcSSc) patients [age, 62 years (55.5–73); disease duration, 6 years (2.3–10.8)]. All dcSSc and 25 lcSSc patients fulfilled the criteria proposed by the American College of Rheumatology [1]. Seven lcSSc patients not meeting these criteria had sclerodactyly and at least two other features, including calcinosis, Raynaud's phenomenon, esophageal dysmotility, and telangiectasia. The whole study was performed according to the Declaration of Helsinki and approved by an ethical committee (University of Tokyo Graduate School of Medicine).

### The measurement of serum CXCL5 levels

Specific enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) were used to measure serum CXCL5 levels. Briefly, polystyrene 96-well plates coated with anti-CXCL5 antibodies were incubated with diluted sera at room temperature for 1 h. Then, the wells were washed and incubated at room temperature for 1 h with horseradish peroxidase-conjugated anti-CXCL5 antibodies. Next, the wells were washed again, added with tetramethylbenzidine, and incubated at room temperature for 20 min. Finally, sulfuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum CXCL5 levels were calculated using a standard curve.

### Clinical assessment

Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time of blood sampling. The clinical and laboratory data were obtained when the blood samples were drawn. Skin score was measured using modified Rodnan total skin thickness score (MRSS) [9]. The details of assessment are briefly summarized in the legends of Table 1.

### Immunohistochemistry

Skin samples were obtained from forearms of five dcSSc patients (<1 year) and five closely matched healthy controls. Murine skin sections were prepared using a 6-mm-diameter punch biopsy device from back skin of wild type C57BL/6 mice or endothelial cell (EC)-specific Fli1 knockout (Fli1 ECKO) mice generated by crossing $Fli1^{flox/flox}$ mice and Tie2-Cre transgenic mice [4]. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Five-micrometer-thick