# Decrease in matrix metalloproteinase-3 activity in systemic sclerosis fibroblasts causes α2-antiplasmin and extracellular matrix deposition, and contributes to fibrosis development

HIROFUMI NIWA[1], YOSUKE KANNO[1,2], EN SHU[1] and MARIKO SEISHIMA[1]

[1]Department of Dermatology, Gifu University Graduate School of Medicine, Gifu 501-1194;
[2]Department of Clinical Pathological Biochemistry, Faculty of Pharmaceutical Science, Doshisha Women's College of Liberal Arts, Kyoto 610-0395, Japan

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**Abstract.** Systemic sclerosis (SSc) is a connective tissue disease of autoimmune origin characterized by fibrosis of the skin and visceral organs, and peripheral circulatory disturbance. α2-antiplasmin (α2AP) is the major circulating inhibitor of plasmin and is a key regulator of fibrinolysis. It has been demonstrated that the expression of α2AP is elevated in dermal fibroblasts obtained from patients with SSc patients. It has also been determined that α2AP is associated with the development and progression of fibrosis in SSc. The present study assessed the relationship between α2AP and matrix metalloproteinase-3 (MMP-3), an extracellular matrix (ECM)-degrading enzyme. Serum levels of α2AP and MMP-3 were measured in healthy controls and patients with SSc using ELISA. No significant differences were determined between these two groups. α2AP, MMP-3 and tissue inhibitor of metalloproteinase-1 (TIMP-1) expression was subsequently evaluated in normal and SSc fibroblasts via western blotting. The results revealed that α2AP expression increased in SSc dermal fibroblasts, while the ratio of MMP-3/TIMP-1 decreased. Additionally, incubation of recombinant α2AP with MMP-3 caused α2AP degradation. The mixture of recombinant α2AP with MMP-3 was subsequently added to normal fibroblasts prior to western blotting. The results revealed decreased α-smooth muscle actin (α-SMA; a marker of the myofibroblast phenotype) and type I collagen expression. The stimulation of SSc fibroblasts with MMP-3 decreased protein levels of α2AP, α-SMA and type I collagen, thus reversing the pro-fibrotic phenotype of SSc fibroblasts. SSc fibroblast transfection with microRNA-29a resulted in a decreased TIMP-1 expression, but also decreased the protein expression of α2AP. The results indicated that MMP-3 attenuated fibrosis progression by degrading α2AP and ECM, and might therefore contribute to a novel therapeutic approach for SSc treatment.

*Correspondence to:* Dr Hirofumi Niwa, Department of Dermatology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan
E-mail: h_niwa@gifu-u.ac.jp

*Abbreviations:* SSc, systemic sclerosis; α2AP, α2-antiplasmin; ECM, extracellular matrix; TIMP-1, tissue inhibitor of metalloproteinase-1

*Key words:* alpha2-antiplasmin, Systemic sclerosis, Fibrosis, matrix metallopeptidase-3, tissue inhibitor of metalloproteinase-1

## Introduction

Systemic sclerosis (SSc) is a chronic connective tissue disease that causes widespread microvascular damage and excessive collagen deposition in the skin and internal organs (1). However, the etiology, pathogenesis, and progression of this disease are not fully understood.

Alpha2-antiplasmin (α2AP), is a 65-70 kDa protein that inactivates plasmin and thereby inhibits fibrinolysis (2,3). α2AP exists in various tissues, such as the liver, kidney, intestine, spleen, lung, muscle, ovary, testis, cerebral cortex, hippocampus, cerebellum, bone, skin, and placenta of murine tissue (4). Apart from the inhibition of plasmin, α2AP regulates various cell functions, including proliferation, differentiation, and cytokine production, and also associates with angiogenesis, tissue repair, vascular remodeling, and fibrosis progression (5-13). In patients with rheumatic diseases, including SSc, plasma levels of the plasmin-α2AP complex are increased (14,15). α2AP affects myofibroblast differentiation, extracellular matrix (ECM) production, vascular dysfunction, and progression of SSc. In addition, we have shown that α2AP levels are elevated in an SSc mouse model and dermal fibroblasts (16,17).

Matrix metalloproteinase-3 (MMP-3) plays a pivotal role in ECM turnover as it can degrade ECM components, including proteoglycans, collagen III, IV, V, and IX, laminin, fibronectin, gelatin, and elastin (18,19). MMP-3 is expressed by fibroblasts, chondrocytes, osteoblasts, endothelial cells, smooth muscle cells and macrophages (20). Jinnin *et al* (21) observed similar MMP-3 serum levels in SSc patients and healthy controls; however, serum levels of anti-MMP-3 autoantibody and tissue inhibitors of metalloproteinase-1 (TIMP-1) were higher in SSc

patients, suggesting that MMP-3 activity may be decreased in SSc (20,22). Since it has been reported that cleavage by MMP-3 inactivates α2AP (23), the suppression of MMP-3 activity in SSc may promote the activation of α2AP.

In the present study, we investigated the relationship between α2AP and MMP-3 to gain insights into the pathogenesis of SSc.

## Materials and methods

The experiments with human samples in this study were approved by the Gifu University Graduate School of Medicine Ethics Committee (Approved ID:29-152). We received written informed consent from the patients and volunteers involved.

*Cell culture*. Human normal and SSc dermal fibroblasts were obtained from seven patients with SSc and four healthy controls as previously described (6). Fibroblasts were seeded onto 60-mm diameter dishes and cultured in 2 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37˚C in a humidified atmosphere with 5% $CO_2$/95% air. After 6 days, the medium was replaced with serum-free DMEM. Human normal dermal fibroblasts were stimulated by α2AP, MMP-3 or mixture of α2AP and MMP-3 for 24 h. In other studies, human SSc dermal fibroblasts were stimulated by MMP-3 for 24 h.

*Western blot analysis*. Cells were washed twice with cold PBS, harvested, and then sonicated in lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5), 1% SDS, 1% Triton X-100, and a protease inhibitor cocktail (Roche Diagnostics GmbH). The skin samples from the subjects were homogenized and sonicated in the lysis buffer. The protein concentration in each lysate was measured using a BCA protein assay kit (Pierce; Thermo Fisher Scientific). Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. We detected each protein by incubation with the relevant primary antibodies followed by horseradish peroxidase-conjugated antibodies to IgG.

*Enzyme-linked immunosorbent assay (ELISA)*. Blood samples were obtained from 10 SSc patients and 10 healthy volunteers, and were subsequently centrifuged for 10 min at 1,600 x g. The supernatant was then collected and used for the assay. The serum levels of α2AP and MMP-3 were determined using ELISA kits, Human Serpin F2/α 2-Antiplasmin (R&D Systems, MN, USA) and Human Total MMP-3 Immunoassay (R&D Systems), respectively. The absorbance was measured at 450 nm using an iMark Microplate Reader (Bio-Rad Laboratories, Inc.).

*miRNA study*. SSc dermal fibroblasts were transfected with miR-29a (sequence: ACUGAUUUCUUUUGGUGUUCAG, Bioneer) or negative control miRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were harvested 48 h after transfection for further analysis.

*Statistical analysis*. All data were expressed as mean ± SEM and analyzed using Statmate III version 3.06 (ATMS Co., Ltd.).

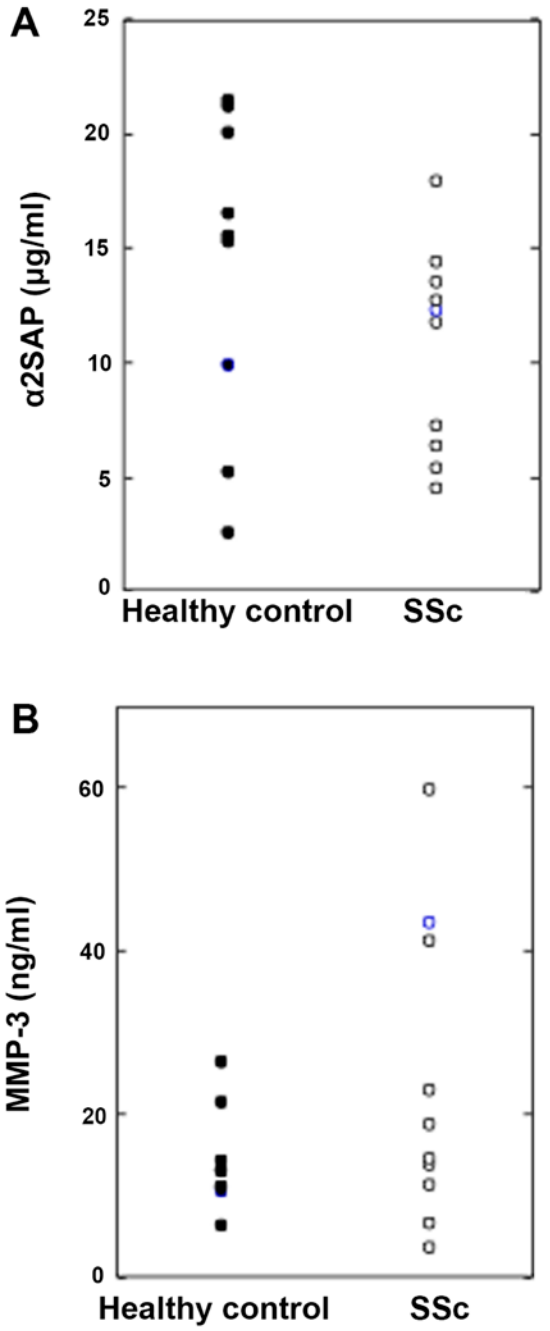


Figure 1. Serum levels of α2AP and MMP-3 in patients with SSc and healthy controls. Blood samples were obtained from 10 patients with SSc and 10 healthy volunteers, and subsequently centrifuged for 10 min at 1,600 x g and 4˚C. Supernatants were then collected and used for assessment. The serum levels of (A) α2AP and (B) MMP-3 were measured using ELISA. No significant differences were identified between patients and healthy controls. Data are presented as the mean ± SEM. α2AP, α2-antiplasmin; MMP-3, matrix metallopeptidase-3; SSc, systemic sclerosis.

The statistical analysis was conducted with unpaired t-test for two-group comparisons, with one-way ANOVA Tukey's for multiple comparison. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Serum levels of a2AP and MMP-3 are similar in healthy controls and SSc patients*. We examined the levels of α2AP

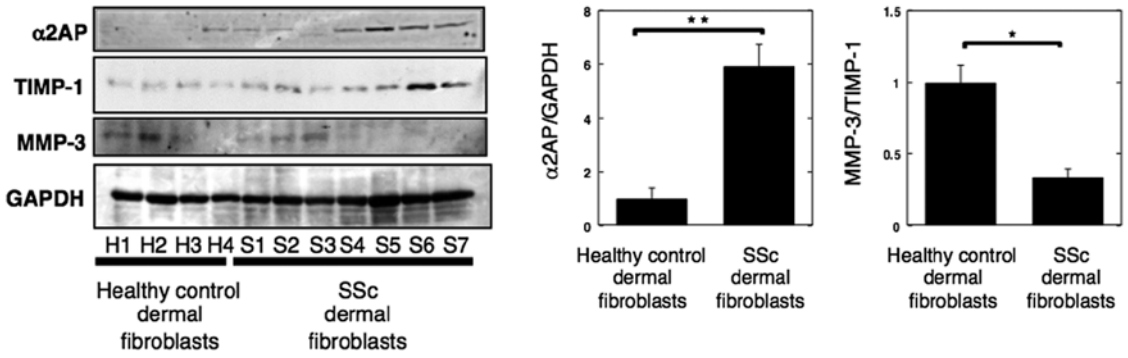


Figure 2. α2AP expression and MMP-3/TIMP-1 ratio in human normal and SSc dermal fibroblasts. Protein levels of α2AP, MMP-3 and TIMP-1 were measured via western blot analysis. The histogram provides quantitative representations of α2AP and MMP-3/TIMP-1 ratio (normal fibroblasts, n=4; SSc fibroblasts, n=7). Data are presented as the mean ± SEM. *P<0.01 and **P<0.05 as indicated. α2AP, α2-antiplasmin; MMP-3, matrix metallopeptidase-3; TIMP-1, tissue inhibitor of metalloproteinase-1; SSc, systemic sclerosis.

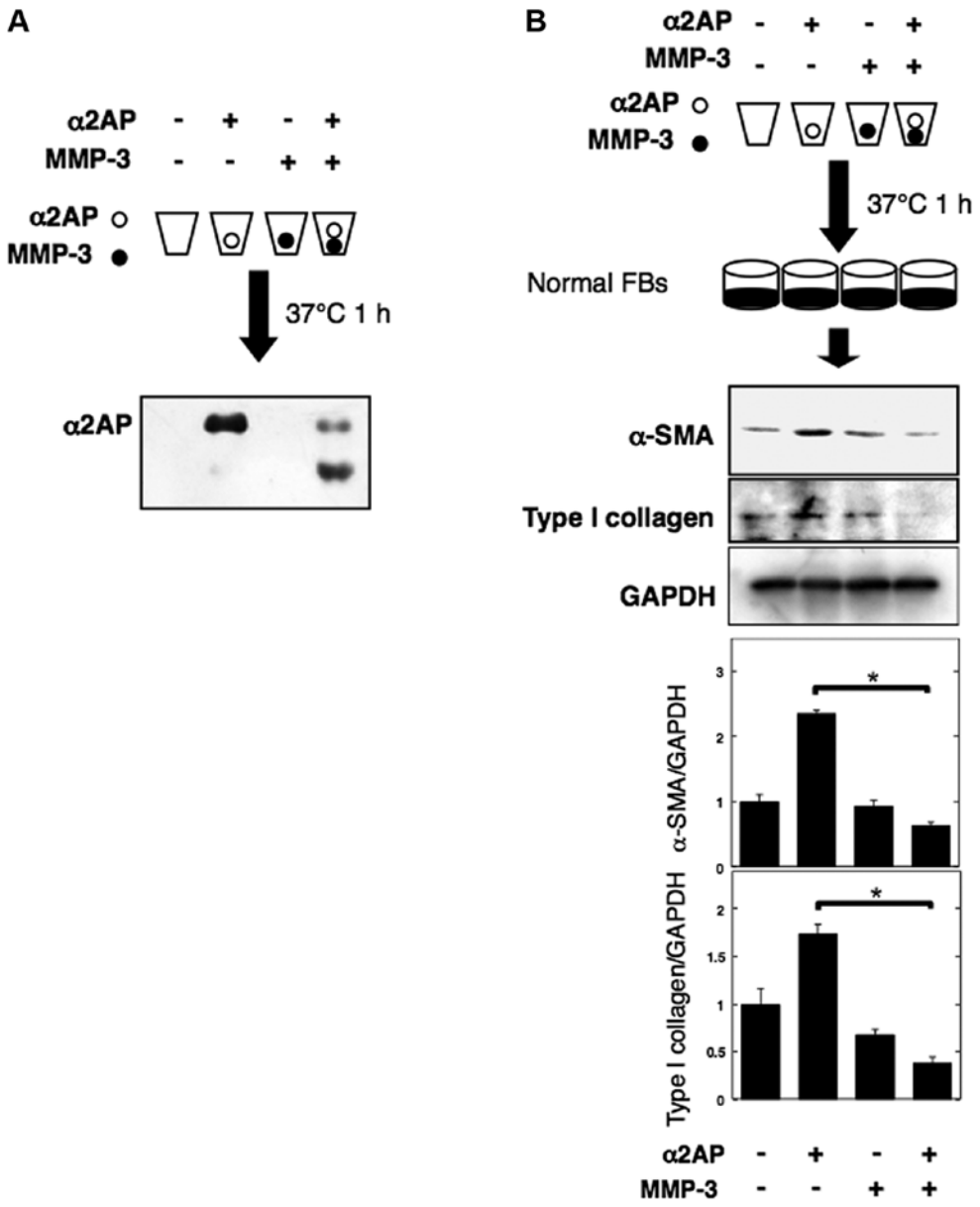


Figure 3. MMP-3-mediated α2AP degradation inhibits the pro-fibrotic effects of α2AP. (A) Recombinant α2AP was incubated with or without MMP-3 for 1 h at 37˚C, after which levels of α2AP were examined via western blotting. (B) Recombinant α2AP was incubated with or without MMP-3 in a tube for 1 h at 37˚C. Normal human dermal fibroblasts were then stimulated for 24 h. The expression of each protein was examined via western blotting. The histogram provides quantitative representations of each protein (n=4). Data are presented as the mean ± SEM. *P<0.01 as indicated. MMP-3, matrix metallopeptidase-3; α2AP, α2-antiplasmin; αSMA, α smooth muscle actin; FB, fibroblast.

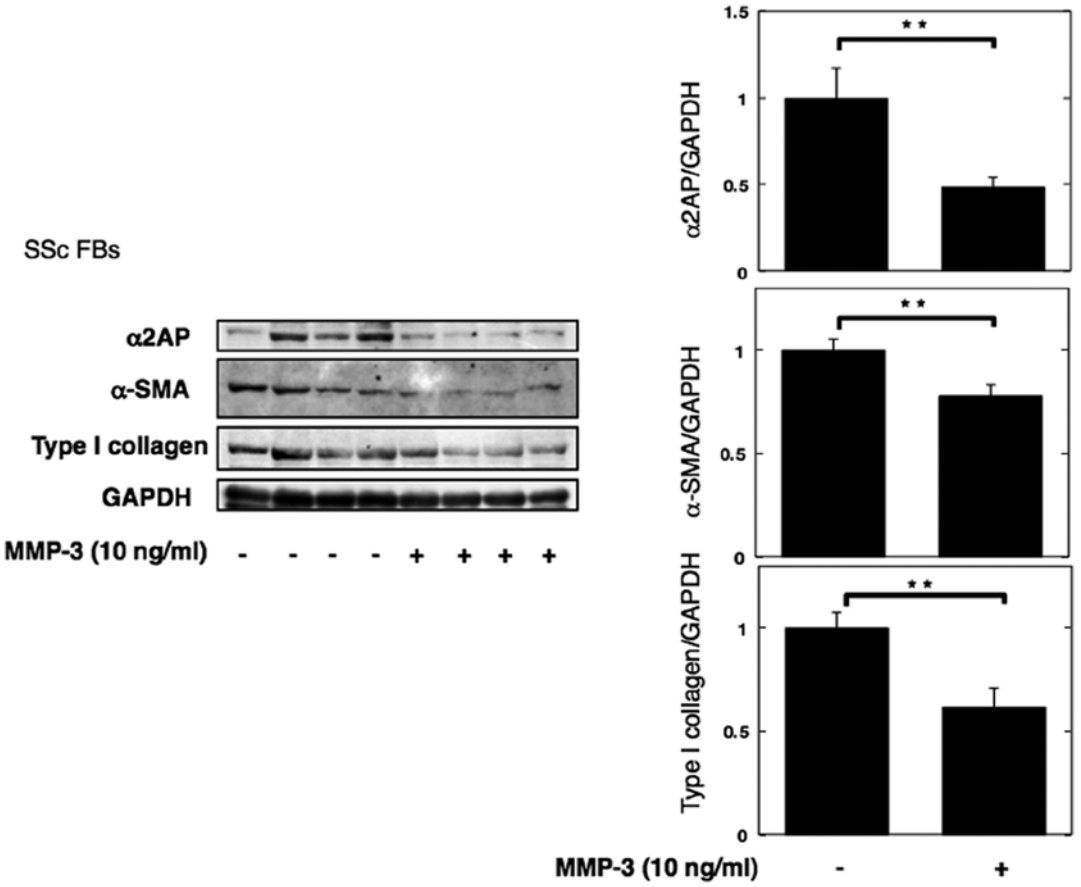


Figure 4. MMP-3 reverses the pro-fibrotic phenotype of SSc dermal fibroblasts. Human SSc dermal fibroblasts were stimulated by MMP-3 (10 ng/ml) for 24 h. The expression of each protein was subsequently determined via western blotting. The histogram provides quantitative representations of each protein (n=4). Data are presented as the mean ± SEM. **P<0.05 as indicated. MMP-3, matrix metallopeptidase-3; SSc, systemic sclerosis; α2AP, α2-antiplasmin; αSMA, α smooth muscle actin; FB, fibroblast.

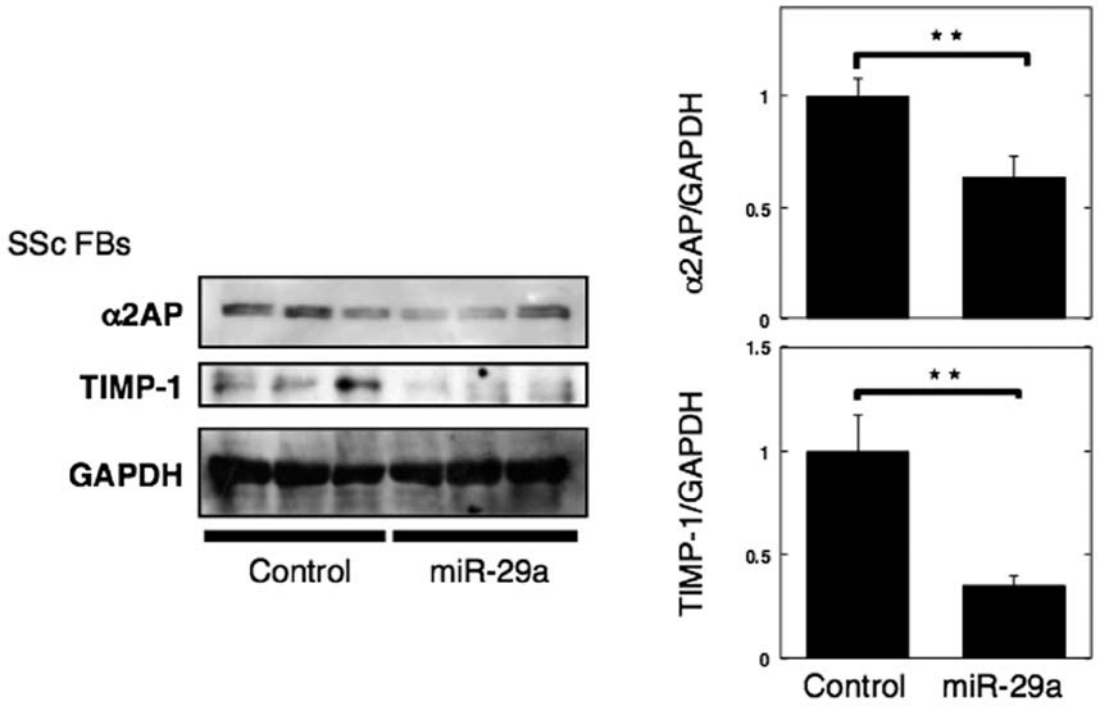


Figure 5. miRNA-29a attenuates α2AP deposition in SSc dermal fibroblasts by suppressing TIMP-1 expression. Human SSc dermal fibroblasts were transfected with control or miRNA-29a and harvested 48 h after transfection. The expression of each protein was determined via western blotting. The histogram provides quantitative representations of each protein (n=3). Data are presented as the mean ± SEM. **P<0.05 as indicated. miRNA or miR, microRNA; α2AP, α2-antiplasmin; SSc, systemic sclerosis; TIMP-1, tissue inhibitor of metalloproteinase-1; αSMA, α smooth muscle actin.

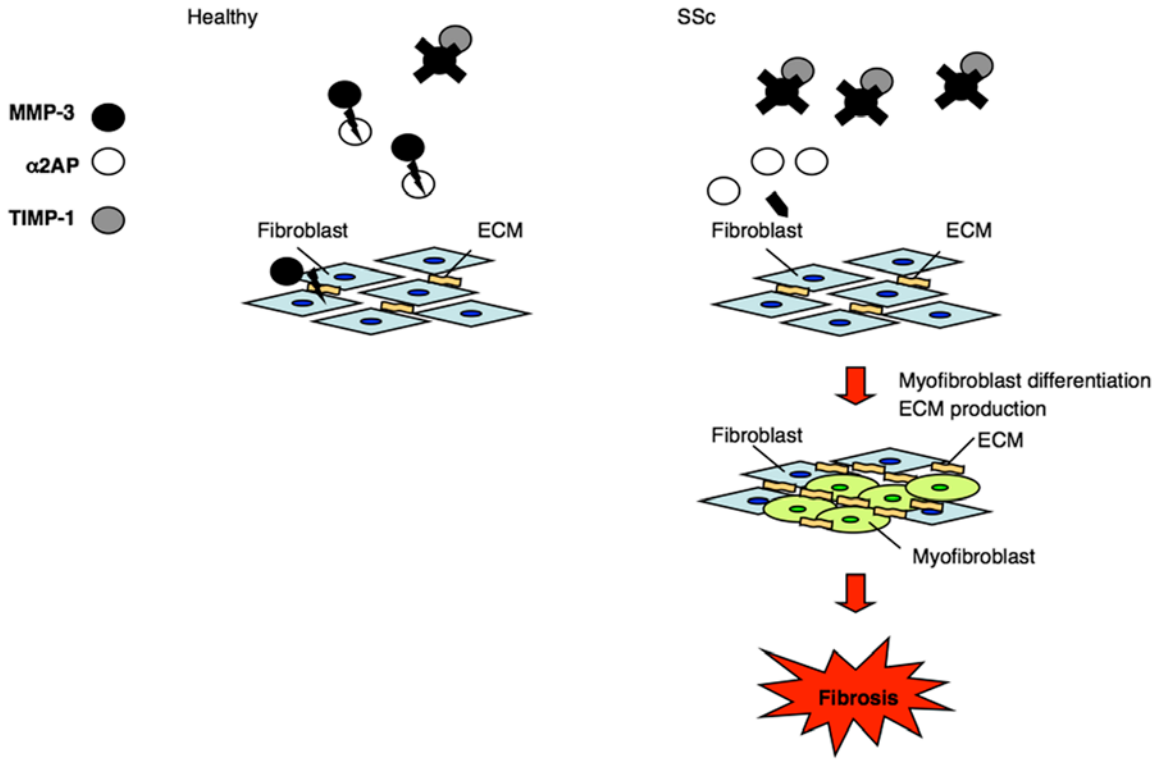


Figure 6. Role of MMP-3 and α2AP in the development of SSc fibrosis. Decreased MMP-3 activity in SSc dermal fibroblasts may promote the development of fibrosis through the suppression of ECM and α2AP degradation. MMP-3, matrix metallopeptidase-3; α2AP, α2-antiplasmin; SSc, systemic sclerosis; TIMP-1, tissue inhibitor of metalloproteinase-1; ECM, extracellular matrix.

and MMP-3 in the sera from healthy controls and SSc patients by ELISA and found no significant differences (healthy controls: n=10, SSc patients: n=10) (Fig. 1).

*α2AP expression increased while MMP-3/TIMP-1 ratio decreased in SSc dermal fibroblasts*. Bonaventura *et al* (24) reported that the ratio of MMP-3/TIMP-1 indicates the activity of MMP-3. Hence, we next assessed α2AP expression and MMP-3/TIMP-1 ratio in normal human and SSc dermal fibroblasts by western blotting (normal fibroblasts: n=4, SSc fibroblasts: n=7). As shown in Fig. 2, we found that α2AP expression in dermal fibroblasts was increased in SSc, whereas the MMP-3/TIMP-1 ratio was reduced.

*MMP-3-mediated degradation inhibits pro-fibrotic effects of α2AP*. Cleavage by MMP-3 inactivates α2AP (23). We thus incubated α2AP for 1 h at 37˚C in the presence or absence of MMP-3 and showed that the enzyme degraded α2AP (Fig. 3A). To assess the effect of MMP-3-mediated degradation on α2AP, we performed the same reactions and added the mixtures to normal human fibroblasts. Pre-incubation of α2AP with MMP-3 led to attenuation of the expression of α-smooth muscle actin (α-SMA) (a marker of the myofibroblast phenotype) and type I collagen (Fig. 3B).

*MMP-3 reverses pro-fibrotic phenotype of SSc dermal fibroblasts*. Next, we stimulated SSc dermal fibroblasts with MMP-3 to investigate its anti-fibrotic effect. Consistent with the previous results, stimulation with MMP-3 decreased the expression of α2AP, αSMA, and type I collagen (Fig. 4).

*MicroRNA-29a attenuates α2AP deposition in SSc dermal fibroblasts by inhibiting TIMP-1*. Ciechomska *et al* (25) showed that miR-29a, decreased TIMP-1 expression and reversed the pro-fibrotic phenotype of SSc dermal fibroblasts. In the present study, we confirmed that miR-29a transfection in SSc fibroblasts caused a decrease in TIMP-1 expression, in line with the previous report (Fig. 5). We also showed that miR-29a caused a decrease in α2AP expression in SSc fibroblasts (Fig. 5).

## Discussion

SSc causes fibrosis of the skin and internal organs. Previously, we showed that α2AP induces TGF-β production through adipose triglyceride lipase, and is associated with myofibroblast differentiation and ECM production (9). We also showed that the expression of α2AP is elevated in SSc model mice and SSc fibroblasts, and that its inactivation attenuates disease severity in SSc model mice and SSc fibroblasts (16,17). These findings suggest that α2AP contributes to the development of fibrosis in SSc. MMP-3, an ECM-degrading enzyme, inactivates α2AP by cleaving its Pro19-Leu20 peptide bond (23). Here, we focused on α2AP and MMP-3 to clarify their roles in the pathogenesis of SSc.

In this study, we showed that serum levels of α2AP and MMP-3 did not vary between healthy controls and SSc patients. However, consistent with our previous findings, α2AP expression in SSc dermal fibroblasts was increased. To determine whether MMP-3 contributes to the high expression of α2AP in SSc fibroblasts, we measured the ratio of MMP-3/TIMP-1 as

an indication of MMP-3 activity. Our results showed that this ratio was low in SSc dermal fibroblasts. Taken together, these data suggest that the decrease in MMP-3 activity might induce α2AP expression in SSc fibrotic tissue. Moreover, skin-specific induction and development of fibrosis may be due to increased α2AP expression and decreased MMP-3 activity in tissue but not in serum.

MMP-3 inactivates α2AP by proteolytic cleavage (23). Here, we confirmed that MMP-3 cleaved α2AP into two fragments and attenuated the α2AP-induced pro-fibrotic effects, such as myofibroblast differentiation and collagen production in normal fibroblasts. In addition, treatment with MMP-3 suppressed the pro-fibrotic response of SSc fibroblasts by reducing the expression of collagen and myofibroblast markers. Moreover, MMP-3 is known to degrade ECM components, such as collagen. The decrease in MMP-3 activity in SSc dermal fibroblasts not only attenuates α2AP inactivation but also promotes ECM deposition, and contributes to SSc progression (Fig. 6).

miR-29a represses TIMP-1 expression, thereby reversing the pro-fibrotic phenotype of SSc dermal fibroblasts (25). In the present study, we showed that miR-29a-transfected SSc fibroblasts exhibited a low α2AP and TIMP-1 expression. Collectively, our data suggest that TIMP-1-induced MMP-3 inhibition may lead to α2AP deposition in SSc.

In conclusion, we found that the activity of MMP-3 was decreased and that α2AP expression was increased in SSc fibroblasts. Moreover, treatment with MMP-3 or suppression with a MMP-3 inhibitor, TIMP-1, reversed the pro-fibrotic phenotype of SSc dermal fibroblasts. Our results suggest that decrease in MMP-3 activity in SSc fibroblasts causes α2AP and ECM deposition, and contributes to the development of fibrosis. Thus, our findings might contribute to a novel therapeutic approach for treatment of SSc.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

HN, YK, ES and MS designed the current study. HN, YK and ES performed the experiments and analyzed data. HN, YK, ES and MS wrote and edited the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The experiments utilizing human samples in the current study were approved by the Gifu University Graduate School of Medicine Ethics Committee (approval no. 29-152). Written informed consent was obtained from the patients and volunteers involved.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that there are no competing interests.

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