# microRNA-30c attenuates fibrosis progression and vascular dysfunction in systemic sclerosis model mice

**Yosuke Kanno** ( ykanno@dwc.doshisha.ac.jp )
Doshisha Women's College of Liberal Arts: Doshisha Joshi Daigaku https://orcid.org/0000-0001-9741-2113
**En Shu**
Gifu University School of Medicine Graduate School of Medicine: Gifu Daigaku Igakubu Daigakuin Igakukei Kenkyuka
**Hirofumi Niwa**
Gifu University School of Medicine Graduate School of Medicine: Gifu Daigaku Igakubu Daigakuin Igakukei Kenkyuka
**Mariko Seishima**
Gifu University School of Medicine Graduate School of Medicine: Gifu Daigaku Igakubu Daigakuin Igakukei Kenkyuka
**Kei-ichi Ozaki**
Doshisha Women's College of Liberal Arts: Doshisha Joshi Daigaku

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# Abstract

Systemic sclerosis (SSc) is characterized by the fibrosis of skin and visceral organs, and peripheral circulatory disturbance. We recently demonstrated that α2-antiplasmin (α2AP) is elevated in SSc dermal fibroblasts and SSc model mice, and is associated with fibrosis progression and vascular dysfunction. In the present study, we predicted that α2AP could be a target of microRNA-30c (miR-30c) using TargetScan online database, and investigated the effect of miR-30c on the pathogenesis of SSc using a bleomycin-induced SSc model mice. miR-30c attenuated α2AP expression, and prevented the pro-fibrotic changes (increased dermal thickness, collagen deposition, myofibroblast accmulation) and the vascular dysfunction (the reduction of vascular endothelial cells (ECs) and blood flow) in the skin of SSc model mice. Furthermore, miR-30c suppressed pulmonary fibrosis progression in the SSc model mice. These findings suggest that miR-30c exerts the anti-fibrotic and anti-angiopathy effects on SSc model mice, and might provide a basis for clinical strategies for SSc.


# Introduction

Systemic sclerosis (SSc) is characterized by the fibrosis of skin and visceral organs, and peripheral circulatory disturbance (1). Vascular damage, such as the reduction of blood vessels and blood flow, occurs in the early stages of the disease, and is associated with fibrosis progression (2). This process usually occurs over many months and years, and can lead to organ dysfunction or death. However, the detailed mechanism of SSc pathogenesis remains unclear.

Alpha2-antiplasmin (α2AP; SERPINF2) is the principal inhibitor of plasmin and inhibits the fibrinolysis (3, 4). α2AP rapidly inactivates plasmin, resulting in the formation of a stable inactive complex, plasmin-α2AP. On the other hand, α2AP can bind and activate adipose triglyceride lipase (ATGL) (5), and is known to have various functions, such as inflammation responses, cytokine production, cell growth, differentiation and recruitment (6–12). It has been reported that the level of PAP in plasma is elevated in SSc patients (13) and the expression of α2AP is elevated in the serum and skin of SSc model mice and SSc dermal fibroblasts (14–16), and the blockade of α2AP prevents pro-fibrotic changes and vascular damage in SSc model mice, and reversed a pro-fibrotic phenotype of SSc dermal fibroblasts (12, 15, 17). Furthermore, α2AP regulates myofibroblast differentiation and collagen production, and is associated with the development of fibrosis (18–20). The changes in α2AP expression may contribute to fibrosis progression in SSc.

MicroRNAs (miRNAs) are endogenous non-coding RNAs of 18–24 nucleotides in length, and can induce mRNA degradation or the translational repression of target genes through complementary pairing with the 3' untranslated region (UTR) of target mRNA (21). It has been reported that miRNA expression is associated with the development of autoimmune diseases including SSc (22). As a member of miR-30 family, miR-30c is generally recognized as a multifunctional regulator of cell growth, differentiation, apoptosis (23), and regulates inflammation, epithelial-to-mesenchymal transition (EMT) and cytokine production, and suppresses the development of renal and cardiac fibrosis (24–27).

In the present study, we predicted that α2AP could be a target of microRNA-30c (miR-30c) using TargetScan online database, and investigated the effect of miR-30c on the pathogenesis of SSc.

# Materials And Methods

microRNA target prediction

TargetScan online database (http://www.targetscan.org/index.html) were used to identify potential miRNA targets.

Mice experiment

The mice experiments in this study were approved by the Animal Research Committee of Doshisha Women's College of Liberal Arts (Approval ID: Y19-025).

The saline or bleomycin were injected subcutaneously into the back of the mice (male 8 weeks old C57BL/6J mice) as previously described (19). The saline or bleomycin was injected subcutaneously daily for up to 2 weeks. The miRNA negative control or miR-30c (sequence: UGUAAACAUCCUACACUCUCAGC Bioneer, CA, USA)(10 mg/body) was injected subcutaneously every a week for up to 2 weeks, using JetPEI transfection reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions.

Western blot analysis

We performed a Western blot analysis as previously described (28). The skin samples from mice were homogenized and sonicated in the lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5), 1% SDS, 1% Triton X-100. The protein concentration in each lysate was measured using a BCA protein assay kit (Pierce, IL, USA). Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. We detected α2AP, type I collagen and GAPDH by incubation with anti-α2AP antibodies (Santa Cruz Biotechnology, CA, USA), anti-type I collagen antibodies (Bioss antibodies, MA, USA), anti-α-SMA antibody (Genetex, CA, USA), anti-VE-cadherin antibody (Santa Cruz Biotechnology, CA, USA) and anti-GAPDH antibodies (Sigma-Aldrich, MO, USA) followed by incubation with horseradish peroxidase-conjugated antibodies to rabbit IgG (Amersham Pharmacia Biotech, Uppsala, Sweden).

Measurement of dermal thickness

The dermal thickness (distance from the epidermal-dermal junction to dermal-subcutaneous junction) was determined by calculating the average of three-point measurement in each skin section. The measurements were carried out in a blinded fashion. The dermal thickness was measured in the skin sections from each group of mice (n = 3).

Collagen content in skin and lung (The sircol biochemical assay)

The collagen content was assessed using sirius red staining as previously described (29). The stained images obtained from separate fields on the specimens were analyzed by using ImageJ. The collagen

content was determined as the percent ratio of the sirius red-positive area in saline plus control miRNA-injected mice.

Immunohistochemical staining of α-SMA and VE-cadherin

The immunohistochemical staining was performed as previously described (30). Paraffin sections were labeled with anti-α-SMA antibody (GeneTex, CA, USA) or anti-VE-cadherin (Santa Cruz Biotechnology, CA, USA), then secondarily labeled with Cy3-conjugated anti-rabbit IgG (Thermo Scientific, CA, USA). The signals were then detected using a laser-scanning microscope.

Blood flow in the skin

Blood flow in the skin was measured for 10 seconds using a laser Doppler flow meter (BRL-100; Bio Research Center, Tokyo, Japan), and determined by calculating the average of three-time measurements in each skin.

Assessment of lung fibrosis score

The lung fibrosis score was assessed as described by Ashcroft et al (31). The lung fibrosis was graded on a scale of 0–8 by examining randomly chosen fields. The criteria for grading lung fibrosis was as follows: 0; normal lung, grade 1; minimal fibrous thickening of alveolar or bronchiolar walls, grade 3; moderate thickening of walls without obvious damage to lung architecture, grade 5; increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses, grade 7; severe distortion of structure and large fibrous area, 8; total fibrous obliteration of the field. Grade 2, 4, and 6 were used as intermediate pictures between the aforementioned criteria.

# Statistical Analysis

All data were expressed as mean ± SEM. The statistical analysis was conducted with one-way ANOVA followed by Tukey test for multiple comparison. Statistical significance was defined as a $P$ value of $< 0.05$.

## Results

# Identification of miRNA for targeting α2AP using online database

TargetScan online database predicted that microRNA-30c (miR-30c) can target α2AP mRNA (Fig. 1).

# The effect of miR-30c on the development of dermal fibrosis in SSc model mice

The injection of miR-30c in the bleomycin-induced SSc model mice attenuated the increased dermal thickness (Fig. 2A, B), collagen production (Fig. 2A, C). In addition, we showed the histological findings of α-smooth muscle actin (α-SMA) (a hallmark of the myofibroblast phenotype) and VE-cadherin (a hallmark of the vasuclar endothelial cell (EC) phenotype), and miR-30c reversed the bleomycin-induced α-SMA expression and VE-cadherin expression reduction in the dermis (Fig. 2D, E). Furthermore, we investigated the effect of miR-30c on the change of a2AP, type I collagen, α-SMA and VE-cadherin expression in the skin of SSc model mice (Fig. 3). miR-30c reversed the bleomycin-induced a2AP, type I collagen, α-SMA expression and VE-cadherin expression reduction in the skin of SSc model mice (Fig. 3).

# The effect of miR-30c on vascular dysfunction in SSc model mice

We investigated the effects of miR-30c on blood flow in the bleomycin-induced SSc model mice. miR-30c reversed the bleomycin-induced blood flow reduction in the back skin of SSc model mice (Fig. 4).

# The effect of miR-30c on the development of pulmonary fibrosis in SSc model mice

The treatment of bleomycin is known to induce pulmonary fibrosis, which shares central features of human SSc. We invetigated the effect of miR-30c on pulmonary fibrosis progression in SSc model mice. miR-30c reversed the bleomycin-induced the increased lung fibrosis score (Fig. 5A, B) and collagen production (Fig. 5A, C) in the lung of SSc model mice.

## Discussion

SSc results in fibrosis of the skin and internal organs, and vascular damage (1). Recently, we showed the expression of α2AP is elevated in the serum and skin of SSc model mice and SSc dermal fibroblasts (14–16), and the increased in α2AP expression affects vascular dysfunction and fibrosis progression in SSc model mice and SSc dermal fibroblasts (15, 17, 19). In the present study, we focused on α2AP, and predicted that α2AP could be a target of miR-30c according to TargetScan online database (Fig. 1).

miR-30c suppressed α2AP expression, and prevented pro-fibrotic changes (increased dermal thickness, collagen and myofibroblast deposition) and vascular dysfunction (blood vessels and blood flow reduction) in the skin of SSc model mice (Fig. 2–4). In addition, miR-30c suppressed pulmonary fibrosis progression in the SSc model mice (Fig. 5). Vascular damage is an early and initiating event in SSc, and is caused by EC apoptosis, defective angiogenesis, defective vasculogenesis, endothelial-to-mesenchymal transition (EndoMT), and coagulation abnormalities (12). Vascular damage is also known to cause the development of interstitial lung disease, pulmonary arterial hypertension, and fibrosis (12, 32). α2AP is associated with EC apoptosis, angiogenesis, and vascular remodeling (6, 10), and the treatment of a2AP causes vascular dysfunction in mice (17). α2AP is also associated with EMT progression (18). EndoMT exhibits features similar to those of EMT, α2AP may also affect EndoMT

progression. Futhermore, plasmin regulates fibrinolysis, coagulation, ECM degradation and myofibroblast apoptosis, and contributes to various cardiovascular diseases and fibrosis (33–35). The α2AP-induced plasmin inhibition may be associated with vascular dysfunction and fibrosis progression. The regulation of a2AP expression by miR-30c may prevent the SSc-associated various events through multiple mechanisms, and alleviate the disease severity.

In conclusion, miR-30c suppresses α2AP expression, and exerts the anti-fibrotic and anti-angiopathy effects on SSc model mice. These findings provide a basis for therapeutic strategies for SSc.

# Declarations

### Conflict of interest

There are no competing interests.

### Author's contributions

YK conceived and designed the experiment. YK, ES and HN were involved in the experiments. YK, ES, MS and KO were involved in data interpretation and writing of the manuscript.


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# Figures

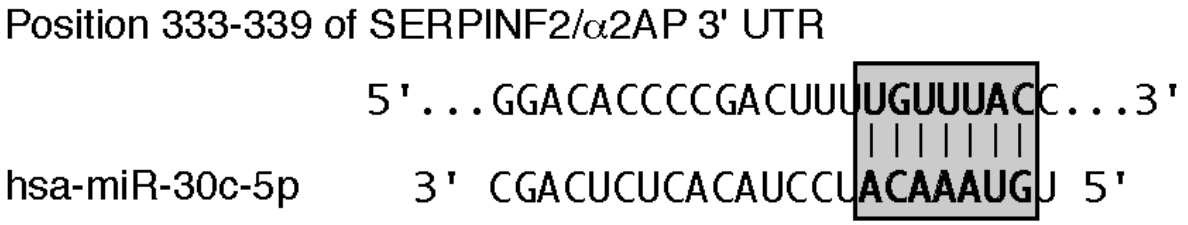
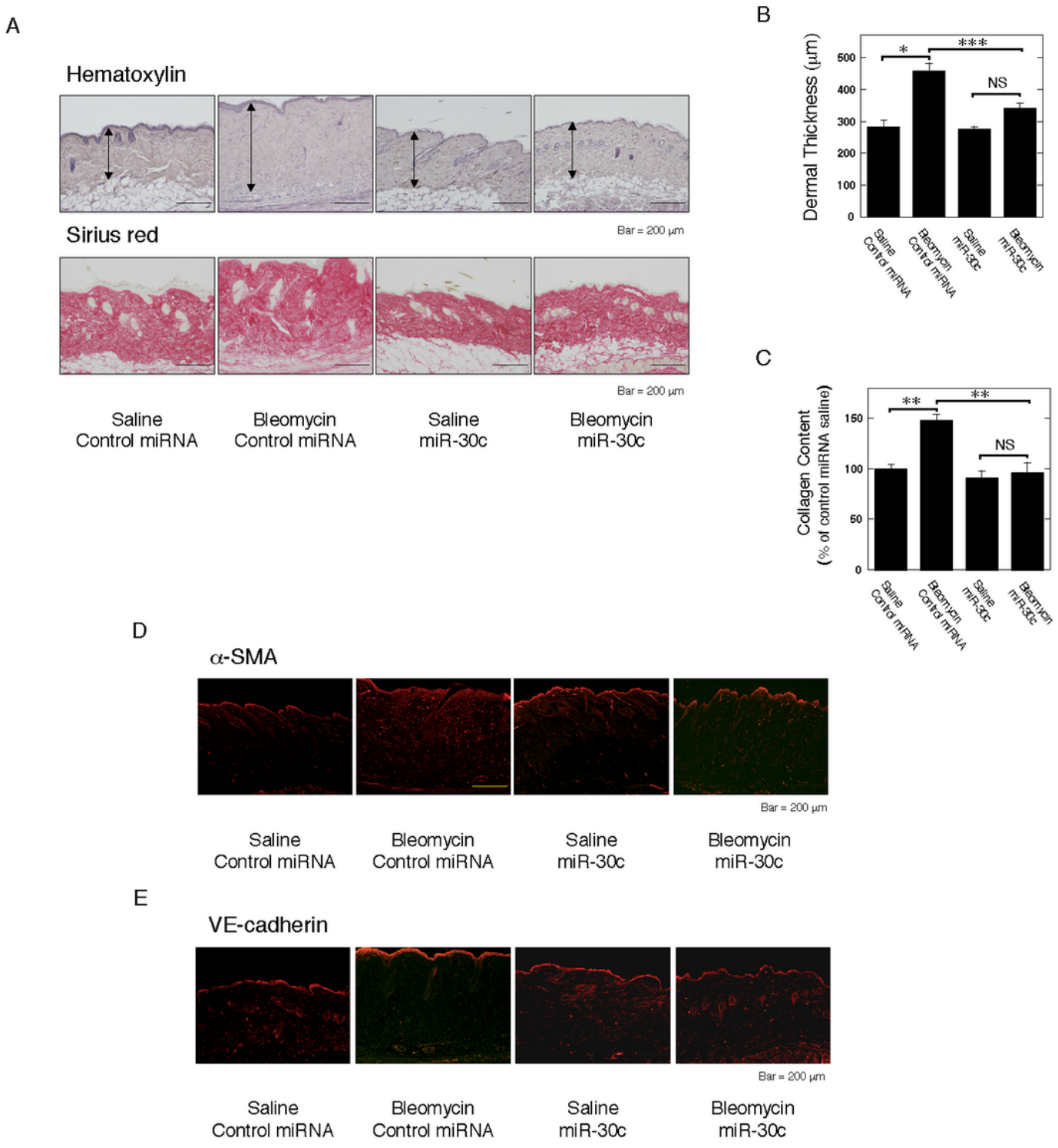



**Figure 1**

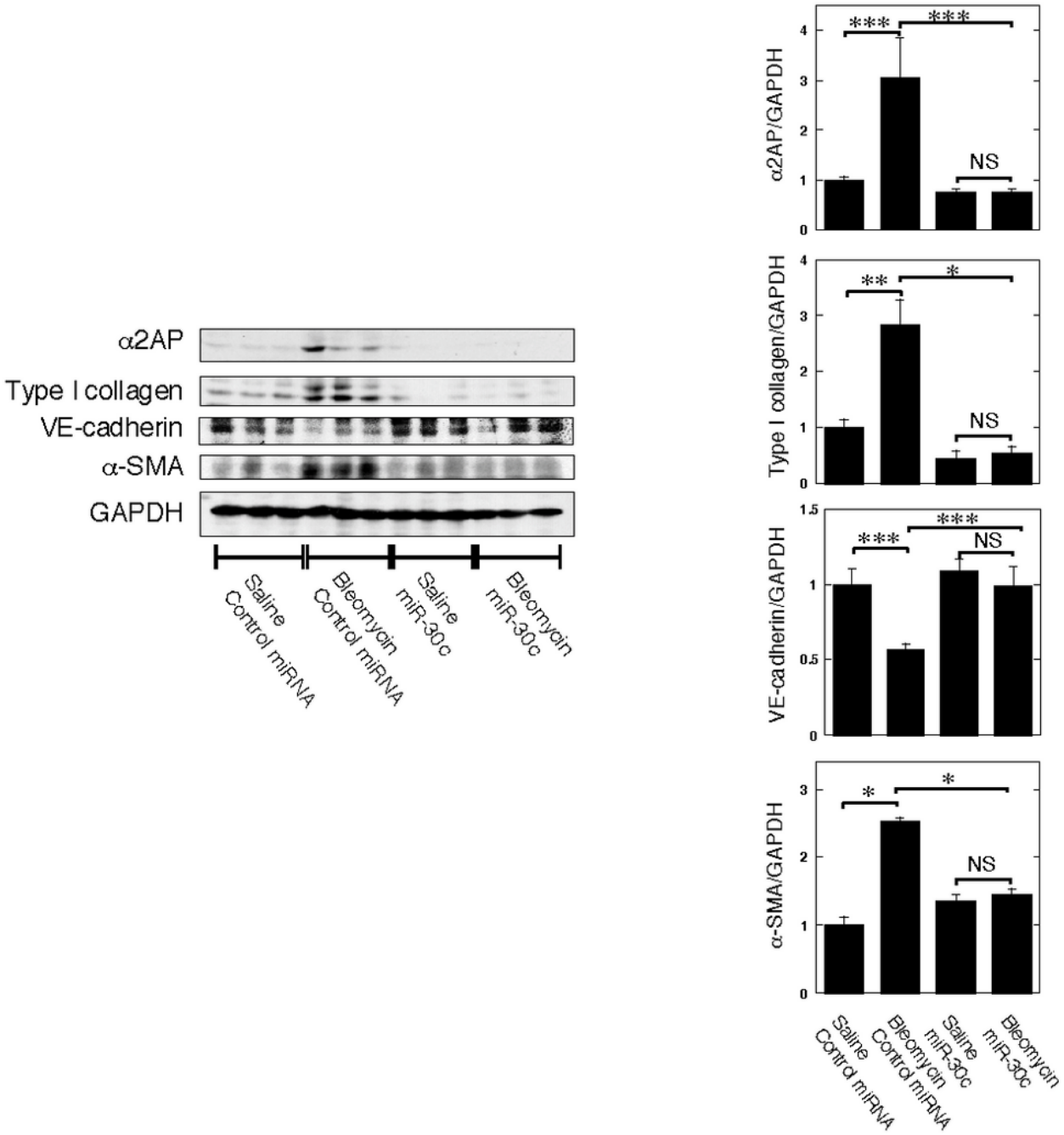
Identification of miRNA for targeting α2AP using online database Potential binding sites of miR-30c and α2AP (SERPINF2) were predicted by TargetScan online database.




## Figure 2

The effect of miR-30c on the development of dermal fibrosis in SSc model mice (A) Represetative skin sections from each group of mice (Hematoxylin and Sirius red stain). Double head arrows indicate the dermal thickness. (B) The dermal thickness in the skin sections from each group of mice (n=3). (C) The collagen content in the skin from each group of mice (n=3). (D) Paraffin sections were stained with α-

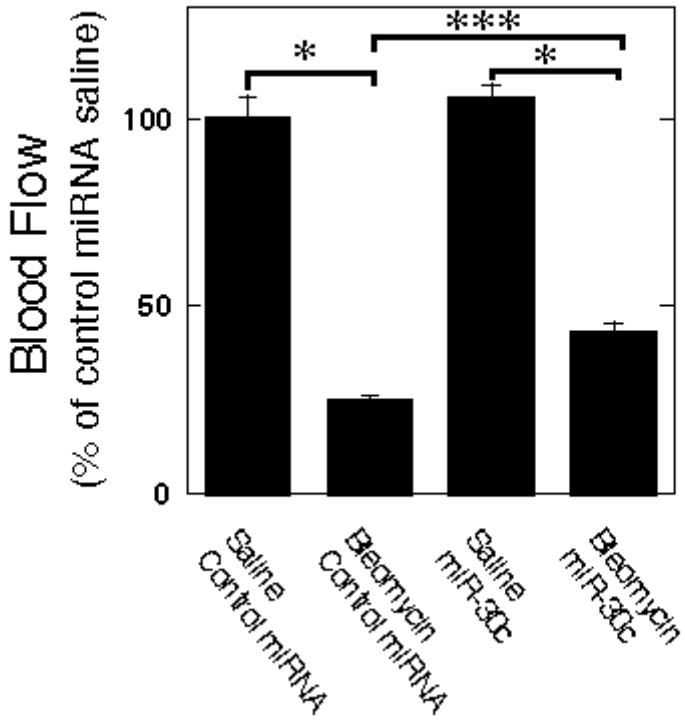
SMA antibodies. (E) Paraffin sections were stained with VE-cadherin antibodies. The data represent the mean ± SEM. *, P<0.001; **, P<0.01; ***, P<0.05. NS, not significant. Scale bar = 200 μm.




## Figure 3

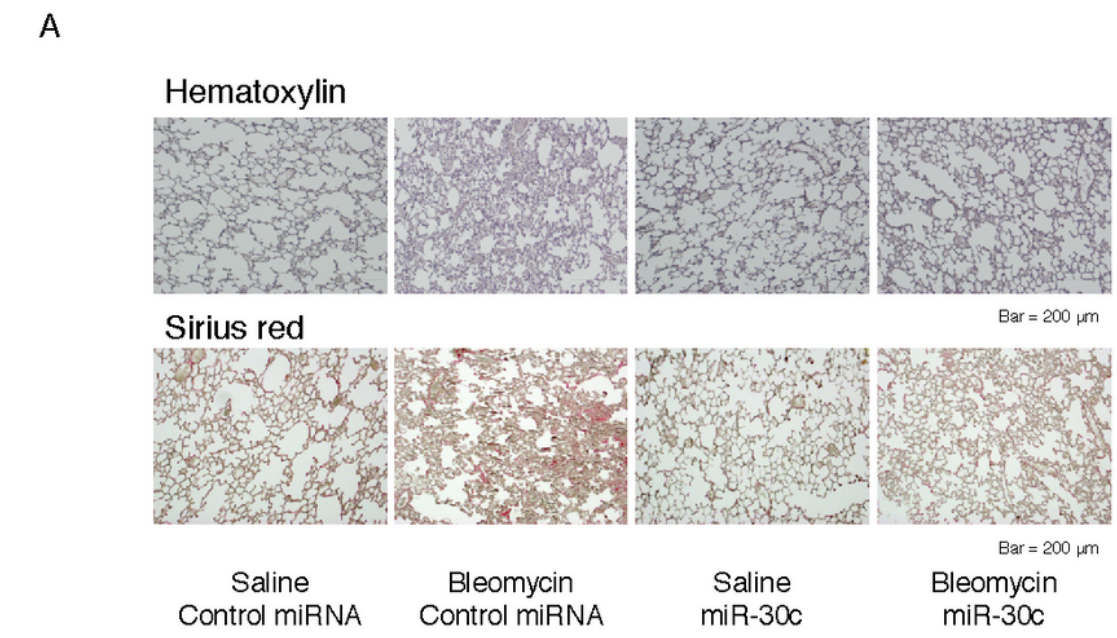
The effect of miR-30c on the change of a2AP and fibrotic-associated protein expression in the skin of SSc model mice The expression of each protein in the skin from mice was examined by a Western blot

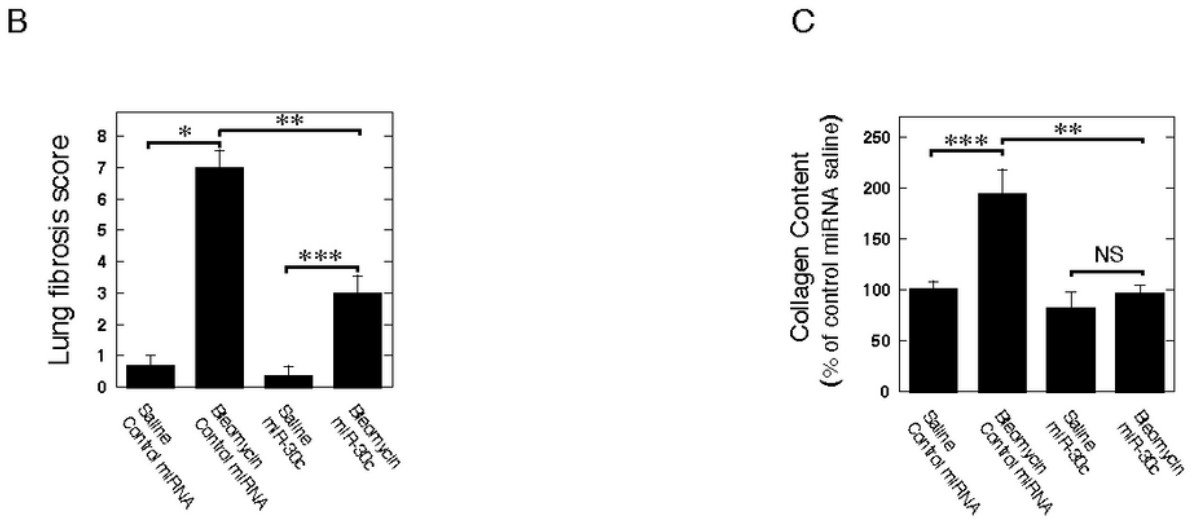
analysis.  The histogram shows quantitative representations of each protein (n=3).  The data represent the mean ± SEM.  *, P<0.001; **, P<0.01; ***, P<0.05. NS, not significant




## Figure 4

The effect of miR-30c on blood flow in SSc model mice Blood flow in the back skin of each group of mice (n=3).  The data represent the mean ± SEM.  *, P<0.001; ***, P<0.05.




## Figure 5

The effect of miR-30c on the development of pulmonary fibrosis in SSc model mice (A) Represetative lung sections from each group of mice (Hematoxylin and Sirius red stain). (B) Lung fibrosis score in the lung sections from each group of mice (n=3). (C) The collagen content in the lung from each group of mice (n=3). The data represent the mean ± SEM. *, P<0.001; **, P<0.01; ***, P<0.05. NS, not significant. Scale bar = 200 mm.