than in those of healthy control subjects.[10] The overexpressed TSP-1 in SSc fibroblasts is thought to activate latent TGF-β by its RFK motif and to contribute to accumulation of ECM.[11] In addition, COMP expression is reported to be increased in SSc dermal fibroblasts, and serum levels of COMP are correlated with severity of changes in skin thickness.[12,13]

TSP-2 has also been implicated in regulation of ECM synthesis, cell behavior, and angiogenesis. Markedly increased TSP-2 synthesis has been found in healing wounds in mice.[14] In TSP-2 null mice, the attachment of fibroblasts to various ECM molecules, as well as TSP-2 itself, is impaired,[15] and the dermis shows abnormal organization of collagen fibers.[14] Moreover, TSP-2 can inhibit the proliferation of microvascular endothelial cells.[16] However, the expression of TSP-2 in SSc dermal fibroblast has not been investigated previously. The present study was undertaken to clarify the expression pattern of TSP-2 in SSc and its role in pathogenesis of the disease.

Materials and Methods

Patient Materials

Serum samples were obtained from 33 patients with SSc (6 male and 27 female; age range, 24 to 87 years; mean age, 60.1 years). Of these, 14 patients had diffuse cutaneous SSc (3 male and 11 female) and 19 patients had limited cutaneous SSc (3 male and 16 female).[17,18] Control serum samples were also collected from 14 healthy age- and sex-matched volunteers. Skin samples were obtained from lesional skin of six patients with diffuse cutaneous SSc. Control fibroblasts were obtained by skin biopsy from eight healthy donors. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki before the patients and healthy volunteers were entered into the present study.

Cell Culture

Human dermal fibroblasts were obtained by skin biopsy of the affected areas (dorsal forearm) from five patients with dcSSc who had <2 years of skin thickening, as described previously.[19] Control fibroblasts were obtained by skin biopsies from five healthy donors.[20] Before experiments, cells were serum-starved for 12 to 24 hours.

Cell Lysis and Immunoblotting

Fibroblasts were cultured until they were confluent, and then conditioned medium (normalized for cell numbers at the time of harvest) and cell lysates (normalized for protein concentration) were analyzed by immunoblotting.[21] Antibodies for β-actin, type I collagen, TSP-2, matrix metalloproteinase (MMP)-1, and MMP-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Southern-Biotech (Birmingham, AL), BD Biosciences (Bedford, MA), Millipore (Temecula, CA), and Daiichi Fine Chemical (Takaoka, Japan), respectively.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from cultured cells with ISOGEN (Nippon Gene, Tokyo, Japan) and from paraffin-embedded sections with an RNeasy FFPE kit (Qiagen, Valencia, CA). cDNA synthesis and quantitative real-time PCR were performed as described previously.[21]

Primer sets for TSP-2 (PPH00238E, designed against exon 23) and GAPDH (PPH00150E, designed against exon 9) were obtained from Qiagen-SABiosciences (Frederick, MD); the α2(I) collagen primer (sense 5′-GAGGGCAACAGCAGGTTCACTTA-3′ and antisense 5′-TCAGCACCACCGATGTCCA-3′) was obtained from Takara Bio (Otsu, Japan). DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C.

Measurement of MMP-1 and MMP-2 Activity

Levels of MMP-1 and MMP-2 activity were measured with a fluorescent activity assay kit (AnaSpec, Fremont, CA) as described previously.[22]

Immunohistochemistry

Wax-embedded sections were dewaxed in xylene and rehydrated in graded alcohols. Antigens were retrieved by incubation with antigen retrieval solution (pH 9; Nichirei, Tokyo, Japan) for 10 minutes at 121°C. Immunohistochemistry was performed with TSP-2 antibody (1:150).[23]

Intradermal Treatment of Mice with Bleomycin

Bleomycin (300 μg; Nippon Kayaku, Tokyo, Japan) or PBS was injected intradermally into the shaved back of 6-week-old C57BL/6 mice daily for 4 weeks, as described previously.[24,25] The back skin was removed 1 day after the final bleomycin injection, fixed in 4% paraformaldehyde, and embedded in paraffin.

Transient Transfection

Cells were transfected with either MMP-2 siRNA (L-005959-00; Thermo Scientific, Bremen, Germany; a mixture of four siRNAs: 5′-UCAAGGACCGGUUCAUUUG-3′, 5′-GCGAGUGGAUGCCGCCUUU-3′, 5′-GGAAUGCCAUCCCCGAUAA-3′ and 5′-ACAAGAACCAGAUCACAUA-3′), TSP-2 siRNA (sc-37031; Santa Cruz Biotechnology; a mixture of three siRNAs; 5′-CGUCAGAUGUGCAACAAGA-3′, 5′-GAAGCAGCCAAGACGGAAA-3′, and 5′-GCAAGGACAAGACACACAA-3′), miR-7 mimic (MSY0000252; Qiagen; 5′-UGGAAGACUAGUGAUUUUGUUGU-3′), or miR-7 inhibitor (MIN0000252; Qiagen; 5′-UGGAAGACUAGUGAUUUUGUUGU-3′) mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) as a transfection reagent. Cells were incubated at 37°C in 5% CO_2. Control siRNA (Dharmacon D-001810-10-05; Thermo Scientific, Lafayette, CO; or SC-37007; Santa Cruz Biotechnology), control miRNA mimic (1027281; Qiagen), or control

miRNA inhibitor (1027271; Qiagen) were used as controls.[26–29]

miRNA Isolation, Reverse Transcription, and PCR Analysis of miRNA Expression

Isolation of miRNA from total RNA was performed using an RT^2 qPCR-grade miRNA isolation kit (SABiosciences). For the miRNA PCR array, miRNAs were reverse-transcribed into first-strand cDNA using an RT^2 miRNA first-strand kit (SABiosciences). The cDNA was mixed with RT^2 SYBR Green/ROX qPCR master mix, and the mixture was added into a 96-well RT^2 human cell development and differentiation miRNA PCR array (MAH-103A; SABiosciences). PCR was performed on a Takara thermal cycler (Dice model TP800). The threshold cycle C_T value for each miRNA was extracted using Takara thermal cycler Dice real-time system software, version 2.10B. The raw C_T values for each miRNA were normalized using the values of small RNA housekeeping gene.

For quantitative real-time PCR, a Mir-X miRNA first-strand synthesis kit (638313; Takara) was used to synthesize first-strand cDNA. The U6 primer was included in the kit. The miR-7 primer (5′-UGGAAGACUAGUGAUUUUGUUGU-3′) was obtained from Takara. DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 20 seconds at 60°C. The miR-7 transcript levels were normalized to those of U6.

Cell Count and Migration Assay

Normal fibroblasts transfected with control or TSP-2 siRNA as described above were incubated for 120 hours.

Cell counting was performed with a Coulter particle counter (Beckmann Coulter, Fullerton, CA) as described previously.[30] For the migration assay, cells were added to the upper chamber of Transwell inserts coated with Matrigel membrane matrix (BD Biosciences). After 12 hours, the cells remaining on the upper surface were wiped off, and those that had migrated to the lower surface were stained with hematoxylin and counted.[31]

Measurement of Serum TSP-2 Concentrations

Levels of serum TSP-2 were measured with a specific enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

In Situ *Hybridization*

In situ hybridization was performed with 5′-locked digoxigenin-labeled nucleic acid (LNA) probes complementary to human mature miR-7 (38485–01; 5′-ACAACAAAATCACTAGTCTTCCA-3′) and a scrambled negative control (99004–01, 5′-GTGTAACACGTCTATACGCCCA-3′; Exiqon, Vedbaek, Denmark).[32,33] Briefly, human tissues were deparaffinized and deproteinized with protease K for 5 minutes. Slides were then washed in 0.2% glycine in PBS and fixed with 4% paraformaldehyde. Hybridization was performed at 50°C for 48 hours, followed by blocking with 2% fetal bovine serum and 2% bovine serum albumin in PBS and 0.1% Tween 20 (PBST) for 1 hour. The probe-target complex was detected immunologically by a digoxigenin antibody conjugated to alkaline phosphatase acting on the chromogen Nitro Blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany). Slides were counterstained with nuclear Fast Red and were examined under a light microscope (BX50; Olympus, Tokyo, Japan).

Reagents

Anti-TGF-β neutralizing antibody and recombinant human TSP-2 protein were obtained from R&D Systems, the pan-MMP inhibitor GM6001 from Merck (Darmstadt, Germany), the recombinant MMP-2 protein from Enzo Life Sciences (Plymouth, PA), and brefeldin A (BFA) from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

Statistical analyses were performed with a Mann-Whitney *U*-test for comparison of medians and Fisher's exact probability test for analysis of frequency. Correlations were assessed by Pearson's correlation coefficient. A P value of <0.05 was considered significant. Data are expressed as means ± SD of at least three independent experiments.

Results

TSP-2 Synthesis Is Decreased at the Transcriptional Level in Cultured SSc Dermal Fibroblasts

First, we compared the TSP-2 expression levels between normal and SSc fibroblasts in the presence or absence of exogenous TGF-β. Immunoblotting revealed that the amount of TSP-2 in cell lysates from SSc fibroblasts was significantly decreased, compared with that from normal cells (Figure 1, A and B), indicating that TSP-2 protein synthesis was down-regulated in SSc fibroblasts. On the other hand, the TSP-2 protein levels in both normal and SSc fibroblasts were not significantly affected by the stimulation with exogenous TGF-β (Figure 1, A and C).

The TSP-2 mRNA levels were also down-regulated in SSc fibroblasts (Figure 1D). Thus, the decreased TSP-2 protein synthesis in SSc fibroblasts may result from the down-regulation of TSP-2 mRNA expression. In addition, consistent with our findings on intracellular TSP-2 protein levels (Figure 1C), treatment with an anti-TGF-β neutralizing antibody (10 μg/mL) had little effect on the TSP-2 mRNA expression in either normal or SSc fibroblasts (Figure 1E), suggesting that the down-regulation of TSP-2 mRNA in SSc fibroblasts is likely to be independent of TGF-β signaling.

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. To determine whether the decrease of TSP-2 mRNA in

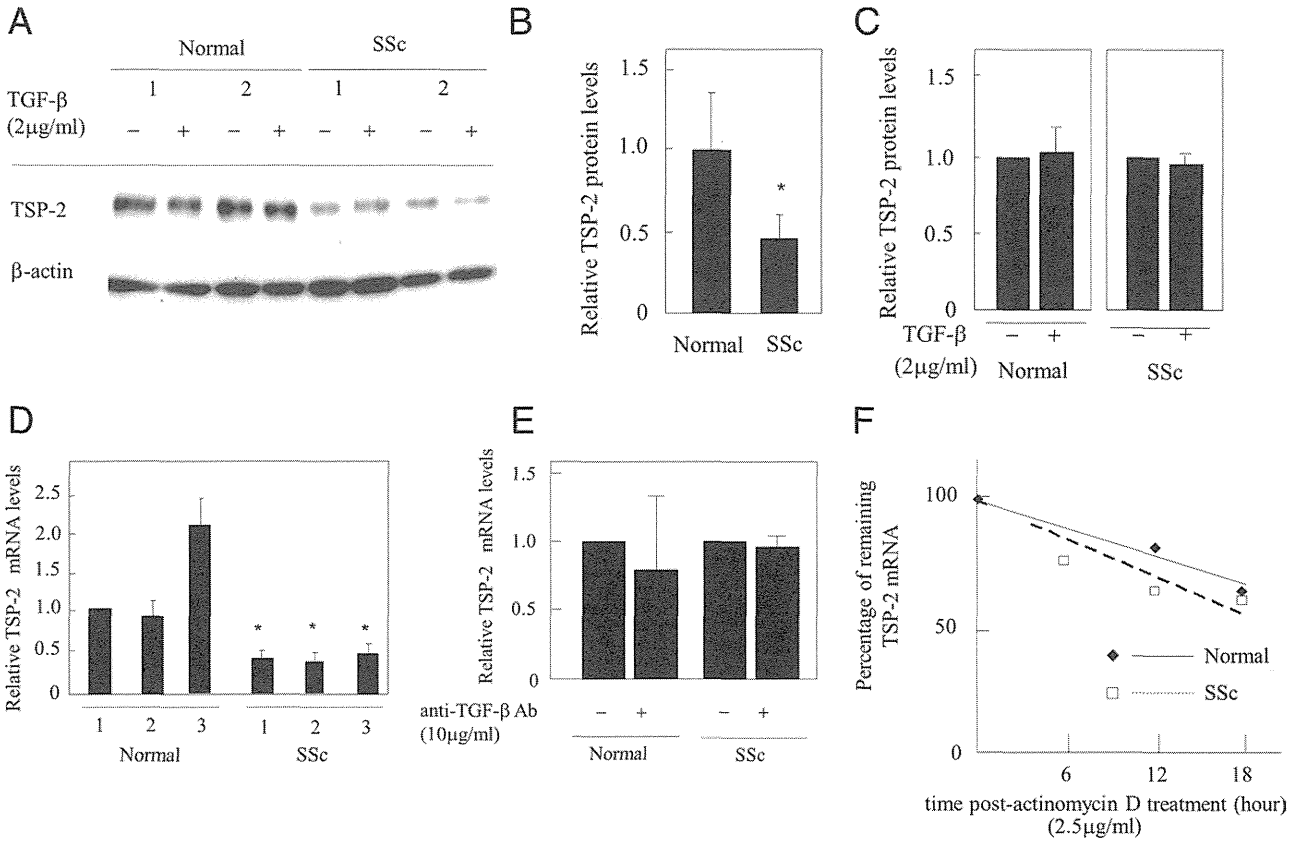

Figure 1. TSP-2 protein synthesis in cultured SSc dermal fibroblasts. **A:** A representative result of immunoblotting for lysates from five normal and SSc fibroblasts (1 and 2) shown from three independent experiments. Cells were incubated in the presence or absence of TGF-β for 24 hours. **B:** Intracellular TSP-2 protein levels in normal and SSc fibroblasts were quantified by scanning densitometry and corrected for β-actin levels in the same samples. $^*P < 0.05$ versus normal cells ($n = 3$). **C:** Intracellular TSP-2 protein levels in normal and SSc fibroblasts in the presence or absence of TGF-β quantified by scanning densitometry and corrected for β-actin levels in the same samples ($n = 3$). **D:** Relative amounts of TSP-2 transcripts (normalized to GAPDH) in three different normal and SSc fibroblasts (1, 2, and 3) were determined by real-time PCR ($n = 3$). **E:** Normal and SSc cells were treated with an anti-TGF-β antibody (10 μg/mL) or control IgG for 48 hours. TSP-2 mRNA levels were quantitated by real-time PCR ($n = 3$). **F:** Normal and SSc fibroblasts were incubated with 2.5 μg/mL actinomycin D for 6, 12, and 18 hours. TSP-2 mRNA expression was analyzed by real-time PCR ($n = 3$). Values in untreated fibroblasts were set at 100%.

SSc fibroblasts takes place at the transcriptional or post-transcriptional level, *de novo* mRNA synthesis was blocked by actinomycin D, a RNA synthesis inhibitor, in normal and SSc fibroblasts. After actinomycin D treatment, there were no significant differences in decrease of TSP-2 mRNA between normal and SSc fibroblasts (Figure 1F). Taken together, these findings indicate that the stability of TSP-2 mRNA in SSc fibroblasts was not altered and that the TSP-2 expression was likely decreased at the transcriptional level.

Accumulation of Extracellular TSP-2 Protein Is Up-Regulated in SSc Fibroblasts

We then determined whether the TSP-2 protein in the conditioned medium of SSc fibroblasts is also decreased. Immunoblotting showed that the extracellular TSP-2 accumulation in SSc fibroblasts was significantly increased, compared with that in normal fibroblasts (Figure 2, A and B). We also found that matrix metalloproteinase-2 (MMP-2) expression and activity were significantly decreased in the SSc medium (Figure 2, A and B), as reported previously.[34] Given that TSP-2 is one of the substrates of MMP-2,[35] we hypothesized that degradation of TSP-2 protein in medium of SSc fibroblasts is decreased. In fact, transfection of MMP-2 siRNA into normal fibroblasts increased the accumulation of TSP-2 in the medium (Figure 2C). Furthermore, treatment with a pan-MMP inhibitor, GM6001 (20 μmol/L), also induced TSP-2 accumulation in normal fibroblasts (Figure 2D). On the other hand, supplementation of recombinant MMP-2 protein (10 nmol/L) in the SSc medium down-regulated the increased TSP-2 accumulation (Figure 2E).

Accordingly, in SSc fibroblasts, the decreased degradation of TSP-2 protein outside the cells may compensate for the decreased synthesis of TSP-2 inside the cells, resulting in the increased post-transcriptional accumulation of extracellular TSP-2 protein. To examine this hypothesis, we also investigated the TSP-2 protein levels inside SSc fibroblasts treated with BFA, which blocks extracellular export of protein. Brefeldin A (BFA; 10 μg/mL) caused a time-dependent accumulation of intracellular TSP-2 in SSc fibroblasts (Figure 2F); however, accumulation of TSP-2 was still decreased in SSc lysates, compared with normal lysates (Figure 2G), indicating that TSP-2 down-regulation inside SSc fibroblasts was independent of extracellular export of the protein. Moreover, after the addition of BFA, we determined the stability of

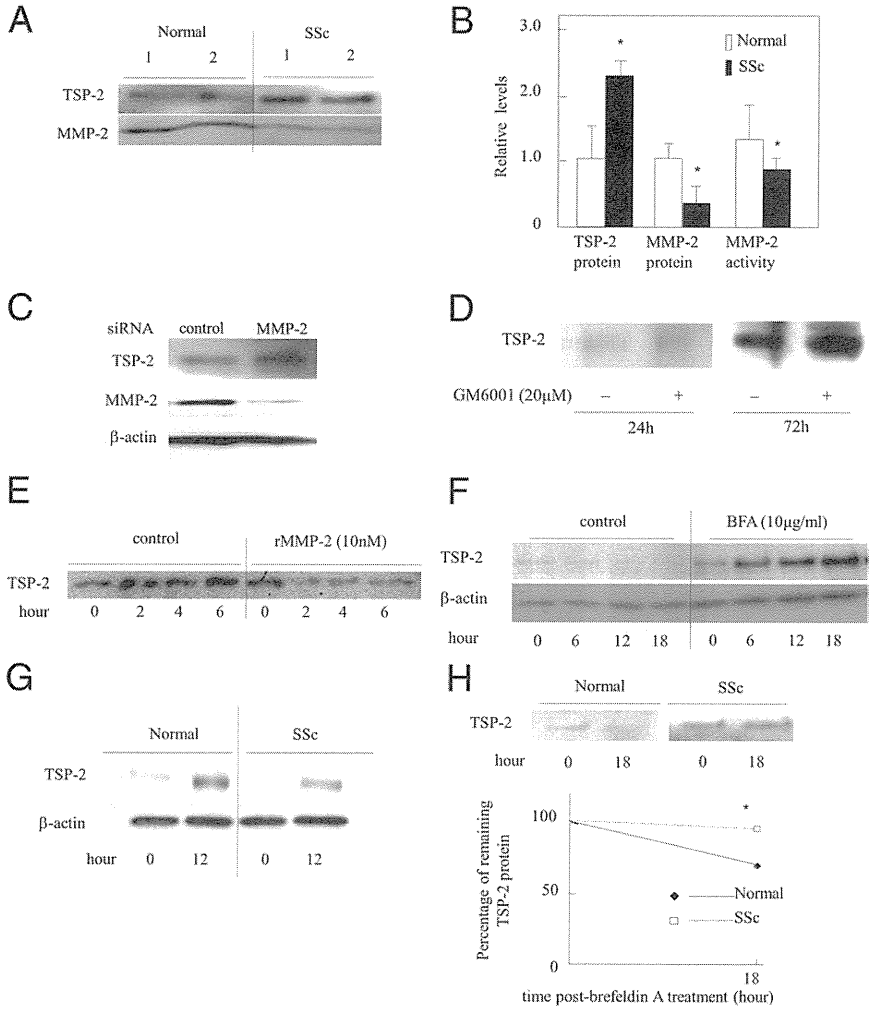

Figure 2. TSP-2 protein accumulation in culture medium of SSc dermal fibroblasts. **A:** Cells were incubated in serum-free medium for 24 hours. Aliquots of conditioned medium (normalized for cell numbers at the time of harvest) were analyzed by immunoblotting. The representative results from five normal and SSc fibroblasts (1 and 2) are shown from three independent experiments. **B:** Extracellular TSP-2 and MMP-2 protein levels were quantitated by scanning densitometry, and the MMP-2 activity was determined by a fluorescent activity assay kit in normal and SSc fibroblasts (corrected for cell numbers at the time of harvest). $^*P < 0.05$ versus normal cells ($n = 3$). **C:** Normal fibroblasts were transfected with MMP-2 siRNA. Expression of TSP-2 (culture medium) and MMP-2 (lysates) was determined by immunoblotting. Results of one experiment representative of five independent experiments are shown. **D:** Normal fibroblasts were treated with GM6001 (20 μmol/L) for 24 or 72 hours. TSP-2 expressions levels in the medium (corrected for cell numbers at the time of harvest) were analyzed by immunoblotting. Results of one experiment representative of five independent experiments are shown. **E:** SSc fibroblasts were supplemented with recombinant human MMP-2 (rMMP-2; 10 nmol/L), and the medium was subjected to immunoblotting. Results of one experiment representative of three independent experiments are shown. **F:** SSc fibroblasts were incubated with brefeldin A (BFA; 10 μg/mL). Dimethyl sulfoxide was used as a control. Cell lysates were subjected to immunoblotting. Results of one experiment representative of three independent experiments are shown. **G:** Before and after BFA treatment for 12 hours, the lysates from normal and SSc fibroblasts were subjected to immunoblotting. Results of one experiment representative of three independent experiments are shown. **H:** Comparison of TSP-2 protein degradation in the medium after BFA treatment between normal and SSc fibroblasts as determined by immunoblotting. TSP-2 protein levels were quantified by scanning densitometry. Values in untreated fibroblasts were set at 100% ($n = 3$).

the TSP-2 protein in medium (Figure 2H). The protein half-life of TSP-2 outside SSc fibroblasts was significantly up-regulated, compared with that of normal fibroblasts, which is consistent with the above hypothesis.

Expression Levels of TSP-2 in SSc Skin

Consistent with our *in vitro* results (Figure 1D), real-time PCR using total RNA extracted from skin tissue samples showed that the mean relative TSP-2 transcript levels in SSc skin were significantly lower than those in healthy control subjects (Figure 3A).

On the other hand, immunohistochemical staining using paraffin-embedded sections revealed that extracellular TSP-2 expression was barely detected in normal dermal fibroblasts (Figure 3B), but was strongly detected in SSc fibroblasts between the thickened collagen bundles (Figure 3C). There was no immunoreactivity for the protein in epidermis or blood vessels of either normal or SSc dermal sections.

To further investigate the TSP-2 expression pattern *in vivo*, paraffin-embedded sections from the skin of a bleomycin-treated mouse fibrotic skin model were stained for TSP-2. Increased numbers of spindle-shaped fibroblasts positive for TSP-2 staining were found in bleomycin-induced thickened skin (Figure 3E), compared with control skin treated with phosphate buffered saline (PBS) (Figure 3D).

Moreover, when the number of TSP-2$^+$ fibroblasts was counted in normal and SSc skin tissue, cell numbers were significantly increased in SSc skin, compared with normal skin (38.2 ± 25.1 versus 3.2 ± 3.0, $P < 0.01$, Mann-Whitney *U*-test; Figure 3F). Similarly, TSP-2$^+$ cell numbers were significantly increased in bleomycin-treated mouse skin, compared with control mouse skin (137.7 ± 64.6 versus 27.3 ± 16.2, $P < 0.05$; Figure 3G). These results suggest that extracellular TSP-2 protein was increased under fibrotic conditions. The decreased mRNA expression and increased extracellular protein accumulation of TSP-2 in SSc skin *in vivo* were similar to *in vitro* findings (Figures 1 and 2), and such increased accumulation of TSP-2 outside the cells may be associated with pathogenesis of SSc.

We next determined whether serum TSP-2 levels can be a disease marker in SSc patients. The serum concentration of TSP-2 was significantly higher in patients with SSc than in healthy control subjects (36.3 ± 13.5 versus 27.1 ± 7.6 mg/dL, $P < 0.02$; Figure 3H). Furthermore,

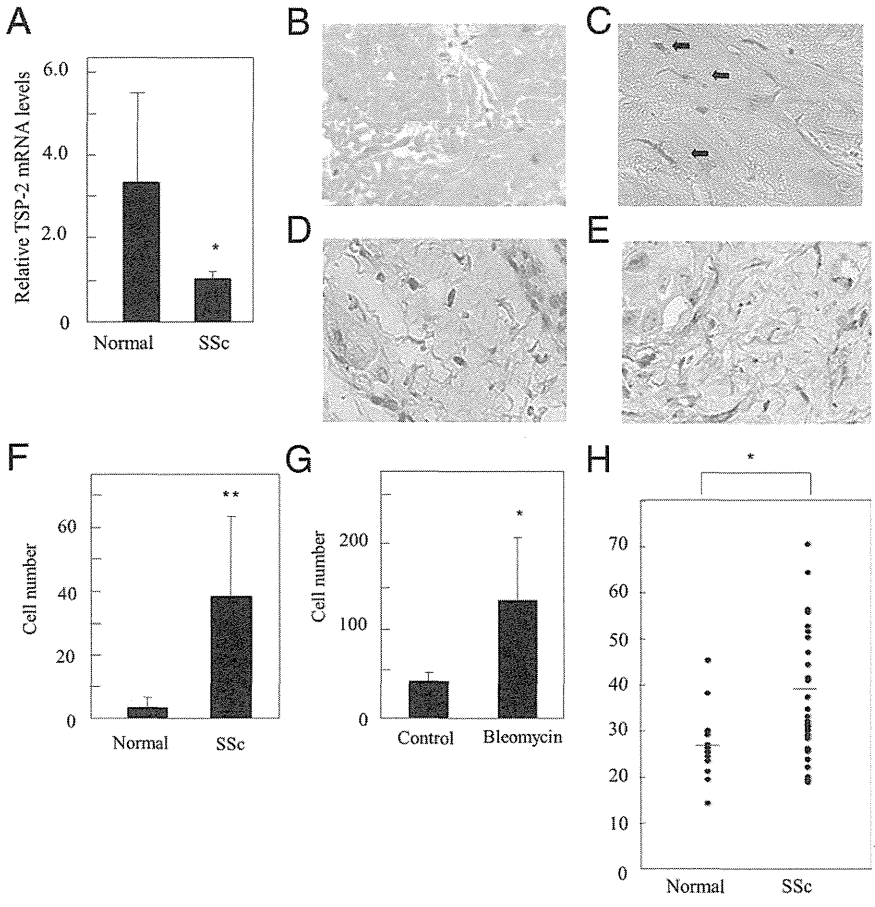

Figure 3. mRNA and protein expression levels of TSP-2 in SSc skin *in vivo* and serum TSP-2 levels in SSc patients. **A:** Mean relative transcript levels of TSP-2 (normalized to GAPDH) in skin tissue samples from healthy control subjects and diffuse cutaneous SSc patients were determined by real-time PCR ($n = 3$). Transcript levels in samples from SSc patients were set at 1. $^{*}P < 0.05$ versus control subjects. **B–E:** TSP-2 expression in normal (**B**) and SSc (**C**) dermal tissue and in murine tissue treated with PBS (**D**) or bleomycin (**E**). Paraffin sections were subjected to immunohistochemical analysis. Original magnification, ×200. Note immunoreactivity for TSP-2 in SSc dermal tissue (**arrows**, **C**). Results shown are representative of five normal and SSc skin samples and three control and three bleomycin-treated mouse skin samples. **F:** Numbers of TSP-2^{+} fibroblasts per five high-power fields (×400) were counted under a microscope. Samples were prepared from healthy control subjects and diffuse cutaneous SSc patients. $^{**}P < 0.01$ versus control subjects ($n = 5$). **G:** Comparison of the number of TSP-2^{+} fibroblasts per five high-power fields (×400) between tissue samples from control mice treated with PBS and those from mice treated with bleomycin ($n = 3$). $^{*}P < 0.05$ versus controls. **H:** Serum concentrations of TSP-2 in 33 SSc patients and 14 normal control subjects as determined by enzyme-linked immunosorbent assay. Horizontal bars indicate means. $^{*}P < 0.05$ versus control subjects.

SSc patients with elevated serum TSP-2 levels tended to have pitting scars and/or ulcers ($P = 0.08$; Table 1). Thus, higher serum TSP-2 levels may correlate with vascular damage in SSc patients.

Knockdown of TSP-2 Results in Down-Regulation of Collagen Expression in Dermal Fibroblasts

Next, to clarify the role of TSP-2 in SSc, fibroblasts were treated with siRNA specific for TSP-2, and changes in the expression pattern of ECM proteins and/or cellular behaviors were evaluated.

In both normal and SSc fibroblasts transfected with TSP-2 siRNA, type I collagen synthesis was down-regulated, but levels of extracellular MMP-1 and MMP-2 expression were unchanged (Figure 4, A and B). In addition, activity of MMP-1 and MMP-2 was not affected by the TSP-2 siRNA in normal fibroblasts (Figure 4C). In contrast, the addition of recombinant human TSP-2 protein (25 ng/mL) to normal and SSc fibroblasts resulted in up-regulation of type I collagen expression (Figure 4D).

In addition, migratory activity of normal cells was significantly up-regulated by the transfection of TSP-2 siRNA, compared with control siRNA (Figure 4E), although cell proliferation was not affected (Figure 4F), indicating that increased migration by TSP-2 siRNA is independent of increased cell number. Taken together, our results suggest that TSP-2 itself has an inducible effect on collagen synthesis. Increased accumulation of extracellular TSP-2 protein in tissue probably contributes to fibrosis in SSc by induction of collagen synthesis. On the other hand, down-regulation of TSP-2 synthesis inside SSc fibroblasts may be one of the negative feedback mechanisms against increased extracellular TSP-2 and/or fibrosis.

Mechanisms Regulating Collagen Expression by TSP-2

Finally, we tried to clarify the regulatory mechanisms by which TSP-2 affects type I collagen synthesis. mRNA levels of α2(I) collagen were not affected by TSP-2 siRNA in normal fibroblasts (Figure 5A). Thus, TSP-2 is thought to regulate collagen expression post-transcriptionally. We hypothesized that miRNAs are involved in this process, because miRNAs usually inhibit transcription of their target genes and do not cause degradation of the target transcript. According to miRNA target gene predictions using the online tool TargetScan (release 5.1; available at *http://www.targetscan.org*), a leading program in the field,[36] we found several miRNAs, including miR-7, 25, 26, 29, 32, 92, 98, 129-5p, 133, 143, 196, 363, 367, and 1297, and let-7, that are putative regulators of α1(I) and/or α2(I) collagen. In addition, miR-21, 150, 192, and 194 have previously been shown to regulate type I collagen under fibrotic conditions.[37–40] Among these,

Table 1. Correlation of Serum TSP-2 Levels with the Clinical and Serological Features in SSc Patients

Clinical and serological features	Patients with elevated TSP-2 levels	Patients with normal TSP-2 levels	*P* value
Sample size	*n* = 24	*n* = 9	
Mean age at the time of serum sampling (years)	61.3	56.9	0.363
Mean age at onset (years)	53.9	52.2	0.539
Mean duration of disease (months)	66.1	57.1	0.906
Type (no. diffuse:no. limited)	14:10	3:6	0.188
MRSS (points)	11.4	12.0	0.943
Clinical features (%)			
Pitting scars/ulcers	57.1	22.2	0.086
Nailfold bleeding	50.0	55.5	0.554
Raynaud's phenomenon	100	100	
Telangiectasia	22.7	0	0.227
Contracture of phalanges	100	83.3	0.627
Calcinosis	10.0	0	0.667
Diffuse pigmentation	35.7	33.3	0.664
Short SF	85.7	83.3	0.681
Sicca symptoms	42.9	71.4	0.440
Organ involvement			
Pulmonary fibrosis (%)	28.6	44.4	0.896
Mean %VC	20.0	22.2	0.743
Mean $\%D_{LCO}$	40.0	55.5	0.353
Pulmonary hypertension (%)	0	11.1	0.657
Esophagus	30.0	11.1	0.273
Heart	27.3	33.3	0.783
Kidneys	0	0	
Joints	44.4	66.6	0.378
ANA specificity (%)			
Anti-topo I	33.3	14.3	0.327
Anti-centromere	57.1	42.9	0.412
Anti-U1 RNP	23.8	0	0.252

ANA, antinuclear antibodies; D_{LCO}, diffusion capacity for carbon monooxidase; MRSS, modified Rodnan total skin thickness score; SF, sublingual frenulum; VC, vital capacity.

only expression of miR-7, a putative regulator of α2(I) collagen based on the prediction, was significantly up-regulated by the transfection of TSP-2 siRNA in normal fibroblasts (Figure 5B). In normal fibroblasts, protein levels of α2(I) collagen were down-regulated by the transfection of miR-7 mimic (Figure 5C), confirming that α2(I) collagen is a target of miR-7. Of note, the overexpression of the miR-7 mimic also decreased α1(I) collagen expression. These results suggest that miR-7 also regulates α1(I) collagen directly or indirectly, and that TSP-2 siRNA down-regulates type I collagen expression via the induction of miR-7. Consistent with this hypothesis, miR-7 inhibitor attenuated the TSP-2 siRNA-mediated type I collagen down-regulation in normal cells (Figure 5D).

We expected that accumulated TSP-2 down-regulates miR-7 expression in SSc fibroblasts and that down-regulated miR-7 contributes to the excessive collagen expression in these cells. However, a miRNA PCR array, consisting of 88 miRNAs involved in human cell differentiation and development, identified six miRNAs that were overexpressed in SSc fibroblasts, compared with normal fibroblasts (Table 2). miR-7 was one of the overexpressed miRNAs (13.3-fold increase by the $\Delta\Delta$CT method). Transfection of mimics of the other five miRNAs led to a less effective decrease in type I collagen expression (data not shown). Consistent with the array data, miR-7 expression was increased in SSc cultured fibroblasts *in vitro* (Figure 5E). Moreover, *in situ* hybridization experiments showed that the signal for miR-7 was barely detected in fibroblasts of normal skin, but was clearly evident in SSc fibroblasts (Figure 5F).

Accordingly, miR-7 in SSc fibroblasts may not be regulated by accumulated TSP-2 outside the cells, but rather by decreased intracellular TSP-2. We hypothesize that, in SSc fibroblasts, decreased TSP-2 inside the cell may stimulate expression of miR-7, which has an inhibitory effect on collagen synthesis, probably because of negative feedback against fibrosis. Nonetheless, this negative feedback seems to be insufficient to normalize the excessive collagen synthesis of SSc fibroblasts. Consistent with this hypothesis, we found that type I collagen expression levels in SSc fibroblasts were strongly correlated with relative extracellular TSP-2 levels (r = 0.645), but inversely correlated with miR-7 levels (R = −0.753; Figure 5G).

Discussion

Three major findings demonstrate the role of TSP-2 in matrix regulation and its contribution to the SSc phenotype.

First, we found that TSP-2 synthesis in cell lysates from cultured SSc fibroblasts was decreased at the transcriptional level, whereas accumulation of TSP-2 protein in the medium was increased in SSc fibroblasts *in vitro*. Exposure to ectopic TGF-β or a TGF-β neutralizing antibody did not affect TSP-2 expression. *In vivo*, TSP-2 mRNA

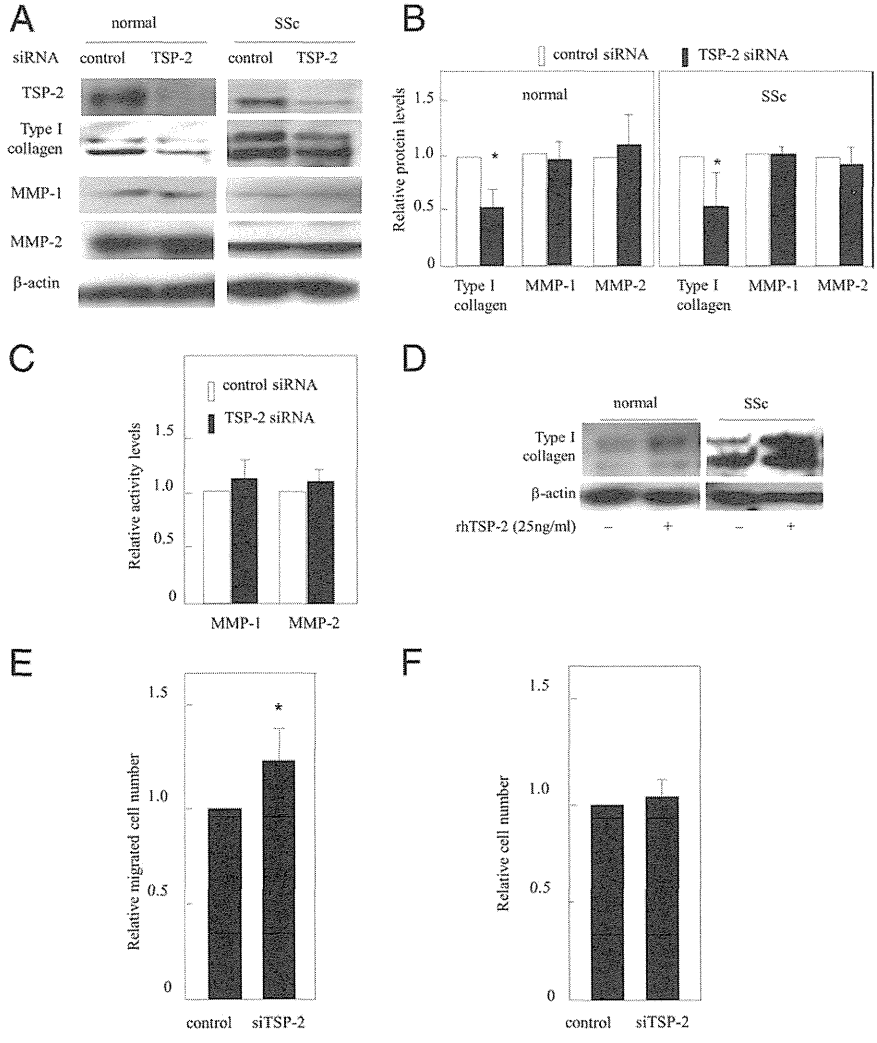

Figure 4. Effect of TSP-2 knockdown by specific siRNA on the expression of type I collagen or MMPs. **A:** Normal and SSc fibroblasts were transfected with control or TSP-2 siRNA. After 120 hours, the intracellular TSP-2 or type I collagen and extracellular MMP-1 or MMP-2 levels were analyzed by immunoblotting. Results of one experiment representative of three independent experiments are shown. **B:** Relative amounts of protein levels in normal and SSc fibroblasts were quantified by scanning densitometry (n = 3). **C:** Normal fibroblasts were transfected with control or TSP-2 siRNA. Activity levels of MMP-1 and MMP-2 were determined with a fluorescent activity assay kit (n = 3). **D:** Normal and SSc fibroblasts were treated with recombinant human TSP-2 protein (rhTSP-2; 25 ng/mL) for 24 hours. Collagen expression levels in cell lysates were analyzed by immunoblotting. Results of one experiment representative of five independent experiments are shown. **E:** Normal fibroblasts were transfected with control or TSP-2 siRNA (siTSP-2). After 120 hours, the number of cells that had migrated was evaluated using Transwell inserts coated with BD Matrigel (n = 3). **F:** Normal fibroblasts were transfected with control or TSP-2 siRNA (siTSP-2). After 120 hours, the number of normal fibroblasts was counted with a particle counter (n = 3).

expression level was significantly decreased, but deposition of TSP-2 protein in SSc skin and serum was increased. This discrepancy between intracellular TSP-2 synthesis and extracellular accumulation can be explained by the low degradation of TSP-2 outside cells (a result of down-regulation of MMP-2) in SSc. We previously found that TSP-1 accumulation in medium was increased in SSc patients at the post-transcriptional level.[11] Stimulation of TGF-β increased TSP-1 expression in normal fibroblasts, and treatment with a TGF-β neutralizing antibody normalized the up-regulated TSP-1 expression only in SSc fibroblasts. Thus, TSP-1 and TSP-2 are most likely regulated independently. As noted in the *Introduction*, even though TGF-β stimulation contributes to the SSc phenotype, several components are thought to be independent of TGF-β signaling in SSc fibroblasts. For example, the expression of connective tissue growth factor (CTGF/CCN2) is up-regulated in SSc fibroblasts, but was not normalized by a TGF-β receptor type I (ALK-5) kinase inhibitor or a TGF-β neutralizing antibody.[41,42] TSP-2 may also be such a component.

Second, we showed the putative function of TSP-2 in dermal fibroblasts. Our data suggest that TSP-2 itself may induce type I collagen expression and inhibit cell migration. Considering that TGF-β stimulation is known to induce collagen expression and inhibit cell migration in fibroblasts,[43] TSP-2 may have a similar effect as TGF-β, and extracellularly accumulated TSP-2 may contribute to tissue fibrosis in SSc patients (Figure 6). However, intracellular TSP-2 synthesis was down-regulated in SSc fibroblasts. We hypothesize that such down-regulation is due to a negative feedback mechanism against increased extracellular TSP-2 and/or tissue fibrosis. This hypothesis is supported by our results showing that miR-7, a negative regulator of collagen expression, was increased in SSc fibroblasts (Table 2 and Figure 5, E and F).

miRNAs are short ribonucleic acid molecules (only 22 nucleotides long, on average) acting as post-transcriptional regulators that bind to complementary sequences in the 3′ untranslated regions (3′ UTRs) of mRNAs and so leading to gene silencing. Recent vigorous efforts in this field indicate that miRNAs play a role in angiogenesis, as well as in immune response and/or carcinogenesis *in vivo*.[44–48] The present findings suggest that miRNAs are also involved in the regulation of the ECM. Given that the collagen expression levels were inversely correlated

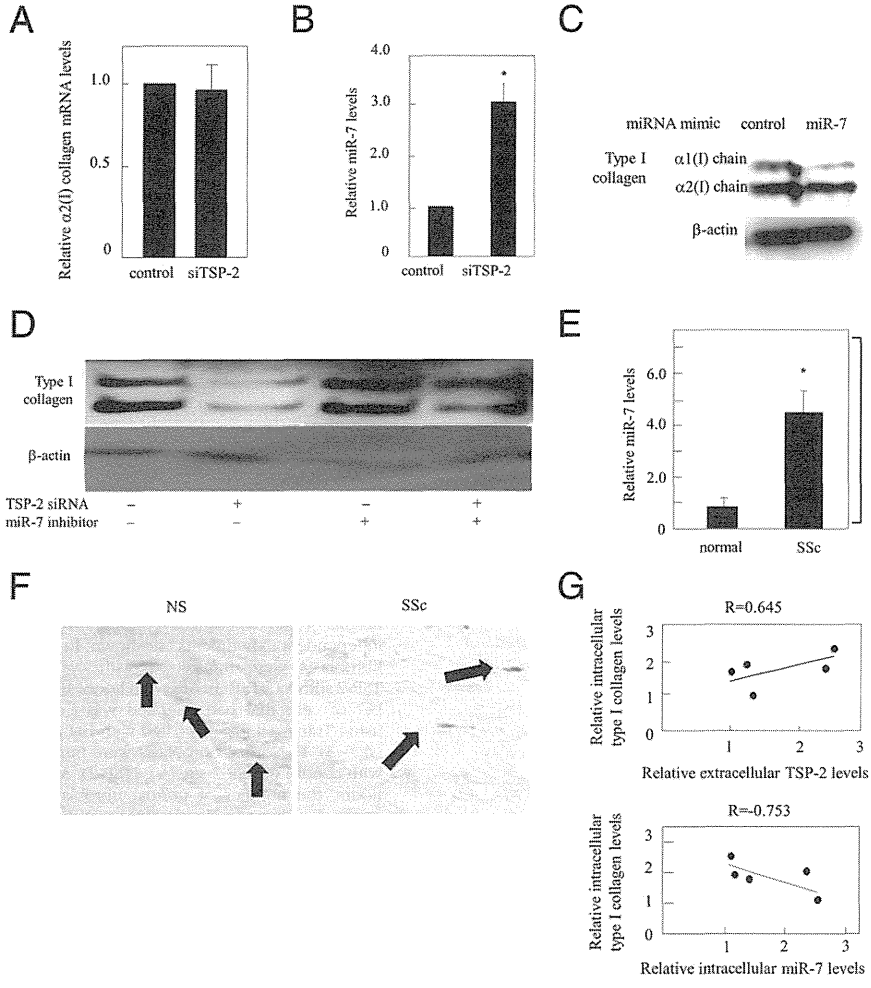

Figure 5. miR-7 mediates the effects of TSP-2 on type I collagen. **A:** Normal fibroblasts were transfected with control or TSP-2 siRNA (siTSP-2). After 12 hours, α2(I) collagen mRNA levels were determined by real-time PCR (n = 3). **B:** Normal fibroblasts were transfected with control or TSP-2 siRNA (siTSP-2). After 12 hours, miR-7 expression was determined by real-time PCR (n = 7). **C:** Normal dermal fibroblasts were transfected with a control or miR-7 mimic. Cell lysates were subjected to immunoblotting. Results of one experiment representative of three independent experiments are shown. **D:** Normal dermal fibroblasts were transfected with control or TSP-2 siRNA in the presence of a control or miR-7 inhibitor. After 72 hours, cells were harvested. Cell lysates were subjected to immunoblotting. Results of one experiment representative of five independent experiments are shown. **E:** Mean relative transcript levels of miR-7 in normal and SSc cultured dermal fibroblasts were determined by quantitative real-time PCR (n = 3). $^*P < 0.05$ versus normal cells. **F:** *In situ* detection of miR-7 in paraffin-embedded, formalin-fixed tissues of normal and SSc skin. Nuclei were counterstained with nuclear Fast Red. The miR-7 stained brown. Dermal fibroblasts are indicated by **arrows**. Original magnification, ×200. Representative results of three normal and three SSc skin samples are shown. **G:** Correlation between relative type I collagen levels and extracellular TSP-2 levels (analyzed by immunoblotting and quantitated by scanning densitometry) or between type I collagen levels and intracellular miR-7 levels (analyzed by real-time PCR) in SSc fibroblasts (n = 5). The minimum value was set at 1.

with the miR-7 levels (Figure 5G), the increase in miR-7 may be the result of increased type I collagen expression as part of negative feedback system in SSc fibroblasts. Leask et al[49] reported overexpression of endoglin in SSc fibroblasts and concluded that it may be a compensatory mechanism, because of the ability of endoglin to suppress canonical TGF-β signaling. Thus, there may be several negative feedback pathways in SSc cells that aim at suppressing the SSc phenotype. On the other hand, we described previously that silencing TSP-1 expression had an inhibitory effect on up-regulated α2(I) collagen in SSc fibroblasts by suppression of TGF-β signaling, indicating that constitutive overexpression of TSP-1 may play an important role in activated TGF-β signaling and in accumulation of ECM in SSc fibroblasts. Nonetheless, TSP-2 seems to be unable to activate latent TGF-β, because TSP-1 (but not TSP-2) contains a RFK motif.[50]

Table 2. Summary of Up-Regulated miRNAs in SSc Dermal Fibroblasts Determined by Microarray Analysis

Gene	Fold change
miR-378	249.0
miR-219-5p	48.2
miR-1	38.3
miR-518b	31.6
miR-194	31.6
miR-7	13.3

A mixture of equal amounts of miRNAs from five normal fibroblasts or five SSc fibroblasts was prepared, and the miRNA expression profile of each cell type was evaluated using a PCR array. The raw threshold cycle C_T was normalized using values for small RNA housekeeping gene. The fold change was calculated as $1/2^{Ct1 - Ct2}$, where Ct1 is the raw C_T for each miRNA and Ct2 is the raw C_T for small RNA housekeeping gene.

Third, we showed that SSc patients had significantly higher serum TSP-2 levels than healthy control subjects, and that patients with high serum TSP-2 levels have a tendency toward pitting scars and/or ulcers, compared with patients whose TSP-2 levels are normal. A previous report described that TSP-2-deficient mice show increased numbers of blood vessels in the skin.[51] In addition, when human A431 squamous cell carcinoma cells were stably overexpressed with TSP-2, tumor growth and angiogenesis were inhibited.[52] TSP-2 is generally thought to be an inhibitor of angiogenesis, and so high serum TSP-2 levels may cause vascular damage in patients with SSc. Circulating TSP-1 was also reported to be elevated in SSc patients, and correlated with angiopathy.[10] Thus, TSP-2 (as well as TSP-1) may be associated with angiopathy in this disease.

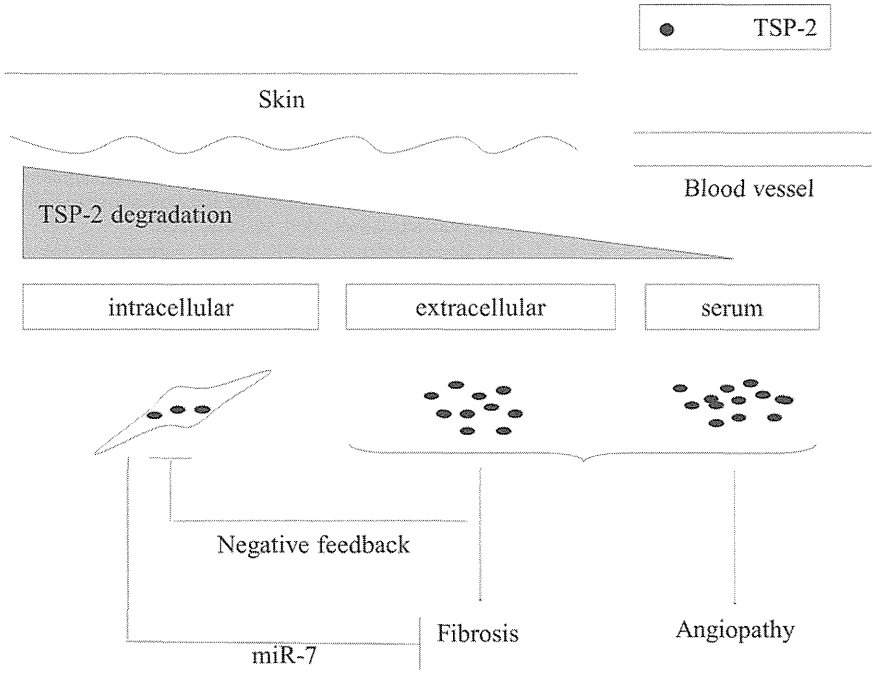

Figure 6. Expression pattern and role of TSP-2 in SSc. In SSc, TSP-2 synthesis inside cells is down-regulated, but extracellular accumulation of TSP-2 protein in tissue and serum is increased, because of the decreased degradation of extracellular TSP-2 protein. Extracellular increase in TSP-2 deposition causes tissue fibrosis, as a result of its inductive effect on collagen expression and the angiopathy resulting from its inhibitory effect on angiogenesis. On the other hand, down-regulation of TSP-2 synthesis inside SSc fibroblasts is one of the negative feedback mechanisms to prevent increased extracellular TSP-2 and/or tissue fibrosis. Decreased TSP-2 inside the cell stimulates the expression of miR-7, which has an inhibitory effect on collagen synthesis.

Taken together, our findings can be expressed schematically in a hypothetical model (Figure 6). In cases of SSc, TSP-2 synthesis inside the fibroblasts is down-regulated at the transcriptional level, whereas extracellular accumulation of the TSP-2 protein in tissue and serum is increased because of decreased degradation of extracellular TSP-2 protein. The increase in extracellular TSP-2 deposition contributes to tissue fibrosis by its inductive effect on collagen expression similar to TGF-β and contributes to angiopathy by its inhibitory effect on angiogenesis. On the other hand, down-regulation of TSP-2 synthesis inside SSc fibroblasts is one of the negative feedback mechanisms against increased extracellular TSP-2 and/or tissue fibrosis. Decreased expression of TSP-2 inside the cell stimulates expression of miR-7, which has an inhibitory effect on collagen synthesis. Nonetheless, this negative feedback seems to be insufficient to normalize the excessive collagen synthesis of SSc fibroblasts.

BFA had similar effects in normal cells (Figure 2G) as in SSc fibroblasts (Figure 2F). Likewise, expression levels of type I collagen, miR-7, and TSP-2 in SSc fibroblasts (Figure 5G) were similar to those in normal fibroblasts using TSP-2 siRNA (Figure 4A), recombinant TSP-2 protein (Figure 2D), or miR-7 mimic (Figure 5C). These results are therefore thought to be applicable to both normal and SSc fibroblasts. In addition, our findings on the effects of TSP-2 siRNA or miR-7 on gene expression and cellular activity in normal cells (Figures 4, C–F, and 5, A–D) may also be applicable to SSc cells, because the suppressive effect of TSP-2 siRNA on collagen expression was similar in normal and SSc fibroblasts (Figure 4, A and B). We also assume that the effects of the MMP-2 siRNA/inhibitor and MMP-2 recombinant protein (Figure 2, C–E) would not differ between normal and SSc fibroblasts; however, this needs to be confirmed in future study.

As another limitation of the present study, the detailed mechanisms underlying the miR-7-mediated regulation of collagen in dermal fibroblasts need to be elucidated. Although we found that both α1(I) and α2(I) collagens were target genes of miR-7, α1(I) collagen is not listed as a target of miR-7 by the prediction program. Therefore, the effect of miR-7 on α1(I) collagen may be indirect, and there may be some mediators that control this effect. Further studies are needed to explain the contradictory results, that increased extracellular TSP-2 overcomes the increased miR-7-mediated negative feedback system against excessive collagen expression in SSc fibroblasts. Investigations of the overall regulatory mechanisms of fibrosis and angiogenesis by TSP-2 may lead to a new therapeutic approach for systemic sclerosis.

Acknowledgments

We thank Junko Suzuki, Chiemi Shiotsu, Tomomi Etoh, and Faith C. Muchemwa for valuable technical assistance.

References

1. Korn JH: Immunologic aspects of scleroderma. Curr Opin Rheumatol 1989, 1:479–484
2. Mauch C, Kreig T: Fibroblast-matrix interactions and their role in the pathogenesis of fibrosis. Rheum Dis Clin North Am 1990, 16:93–107
3. Mauch C, Kozlowska E, Eckes B, Krieg T: Altered regulation of collagen metabolism in scleroderma fibroblasts grown within three-dimensional collagen gels. Exp Dermatol 1992, 1:185–190
4. Jelaska A, Arakawa M, Broketa G, Korn JH: Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts. Increased

proportion of high collagen-producing cells in systemic sclerosis fibroblasts. Arthritis Rheum 1996, 39:1338–1346
5. Massagué J: The transforming growth factor-beta family. Annu Rev Cell Biol 1990, 6:597–641
6. LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM: A strategy for determining the pathogenesis of systemic sclerosis. Is transforming growth factor beta the answer? Arthritis Rheum 1989, 32:817–825
7. LeRoy EC: Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest 1974, 54:880–889
8. Jimenez SA, Feldman G, Bashey RI, Bienkowski R, Rosenbloom J: Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. Biochem J 1986, 237:837–843
9. Bornstein P, Sage EH: Thrombospondins. Methods Enzymol 1994, 245:62–85
10. Macko RF, Gelber AC, Young BA, Lowitt MH, White B, Wigley FM, Goldblum SE: Increased circulating concentrations of the counteradhesive proteins SPARC and thrombospondin-1 in systemic sclerosis (scleroderma). Relationship to platelet and endothelial cell activation. J Rheumatol 2002, 29:2565–2570
11. Mimura Y, Ihn H, Jinnin M, Asano Y, Yamane K, Tamaki K: Constitutive thrombospondin-1 overexpression contributes to autocrine transforming growth factor-beta signaling in cultured scleroderma fibroblasts. Am J Pathol 2005, 166:1451–1463
12. Hesselstrand R, Kassner A, Heinegård D, Saxne T: COMP: a candidate molecule in the pathogenesis of systemic sclerosis with a potential as a disease marker. Ann Rheum Dis 2008, 67:1242–1248
13. Farina G, Lemaire R, Korn JH, Widom RL: Cartilage oligomeric matrix protein is overexpressed by scleroderma dermal fibroblasts. Matrix Biol 2006, 25:213–222
14. Bornstein P, Agah A, Kyriakides TR: The role of thrombospondins 1 and 2 in the regulation of cell-matrix interactions, collagen fibril formation, and the response to injury [Erratum appeared in Int J Biochem Cell Biol 2005, 37:239–240]. Int J Biochem Cell Biol 2004, 36:1115–1125
15. Yang Z, Kyriakides TR, Bornstein P: Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. Mol Biol Cell 2000, 11:3353–3364
16. Armstrong LC, Björkblom B, Hankenson KD, Siadak AW, Stiles CE, Bornstein P: Thrombospondin 2 inhibits microvascular endothelial cell proliferation by a caspase-independent mechanism. Mol Biol Cell 2002, 13:1893–1905
17. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Rowell N, Wollheim F: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol 1988, 15:202–205
18. Jinnin M, Makino T, Kajihara I, Honda N, Makino K, Ogata A, Ihn H: Serum levels of soluble vascular endothelial growth factor receptor-2 in patients with systemic sclerosis. Br J Dermatol 2010, 162:751–758
19. Ihn H, LeRoy EC, Trojanowska M: Oncostatin M stimulates transcription of the human alpha2(I) collagen gene via the Sp1/Sp3-binding site. J Biol Chem 1997, 272:24666–24672
20. Makino T, Jinnin M, Muchemwa FC, Fukushima S, Kogushi-Nishi H, Moriya C, Igata T, Fujisawa A, Johno T, Ihn H: Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways. Br J Dermatol 2010, 162:717–723
21. Igata T, Jinnin M, Makino T, Moriya C, Muchemwa FC, Ishihara T, Ihn H: Up-regulated type I collagen expression by the inhibition of Rac1 signaling pathway in human dermal fibroblasts. Biochem Biophys Res Commun 2010, 393:101–105
22. Handy JA, Saxena NK, Fu P, Lin S, Mells JE, Gupta NA, Anania FA: Adiponectin activation of AMPK disrupts leptin-mediated hepatic fibrosis via suppressors of cytokine signaling (SOCS-3) [Erratum appeared in J Cell Biochem 2011 Feb;112(2):734]. J Cell Biochem 2010, 110:1195–1207
23. Higashi-Kuwata N, Jinnin M, Makino T, Fukushima S, Inoue Y, Muchemwa FC, Yonemura Y, Komohara Y, Takeya M, Mitsuya H, Ihn H: Characterization of monocyte/macrophage subsets in the skin and peripheral blood derived from patients with systemic sclerosis. Arthritis Res Ther 2010, 12:R128
24. Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, Nishioka K: Animal model of sclerotic skin. I: local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol 1999, 112:456–462
25. Tanaka C, Fujimoto M, Hamaguchi Y, Sato S, Takehara K, Hasegawa M: Inducible costimulator ligand regulates bleomycin-induced lung and skin fibrosis in a mouse model independently of the inducible costimulator/inducible costimulator ligand pathway. Arthritis Rheum 2010, 62:1723–1732
26. Pacheco CD, Kunkel R, Lieberman AP: Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects. Hum Mol Genet 2007, 16:1495–1503
27. Su X, Ao L, Zou N, Song Y, Yang X, Cai GY, Fullerton DA, Meng X: Post-transcriptional regulation of TNF-induced expression of ICAM-1 and IL-8 in human lung microvascular endothelial cells: an obligatory role for the p38 MAPK-MK2 pathway dissociated with HSP27. Biochim Biophys Acta 2008, 1783:1623–1631
28. Yan H, Choi AJ, Lee BH, Ting AH: Identification and functional analysis of epigenetically silenced microRNAs in colorectal cancer cells. PLoS One 2011, 6:e20628
29. Nakashima T, Jinnin M, Etoh T, Fukushima S, Masuguchi S, Maruo K, Inoue Y, Ishihara T, Ihn H: Down-regulation of mir-424 contributes to the abnormal angiogenesis via MEK1 and cyclin E1 in senile hemangioma: its implications to therapy. PLoS One 2010, 5:e14334
30. Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K: Platelet derived growth factor induced tenascin-C transcription is phosphoinositide 3-kinase/Akt-dependent and mediated by Ets family transcription factors. J Cell Physiol 2006, 206:718–727
31. Albini A, Benelli R, Noonan DM, Brigati C: The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes. Int J Dev Biol 2004, 48:563–571
32. Martin MM, Buckenberger JA, Jiang J, Malana GE, Nuovo GJ, Chotani M, Feldman DS, Schmittgen TD, Elton TS: The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microRNA-155 binding. J Biol Chem 2007, 282:24262–24269
33. Nuovo GJ: In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations. Methods 2008, 44:39–46
34. Kuroda K, Shinkai H: Gene expression of types I and III collagen, decorin, matrix metalloproteinases and tissue inhibitors of metalloproteinases in skin fibroblasts from patients with systemic sclerosis. Arch Dermatol Res 1997, 289:567–572
35. Prudova A, auf dem Keller U, Butler GS, Overall CM: Multiplex N-terminome analysis of MMP-2 and MMP-9 substrate degradomes by iTRAQ-TAILS quantitative proteomics. Mol Cell Proteomics 2010, 9:894–911
36. Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005, 120:15–20
37. Maurer B, Stanczyk J, Jüngel A, Akhmetshina A, Trenkmann M, Brock M, Kowal-Bielecka O, Gay RE, Michel BA, Distler JH, Gay S, Distler O: MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum 2010, 62:1733–1743
38. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, Abraham E: miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. J Exp Med 2010, 207:1589–1597
39. Venugopal SK, Jiang J, Kim TH, Li Y, Wang SS, Torok NJ, Wu J, Zern MA: Liver fibrosis causes downregulation of miRNA-150 and miRNA-194 in hepatic stellate cells, and their overexpression causes decreased stellate cell activation. Am J Physiol Gastrointest Liver Physiol 2010, 298:G101–G106
40. Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, Natarajan R: MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci USA 2007, 104:3432–3437
41. Chen Y, Shi-wen X, Eastwood M, Black CM, Denton CP, Leask A, Abraham DJ: Contribution of activin receptor-like kinase 5 (transforming growth factor beta receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. Arthritis Rheum 2006, 54:1309–1316
42. Holmes AM, Ponticos M, Shi-Wen X, Denton CP, Abraham DJ: Elevated CCN2 expression in scleroderma: a putative role for the TGFβ accessory receptors TGFβRIII and endoglin. J Cell Commun Signal 2011, 5:173–177
43. Ellis I, Grey AM, Schor AM, Schor SL: Antagonistic effects of TGF-beta 1 and MSF on fibroblast migration and hyaluronic acid synthesis.

Possible implications for dermal wound healing. J Cell Sci 1992, 102:447–456
44. Kuehbacher A, Urbich C, Dimmeler S: Targeting microRNA expression to regulate angiogenesis. Trends Pharmacol Sci 2008, 29:12–15
45. Chen Y, Gorski DH: Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. Blood 2008, 111:1217–1226
46. Furer V, Greenberg JD, Attur M, Abramson SB, Pillinger MH: The role of microRNA in rheumatoid arthritis and other autoimmune diseases. Clin Immunol 2010, 136:1–15
47. Lu LF, Liston A: MicroRNA in the immune system, microRNA as an immune system. Immunology 2009, 127:291–298
48. Davidson-Moncada J, Papavasiliou FN, Tam W: MicroRNAs of the immune system: roles in inflammation and cancer. Ann N Y Acad Sci 2010, 1183:183–194
49. Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, Shi-Wen X, Chen Y, Venstrom K, Dou X, Ponticos M, Black C, Bernabeu C, Jackman JK, Findell PR, Connolly MK: Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts [Erratum appeared in Arthritis Rheum 2002, 46:2830]. Arthritis Rheum 2002, 46:1857–1865
50. Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Krutzsch HC, Roberts DD, Murphy-Ullrich JE: Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. J Biol Chem 1995, 270:7304–7310
51. Kyriakides TR, Zhu YH, Smith LT, Bain SD, Yang Z, Lin MT, Danielson KG, Iozzo RV, LaMarca M, McKinney CE, Ginns EI, Bornstein P: Mice that lack thrombospondin 2 display connective tissue abnormalities that are associated with disordered collagen fibrillogenesis, an increased vascular density, and a bleeding diathesis. J Cell Biol 1998, 140:419–430
52. Streit M, Riccardi L, Velasco P, Brown LF, Hawighorst T, Bornstein P, Detmar M: Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. Proc Natl Acad Sci USA 1999, 96: 14888–14893

TGF-β–Mediated Downregulation of MicroRNA-196a Contributes to the Constitutive Upregulated Type I Collagen Expression in Scleroderma Dermal Fibroblasts

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Previous reports indicated the significance of the TGF-β signaling in the pathogenesis of systemic sclerosis. We tried to evaluate the possibility that microRNAs (miRNAs) play a part in the type I collagen upregulation seen in normal fibroblasts stimulated with exogenous TGF-β and systemic sclerosis (SSc) fibroblasts. miRNA expression profile was evaluated by miRNA PCR array and real-time PCR. The protein expression of type I collagen was determined by immunoblotting. In vivo detection of miRNA in paraffin section was performed by in situ hybridization. Several miRNAs were found to be downregulated in both TGF-β–stimulated normal fibroblasts and SSc fibroblasts compared with normal fibroblasts by PCR array. Among them, miR-196a expression was decreased in SSc both in vivo and in vitro by real-time PCR or in situ hybridization. In SSc fibroblasts, miR-196a expression was normalized by TGF-β small interfering RNA. miR-196a inhibitor leads to the overexpression of type I collagen in normal fibroblasts, whereas overexpression of the miRNA resulted in the downregulation of type I collagen in SSc fibroblasts. In addition, miR-196a was detectable and quantitative in the serum of SSc patients. Patients with lower serum miR-196a levels had significantly higher ratio of diffuse cutaneous SSc:limited cutaneous SSc, higher modified Rodnan total skin thickness score, and higher prevalence of pitting scars than those without. miR-196a may play some roles in the pathogenesis of SSc. Investigation of the regulatory mechanisms of type I collagen expression by miR-196a may lead to new treatments using miRNA. *The Journal of Immunology*, **2012, 188: 3323–3331.**

Systemic sclerosis (SSc) or scleroderma is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to the activation of fibroblasts and disturbed interactions with different components of the extracellular matrix (ECM) (1, 2). Thus, abnormal SSc fibroblasts that are responsible for fibrosis may develop from a subset of cells that have escaped from normal control mechanisms (3, 4).

Although the mechanism of fibroblast activation in SSc is presently unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β1 (5, 6). The principal effect of TGF-β1 on mesenchymal cells is its stimulation of ECM deposition. Fibroblasts from affected SSc skin cultured in vitro produce excessive amounts of various collagens, mainly type I collagen, which consists of α1(I) and α2(I) collagen (7, 8), suggesting that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF-β signaling. This notion is supported by our following recent findings: 1) although the transcriptional activity of the α2(I) collagen gene in SSc fibroblasts was constitutively higher than that in normal fibroblasts, the responsiveness to ectopic TGF-β1 was decreased in SSc cells (9); 2) phosphorylated levels and DNA-binding activity of Smad3, a mediator of TGF-β1 signaling, were constitutively upregulated in SSc fibroblasts (10); and 3) the blockade of TGF-β1 signaling with anti–TGF-β1–neutralizing Abs abolished the increased expression of human α2(I) collagen mRNA in SSc fibroblasts (11).

Recently, epigenetics has attracted attention for its involvement in the various cellular behavior, including cell development, cell differentiation, immune response, organogenesis, growth control, and apoptosis. microRNAs (miRNAs), short RNA molecules on average only 22 nt long, are posttranscriptional regulators that bind to complementary sequences in the 3′ untranslated regions (3′UTRs) of mRNAs, leading to gene silencing (12–14). Because there are >1000 miRNAs in the human genome, which may target ~60% of mammalian genes (15), miRNAs are thought to be the most abundant class of regulators. Accordingly, miRNAs have been implicated in the pathogenesis of various human diseases, such as immunological disorders, cancers, and metabolic disorders (16–21). However, little is known about the role of miRNAs in SSc. In the current study, we tried to evaluate the possibility that miRNAs may also play some roles in the constitutive upregulation of type I collagen expression seen in SSc.

Materials and Methods

Cell culture

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of five patients with diffuse cutaneous SSc and <2 y of

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Received for publication March 25, 2011. Accepted for publication January 22, 2012.

This work was supported in part by a grant for scientific research from the Japanese Ministry of Education, Science, Sports, and Culture and by a grant for project research on intractable diseases from the Japanese Ministry of Health, Labor, and Welfare.

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Abbreviations used in this article: Ct, threshold cycle; dcSSc, diffuse cutaneous systemic sclerosis; ECM, extracellular matrix; EIA, enzyme immunoassay; lcSSc, limited cutaneous systemic sclerosis; miRNA, microRNA; MRSS, modified Rodnan total skin thickness; PIP, procollagen type I C-terminal peptide; siRNA, small interfering RNA; SSc, systemic sclerosis; UTR, untranslated region.

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skin thickening, as described previously (22). Control fibroblasts were obtained by skin biopsies from five healthy donors. Control donors were each matched with SSc patients for age, sex, and biopsy site. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm^2 culture flasks in MEM supplemented with 10% FCS and antibiotic-antimycotic (Invitrogen, Carlsbad, CA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO_2 in air. Fibroblasts between the third and six subpassages were used for experiments. Before experiments, cells were serum starved for 12–24 h.

Patient material

Skin samples were obtained from affected skin of 10 SSc patients. Patients were grouped into 5 diffuse cutaneous SSc (dcSSc) and 5 limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy et al. (23). Patients with SSc fulfilled the criteria proposed by the American College of Rheumatology (24, 25). Control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin graft. Immediately after removal, skin samples were fixed by formalin and embedded in paraffin (26).

Serum samples were obtained from 40 patients with SSc (20 dcSSc and 20 lcSSc). Control samples were collected from 25 healthy age- and sex-matched volunteers. Clinical and laboratory data reported in this study were obtained at the time of serum sampling. All serum samples were stored at −80°C prior to use. Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki.

Mice

Heterozygous TSK/+ mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free barrier facility and sacrificed at 8 wk. The Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science approved all studies and procedures (27).

In situ hybridization

In situ hybridization was performed with 5′-locked digoxigenin-labeled nucleic acid probes complementary to human mature miR-196a and scrambled negative control (Exiqon, Vedbeak, Denmark) (28, 29). Briefly, human tissues were deparaffinized and deproteinized with protease K for 5 min. Slides were then washed in 0.2% glycine in PBS and fixed with 4% paraformaldehyde. Hybridization was performed at 50°C overnight, followed by blocking with 2% FBS and 2% BSA in PBS and 0.1% Tween 20 for 1 h. The probe–target complex was detected immunologically by a digoxigenin Ab conjugated to alkaline phospatase acting on the chromogen nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany). Slides were counterstained with nuclear fast red, and examined under a light microscope (Olympus BX50, Tokyo, Japan).

Table I. Summary of microRNA expression in normal and SSc fibroblasts by PCR array analysis

miRNA Name	NS	NS + TGF-β	SSc	miRNA Name	NS	NS + TGF-β	SSc
let-7a	−6.69	−4.95	−4.44	miR-132	−1.53	ND	0.38
let-7b	−6.34	−4.77	−4.27	miR-133b	8.39	8.76	8.54
let-7c	−1.05	0.79	1.43	miR-134	−1.23	0.98	1.71
let-7d	1.17	1.84	2.82	miR-137	0.15	2.17	2.44
let-7e	−2.28	−1.16	−0.39	miR-141	2.75	10.10	5.59
let-7f	3.58	12.17	6.08	miR-142-3p	4.29	12.96	6.35
let-7g	−3.02	−1.73	−1.09	miR-142-5p	6.10	11.55	8.49
let-7i	−2.34	−1.61	−1.76	miR-146a	−1.99	1.31	1.47
miR-1	9.33	ND	4.77	miR-146b-5p	−1.01	2.22	3.53
miR-7	11.33	5.10	8.30	miR-150	−0.46	1.80	2.63
miR-9	8.83	ND	6.69	miR-155	0.58	3.24	3.19
miR-10a	−2.85	−1.46	2.37	miR-181a	2.50	ND	0.94
miR-10b	−2.75	−0.31	0.13	miR-182	2.29	6.08	6.64
miR-15a	1.20	ND	2.44	miR-183	2.66	4.23	9.75
miR-15b	−4.97	−2.57	−1.54	miR-185	1.41	ND	5.23
miR-16	−6.67	−4.67	−3.29	miR-192	−0.90	5.06	3.44
miR-17	−1.70	0.08	0.14	miR-194	7.31	6.01	3.03
miR-18a	2.98	3.23	3.13	miR-195	−5.83	−3.65	−2.64
miR-18b	5.68	6.15	6.01	miR-196a	−2.61	−0.55	5.61
miR-20a	−0.58	−0.51	0.57	miR-205	1.40	4.19	4.43
miR-20b	4.06	3.63	5.58	miR-206	3.01	6.93	6.69
miR-21	−9.32	−8.06	−7.14	miR-208a	−0.24	2.55	3.50
miR-22	−1.45	ND	−0.83	miR-210	−0.93	1.58	1.51
miR-23b	−5.64	−4.68	−4.41	miR-214	−3.61	−2.33	−1.79
miR-24	−5.01	−3.72	−4.35	miR-215	7.37	10.67	7.66
miR-26a	−5.35	−4.04	−3.67	miR-218	−1.18	1.25	2.53
miR-33a	8.38	6.00	7.76	miR-219-5p	9.58	4.40	4.69
miR-92a	−3.00	−1.17	−1.22	miR-222	−0.83	−0.83	−0.29
miR-93	−0.39	0.19	1.93	miR-223	7.30	3.20	4.73
miR-96	1.24	ND	3.42	miR-301a	8.71	3.73	9.20
miR-99a	−2.82	−1.27	0.13	miR-302a	8.74	5.61	10.10
miR-100	−6.65	−4.80	−3.64	miR-302c	8.99	10.85	8.45
miR-101	0.57	1.64	1.35	miR-345	3.46	3.45	2.71
miR-103	−0.44	0.45	0.14	miR-370	1.65	4.14	4.33
miR-106b	0.60	1.44	2.82	miR-371-3p	2.57	4.36	4.63
miR-122	10.31	ND	11.82	miR-375	−1.84	1.50	1.90
miR-124	0.01	2.61	2.03	miR-378	11.66	5.06	4.40
miR-125a-5p	−5.13	−3.27	−2.29	miR-424	−3.07	−3.05	−3.06
miR-125b	−9.49	−3.62	−6.21	miR-452	4.82	8.88	5.92
miR-126	1.83	3.08	5.19	miR-488	7.50	9.06	9.57
miR-127-5p	9.71	7.52	7.75	miR-498	8.28	8.82	8.00
miR-128	0.26	3.61	2.80	miR-503	2.26	3.68	4.83
miR-129-5p	−0.70	1.45	1.86	miR-518b	12.04	8.31	7.76
miR-130a	1.93	6.77	2.89	miR-520g	9.18	13.74	11.03

A mixture of equal amounts of microRNAs from five normal fibroblasts (NS), five normal fibroblasts stimulated with TGF-β (NS + TGF-β), or five scleroderma fibroblasts (SSc) was prepared, and miRNA expression profile in each cell group was evaluated using PCR array. The ΔΔCt (the raw Ct value of each miRNA − Ct value of small RNA housekeeping gene) is shown.

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Transient transfection

Control small interfering RNA (siRNA) and TGF-β siRNA were purchased from Santa Cruz Biotechnology. miRNA inhibitors, mimics, and miScript Target protectors for control or miR-196a were purchased from Qiagen. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used as transfection reagent. For reverse transfection, siRNAs, miRNA inhibitors (50 nM), mimics (5 nM), and protectors (500 nM) mixed with transfection reagent were added when cells were plated, followed by the incubation for 48–96 h at 37°C in 5% CO_2. Control experiments showed transcript levels for target of miRNA inhibitors to be reduced by >80%, and expression of miRNAs was increased at least 5-fold by the transfection of mimics (data not shown).

Cell lysis and immunoblotting

Fibroblasts were washed with cold PBS twice and lysed in Denaturing Cell Extraction Buffer (Biosource, Camarillo, CA). Aliquots of cell lysates (normalized for protein concentrations) were subjected to electrophoresis on 10% $NaDodSO_4$-polyacrylamide gels and transferred onto nitrocellulose filters. The nitrocellulose filters were then incubated with Ab against type I collagen, fibronectin, or β-actin. Then the filters were incubated with secondary Ab, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences, Arlington Heights, IL), according to the manufacturer's recommendations.

Measurement of type I collagen concentrations

Levels of type I collagen in cell lysate were measured with enzyme immunoassay (EIA) kit for procollagen type I C-terminal peptide (PIP; Takara Bio, Shiga, Japan). Briefly, anti-PIP mAbs were precoated onto microtiter wells. Aliquots of cell lysate were added to each well, followed by peroxidase-conjugated Abs to PIP. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase, and the absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 540 nm. The concentration of PIP in each sample was determined by interpolation from a standard curve.

miRNA extraction and PCR array analysis

miRNA isolation from cultured cells or skin tissue was performed using RT^2 qPCR-Grade miRNA Isolation Kit (SA Bioscience) or miRNeasy FFPE kit (Qiagen, Valencia, CA), respectively. miRNAs were reverse transcribed into first-strand cDNA using RT^2 miRNA First Strand Kit (SA Bioscience). For RT^2 Profiler PCR array, the cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix, and the mixture was added into 96-well RT^2 miRNA PCR array (SA Bioscience) that includes primer pairs for 88 human miRNAs. PCR was performed on a Takara Thermal Cycler Dice (TP800) following the manufacturer's protocol. Threshold cycle (Ct) for each miRNA was extracted using Thermal Cycler Dice Real Time System ver2.10B (Takara Bio). The raw Ct was normalized using the values of small RNA housekeeping genes.

For quantitative real-time PCR, primers for miR-196a, U6, or Snord68 (SA Bioscience) and templates were mixed with the SYBR Premix Ex *Taq*II (Takara Bio). DNA was amplified for 40 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 60°C. Data generated from each PCR were analyzed using Thermal Cycler Dice Real Time System ver2.10B. Transcript levels of miR-196a were normalized to U6 for humans and Snord68 for mice.

miRNA extraction from serum and PCR analysis of miRNA expression

miRNA isolation from serum samples was performed with miRNeasy RNA isolation kit (Qiagen) following the manufacturer's instructions with minor modification (30). Briefly, 100 μl serum was supplemented with 5 μl 5 fmol/μl synthetic nonhuman miRNA (*Caenorhabditis elegans* miR-39; Takara Bio) as controls, providing an internal reference for normalization of technical variations between samples. After Qiazol solution (1 ml) was added and mixed well by vortexing, then samples were incubated at room temperature for 5 min. Aqueous and organic phase separation was achieved by the addition of chloroform. The aqueous phase was applied to

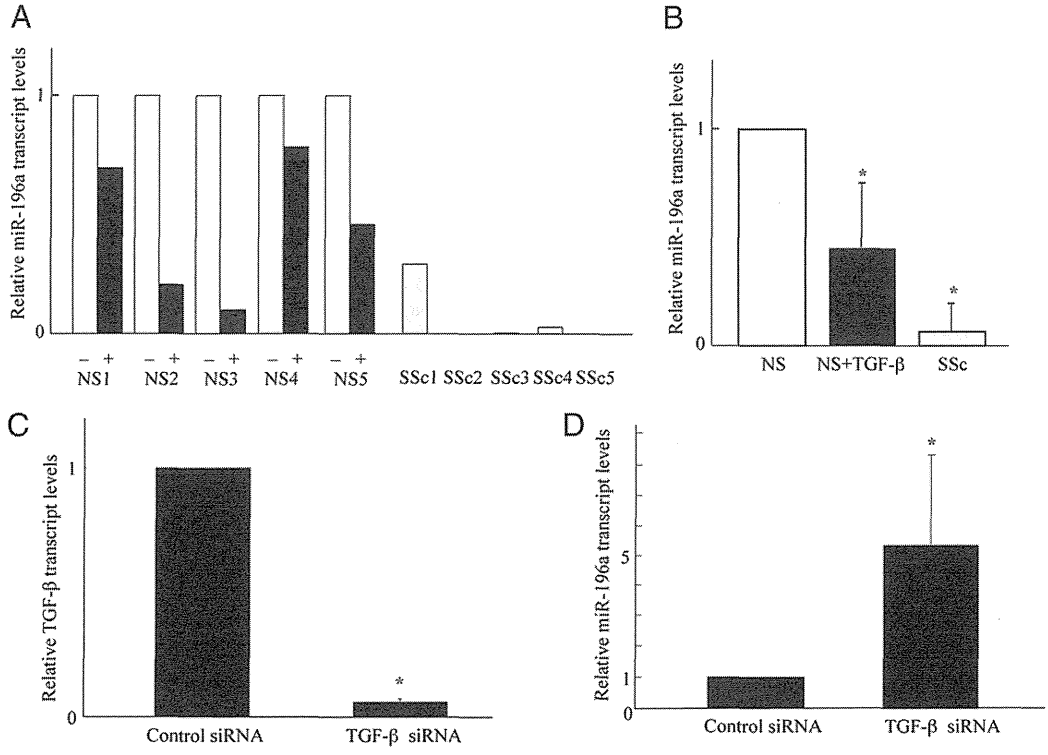

FIGURE 1. Expression levels of miR-196a in normal and scleroderma fibroblasts. (**A**) Five normal and scleroderma fibroblasts were serum starved for 24 h and incubated in the presence or absence of TGF-β1 (2 ng/ml) for 6 h. Total miRNA was extracted, and the relative level of miR-196a (normalized to U6) in each of normal (NS, white bars) and scleroderma fibroblast (SSc, gray bars) was determined by quantitative real-time PCR, as described in *Materials and Methods*. The values in normal fibroblasts were set at 1. (**B**) Mean relative transcript levels of miR-196a in normal fibroblast (NS, white bars), normal fibroblasts stimulated with TGF-β (NS + TGF-β, black bars), and scleroderma fibroblasts (SSc, gray bars). The data are expressed as mean ± SE of independent experiments using five NS and five SSc fibroblasts. *$p < 0.05$ as compared with the value in untreated NS cells (1.0). (**C** and **D**) Scleroderma dermal fibroblasts were cultured in 6-well plates and transfected with TGF-β1 siRNA or control siRNA, as described in *Materials and Methods*. Then, relative transcript levels of TGF-β1 (C, normalized to GAPDH) and miR-196a (D, normalized to U6) were determined by real-time PCR. *$p < 0.05$ as compared with the values in fibroblasts transfected with control siRNA (1.0).

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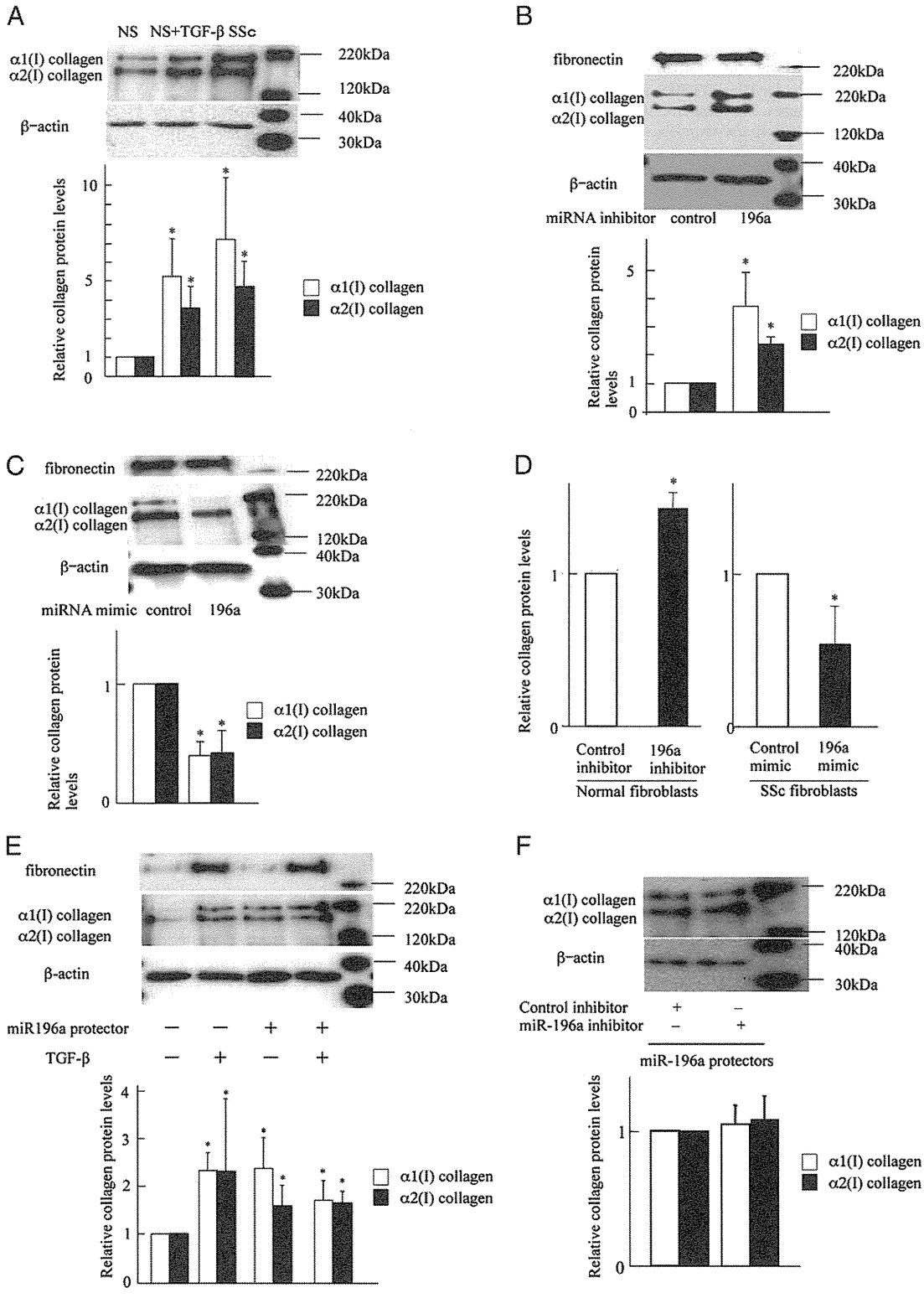

FIGURE 2. miR-196a regulates type I collagen expression in vitro. (**A**) *Upper panel*, Normal (NS) and scleroderma (SSc) fibroblasts were cultured independently under the same conditions until they were confluent, and serum starved for 24 h. Then cells were incubated in the presence or absence of TGF-β1 (2 ng/ml) for additional 24 h. Cell lysates were subjected to immunoblotting with Ab for type I collagen. The same membrane was reprobed with anti–β-actin Ab as a loading control. The representative results for five normal and SSc fibroblasts are shown. Molecular mass of marker proteins is shown on the *right*. *Lower panel*, α1(I) collagen (white bars) and α2(I) collagen (black bars) protein levels quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the level in normal fibroblast (1.0). The data are expressed as mean ± SE of three independent experiments. $*p < 0.05$ as compared with the value in normal cells. (**B**) *Upper panel*, Normal human fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control inhibitor or miR-196a inhibitor for 96 h. Cell lysates were subjected to immunoblotting using Ab for type I collagen, fibronectin, and β-actin. *Lower panel*, α1(I) collagen (white bars) and α2(I) collagen (black bars) protein levels quantitated by scanning densitometry and corrected for the levels of β-actin in the same samples are shown relative to the level in cells transfected with control inhibitor (1.0). The data are expressed as mean ± SE of three independent experiments. $*p < 0.05$ as compared with the value in cells with control (*Figure legend continues*)

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RNeasy spin column and RNeasy MinElute spin column. miRNA was eluted from the column with nuclease-free water.

cDNA was synthesized from miRNA with Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio). Quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800) used primers and templates mixed with the SYBR Premix. The sequence of hsa-miR-196a primer was designed based on miRBase (http://www.mirbase.org): 5′-TAGGTAGTTTCATGTTGTTGGG-3′. The primer set was prevalidated to generate single amplicons. DNA was amplified for 40 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. Data generated from each PCR were analyzed using Thermal Cycler Dice Real Time System ver2.10B (Takara Bio). Specificity of reactions was determined by dissociation curve analysis. The relative gene expression of hsa-miR-196a and cel-miR-39 was calculated by standard curve method. Transcript level of miR-196a was normalized to that of cel-miR-39.

Statistical analysis

Statistical analysis was carried out with a Student *t* test or Mann–Whitney *U* test for comparison of means, and Fisher's exact probability test for the analysis of frequency. The *p* values <0.05 were considered significant. This research was approved by the Ethics Committee at Kumamoto University (No.177).

Results

Several miRNAs are downregulated in normal fibroblasts stimulated with TGF-β and in SSc fibroblasts

As an initial experiment, to determine which miRNAs were involved in the pathogenesis of SSc, we performed miRNA PCR array analysis, consisting of 88 miRNAs involved in human cell differentiation and development. A mixture of equal amounts of miRNAs from five normal fibroblasts, five normal fibroblasts stimulated with exogenous TGF-β1, and five SSc fibroblasts was prepared, and miRNA expression profile in each cell group was evaluated using the PCR array. There were several miRNAs downregulated in both TGF-β–stimulated normal fibroblasts and SSc fibroblasts compared with normal fibroblasts (Table I; the complete dataset is available at the Gene Expression Omnibus microarray data repository, www. ncbi.nlm.nih.gov/geo/, accession number GSE34827). Among them, we focused on miR-196a as the regulator of both α1(I) and α2(I) collagen, according to miRNA target gene predictions using the TargetScan (version 5.1, http://www.targetscan.org/), MiRanda (August 2010 Release, http://www.microrna.org/), DIANAmicroT (version 3.0, http://diana.cslab.ece.ntua.gr/microT/), and PicTar (http://pictar.mdc-berlin.de/), leading programs in the field (31–34).

The array showed the expression of miR-196a was downregulated in normal fibroblasts stimulated with TGF-β1 (2.06-cycle difference; 4.17-fold change in ΔΔCt method) and SSc fibroblasts (8.22-cycle difference; 298.17-fold change). To confirm the results obtained by miRNA PCR array, we performed quantitative real-time PCR analysis using the specific primer for miR-196a. As expected, miR-196a was decreased in all five normal fibroblasts in the presence of TGF-β and all five SSc fibroblasts (Fig. 1A), and the decrease of miR-196a in these cells was statistically significant ($p < 0.05$, Fig. 1B). Interestingly, when TGF-β signaling was inhibited by the specific siRNA in SSc fibroblasts (Fig. 1C), real-time PCR revealed that the miR-196a expression was significantly upregulated (Fig. 1D). The recovery of miR-196a expression by TGF-β1 siRNA in SSc fibroblasts suggests that downregulation of miR-196a is due to the stimulation of TGF-β signaling in that cell type, as described above.

Low miR-196a expression leads to the upregulation of type I collagen protein

As described above, the expression of both α1(I) and α2(I) collagen protein was increased in SSc fibroblasts as well as normal fibroblasts stimulated with TGF-β, compared with untreated normal fibroblasts (Fig. 2A). Next, we determined the contribution of downregulated miR-196a to the upregulation of type I collagen in TGF-β–treated normal fibroblasts and SSc fibroblasts, using miRNA inhibitor and mimic: miRNA inhibitor is chemically synthesized, ssRNA, which has complementary sequence of the target miRNA, and specifically inhibits the target miRNA by the transfection into cells. miRNA mimic is also chemically synthesized RNA that mimics mature endogenous miRNA. The downregulation of miR-196a by the transfection of specific miRNA inhibitor in normal fibroblasts resulted in statistically significant increase of protein expression of α1(I) and α2(I) collagen (Fig. 2B). In contrast, the overexpression of miR-196a using miR-196a mimic normalized the upregulated type I collagen expression in SSc fibroblasts (Fig. 2C). Fibronectin, another ECM that is also increased in SSc and induced by TGF-β stimulation (35, 36), but not the target of miR-196a, was not affected by the miRNA inhibitor or mimic (Fig. 2B, 2C), suggesting that the effect of miR-196a inhibitor or mimic is specific to type I collagen. These results indicated the possibility that α1(I) and α2(I) collagen is the target of miR-196a. The inducible or suppressive effect of miR-196a inhibitor or mimic on the collagen expression was confirmed by the EIA experiment determining PIP (Fig. 2D).

Furthermore, because the construct of 3′UTR of type I collagen for reporter assay was not available, to determine the direct interaction between miR-196a with α1(I) or α2(I) collagen 3′UTR, we used miScript Target Protector (Qiagen), ssRNA complementary to the miR-196a binding site on the collagen mRNA: the protector covers the flanking region of the binding site and specifically interferes with the direct interaction between miRNA and mRNA (http://www.qiagen.com/products/miscripttargetprotectors.aspx) (37). As shown in Fig. 2E, transfection of the miR-196a protector resulted in the upregulation of type I collagen expression, but not fibronectin, suggesting the direct interaction between miR-196a and collagen 3′UTR. Furthermore, in the presence of miR-196a protector, TGF-β1 could not increase the collagen expression, probably because the binding of miR-196a to collagen 3′UTR was already inhibited by the protector and TGF-β1–me-

inhibitor. (**C**) SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA or miR-196a mimic for 96 h. Cell lysates were subjected to immunoblotting, and the protein levels were quantitated, as described in (B). (**D**) *Left panel*, Normal human fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control inhibitor or miR-196a inhibitor for 96 h. Cell lysates were subjected to EIA experiment, as described in *Materials and Methods*. Type I collagen protein levels are shown relative to the level in cells transfected with control inhibitor (1.0). The data are expressed as mean ± SE of independent experiments using five NS fibroblasts. $*p < 0.05$ as compared with the value in cells with control inhibitor. *Right panel*, SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control mimic or miR-196a mimic for 96 h. Cell lysates were subjected to EIA experiment described in *Materials and Methods*. (**E**) Normal fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control or specific miScript protector for the miR-196a binding site on α1(I) and α2(I) collagen for 48 h. Then cells were incubated in the presence or absence of TGF-β1 (2 ng/ml) for additional 24 h. Cell lysates were subjected to immunoblotting, and the protein levels were quantitated, as described in (B). (**F**) Normal fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control or miR-196a inhibitor in the presence of miScript Target protector for the miR-196a binding site on α(I) and α2(I) collagen for 96 h. Cell lysates were subjected to immunoblotting, and the protein levels were quantitated, as described in (B).

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diated downregulation of miR-196a had no effect on the collagen expression. Consistent with this, cotransfection of the 196a protector blocked the miR-196a inhibitor-mediated upregulation of α1(I) and α2(I) collagen (Fig. 2F). Taken together, miR-196a may directly contribute to the constitutively upregulated type I collagen expression in SSc fibroblasts.

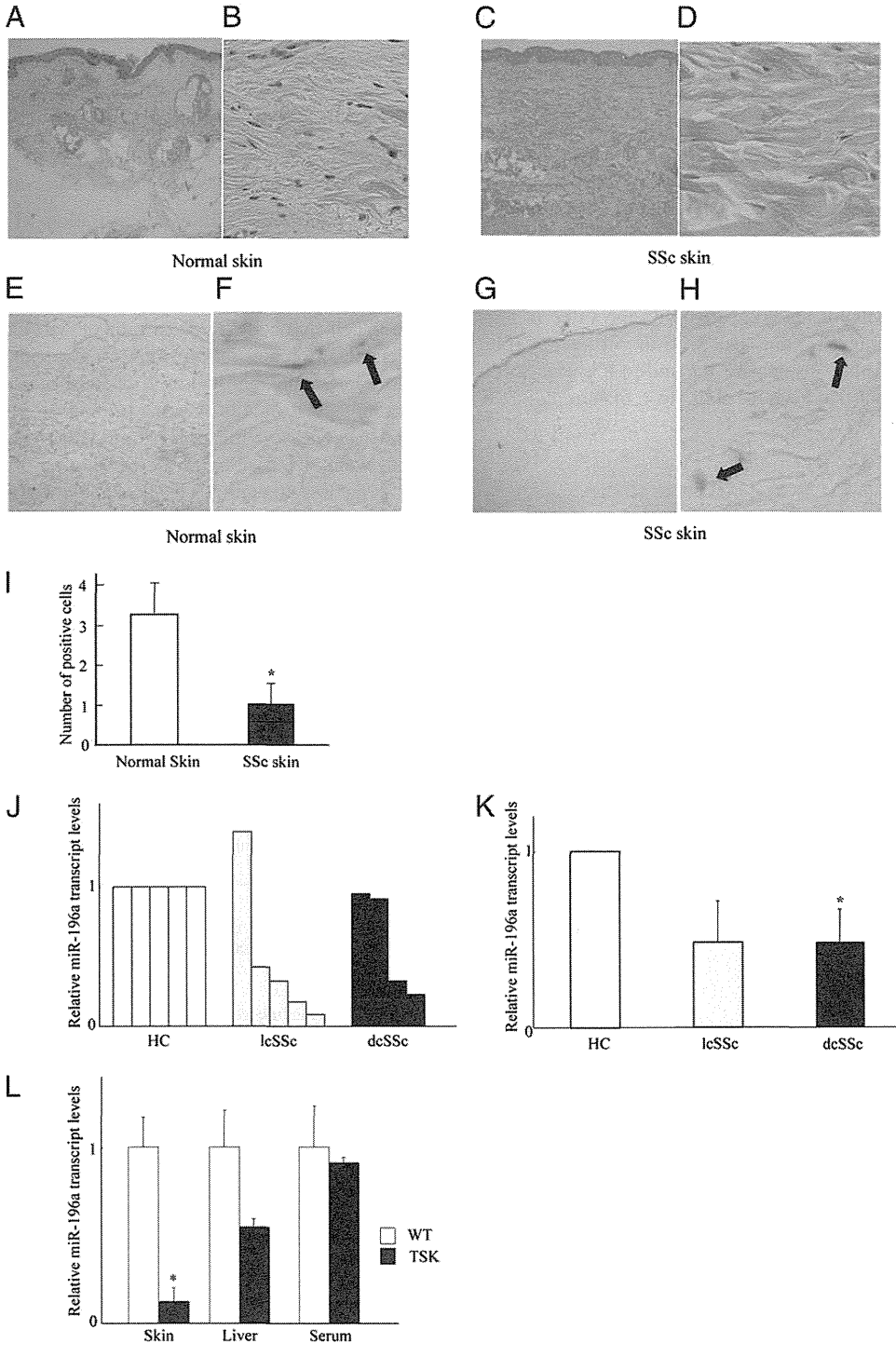

FIGURE 3. In vivo expression of miR-196a in SSc. (**A–D**) H&E staining of normal skin (A, B) and SSc skin (C, D). Original magnification ×40 (A, C), ×400 (B, D). (**E–H**) In situ detection of miR-196a in paraffin-embedded, formalin-fixed tissues of normal skin (E, F) and SSc skin (G, H). Nucleus was counterstained with nuclear fast red. The miR-196a stained blue. The dermal fibroblasts are indicated by arrows. Original magnification ×40 (E, G), ×800 (F, H). Representative result of three normal and three SSc skin is shown. (**I**) The number of miR-196a–positive nuclei by in situ hybridization was counted at five high-power fields (original magnification ×400) in the skin specimens from three normal and three SSc skin. $*p < 0.05$ as compared with the values in normal skin. (**J**) The levels of miR-196a expression in each tissue of five healthy control (HC, white bars), five lcSSc (gray bars), and five dcSSc (black bars) were determined by real-time PCR, as described in *Materials and Methods*. The transcript levels in samples from healthy controls were set at 1. (**K**) Mean relative transcript levels of miR-196a in tissues from five healthy control (HC, white bars), five lcSSc (gray bars), and five dcSSc (black bars). The data are expressed as mean ± SE. $*p < 0.05$ as compared with the value in samples from HC (1.0). (**L**) Total miRNA was extracted from the skin, liver, and serum of three TSK mice (TSK, black bars) and three wild-type mice (WT, white bars), and relative transcript levels of miR-196a (normalized to Snord68) were determined by real-time PCR, as described in *Materials and Methods*. $*p < 0.05$, as compared with the values in samples from WT mice (1.0).

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The association between dermal fibrosis and miR-196a expression in vivo

We then tried to determine the role of miR-196a in tissue fibrosis in vivo. Histopathologically, compared with normal tissue (Fig. 3A, 3B), SSc skin is characterized by dermal fibrosis due to thickened and increased collagen fibers (Fig. 3C, 3D). In situ hybridization showed that signal for miR-196a was evident in fibroblasts of normal skin (Fig. 3E, 3F), but hardly detected in SSc fibroblasts between the thickened collagen bundles (Fig. 3G, 3H). Actually, the number of miR-196a–positive nuclei by in situ hybridization was significantly decreased in SSc skin (Fig. 3I). In addition, we performed quantitative analysis of the miR-196a expression in normal and SSc skin. Compared with five normal skin, four of the five lcSSc skin and all five dcSSc skin showed decreased miR-196a expression (Fig. 3J), and the decrease of miR-196a in dcSSc skin was statistically significant compared with the value in normal skin (Fig. 3K), which is consistent with in vitro result (Fig. 1A, 1B). Also, to further investigate the miR-196a expression in vivo, miRNA was purified from the skin, liver, or serum of TSK mice, the animal fibrotic skin model: in the TSK mice skin, collagen expression is reported to be upregulated (38–40). Consistent with this, real-time PCR revealed that mean miR-196a levels in the skin of TSK mouse were significantly lower than those in wild-type mice ($p < 0.05$, Fig. 3L). In contrast, the miR-196a expression in the liver and serum was also slightly decreased in TSK mice compared with wild type, but not significant. Therefore, both in vitro and in vivo, the expression of miR-196a was likely to be decreased in fibrotic skin.

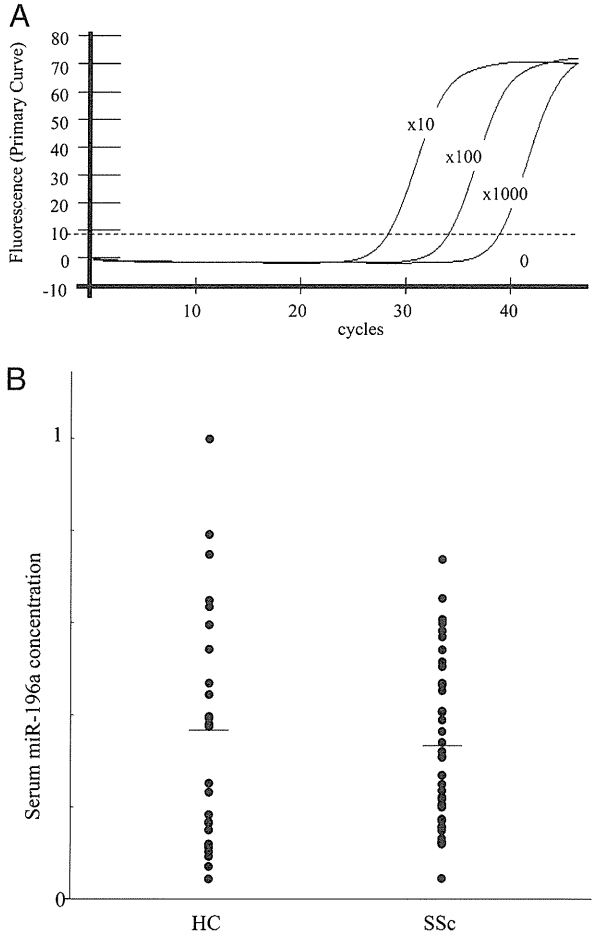

FIGURE 4. Serum miR-196a levels in patients with dcSSc or lcSSc and healthy control subjects (HC). (**A**) miR-196a is present in serum sample. Serial dilution of cDNA (10-fold dilution, 100-fold dilution, 1000-fold dilution, and water) synthesized from serum-derived miRNA was used as template for real-time PCR, as described in *Materials and Methods*. Amplification curves of gene-specific transcripts are shown to illustrate the process of exponential increase of fluorescence. Horizontal dotted line indicates the threshold. (**B**) Serum miR-196a levels (normalized to cel-miR-39) were measured by quantitative real-time PCR. miR-196a concentrations are shown on the ordinate. Bars show means. The maximum value in the healthy controls (HC) was set at 1.

Correlation of serum miR-196a levels with clinical manifestations and laboratory data in SSc patients

We also determined serum concentration of miR-196a in SSc patients and evaluated the possibility that serum miR-196a levels can be a disease marker.

There has been no report demonstrating the expression of miR-196a in cell-free body fluid. To validate that the miRNA is indeed detectable in human serum, miRNA was extracted from sera of healthy individual, and the level of miR-196a was determined by quantitative real-time PCR using primer set specific for miR-196a (Fig. 4A). The amplification of miR-196a was observed, and Ct values were increased by the serial dilution of the miRNA. Thus, miR-196a was thought to be detectable and quantitative in the serum using our method.

Serum samples were obtained from 40 patients with SSc (9 men and 31 women); 20 patients had dcSSc, and 20 patients had lcSSc. Twenty-five healthy subjects were also included in this study. There was no statistically significant difference between healthy control subjects and SSc patients ($p = 0.90$ by Mann–Whitney U test,

Table II. Correlation of serum miR-196a levels with clinical and serological features in SSc patients

Clinical and Serological Features	Patients with Normal miR-196a Levels ($n = 18$)	Patients with Lower miR-196a Levels ($n = 22$)
Age at onset (year)	63.4	56.5
Duration of disease (month)	48.5	64.8
Type (diffuse: limited)	4:14	16:6*
MRSS (point)	7.7	15.9*
Clinical features		
Pitting scars/ulcers	25.0	70.6*
Nailfold bleeding	60.0	33.3
Raynoud's phenomenon	100	84.2
Telangiectasia	40.0	35.3
Contracture of phalanges	100	88.9
Calcinosis	0	0
Diffuse pigmentation	33.3	57.1
Short SF	80.0	80.0
Sicca symptoms	50.0	70.0
Organ involvement		
Pulmonary fibrosis	37.5	40.0
Mean %VC	94.7	92.6
Mean %DLCO	78.6	75.9
Pulmonary hypertension	55.5	54.5
Esophagus	7.1	31.5
Heart	40.0	50.0
Kidney	6.7	0
Joint	27.7	13.6
ANA specificity		
Anti-topo I	33.3	27.2
Anti-centromere	66.6	40.0
Anti-U1 RNP	11.1	9.1

Unless indicated, values are percentages.

*$p < 0.05$ versus patients with normal serum miR-196a levels using Fisher's exact probability test or Mann–Whitney U test.

ANA, anti-nuclear Ab; anti-centromere, anti-centromere Ab; anti-topo I, anti-topoisomerase I Ab; DLCO, diffusion capacity for carbon monooxidase; RNP, ribonucleoprotein; SF, sublingual frenulum; VC, vital capacity.

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Fig. 4B). However, by the analysis of the association between miR-196a levels and the clinical or laboratory features, we found that patients with lower miR-196a levels had significantly higher ratio of dcSSc:lcSSc (16:6 versus 4:14, $p < 0.05$) and significantly higher modified Rodnan total skin thickness score (MRSS) (15.9 versus 7.7, $p < 0.05$) than those without (Table II). Also, the patients with lower miR-196a levels were accompanied with pitting scars at the significantly higher prevalence than those without (70.6% versus 25.0%, $p < 0.05$).

Discussion

This study demonstrated the role of miR-196a in type I collagen expression and its contribution to the pathogenesis of SSc by three major findings.

First, we identified several overexpressed or suppressed miRNAs specifically in SSc fibroblasts as well as TGF-β–stimulated normal fibroblasts, compared with untreated normal fibroblasts by miRNA PCR array consisting of 88 miRNAs involved in human cell differentiation and development. We focused on miR-196a as the regulator of both α1(I) and α2(I) collagen. Although the expression of miR-196a has been evaluated in glioblastoma and breast cancer (41, 42), to our knowledge our study is the first to demonstrate the expression of miR-196a in autoimmune diseases, including SSc.

Second, in this study, we also found the new miRNA–target interactions in dermal fibroblasts: downregulated miR-196a leads to the overexpression of type I collagen in normal fibroblasts, whereas overexpression of the miRNA resulted in the downregulation of type I collagen in SSc fibroblasts. Also, our results suggest exogenous TGF-β stimulation upregulated type I collagen expression via miR-196a downregulation in normal fibroblasts, at least partly. Although Smad proteins are known to be key intermediates in the TGF-β signaling process, recent reports have identified other pathways, including protein kinase C-δ, phosphatidylcholine-specific phospholipase C, geranylgeranyl transferase I, or p38 MAPK, as the participants in the regulation of various ECM expressions by TGF-β family (43, 44). miR-196a may also be one of such downstream targets to mediate the effect of TGF-β. The downregulation of miR-196a seen in SSc fibroblasts may result from activated endogenous TGF-β signaling, and may play a role in the constitutive upregulation of type I collagen in these cells.

Recently, Maurer et al. (45) have reported that downregulation of miR-29a contributes to the ECM overexpression in SSc fibroblasts. Our study supports the idea that miRNAs are involved in the pathogenesis of SSc. Furthermore, we investigated tissue and serum miRNA levels in SSc. To our knowledge, this is the first report showing that miR-196a is detectable and quantitative in the serum using our method. Our results indicate that SSc patients with lower serum miR-196a levels had significantly higher ratio of dcSSc:lcSSc, significantly higher MRSS, and significantly higher prevalence of pitting scar. Thus, serum miR-196a levels can be a disease marker, reflecting the activity of type I collagen production. However, we could not find statistically significant difference in serum miR-196a levels between SSc patients and healthy controls. This may be because of the small number of patients. Larger studies are needed in the future.

In conclusion, SSc may be the good model for tissue fibrosis. There are thought to be so many factors regulating the fibrotic process in SSc, and miR-196a may also play some roles in the pathogenesis of this disease. Investigation of the regulatory mechanisms of collagen expression by miRNAs may lead to new treatments using miRNA by the transfection into the fibrotic lesion.

Acknowledgments

We thank Junko Suzuki, Chiemi Shiotsu, Tomomi Etoh, and F.C. Muchemwa for valuable technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Korn, J. H. 1989. Immunologic aspects of scleroderma. *Curr. Opin. Rheumatol.* 1: 479–484.
2. Mauch, C., and T. Kreig. 1990. Fibroblast-matrix interactions and their role in the pathogenesis of fibrosis. *Rheum. Dis. Clin. North Am.* 16: 93–107.
3. Mauch, C., E. Kozlowska, B. Eckes, and T. Krieg. 1992. Altered regulation of collagen metabolism in scleroderma fibroblasts grown within three-dimensional collagen gels. *Exp. Dermatol.* 1: 185–190.
4. Jelaska, A., M. Arakawa, G. Broketa, and J. H. Korn. 1996. Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts: increased proportion of high collagen-producing cells in systemic sclerosis fibroblasts. *Arthritis Rheum.* 39: 1338–1346.
5. Massagué, J. 1990. The transforming growth factor-beta family. *Annu. Rev. Cell Biol.* 6: 597–641.
6. Leroy, E. C., E. A. Smith, M. B. Kahaleh, M. Trojanowska, and R. M. Silver. 1989. A strategy for determining the pathogenesis of systemic sclerosis: is transforming growth factor beta the answer? *Arthritis Rheum.* 32: 817–825.
7. LeRoy, E. C. 1974. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. *J. Clin. Invest.* 54: 880–889.
8. Jimenez, S. A., G. Feldman, R. I. Bashey, R. Bienkowski, and J. Rosenbloom. 1986. Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. *Biochem. J.* 237: 837–843.
9. Kikuchi, K., C. W. Hartl, E. A. Smith, E. C. LeRoy, and M. Trojanowska. 1992. Direct demonstration of transcriptional activation of collagen gene expression in systemic sclerosis fibroblasts: insensitivity to TGF beta 1 stimulation. *Biochem. Biophys. Res. Commun.* 187: 45–50.
10. Asano, Y., H. Ihn, K. Yamane, M. Kubo, and K. Tamaki. 2004. Impaired Smad7-Smurf-mediated negative regulation of TGF-beta signaling in scleroderma fibroblasts. *J. Clin. Invest.* 113: 253–264.
11. Ihn, H., K. Yamane, M. Kubo, and K. Tamaki. 2001. Blockade of endogenous transforming growth factor beta signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor beta receptors. *Arthritis Rheum.* 44: 474–480.
12. Bartel, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281–297.
13. Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting, and G. J. Hannon. 2004. Processing of primary microRNAs by the microprocessor complex. *Nature* 432: 231–235.
14. Farh, K. K., A. Grimson, C. Jan, B. P. Lewis, W. K. Johnston, L. P. Lim, C. B. Burge, and D. P. Bartel. 2005. The widespread impact of mammalian microRNAs on mRNA repression and evolution. *Science* 310: 1817–1821.
15. Friedman, R. C., K. K. Farh, C. B. Burge, and D. P. Bartel. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 19: 92–105.
16. Bostjancic, E., and D. Glavac. 2008. Importance of microRNAs in skin morphogenesis and diseases. *Acta Dermatovenerol. Alp. Panonica Adriat.* 17: 95–102.
17. Herrera, B. M., H. E. Lockstone, J. M. Taylor, M. Ria, A. Barrett, S. Collins, P. Kaisaki, K. Argoud, C. Fernandez, M. E. Travers, et al. 2010. Global microRNA expression profiles in insulin target tissues in a spontaneous rat model of type 2 diabetes. *Diabetologia* 53: 1099–1109.
18. Kuehbacher, A., C. Urbich, and S. Dimmeler. 2008. Targeting microRNA expression to regulate angiogenesis. *Trends Pharmacol. Sci.* 29: 12–15.
19. Chen, Y., and D. H. Gorski. 2008. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood* 111: 1217–1226.
20. Furer, V., J. D. Greenberg, M. Attur, S. B. Abramson, and M. H. Pillinger. 2010. The role of microRNA in rheumatoid arthritis and other autoimmune diseases. *Clin. Immunol.* 136: 1–15.
21. Davidson-Moncada, J., F. N. Papavasiliou, and W. Tam. 2010. MicroRNAs of the immune system: roles in inflammation and cancer. *Ann. N. Y. Acad. Sci.* 1183: 183–194.
22. Ihn, H., E. C. LeRoy, and M. Trojanowska. 1997. Oncostatin M stimulates transcription of the human alpha2(I) collagen gene via the Sp1/Sp3-binding site. *J. Biol. Chem.* 272: 24666–24672.
23. LeRoy, E. C., C. Black, R. Fleischmajer, S. Jablonska, T. Krieg, T. A. Medsger, Jr., N. Rowell, and F. Wollheim. 1988. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J. Rheumatol.* 15: 202–205.
24. A. T. Masi; Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. 1980. Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum.* 23: 581–590.
25. Hochberg, M. C. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 40: 1725.

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26. Ihn, H., S. Sato, M. Fujimoto, K. Kikuchi, A. Igarashi, Y. Soma, K. Tamaki, and K. Takehara. 1996. Measurement of anticardiolipin antibodies by ELISA using beta 2-glycoprotein I (beta 2-GPI) in systemic sclerosis. *Clin. Exp. Immunol.* 105: 475–479.
27. Matsushita, T., M. Fujimoto, M. Hasegawa, Y. Matsushita, K. Komura, F. Ogawa, R. Watanabe, K. Takehara, and S. Sato. 2007. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. *J. Invest. Dermatol.* 127: 2772–2780.
28. Martin, M. M., J. A. Buckenberger, J. Jiang, G. E. Malana, G. J. Nuovo, M. Chotani, D. S. Feldman, T. D. Schmittgen, and T. S. Elton. 2007. The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microrna-155 binding. *J. Biol. Chem.* 282: 24262–24269.
29. Nuovo, G. J. 2008. In situ detection of precursor and mature microRNAs in paraffin embedded, formalin fixed tissues and cell preparations. *Methods* 44: 39–46.
30. Kroh, E. M., R. K. Parkin, P. S. Mitchell, and M. Tewari. 2010. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods* 50: 298–301.
31. Lewis, B. P., C. B. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120: 15–20.
32. John, B., A. J. Enright, A. Aravin, T. Tuschl, C. Sander, and D. S. Marks. 2004. Human microRNA targets. *PLoS Biol.* 2: e363.
33. Kiriakidou, M., P. T. Nelson, A. Kouranov, P. Fitziev, C. Bouyioukos, Z. Mourelatos, and A. Hatzigeorgiou. 2004. A combined computational-experimental approach predicts human microRNA targets. *Genes Dev.* 18: 1165–1178.
34. Krek, A., D. Grün, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel, and N. Rajewsky. 2005. Combinatorial microRNA target predictions. *Nat. Genet.* 37: 495–500.
35. Rudnicka, L., J. Varga, A. M. Christiano, R. V. Iozzo, S. A. Jimenez, and J. Uitto. 1994. Elevated expression of type VII collagen in the skin of patients with systemic sclerosis: regulation by transforming growth factor-beta. *J. Clin. Invest.* 93: 1709–1715.
36. Hocevar, B. A., T. L. Brown, and P. H. Howe. 1999. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J.* 18: 1345–1356.
37. Long, J. M., and D. K. Lahiri. 2011. MicroRNA-101 downregulates Alzheimer's amyloid-β precursor protein levels in human cell cultures and is differentially expressed. *Biochem. Biophys. Res. Commun.* 404: 889–895.
38. Jimenez, S. A., A. Millan, and R. I. Bashey. 1984. Scleroderma-like alterations in collagen metabolism occurring in the TSK (tight skin) mouse. *Arthritis Rheum.* 27: 180–185.
39. Jimenez, S. A., C. J. Williams, J. C. Myers, and R. I. Bashey. 1986. Increased collagen biosynthesis and increased expression of type I and type III procollagen genes in tight skin (TSK) mouse fibroblasts. *J. Biol. Chem.* 261: 657–662.
40. Hasegawa, M., Y. Matsushita, M. Horikawa, K. Higashi, Y. Tomigahara, H. Kaneko, F. Shirasaki, M. Fujimoto, K. Takehara, and S. Sato. 2009. A novel inhibitor of Smad-dependent transcriptional activation suppresses tissue fibrosis in mouse models of systemic sclerosis. *Arthritis Rheum.* 60: 3465–3475.
41. Guan, Y., M. Mizoguchi, K. Yoshimoto, N. Hata, T. Shono, S. Suzuki, Y. Araki, D. Kuga, A. Nakamizo, T. Amano, et al. 2010. MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance. *Clin. Cancer Res.* 16: 4289–4297.
42. Li, Y., M. Zhang, H. Chen, Z. Dong, V. Ganapathy, M. Thangaraju, and S. Huang. 2010. Ratio of miR-196s to HOXC8 messenger RNA correlates with breast cancer cell migration and metastasis. *Cancer Res.* 70: 7894–7904.
43. Kucich, U., J. C. Rosenbloom, G. Shen, W. R. Abrams, A. D. Hamilton, S. M. Sebti, and J. Rosenbloom. 2000. TGF-beta1 stimulation of fibronectin transcription in cultured human lung fibroblasts requires active geranylgeranyl transferase I, phosphatidylcholine-specific phospholipase C, protein kinase C-delta, and p38, but not erk1/erk2. *Arch. Biochem. Biophys.* 374: 313–324.
44. Kucich, U., J. C. Rosenbloom, G. Shen, W. R. Abrams, M. A. Blaskovich, A. D. Hamilton, J. Ohkanda, S. M. Sebti, and J. Rosenbloom. 1998. Requirement for geranylgeranyl transferase I and acyl transferase in the TGF-beta-stimulated pathway leading to elastin mRNA stabilization. *Biochem. Biophys. Res. Commun.* 252: 111–116.
45. Maurer, B., J. Stanczyk, A. Jüngel, A. Akhmetshina, M. Trenkmann, M. Brock, O. Kowal-Bielecka, R. E. Gay, B. A. Michel, J. H. Distler, et al. 2010. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. *Arthritis Rheum.* 62: 1733–1743.

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