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miR-150 Down-Regulation Contributes to the Constitutive Type I Collagen Overexpression in Scleroderma Dermal Fibroblasts via the Induction of Integrin $\beta 3$

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Overexpression of integrins in dermal fibroblasts is thought to play a key role in the pathogenesis of systemic sclerosis (SSc), but the mechanism is unknown. We evaluated the possibility that microRNAs (miRNAs) are involved in the regulation of integrin $\beta 3$ in these cells. The miRNA expression profile was determined by miRNA PCR array and real-time PCR. Protein expression of integrin $\beta 3$ was determined by immunoblotting. *In vivo* detection of miRNA in paraffin section was performed by *in situ* hybridization. miR-150 expression was decreased in SSc fibroblasts both *in vivo* and *in vitro*. The transfection of miR-150 inhibitor into normal fibroblasts induced expression of integrin $\beta 3$, phosphorylated Smad3, and type I collagen, whereas forced overexpression of the miRNA resulted in their down-regulation in SSc fibroblasts. Treatment of SSc fibroblasts with 5-AdC revealed that miR-150 down-regulation in these cells is caused by DNA methylation. In addition, we found that miR-150 is detectable and quantitative in serum. Serum miR-150 levels were decreased in SSc patients, and the SSc patients with lower serum miR-150 levels tended to have more severe clinical manifestations. miR-150 may play an important role in the pathogenesis of SSc via overexpression of integrin $\beta 3$. Investigation of the regulatory mechanisms of tissue fibrosis by miR-150 could lead to development of new diagnostic tools and new treatments using miRNA. (*Am J Pathol* 2013, 182: 206–216; <http://dx.doi.org/10.1016/j.ajpath.2012.09.023>)

Systemic sclerosis (SSc), or scleroderma, is characterized by tissue fibrosis of the skin and internal organs. Inflammation, autoimmune attack, and vascular damage are thought to trigger the activation of fibroblasts and excess production of extracellular matrix.^{1,2} Thus, abnormal SSc fibroblasts 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 skin is at present unknown, many of the characteristics of SSc fibroblasts resemble those of normal fibroblasts stimulated by transforming growth factor β -1 (TGF- β 1).^{5,6} One of the diverse effects of TGF- β 1 on mesenchymal cells is stimulation of extracellular matrix deposition. Cultured dermal fibroblasts from affected SSc skin *in vitro* produce excessive amounts of various collagens, mainly type I collagen, which consists of α 1(I) and α 2(I) collagen,^{7,8} suggesting that the activation of SSc fibroblasts is a result

of stimulation by TGF- β 1 signaling. This notion is supported by our previous findings, that i) the transcriptional activity of the α 2(I) collagen gene in SSc fibroblasts is constitutively up-regulated compared with that in normal fibroblasts but the responsiveness to ectopic TGF- β 1 is decreased in SSc cells,⁹ ii) Smad3, a downstream mediator of TGF- β , is constitutively phosphorylated and translocated into the nucleus in SSc fibroblasts,¹⁰ and iii) the blockade of TGF- β 1 signal using neutralizing antibody abolishes the increased expression of human α 2(I) collagen mRNA in SSc fibroblasts.¹¹ On the other hand, we have previously reported that TGF- β 1 levels in the culture medium of SSc fibroblasts

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are not increased.¹¹ Thus, the activation of dermal fibroblasts in SSc may result from self-activation of TGF- β 1 signaling, without any increase in TGF- β 1 concentration.

Bioactive peptide of TGF- β 1 is usually captured by latency-associated peptide- β 1 (LAP- β 1) noncovalently, forming the small latent complex (SLC). In this configuration, TGF- β 1 is inactive and cannot bind to its receptors. TGF- β 1 activation is a complex process involving conformational changes induced by cleavage of LAP- β 1 either by various proteases (eg, plasmin) or by physical interactions of LAP- β 1 with other proteins (eg, integrins and thrombospondin-1), leading to the release of bioactive TGF- β 1.^{12–15}

In explanation of how TGF- β 1 is activated in SSc fibroblasts without any quantitative increase, we recently reported constitutive overexpression of integrin β 3 in these cells. The forced overexpression of integrins, including the β 3 subunit, in dermal fibroblasts results in the up-regulation of collagen expression.^{14,16–18} In SSc fibroblasts, the integrins may recruit and activate the SLC on the cell surface, resulting in the release of active TGF- β 1 in the pericellular region. This may enhance the incidence of interaction between active TGF- β 1 and its receptors, leading to the self-activation of SSc fibroblasts without increasing the amount of TGF- β 1.^{14,16–18} Integrin overexpression is therefore thought to be the most upstream event of TGF- β 1 activation and collagen up-regulation in SSc fibroblasts. Nonetheless, the mechanism of integrin overexpression in SSc is still unknown.

Recently, epigenetics has attracted attention for involvement in various cellular behaviors, including cell differentiation, immune response, and organogenesis. microRNAs (miRNAs), short ribonucleic acid molecules on average only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the 3' untranslated regions (3' UTRs) of mRNAs, leading to gene silencing.^{19,20} miRNAs have been implicated in the pathogenesis of various human diseases, including immunological disorders, cancers, and metabolic disorders.^{21–24}

In the present study, our objective was to evaluate the possibility that miRNAs may play some roles in the constitutive up-regulation of integrin β 3 in SSc.

Materials and Methods

Cell Culture

Human dermal fibroblasts were obtained by skin biopsy from the affected area (dorsal forearm) of five patients with diffuse cutaneous SSc and <2 years of skin thickening, as described previously.²⁵ Control fibroblasts were obtained by skin biopsies from five healthy donors. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki.

Primary explant cultures were established in 25-cm² culture flasks in modified Eagle's medium supplemented with 10% fetal calf serum and antibiotic-antimycotic (Invitrogen; Life Technologies, Carlsbad, CA). Monolayer

cultures independently isolated from different individuals were maintained at 37°C in 5% CO₂-enriched air. Fibroblasts between the third and sixth subpassages were used for experiments. Cells were serum-starved for 12 to 24 hours before experiments.

Patient Material

Skin samples were obtained from affected skin of five SSc patients. Control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin graft.²⁶

Serum samples were obtained from 40 patients with SSc; 20 cases were classified as diffuse cutaneous SSc (dcSSc) and 20 as limited cutaneous SSc (lcSSc) according to the classification system proposed by LeRoy et al.²⁷ Control samples were collected from 20 healthy age- and sex-matched volunteers. Five patients with dermatomyositis or systemic lupus erythematosus were also included in the study as disease controls. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki before patients and healthy volunteers were entered into the study.

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. The Committee on Animal Experimentation of Kanazawa University Graduate School of Medical Science approved all studies and procedures.²⁸

In Situ Hybridization

In situ hybridization was performed with 5'-locked digoxigenin-labeled nucleic acid probes complementary to human mature miR-150 and scrambled negative control (Exiqon, Vedbaek, Denmark).^{29–31}

Cell Lysis and Immunoblotting

Fibroblasts were washed with cold PBS twice and lysed in denaturing cell extraction buffer (Biosource International, Camarillo, CA). Aliquots of cell lysates, normalized for protein concentrations, were subjected to electrophoresis as described previously.³²

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from cultured cells using ISOGEN reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized from total RNA with a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan). The primer set for GAPDH was purchased from SA Biosciences (Frederick, MD), and the primers for TGF- β 1 were from Takara Bio. DNA was

amplified for 40 cycles of denaturation for 15 seconds at 95°C and annealing for 30 seconds at 65°C.

miRNA Extraction and PCR Array Analysis

miRNA isolation from cultured cells or skin tissue was performed using an RT² qPCR-grade miRNA isolation kit (SABiosciences, Frederick, MD) or a miRNeasy FFPE kit (Qiagen, Valencia, CA), respectively. miRNAs were reverse-transcribed into first-strand cDNA using an RT² miRNA first-strand kit (SABiosciences). For the RT² Profiler PCR array (SABiosciences), the cDNA was mixed with RT² SYBR Green/ROX qPCR master mix, and the mixture was added into a 96-well RT² miRNA PCR array (SABiosciences), which included primer pairs for 88 human miRNAs. PCR was performed on a Takara thermal cycler Dice real-time system (model TP800) according to the manufacturer's protocol, and C_T values for each miRNA were extracted using thermal cycler Dice real-time system software version 2.10B (Takara Bio).

For quantitative real-time (q)PCR, primers for miR-150 or U6 (SABiosciences) and templates were mixed with SYBR Premix Ex Taq II (Takara Bio). DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C. Data generated from each PCR reaction were analyzed using thermal cycler Dice real-time system software version 2.10B (Takara Bio). Transcript levels of miR-150 were normalized to U6.

miRNA Extraction from Serum and PCR Analysis of miRNA Expression

miRNA isolation from serum samples was performed with a miRNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions, with minor modification.³³ Briefly, 100 µL of serum was supplemented with 5 µL of 5 fmol/µL synthetic nonhuman miRNA (*Caenorhabditis elegans* miR-39; Takara) as control, providing an internal reference for normalization of technical variation between samples. QIAzol lysis reagent (1 mL) was added and mixed well by vortexing, and then samples were incubated at room temperature for 5 minutes. Aqueous and organic phase separation was achieved by the addition of chloroform. The aqueous phase was applied to an RNeasy spin column and an RNeasy MinElute spin column (Qiagen). miRNA was eluted from the column with nuclease-free water.

cDNA was synthesized from miRNA with a Mir-X miRNA first-strand synthesis and SYBR qRT-PCR kit (Takara Bio). qPCR with a Takara thermal cycler Dice system (model TP800) used primers and templates mixed with the SYBR Premix. The sequence of the hsa-miR-150 primer (5'-TC-TCCCAACCCCTTGTAACAGTG-3') was designed based on the miRBase archive (<http://www.mirbase.org>, last accessed November 2011).^{34–37} DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 20 seconds at 60°C. The relative gene expression of hsa-miR-150

and of cel-miR-39 was calculated by the standard curve method. The transcript level of miR-150 was normalized to that of cel-miR-39.

Transient Transfection

miRNA inhibitors, mimics, and miScript Target protectors were purchased from Qiagen. Lipofectamine RNAiMAX (Invitrogen; Life Technologies) was used as transfection reagent. For reverse transfection, miRNA inhibitors (50 nmol/L), mimics (5 nmol/L), and protectors (500 nmol/L) mixed with transfection reagent were added when cells were plated, followed by incubation for 96 hours at 37°C in 5% CO₂-enriched air. Control experiments showed transcript levels for target of miRNA inhibitors to be reduced by >80%, and expression of miRNAs was increased at least fivefold by the transfection of mimics (data not shown).

Luciferase Reporter Assay

A luciferase reporter plasmid containing the integrin β3 3'UTR was purchased from GeneCopoeia (HmiT009818-MT01; Rockville, MD). Lipofectamine 2000 (Invitrogen; Life Technologies) was used as transfection reagent. miRNA inhibitors (50 nmol/L), mimics (5 nmol/L), and reporter plasmid mixed with transfection reagent were added when cells were plated, followed by incubation for 48 hours at 37°C in 5% CO₂-enriched air. A Luc-Pair miR luciferase assay (GeneCopoeia) and a FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA) were used to analyze luciferase expression according to the manufacturer protocols.

Demethylation of DNA by 5-AdC

SSc fibroblasts were treated with or without 5 µmol/L 5-azadeoxycytidine (5-AdC; Wako Pure Chemical Industries, Osaka, Japan) for 4 days.³⁸ Cells were subjected to miRNA expression analysis.

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test, *U*-test, or Fisher's exact probability test. *P* values of <0.05 were considered significant.

Results

Several miRNAs Are Down-Regulated in SSc Fibroblasts

As an initial experiment, we performed miRNA PCR array analysis with 88 miRNAs involved in human cell differentiation and development. A mixture of equal amounts of miRNAs from five normal fibroblasts and five SSc fibroblasts was prepared, and miRNA expression profile in each cell group was evaluated using PCR array. Several miRNAs

were up-regulated or down-regulated in SSc fibroblasts, compared with normal fibroblasts (Table 1). From among these, we focused on miR-150 as the regulator of integrin $\beta 3$, according to miRNA target gene prediction. Several leading

online software tools identified integrin $\beta 3$ as a putative target of miR-150, including TargetScan version 6.2 (<http://www.targetscan.org>, last accessed June 1, 2012),^{39–42} MiRanda (<http://www.microrna.org>, last accessed August 1,

Table 1 miRNA Expression in SSc Fibroblasts Relative to Normal Fibroblasts, by PCR Analysis

miRNA name	Mean fold-change*	P value [†]	miRNA name	Mean fold-change*	P value [†]
Down-regulated miRNAs in SSc fibroblasts					
let-7a	0.34 ± 0.12	0.037	miR-133b	0.33 ± 0.28	0.037
let-7b	0.25 ± 0.23	0.037	miR-134	0.13 ± 0.05	0.037
let-7c	0.65 ± 0.44	0.487	miR-137	0.23 ± 0.04	0.037
let-7d	0.21 ± 0.10	0.037	miR-141	0.09 ± 0.03	0.037
let-7e	0.88 ± 0.45	0.487	miR-142-3p	0.09 ± 0.08	0.037
let-7f	0.20 ± 0.03	0.037	miR-142-5p	0.11 ± 0.05	0.037
let-7g	0.31 ± 0.05	0.037	miR-146a	0.06 ± 0.02	0.037
let-7i	0.75 ± 0.19	0.487	miR-150	0.18 ± 0.06	0.037
miR-10a	0.19 ± 0.09	0.037	miR-155	0.23 ± 0.13	0.037
miR-15a	0.32 ± 0.10	0.037	miR-182	0.05 ± 0.01	0.037
miR-15b	0.31 ± 0.14	0.037	miR-183	0.03 ± 0.01	0.037
miR-17	0.15 ± 0.07	0.037	miR-185	0.04 ± 0.02	0.037
miR-18a	0.37 ± 0.27	0.037	miR-192	0.16 ± 0.06	0.037
miR-18b	0.34 ± 0.23	0.037	miR-196a	0.02 ± 0.01	0.037
miR-20a	0.20 ± 0.13	0.037	miR-205	0.05 ± 0.06	0.037
miR-20b	0.14 ± 0.11	0.037	miR-206	0.05 ± 0.01	0.037
miR-21	0.36 ± 0.09	0.037	miR-208a	0.07 ± 0.03	0.037
miR-22	0.61 ± 0.29	0.487	miR-210	0.08 ± 0.05	0.037
miR-23b	0.95 ± 0.38	0.487	miR-214	0.62 ± 0.28	0.487
miR-24	0.89 ± 0.33	0.487	miR-215	0.34 ± 0.24	0.037
miR-26a	0.55 ± 0.40	0.487	miR-222	0.40 ± 0.17	0.037
miR-33a	0.58 ± 0.48	0.487	miR-301a	0.36 ± 0.19	0.037
miR-93	0.09 ± 0.06	0.037	miR-302a	0.20 ± 0.10	0.037
miR-96	0.09 ± 0.07	0.037	miR-302c	0.52 ± 0.47	0.487
miR-100	0.93 ± 0.46	0.487	miR-345	0.58 ± 0.55	0.487
miR-101	0.25 ± 0.17	0.037	miR-370	0.11 ± 0.03	0.037
miR-103	0.57 ± 0.09	0.037	miR-371-3p	0.10 ± 0.07	0.037
miR-106b	0.13 ± 0.05	0.037	miR-375	0.08 ± 0.01	0.037
miR-122	0.13 ± 0.11	0.037	miR-424	0.77 ± 0.21	0.037
miR-124	0.10 ± 0.07	0.037	miR-452	0.17 ± 0.15	0.037
miR-125a-5p	0.76 ± 0.35	0.487	miR-488	0.11 ± 0.06	0.037
miR-129-5p	0.07 ± 0.05	0.037	miR-498	0.42 ± 0.40	0.487
miR-130a	0.02 ± 0.16	0.037	miR-503	0.10 ± 0.04	0.037
miR-132	0.37 ± 0.07	0.037	miR-520g	0.10 ± 0.09	0.037
Up-regulated miRNAs in SSc fibroblasts					
miR-1	8.13 ± 7.73	0.487	miR-128	1.08 ± 0.96	0.487
miR-7	2.75 ± 2.71	0.487	miR-146b-5p	1.15 ± 1.09	0.487
miR-9	1.56 ± 1.42	0.487	miR-181a	1.00 ± 0.97	0.487
miR-10b	1.42 ± 0.77	0.487	miR-194	6.73 ± 6.34	0.487
miR-16	1.72 ± 1.05	0.487	miR-195	1.10 ± 0.53	0.487
miR-92a	5.69 ± 2.74	0.487	miR-218	2.80 ± 1.83	0.487
miR-99a	3.21 ± 2.22	0.487	miR-219-5p	9.97 ± 9.83	0.487
miR-125b	1.66 ± 0.87	0.487	miR-223	2.02 ± 1.96	0.487
miR-126	1.08 ± 0.56	0.487	miR-378	51.1 ± 51.0	0.487
miR-127-5p	1.39 ± 1.25	0.037	miR-518b	6.49 ± 6.47	0.487

A mixture of equal amounts of miRNAs from normal ($n = 5$) or SSc ($n = 5$) fibroblasts was prepared, and the miRNA expression profile in each cell group was evaluated using PCR array. The miRNA of interest for this study (miR-150, the regulator of integrin $\beta 3$) is highlighted in bold.

*Fold change was calculated as one half of the difference between the raw C_T value of each miRNA and the C_T value of the small RNA housekeeping gene in SSc fibroblasts, divided by one half of the difference between the raw C_T value of each miRNA and the C_T value of the small RNA housekeeping gene in normal fibroblasts. Data are expressed as mean fold change ± SE of three independent experiments.

[†]P values were calculated using the *U*-test.

2010),^{43–46} DIANA microT version 3.0 (<http://diana.cslab.ece.ntua.gr>, last accessed February 1, 2009),^{47,48} and miRDB (<http://mirdb.org/miRDB>, last accessed January 1, 2012).^{49,50}

The expression of miR-150 was down-regulated in SSc fibroblasts (0.186-fold change according to the $\Delta\Delta C_T$

method) in the array. To confirm the results obtained by miRNA PCR array, we performed qPCR analysis using a specific primer for miR-150. As expected, miR-150 was decreased in all five SSc fibroblast samples (Figure 1A), and the decrease of miR-150 in these cells was statistically significant, compared with normal cells (Figure 1B).

Figure 1 The effects of miR-150 inhibitor on the expression of integrin $\beta 3$ and type I collagen in normal dermal fibroblasts. **A:** Normal ($n = 5$) and SSc ($n = 5$) fibroblasts were serum starved for 24 hours. Total miRNA was extracted, and the relative level of miR-150 (normalized to U6) in each of the NS and SSc fibroblast samples was determined by qPCR. The values in NS fibroblasts were set at 1. **B:** The mean relative transcript level of miR-150 was significantly lower in SSc fibroblasts. **C:** NS and SSc fibroblasts were cultured independently under the same conditions until they were confluent, and then were serum starved for 24 hours. Cell lysates were subjected to immunoblotting with antibody for integrin $\beta 3$ or type I collagen. The same membrane was re probed with anti- β -actin antibody as a loading control. Both collagen and integrin expression was increased in SSc fibroblasts. Representative results are shown. **D:** Normal fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA inhibitor or miR-150 inhibitor in the presence of control or integrin $\beta 3$ siRNA for 96 hours. Cell lysates were subjected to immunoblotting as described for panel C. Protein levels of integrin $\beta 3$ and type I collagen quantitated by scanning densitometry and corrected for β -actin levels in the same samples are shown relative to the level in cells transfected with control inhibitor (set at 1.0). Silencing increased integrin $\beta 3$ expression and induced collagen expression. **E:** Normal human fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA inhibitor or miR-150 inhibitor for 96 hours. Total mRNA was extracted, and the relative level of TGF- $\beta 1$ (normalized to GAPDH) was determined by qPCR as described under *Materials and Methods*. The values in cells with control miRNA inhibitor were set at 1. Silencing did not affect TGF- $\beta 1$ transcript levels. **F:** Normal human fibroblasts at a density of 1×10^4 cells/well in 96-well culture plates were transfected with luciferase reporter containing the 3'UTR segment of integrin $\beta 3$ and miR-150 inhibitor for 24 hours. Control miRNA inhibitor and control RNA oligo to an unrelated region of the integrin 3'UTR were also included. The miR-150 inhibitor induced luciferase activity. **G:** Normal fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control or miR-150 inhibitor in the presence of miScript Target protector specific for the miR-150 binding site on the integrin $\beta 3$ 3'UTR for 96 hours. Cell lysates were subjected to immunoblotting as described for panel C and the signals were quantitated as described for panel D. Cotransfection of the protector blocked the miR-150 inhibitor-induced up-regulation of integrin $\beta 3$. Data are expressed as means $\pm$ SE of three independent experiments. $n = 5$ per group. * $P < 0.05$ versus NS or control.

miR-150 Regulates Type I Collagen Expression via the Direct Interaction with Integrin $\beta 3$ in Normal Fibroblasts

The expression of integrin $\beta 3$ and of both $\alpha 1(I)$ and $\alpha 2(I)$ collagen protein was increased in SSc fibroblasts (Figure 1C). Next, we determined the contribution of miR-150 to the expression of integrin $\beta 3$ and type I collagen. Silencing miR-150 by the transfection of specific inhibitor for miR-150 into normal fibroblasts resulted in the increase of integrin $\beta 3$

expression (Figure 1D). Type I collagen expression was also induced by miR-150 inhibitor (Figure 1D), probably subsequent to the overexpression of integrin $\beta 3$ and phosphorylated levels of Smad3. Consistent with the hypothesis, in the presence of integrin $\beta 3$ siRNA, miR-150 inhibitor could not increase expression of phosphorylated Smad3 and type I collagen. Additionally, transfection of miR-150 inhibitor had no effect on TGF- β transcript levels (Figure 1E). Thus, miR-150 regulates TGF- β activation via the integrin without changing TGF- β levels directly. These results indicate that

Figure 2 The effect of miR-150 mimic on the expression of integrin $\beta 3$ and type I collagen in SSc fibroblasts. **A:** SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA mimic or miR-150 mimic for 96 hours, which resulted in a decrease of up-regulation of both integrin $\beta 3$ and type I collagen. Cell lysates were subjected to immunoblotting and the signals were quantitated as described for Figure 1D. **B:** SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA mimic or miR-150 mimic for 96 hours. Total mRNA was extracted, and the relative level of TGF- β (normalized to GAPDH) was determined by qPCR. No significant differences were revealed. The values in cells with control miRNA mimic were set at 1. **C:** SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control siRNA or integrin $\beta 3$ siRNA for 96 hours, which resulted in a decrease in up-regulation of phosphorylated Smad3 and type I collagen. Cell lysates were subjected to immunoblotting as described for Figure 1D. **D:** SSc fibroblasts at a density of 1×10^4 cells/well in 96-well culture plates were transfected with luciferase reporter containing the 3'UTR segment of integrin $\beta 3$ and miR-150 mimic for 24 hours, which resulted in a decrease of luciferase activity. Control miRNA mimic and control RNA oligo to an unrelated region of the integrin 3'UTR were included. **E:** SSc dermal fibroblasts were cultured in six-well plates and treated with 5 μ mol/L 5-AdC, which increased expression of miR-150 (normalized to U6) more than 10-fold, as determined by real-time PCR. Data are expressed as means $\pm$ SE of three independent experiments. * $P < 0.05$ versus control.

normal fibroblasts transfected with miR-150 inhibitor resemble SSc fibroblasts.

To determine whether miR-150 directly regulates the expression of integrin $\beta 3$, we performed reporter assay using luciferase construct containing the integrin $\beta 3$ 3'UTR. miR-150 inhibitor induced the luciferase activity, in contrast to control inhibitor and control RNA oligo to an unrelated region of the integrin $\beta 3$ 3'UTR (Figure 1F). In addition, we used miRNA inhibitor and miScript Target protector for the miR-150 binding site on the integrin $\beta 3$ 3'UTR (Qiagen), single-stranded modified RNAs designed to specifically interfere with their interaction.⁵¹ Cotransfection of the protector (Figure 1G) blocked the miR-150 inhibitor-induced up-regulation of integrin $\beta 3$ (Figure 1D), suggesting direct interaction between them.

Low miR-150 Expression Leads to Up-Regulation of $\beta 3$ Integrin in SSc Fibroblasts

We then determined whether supplementation with miR-150 can normalize the SSc phenotype in SSc fibroblasts. Forced overexpression of miR-150 using miR-150 mimic decreased the up-regulated expression of integrin $\beta 3$, phosphorylated Smad3, and type I collagen in SSc fibroblasts (Figure 2A). Consistent with the data presented in Figure 1E, the transfection of miR-150 mimic into SSc fibroblasts had no effect on TGF- β levels (Figure 2B). Transfection of integrin $\beta 3$

siRNA into SSc fibroblasts (Figure 2C) showed effects on the expression of phosphorylated Smad3 and type I collagen similar to those of miR-150 mimic (Figure 2A). These results also suggest that miR-150 plays some roles in the constitutively up-regulated collagen expression in SSc fibroblasts via the induction of integrin $\beta 3$ and the activation of TGF- β signaling. miR-150 mimic significantly decreased the luciferase activity of integrin in SSc fibroblasts, indicating that down-regulated miR-150 in SSc fibroblasts directly up-regulates integrin $\beta 3$ (Figure 2D).

Several reports indicate that hypermethylation of miRNA loci is involved in the regulation of miRNA expression.⁵² We therefore determined the effect of 5-AdC, a cytosine analog refractory to methylation and used to relieve the inhibitory effects by DNA methylation *in situ*,³⁸ on the down-regulated miR-150 in SSc fibroblasts. The expression of miR-150 in SSc fibroblasts treated with 5-AdC was significantly increased, compared with controls (Figure 2E). Thus, the down-regulation of miR-150 in SSc fibroblasts is likely to be regulated, at least in part, by DNA methylation.

miR-150 Expression in SSc Fibroblasts *in Vivo*

We then tried to determine the role of miR-150 in tissue fibrosis *in vivo*. Histopathologically, compared with normal tissue (Figure 3, A and B), SSc skin is characterized by dermal fibrosis due to thickened and increased collagen fibers (Figure 3, C and D). *In situ* hybridization showed that

Figure 3 *In vivo* expression of miR-150 in SSc skin. **A–D:** H&E staining of normal (**A** and **B**) and SSc (**C** and **D**) skin. **E–H:** *In situ* detection of miR-150 (**E–H**) in paraffin-embedded, formalin-fixed tissues of normal (**E** and **F**) and SSc (**G** and **H**) skin. Nuclei were counterstained with Nuclear Fast Red. The miR-150 stained blue. Dermal fibroblasts are indicated by **arrows**. Negative controls using scrambled probe are also shown (**I–L**). **Boxed** regions correspond to the adjacent higher-power image. **M** and **N:** Expression levels of miR-150 were decreased in five SSc skin samples, compared with five healthy control (HC) skin samples, as determined by real-time PCR. The transcript levels in samples from HC skin were set at 1. **O:** Total miRNA was extracted from skin tissue derived from five TSK mice (TSK) and three normal mice (WT). Expression levels of miR-150 were significantly lower in TSK mice, compared with WT (set at 1.0), as determined by real-time PCR. Data are expressed as means $\pm$ SE. * $P < 0.05$ versus control. Original magnification: $\times 40$ (**A**, **C**, **E**, **G**, **I**, and **K**); $\times 400$ (**B**, **D**, **F**, **H**, **J**, and **L**). Scale bars: 200 μ m (**A**, **C**, **E**, **G**, **I**, and **K**); 20 μ m (**B**, **D**, **F**, **H**, **J**, and **L**).

Figure 4 **A:** Serum miR-150 levels were lower in SSc patients. Serum miR-150 levels (normalized to cel-miR-39) were measured in 20 healthy control (HC), 20 limited cutaneous SSc (lcSSc), 20 diffuse cutaneous SSc (dcSSc), 5 dermatomyositis (DM), and 5 systemic lupus erythematosus (SLE) samples by qPCR. The maximum value in HC was set at 1. Means are indicated by horizontal bars. **B:** Model of the role of miR-150 in SSc fibroblasts. In SSc fibroblasts, miR-150 expression is constitutively down-regulated, probably because of hypermethylation of the promoter. Decreased miR-150 causes up-regulation of $\beta 3$ integrin, which results in the activation of TGF- $\beta 1$ on the cell surface by the removal of latency-associated peptide (LAP). The bioactive TGF- $\beta 1$ binds to its receptor and stimulates Smad3 phosphorylation/translocation into the nucleus, leading to the abnormal transcriptional activation of collagen gene in SSc fibroblasts and tissue fibrosis. * $P < 0.05$ versus HC.

signal for miR-150 was evident in fibroblasts of normal skin (Figure 3, E and F), but was scarcely detected in SSc fibroblasts between the thickened collagen bundles (Figure 3, G and H). No signal was found in negative controls (Figure 3, I–L). The expression of miR-150 in lymphocytes, endothelial cells, and fat cells did not differ between normal skin and SSc skin (not shown). We also performed quantitative analysis of the miR-150 expression in normal and SSc skin. Compared with five normal skin samples, all five SSc skin samples showed decreased miR-150 expression (Figure 2M), and the decreases of miR-150 in SSc skin were statistically significant, relative to the value in normal skin (Figure 2N), which is consistent with the *in vitro* results (Figure 1, A and B).

To further investigate the miR-150 expression *in vivo*, miRNA was purified from paraffin-embedded sections derived from TSK mice, the animal fibrotic skin model. Real-time PCR revealed that mean miR-150 levels in the skin of TSK mice were significantly lower than those in wild-type mice (Figure 2O). Therefore, both *in vitro* and *in vivo*, the expression of miR-150 was likely to be decreased in fibrotic conditions.

Correlation of Serum miR-150 Levels with Clinical Manifestations and Laboratory Data in SSc Patients

Recent reports indicate that serum miRNAs can be novel biomarkers in various diseases. Serum miRNAs are thought to be stable, because they can be encapsulated in microvesicles shed from cell plasma membrane and protected from RNase.^{53–57} Furthermore, miRNAs in the microvesicles can be incorporated into other cells, and may alter gene expression.⁵⁷ Thus, serum miRNA levels may not be merely secreted from apoptotic cells, but may exert some biological effects. We determined serum concentration of miR-150 in SSc patients and evaluated the possibility that serum miR-150 levels can be a disease marker.

There has been no previous report demonstrating the expression of miR-150 in cell-free body fluid. To validate that this miRNA is indeed detectable in human serum,

Table 2 Correlation of Serum miR-150 Levels with Clinical and Serological Features in SSc Patients

Clinical and serological features	Patients with normal miR-150 levels ($n = 13$)	Patients with lower miR-150 levels ($n = 27$)
Age at onset (years)	63.0	58.0
Duration of disease (years)	5.26	4.46
Type, diffuse:limited ($n:n$)	5:8	15:12
MRSS (score)	8.3	14.0
Clinical features (%)		
Pitting scars or ulcers	12.5	58.3*
Nailfold bleeding	30.0	50.0
Raynaud's phenomenon	80.0	95.6
Telangiectasia	33.3	44.4
Contracture of phalanges	87.5	84.6
Calcinosis	0	0
Diffuse pigmentation	33.3	44.4
Short sublingual frenulum	87.5	84.6
Sicca symptoms	50.0	71.4
Organ involvement (%)		
Pulmonary fibrosis	45.4	28.0
Mean vital capacity	92.0	94.2
Mean DLCO	73.5	78.6
Pulmonary hypertension	50.0	68.0
Esophagus	30.0	17.3
Heart	45.5	33.3
Kidney	0	10.5
Joint	66.6	28.6
Thrombosis	0	0
ANA specificity (%)		
Anti-topoisomerase I	0	43.4*
Anti-centromere	60.0	47.8
Anti-U1 RNP	10.0	17.4

The cutoff value was set at the mean value of the patients.

* $P < 0.05$ versus patients with normal serum miR-150 levels; Fisher's exact probability test or *U*-test.

ANA, antinuclear antibodies; DLCO, diffusion capacity of the lung for carbon monoxide; MRSS, Modified Rodnan Skin Score; RNP, ribonucleoprotein.

miRNA was extracted from sera of healthy individuals and the level of miR-150 was determined by qPCR using a primer set specific for miR-150 (Supplemental Figure S1). The amplification of miR-150 was observed, and C_T values were increased by the serial dilution of the miRNA. Thus, miR-150 was shown to be detectable and quantifiable in the serum using our method.

Serum samples were obtained from 40 patients (9 men, 31 women) with SSc (20 dcSSc, 20 lcSSc). Serum samples were also obtained from 5 systemic lupus erythematosus patients, 5 dermatomyositis patients, and 20 healthy subjects. Real-time PCR revealed that miR-150 levels in the sera of SSc patients were significantly lower than those in healthy subjects (Figure 4A). When SSc patients were considered separately by classification, both dcSSc patients and lcSSc patients showed decreased miR-150 concentration. The values in dermatomyositis patients and systemic lupus erythematosus patients were slightly decreased compared with those in healthy controls, but there were no significant differences.

Next, to determine whether serum miR-150 levels contribute to the pathogenesis of SSc, we analyzed the association between miR-150 levels and various clinical or laboratory features (Table 2). Although the difference did not reach statistical significance, patients with lower miR-150 levels had a higher ratio of dcSSc to lcSSc (15:12 versus 5:8) and a higher Modified Rodnan Skin Score (MRSS; 14.0 versus 8.3), compared with patients with normal levels of miR-150. A significantly higher incidence of anti-topoisomerase I antibody and a higher prevalence of pitting scars were seen in patients with lower miR-150 levels than in those without (58.3% versus 0% and 58.3% versus 12.5%, respectively; $P < 0.05$). Thus, our results suggest that patients with low miR-150 levels may exhibit more severe clinical manifestations.

Discussion

Recently, Maurer et al⁵⁸ reported that down-regulation of miR-29a contributes to overexpression of extracellular matrix in SSc fibroblasts *in vitro*. Our present findings support the idea that miRNAs are involved in the pathogenesis of SSc. In addition, with this study we have demonstrated three major new findings.

First, we identified several miRNAs that were overexpressed or suppressed specifically in SSc fibroblasts, compared with normal fibroblasts, by miRNA PCR array. Among the down-regulated miRNAs, we focused on miR-150 as the regulator of integrin $\beta 3$. Although the expression of miR-150 has been evaluated in gastric cancer and in chronic lymphocytic leukemia,^{59,60} the present study is the first to demonstrate the expression of miR-150 in rheumatic diseases (of which SSc is one).

Second, in the present study we also found new miRNA-target interactions in dermal fibroblasts. Down-regulated

miR-150 leads to overexpression of integrin $\beta 3$ in normal fibroblasts, and overexpression of the miRNA leads to down-regulation of the integrin in SSc fibroblasts. Although integrin $\beta 3$ is thought to be the upstream key event in the TGF- β activation process of SSc, the mechanism of integrin overexpression in SSc is unknown. Our results suggest that the down-regulation of miR-150, due to DNA methylation, can cause the overexpression of integrin directly, and can activate TGF- β signaling without changing TGF- $\beta 1$ levels in SSc fibroblasts. Activated TGF- β signaling induces phosphorylation of Smad3, which stimulates the transcriptional activation of collagen gene and the tissue fibrosis seen in SSc.¹⁰

Lastly, we investigated tissue and serum miRNA levels in SSc. To our knowledge, the present study is the first to show that miR-150 is detectable and quantifiable in serum. The miR-150 levels in serum from lcSSc and dcSSc patients were significantly lower than those in serum from healthy subjects. Also, SSc patients with lower serum miR-150 levels tended to have more severe clinical manifestation. Thus, serum miR-150 levels can be a disease marker. The diagnosis of SSc presents little problem when the clinical features have fully developed. It may be difficult to distinguish lcSSc from healthy skin, however, because skin sclerosis is sometimes not apparent in lcSSc, especially in very early stages.⁶¹ Serum levels of miR-150 may be useful for the differentiation of lcSSc from healthy skin. Serial time-course measurement of serum miR-150 concentration in suspect cases could lead to early detection of developing SSc.

Taken together, our results yield a hypothetical model of an miR-150-mediated SSc-phenotype (Figure 4B). The down-regulation of miR-150 may be the most upstream event in the TGF- β activation process of SSc. Clarifying the involvement of miRNAs in the pathogenesis of SSc could lead to development of new diagnostic tools and new treatments using miRNA (eg, by transfection into the fibrotic lesion).

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.09.023>.

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