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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 β3

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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 β3 in these cells. The miRNA expression profile was determined by miRNA PCR array and real-time PCR. Protein expression of integrin β3 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 β3, 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 β3. 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-β1).[5,6] One of the diverse effects of TGF-β1 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 α1(I) and α2(I) collagen,[7,8] suggesting that the activation of SSc fibroblasts is a result of stimulation by TGF-β1 signaling. This notion is supported by our previous findings, that i) the transcriptional activity of the α2(I) collagen gene in SSc fibroblasts is constitutively up-regulated compared with that in normal fibroblasts but the responsiveness to ectopic TGF-β1 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-β1 signal using neutralizing antibody abolishes the increased expression of human α2(I) collagen mRNA in SSc fibroblasts.[11] On the other hand, we have previously reported that TGF-β1 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-β1 signaling, without any increase in TGF-β1 concentration.

Bioactive peptide of TGF-β1 is usually captured by latency-associated peptide-β1 (LAP-β1) noncovalently, forming the small latent complex (SLC). In this configuration, TGF-β1 is inactive and cannot bind to its receptors. TGF-β1 activation is a complex process involving conformational changes induced by cleavage of LAP-β1 either by various proteases (eg, plasmin) or by physical interactions of LAP-β1 with other proteins (eg, integrins and thrombospondin-1), leading to the release of bioactive TGF-β1.[12–15]

In explanation of how TGF-β1 is activated in SSc fibroblasts without any quantitative increase, we recently reported constitutive overexpression of integrin β3 in these cells. The forced overexpression of integrins, including the β3 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-β1 in the pericellular region. This may enhance the incidence of interaction between active TGF-β1 and its receptors, leading to the self-activation of SSc fibroblasts without increasing the amount of TGF-β1.[14,16–18] Integrin overexpression is therefore thought to be the most upstream event of TGF-β1 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 β3 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-cm^2 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% CO_2-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-β1 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 RT^2 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 RT^2 miRNA first-strand kit (SABiosciences). For the RT^2 Profiler PCR array (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 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′-TCTCCCAACCCTTGTACCAGTG-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% CO_2-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% CO_2-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-aza-deoxycytidine (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 β3, according to miRNA target gene prediction. Several leading online software tools identified integrin β3 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	0487
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 b3) 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] DIANAmicroT 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).

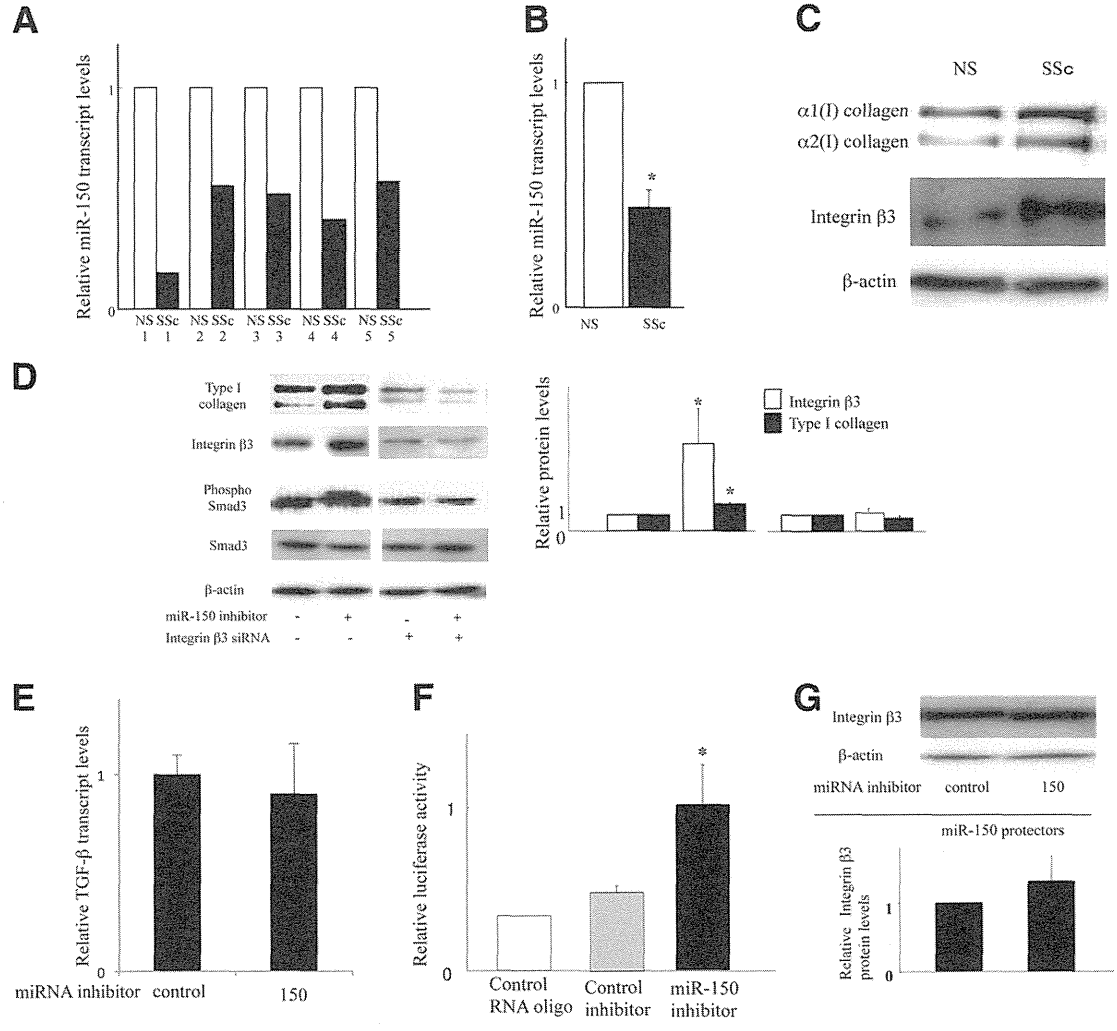

Figure 1 The effects of miR-150 inhibitor on the expression of integrin β3 and type I collagen in normal dermal fibroblasts. **A**: Normal ($n = 5$) and SSc ($n = 5$) fibroblasts were serum starved for 24 hours. Total miRNA was extracted, and the relative level of miR-150 (normalized to U6) in each of 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 β3 or type I collagen. The same membrane was reprobed 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 β3 siRNA for 96 hours. Cell lysates were subjected to immunoblotting as described for panel **C**. Protein levels of integrin β3 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 β3 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-β1 (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-β1 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 β3 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 β3 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 β3. Data are expressed as means ± 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 β3 in Normal Fibroblasts

The expression of integrin β3 and of both α1(I) and α2(I) collagen protein was increased in SSc fibroblasts (Figure 1C). Next, we determined the contribution of miR-150 to the expression of integrin β3 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 β3 expression (Figure 1D). Type I collagen expression was also induced by miR-150 inhibitor (Figure 1D), probably subsequent to the overexpression of integrin β3 and phosphorylated levels of Smad3. Consistent with the hypothesis, in the presence of integrin β3 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

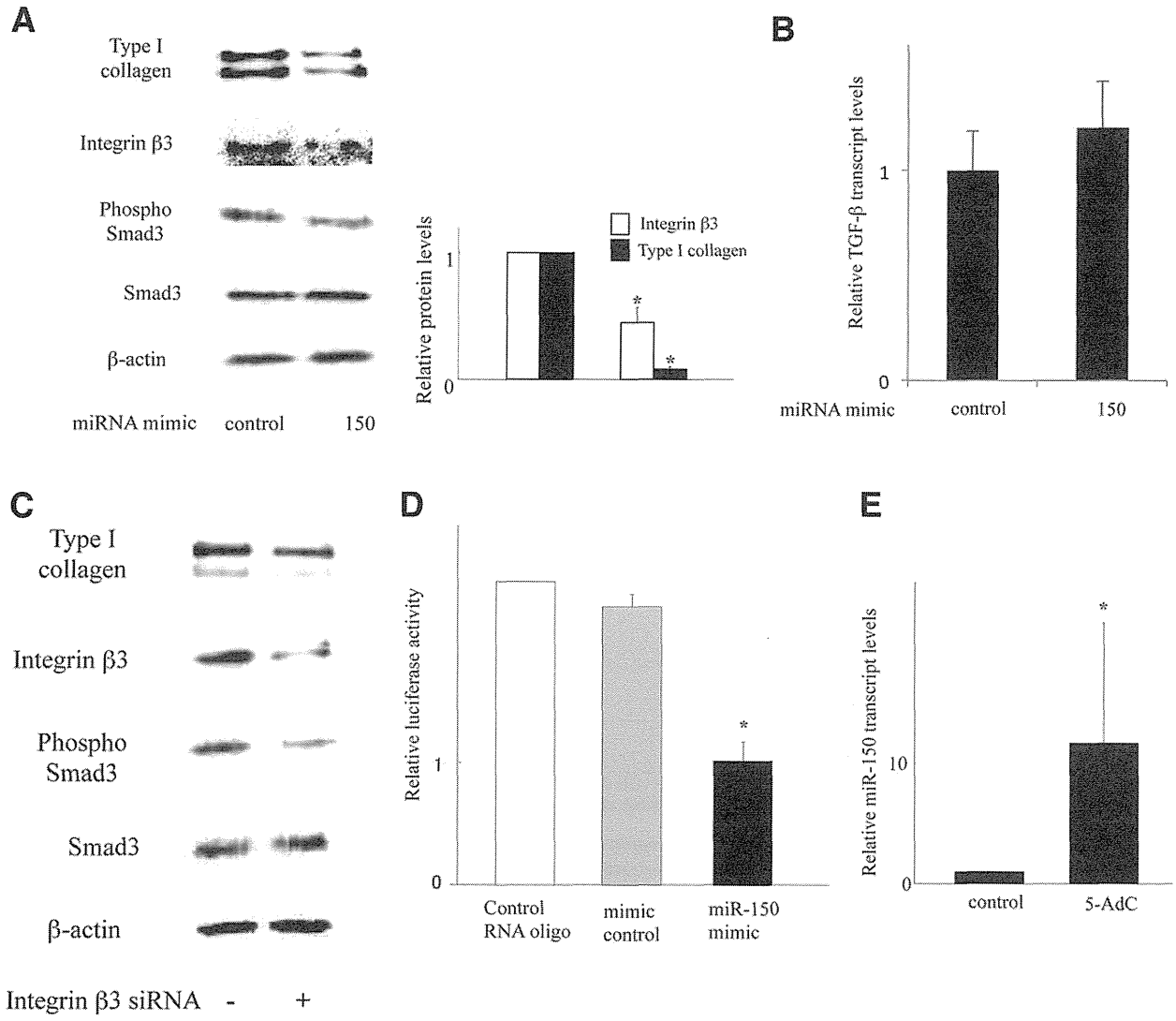

Figure 2 The effect of miR-150 mimic on the expression of integrin β3 and type I collagen in SSc fibroblasts. **A:** SSc fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA mimic or miR-150 mimic for 96 hours, which resulted in a decrease of up-regulation of both integrin β3 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 β3 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 β3 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 μmol/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 ± 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 β3, we performed reporter assay using luciferase construct containing the integrin β3 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 β3 3′UTR (Figure 1F). In addition, we used miRNA inhibitor and miScript Target protector for the miR-150 binding site on the integrin β3 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 β3 (Figure 1D), suggesting direct interaction between them.

Low miR-150 Expression Leads to Up-Regulation of β3 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 β3, 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 β3 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 β3 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 β3 (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

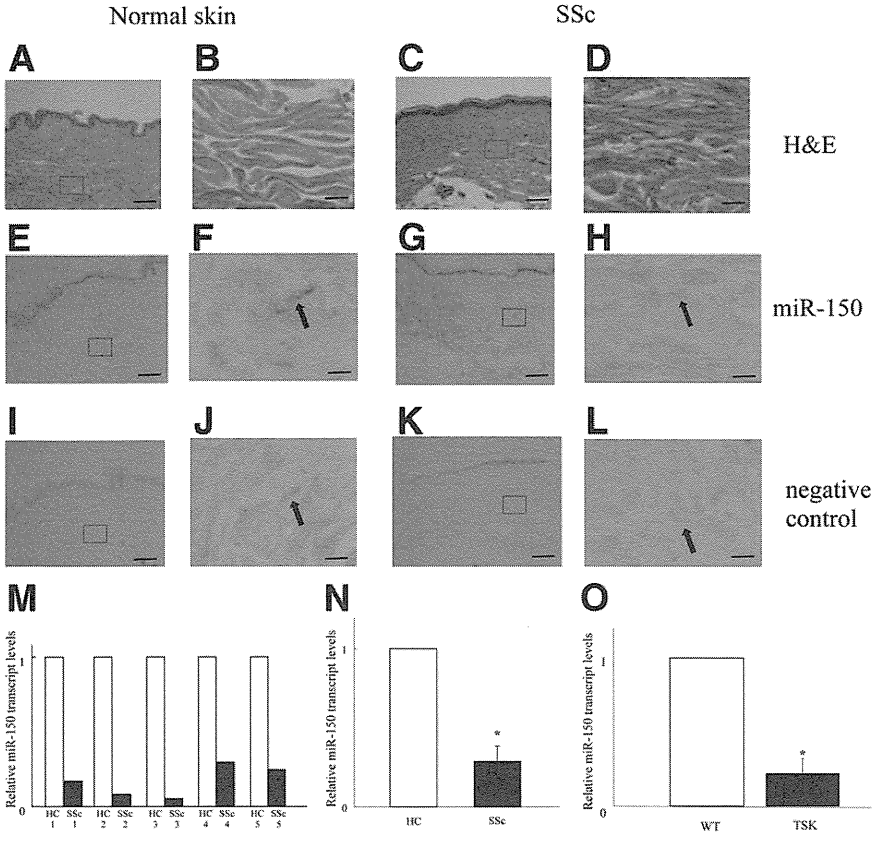

Figure 3 *In vivo* expression of miR-150 in SSc skin. **A**–**D**: H&E staining of normal (**A** and **B**) and SSc (**C** and **D**) skin. **E**–**L**: *In situ* detection of miR-150 (**E**–**H**) in paraffin-embedded, formalin-fixed tissues of normal (**E** and **F**) and SSc (**G** and **H**) skin. 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 ± SE. $^{*}P < 0.05$ versus control. Original magnification: ×40 (**A**, **C**, **E**, **G**, **I**, and **K**); ×400 (**B**, **D**, **F**, **H**, **J**, and **L**). Scale bars: 200 μm (**A**, **C**, **E**, **G**, **I**, and **K**); 20 μm (**B**, **D**, **F**, **H**, **J**, and **L**).

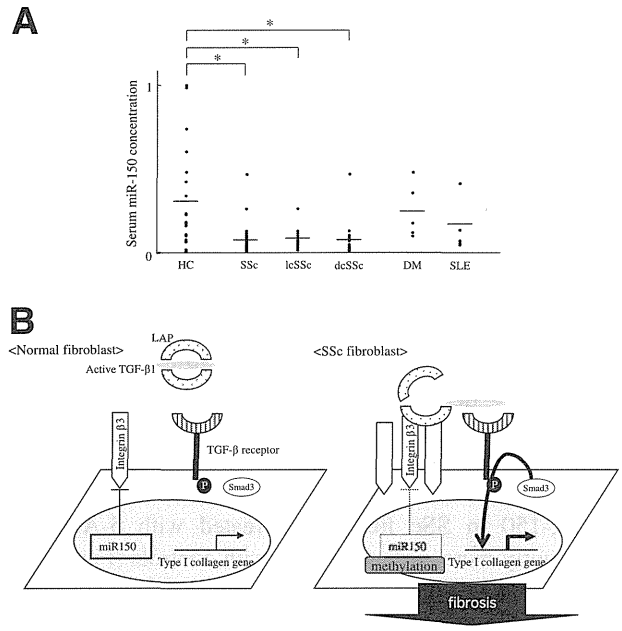

Figure 4 **A**: Serum miR-150 levels were lower in SSc patients. Serum miR-150 levels (normalized to cel-miR-39) were measured in 20 healthy control (HC), 20 limited cutaneous SSc (lcSSc), 20 diffuse cutaneous SSc (dcSSc), 5 dermatomyositis (DM), and 5 systemic lupus erythematosus (SLE) samples by qPCR. 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 β3 integrin, which results in the activation of TGF-β1 on the cell surface by the removal of latency-associated peptide (LAP). The bioactive TGF-β1 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 al[58] 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 β3. 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 β3 in normal fibroblasts, and overexpression of the miRNA leads to down-regulation of the integrin in SSc fibroblasts. Although integrin β3 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-β1 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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Review

Proangiogenic hematopoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis

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Summary. New blood vessel formation is critical, not only for organ development and tissue regeneration, but also for various pathologic processes, such as tumor development and vasculopathy. The maintenance of the postnatal vascular system requires constant remodeling, which occurs through angiogenesis, vasculogenesis, and arteriogenesis. Vasculogenesis is mediated by the *de novo* differentiation of mature endothelial cells from endothelial progenitor cells (EPCs). Early studies provided evidence that bone marrow-derived $CD14^+$ monocytes can serve as a subset of EPCs because of their expression of endothelial markers and ability to promote neovascularization *in vitro* and *in vivo*. However, the current consensus is that monocytic cells do not give rise to endothelial cells *in vivo*, but function as support cells, by promoting vascular formation and repair through their immediate recruitment to the site of vascular injury, secretion of proangiogenic factors, and differentiation into mural cells. These monocytes that function in a supporting role in vascular repair are now termed monocytic pro-angiogenic hematopoietic cells (PHCs). Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by excessive fibrosis and microvasculopathy, along with poor vascular formation and repair. We recently showed that in patients with SSc, circulating monocytic PHCs increase dramatically and have enhanced angiogenic potency. These effects may be induced in response to defective vascular repair machinery. Since $CD14^+$ monocytes can also differentiate into fibroblast-like cells that produce extracellular matrix proteins, here we propose a new hypothesis that aberrant monocytic PHCs, once mobilized into circulation, may also contribute to the fibrotic process of SSc.

Key words: Angiogenesis, Endothelial progenitor cells, Monocytes, Scleroderma, Vasculogenesis

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Introduction

Postnatal blood vessel formation is important for tissue repair and regeneration, but the regulation of this critical process is not fully understood. Maintenance of the postnatal vascular system requires constant remodeling in response to injury and senescence. This may occur by synergic effects of three distinct processes: (i) angiogenesis, which refers to the formation of new blood vessels that sprout from preexisting vessels by a process involving the proliferation and migration of mature endothelial cells (ECs); (ii) vasculogenesis, which refers to the *de novo* differentiation of mature ECs through the recruitment and differentiation of endothelial progenitor cells (EPCs); and (iii) arteriogenesis, which refers to the remodeling of nascent vessels via the recruitment of mesenchymal cells, such as pericytes and smooth muscle cells (Fisher et al., 2006). Since the first description of EPCs as circulating primitive cells that contribute to postnatal vasculogenesis (Asahara et al., 1997), numerous *in vitro* and *in vivo* studies have been carried out to clarify the mechanisms of postnatal vascular formation and repair, as well as the contribution of EPCs to the pathogenesis of vascular diseases, and to develop potential therapeutic strategies that promote tissue regeneration or attenuate pathologic neovascularization. However, a great deal of controversy about EPCs and their roles in postnatal vascular

formation has arisen because of discrepancies in how EPCs are defined (Watt et al., 2010).

The major problem in defining EPCs derives from the lack of specific markers. In the landmark paper by Asahara et al, EPCs were characterized using EC marker-positive cells, which were selected as a cell fraction from peripheral blood mononuclear cells that was enriched in cells expressing CD34 or vascular endothelial growth factor (VEGF) receptor type 2 (VEGFR-2). These cells contributed to the revascularization and salvage of ischemic hind limbs in animal models (Asahara et al., 1997). Currently, it is widely accepted that there are at least two types of EPCs that can be discriminated based on their surface antigen expression, proliferation potential, and time of emergence in the cell culture system (Prater et al., 2007). The first subset is endothelial colony-forming cells (ECFCs) or late-outgrowth EPCs, which are regarded as "true EPCs," based on their potential for clonogenic expansion *in vitro* and their ability to form vessels *in vitro* and *in vivo* (Prater et al., 2007). Circulating precursors of ECFCs have not been identified yet, but they are known to express CD34 and CD31, and to lack the expression of CD133, CD45, and CD14 (Estes et al., 2010).

The cells originally identified as EPCs in various assays are in fact hematopoietic lineage cells that display pro-angiogenic properties, and are now termed pro-angiogenic hematopoietic cells (PHCs). PHCs include several different circulating cell types that are identified in the literature as circulating angiogenic cells (CACs), circulating endothelial precursors, monocytic EPCs, early-outgrowth EPCs, and colony-forming unit (CFU)-ECs. They are hematopoietic progenitors derived from the bone marrow (BM) that fall into at least two distinct major subsets: $CD14^+$ monocytic PHCs (the dominant population) and $CD14^-$ non-monocytic PHCs, which are primitive cells positive for CD34, CD133, and VEGFR-2 (Peichev et al., 2000). Currently, it is generally accepted that PHCs do not give rise to ECs, but rather work as pro-angiogenic support cells (Richardson and Yoder, 2011).

In this review, we focus on the vascular regenerative functions of PHCs originating from the monocytic lineage and their potential roles in the pathogenesis of systemic sclerosis (SSc), a multisystem connective tissue disease characterized by excessive fibrosis and widespread microvasculopathy.

Pro-angiogenic capacity of $CD14^+$ monocytes

EC-like features of $CD14^+$ monocyte-derived cells have been reported ever since Asahara et al.'s 1997 paper was published. Fernandez et al. described a subset of $CD14^+$ monocytes that become adherent within 24 hours of the culture and change their morphology to that of EC-like cells with Weibel-Palade bodies (Fernandez et al., 2000). When cultured with multiple pro-angiogenic growth factors, these $CD14^+$ monocytes gradually lose their expression of hematopoietic markers, such as CD14 and CD45, and display an up-regulated expression of EC markers, including von Willebrand factor (vWF), CD144, CD105, CD34, CD36, acetylated low-density lipoprotein-receptor, endothelial nitric oxide synthase, VEGF receptor type 1 (VEGFR-1), and VEGFR-2 (Fernandez et al., 2000; Schmeisser et al., 2001). In these reports, the cultured EC-like cells formed tubular structures in three-dimensional gel cultures that consisted of short sprouts from the EC-like colonies.

Subsequently, the *in vivo* functional capacity of monocytes was evaluated using animal models for neovascularization. In a study by Urbich et al, peripheral blood-derived $CD14^+$ monocytes were incubated on a fibronectin-coated plate under pro-angiogenic conditions for 4 days, and the recovered adherent monocytes were transplanted into the hind-limb ischemia mouse model (Urbich et al., 2003). The transplanted monocyte-derived cells were incorporated into the vascular structure and promoted neovascularization. In another study, peripheral blood- or BM-derived $CD34^-CD14^+$ monocyte lineage cells accelerated re-endothelialization in a monocyte chemoattractant protein 1 (MCP-1)-dependent manner in a rat model for balloon-injured artery (Fujiyama et al., 2003). These findings together suggest that a subset of $CD14^+$ monocytes can differentiate into the endothelial lineage and contribute to *in vivo* neovascularization and vascular repair (Urbich and Dimmeler, 2004).

A specific marker for this unique monocyte subset has not been identified, but the expression of VEGFR-2 in circulating $CD14^+$ monocytes is essential for their capacity to differentiate into the EC lineage (Elsheikh et al., 2005). Upon vascular injury, a subset of $CD14^+$ monocytes is mobilized into the circulation, adheres to the injured endothelium, and differentiates into EC-like cells, although whether or not monocyte-derived EC-like cells are integrated properly into the endothelium and serve as fully functional ECs has not been confirmed.

Circulating $CD14^+$ monocytes as a primary source of PHCs

The cultivation of circulating mononuclear cells in medium favoring endothelial differentiation has been used to identify EPCs and to expand circulating EPCs. In these cultures, it is difficult to determine which precursor cells give rise to the EPCs, because the starting cell population is heterogeneous, and cellular phenotypes change over time in culture. In the original protocol by Asahara et al. peripheral blood mononuclear cells were cultured on fibronectin for 7 days (Asahara et al., 1997). Currently, CACs are described as the cell type of origin for these cultured cells (Hirschi et al., 2008). Typically, these cells do not form colonies in culture, but they have EC features, including the ability to bind Ulex lectin Europeus Agglutinin-1, to take up acetylated low-density liproprotein, and to express CD31, CD105,

VEGFR-2, and vWF. The vast majority of the cells recovered in these cultures express both CD45 and CD14, indicating their monocytic origin.

In contrast, Hill et al. developed a semi-solid clonogenic assay, in which peripheral blood mononuclear cells that did not adhere to fibronectin within 48 hours were reseeded on fibronectin, and formed cell clusters (Hill et al., 2003). These cells are termed CFU-ECs or CFU-Hill, and express EC markers, including CD31, CD105, CD146, VEGFR-2, CD144, and vWF (Hill et al., 2003). However, unlike the CAC-derived cells, nearly all the cells within the CFU-EC clusters express the hematopoietic marker CD45, but only a tiny fraction express CD34 (Rohde et al., 2006). In addition, the depletion of $CD14^+$ monocytes from the mononuclear cells before seeding effectively prevents colony formation. CACs and CFU-ECs are primarily derived from $CD14^+$ monocytes, and thus are now categorized together as PHCs or early-outgrowth EPCs (Prater et al., 2007). Most importantly, PHCs cannot proliferate or form tubular structures *in vitro* without a co-culture with mature ECs. Several studies reported that PHCs can integrate into tubular structures and differentiate into EC-like cells *in vivo* (Elsheikh et al., 2005; Kuwana et al., 2006), but it is uncertain whether the EC-like cells can exert the full range of endothelial functioning.

PHCs are distinct from ECFCs or late-outgrowth EPCs, which appear 10-21 days after circulating mononuclear cells are plated in medium favoring endothelial differentiation (Ingram et al., 2004; Yoder et al., 2007). These cultured cells display a cobblestone morphology and express EC markers but not hematopoietic markers. Circulating precursor cells that give rise to ECFCs display clonal proliferative potential, self-renewal, and the ability to form vessels *in vivo*, compatible with features of traditional EPCs. A recent genome-wide transcriptional profiling of early- and late-outgrowth EPCs revealed strikingly different gene expression signatures between these cell populations, which provided evidence that the early-outgrowth EPCs are hematopoietic cells with a molecular phenotype linked to monocytes, whereas late-outgrowth EPCs exhibit commitment to the endothelial lineage (Medina et al., 2010). Based on these findings, it has been proposed that the term EPCs should be reserved for ECFCs (Prater et al., 2007; Watt et al., 2010; Richardson and Yoder, 2011). Whether rare ECFCs are derived from hemangioblasts in the BM or from endothelial stem cells that reside in the endothelium remains undetermined (Yoder, 2010).

Roles of monocytic PHCs in neovascularization

PHCs, whether in the monocytic or non-monocytic lineage, are no longer defined as "true EPCs," although they clearly participate in blood vessel formation and vascular repair, and thereby contribute to the maintenance of vascular homeostasis. A function in vascular regeneration was suggested for monocytic PHCs in a vascular injury model, in which green fluorescent-labeled $CD14^+$ monocytes integrated into the endothelium and improved the re-endothelialization (Elsheikh et al., 2005). Indeed, monocytic PHCs are widely accepted to function in a supporting role in vascular repair, and several different mechanisms for their involvement have been described.

First, monocytic PHCs can release a variety of potent, soluble pro-angiogenic growth factors, including VEGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and stromal cell-derived factor-1 (SDF-1) (Rehman et al., 2003; Urbich et al., 2005). When secreted locally, these factors induce increased vascular permeability, the enhanced proliferation and migration of mature ECs, and the recruitment of progenitor and inflammatory cells from the BM.

Second, immunohistochemical studies in mouse have revealed that monocytic cells attach to the injured vascular lumen immediately after injury and change their morphology to EC-like cells; some of these cells then behave like ECs (Fujiyama et al., 2003; Elsheikh et al., 2005), although it is still unclear if monocytic PHCs are truly integrated into the vascular structures or simply localize there because of their adhesive characteristics. These EC-like cells may supplement the function of impaired ECs at the site of vascular injury, until they are replaced by mature ECs differentiated from ECFCs.

Finally, several lines of evidence have shown that monocytic cells contribute to arteriogenesis (Heil and Schaper, 2004). Mural cells, including pericytes and smooth muscle cells (SMCs), are essential for vessel maturation and stability, but their origin is not fully understood. In a chimeric mouse model for neovascularization, most BM-derived peri-endothelial cells were positive for CD45, CD11b (a monocyte marker), and NG2 proteoglycan (a pericyte marker) (Rajantie et al., 2004), indicating that the pericyte and monocyte lineages have a common origin. Pericyte precursors can differentiate into various mesenchymal cells, including SMCs, fibroblasts, and myofibroblasts (Diaz-Flores et al., 2009), an ability shared by circulating monocytes, which are now considered oligopotent progenitor cells (Seta and Kuwana, 2010). These findings together suggest that monocytic PHCs differentiate into EC-like cells as well as other elements of the vasculature, such as pericytes and SMCs, during the vascular repair process. In addition, monocytic PHCs comprise approximately 0.1% to 2% of peripheral blood mononuclear cells (Dimmeler et al., 2001; Elsheikh et al., 2005; Prater et al., 2007), although the frequency of monocytic PHCs varies depending on the method used to define them. Regardless, monocytic PHCs clearly predominate over non-monocytic PHCs and ECFCs in their absolute numbers in circulation (Prater et al., 2007). The potential mechanisms by which monocytic PHCs provide supportive functions in the neovascular microenvironment are summarized in Fig. 1. During this

process, the monocytic PHCs work in concert with platelets, residential ECs, non-monocytic PHCs, and ECFCs to form new blood vessels (Semenza, 2007).

Monocytic PHCs as oligopotent progenitors

Circulating $CD14^+$ monocytes exhibit heterogeneity in terms of their surface markers, phagocytic activity, and differentiation potential. They are committed precursors in transit from the BM to their ultimate sites of activity. Until recently, monocytes were believed to differentiate only into phagocytic and/or antigen-presenting cells, such as macrophages, dendritic cells, and osteoclasts. However, accumulating evidence indicates that circulating monocytes may differentiate into a variety of other cell types as well, including mesenchymal or endothelial lineage cells (Seta and Kuwana, 2010). Specifically, we described a primitive cell population termed monocyte-derived multipotential cells (MOMCs), which have a fibroblast-like morphology and a unique molecular phenotype positive for CD14, CD45, CD34, and type I collagen in culture (Kuwana et al., 2003). MOMCs include progenitors that differentiate into a variety of non-phagocytes, including bone, cartilage, fat, skeletal and cardiac muscle, neurons, and endothelium (Kuwana et al., 2003, 2006; Kodama et al., 2005, 2006).

At present, several distinct human cell populations derived from circulating $CD14^+$ monocytes have been reported to differentiate into non-phagocytes. Zhao and colleagues demonstrated that pluripotent stem cells generated from circulating monocytes by repeated stimulation with a high concentration of macrophage-colony stimulating factor and phorbol myristate acetate differentiate along several distinct cell lineages, including macrophages, T cells, epithelial cells, endothelial cells, neuronal cells, and hepatocytes (Zhao et al., 2003). Monocytic EPCs also differentiate into cardiomyocytes (Badorff et al., 2003), and monocytic EPCs residing within the circulating $CD14^+CD34^{low}$ cell population differentiate not only into endothelial cells, but also into osteoblasts, adipocytes, or neurons (Romagnani et al., 2005). Finally, fibrocytes are identified as circulating BM-derived cells, which home to sites of tissue injury, differentiate into fibroblasts, and contribute to tissue repair and fibrosis (Bucala et al., 1994). The origin of the fibrocytes is a subpopulation of circulating $CD14^+$ monocytes (Abe et al., 2001).

A variety of $CD14^+$ monocyte-derived cultured cell populations with distinct phenotypes and differentiation potentials have been reported in the literature, but their circulating precursors among the $CD14^+$ monocytes have not been identified to date. All of these cell populations can be enriched by the short-term culturing of circulating monocytes in medium containing different soluble factors and on plates coated with specific matrix proteins. Circulating fibrocytes express the chemokine receptors CCR3, CCR5, CCR7, and CXCR4 (Strieter et al., 2007), but the monocytic precursors of MOMCs are in the $CD14^+CXCR4^{high}$ population. A recent report showed that fibrocytes generated in the absence or presence of fetal calf serum exhibit different morphologies and gene expression profiles (Curnow et al., 2010).

Since circulating $CD14^+$ monocytes change their morphology, gene expression profiles, and function over

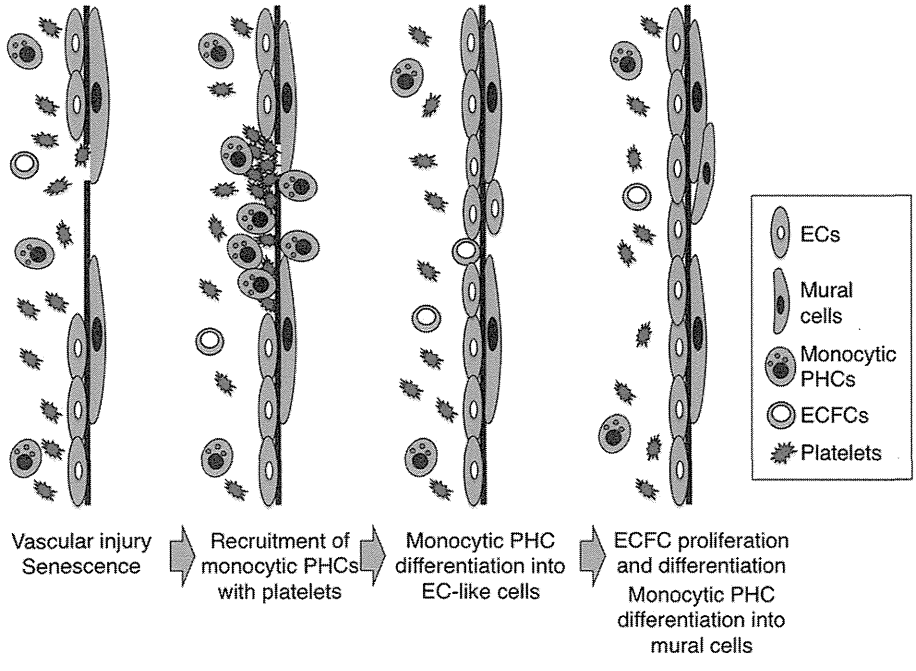

Fig. 1. Potential roles of monocytic PHCs in neovascularization. Monocytic PHCs are recruited to the site of vascular injury, differentiate into EC-like cells, and function as ECs by being incorporated into the vascular structure until ECFCs differentiate into mature ECs. Monocytic PHCs also provide supportive functions by releasing angiogenic factors, chemokines, and proteases to enhance the proliferation, migration, and maturation of the cells required for vascular regeneration. In the late phase of vascular recovery, monocytic PHCs differentiate into mural cells.

time *in vitro* (Seta and Kuwana, 2010), the heterogeneity among culture-enriched monocytic cells does not necessarily indicate that they originate from different circulating precursors. In other words, it is possible that culturing the same $CD14^+$ monocyte precursors in different conditions can generate cell progenies with different characteristics. In fact, monocyte-derived oligopotent cells have common characteristics, including a spindle shape, the expression of CD34 when cultured on fibronectin or type I collagen, and a low proliferative capacity (Seta and Kuwana, 2007). These characteristics are shared by monocytic PHCs, which are oligopotent for differentiation into mesenchymal cells other than EC-like cells. It is possible that monocytic PHCs and other monocyte-derived primitive cells, such as MOMCs and fibrocytes, are all derived from circulating $CD14^+$ precursors.

Roles of monocytic PHCs in neovascular responses in SSc

Given the critical role of monocytic PHCs in postnatal vascular formation and repair, alterations in their numbers and/or functions may contribute to the pathogenic processes of various vascular diseases. In this regard, we focused on SSc, which is characterized by excessive fibrosis and microvascular abnormalities. SSc vasculopathy mainly affects small arteries and causes reduced blood flow and tissue ischemia, leading to clinical manifestations such as digital ulcers and pulmonary arterial hypertension (LeRoy, 1996). Two types of vascular pathology are progressive intimal proliferation and fibrosis, and the loss of capillaries. The mechanism of SSc vasculopathy is not fully understood, but increasing evidence indicates that an endothelial injury is a primary event in the pathogenesis of scleroderma (Guiducci et al., 2007). The persistent increase in pro-angiogenic factors, such as VEGF, platelet-derived growth factor, and SDF-1 observed in SSc patients indicates a strong pro-angiogenic response to vascular damage (Liakouli et al., 2011). Nailfold capillaroscopic findings reveal giant capillaries in the early phase of the disease, and the loss of capillaries and vascular disorganization in the late phase (Herrick and Cutolo, 2010). Severe capillary loss may result from vascular damage, but there is almost no evidence of vascular recovery. In addition, the formation of abnormal blood vessels like giant and bushy capillaries indicates an inadequate vascular repair process. These findings together suggest that, in patients with SSc, the vascular repair machinery does not work properly, and the disease progresses toward irreversible structural changes, despite the strong neovascular push. Thus, impaired angiogenesis and vasculogenesis were proposed in an intriguing hypothesis to explain the pathogenesis of SSc vasculopathy (Manetti et al., 2010).

To test this hypothesis, several studies have been conducted to quantify the circulating $CD14^-CD34^+$ $CD133VEGFR^+$ EPCs, which are now regarded as a non-monocytic subset of PHCs, in patients with SSc. We first reported that there is a reduced number of non-monocytic PHCs in SSc patients (Kuwana et al., 2004). In subsequent analyses by other groups, some confirmed our finding (Zhu et al., 2008; Mok et al., 2010), but

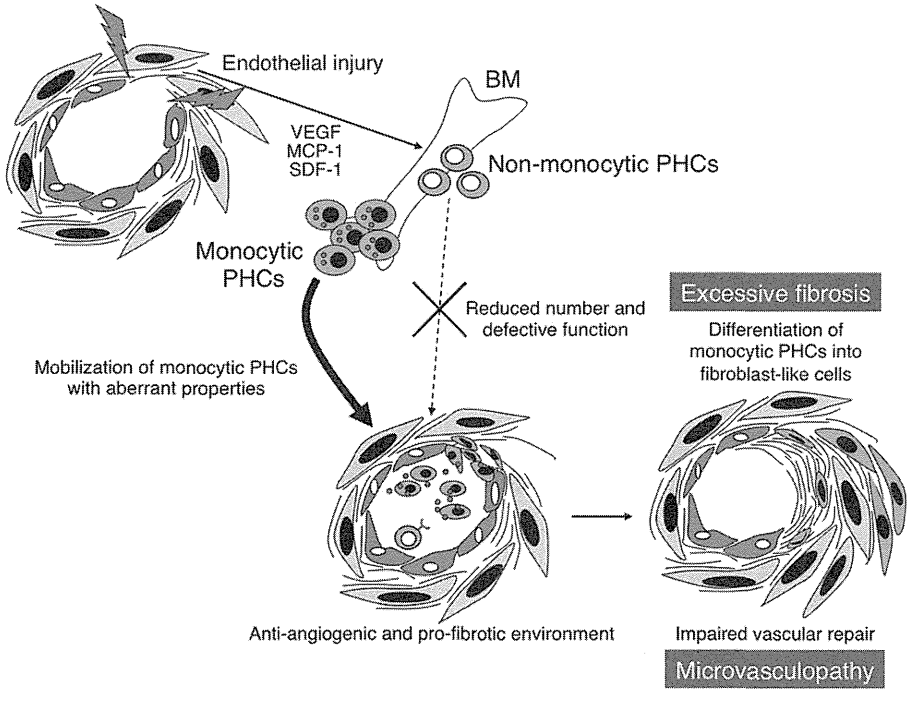

Fig. 2. Potential roles of monocytic PHCs in the pathogenesis of SSc. Growth factors and chemokines produced at the site of endothelial injury mobilize a variety of progenitor cells, including monocytic PHCs. The strong anti-angiogenic environment at the affected site prevents adequate vascular repair, leading to microvasculopathy. In the pro-fibrotic environment, accumulated monocytic PHCs differentiate into fibroblast-like cells and promote excessive fibrosis.

others showed an increase in non-monocytic PHCs in SSc patients (Del Papa et al., 2006; Avouac et al., 2008). Thus, the effect of SSc on the number of circulating non-monocytic PHCs remains a matter of debate (Kuwana and Okazaki, 2012). On the other hand, there is little information on the roles of monocytic PHCs in SSc vasculopathy.

We recently evaluated the number of monocytic PHCs in SSc patients using a culture system previously developed to enrich for MOMCs (Yamaguchi et al., 2010). The MOMCs enriched in this culture can differentiate into EC-like cells and promote blood-vessel formation *in vitro* and *in vivo* (Kuwana et al., 2006), and thus correspond to monocytic PHCs. Unexpectedly, we observed a paradoxical increase in monocytic PHCs in SSc patients compared with healthy controls. Intriguingly, the monocytic PHCs derived from SSc patients showed enhanced *in vitro* tubular structure formation compared with those from healthy controls. Furthermore, in a murine tumor neovascularization model, the transplantation of SSc-derived monocytic PHCs dramatically promoted tumor growth and tumor vessel formation *in vivo*, indicating that monocytic PHCs have enhanced angiogenic activity in SSc patients, an effect that has also been observed in a chick embryo chorioallantoic membrane assay (Ribatti et al., 1998) and in the SCID mouse skin xenograft model (Liu et al., 2005), in which the normal tissue surrounding an SSc skin graft showed a prominent increase in new blood vessel formation. The increased number and enhanced angiogenic potency of the monocytic PHCs are likely to be compensatory responses to damaged vessels.

Despite the robust pro-angiogenic responses, appropriate blood vessel formation does not occur in patients with SSc. The neovascular process consists of a sequence of highly regulated events, including angiogenesis, vasculogenesis, and arteriogenesis, which are tightly controlled by pro- and anti-angiogenic signals (Semenza, 2007). In this regard, the SSc-affected tissues, such as skin and lungs, exhibit dysregulated endothelial features. In microvascular ECs isolated from the skin of SSc patients, metalloproteinase (MMP)-12 is over-expressed and cleaves urokinase-type plasminogen activator receptor, causing inhibition of the invasion/migration capacities of ECs (D'Alessio et al., 2004; Margheri et al., 2006). Furthermore, the reduction of tissue kallikreins 9, 11, and 12, which exert a mitogenic effect on ECs, and the up-regulation of anti-angiogenic kallikrein 3 were reported in SSc skin (Giusti et al., 2005). In addition, in SSc lesions, ECs lose their expression of VE-cadherin, which is required for vascular tube formation (Fleming et al., 2008). Finally, selective up-regulation of the anti-angiogenic VEGF b isoform was observed in the circulation and skin of SSc patients, indicating a switch from the pro-angiogenic to the anti-angiogenic VEGF isoform in these patients (Manetti et al., 2011). These dysregulated endothelial features at the site of SSc organ involvement are responsible for the disease-related defects in angiogenesis and prevent vascular repair. Together, these data suggest that the balance between pro- and anti-angiogenic responses favors anti-angiogenesis in SSc patients.

Pathogenic roles of monocytic PHCs in SSc

Current data on the functions of monocytic PHCs provide strong hints about their roles in the pathogenesis of SSc. Circulating monocytic PHCs are mobilized from the BM and recruited to SSc-induced lesions in response to chemokines such as MCP-1 and SDF-1, which are up-regulated in the affected skin of SSc patients (Distler et al., 2001; Cipriani et al., 2006). In addition, the hypoxic condition of the affected tissues of SSc patients appears to potentiate the in situ differentiation of circulating monocytic cells into EC-like cells (Bellik et al., 2008). Thus, functionally altered monocytic PHCs accumulate at SSc lesions.

Since monocytic PHCs are oligopotent in terms of their capacity to differentiate into mesenchymal lineage cells (Badorff et al., 2003; Kuwana et al., 2003; Kodama et al., 2005; Romagnani et al., 2005), they may differentiate into fibroblast-like cells, produce collagens and other extracellular matrix proteins, and participate in the fibrotic process. In this regard, recent lines of evidence indicate that $CD14^+$ monocytes are involved in fibrogenesis. For instance, fibrocytes derived from $CD14^+$ monocytes home to the site of tissue injury and contribute to tissue repair and fibrosis by differentiating into myofibroblasts that express αSMA (Abe et al., 2001). In addition, $CD14^+$ circulating monocytes acquire the ability to produce extracellular matrix components, such as type I collagen, in an MCP-1/CCR2-dependent amplification loop (Sakai et al., 2006). Furthermore, an enhanced profibrotic phenotype of circulating $CD14^+$ monocytes was reported in SSc patients with interstitial lung disease (Mathai et al., 2010). Another report described a correlation between fibrotic clinical features and the increased proportion of $CXCR4^+$ circulating cells with monocytic and endothelial markers in SSc patients (Campioni et al., 2008). Therefore, monocytic PHCs may acquire pro-fibrotic characteristics and contribute to the promotion of fibrosis at sites affected by SSc that have a strong anti-angiogenic and pro-fibrotic environment (Fig. 2).

Conclusions

In summary, monocytic PHCs contribute to postnatal blood vessel formation and vascular repair, mainly through their immediate recruitment to the site of vascular injury, their secretion of a variety of pro-angiogenic factors, and their differentiation into mural cells. These cells are also oligopotent; that is, they can differentiate into various cell types in the mesenchymal lineage. This unique feature raises the intriguing hypothesis that monocytic PHCs are involved in the pathogenesis of SSc by participating in two major

pathological features, microvasculopathy and excessive fibrosis. Understanding the roles of monocytic PHCs in the progression of SSc may be key to dissecting its pathogenesis and to developing novel therapeutic strategies for this intractable condition.

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