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Impairment of Endothelial Cell Differentiation From Bone Marrow–Derived Mesenchymal Stem Cells

New Insight Into the Pathogenesis of Systemic Sclerosis

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Objective. **Systemic sclerosis (SSc) is a disorder characterized by vascular damage and fibrosis of the skin and internal organs. Despite marked tissue hypoxia, there is no evidence of compensatory angiogenesis. The ability of mesenchymal stem cells (MSCs) to differentiate into endothelial cells was recently demonstrated. The aim of this study was to determine whether impaired differentiation of MSCs into endothelial cells in SSc might contribute to disease pathogenesis by decreasing endothelial repair.**

Methods. **MSCs obtained from 7 SSc patients and 15 healthy controls were characterized. The number of colony-forming unit–fibroblastoid colonies was determined. After culture in endothelial-specific medium, the endothelial-like MSC (EL-MSC) phenotype was assessed according to the surface expression of vascular endothelial growth factor receptors (VEGFRs). Senescence, chemoinvasion, and capillary morphogenesis studies were also performed.**

Results. **MSCs from SSc patients displayed the same phenotype and clonogenic activity as those from controls. In SSc MSCs, a decreased percentage of VEGFR-2+, CXCR4+, VEGFR-2+/CXCR4+ cells and early senescence was detected. After culturing, SSc EL-MSCs showed increased expression of VEGFR-1, VEGFR-2, and CXCR4, did not express CD31 or annexin V, and showed significantly decreased migration after specific stimuli. Moreover, the addition of VEGF and stromal cell–derived factor 1 to cultured SSc EL-MSCs increased their angiogenic potential less than that in controls.**

Conclusion. **Our data strongly suggest that endothelial repair may be affected in SSc. The possibility that endothelial progenitor cells could be used to increase vessel growth in chronic ischemic tissues may open up new avenues in the treatment of vascular damage caused by SSc.**

Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by vascular signs and symptoms (e.g., Raynaud's phenomenon, fingertip ulcers, and gangrene) due to endothelial damage. This event precedes the development of skin fibrosis and leads to vessel wall intimal proliferation and obliteration and decreased capillary density due to both inflammatory immune processes and ischemia-reperfusion damage (1–3). Usually, tissue hypoxia induces the formation of new blood vessels that sprout from existing vessels (angiogenesis), but in SSc, where there is marked tissue hypoxia, there is evidence of loss of angiogenesis.

We now know that new vessels arise not only from angiogenesis, but also from vasculogenesis, a process in which endothelial progenitor cells (EPCs) are mobilized from the bone marrow to the site of neovascularization, with differentiation into mature endothelial cells. This process takes place in response to cytokines and/or tissue ischemia, and it occurs independently of the preexisting vessels. The number of circulating EPCs may be modified under physiologic and pathologic conditions (4–6). In SSc, a lower number of EPCs has been

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Table 1. Clinical and demographic features of the 7 SSc study patients*

Sex/age	Year of SSc onset	MRSS	Autoantibodies	Lung involvement by HRCT/PFT	Heart and kidney involvement	Previous treatment
F/44	2002	38	ANAs, Scl-70	Ground glass/normal	No heart involvement; SSc renal crisis while taking cyclosporine	Cyclosporine stopped because of renal crisis; IV CYC (2 gm total)
M/44	2004	36	ANAs, Scl-70	Ground glass/normal	Absent	No previous treatment
F/18	2004	18	ANAs, Scl-70	Ground glass/normal	Absent	Calcium-channel blockers
F/36	2003	38	ANAs, Scl-70	Ground glass/normal	Absent	CYC (5 gm total)
F/42	2004	22	ANAs, Scl-70	Ground glass/normal	Absent	Calcium-channel blockers
F/27	2003	15	ANAs, Scl-70	Ground glass/normal	Absent	Prostanoids; IVIG
F/42	2004	26	ANAs, Scl-70	Ground glass/normal	Absent	Prostanoids

* SSc = systemic sclerosis; MRSS = modified Rodnan skin thickness score (maximum possible score 51); HRCT = high-resolution computed tomography; PFT = pulmonary function testing; ANAs = antinuclear antibodies; IV = intravenous; CYC = cyclophosphamide; IVIG = intravenous immunoglobulin.

detected and linked with the clinical features of vascular involvement, such as pitting scars and fingertip ulcers (7).

Human mesenchymal stem cells (MSCs) are multipotent cells that are present in the bone marrow of adults. They differentiate into several cell lineages of mesenchymal tissues. MSCs have no specific markers but are generally considered to be plastic-adherent, clonogenic, nonphagocytic cells that have a fibroblast-like morphology. These cells bear certain surface antigens (CD29, CD44, CD73, CD90, CD105, and CD166), are negative for hematopoietic markers (CD14, CD34, and CD45), and do not express costimulation molecules such as CD80, CD86, or CD40. Usually, they display at least a trilineage potential (bone, cartilage, and adipose tissue) (8,9). Human MSCs may be an alternative source of EPCs. In fact, MSCs display some features of mature endothelial cells, such as the expression of von Willebrand factor (vWF), vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2, VE-cadherin, and vascular cell adhesion molecule 1 (VCAM-1), but do not express CD31 and CD34 (10).

Failure of endothelial repair following SSc-related damage might be linked to an alteration in the endothelial differentiation of MSCs. Therefore, the aim of our study was to investigate the characteristics of MSCs in SSc, their differentiation into endothelial cells, and their angiogenic potential.

PATIENTS AND METHODS

Patients. Seven patients who were classified as having severe diffuse cutaneous SSc with rapidly progressive disease according to the criteria of LeRoy et al (11) and fulfilled the Autologous Stem cell Transplantation International Scleroderma (ASTIS) trial enrollment criteria (12) underwent autologous hematopoietic stem cell transplantation (HSCT). The ASTIS trial targets patients with early diffuse SSc who are at risk of early mortality, with a disease duration of $\leq$4 years, a modified Rodnan skin thickness score (MRSS) of at least 15 (of a maximum of 51), and evidence of heart, lung, or kidney disease, as well as patients with a maximum disease duration of 2 years, an MRSS of $\geq$20, and laboratory signs of an acute-phase reaction. Exclusion criteria are end-stage organ failure and extensive pretreatment with cyclophosphamide.

The mean age of our patients was 42 years (range 18–44 years) and the mean disease duration was 22 months (range 18–44 months). Before HSCT, patients underwent physical examination, laboratory testing, and instrumental examination to evaluate internal organ involvement. All but 1 of the SSc patients received intravenous prostanoids. Aspirations from the posterior superior iliac crest to collect MSCs for assay were performed just before the prostanoid infusion and at least 1 month after the previous infusion. One patient received cyclosporine, which had been discontinued 18 months before study. Angiotensin-converting enzyme inhibitors and calcium-channel blockers were discontinued at least 3 weeks before collection of MSCs. All patients were assessed for disease severity according to international guidelines (13), and their clinical profiles are summarized in Table 1.

Isolation and culture of MSCs. After ethics committee approval and informed consent was given, human bone marrow cells were obtained from 7 SSc patients before HSCT and from 15 healthy donors (14 women and 1 man; mean age 41 years [age range 22–46 years]) by aspiration from the posterior superior iliac crest. Samples were collected in tubes containing acid citrate dextrose. In order to enrich the total nucleated cell fraction, an aliquot was centrifuged for 10 minutes at 700*g*. The interface between plasma and the red cell pellet (the buffy coat) was recovered, diluted 1:10 in Hanks' balanced salt solution (EuroClone, Milan, Italy), and then counted.

These cells were plated in 75-cm^2 flasks (1.6×10^5 total nucleated cells/cm^2) in Iscove's modified Dulbecco's medium (IMDM; with L-glutamine and HEPES 25 m*M*; EuroClone) with 50 μg/ml of gentamicin (Schering-Plough, Milan, Italy), 10% fetal bovine serum (FBS; Hyclone, South Logan, UT), and 2% Ultroser G (Pall BioSepra, Cergy-St. Christophe, France), and incubated at 37°C in a humidified

atmosphere containing 95% air and 5% CO_2. Half of the complete medium was changed after 1 week, and thereafter, the entire medium was changed every 3–4 days. When ~80% of the flask surface was covered, the adherent cells were incubated for 5–10 minutes at 37°C with 0.05% trypsin–0.02% EDTA (Eurobio, Courtaboeuf, France), harvested, washed, and resuspended in complete medium (primary culture [P0]). Cells were then reseeded (P1). Expansion of the cells was obtained with successive cycles of trypsinization and reseeding.

Determination of colony-forming unit–fibroblastoid (CFU-F) frequency. The number of CFU-F colonies was used as a surrogate marker for MSC progenitor frequency. Two dishes measuring 100-mm in diameter were seeded with 5×10^5 total nucleated cells (1:10 dilution in IMDM culture medium) from the bone marrow buffy coat. After incubation for 14 days, visible colonies formed by 50 or more cells were counted and reported as the number of CFU-F colonies/10^6 total nucleated cells seeded.

Analysis of the osteogenic differentiation of MSCs. MSCs (10^4 cells/cm^2) were grown to near confluence in 35-mm–diameter dishes and then incubated in osteogenic medium (IMDM with 10% FBS, 10 n*M* dexamethasone, 100 μg/ml ascorbic acid, and 10 m*M* β-glycerophosphate; all from Sigma, St. Louis, MO). After 21 days, the deposition of mineral nodules was revealed with alizarin red S staining. The extracellular matrix mineral–bound staining was examined using light microscopy and photographed.

Adipogenic differentiation of MSCs. MSCs (10^4 cells/cm^2) were grown to near confluence in 35-mm–diameter dishes and then incubated in adipogenic medium (IMDM with 10% FBS, 0.5 m*M* isobutyl methylxanthine, 1 μM dexamethasone, 10 μg/ml of insulin, and 70 μM indomethacin; all from Sigma). After 21 days, accumulation of lipid-containing vacuoles was revealed with oil red O staining and photographed under light microscopy.

Immunophenotyping of MSCs by flow cytometric analysis. First-passage MSCs were analyzed for the expression of a number of surface antigens by flow cytometry. Aliquots were incubated with the following conjugated monoclonal antibodies: phycoerythrin (PE)–conjugated CD34, fluorescein isothiocyanate (FITC)–conjugated CD45, PE-conjugated CD14; PE-conjugated CD29, FITC-conjugated CD44, PE-conjugated CD166, PE-conjugated CD90, PE-conjugated CD73, FITC-conjugated HLA–DP, DQ, and DR, and FITC-conjugated HLA–A, B, and C (all from BD PharMingen, San Diego, CA) and PE-conjugated CD105 (Ancell, Bayport, MN). Nonspecific fluorescence and morphologic features of the cells were determined by incubation of the same cell aliquot with isotype-matched mouse monoclonal antibodies (BD PharMingen). Gating acquisition was performed according to previously described methods (14).

Endothelial cell differentiation. Third-passage MSCs derived from SSc patients and controls were used for these studies. MSCs were cultivated in the presence or absence of endothelial growth medium (Clonetics, San Diego, CA) supplemented with 2% FBS and 50 ng/ml of VEGF (PromoCell, Heidelberg, Germany) for 7 days (10). Medium was changed every 2 days.

Flow cytometric analysis of endothelial cells. After trypsin treatment, detached cells were stained with the specific monoclonal antibody for the endothelial cell surface markers anti–VEGFR-2 (Sigma) and anti–VEGFR-1 (Sigma). Anti-CXCR4 and anti-CD31 (Becton Dickinson, San Diego, CA) staining was also used to assess the surface expression of these molecules. Annexin V expression was used to detect apoptotic cells. Analyses were performed using CellQuest software (Becton Dickinson).

Chemoinvasion assays of MSCs and endothelial-like MSCs (EL-MSCs). A Boyden chamber was used to evaluate cell migration. This method is based on the passage of cells across porous filters against a concentration gradient of the migration effector. A 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) was used. The 2 compartments were separated by a polyvinylpyrrolidone-free polycarbonate filter with an 8-μm pore size (Neuro Probe). To evaluate chemoinvasion, the filter was coated with Matrigel (50 μg/filter; Becton Dickinson, Bedford, MA). Fifty microliters of cell suspension (2×10^5 cells) was placed in the upper compartment of the Boyden chamber. Test solutions were dissolved in serum-free medium and placed in wells of the lower compartment. VEGF-A at 10 ng/ml and 50 ng/ml and stromal cell–derived factor 1 (SDF-1) at 50 ng/ml and 250 ng/ml, with or without anti-CXCR4 antibody at 2 μg/ml, were tested. Irrelevant IgG was used to verify the specificity of the effect.

The chamber was incubated at 37°C for 5 hours. The filter was then removed and fixed with methanol. Nonmigrating cells on the upper surface of the filter were removed by a cotton swab. Cells were stained with Diff-Quick (Mertz-Dade/Dade International, Milan, Italy) (15). The number of SSc and control MSCs that had migrated was counted at 100× magnification. Results were expressed as a chemotactic index, which was calculated as the average number of migrated cells in stimulated wells divided by the average number of migrated cells in control wells. Values are reported as the mean ± SD of 3 different experiments, each of which was performed in triplicate.

In vitro capillary morphogenesis assay. Matrigel (0.5 ml at a concentration of 10–12 mg/ml) was pipetted into 13-mm–diameter tissue culture wells and polymerized at 37°C for 30 minutes to 1 hour (15,16). MSCs and EL-MSCs from SSc patients and controls were plated (6×10^4 cells/ml) in IMDM with 1% FBS. Positive controls were obtained upon stimulation of capillary morphogenesis with VEGF (50 ng/ml) using microvascular endothelial cells. Fibroblast-like synoviocytes were used as negative controls. Both MSCs and EL-MSCs were cultured with VEGF-A (50 ng/ml) or SDF-1 (50 ng/ml and 250 ng/ml), with or without anti-CXCR4 antibody (2 μg/ml). After 24 hours, plates were photographed.

Angiogenesis was evaluated by measurement of tubule area, using AngioSys software (TCS CellWorks, Botolph Claydon, UK) according to the manufacturer's instructions. Six to nine photographic fields from 3 plates were scanned for each point. The amount of tubule area in SSc patients and controls was quantified by measuring the percentage of the photographic field occupied by tubule structures, with reference to the field occupied by microvascular endothelial cells, which were considered to perform 100% of capillary morphogenesis.

Telomerase activity assay. Third-passage MSCs and EL-MSCs derived from SSc patients and controls were harvested by trypsinization and lysed at 4°C in cell lysis buffer. Aliquots of the lysate (equivalent to 1.5 μg of protein) were assayed for telomerase activity by a modified telomeric-repeat amplification protocol (TRAPeze; Intergen, Purchase, NY).

Table 2. Surface phenotype markers expressed by MSCs and EL-MSCs derived from SSc patients and healthy controls*

	MSCs			EL-MSCs		
	SSc patients	Healthy controls	*P*	SSc patients	Healthy controls	*P*
VEGFR-1	2.61 (0.16–11)	3.07 (1–13.4)	NS	21 (16–56.4)	25.4 (13.9–67)	NS
VEGFR-2	1.6 (0.04–3)	2.79 (2.5–12.1)	<0.05	46.39 (6–48)	15 (2.33–28)	<0.007
CXCR4	0.6 (0–1.6)	3.47 (2.5–5.2)	<0.007	14 (13.3–17)	18 (4–22)	NS
VEGFR-2/CXCR4	0.6 (0–0.78)	2.23 (1.86–3.6)	<0.007	14 (13.3–17)	15 (2.33–19.7)	NS
% VEGFR-2	37.5 (32.5–43.7)	74 (62.8–76.2)	NS	30 (27–39.4)	82 (70.5–100)	<0.007
CD31	0 (0–0.9)	0.2 (0–1.1)	NS	0 (0–0.5)	0 (0–0.3)	NS
Annexin V	0.24 (0–0.33)	0 (0–0.8)	NS	0 (0–0.6)	0.8 (0–1.1)	NS

* Mesenchymal stem cells (MSCs) were unstimulated, and endothelial-like mesenchymal stem cells (EL-MSC) were stimulated with endothelial growth medium supplemented with 2% fetal bovine serum and 50 ng/ml of vascular endothelial growth factor. Values are the median (range). SSc = systemic sclerosis; VEGFR-1 = vascular endothelial growth factor receptor 1; NS = not significant.

Results are expressed in arbitrary units and were normalized to the signal obtained from an extract of 500 HeLa cells routinely assayed in parallel. Telomerase activity is shown quantitatively, reflecting the ratio of the TRAP product ladder bands to the internal control band, and was calculated according to the formula supplied in the manufacturer's manual.

Statistical analysis. Results are expressed as mean ± SD. Multiple comparisons were performed by the Student-Newman-Keuls test after demonstration of significant differences among medians by nonparametric variance analysis using the Kruskal-Wallis test. *P* values less than 0.05 were considered significant.

RESULTS

Expansion of SSc patient and control MSCs in culture. Human bone marrow–derived MSCs from SSc and controls were expanded in culture. Culture-expanded confluent MSCs displayed both spindle-shaped cells and large flat cells. Morphologic features were typical of MSCs.

The third-passage cellular expansion capability of SSc MSCs was not different from that of controls. Primary cultured cells were repeatedly trypsinized and replated, reaching a mean ± SD cellular expansion of $3 \pm 1.3/10^6$ at the third passage. MSCs from 3 of the SSc patients slowed down after this point, whereas all others continued to expand. Control MSCs reached a mean cellular expansion of $5.1 \pm 2.3 \times 10^5$ at the third passage.

Findings of the CFU-F assay. Total nucleated cells from the bone marrow buffy coat were used in the CFU-F assay. The mean ± SD number of CFU-F colonies, a surrogate marker of MSC progenitor frequency, in cells from the SSc patients ($51 \pm 26/10^6$ total nucleated cells) was not different from that in cells from the controls ($53 \pm 11/10^6$ total nucleated cells).

Immunophenotype of MSCs. MSCs from SSc patients and controls were uniformly positive for CD29, CD44, CD166, CD90, CD73, HLA–A, B, and C, and CD105; HLA–DP, DQ, and DR were expressed in <4% of the population. There was no contamination by hematopoietic cells, as indicated by negative findings on flow cytometry for markers of hematopoietic lineage, including CD14, CD34, and CD45. There was no statistically significant difference in the immunophenotype of MSCs from SSc patients as compared with controls.

Osteogenic differentiation of MSCs. Osteogenic differentiation of MSCs was determined after 21 days of stimulation. Alizarin red S staining showed aggregates or nodules of hydroxyapatite-mineralized matrices that were intensely red-stained in both SSc patients and controls.

Adipogenic differentiation of MSCs. MSCs treated with adipogenic medium were successfully differentiated toward adipogenic lineages in both SSc patients and controls. Lipid vacuoles stained orange-red after 21 days.

Findings of flow cytometry for endothelial cell differentiation. The results of flow cytometry for surface phenotype markers expressed by MSCs and EL-MSCs from SS patients and controls are summarized in Table 2. In MSCs from the SSc patients, there was a significant decrease in the percentages of VEGFR-2+ cells, CXCR4+ cells, and VEGFR-2+/CXCR4+ cells as compared with MSCs from the controls (median 1.6 [range 0.04–3] versus 2.79 [range 2.5–12.1] [$P < 0.05$] for VEGFR-2; 0.6 [range 0–1.6] versus 3.47 [range 2.5–5.2] [$P < 0.007$] for CXCR4; and 0.6 [range 0–0.78] versus 2.23 [range 1.86–3.6] [$P < 0.007$] for VEGFR-2/CXCR4). Furthermore, neither SSc MSCs nor control MSCs expressed surface CD31 (median 0.24 [range 0–0.89] versus 0 [range 0–0.56]; *P* not significant). No difference in the percentage of VEGFR-1+ cells was found in SSc patients compared with controls (median

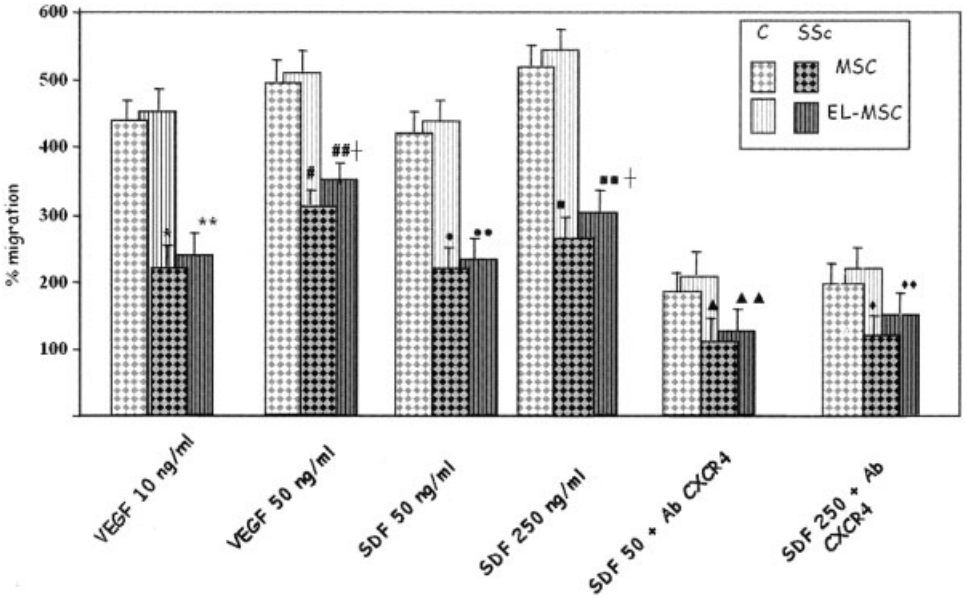

Figure 1. Effects of vascular endothelial growth factor (VEGF) and stromal cell–derived factor 1 (SDF-1) stimuli on cell migration. Chemoinvasion of mesenchymal stem cells (MSCs) from patients with systemic sclerosis (SSc) was significantly less responsive at each concentration of stimulus studied as compared with MSCs from healthy control (C) subjects ($* = P < 0.001$, $\# = P < 0.001$, $\bullet = P < 0.001$, $\blacksquare = P < 0.001$, $\blacktriangle = P < 0.001$, and $\blacklozenge = P < 0.001$). Similarly, endothelial-like MSCs (EL-MSCs) from SSc patients were significantly less responsive to stimuli as compared with EL-MSCs from healthy controls ($** = P < 0.001$, $\#\# = P < 0.001$, $\bullet\bullet = P < 0.001$, $\blacksquare\blacksquare = P < 0.001$, $\blacktriangle\blacktriangle = P < 0.001$, and $\blacklozenge\blacklozenge = P < 0.001$). Of note, the specific stimulation did not significantly increase the performance of MSCs from either SSc patients or controls over that of SSc patient MSCs cultured under massive stimulation (50 ng/ml of VEGF and 250 ng/ml of SDF-1) ($\dagger = P = 0.03$ for both stimulations versus healthy controls). Values are the mean and SD of triplicate determinations.

2.61 [range 0.16–11] versus 3.07 [range 1–13.4]; *P* not significant).

SSc and control EL-MSCs displayed an increased percentage of VEGFR-1, VEGFR-2, and CXCR4. Furthermore, almost 50% of cultured SSc cells expressed the specific endothelial marker VEGFR-2, with a 25-fold increase as compared with unstimulated cells, whereas a lower increase in VEGFR-2+ cells was observed in the controls (median 21 [range 16–56.4] versus 25.4 [range 13.9–67] [*P* not significant] for VEGFR-1; 46.39 [range 6–48] versus 15 [range 2.33–28] [$P < 0.007$] for VEGFR-2; 14 [range 13.3–17] versus 18 [range 4–22] [*P* not significant] for CXCR4; and 14 [range 13.3–17] versus 15 [range 2.33–19.7] [*P* not significant] for VEGFR-2/CXCR4). In SSc MSCs, only a minority of the total VEGFR-2+ cells displayed CXCR4; almost all control EL-MSCs displayed both receptors (median 30 [range 27–39.4] in SSc patients versus 82 [range 70.5–100] in controls; $P < 0.007$). After VEGF stimulation, no variation was found in the percentage of cells expressing CD31, either in the SSc patients or the controls.

In 4 SSc patients and 3 controls, we further assessed the expression of vWF in MSCs and in EL-MSCs by flow cytometry. Conflicting results were obtained, with an increased expression of vWF in 2 SSc patients and 2 controls after differentiation to EL-MSCs, and no variation in the other subjects (data not shown).

No apoptotic cells were observed in SSc or controls cultures, regardless of the culture media used (Table 2).

Findings of MSC and EL-MSC chemoinvasion analyses. Results of the chemoinvasion analyses are summarized in Figure 1. The effect of VEGF and SDF-1 on chemoinvasion was dose-dependent, with the maximal effect at 50 ng/ml of VEGF and 250 ng/ml of SDF-1, in both control and SSc MSCs, with a 3-fold increase over basal values. Furthermore, a significant difference between SSc patients and controls was observed at each concentration of VEGF and SDF-1 analyzed. The increased invasion observed after a 5-hour incubation with 250 ng/ml of SDF-1 was counteracted by incubation with anti-CXCR4 antibody (Figure 1), confirming that the

Table 3. Percentage of the well surface covered by tubular-like structures under basal conditions and after addition of stimuli to cultures of MSCs and EL-MSCs derived from SSc patients and healthy controls*

		Stimulus		
	Basal	VEGF, 50 ng/ml	SDF-1, 250 ng/ml	SDF-1, 250 ng/ml, plus anti-CXCR4, 2 μg/ml
MSCs				
Healthy controls	44 (27–65)	88 (78–99)	82 (74.8–89.2)	15 (9.9–20.1)
SSc patients	10.3 (0–29)	29 (22.8–35.2)	36 (31.7–40.3)	8.7 (4.9–12.5)
EL-MSCs				
Healthy controls	87 (78.8–95.2)	101 (91.7–110.3)	98 (90.5–105.5)	33 (25.6–40.4)
SSc patients	11 (7.8–14.2)	75 (67.8–82.2)	73 (66.1–79.9)	13 (7.9–18.1)

* Values are the median percentages (range). MSCs = mesenchymal stem cells; EL-MSCs = endothelial-like mesenchymal stem cells; SSc = systemic sclerosis; VEGF = vascular endothelial growth factor; SDF-1 = stromal cell–derived factor 1.

In analyses of capillary morphogenesis of MSCs and EL-MSCs from SSc patients and controls, cells stimulated with VEGF, SDF-1α, or SDF-1α + anti-CXCR4 were compared with their own unstimulated (basal) cells, as follows. For healthy control MSCs versus basal healthy control MSCs, $P < 0.005$ for VEGF and for SDF-1α treatment, and $P < 0.001$ for SDF-1α + anti-CXCR4 treatment. For SSc MSCs versus basal SSc MSCs, $P < 0.05$ for VEGF treatment, $P < 0.001$ for SDF-1α treatment, and P not significant [NS] for SDF-1α + anti-CXCR4 treatment. For healthy control EL-MSCs versus basal healthy control EL-MSCs, $P < 0.05$ for VEGF treatment, P NS for SDF-1α treatment, and $P < 0.001$ for SDF-1α + anti-CXCR4 treatment. For SSc EL-MSCs versus basal SSc EL-MSCs, $P < 0.001$ for each treatment.

In analyses of capillary-like structures in MSCs and EL-MSCs from SSc patients and controls, cells were left unstimulated or were stimulated with VEGF, SDF-1α, or SDF-1α + anti-CXCR4 and were then compared between the 2 groups of study subjects, as follows. For basal MSCs from SSc patients versus controls, $P < 0.01$. For VEGF-treated and SDF-1α–treated MSCs from SSc patients versus controls, $P < 0.005$ for each treatment. For SDF-1α + anti-CXCR4–treated MSCs from SSc patients versus controls, P NS. For basal EL-MSCs from SSc patients versus controls, $P < 0.001$. For VEGF-treated, SDF-1α–treated, and SDF-1α + anti-CXCR4–treated EL-MSCs from SSc patients versus controls, $P < 0.01$ for each treatment.

In analyses of capillary-like structures after differentiation to EL-MSCs, MSCs and EL-MSCs from SSc patients and controls were left unstimulated or were stimulated with VEGF, SDF-1α, or SDF-1α + anti-CXCR4 and were then compared, as follows. For basal healthy control EL-MSCs versus healthy control MSCs, $P < 0.001$. For VEGF-treated healthy control EL-MSCs versus healthy control MSCs, $P < 0.05$. For SDF-1α–treated and SDF-1α + anti-CXCR4–treated healthy control EL-MSCs versus healthy control MSCs, P NS. For basal SSc EL-MSCs versus SSc MSCs, P NS. For VEGF-treated and SDF-1α–treated SSc EL-MSCs versus SSc MSCs, $P < 0.001$ for each treatment. For SDF-1α + anti-CXCR4–treated SSc EL-MSCs versus SSc MSCs, $P < 0.001$.

SDF-1/CXCR4 interaction is required for the proinvasive effect of SDF-1 on control and SSc MSCs.

In EL-MSCs, the effects of VEGF and SDF-1 on chemoinvasion mirrored those observed in MSCs. Furthermore, no difference in the chemotactic index between control MSCs and EL-MSCs was observed. However, SSc EL-MSCs displayed a statistically significant increase in chemoinvasive ability over SSc MSCs (mean ± SD chemotactic index in SSc MSCs 2.11 ± 0.13 versus 2.64 ± 0.15 [$P = 0.012$] at 50 ng/ml of VEGF and mean ± SD chemotactic index in SSc EL-MSCs 1.32 ± 0.12 versus 1.69 ± 0.16 [$P = 0.034$] at 250 ng/ml of SDF-1).

Capillary morphogenesis of unstimulated and stimulated MSCs in Matrigel. Results of the capillary morphogenesis studies are summarized in Table 3 and Figure 2. Control MSCs seeded on Matrigel formed tubular structures within 1 day, while no tubular network appeared in SSc MSC samples (median 10.3% [range 0–29%] in SSc patients versus 44% [range 27–65%] in controls; $P < 0.005$). Addition of VEGF and SDF-1 significantly increased the in vitro morphogenesis of both control and SSc MSCs, with a significant between-group difference in capillary-forming ability still observable under each condition studied. Blocking of the interaction between SDF-1 and CXCR4 resulted in a decrease in capillary morphogenesis (Figure 2A).

EL-MSCs from controls formed tubular structures within 1 day, while EL-MSCs from SSc patients showed a lower ability to form tubular structures. Addition of VEGF and SDF-1 increased the angiogenic potential of EL-MSCs from both SSc patients and controls. Unexpectedly, SSc EL-MSCs displayed a much

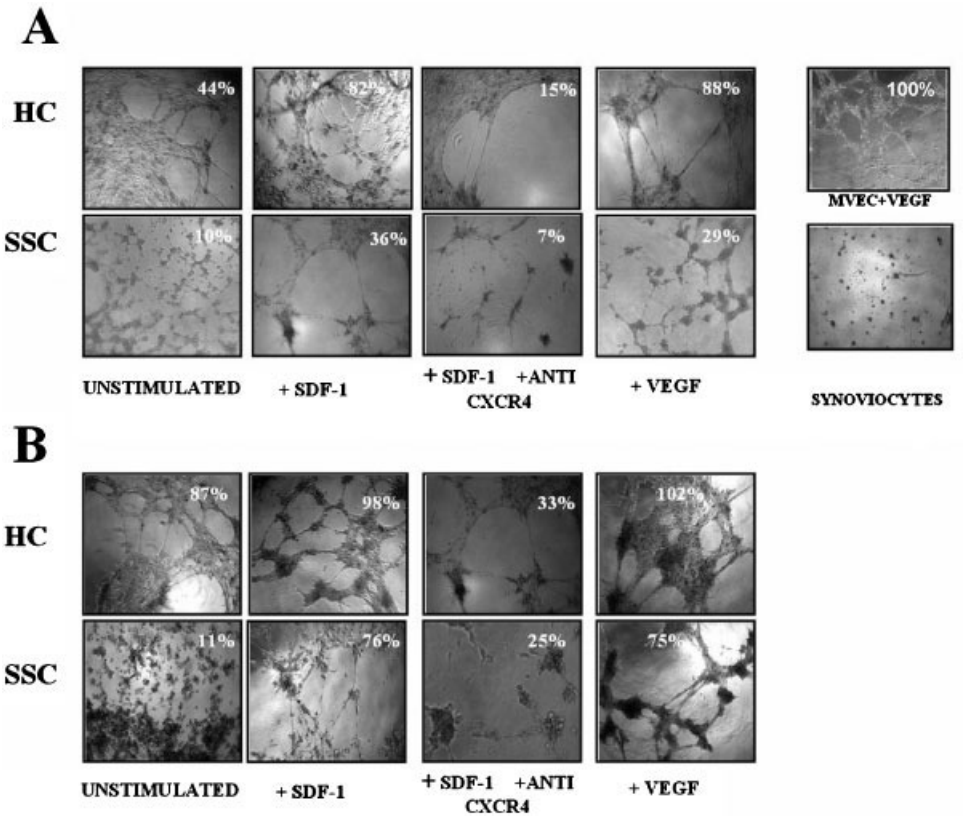

Figure 2. Light microscopic analysis of **A,** mesenchymal stem cell (MSC) and **B,** endothelial-like MSC (EL-MSC) capillary network formation on semisolid medium, both spontaneously and in the presence of vascular endothelial growth factor (VEGF) and stromal cell–derived factor 1 (SDF-1) as stimuli. Shown are photomicrographs of MSCs and EL-MSCs from a patient with systemic sclerosis (SSc) and from a healthy control (HC) subject. Results are representative of all experiments. Values in the upper right of each photomicrograph are the percentage of the well surface covered, with reference to microvascular endothelial cells (MVECs), as determined with the use of AngioSys software (see Patients and Methods for details). (Original magnification × 20.)

stronger in vitro ability to perform capillary morphogenesis after stimulation than did either the controls or the stimulated MSCs from SSc patients. Blocking the interaction of SDF-1 with CXCR4 reduced capillary morphogenesis (Figure 2B).

Endothelial cell morphology and telomerase assay results. SSc MSCs cultured in the presence of IMDM presented a flattened morphology, with an increase in vacuoles and cytoplasmic granules (Figure 3A), suggesting a senescent phenotype. The same cells in the presence of VEGF lost their intense intracytoplasmic granulation and developed a fusiform morphology, similar to control MSCs and resembling the morphology of cultured endothelial cells.

We measured the telomerase activity in MSCs and EL-MSCs at the third passage, after 14 days of culture. Telomerase activity in MSCs from SSc patients was significantly reduced as compared with that in MSCs from the controls (median 47 arbitrary units [range 21–57] versus 93 arbitrary units [range 49–96], respectively; $P < 0.05$). After endothelial differentiation, both subsets displayed decreased activity, with a stronger decrease in EL-MSCs from SSc patients as compared with those from controls (median 25 arbitrary units [range 12–36] versus 54 arbitrary units [range 37–66], respectively; $P < 0.05$) (Figures 3B and C).

DISCUSSION

In this study we provide evidence that the in vitro differentiative potential of MSCs in patients with SSc also includes EL-MSCs, but these cells display both an early senescence and decreased capacity to perform specific endothelial activities, such as capillary morpho-

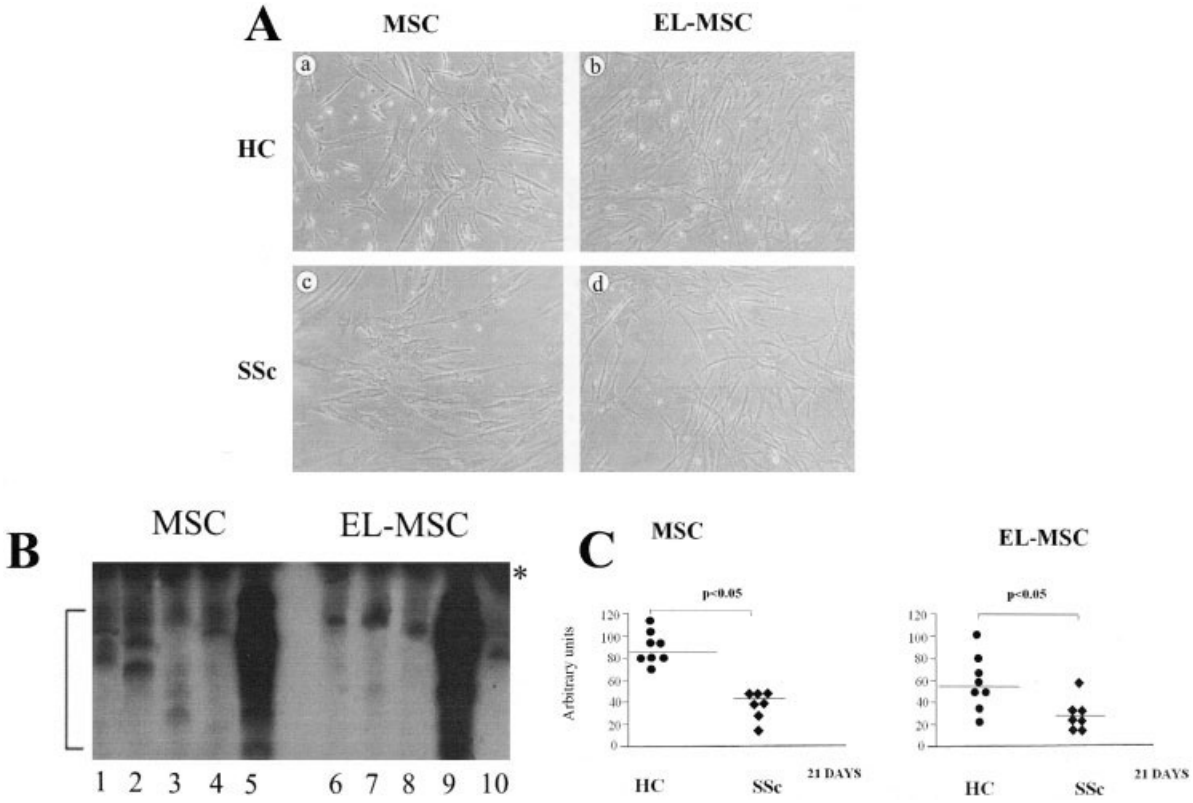

Figure 3. A, Phase-contrast microscopy of bone marrow–derived mesenchymal stem cells (MSCs) and endothelial-like MSCs (EL-MSCs). **a,** Healthy control (HC) MSCs grown in Iscove's modified Dulbecco's medium (IMDM) show a flattened morphology. **b,** Healthy control EL-MSCs show a fusiform morphology. **c,** Systemic sclerosis (SSc) patient MSCs grown in IMDM show a more prominent flattened morphology. **d,** SSc patient EL-MSCs subjected to specific culture show a more fusiform morphology compared with control cells. (See Patients and Methods for culture and treatment details.) (Original magnification × 30.) **B,** Telomerase activity was assessed by the telomeric-repeat amplification protocol (TRAP). Lanes 1 and 2, MSCs from 2 different control subjects; lanes 3 and 4, MSCs from 2 different SSc patients; lane 5, unrelated MSCs with stable expression of telomerase reverse transcriptase activity (positive control); lanes 6 and 7, EL-MSCs from the same 2 SSc patients; lanes 8 and 9, EL-MSCs from the same 2 healthy control subjects; lane 10, unrelated EL-MSCs with stable expression of telomerase reverse transcriptase activity (positive control). Results are representative of all experiments. The asterisk at right shows the polymerase chain reaction product that serves as the internal positive control for the reaction. The bracket at left shows the amplified telomeric repeats indicative of the presence of telomerase activity. **C,** Quantification of telomerase activity (expressed in arbitrary units), reflecting the ratio of the TRAP product ladder bands to the internal control band (see Patients and Methods for details). The activity of MSCs from SSc patients was significantly reduced as compared with that of MSCs from healthy controls. After stimulation with VEGF, the EL-MSCs showed a further decrease in activity, both in SSc patients and controls, with a significant reduction in SSc patients versus controls.

genesis and chemoinvasion. Furthermore, the possibility that this impairment can be partially reversed suggests that these cells might be helpful for future regenerative therapies.

Of note, although the basal number of MSCs expressing endothelial markers such as VEGFR-2 was lower in SSc patients, suggesting that this differentiative ability in vivo seems to be partially impaired, stimulation with a specific growth factor, such as VEGF, induced a significant overexpression of VEGFR-2 as compared with control MSCs. However, in SSc patients, increased levels of VEGF can be detected both in the sera and skin and, during the early phase of SSc, is inversely correlated with the number of pitting ulcers and the severity of Raynaud's phenomenon (17). Based on current limited knowledge of both vasculogenesis and angiogenesis, we cannot explain why only a very small portion of SSc MSCs constitutively display VEGFR-2, when persistently increased levels of VEGF may be demonstrated. We hypothesize that some local factors in the bone marrow might be involved. When MSCs from SSc patients were cultured in the presence of VEGF, almost half of the cells expressed VEGFR-2 on their surface, largely exceeding the expression observed on control cells.

It has been reported that MSCs constitutively display VEGFR-1 and that their ability to migrate in the presence of VEGF is strongly related to this receptor

(18). In SSc MSCs, we confirmed this constitutive expression, although lower levels were detectable as compared with controls.

Interestingly, SSc MSCs cultured in the presence of IMDM showed an increase in the number of vacuoles and cytoplasmic granules, suggesting an increased apoptotic rate and/or an early senescent phenotype. However, the same cells supplemented with 50 ng/ml of VEGF lost the intense intracytoplasmic granulation and assumed a fusiform morphology, similar to the phenotype of control MSCs. The increased number of vacuoles and cytoplasmic granules observed in SSc MSCs were not linked to increased apoptosis, which might be induced by the chronic hypoxia of SSc tissues (19), as demonstrated by the undetectable surface expression of annexin V under basal conditions as well as after VEGF stimulation.

Somatic cells undergo a finite number of cell divisions, ultimately entering a nondividing state of senescence (20). Loss of telomerase activity constitutes the molecular clock that triggers cellular senescence (21), and emerging evidence suggests that senescence is involved in MSC dysfunction (22,23). Intriguingly, MSCs from SSc patients displayed a significantly reduced telomerase activity after 21 days of culture, suggesting that these cells, although demonstrating regenerative potential, seem to be lineage-committed, with a possible predetermined lifespan. Furthermore, after specific stimulation, the cells displayed a further decrease in telomerase activity, similar to that of fully differentiated cells. These data suggest that MSCs from patients with SSc display early senescence, probably related to several pathologic stimuli encountered by these cells during their lifetimes, and VEGF might offer a preferential survival stimulus to the precommitted MSCs, which show normal morphology in culture with stimuli, and a possible loss of other differently precommitted cells, which do not use VEGF as their specific growth signal.

It was also recently shown that CXCR4, which is present on the surface of a small subset of human MSCs, is important in mediating the specific migration of these cells (24). In fact, SDF-1 and its specific ligand CXCR4 play an important role in the recruitment of cells in specific tissues, including bone marrow (25). SDF-1 also induces the migration of endothelial cells into tissues and regulates vascular remodeling, as recently shown in a rat model of myocardial infarction, in which SDF-1 may induce homing of bone marrow–derived stem cells into the injured myocardium (26). Therefore, the chemotactic interaction of SDF-1 and CXCR4 seems to facilitate MSC homing to hypoxic sites.

In this study, we showed a significantly lower expression of CXCR4 in SSc MSCs than in control MSCs. After differentiation into EL-MSCs, an increase in the surface expression of CXCR4 was observed both in SSc and control EL-MSCs. Of note, in SSc EL-MSCs, only a minority of the total VEGFR-2+ cells coexpressed CXCR4, whereas almost all control EL-MSCs simultaneously displayed both receptors. Recent studies have shown that MSCs may display lower levels of CXCR4 on their surface, with large amounts found intracellularly. Several cytokines and chemokines seem to be responsible for this phenomenon through posttranscriptional regulation (27). It remains to be elucidated whether intracellular storage and/or mobilization of the internalized receptor might be impaired in SSc MSCs. Consistent with these data, the migratory ability of SSc MSCs in response to SDF-1 seems to be impaired. In SSc, differentiation into EL-MSCs largely increased the chemotactic response to SDF-1–conditioned medium, thus implicating a functionally active CXCR4 receptor in the mediation of the migratory signal (24).

Among VEGF family members, it has been shown that VEGF-A induces a dose-dependent migratory response in human bone marrow MSCs, whereas VEGF-E, which mediates its effects via VEGFR-2, does not stimulate a chemotactic response in these cells (28). Our results confirm that VEGF-A induces relevant cell migration of MSCs and EL-MSCs, which, both in SSc patients and controls, display a functional VEGFR-1 to modulate their migratory activity. Furthermore, the chemotactic response of SSc MSCs and EL-MSCs to VEGF-A was significantly lower than of controls, probably due to the reduced expression of VEGFR-1 in these cells. In controls, VEGF-conditioned medium from EL-MSCs did not significantly increase migratory ability as compared with that of MSCs, although they showed a considerably increased surface expression of VEGFR-1 after differentiative stimulation.

It is well known that the majority of biologic effects of VEGF in mature endothelial cells, including migration, proliferation, and angiogenesis, are mediated primarily via VEGFR-2 (29), which suggests a different role for VEGF receptors on endothelial cells as compared with MSCs. It could be hypothesized that control EL-MSCs are in a relatively early stage of differentiation toward an endothelial lineage (30,31), given the lack of expression of CD31, features of mature endothelial cells, and lack of CD105-specific and CD166-specific markers of MSCs (32). Our preliminary results concerning the expression of vWF might confirm that this molecule can be expressed by MSCs after specific proendothelial

stimuli, although the reason for the differences observed among SSc patients is not clear.

Furthermore, these results support the hypothesis that EL-MSCs are in an early differentiative stage. In this context, the surface increase in VEGFR-1 might not mediate additional migratory stimuli in quiescent MSCs, as one would expect, nor would the observed increase in surface VEGFR-2 mediate a better chemotactic response, such as that seen in mature endothelial cells. The impairment of the migratory response to chemotactic stimuli of both MSCs and EL-MSCs from SSc patients suggests a defect in the recruitment of bone marrow–derived progenitor cells. Of note, the chemoinvasive activity of control MSCs, in the presence of VEGF as well as SDF-1, was not increased by differentiation into EL-MSCs. Instead, in SSc patients, EL-MSC differentiation significantly improved chemoinvasive performance after specific stimuli, although it did not reach the chemoinvasive activity of control cells.

When seeded on Matrigel, control MSCs spontaneously formed capillary-like structures, whereas SSc MSCs did not. Recent studies have shown that murine stromal cells can also differentiate into vasculature-forming cells under hypoxic conditions or when genetically transduced to express VEGF (33,34), and human bone marrow MSCs form tubular structures when cultivated in a semisolid medium. The presence of VEGF markedly enhances this behavior (34). In our study, VEGF and SDF-1 markedly increased the ability of control MSCs to form tubular structures, which were fewer and less organized in MSCs from SSc patients. After differentiation, a substantial formation of capillary structures was seen in control EL-MSCs, with minor changes after VEGF and SDF-1 stimulation. In SSc EL-MSCs, no endothelial network was observed, but after stimulation with VEGF and with SDF-1, a large improvement in angiogenic ability was observed.

Both VEGF and SDF-1 take part in a complex signaling system involved in the process of angiogenesis, vasculogenesis, and endothelial repair after damage. In SSc, only a minority of VEGFR-2+ cells simultaneously displayed CXCR4. VEGF stimulation did not reverse the expression of these receptors after differentiation into EL-MSCs; however, almost all control EL-MSCs displayed both receptors. This may explain the impaired ability of SSc cells to form capillary structures.

Recently, another group of investigators examined bone marrow stromal cells obtained from SSc patients for their ability to differentiate to mature endothelial cells (35). Unlike the findings of our study, those investigators found decreased clonogenic activity in MSCs from the SSc patients as compared with healthy controls. Although both our study and theirs included too few patients to allow definitive conclusions, our investigation was planned with the consideration that a strongly homogeneous population of patients (with early aggressive diffuse cutaneous SSc) referred to an autologous stem cell transplantation program, who had no clinical signs or symptoms of hematologic involvement, were the most eligible for transplantation. In contrast, no information about the clinical setting of the other study was reported.

With regard to the EL phenotype observed in MSCs, some findings of our study mirror those of the other study (35). However, 2 main results differentiate our study. First, we clearly demonstrated that, at least in patients with rapidly progressive diffuse cutaneous SSc, independently of the acquired phenotype, some of the endothelial functions of these endothelial-oriented cells are impaired, confirming that not only the phenotype, but also the function is pivotal to understanding the role of MSCs in the altered repair of SSc. Furthermore, the improvement after EL differentiation opens some possibilities for planning strategies for regenerative therapy, which MSCs seem to offer. Second, these cells are in early senescence, as shown by the decreased telomerase activity, and it is well known that aged human MSCs show a decline in differentiation potential as well as in the proliferation rate. These data support the previously proposed concept that the inevitable reduction of telomerase activity in human committed or stressed MSCs must be challenged and that the regenerative properties of "rejuvenated" MSCs might be enhanced, with consequent therapeutic implications (36,37).

In conclusion, we demonstrated an impairment of MSCs from SSc patients to acquire the full functions of mature endothelial cells, despite their endothelial phenotype. This finding may explain the difficulty to produce sufficient vasculogenesis in SSc. This evidence adds new insight into the pathogenesis of SSc, and the possibility of reversing this impairment opens new perspectives for regenerative cellular therapy for the vascular damage of this disease.

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AUTHOR CONTRIBUTIONS

Dr. Giacomelli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Cipriani, Tyndall, Giacomelli, Cerinic.

Acquisition of data. Cipriani, Guiducci, Cinelli, Miniati, Urbani, Marrelli, Dolo, Pavan, Saccardi.
Analysis and interpretation of data. Cipriani, Guiducci, Urbani, Marrelli, Giacomelli.
Manuscript preparation. Cipriani, Guiducci, Tyndall, Giacomelli, Cerinic.
Statistical analysis. Cipriani, Giacomelli.

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