ONLINE SUPPLEMENTARY MATERIAL

Manetti M, *et al.* **"Endothelial-to-mesenchymal transition contributes to endothelial dysfunction and dermal fibrosis in systemic sclerosis"**

MATERIALS AND METHODS

Cell culture and reagents

Primary cultures of dermal microvascular endothelial cells (dMVECs) were established by explantation from biopsies of the lesional forearm skin from 6 patients with early diffuse cutaneous systemic sclerosis (dcSSc; disease duration <2 years from first non-Raynaud symptom) [1] and from 6 healthy age-matched and gender-matched adult subjects under protocols approved by the Institutional Review Board of the Azienda Ospedaliero-Universitaria Careggi (AOUC), Florence, Italy, as described elsewhere [2,3]. At the time of biopsy, patients were not on immunosuppressive or disease-modifying drugs. Patient characteristics are summarised in online supplementary table S1. Skin biopsies were mechanically cleaned of epidermis and adipose tissue in order to obtain a pure specimen of vascularised dermis, and were processed as previously described [2,3]. Adherent cells were detached and subjected to CD31 immunomagnetic isolation by incubation with anti-CD31 conjugated-microbeads [2,3]. Isolated cells were further identified as endothelial cells by labelling with anti-factor VIII-related antigen (von Willebrand factor) and anti-CD105, followed by reprobing with anti-CD31 antibodies (see online supplementary figure S1). dMVECs from healthy subjects (H-dMVECs) and SSc patients (SSc-dMVECs) were maintained in MCDB 131 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 30% fetal bovine serum (FBS), 20 μg/ml endothelial cell growth supplement (Calbiochem, Nottingham, UK), 10 μg/ml hydrocortisone, 15 IU/ml heparin, and antibiotics in a humidified atmosphere of 5% CO_2 at 37°C, and used between the third and seventh passages in culture. In selected experiments, H-dMVECs were grown to confluence, and then were washed three times with serum-free medium and serum-starved overnight in MCDB 131 medium supplemented with 2% FBS. Medium was subsequently removed and cells were incubated with MCDB 131 medium containing 2% FBS and recombinant human transforming growth factor-β1 (TGFβ1) (10 ng/ml; PeproTech, Rocky Hill, NJ, USA) or 10% serum from early dcSSc patients (n=6) and healthy subjects (n=6) for 24, 48 and 72 hours. Each serum sample was tested individually. The medium was changed and additives replenished every day. In some experimental points, sera were preincubated with the matrix metalloproteinase-12 (MMP-12) specific inhibitor MMP408 (10 nM; Sigma-Aldrich) before cell stimulation. Phase-contrast images were obtained under a Leica inverted microscope (Leica Microsystems, Mannheim,

Germany) to assess cell morphology. The proportion of spindle-shaped cells relative to polygonal-shaped endothelial cells was assessed in at least ten randomly selected fields (x10 original magnification) per sample employing the ImageJ software (NIH, Bethesda, MD, USA). Cells with a diameter at their longest axis that was two-fold greater than the average diameter of untreated cobblestone H-dMVECs were considered spindle-shaped.

Fluorescence immunocytochemistry

H-dMVECs and SSc-dMVECs were seeded onto glass coverslips. In some experiments, H-dMVECs were treated as described above for 72 hours. At the end of the experiments, cells were fixed with 3.7% buffered paraformaldehyde and permeabilised with 0.1% Triton X-100 in phosphate buffered saline (PBS). Slides were washed with PBS and blocked with 1% bovine serum albumin in PBS for 1 hour at room temperature, and were then incubated overnight at 4°C with primary antibodies against CD31 (catalogue number ab9498; Abcam, Cambridge, UK) at 1:50 dilution, vascular endothelial (VE)-cadherin (catalogue number ab33168; Abcam) at 1:50 dilution, α-smooth muscle actin (α-SMA) (catalogue number ab7817; Abcam) at 1:100 dilution, S100A4/fibroblast-specific protein-1 (FSP1) (catalogue number ab124805; Abcam) at 1:100 dilution, type I collagen (catalogue number ab90395; Abcam) at 1:100 dilution, or Snail1 (catalogue number ab167609; Abcam) at 1:50 dilution, followed by incubation for 45 minutes at room temperature in the dark with Alexa Fluor-488-conjugated or Rhodamine Red-X-conjugated secondary antibodies at 1:200 dilution (Invitrogen, Carlsbad, CA, USA). Double immunofluorescence staining was performed by mixing mouse anti-α-SMA (1:100 dilution; catalogue number ab7817; Abcam) and rabbit anti-CD31 (1:20 dilution; catalogue number ab28364; Abcam) primary antibodies and subsequently mixing fluorochrome-conjugated secondary antibodies. Irrelevant isotype-matched and concentration-matched mouse and rabbit IgG (Sigma-Aldrich) were used as negative controls. In some specimens, Alexa 488-labelled phalloidin (1:40 dilution; Invitrogen) was applied to the cells to visualise the arrangement of the F-actin cytoskeleton. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Immunolabelled cells were examined with a Leica DM4000 B microscope (Leica Microsystems) and fluorescence images were captured with a Leica DFC310 FX 1.4-megapixel digital colour camera equipped with the Leica software application suite LAS V3.8 (Leica Microsystems).

RNA isolation and quantitative real-time PCR

At the end of the experiments, cultures were harvested, and total RNA was isolated using the RNeasy Micro Kit (Qiagen, Milan, Italy). First strand cDNA synthesis and mRNA quantification by

SYBR Green real-time PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, Milan, Italy) were performed as reported elsewhere [3]. Predesigned oligonucleotide primer pairs were obtained from Qiagen (QuantiTect Primer Assay). The assay IDs were Hs_PECAM1_1_SG (CD31; catalogue number QT00081172), Hs_CDH5_1_SG (VE-cadherin; catalogue number QT00013244), Hs_ACTA2_1_SG (α-SMA; catalogue number QT00088102), Hs_S100A4_1_SG (catalogue number QT00014259), Hs_COL1A1_1_SG (catalogue number QT00037793), Hs_COL1A2_1_SG (catalogue number QT00072058), Hs_SNAI1_1_SG (Snail1; catalogue number QT00010010), Hs_FLI1_1_SG (catalogue number QT00078372), and Hs_RPS18_1_SG (catalogue number QT00248682). Ribosomal protein S18 (RPS18) mRNA was measured as an endogenous control to normalise for the amounts of loaded cDNA. Differences were calculated with the threshold cycle (Ct) and comparative Ct method for relative quantification. All measurements were performed in triplicate.

Immunoblotting

Whole cell protein lysates from dMVECs were subjected to immunoblot analysis as described elsewhere [3]. The following antibodies were used at 1:1000 dilution: anti-CD31 (catalogue number ab9498; Abcam), anti-VE-cadherin (catalogue number ab33168; Abcam), anti-α-SMA (catalogue number ab7817; Abcam), anti-S100A4/FSP1 (catalogue number ab124805; Abcam), anti-type I collagen (catalogue number ab90395; Abcam), anti-Snail1 (catalogue number ab167609; Abcam), anti-Friend leukemia integration-1 (Fli1) (catalogue number ab180902; Abcam), and anti-α-tubulin (catalogue number #2144; Cell Signaling Technology, Danvers, MA, USA). Anti-urokinase-type plasminogen activator receptor (uPAR) domain 1 (D1) (catalogue number 3931; American Diagnostica, Stamford, CT, USA) and anti-uPAR domain 2 (D2) (catalogue number 3932; American Diagnostica) antibodies were used at 1:200 dilution. Immunodetection was performed using the Western Breeze Chromogenic Western Blot Immunodetection Kit (Invitrogen). Band intensities were quantified with the ImageJ software (NIH) and normalised with α-tubulin in each sample.

Collagen gel contraction assay

Collagen gel contraction assays were performed using the CytoSelect™ 24-Well Cell Contraction Assay Kit (Floating Matrix Model; catalogue number CBA-5020; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. H-dMVECs and SSc-dMVECs were harvested, pelleted and resuspended in serum-free medium at 5×10^6 cells/ml. In some experimental points, H-dMVECs were treated with recombinant human TGFβ1 (10 ng/ml; PeproTech) or 10% serum from

early dcSSc patients (n=6) and healthy subjects (n=6) for 72 hours before the assays. For each assay, 100 µl of the cell suspension was mixed with 400 µl of cold neutralised collagen gel solution and subsequently added to one well of the adhesion resistant matrix-coated 24-well cell contraction plate. Gels were allowed to solidify for 1 hour at 37°C and 5% CO_2. After polymerisation, 1 ml of basal media or media containing different stimuli (i.e. TGFβ1, early dcSSc sera and healthy sera) was added to the top of each collagen gel lattice. Gels without cells were included as negative controls. Each experimental point was performed in triplicate. After 24 hours, the culture dish was scanned and the area of each collagen gel was measured by ImageJ software (NIH).

Enzyme-linked immunosorbent assay

Levels of MMP-12 in serum samples were measured by commercial quantitative colorimetric sandwich enzyme-linked immunosorbent assay (Human Matrix Metallopeptidase 12 ELISA Kit; Antibodies-online, Atlanta, GA, USA) according to the manufacturer's protocol. The detection range was 0.156-10 ng/ml. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer. Each sample was measured in duplicate.

Fluorescence immunohistochemistry on human and mouse skin

To assess the presence of endothelial-to-mesenchymal transition (EndoMT) in the skin, paraffin-embedded sections of lesional forearm skin biopsies were obtained from 12 SSc patients (10 women and 2 men; n=4 with limited cutaneous SSc and n=8 with dcSSc) [1] and 10 age- and sex-matched healthy donors, as described elsewhere [2,3]. Biopsies were obtained under protocols approved by the Institutional Review Board of the AOUC, Florence, Italy. After antigen retrieval, quenching of autofluorescence and blocking of nonspecific binding sites [4], skin sections (5 µm thick) were examined by double-label immunofluorescence using antibodies against α-SMA (1:50 dilution; catalogue number ab7817; Abcam) and CD31 (1:50 dilution; catalogue number ab28364; Abcam) or VE-cadherin (1:50 dilution; catalogue number ab33168; Abcam), followed by fluorochrome-conjugated secondary antibodies (Invitrogen) as well as DAPI to identify nuclei. Negative controls stained without primary antibody were used to confirm specificity. Images were acquired on a Leica DM4000 B microscope (Leica Microsystems) equipped with a Leica DFC310 FX 1.4-megapixel digital colour camera and the Leica software application suite LAS V3.8 (Leica Microsystems). The percentage of dermal vessels displaying CD31/α-SMA and VE-cadherin/α-SMA colocalisation was determined in five randomly selected high-power fields (hpf; x40 original magnification) of the dermis from each of three sections per sample by two independent blinded observers. To examine the presence of EndoMT in vivo, skin sections from two mouse models of dermal fibrosis were

used. First, 6 week-old male C57BL/6 mice (Charles River Laboratories, Calco, Lecco, Italy) received subcutaneous injections of 100 μl of bleomycin dissolved in 0.9% NaCl (saline solution) at a concentration of 0.5 mg/ml every other day for 4 weeks in well-defined areas (1 cm^2) of the upper back. Subcutaneous injections of 0.9% NaCl served as controls [5]. The second model consisted of 12 week-old male uPAR-deficient mice and wild-type littermates as described elsewhere [6,7]. All animal protocols were performed in accordance with DL 116/92 and approved by the Institutional Animal Care and Use Committee of the University of Florence. Each experimental group consisted of at least six mice. At the end of the experiments, mice were anesthetised intraperitoneally with cloralium hydrate (400 mg/kg) and sacrificed by cervical dislocation. Lesional skin was harvested, and double immunofluorescence using antibodies against α-SMA (1:50 dilution; catalogue number ab7817; Abcam) and CD31 (1:50 dilution; catalogue number ab28364; Abcam) or VE-cadherin (1:50 dilution; catalogue number ab33168; Abcam), followed by incubation with Alexa Fluor-488-conjugated and Rhodamine Red-X-conjugated IgG (Invitrogen) and DAPI, was performed. Irrelevant IgG were used as negative controls. Sections were imaged at x40 original magnification at five randomly selected hpf spanning the dermis under a Leica DM4000 B microscope (Leica Microsystems). The proportion of vessels with CD31/α-SMA and VE-cadherin/α-SMA colocalisation was scored in at least five hpf of the dermis from each of three sections per mouse by two independent blinded observers.

Transmission electron microscopy

Ultrathin sections (~70 nm thick) from skin biopsies from 5 dcSSc patients and 5 healthy controls were examined and photographed under a JEOL JEM-1010 electron microscope (Jeol, Tokyo, Japan) equipped with a MegaView III high-resolution digital camera and imaging software (Jeol), as described elsewhere [8]. At least eight capillary vessels from each of three ultrathin sections per sample were analysed.

Statistical analysis

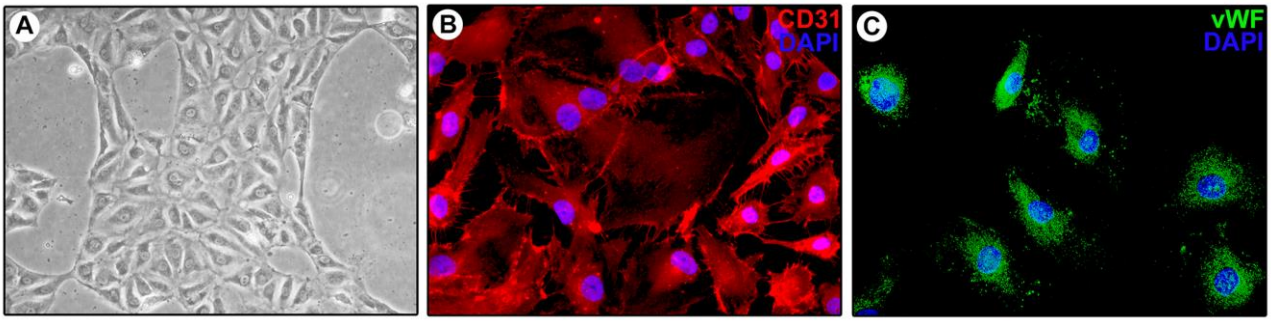
Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software for Windows, V.20.0 (SPSS, Chicago, IL, USA). Data are expressed as means and standard errors of the mean (SEM). The Student's t-test was used for statistical evaluation of the differences between two independent groups. A p value of <0.05 according to a two-tailed distribution was considered statistically significant.

REFERENCES

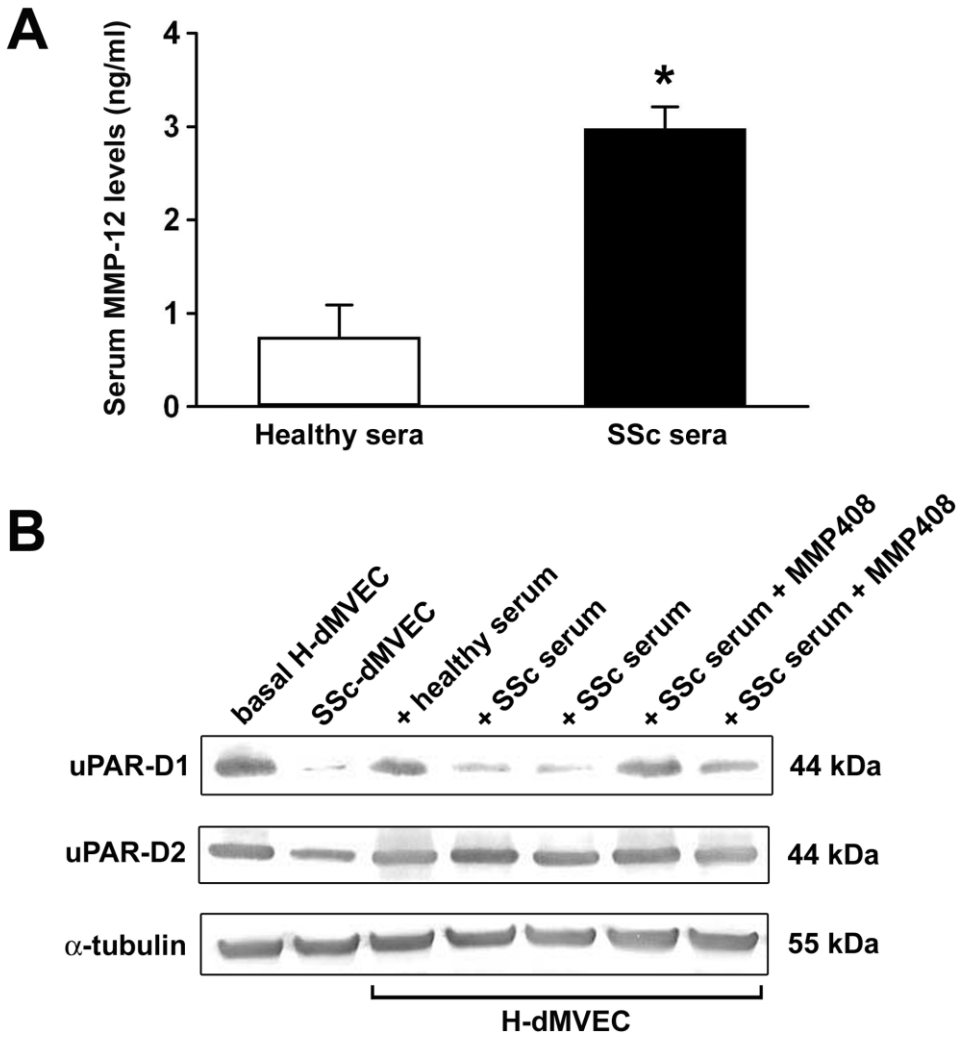
1. van den Hoogen F, Khanna D, Fransen J, *et al.* 2013 classification criteria for systemic sclerosis: an American college of rheumatology/European league against rheumatism collaborative initiative. *Ann Rheum Dis* 2013;72:1747-55.
2. Manetti M, Guiducci S, Romano E, *et al.* Overexpression of VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, leads to insufficient angiogenesis in patients with systemic sclerosis. *Circ Res* 2011;109:e14-26.
3. Romano E, Chora I, Manetti M, *et al.* Decreased expression of neuropilin-1 as a novel key factor contributing to peripheral microvasculopathy and defective angiogenesis in systemic sclerosis. *Ann Rheum Dis* 2016;75:1541-9.
4. Manetti M, Guiducci S, Romano E, *et al.* Decreased expression of the endothelial cell-derived factor EGFL7 in systemic sclerosis: potential contribution to impaired angiogenesis and vasculogenesis. *Arthritis Res Ther* 2013;15:R165.
5. Distler JH, Jüngel A, Huber LC, *et al.* Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. *Arthritis Rheum* 2007;56:311-22.
6. Manetti M, Rosa I, Milia AF, *et al.* Inactivation of urokinase-type plasminogen activator receptor (uPAR) gene induces dermal and pulmonary fibrosis and peripheral microvasculopathy in mice: a new model of experimental scleroderma? *Ann Rheum Dis* 2014;73:1700-9.
7. Manetti M, Rosa I, Fazi M, *et al.* Systemic sclerosis-like histopathological features in the myocardium of uPAR-deficient mice. *Ann Rheum Dis* 2016;75:474-8.
8. Manetti M, Guiducci S, Ruffo M, *et al.* Evidence for progressive reduction and loss of telocytes in the dermal cellular network of systemic sclerosis. *J Cell Mol Med* 2013;17:482-96.

Supplementary table S1. Demographic and clinical characteristics of patients with early diffuse cutaneous systemic sclerosis enrolled for isolation of dermal microvascular endothelial cells and collection of serum samples.

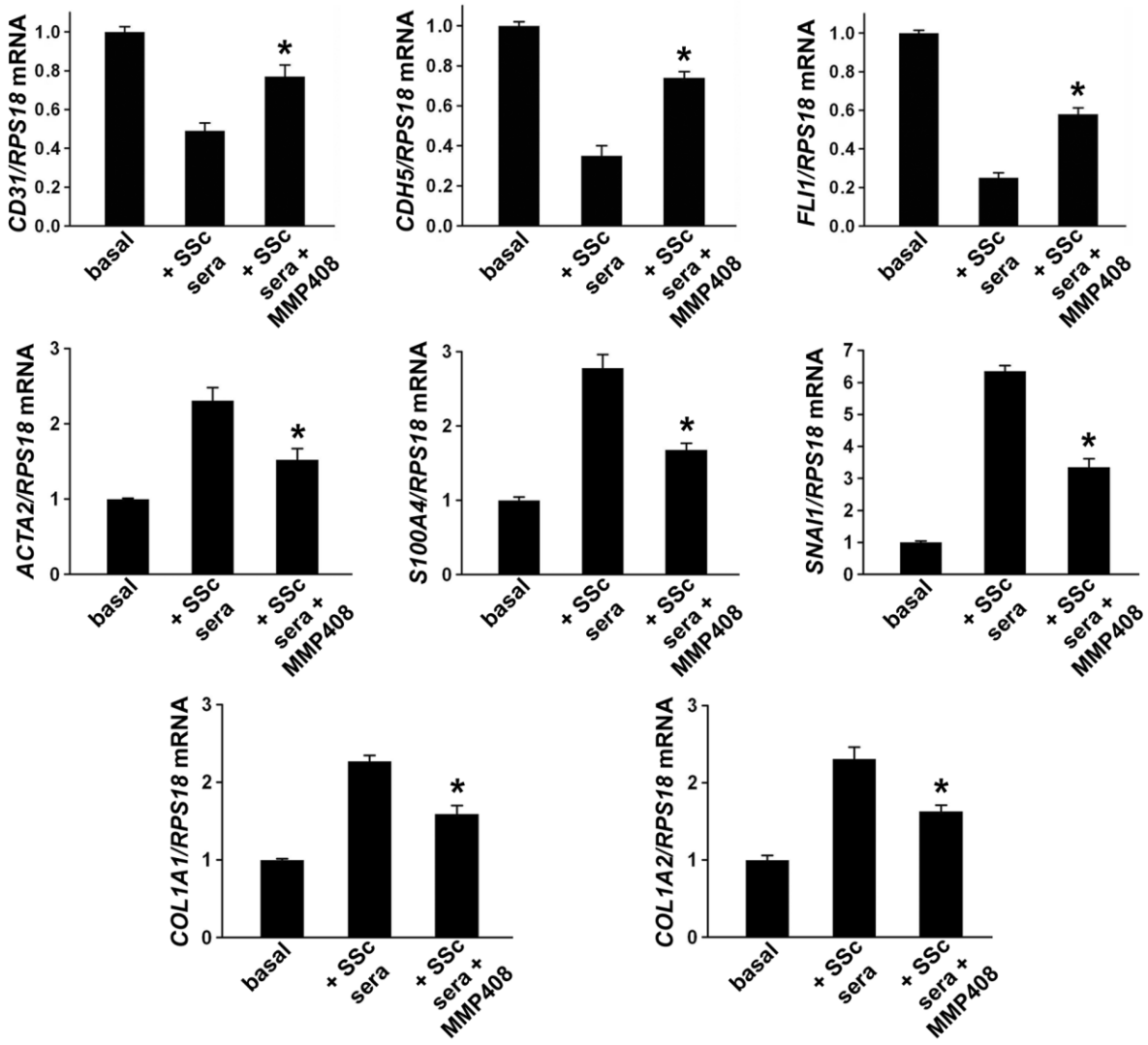
Characteristics	**Patients (n = 6)**
Mean age, years (range)	37.5 (26-49)
Gender, male/female	1/5
Mean disease duration, months (range)	15.5 (10-22)
Antinuclear antibodies, n	6
Anti-topoisomerase I antibodies, n	4
Mean modified Rodnan skin score (range)	16.2 (10-21)



Supplementary figure S1. (A) Representative phase-contrast microphotograph of dermal microvascular endothelial cells (dMVECs) isolated from forearm skin biopsies and subjected to CD31 immunomagnetic isolation by incubation with anti-CD31 conjugated-microbeads. (B and C) Representative fluorescence microphotographs of purified dMVECs immunostained for the endothelial cell markers CD31 (red) (B) and von Willebrand factor (vWF; green) (C). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue).



Supplementary figure S2. (A) Levels of matrix metalloproteinase-12 (MMP-12) in serum samples from healthy subjects (n=6) and systemic sclerosis (SSc) patients (n=6) measured by quantitative colorimetric sandwich enzyme-linked immunosorbent assay. Data are mean±SEM. *$p<0.05$ versus healthy sera. (B) Protein levels of urokinase-type plasminogen activator receptor (uPAR) domain 1 (uPAR-D1) and uPAR domain 2 (uPAR-D2) in dermal microvascular endothelial cells (dMVECs) from healthy subjects (H-dMVECs) and SSc patients (SSc-dMVECs) at basal conditions, and in H-dMVECs treated for 24 hours with sera from healthy subjects (n=6), sera from SSc patients (n=6) or SSc sera (n=6) preincubated with the MMP-12 specific inhibitor MMP408. Treatment of H-dMVECs with SSc sera results in uPAR-D1 cleavage similarly to SSc-dMVECs. Healthy sera do not affect uPAR integrity in H-dMVECs. uPAR-D1 cleavage is effectively prevented by preincubation of SSc sera with MMP408. Representative immunoblots are shown. Molecular weight values (kDa) are indicated. Protein expression of α-tubulin was measured as a loading control. Results are representative of three independent experiments performed with three H-dMVEC and three SSc-dMVEC lines.



Supplementary figure S3. Preincubation with the matrix metalloproteinase-12 specific inhibitor MMP408 effectively attenuates the effects of sera from patients with systemic sclerosis (SSc) on mRNA expression levels of endothelial and mesenchymal cell markers in healthy dermal microvascular endothelial cells (H-dMVECs). H-dMVECs were treated for 48 hours with sera from SSc patients (n=6), preincubated or not preincubated with MMP408, and subsequently assayed for mRNA expression levels of *CD31*, *CDH5* (VE-cadherin), *FLI1*, *ACTA2* (α-SMA), *S100A4*, *SNAI1* (Snail1), *COL1A1* and *COL1A2* genes by quantitative real-time PCR. Ribosomal protein S18 (*RPS18*) mRNA was measured as an endogenous control for normalisation. The relative values compared with basal H-dMVECs are expressed as mean±SEM of three independent experiments performed with three H-dMVEC lines. *p<0.05 versus H-dMVECs treated with SSc sera not preincubated with MMP408.