Vascular Biology, Atherosclerosis and Endothelium Biology

Endothelial Fli1 Deficiency Impairs Vascular Homeostasis

A Role in Scleroderma Vasculopathy

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Systemic sclerosis or scleroderma (SSc) is a complex autoimmune connective tissue disease characterized by obliterative vasculopathy and tissue fibrosis. The molecular mechanisms underlying SSc vasculopathy are largely unknown. Friend leukemia integration factor 1 (Fli1), an important regulator of immune function and collagen fibrillogenesis, is expressed at reduced levels in endothelial cells in affected skin of patients with SSc. To develop a disease model and to investigate the function of Fli1 in the vasculature, we generated mice with a conditional deletion of Fli1 in endothelial cells (Fli1 CKO). Fli1 CKO mice showed a disorganized dermal vascular network with greatly compromised vessel integrity and markedly increased vessel permeability. We show that Fli1 regulates expression of genes involved in maintaining vascular homeostasis including VE-cadherin, platelet endothelial cell adhesion molecule 1, type IV collagen, matrix metalloproteinase 9, platelet-derived growth factor B, and SIP₁ receptor. Accordingly, Fli1 CKO mice are characterized by down-regulation of VE-cadherin and platelet endothelial cell adhesion molecule 1, impaired development of basement membrane, and a decreased presence of α -smooth muscle actin-positive cells in dermal microvessels. This phenotype is consistent with a role of Fli1 as a regulator of vessel maturation and stabilization. Importantly, vascular characteristics of Fli1 CKO mice are recapitulated by SSc microvasculature. Thus, persistently reduced levels of Fli1 in endothelial cells may play a critical role in the development of SSc vasculopathy. (*Am J Pathol* 2010, 176:1983–1998; DOI: 10.2353/ajpath.2010.090593)

Systemic sclerosis or scleroderma (SSc) is a complex autoimmune connective tissue disease characterized by obliterative vasculopathy and fibrosis of the skin and internal organs.^{1,2} Numerous vascular abnormalities have been documented in SSc, with ultrastructural studies performed >40 years ago demonstrating a decrease in the number of normal capillaries, microvascular endothelial cell swelling, and increase in thickness and reduplication of the capillary basement membrane (BM).^{3,4} Morphological alterations in dermal microvessels are observed in the nailfold capillaries⁵ where they appear to reflect the severity of skin and internal organs affected by SSc.⁶ Disease progression is characterized by a reduction in the number of capillaries and severe morphological changes in the vessels occurring in parallel with tissue fibrosis. Although there is evidence for new capillary growth taking place in SSc lesions,⁷ vessel regression prevails. The mechanisms underlying the pathological changes in the SSc blood vessels are unclear. Current hypotheses suggest that apoptosis of endothelial cells, which may be caused by infectious agents, antienothelial cell autoantibodies, or cytotoxic T cells, contribute to vessel degeneration.¹ However, a recent comprehensive study of SSc skin vasculature found no evidence of endothelial cell death.⁸ The authors of the latter study have shown that SSc vessels display an “antiangiogenic phenotype” characterized by reduced levels of VE-cadherin, activation of the interferon- α signaling pathway, and elevated levels of the pericyte (PC) marker, Rgs5. Other proposed mechanisms contributing to endothelial

Supported in part by grants from National Institutes of Health AR042334 and PO1 CA78582. Y.A. was supported by a postdoctoral fellowship from Japan Society for the Promotion of Science.

Accepted for publication December 1, 2009.

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cell injury in SSc involve the production of nitric oxide-related free radicals⁹ or granzyme.¹⁰ Additional studies suggest that insufficient vascular repair due to impairment of vasculogenesis may also be a contributing factor.¹¹ Interestingly, analyses of the microvascular cells isolated from SSc skin suggest that endothelial cells themselves may be defective.¹²

Activation of microvascular PCs has also been reported in early SSc and autoimmune Raynaud's phenomenon.^{13,14} In SSc lesions, PCs were characterized by the expression of platelet-derived growth factor (PDGF) β receptors and high molecular weight-melanoma-associated antigen, a marker for activated PCs. Interestingly, previous studies in dermal scarring have shown that, *in vivo*, microvascular PCs that express both high molecular weight-melanoma-associated antigen and PDGF β receptors can migrate from the microvasculature and undergo a gradual phenotypic transition to collagen-synthesizing fibroblasts.¹⁵ Taken together, these studies suggest that endothelial cells and PCs both undergo changes during early stages of SSc. Presently, however, the mechanism(s) responsible for the progressive microvessel injury in SSc remains elusive. An impairment of neoangiogenesis despite tissue ischemia and increased amounts of proangiogenic factors, including vascular endothelial growth factor (VEGF),¹⁶ represents a major unresolved issue in understanding the pathogenesis of SSc.

Friend leukemia integration factor 1 (Fli1) is a member of the Ets family of transcription factors characterized by the presence of the evolutionary conserved DNA-binding (ETS) domain, which recognizes the purine-rich GGA/T core sequence.¹⁷ Fli1 is preferentially expressed in hematopoietic cell lineages,¹⁸ and it is known to play a key role in megakaryocytic differentiation.^{19,20} Fli1 is also involved in myelomonocytic, erythroid, natural killer, and B cell development.^{21,22} Although Fli1 expression in dermal fibroblasts is relatively low, recent studies have shown that Fli1 plays a pivotal role in the regulation of extracellular matrix genes, including type I collagen^{23–26} and the multifunctional matricellular factor CTGF/CCN2.²⁷ Importantly, Fli1 has been shown to be a potent inhibitor of collagen biosynthesis in dermal fibroblasts and its aberrant expression has been implicated in the pathogenesis of cutaneous fibrosis in SSc.^{25,28} The role of Fli1 in the vasculature has not been fully characterized²⁹; however, recent studies of zebrafish and *Xenopus* embryos have shown that Fli1 functions as a master regulator of the transcriptional network driving blood and endothelial cell lineages.³⁰ Consistent with the role of Fli1 in vascular development, mice with targeted deletion of Fli1 gene die at 11.5 days post coitum (dpc) as a result of cranial and spinal hemorrhages.³¹ In humans, Fli1 is expressed in the healthy skin microvasculature; however, its presence is greatly reduced in endothelial and periendothelial cells in SSc skin.²⁵

Despite intensive studies, the causes of endothelial cell dysfunction in SSc are not well understood. The absence of an animal model that recapitulates the major features of SSc vasculopathy has hindered progress in this area. Given the important regulatory role of Fli1 during vascular development and the marked reduction of

Fli1 expression in the vascular compartment in SSc skin, we investigated the role of Fli1 in adult skin vasculature *in vivo*. Because of the early lethality of Fli1 null mice, we generated mice with a conditional deletion of Fli1 in endothelial cells, and we show that Fli1 is a critical regulator of vascular homeostasis in the skin. Importantly, vascular defects observed in SSc vasculature are also reproduced in these mice, supporting the notion that conditional Fli1-deficient mice may represent a useful model to investigate the molecular mechanisms involved in SSc vasculopathy.

Materials and Methods

Generation of Endothelial Cell-Specific Fli1-Knockout Mice

To generate Fli1 CKO mice, Fli1 genomic clones were isolated from a mouse 129SvEv bacterial artificial chromosome (BAC) library (CHORI-Children's Hospital Oakland Research Institute, Oakland, CA, BACPAC resources, RP-22). The Apal-XhoI fragment containing exons 3 and 4 was subcloned into a pZero-2 vector (Invitrogen, Carlsbad, CA). An frt-neo-frt-loxP cassette (vector provided by Dr. R. Depinho, Harvard University, Cambridge, MA) was inserted into the EcoRI site downstream of exon 4.

A second loxP element was cloned into the Scal site just upstream of exon 3. The correct orientation of the inserted elements was confirmed by DNA sequencing. The targeting vector was electroporated into embryonic stem (ES) cells originating from the 129/Ola mouse strain (R. J. Thresher, University of North Carolina Animal Models Core Facility, Chapel Hill, NC). Using long-range PCR and Southern blotting, two out of 96 ES clones were identified as homologous recombinants also harboring the distal loxP element relative to the selection marker. To remove the neo cassette, the cells were transfected with a Flpe expression plasmid. Out of the four G418-sensitive ES clones, one showed the expected gene structure. ES cells from this line were injected into BL6 blastocysts, and 20 chimeric mice were obtained. Mice were backcrossed to B6 for at least six generations before use in the proposed *in vivo* studies.

Mice expressing the Cre recombinase under the control of the endothelium-specific Tie2 (Tek) receptor promoter were purchased from The Jackson Laboratory (Bar Harbor, ME) (B6.Cg-Tg(Tek-cre)12Fiv/J) and crossed with Fli1^{fllox/fllox} mice.

Patients

The study group consisted of seven patients with diffuse cutaneous SSc and seven healthy volunteers. Biopsy specimens were obtained from dorsal forearm of SSc patients with diffuse cutaneous disease and from age, race, and gender matched healthy donors, on informed consent and in compliance with the Institutional Review Board. All patients fulfilled the criteria of the American College of Rheumatology for diffuse cutaneous SSc.³²

Reagents and Antibodies

Antibodies for Fli1 (C-19), VE-cadherin (C-19), platelet endothelial cell adhesion molecule 1 (PECAM1) (M-20), matrix metalloproteinase (MMP)-2 (C-19), MMP-9 (C-20), PDGFB (H-55), S1P₁ (P-20), N-cadherin (H-63), and Tie-2 (H-176) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-type IV collagen antibody (PRO10760) was purchased from Fitzgerald Industries International, Inc. (Concord, MA). Anti-glyceraldehyde-3-phosphate dehydrogenase antibody (MAB374) was obtained from Millipore (Bedford, MA). Anti- α -smooth muscle actin (α -SMA) antibody was purchased from NeoMarkers (Freemont, CA). FITC-dextran was obtained from Sigma-Aldrich (St. Louis, MO). Mustard oil and mineral oil were obtained from Tattva's Herbs (Seattle, WA) and Fisher Scientific (Pittsburgh, PA), respectively.

Immunohistochemistry

Skin samples were obtained from the back skin of 3-month-old mice using a 6-mm-diameter punch biopsy device. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Five-micrometer-thick sections were mounted on APES (aminopropyl-triethoxy-silane)-coated slides, deparaffinized with histoclear, and rehydrated through a graded series of ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide for 30 minutes. The sections were then incubated with indicated antibodies diluted with blocking buffer overnight at 4°C, followed by the incubation with biotinylated secondary antibody. The concentration of each primary antibody was first tested to determine the optimal sensitivity range. The following antibody dilutions were used for immunostaining: VE-cadherin 1/100, PECAM1 1/400, type IV collagen 1/100, and α -SMA 1/1600. The immunoreactivity was visualized with diaminobenzidine and the sections were counterstained with hematoxylin or methyl green. Arteries, capillaries, and venules were distinguished based on their histological features. Arteries were determined based on the presence of internal elastic lamina and tunica media and bulged nucleus into the lumen. Capillaries and venules were determined based on their diameter. Blood vessels with diameter almost equal to or less than that of erythrocyte were classified as capillaries, and the others were classified as venules. The determination of blood vessel type was performed by two independent dermatologists (T. Hattori and Y. Asano).

Immunofluorescence

Paraffin-embedded sections were deparaffinized and rehydrated as described above. Then, sections were incubated with anti-type IV collagen antibody diluted with blocking buffer overnight at 4°C. After washing with PBS, sections were incubated with FITC-conjugated secondary antibody and positive signals were detected under fluorescent microscope.

Visualization of Subcutaneous Vascular Network with FITC-Dextran Injection

Mice were anesthetized, and 200 μ l of FITC-conjugated dextran (2000 kDa, 20 mg/ml in PBS) was injected directly into the heart through a 28-gauge needle. After 5 minutes, mice were sacrificed and a full thickness piece of back skin (4 $\times$ 2 cm) was prepared. The skin specimen was placed directly on the slide (epidermis side up) and the structure of vascular network in the skin was visualized by fluorescence microscopy.

Cell Culture

Human dermal microvascular endothelial cells were purchased from Cambrex (East Rutherford, NJ). Mouse dermal microvascular endothelial cells (MDMECs) were isolated from the ear dermis of 5- to 8-week-old mice as described previously.^{33,34} Endothelial cells were cultured on collagen-coated tissue culture plates in endothelial cell basal medium-2 (Cambrex) supplemented with endothelial cell growth media-2 SingleQuots (human VEGF, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor-1, ascorbic acid, gentamicin, and heparin; Cambrex) and 5% heat-inactivated FBS. Experiments were conducted with human dermal microvascular endothelial cells and MDMECs in passages 3–5 and in passage 1, respectively. Cultured cells were characterized by a cobblestone appearance and specific staining for VE-cadherin and PECAM1.

RNA Isolation, Quantitative RT-PCR

RNA isolation and quantitative RT-PCR were performed as described previously. Briefly, 2 μ g of RNA isolated from cells using Tri reagent (Molecular Research Center, Inc., Cincinnati, OH) was reverse transcribed in 20 μ l reaction volume using random primers and Transcriptor First Strand synthesis kit (Roche, Basel, Switzerland). Real-time quantitative PCR was performed using Sybr green master mix (Bio-Rad, Hercules, CA) on iCycler machine (Bio-Rad) in triplicates. The sequence of primers for each target gene is described in Table 1. PCR conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds and 58°C for 1 minute. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

Immunoblotting

Whole-cell extracts were prepared from MDMECs using lysis buffer with the following composition: 1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 3 mmol/L MgCl₂, 1 mmol/L CaCl₂, proteinase inhibitor mixture (Roche), and 1 mmol/L phenylmethyl sulfonyl fluoride. Protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight with primary antibody, washed, and incubated for 1 hour with secondary antibody. After

Table 1. Primers for Quantitative Real-Time PCR

m-Fli1	Forward:	5'-ACTTGGCCAAATGGACGGGACTAT-3'
	Reverse:	5'-CCCGTAGTCAGGACTCCCG-3'
m-VE-cadherin	Forward:	5'-GTCAAGTTTGCCCTGAAGAA-3'
	Reverse:	5'-GTGATGTTGGCGGTGTTGT-3'
m-PECAM-1	Forward:	5'-CGGTGTTACGCGAGATCC-3'
	Reverse:	5'-CGACAGGATGGAAATCACA-3'
m-COL4A1	Forward:	5'-TTAAAGGACTCCAGGGACCAC-3'
	Reverse:	5'-CCCCTGAGCCTGTACAC-3'
m-MMP-2	Forward:	5'-AACTTTGAGAAGGATGGCAAGT-3'
	Reverse:	5'-TGCCACCCATGGTAAACAA-3'
m-MMP-9	Forward:	5'-ACGACATAGACGGCATCCA-3'
	Reverse:	5'-GCTGTGGTTTCACTTGTGGTG-3'
m-PDGF-B	Forward:	5'-CGGCTGTGACTAGAAAGTCC-3'
	Reverse:	5'-GAGCTTGAGGCGTCTTG-3'
m-S1P1	Forward:	5'-CGGTGTAGACCCAGAGTCCT-3'
	Reverse:	5'-AGCTTTTCTCTTGGCTGGAG-3'
m-N-cadherin	Forward:	5'-CCTCCATGTGCCGGATAG-3'
	Reverse:	5'-CACCAGAAGCCTCCACAGAC-3'
m-Tie2	Forward:	5'-CATAGGAGAAACCTGTTTACC-3'
	Reverse:	5'-CCCACTTCTGAGCTTCACATC-3'
h-Fli1	Forward:	5'-GGATGGCAAGGAAGTGTGTA-3'
	Reverse:	5'-GGTTGTATAGGCCAGCAG-3'
h-VE-cadherin	Forward:	5'-AAGCCTCTGATTGGCACAGT-3'
	Reverse:	5'-CTGGCCCTTGTCACTGGT-3'
h-PECAM-1	Forward:	5'-AGAGTACCAGCTGTTGGTGGA-3'
	Reverse:	5'-CACCTTGGATGGCCTCTTT-3'
h-COL4A1	Forward:	5'-CGGTATCCAGGACTCATAG-3'
	Reverse:	5'-GGACCTGCTTACCCCTTTTC-3'
h-MMP-2	Forward:	5'-TACGATGACGACCGCAAG-3'
	Reverse:	5'-GGTCTTGGGAGTGCTCCAG-3'
h-MMP-9	Forward:	5'-GAACCAATCTCACCACAGG-3'
	Reverse:	5'-GCCACCCGAGTGTAACCAT-3'
h-PDGF-B	Forward:	5'-TGATCTCCAACGCTGCT-3'
	Reverse:	5'-TCATGTTTCAAGTCCCACTCG-3'
h-S1P1	Forward:	5'-AACTTCGCCCTGCTTGAG-3'
	Reverse:	5'-CCAGGCTTTTGTGTAGCTTTT-3'
h-N-cadherin	Forward:	5'-GGTGGAGGAGGAGAAGACCA-3'
	Reverse:	5'-GGCATCAGGCTCCACAGT-3'
h-Tie2	Forward:	5'-TCCAAGGATGTCTCTGCTCTC-3'
	Reverse:	5'-TTGGGGTCATCCTCGGTAT-3'

washing, visualization was performed by enhanced chemiluminescence (Pierce, Rockford, IL).

Bromodeoxyuridine Labeling

In vivo bromodeoxyuridine (BrdU) labeling of mice was performed by i.p. injection of 0.01 ml of a 10 mg/ml solution of BrdU triphosphate (Sigma-Aldrich) per gram of mouse.³⁵ Mice were sacrificed 2 hours after the BrdU injection. Skin samples were fixed, embedded, and sectioned. After dewaxing, the sections were microwaved in citrate buffer (pH 6) for 10 minutes and then placed in 4 N HCl for 30 minutes at 37°C. Sections were incubated overnight at 4°C with a biotinylated anti-BrdU antibody, then washed in PBS and incubated with a streptavidin-coupled horseradish peroxidase. Sections were visualized with diaminobenzidine, counterstained with methyl green, and mounted. BrdU-positive endothelial cells were counted.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

Cells undergoing apoptosis were detected using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore), as specified by the manufacturer. Briefly, paraffin-embedded sections were deparaffinized and pretreated with proteinase K (20 µg/ml) for 15 minutes. Equilibration buffer was added directly onto the specimen, after which

terminal deoxynucleotidyl transferase enzyme in reaction buffer was added for 1 hour at 37°C. Sections were washed in working strength Stop/Wash buffer for 10 minutes. Prewarmed working strength antidigoxigenin conjugate (horseradish peroxidase) was added to the sections and incubated at room temperature for 30 minutes. The samples were washed with PBS, visualized with diaminobenzidine, counterstained, and mounted.

Vascular Permeability Assay

Evans blue dye (0.5%) in 200 µl of 0.9% saline was injected into the tail vein and allowed to circulate for 30 minutes. Mineral oil (control) or mustard oil (5% diluted in mineral oil) were topically applied with a cotton bud onto both side of the ear. A second topical application was administered at 15 minutes. After 30 minutes, mice were sacrificed and intravascular Evans blue dye was removed by flushing the systemic vasculature with 30 ml of saline through a cannula placed in the aorta. Ears and 6-mm full thickness pieces of back skin were removed, dried up, weighed, and homogenized. The dye was extracted with formamide (1.5 ml) and quantified by spectrophotometer (absorbance 620 nm).

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed essentially as described previously.³⁶ Briefly, MDMECs were treated with 1% formaldehyde for 10 minutes. The cross-linked chromatin was then prepared and sonicated to an average size of 300 to 500 bp. The DNA fragments were immunoprecipitated overnight with or without anti-Fli1 antibody at 4°C. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific regions of target genes. The amplified DNA products were resolved by agarose gel electrophoresis. Putative Fli1 transcription factor binding site was predicted by Tfsitescan. Primer sequences are shown in Table 2.

Statistical Analysis

Data presented as bar graphs are the means ± SD of at least three independent experiments. Statistical significance of data was determined by unpaired Student's

Table 2. Primers for ChIP

VE-cadherin	Forward:	5'-ATCACCCAGTATTTGTAAAGTGAGA-3'
	Reverse:	5'-ACTCTGTAGTATGGTGAACGAAAG-3'
PECAM1	Forward:	5'-AAGGAACGTGGAGGAAGTCA-3'
	Reverse:	5'-TTCCCCCTCTACCTTGGAA-3'
MMP-9	Forward:	5'-GCCTCAGGTCTCCAGTCTT-3'
	Reverse:	5'-CTTATGCCCTGCCACAGT-3'
PDGFB	Forward:	5'-AAGAGGCTAGATTACAGTCACAG-3'
	Reverse:	5'-TTATAAAGGAGAAGGAGAGTGC-3'
S1P1	Forward:	5'-TTCTGGGAGGCTGCTTCTTA-3'
	Reverse:	5'-CTCAGCGGTGCACCTCCAAT-3'
Tie2	Forward:	5'-AAATGCACCCAGAGAACAG-3'
	Reverse:	5'-GGCTTATCTTCTGCTCTGCT-3'

Figure 1. Generation of endothelial cell-specific Fli1 knockout (Fli1 CKO) mice. **A:** Schematic outline of the Fli1 locus (i) and the targeting construct (ii). Neo: neomycin resistance gene. The targeting construct was electroporated into embryonic stem cells and the recombinant allele (iii) was selected by neomycin. Correctly targeted ES cells were incubated with FLP recombinase to remove the Neo cassette. The structures of the targeted allele (iv) and of the mutant allele after *in vivo* Cre-mediated recombination in endothelial cells (v) are illustrated. **B:** Body size of 3-day-old animals was compared between wild-type and Fli1 CKO mice. **C:** Genomic DNA was isolated from MDMECs isolated from 5- to 8-week-old mice. The flox allele was amplified using primers flanking the flox site located in intron 3. The sIL2R allele was amplified as an internal control. The levels of the flox allele were quantified by real-time PCR. * $P < 0.05$ versus wild-type (WT). **D** and **E:** mRNA levels and protein levels of Fli1 were determined in MDMECs isolated from 5- to 8-week-old mice by quantitative real-time PCR and immunoblotting, respectively. Representative results of three littermate pairs are shown. * $P < 0.05$ versus wild-type in each littermate group.

t-test assuming equal variances ($P < 0.05$ was considered significant).

Results

Generation of Mice with Endothelium-Restricted Deletion of Fli1

To determine the role of endothelial Fli1 in vascular homeostasis, mice with the conditional deletion of Fli1 in endothelial cells were generated (Figure 1A). LoxP sites were placed in the introns flanking the essential exons 3 and 4 of Fli1 gene, thereby producing a Fli1^{flox} allele, and mice carrying this allele were made. Fli1^{flox/flox} mice were phenotypically normal, demonstrating functional activity of the Fli1^{flox} allele. Mice expressing the Cre recombinase under the control of the endothelium-specific Tie2 receptor promoter were mated to Fli1^{flox/flox} mice. Fli1^{flox/+}/Tie2Cre^{+/-} animals were born with the expected Mendelian ratio and showed normal life span (data not shown). Male offspring were mated to Fli1^{flox/flox} females to generate Fli1^{flox/flox}/Tie2Cre^{+/-} animals (Fli1 CKO). Fli1 CKO mice were born at Mendelian ratios. However, Fli1 CKO mice were initially smaller than Fli1^{flox/flox}/Tie2Cre^{-/-} (wild-type) littermates (differ-

ence in size at day 3 is shown in Figure 1B, difference in body weight at 3 weeks was 7.69 ± 0.93 g ($n = 12$) versus 9.61 ± 0.65 g ($n = 8$); $P < 0.0001$), but their growth caught up with that of wild-type mice by 3 months. Adult Fli1 CKO mice were fertile, and their gross appearance and behavior did not differ from control littermates. Attempts to breed Fli1^{flox/flox}/Tie2Cre^{+/+} mice with an increased dosage of Cre resulted in significantly smaller litters, likely due to embryonic lethality, as well as loss of additional animals during the early perinatal period.

To assess the efficiency of Cre-mediated recombination at the Fli1 locus in endothelial cells *in vivo*, we isolated MDMECs from wild-type and Fli1 CKO mice and determined the relative abundance of the flox alleles by quantitative real-time PCR. As shown in Figure 1C, the Cre-mediated recombination of the flox allele in endothelial cells from different individuals varied between 50 and 80%. Expression levels of Fli1 gene in MDMECs were also examined at mRNA and protein levels by quantitative real-time PCR and immunoblotting, respectively. Reduction of Fli1 mRNA and protein levels was also variable among individual strains of MDMECs, but there was at least ~50% reduction in the majority of cell strains (Figure 1, D and E).

Figure 2. Evidence of defective vasculature in Fli1 CKO mice. Visualization of dermal vascular network by FITC-dextran in 3 month-old wild-type (**left panel**) and Fli1 CKO mice (**right panel**) (original magnification, $\times 25$; scale bar is $100\ \mu\text{m}$). FITC signals in blood vessels were observed under fluorescence microscope. Note presence of microaneurysm (**solid arrow**), dilation (**dotted arrows**), and stenosis (**arrowheads**) in Fli1 CKO mice. **Insets** 1, 2, and 3 (original magnification, $\times 100$; scale bar is $50\ \mu\text{m}$).

Fli1 CKO Mice Display Severe Abnormalities of the Skin Vasculature

We observed that Fli1 CKO mice were consistently more hemorrhagic against mechanical force, such as cervical dislocation, and surgical procedure (data not shown), suggesting the presence of immature and fragile blood vessels. Because of the potential relevance of the endothelial Fli1 gene deficiency to the microvascular disease in SSc, in this study, we focused on the characterization of the skin vasculature. To evaluate the structure of the vascular network in the skin of Fli1 CKO mice, vessels were visualized by injecting FITC-conjugated dextran. In wild-type mice, the dermal vascular network was well-organized and vessels showed a regular diameter (**left panel** in Figure 2). In contrast, in Fli1 CKO mice, the dermal vascular network was disorganized and most of the vessels had an irregular diameter. Notably, stenosis

of arterioles (arrowheads), microaneurysm formation (a solid arrow), and dilation of capillaries (dotted arrows) were observed in Fli1 CKO mice (**right panel** of Figure 2). Furthermore, background of the FITC signal was markedly increased in Fli1 CKO mice compared with wild-type mice (data not shown), suggesting that there is increased vascular leakage in Fli1 CKO mice. To determine whether dermal small blood vessels are dilated, we compared the diameter of arterioles, capillaries, and venules between wild-type and Fli1 CKO mice. As shown in Figure 3A, the mean vessel diameter of Fli1 CKO mice was increased by 55% in capillaries and venules ($3.54 \pm 2.66\ \mu\text{m}$ ($N = 177$) versus $5.48 \pm 4.50\ \mu\text{m}$ ($N = 177$), $P < 0.00001$) and by 70% in arterioles ($13.59 \pm 8.83\ \mu\text{m}$ ($N = 20$) versus $23.23 \pm 13.30\ \mu\text{m}$ ($N = 22$); $P < 0.01$) compared with wild-type mice. Dilation could be caused by proliferation of endothelial cells; therefore, we next evaluated endothelial cell proliferation in wild-type and Fli1 CKO mice. To this end, we compared the number of endothelial cells between wild-type and Fli1 CKO mice by counting the number of endothelial nuclei per cross-section of dermal small blood vessels. Because endothelial cells and PCs can be easily distinguished in arterioles we focused on arterioles. There was an increase of $\sim 65\%$ in the number of nuclei per cross section of arterioles in Fli1 CKO mice compared with wild-type mice (Figure 3B, 2.46 ± 1.39 ($N = 55$) versus 4.08 ± 2.51 ($N = 55$); $P < 0.01$). Studies of longitudinally sectioned arterioles and serial sections showed that endothelial nuclear shape was not altered in Fli1 CKO mice (data not shown). Given that the arteriols are also larger in Fli1CKO mice, the actual endothelial cell density does not differ between wild-type and Fli1 CKO mice. To further evaluate the proliferation and apoptosis of endothelial cells, we also performed BrdU incorporation assay and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, but no significant differences between control and mutant animals were observed (data not shown). Taken together, these data suggest that reduced levels of Fli1 do not directly affect endothelial cell proliferation rate or apoptosis in dermal vasculature.

Figure 3. Evidence of vessel dilation in Fli1 CKO mice. **A:** The diameter of arterioles, capillaries, and venules was measured by Image J on photographic areas from H&E-stained skin sections of 3-month-old wild-type and Fli1 CKO mice (six mice in each group). **B:** The number of endothelial cell nuclei was counted in cross section of arterioles. A series of sections were prepared from each of six wild-type and six Fli1 CKO mice and total of 177 capillaries and venules (for each strain), 20 arterioles (Fli1 wild-type), and 22 arterioles (Fli1 CKO) were evaluated.

Figure 4. Evidence of increased vascular permeability in Fli1 CKO mice. **A** and **B**: Evans blue dye was injected into the tail vein of 3-month-old mice, and animals were sacrificed in half an hour. Mustard oil and control mineral oil were applied twice in 15-minute intervals to the ears. Macroscopic differences were observed in ears (**A**) and skin (**B**). **C**: Quantification of the Evans blue extravasation in five wild-type (WT) and five Fli1 CKO mice; * $P < 0.05$.

Vascular Permeability Is Increased in Fli1 CKO Mice

We next compared vascular permeability in wild-type and Fli1 CKO animals using a well established method of injection of Evans blue dye. Evans blue dye injection was followed by application of mustard oil to the animal left ears, while mineral oil was used as control and applied to the right ears. Anesthetized animals were cardiac perfused to remove i.v. Evans blue dye and sacrificed, and the extent of Evans blue leakage was evaluated in ears and in skin. As shown in Figure 4A, an extensive leakage of Evans blue dye in the ear at the periphery and along blood vessels was observed in Fli1 CKO mice treated with mineral oil, whereas only minimal leakage was seen

Figure 5. Fli1 regulates a subset of genes involved in endothelial cell-cell interactions, BM remodeling, and endothelial cell-PC interaction. **A**: MDMECs were isolated from 5- to 8-week-old wild-type and Fli1^{+/-} and Fli1 CKO mice. Total cell lysates were subjected to immunoblotting for the indicated antibodies. **B**: Chromatin was isolated from wild-type MDMEC and immunoprecipitated using rabbit anti-Fli1 polyclonal antibody or beads alone (No-Ab). After isolation of bound DNA, PCR amplification was performed using specific primers for the indicated mouse gene promoters. Putative Fli1 binding sites were predicted by Tfsitescan (see Table 4). Input DNA (5%) was taken from each sample before addition of antibody.

in control mice. Topical application of mustard oil, which causes nonspecific inflammation and subsequently increases vascular permeability, moderately increased the leakage of Evans blue dye in both animals. Of note, the degree of leakage was higher in the ear of Fli1 CKO mice treated with mineral oil compared with the ear of wild-type mice treated with mustard oil (113.2 ± 6.6 mg/g (Evans blue/sample weight) versus 74.2 ± 13.8 mg/g; $P < 0.05$). Furthermore, an extensive vascular leakage was observed in the back skin of Fli1 CKO mice, but not in control animals (435.5 ± 44.0 mg/g versus 75.7 ± 33.3 mg/g, $P < 0.0005$) (Figure 4, B and C).

Fli1 Regulates Expression of Genes Involved in Endothelial Cell-Cell Interactions, BM Remodeling, and Endothelial Cell-PC Interactions

To elucidate the nature of the Fli1 target genes underlying the observed vascular alterations, we investigated the effect of Fli1 depletion on the expression levels of genes previously implicated in regulating vessel integrity. Specifically, we focused on genes regulating endothelial cell-cell interaction (VE-cadherin and PECAM1), BM remodeling (type IV collagen, MMP-2, and MMP-9), and endothelial cell-pericyte interaction (PDGFB, S1P₁ receptor, Tie-2/Ang1, and N-cadherin). The analyses included MDMEC isolated from Fli1 CKO, as well as Fli1^{+/-} mice since both strains are characterized by an average of ~50% reduction in Fli1 expression level in comparison with wild-type mice. Representative results of immunoblotting are shown in Figure 5A, and the results of real-time PCR are summarized in Table 3. The protein and mRNA levels of endothelial cell markers, VE-cadherin and PECAM1, were decreased in the mutant

Table 3. The Effect of Fli1 Down-Regulation on the Expression Levels of Various Target Genes

Gene	MDMEC (Fli1 ^{+/-} /wild-type)	MDMEC (Fli1 CKO/wild-type)	HDMEC (Fli1 siRNA/SCR)
<i>Fli1</i>	45.5 ± 13.7*	34.7 ± 10.5*	48.9 ± 7.5*
<i>VE-cadherin</i>	63.8 ± 11.6*	61.7 ± 4.3*	65.2 ± 11.8*
<i>PECAM1</i>	59.2 ± 7.3*	57.5 ± 4.0*	74.8 ± 3.6*
<i>COL4A1</i>	91 ± 6.8	102.4 ± 4.1	102.4 ± 9.0
<i>MMP-2</i>	99.6 ± 2.1	103.0 ± 14.4	97.9 ± 7.9
<i>MMP-9</i>	195.3 ± 27.1*	219.5 ± 8.9*	171.4 ± 15.6*
<i>PDGFB</i>	43.5 ± 1.8*	45.7 ± 12.3*	59.3 ± 9.9*
<i>S1P₁</i>	58.2 ± 6.6*	62.3 ± 11.9*	66.9 ± 4.7*
<i>N-cadherin</i>	137.0 ± 23.7	107.9 ± 14.9	88.6 ± 15.5
<i>Tie2</i>	63.6 ± 9.7*	64.5 ± 2.6*	84.4 ± 4.7*

**P* < 0.05.

Fli1 MDMECs compared with wild-type cells. The protein level of type IV collagen, the main constituent of BM, was markedly decreased in mutant Fli1 MDMECs, whereas mRNA level was not affected, suggesting that Fli1 does not directly regulate expression of type IV collagen gene. Significantly, MMP-9 mRNA and protein expression were markedly increased in mutant Fli1 MDMECs, whereas there was no difference in MMP-2 levels, suggesting a possible disruption of BM in Fli1 mutant mice. Among the genes involved in endothelial cell-PC interactions, the levels of PDGFB and S1P₁ receptor were significantly decreased at both mRNA and protein levels, whereas the expression level of N-cadherin was not affected in MDMECs with reduced Fli1 expression levels. The expression of Tie2 was also moderately decreased in mutant Fli1 MDMECs compared with wild-type MDMECs. Therefore, VE-cadherin, PECAM1, MMP9, PDGFB, S1P₁ receptor, and Tie2 are the potential Fli1 direct target genes in endothelial cells. To further investigate whether Fli1 targets these genes directly, we performed ChIP analysis. Cross-linked chromatin from wild-type MDMECs was immunoprecipitated with anti-Fli1 antibody and the purified genomic DNA was amplified with primers specific to the putative Fli1 binding sites predicted by Tfsitescan (see Table 4) in the promoter of each gene. As shown in Figure 5B, Fli1 occupied the promoter of VE-

cadherin, PECAM1, MMP-9, PDGFB, and S1P₁, indicating that these genes are directly regulated by Fli1. We were not able to detect the Fli1 presence on the Tie2 promoter, suggesting that Fli1 may regulate Tie2 gene expression indirectly or by a regulatory element not encompassed by the Tie2 primers used for ChIP. We also investigated the effect of Fli1 siRNA on the expression levels of these genes in human microvascular endothelial cells, which were depleted of Fli1 using previously described AdenoFli1siRNA.²⁷ Significantly, Fli1 depletion in human endothelial cells showed similar effects on mRNA expression (right column of Table 3), suggesting that Fli1 regulates similar subset of genes in human endothelial cells.

Endothelial Fli1 Deficiency Results in Decreased VE-Cadherin, PECAM1, and Type IV Collagen in Skin Vasculature

The above analyses using Fli1-deficient cultured endothelial cells suggested that Fli1 positively regulates VE-cadherin and PECAM1, two important regulatory proteins that are located in the cell adhesion junctions.^{37,38} We therefore evaluated the expression of these proteins by immunohistochemistry in the skin vasculature of wild-type and Fli1 CKO mice. Small blood vessels were classified

Table 4. Summary of Results of ChIP Analysis for Fli1 in MDMEC

Gene	Predicted putative binding site of Ets transcription factor family	Amplified regions of target gene promoter	<i>In vivo</i> binding of Fli1
<i>VE-Cadherin</i>	-525 to -522	-596 to -414	+
<i>PECAM1</i>	-1099 to -1096	-1184 to -939	+
	-56 to -53	-191 to +16	-
<i>MMP-9</i>	-886 to -883	-958 to -792	-
	-670 to -667	-789 to -612	-
	-491 to -488, -377 to -374	-532 to -333	-
	-289 to -286	-361 to -158	+
<i>PDGFB</i>	-203 to -200	-411 to -178	-
	-111 to -108	-195 to -25	+
<i>S1P₁</i>	-1144 to -1142	-1236 to -999	-
	-573 to -570	-713 to -500	-
	-257 to -254	-342 to -141	+
	-35 to -32	-131 to +70	-
<i>Tie2</i>	-1264 to -1261, -1230 to -1227, -1155 to -1152	-1305 to -1095	-
	-674 to -671	-807 to -591	-
	-463 to -460	-564 to -347	-
	-227 to -224	-339 to -124	-

Figure 6. Abnormal expression of vascular markers in Fli1 CKO mice. **A–F:** Immunodetection of VE-cadherin (**A** and **B**), PECAM1 (**C** and **D**), and type IV collagen (**E** and **F**) in the skin sections of 3-month-old wild-type (**A**, **C**, and **E**) and Fli1 CKO (**B**, **D**, and **F**) mice (original magnification, $\times 100$; scale bar is $100\ \mu\text{m}$). **Insets 1, 2, and 3** (original magnification, $\times 400$; scale bar is $10\ \mu\text{m}$) depict arterioles (**thick arrow**), capillaries (**thin arrow**), and venules (**dotted arrow**), respectively. **G** and **H:** Heparan sulfate proteoglycans (PAS staining) in the skin sections of wild-type (**G**) and Fli1 CKO (**H**) mice (original magnification, $\times 100$; scale bar is $100\ \mu\text{m}$). **Insets 1 and 2** depict representative arterioles (**thick arrow**), **insets 3 and 4** depict representative venules (**dotted arrow**), and **insets 5 and 6** depict representative capillaries (**thin arrow**) (original magnification, $\times 400$; Scale bar is $10\ \mu\text{m}$).

into arterioles, capillaries, and venules based on the criteria described in *Materials and Methods*, and the results were evaluated separately for each type of blood vessel. As shown in Figure 6A, in wild-type mice, VE-cadherin was uniformly detected as a strong, thin, linear staining on endothelial cells, including arterioles (inset 1), capillaries (inset 2), and venules (inset 3). In contrast, in Fli1 CKO mice VE-cadherin expression was markedly reduced in endothelial cells in all types of blood vessels (Figure 6B). Likewise, PECAM1 staining was uniformly present on arterioles (inset 1), capillaries (inset 2), and venules (inset 3) in wild-type mice (Figure 6C) but was markedly decreased in the mutant mice (Figure 6D). These results suggest that endothelial cell junctions do not function properly in Fli1 CKO mice, thus contributing to the observed vessel leakage. Because VE-cadherin also plays an important role in maturation of blood vessels,³⁹ these data support the notion that this process might be impaired in Fli1 CKO mice.

We next investigated BM by immunostaining for type IV collagen, the most abundant constituent of BM, and by periodic acid-Schiff (PAS) staining, which stains heparan sulfate proteoglycans. In wild-type mice (Fig-

ure 6E), vascular BM was abundant with type IV collagen in all types of blood vessels, including arterioles (inset 1, thick arrow), capillaries (inset 2, thin arrow), and venules (insets 2 and 3, dotted arrow), whereas the levels of type IV collagen were uniformly decreased in BM of all types of blood vessels, including arterioles (inset 1), capillaries (inset 2), and venules (inset 3) in Fli1 CKO mice (Figure 6F). Differences were also observed in PAS staining. As shown in Figure 6G, all types of blood vessels showed uniform PAS staining in wild-type mice, whereas the staining was irregular and uneven in Fli1 CKO mice (Figure 6H). For example, although the majority of the arterioles show a normal staining pattern (inset 1), increased PAS-positive BM thickness in arterioles was occasionally observed (inset 2). Most venules were stained unevenly (inset 3) or showed very low levels of staining (inset 4). Most of the capillaries were stained at relatively low levels with PAS (insets 5 and 6). These results demonstrate that the composition of vascular BM is altered in Fli1 CKO mice and may also contribute to the compromised vascular integrity in Fli1 CKO mice.

Figure 7. Reduced presence of α SMA-positive PC/vSMCs in Fli1 CKO mice. Immunodetection of α SMA in the skin sections of 3 month-old wild-type (**A**) and Fli1 CKO (**B**) mice (original magnification, $\times 100$; scale bar is 100 μ m). **Insets** (original magnification, $\times 400$; scale bar is 10 μ m) depict arterioles (**thick arrow**), capillaries (**thin arrow**), and venules (**dotted arrow**), respectively. Representative arterioles (**panel 1**), capillaries (**panel 2**), and venules (**panel 3**) in wild-type mice are shown in **A**. In Fli1 CKO skin (**B**) representative images depict variation in staining intensity for arterioles (**insets 1–3**), capillaries (**insets 4–6**), and venules (**insets 7–9**). Quantitative analysis of PC coverage in small dermal blood vessels is included in Table 5.

Fli1 CKO Mice Have Decreased Pericyte Coverage

The vascular phenotype of Fli1 CKO mice was reminiscent of mice with impaired pericyte/vascular smooth muscle cells (PC/vSMC) coverage.⁴⁰ Consistent with this notion, we showed that PDGFB, a key regulator of PC recruitment during vessel maturation, and S1P₁ receptor, which contributes to vessel stability via N-cadherin-mediated endothelial cell PC interactions,⁴¹ were directly targeted by Fli1 and significantly reduced in Fli1 CKO mice (Figure 5). Therefore, to assess the degree of PC/vSMC coverage in Fli1 CKO mice, we performed immunostaining with a PC/vSMC marker, α SMA. Representative results are shown in Figure 7, and the percentage of PC/vSMC coverage is summarized in Table 5. In wild-type mice (Figure 7A), all of the arterioles were densely covered with PC/vSMCs (inset 1), whereas 50 to 80% of capillaries (inset 2) and venules (inset 3) were well-

covered with PCs. Because capillaries and venules are incompletely covered with PCs, a small number of cross-sections of these vessels showed partial or no α SMA labeling. In contrast, in Fli1 CKO mice (Figure 7B), some arterioles showed weak or partial labeling for α SMA (insets 2 and 3). Furthermore, α SMA staining was not detectable on the majority of capillaries (insets 4–6), and venules (insets 7–9). Of note, a proportion of arterioles (inset 1), capillaries (inset 4), and venules (inset 7) were well-stained with α SMA. Although there was a variation between individual animals, the degree of PC/vSMC coverage, as assessed by presence of α SMA, was consistently decreased in all types of blood vessels in Fli1 CKO mice (Table 5). Taken together, these data strongly suggest that Fli1 deficiency in endothelial cells impairs vascular homeostasis of dermal blood vessels at multiple levels, including endothelial cell-cell interaction, BM composition, and endothelial cell-PC interaction.

Table 5. Evaluation of PC Coverage in Small Dermal Blood Vessels in Wild-Type (WT) and Fli1 CKO Mice

Vessels type	WT-1			WT-2			WT-3			Fli1 CKO-1			Fli1 CKO-2			Fli1 CKO-3		
	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-	+	+/-	-
Arterioles	100	0	0	100	0	0	100	0	0	50	30	20	67	27	6	100	0	0
Capillaries/ Venules	76	14	10	64	18	18	72	14	14	22	13	65	13	14	73	12	11	77

Table 6. Comparison of PC Coverage of Small Dermal Vessels between Normal and Scleroderma Skin

	Age	Sex	Race	Disease duration	TSS	Degree of pericyte coverage		
						Arterioles	Capillaries	Venules
NS 352	37	F	AA			+++	+++	+++
SSc 351	38	F	AA	2 y	3	++	+	++
NS 356	60	F	C			+++	+++	+++
SSc 355	63	F	C	4 m	37	+	+/-	+
NS 360	40	F	AA			+++	+++	+++
SSc 359	42	F	AA	3 y	15	+	+	+
NS 362	44	F	AA			+++	+++	+++
SSc 361	49	F	AA	4 y	24	+	+/-	+
NS 364	54	M	C			+++	+++	+++
SSc 363	50	M	C	1.5 y	3	++	+/-	++
NS 366	48	M	C			+++	+++	+++
SSc 365	42	M	C	2 y	ND	+	+/-	+
NS 368	45	F	C			+++	+++	+++
SSc 367	50	F	C	13 y	34	+++	+/-	++

TSS, total skin score; M, male; F, female; AA, african american; C, caucasian; -, negative; +, slight staining; ++, staining and +++, strong staining.

Vascular Alterations in Fli1 CKO Mice Resemble the Scleroderma (SSc) Phenotype of Dermal Blood Vessels

We have previously reported that Fli1 protein is expressed at reduced levels in SSc skin vasculature,²⁵ suggesting that the abnormal vascular characteristics observed in Fli1 CKO mice may also be present in SSc skin. Consistent with our finding, absence of VE-cadherin and altered composition of type IV collagen were reported in SSc skin vessels.^{8,42} To corroborate these previous observations, we examined expression of VE-cadherin and type IV collagen in seven pairs of SSc and closely matched control skin biopsies. We also characterized expression of vascular Fli1 in these patients. In agreement with our previous study,²⁵ expression of Fli1 was significantly reduced in SSc skin vasculature (data not shown). Because PECAM1 was also down-regulated in Fli1 CKO mice, we included PECAM1 in our analysis. Patient information is provided in Table 6 and representative results are shown in Figure 8. As shown in the previous study,⁸ SSc skin (Figure 8B) showed significantly decreased levels of VE-cadherin in some arterioles (inset 1), capillaries (inset 2), and venules (inset 3) as compared with blood vessels in healthy skin (Figure 8A). Likewise, PECAM1 staining was uniformly present on arterioles (inset 1), capillaries (inset 2), and venules (inset 3) in healthy skin (Figure 8C), but in lesional SSc skin, staining for PECAM1 was decreased on some arterioles (inset 1), capillaries (inset 2), and venules (inset 3) (Figure 8D). The expression of type IV collagen was reduced on all types of blood vessels in SSc skin (compare Figure 8, E and F).

We next examined the presence of the PC/vSMC marker, α -SMA. Representative results are shown in Figure 9 and scoring assessment of α -SMA-positive vessels is summarized in Table 6. The extent of PC/vSMC coverage evaluated by α -SMA staining varied among the individual patients, but in general was consistently decreased in all types of small blood vessels, especially in the capillaries (inset 2) and venules (inset 3) in lesional SSc skin (compare Figure 9, A and C, with Figure 9, B

and D). These results demonstrate similar phenotypic changes in the skin vasculature of Fli1 CKO mice and SSc patients, strongly suggesting that Fli1 deficiency may be, at least in part, involved in the development of scleroderma vasculopathy.

Discussion

In this article, we characterize the phenotype of mice with a conditional knockout of Fli1 in endothelial cells. The vascular phenotype is consistent with the previous observations of the embryonic lethality of Fli1 null mice that die at day 11.5 of embryogenesis with loss of vascular integrity,^{29,31} suggesting that Fli1 is not required for the formation of nascent blood vessels (vasculogenesis), but its absence impairs further vessel remodeling and maturation. In our study, mice with reduced expression level of Fli1 gene in endothelial cells exhibited abnormal skin vasculature with greatly compromised vessel integrity. Markedly increased vessel permeability was clearly evident in the absence of any further treatments. Additional experiments using explanted endothelial cells from wild-type and Fli1 mutant mice demonstrated that Fli1 directly regulates a number of genes important in the maintenance of vascular homeostasis, including VE-cadherin, PECAM1, MMP-9, PDGFB, and S1P₁ receptor. This Fli1 CKO mouse phenotype suggests that Fli1 may play a major role as a key regulator of vessel maturation. Importantly, Fli1 expression is also reduced in dermal vessels of SSc patients. Furthermore, other vascular characteristics of Fli1 CKO mice, including reduced levels of VE-cadherin, PECAM1, and type IV collagen, as well as decreased staining for α -SMA, are also observed in SSc vessels. Thus, persistently reduced levels of Fli1 in endothelial cells may be a critical factor associated with the impairment of SSc microvasculature.

Here we report that reduced levels of Fli1 lead to decreased expression of VE-cadherin both in cultured endothelial cells and in mouse skin *in vivo*. Reduced levels of Fli1 also correlate with reduced expression of VE-cadherin in SSc skin vasculature. Previous studies

Figure 8. Abnormal expression of vascular markers in SSc dermal blood vessels. Skin sections from SSc patients (**B**, **D**, and **F**) and normal controls (**A**, **C**, and **E**) were stained with anti-VE-cadherin antibody (**A** and **B**), anti-PECAM1 antibody (**C** and **D**), and with anti-type IV collagen antibody (**E** and **F**) (original magnification, $\times 100$; scale bar is $100\ \mu\text{m}$). The signal was developed by DAB (**A–D**) or FITC (**E** and **F**). **Insets** (original magnification, $\times 400$; scale bar is $10\ \mu\text{m}$) depict arterioles (**thick arrow**), capillaries (**thin arrow**), and venules (**dotted arrow**).

have characterized two Ets binding sites within the VE-cadherin promoter,⁴³ and subsequent studies demonstrated that two members of the Ets family, Ets1 and Erg, positively regulate expression of VE-cadherin in cultured

endothelial cells.^{44,45} Thus, different Ets factors may regulate VE-cadherin expression in different cellular contexts. Our study conclusively demonstrates that VE-cadherin expression in the skin vasculature *in vivo* depends

Figure 9. Reduced presence of α -SMA-positive PC/vSMCs in SSc skin vasculature. **A:** Skin sections from SSc patients (**A** and **C**) and normal controls (**B** and **D**) were stained with anti- α -SMA antibody. **Insets** (original magnification, $\times 400$; Scale bar is 10 μ m) depict arterioles (**thick arrow**), capillaries (**thin arrow**), and venules (**dotted arrow**). The signal was developed by diaminobenzidine (DAB). **C** and **D:** Comparison of α -SMA staining in control and SSc skin vessel by differential interference contrast (DIC) imaging. **Thin arrows** point to capillaries, and **dotted arrows** point to venules. Summary of pericyte coverage in dermal blood vessels in all SSc and healthy control skin samples is included in Table 6.

on the presence of endothelial Fli1. VE-cadherin is a main constituent of adherens junctions and plays an important role in regulating endothelial barrier function. In addition, recent studies have shown that VE-cadherin also controls tight junctions by regulating expression of its key component, claudin-5.³⁷ Furthermore, VE-cadherin functions in vessel stabilization through its interactions with growth factor receptors. VE-cadherin inhibits VEGF signaling through formation of complexes with VEGF-2 receptor and subsequent inhibition of ERK1/2 signaling, whereas its interaction with the TGF- β receptor complex promotes TGF- β signaling and the antimigratory and antiproliferative effects of this growth factor in endothelial cells.⁴⁶

Embryos lacking VE-cadherin die at day 9.5 to 10.5 *post coitum* and display irregular, hemorrhagic vessels, which eventually regress.⁴⁷ Although VE-cadherin is not required for the process of the capillary plexus formation, its function is necessary to prevent the disassembly of the nascent blood vessels.^{48,49} VEGF-induced vessel permeability is associated with disassembly of adherens junctions through a mechanism that involves β -arrestin-dependent internalization of VE-cadherin into clathrin coated pits and subsequent recycling.⁴⁹ Circulating levels of VEGF are abnormally elevated in patients with SSc,⁵⁰ whereas the consequences of chronic VEGF exposure on adherens junctions have not been fully inves-

tigated, it is unlikely that VEGF is responsible for the reduced levels of VE-cadherin in patients with SSc because it does not target VE-cadherin for degradation.⁴⁹ Absence of VE-cadherin is a distinctive pathological manifestation of SSc vasculopathy, and our study strongly suggests that Fli1 deficiency may be the primary mechanism contributing to this process. On the other hand, elevated levels of VEGF might be protective for microvessels with impaired PC coverage.⁵¹ Consistent with this possibility, in SSc patients, the plasma levels of VEGF inversely correlate with the development of fingertip ulcers, and patients with the highest levels of VEGF are protected from developing fingertip ulcers.⁵⁰

Vessel stability also depends on the presence of intact vascular BM, a specialized extracellular matrix composed of type IV collagen, laminin, heparan sulfate proteoglycans, nidogen, perlecan, and other minor proteins.^{40,52} In agreement with previous studies, we observed that in comparison with healthy skin vessels the levels of type IV collagen was decreased in SSc vascular BM. A similar decrease was also present in the skin of Fli1 CKO mice. Further analyses of cultured endothelial cells demonstrated that type IV collagen protein levels were decreased, whereas mRNA levels were unchanged in Fli1 CKO endothelial cells, suggesting that Fli1 does not directly regulate type IV collagen gene transcription. Importantly, Fli1 deficiency resulted in a marked up-regulation of MMP-9, a key metalloproteinase involved in degradation of type IV collagen.⁵² Whereas MMP-9 is not effective in degrading perlecan or heparan sulfate proteoglycans, proteolysis of a main constituent of BM such as type IV collagen would likely cause a disruption of the entire collagen network. Changes in PAS staining observed in Fli1 CKO mice and SSc patients (data not shown) are consistent with this possibility. Although more studies are needed to carefully evaluate possible additional characteristics of BM in Fli1 CKO mice as well as in SSc patients, our studies support the view that Fli1 deficiency in the endothelium leads to structural abnormalities of vascular BM.

The proper functioning of quiescent vessels in healthy tissues also depends on the presence of PC/vSMCs.^{40,53} Small vessels are variably covered by PCs embedded within the BM of microvessels, whereas larger vessels are surrounded by vSMCs. Genetic studies of mice lacking PC/vSMCs strongly suggest that these specialized cells play a critical role in regulating vascular stability. Four major ligand-receptor systems have been shown to regulate vessel stability through interaction of endothelial cells and PCs. These include PDGFB-PDGFR β , S1P, and S1P₁ receptor, Angiopoietins1 and 2 and Tie2 receptor, as well as the components of the TGF- β ligand/receptor system.^{40,53} We show that Fli1 deficiency in endothelial cells leads to decreased protein and mRNA levels of PDGFB, S1P₁ receptor, and the Tie2 receptor. ChIP analyses demonstrate that Fli1 binds to the promoter of PDGFB and S1P₁ receptor genes, suggesting that these genes are direct targets of Fli1. Although previous studies have shown that Tie2 gene is not expressed in Fli1 null mouse embryonic fibroblasts,²⁹ our results based on a partial deficiency of Fli1 gene suggest that the *Tie2* gene is less sensitive to the decreased dosage of Fli1 in comparison with other Fli1-

regulated genes. Furthermore, we were unsuccessful in localizing Fli1 to the Tie2 promoter, suggesting that Fli1 may regulate the *Tie2* gene through indirect mechanisms. Moreover, reduced levels of VE-cadherin may indirectly affect TGF- β signaling in endothelial cells in Fli1 CKO mice. Consistent with the role of Fli1 as regulator of PDGFB and S1P₁ receptor, vessels of Fli1 CKO mice have a significantly decreased presence of α -SMA-positive cells and as a consequence are fragile and leaky. We have also observed decreased staining for α -SMA in SSc vessels, which may suggest either a decreased number of PCs or an abnormal expression of the α -SMA gene. Interestingly, SSc vasculature is characterized by an increased presence of the PC/vSMC marker, Rgs5,⁸ as well as PDGFR β , a marker of PC progenitors.¹⁴ The specific function of Rgs5 is not well understood, but its expression is induced during active vascular remodeling, eg, during tumor angiogenesis, wound healing, and ovulation.⁵³ Relevant to our study, it was recently shown that absence of Rgs5 had a profound effect on tumor angiogenesis through modulation of PC phenotype.⁵⁴ Absence of Rgs5 did not affect overall PC coverage; however, it modulated expression of PC markers. Hence, Rgs5-positive PCs showed features of immature PCs characterized by expression of PDGFR β and low levels of α -SMA expression, whereas Rgs5-deficient PCs predominantly expressed markers of mature PCs such as α -SMA and NG2.⁵⁴ Furthermore, absence of Rgs5 correlated with PC maturation, vessel normalization and decreased vessel leakage. Thus, a decreased expression of α -SMA in SSc skin vasculature observed in our study could reflect two mechanisms: decreased PC coverage due to the Fli1-dependent reduced expression of genes involved in PC recruitment, including PDGFB and S1P₁ receptor, and an increased presence of immature PCs. Taken together, the phenotype of SSc PC/vSMCs suggests that SSc vasculature might be in a state of pathological perpetual vessel remodeling. Proper associations between endothelial cells, PCs and extracellular matrix are essential for the maintenance of vessel integrity and dysregulation of this process underlies various vascular pathologies, including diabetic retinopathy, age-related macular degeneration, as well as tumor angiogenesis.^{55,56} Our findings of the key role of Fli1 in regulating these associations suggest that its deficiency might also play a role in other diseases. Consistent with this notion we have observed exaggerated tumor growth and increased metastasis in Fli1 CKO mice (L. Stawski and M. Trojanowska, unpublished observations).

Pathological changes in the microvasculature, which are noticeable during the earliest stages of the disease, have long been documented in SSc. Although the initial event causing vascular injury may vary, it triggers a sequel of pathological changes that eventually leads to widespread capillary regression and devascularization of involved organs.¹ Failure to properly respond to this initial injury and to regenerate damaged vessels suggests a deficiency in the process of neovascularization; however, the underlying pathological mechanism has not been elucidated. The evidence presented in this study suggests that persistently reduced levels of Fli1 may contribute to the impairment of vessel remodeling in SSc. Fli1 is highly and uniformly expressed on quiescent vessels,

however its expression is transiently decreased during angiogenesis, eg, during wound healing (Y. Asano and M. Trojanowska, unpublished data). Thus, although in some vessels decreased levels of Fli1 may reflect the ongoing angiogenesis, its persistent down-regulation in the majority of the vessels is likely to interfere with vessel maturation and stabilization. As a result, integrity of the vessels is compromised; they become more permeable and prone to regression in the absence of high levels of VEGF. The factors that lead to the reduced expression of Fli1 are presently not known; however, reduced Fli1 expression in the SSc vasculature parallels its decreased expression in fibroblasts,²⁵ possibly suggesting a common underlying mechanism. Studies in fibroblasts showed that TGF- β degrades Fli1 through an acetylation-mediated mechanism,³⁶ and although the factors regulating Fli1 expression in endothelial cells have not yet been investigated, successful regeneration of capillaries in SSc following immunosuppressive therapy suggests that they may have an immune origin.⁸ Interestingly, epigenetic mechanisms have also been implicated in regulation of Fli1 gene in SSc.²⁸

In conclusion, this study supports the critical role of Fli1 in the process of vessel maturation and strongly suggests that deficiency of Fli1 plays a pathological role in microvascular disease of SSc. However, it remains to be established whether pathological changes observed in SSc vasculature are specific for this disease or whether they are also present in other vasculopathies.

The complexity of vascular changes in SSc will likely require contribution of multiple factors to fully elaborate the disease phenotype. The Fli1 CKO mice should be very useful in further understanding of the molecular mechanisms involved in SSc vasculopathy and could be helpful in characterization of other contributing factors by multiple mutant analyses.

Acknowledgment

We thank Dr. Tien Hsu for a critical reading of the manuscript.

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