Transcription Factor Fos-Related Antigen-2 Induces Progressive Peripheral Vasculopathy in Mice Closely Resembling Human Systemic Sclerosis

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**Background**—Microvascular damage is one of the first pathological changes in systemic sclerosis. In this study, we investigated the role of Fos-related antigen-2 (Fra-2), a transcription factor of the activator protein-1 family, in the peripheral vasculopathy of systemic sclerosis and examined the underlying mechanisms.

**Methods and Results**—Expression of Fra-2 protein was significantly increased in skin biopsies of systemic sclerosis patients compared with healthy controls, especially in endothelial and vascular smooth muscle cells. Fra-2 transgenic mice developed a severe loss of small blood vessels in the skin that was paralleled by progressive skin fibrosis at 12 weeks of age. The reduction in capillary density was preceded by a significant increase in apoptosis in endothelial cells at week 9 as detected by immunohistochemistry. Similarly, suppression of Fra-2 by small interfering RNA prevented human microvascular endothelial cells from staurosporine-induced apoptosis and improved both the number of tubes and the cumulative tube lengths in the tube formation assay. In addition, cell migration in the scratch assay and vascular endothelial growth factor–dependent chemotaxis in a modified Boyden chamber assay were increased after transfection of human microvascular endothelial cells with Fra-2 small interfering RNA, whereas proliferation was not affected.

**Conclusions**—Fra-2 is present in human systemic sclerosis and may contribute to the development of microvasculopathy by inducing endothelial cell apoptosis and by reducing endothelial cell migration and chemotaxis. Fra-2 transgenic mice are a promising preclinical model to study the mechanisms and therapeutic approaches of the peripheral vasculopathy in systemic sclerosis. *(Circulation. 2009;120:2367-2376.)*

**Key Words:** endothelial cells • peripheral vascular diseases • systemic sclerosis

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Systemic sclerosis (SSc) is an autoimmune disease of still-unknown origin with widespread microvascular damage and progressive fibrosis of the skin and internal organs. Among others, infectious agents, tissue injury, and hypoxia/oxidative stress based on a genetic susceptibility are thought to trigger the onset of the disease. Despite the progress in managing complications that occur mostly as a result of organ failure, to date, there is still neither a cure nor a disease-specific treatment.¹

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**Clinical Perspective on p 2376**

Microvascular alterations are one of the earliest phenomena in the pathophysiology of SSc.² Raynaud syndrome and morphological changes on nailfold capillaroscopy might occur even years before the onset of fibrosis, suggesting a crucial role of the microangiopathy in the overall disease process. Microvascular manifestations can be found in different organs. Pulmonary arterial hypertension is among the leading causes of death in patients with SSc.³ The peripheral vasculopathy in the skin is characterized by a progressive reduction in the number of capillaries, resulting in ischemic manifestations such as fingertip ulcers. The peripheral vasculopathy has a high morbidity and is a major burden of the disease for patients with SSc.⁴ Increased endothelial cell apoptosis can be detected in early peripheral vascular lesions and is considered the earliest pathological event in the development of SSc.⁵ However, the molecular mechanisms initiating and perpetuating the peripheral vasculopathy are still unknown. In addition, suitable animal models for the peripheral vasculopathy of SSc allowing pathophysiological studies and preclinical testing of potential drugs are not available.

The transcription factor activator protein-1 is a heterodimeric molecule composed of members of the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, FosB, Fos-related antigen
with Fra-2 small interfering RNA (siRNA) as previously described. HUMECs transfected with control siRNAs (scrambled siRNA, Ambion, Darmstadt, Germany) were used as negative controls.

Real-Time Reverse-Transcription Polymerase Chain Reaction
Gene expression was quantified by SYBR Green real-time polymerase chain reaction with the ABI Prism 7500 Sequence Detection System (PE Applied Biosystems, Rotkreuz, Switzerland).

Western Blot Analysis for Fra-2
Nuclear extracts were prepared according to the protocol of Andrews and Faller. Protein (8 μg) from each sample was separated by 10% SDS-PAGE and electrophoresed onto polyvinylidene difluoride membranes (Roth, Karlsruhe, Germany) as previously described. Membranes were incubated with polyclonal rabbit anti-human Fra-2 antibodies (Sigma) and secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibodies (Dako, Hamburg, Germany). For confirmation of equal loading of proteins, the amount of U2AF65 was determined with the use of mouse anti-human U2AF65 antibodies (Sigma) and goat anti-mouse antibodies (Dako, Glostrup, Denmark).

Microtiter Tetrazolium Assay
To assess cell proliferation, HUMECs transfected with Fra-2 or scrambled siRNA were cultured in 96-well plates and analyzed after 40 hours with an automatic ELISA plate reader (UV MAX, Molecular Devices, Sunnyvale, Calif) at a test wavelength of 570 nm.

Scratch Assay
HUMECs transfected with Fra-2 siRNA or scrambled siRNA were seeded on 6-well plates with or without mitomycin C at a final concentration of 25 μg/mL to control for proliferation. After 24 hours, a single uniform scratch was made in each well with a 1-mL plastic pipet tip. Photographs were taken at 0, 24, and 48 hours. The distance of the wound edges was measured as described recently.

Chemotaxis-Induced Migration Assay
Migration was analyzed with the QCMTM Chemotaxis 96-well cell migration assay (Chemicon/Millipore). HUMECs were transfected with Fra-2 siRNA. Vascular endothelial growth factor was used as the chemoattractant. To exclude misleading effects resulting from proliferation, the experiment was repeated in the presence of mitomycin C as described above. After 24 hours, cell lysates were analyzed with a fluorescence plate reader with the 480/520-nm filter set.

Tube Formation Assay
HUMECs transfected with Fra-2 siRNA or scrambled siRNA were cultured in 6-well plates coated with Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, Calif). Cumulative tube length and number of tubes were quantified after 24 hours as described previously.

Apoptosis Assay
For induction of apoptosis, HUMECs were treated at different time points with increasing concentrations of the protein kinase inhibitor staurosoporine (Sigma). Untreated HUMECs or HUMECs transfected with scrambled siRNA served as controls. Apoptotic cells were detected by annexin V–Fluos/PI staining (Roche) with flow cytometry analysis (fluorescence-activated cell sorter).

Combined Tube Formation and Apoptosis Assay
Apoptosis in tube-forming HUMECs was analyzed by immunocytochemistry with annexin V–Fluos (Roche) staining. After transfection with Fra-2 siRNA or scrambled siRNA, HUMECs were seeded on double-chamber slides coated with Matrigel. Twenty-four hours later, tube formation was analyzed, and cells were treated with staurosoporine. After 2 hours, cells were incubated with annexin V–Fluos labeling solution and stained with DAPI (Sigma). The

Methods
Additional information on methods is provided in the online-only Data Supplement.

Patients and Animals
Skin biopsy specimens from SSc patients (n=12) and healthy donors (n=5 each) were obtained by punch biopsy. All patients fulfilled the criteria for SSc as suggested by LeRoy et al and all subjects signed a consent form approved by the local institutional review boards. Clinical characteristics are summarized in Table I of the online-only Data Supplement. Fra-2tg mice have been described previously and were kindly provided by Erwin F. Wagner.

Immunohistochemistry for Fra-2, von Willebrand Factor, α-Smooth Muscle Antigen, and Smooth Muscle Protein 22α
Primary and secondary antibodies were used for immunohistochemistry: polyclonal rabbit antibodies against Fra-2 (abcam, Cambridge, UK), von Willebrand Factor (vWF; abcam), monoclonal mouse antibodies against smooth muscle protein 22α (abcam), horseradish peroxidase-labeled secondary goat anti-rabbit and goat anti-mouse antibodies, and alkaline phosphatase-conjugated secondary goat anti-mouse antibodies (all from Jackson ImmunoResearch, Soham, UK). Negative controls of all stainings are shown in Figure I of the online-only Data Supplement.

Detection of Apoptosis by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay and Caspase 3 Staining
For immunohistochemical detection and quantification of apoptotic cells, we used different assays: the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technology using the In Situ Cell Death Detection Kit, peroxidase (Roche, Basel, Switzerland), a fluorescent apoptosis kit (ApopTag, Chemicon/Millipore, Zug, Switzerland), and caspase 3 staining with active plus procaspase 3 antibodies (abcam).

Transfection With Small Interfering RNAs Against Fra-2
Human microvascular endothelial cells (HUMECs) were transfected with the HMVEC-L Nucleofector Kit (Amaxa, Cologne, Germany) with Fra-2 small interfering RNA (siRNA) as previously described. HUMECs transfected with control siRNAs (scrambled siRNA, Ambion, Darmstadt, Germany) were used as negative controls.

[Fra]-1, and Fra-2) families. Activator protein-1 family members are immediate early genes that are induced by a variety of stress signals and control subsequent stress responses, including cell proliferation, apoptosis, inflammation, wound healing, and tumorigenesis. Whereas Jun proteins and the majority of Fos proteins are well characterized, little is known about Fra-2 and its functions in vivo. Recently, Fra-2 transgenic (tg) mice have been characterized to develop a severe, proliferative vasculopathy of the lungs resembling pulmonary arterial hypertension. This is followed by progressive lung fibrosis, leading to death of the animals at a median of 17 weeks of age because of respiratory distress. Thus, this animal model links vascular remodeling of the lungs with fibrogenesis and explains the link between the 2 major lung manifestations of SSc. The objective of the present study was to investigate the role of Fra-2 for the peripheral microangiopathy in Fra-2tg mice and human SSc.

Histological Analysis
Skin sections of SSc patients, healthy control subjects, Fra-2tg and wild-type (wt) mice were fixed in 4% formalin and embedded in paraffin for subsequent analysis as described previously. Additional information on methods is provided in the online-only Data Supplement.

Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay and Caspase 3 Staining
For immunohistochemical detection and quantification of apoptotic cells, we used different assays: the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technology using the In Situ Cell Death Detection Kit, peroxidase (Roche, Basel, Switzerland), a fluorescent apoptosis kit (ApopTag, Chemicon/Millipore, Zug, Switzerland), and caspase 3 staining with active plus procaspase 3 antibodies (abcam).
The reduction in small vessels is a hallmark of the peripheral microangiopathy in human SSc. Therefore, we next assessed the vessel density in skin sections of Fra-2 tg mice compared with wt mice. In wt mice of different ages, there was no evident reduction in small dermal arteries and capillaries (Figure 3A). Similarly, Fra-2 tg mice at 9 weeks of age did not differ from wt mice (Figure 3B). However, starting from week 12, a significant decrease in capillary density could be observed in Fra-2 tg mice (Figure 3B). Semi quantitative analysis (Figure 3C) revealed 5.0 (quartiles 1 and 3, 4 and 6) capillaries per HPF in wt mice compared with only 0 (quartiles 1 and 3, 0 and 1) capillaries per HPF in Fra-2 tg mice (P=0.0003) at the 12 weeks of age. A similar difference could be detected at 16 weeks with 3.0 (quartiles 1 and 3, 2 and 3) capillaries per HPF in wt mice compared with 0.5 (quartiles 1 and 3, 0 and 1.75) capillaries per HPF in the dermis of Fra-2 tg mice (P=0.0013).

In contrast to human SSc, dilatation of capillaries was not observed in the skin of Fra-2 tg mice, and there was a decreased capillary diameter at week 16 in Fra-2 tg mice (Figure II of the online-only Data Supplement). Despite the same expression pattern of Fra-2 in the lungs and skin without any obvious difference in the vascular component cell layers (Figure IIIA of the online-only Data Supplement),
Fra-2 tg mice did not show a proliferative vasculopathy in the skin (Figure IIIB of the online-only Data Supplement), whereas they developed simultaneously a severe proliferative vasculopathy in the lungs. These results suggest that the effects of Fra-2 on the vasculopathy might be organ dependent. Increased perivascular inflammatory infiltrates, which are characteristic of early human SSc, were also present in the skin of 9-week-old Fra-2 tg mice compared with wt mice (Figure IV of the online-only Data Supplement) but not in older mice.

The Onset of Microangiopathy in Fra-2 tg Mice Parallels the Development of Progressive Skin Fibrosis

We next aimed to evaluate the temporal relationship between the microangiopathy and skin fibrosis in the Fra-2 tg model. In wt mice, no changes in dermal thickness could be observed over time (Figure 4A). Similar to the microvascular assessment, no differences between wt and Fra-2 tg mice could be detected at 9 weeks of age. However, Fra-2 tg mice showed a time-dependent increase in dermal thickness (Figure 4A, bottom) caused by accumulation of extracellular matrix (Figure 4B, bottom) starting at 12 weeks (dermal thickness, 431 μm; quartiles 1 and 3, 377 and 529 μm) compared with wt littermates (dermal thickness, 326 μm; quartiles 1 and 3, 291 and 354 μm; P = 0.0004; Figure 4A and 4B, top). In Fra-2 tg mice (Figure 4A and 4B, bottom), a progression of skin thickness could be observed at 16 weeks, with a thickness of 552 μm (quartiles 1 and 3, 488 and 552 μm) compared with wt mice with 393 μm (quartiles 1 and 3, 306 and 485 μm;
Figure 4A and 4B, top; $P=0.002$. Figure 4C displays the semiquantitative analysis. Taken together, these data show that the microvascular changes paralleled the development of skin fibrosis in Fra-2 tg mice.

**Apoptosis of Endothelial Cells Precedes the Development of Microangiopathy and Fibrosis in Fra-2 tg Mice**

Apoptosis of endothelial cells is considered one of the earliest phenomena in the development of the microangiopathy in human SSc. Therefore, we evaluated whether apoptosis of endothelial cells might play a role in the observed peripheral microangiopathy in Fra-2 tg mice. Analysis of skin sections by TUNEL assay demonstrated a higher number of apoptotic cells in the skin of Fra-2 tg compared with wt mice (Figure 5A). Semiquantitative analysis of skin sections showed a trend toward an increase in apoptotic cells in Fra-2 tg mice already at 9 weeks, which reached statistical significance at 12 and 16 weeks compared with wt mice (data not shown).

To confirm these findings with an independent method, staining with caspase 3 antibodies was performed as a marker of early apoptosis. Indeed, expression of caspase 3 confirmed the TUNEL assay findings with the same time-dependent expression pattern (Figure 5A). The increased apoptosis in the skin of Fra-2 tg mice was further assessed with an additional TUNEL assay using indirect immunofluorescence. Again, Fra-2 tg mice showed increased numbers of apoptotic cells compared with wt mice (Figure 5C).

Moreover, double staining with the endothelial cell marker vWF identified endothelial cells as the major apoptotic cell fraction. Notably, in contrast to the total cell fraction, endothelial cells in skin sections of Fra-2 tg mice showed a remarkable and significant increase in apoptosis already at 9 weeks compared with wt mice (55 [quartiles 1 and 3, 17 and 100] versus 33 [quartiles 1 and 3, 0 and 75] TUNEL-positive endothelial cells per HPF; $P=0.01$; Figure 5B). Thus, apoptosis of endothelial cells preceded the development of...
microangiopathy and fibrosis in Fra-2 tg mice. These data suggest that similar to human SSc, endothelial cell apoptosis could be one of the initiating events for microangiopathy in this model.

**Fra-2 Knockdown Reduces Apoptosis of HUMECs**

To further confirm the relationship between Fra-2 and increased apoptosis of endothelial cells, functional assays with HUMECs were performed. HUMECs expressed Fra-2 protein under basal conditions as analyzed by Western blot (Figure VA of the online-only Data Supplement). Fra-2 knockdown was achieved by transfection of HUMECs with specific siRNA (Figure VB and VC of the online-only Data Supplement). Twenty-four hours after transfection, HUMECs were stimulated with increasing concentrations of staurosporine (1, 2.5, and 5 μmol/L) at different time points. Treatment with staurosporine led to a dose- and time-dependent increase in apoptosis in all cell lines as analyzed by annexin V/PI staining. Although there was no difference in the spontaneous rate of apoptosis, Fra-2 knockdown prevented staurosporine-induced apoptosis in HUMECs. The effect was most pronounced after 6 hours of treatment, when Fra-2–transfected HUMECs showed an average apoptosis of 18.3% (quartiles 1 and 3, 14% and 22%) compared with wt mice. The adjacent picture shows the respective conventional TUNEL staining to allow better assessment of the structures (right). Experiments were done in triplicate. Magnification ×200. Figures are representative examples of 16 Fra-2 tg mice and 17 wt mice. *Significant (P<0.05).

Figure 5. Apoptosis of endothelial cells precedes the development of the peripheral microvasculopathy. A, Compared with wt littermates (top), a higher number of apoptotic cells could be observed in the dermis of Fra-2 tg mice (bottom; dark brown and black nuclei) as assessed by TUNEL assay or staining for caspase 3. Magnification ×100. B, Semiquantitative analysis revealed a significantly higher percentage of TUNEL-positive endothelial cells in the dermis of Fra-2 tg compared with wt mice starting from week 9. C, With the use of an additional TUNEL assay with indirect immunofluorescence, apoptotic nuclei were stained with FITC (green) and counterstained with DAPI (blue). In Fra-2 tg mice (middle), a higher number of FITC-positive cells could be detected compared with wt mice.
link between Fra-2 expression and apoptosis of microvascular endothelial cells.

**Fra-2 Knockdown Improves Tube Formation by Protecting HUMECs From Apoptosis**

We next aimed to investigate the mechanisms by which Fra-2–induced apoptosis of microvascular endothelial cells leads to rarefaction of capillaries. To do so, we performed a tube formation assay comparing cells transfected with Fra-2 siRNA and scrambled controls. Knockdown of Fra-2 in vitro improved the tube formation capacity of HUMECs (Figure 6A). The cumulative tube length of cells transfected with Fra-2 siRNA was 65 mm (quartiles 1 and 3, 47 and 79 mm) compared with 17.3 mm (quartiles 1 and 3, 11 and 23 mm) from controls (P = 0.0001), and the number of tubes increased to 46 (quartiles 1 and 3, 29 and 54) after Fra-2 knockdown compared with 10.5 (quartiles 1 and 3, 8 and 17) in scrambled controls (P = 0.002; Figure 6A). To prove that the observed high tube formation capacity of HUMECs after Fra-2 knockdown was indeed due to prevention of apoptosis, we combined the tube formation assay with an apoptosis assay. Apoptosis was induced by staurosporine after transfection with Fra-2 or scrambled siRNA and examined by immunocytochemistry using annexin V staining. In scrambled controls, fewer cells were visible because most of them had detached as a result of apoptosis, and the majority of remaining cells (DAPI, blue staining) were apoptotic (FITC, green staining). Fra-2 knockdown resulted in unaffected tube formation with a higher number of attached cells (DAPI, blue) and a much lower percentage of apoptotic cells (FITC, green). Semi quantitative analysis (right) confirmed that Fra-2 knockdown prevented HUMECs from staurosporine-induced apoptosis, with a significantly lower percentage of apoptotic cells in HUMECs transfected with Fra-2 siRNA. Experiments were done in triplicate. *Significant (P < 0.05).
limited cutaneous SSc, frequently in parallel with the onset of fibrosis in the subset of patients with its complications would be an even more promising therapeutic approach. Unfortunately, the initiating factors are often diagnosed late in their disease course, when fibrosis is already established and early disease stages have been passed.20 The UCD 200 chicken model is characterized by an antibody-mediated vasculopathy, early endothelial cell apoptosis, and tissue fibrosis.5 However, its use is restricted by the high variability in the disease course of individual animals, the long generation time of chicken, and the challenges in housing chicken. Other animal models of SSc do not resemble the vascular features of human SSc or are not yet characterized in detail.21,22 Thus, because human samples from early disease stages are not available, there is a clear need for feasible animal models of the peripheral vasculopathy of SSc.

In this study, we provide the first evidence that Fra-2 tg mice develop several features of the human peripheral vasculopathy, which is paralleled by a progressive skin fibrosis similar to that in patients with SSc. We also provide evidence on several experimental levels that apoptosis of endothelial cells of peripheral microvessels is among the earliest features of enhanced Fra-2 expression in both the in vivo animal model and functional in vitro assays with HUMECs. A similar mechanism with endothelial cell apoptosis has been proposed for the early peripheral microvascular changes in the human disease.5 Our experiments extend recent findings by Eferl et al9 showing that Fra-2 tg mice develop a severe proliferative pulmonary vasculopathy resembling pulmonary arterial hypertension followed by overt lung fibrosis leading to premature mortality. Thus, Fra-2 tg mice develop both a peripheral and pulmonary vasculopathy followed by organ fibrosis, which are unique features of SSc.

Effects of Fra-2 on Migration and Proliferation of Endothelial Cells
To address additional aspects of blood vessel formation, we examined the effects of Fra-2 knockdown on the migration and proliferation of HUMECs. HUMECs transfected with Fra-2 siRNA showed an increased migratory capacity in the scratch assay. Differences were most pronounced after 48 hours when HUMECs transfected with Fra-2 siRNA had already closed the scratch (P=0.02; Figure 7A). The effect of Fra-2 on migration was underlined by experiments examining chemotaxis. After transfection with Fra-2 siRNA, HUMECs showed an increased vascular endothelial growth factor–induced chemotaxis (1.7-fold [quartiles 1 and 3, 1.4 and 1.8]) compared with scrambled control cells; P=0.02; Figure 7B).

In contrast to migration, proliferation was not affected by transfection of HUMECs with Fra-2 siRNA as analyzed with the microtiter tetrazolium assay (data not shown). In addition, both the scratch assay and the chemotaxis assay did not reveal different results when the proliferation inhibitor mitomycin C was added. This suggests that differences in the assays were caused by the effects of Fra-2 on migration rather than proliferation.

Discussion
To date, the majority of therapeutic efforts in SSc aim to stop the progression of fibrosis and the resultant organ failures. However, preventing the onset of fibrosis rather than dealing with its complications would be an even more promising therapeutic approach. Unfortunately, the initiating factors leading to the increased production of extracellular matrix proteins and organ fibrosis in SSc remain unclear. Vascular damage has been considered important for the early pathomechanisms of the disease because it often occurs even years before the onset of fibrosis in the subset of patients with limited cutaneous SSc, frequently in parallel with the onset of fibrosis in diffuse cutaneous SSc.18 However, neither the initiating mechanisms leading to the early vascular manifestations nor the link between the vasculopathy and organ fibrosis has been established to date despite a number of proposed hypotheses.19

The limited progress in the understanding of the early pathomechanisms in SSc is caused in part by the lack of human biosamples from early disease stages and the lack of appropriate animal models resembling the human vasculopathy. Data from larger databases confirm that patients with SSc are often diagnosed late in their disease course, when fibrosis is already established and early disease stages have been passed.20 The UCD 200 chicken model is characterized by an antibody-mediated vasculopathy, early endothelial cell apoptosis, and tissue fibrosis.5 However, its use is restricted by the high variability in the disease course of individual animals, the long generation time of chicken, and the challenges in housing chicken. Other animal models of SSc do not resemble the vascular features of human SSc or are not yet characterized in detail.21,22 Thus, because human samples from early disease stages are not available, there is a clear need for feasible animal models of the peripheral vasculopathy of SSc.
Compared with other family members of the Fos and Jun families, Fra-2 is less well investigated. Fra-2 is supposed to exert mainly inhibitory effects on its downstream targets, but its effects have also been shown to depend on various factors such as the respective dimerization partner, the cell type, or even the cellular milieu and cell cycle phase. Interestingly, the induction of fibrosis in animal models of chronic inflammation and interleukin-13/interleukin-13Rα2 signaling has been shown recently to require dimerization of Fra-2 and c-Jun with subsequent induction of transforming growth factor-β. Indeed, staining of sequential skin sections of Fra-2 tg mice by immunohistochemistry revealed strikingly similar expression patterns of c-Jun and Fra-2, suggesting that the Fra-2/c-Jun activator protein-1 dimer might also be of importance in Fra-2 tg mice (data not shown). Although we did not address potential stimulators of Fra-2 expression in the human disease in our study, preliminary experiments indicate that the expression of Fra-2 in HUMECS is independent of major cytokines and growth factors operative in SSc such as transforming growth factor-β, platelet-derived growth factor-B, and vascular endothelial growth factor (data not shown). In addition, our data support the intriguing hypothesis that the pathophysiology of internal organ manifestations in SSc is at least in part different from the skin disease because, in contrast to the skin, the lungs of Fra-2 tg mice did not show any evidence for increased apoptosis as assessed by TUNEL and caspase 3 staining, and no proliferative vasculopathy was detectable in the skin of Fra-2 tg mice. Taken together, these results show that Fra-2 tg mice display various features of the peripheral vasculopathy of human SSc, with a reduction in capillaries and early apoptosis of endothelial cells. Furthermore, Fra-2 tg mice combine clinically evident peripheral and pulmonary vasculopathy with fibrosis of the skin and internal organs, particularly the lungs. We could also show that apoptosis of endothelial cells occurs before the manifestation of fibrosis and contributes to the reduction in capillary density in later stages of the model. Therefore, Fra-2 tg mice can be proposed as an excellent preclinical model that allows us to study the link between vasculopathy and fibrosis. Fra-2 tg mice also enable testing of antifibrotic agents and drugs targeting vasculopathy simultaneously. Because we provided evidence that Fra-2 is present in human SSc, Fra-2 might also represent a promising future target to treat both vasculopathy and fibrosis in SSc patients.

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Supplemental Material

Expression of Fra-2 in Fra-2 tg mice

In the present study, Fra-2 tg and wt littermates from the same breedings of 9 (n=6 each), 12 (n=5, n=6), and 16 weeks of age (n=5, n=6) were analyzed. The murine skin biopsies were all taken from the same localization at the upper back. In Fra-2 tg mice, the murine Fra-2 gene was expressed under the control of the ubiquitous major histocompatibility complex class I antigen H2Kb promoter\(^1\). Fra-2 tg mice\(^1\) were backcrossed from a mixed (C57BL/6 × CBA) genetic background on a pure C57/Bl6 background for six generations.

Histological analysis

Skin sections of SSc patients and healthy controls as well as skin and lung sections of Fra-2 tg and wt mice were fixed in 4 % formalin and embedded into paraffin. Five μm thick sections were stained with hematoxylin and eosin (HE) for determination of dermal thickness. For staining of extracellular matrix, skin sections were additionally processed with 0.1% picrosirius red according to standard protocols. Dermal thickness was analyzed with an Imager1 microscope (Carl-Zeiss, Jena, Germany) at 100 fold magnification by measuring the maximal distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction in three consecutive skin sections as described previously\(^2\). Local inflammation was analyzed by HE staining.
**Immunohistochemistry for Fra-2, von Willebrand factor (vWF), α-SMA (smooth muscle antigen), SM22α (smooth muscle protein 22α)**

For immunohistochemistry, paraffin-embedded tissue sections from human and murine skin and lungs were used. The treatment of tissue sections was performed as described previously. Incubation with the primary antibodies was performed at 4°C overnight, and the following antibodies were used: polyclonal rabbit-antibodies against Fra-2 (abcam, Cambridge, UK), vWF (abcam), monoclonal mouse-antibodies against α-SMA (Sigma-Aldrich, Buchs, Switzerland), monoclonal mouse antibodies against SM22α (abcam). Then, the slides were incubated for 30 min at room temperature with the following secondary antibodies: horseradish peroxidase (HRP)-labeled secondary goat anti-rabbit and goat anti-mouse antibodies, alkaline phosphatise (AP)-conjugated secondary goat anti-mouse antibodies (Jackson ImmunoResearch, Soham, UK). Staining for Fra-2 was visualized with AEC or diaminobenzidine (DAB), using a peroxidase substrate kit, for vWF with VIP, for α-SMA with fast blue or histogreen, using an AP substrate kit, and for SM22α with NT-BCIP using an AP substrate kit (all from Vector, Burlingame, USA). Irrelevant isotype-matched antibodies or IgGs were used as controls.

The analyses of all histological and immunohistochemical stainings were performed by two independent examiners who were blinded with regard to the different groups. Pictures were taken with a digital camera on an Imager1 microscope (Carl-Zeiss AG, Feldbach, Switzerland), using AxioVision software Release 4.6. The analyses included 12 SSc patients and 5 healthy controls as well as 16 Fra-2 tg and 17 wt mice.

To assess potential differences in the expression of Fra-2 between patients with diffuse and limited SSc, Fra-2 positive nuclei in dermal vessels were quantified by
automated counting performed using image analysis software (Image J, NIH) in three randomly chosen HPF/section.

For the semiquantitative analysis of microvessel density, pictures of three randomly chosen HPF/slide at x400 magnification were taken. Then, Fra-2-/vWF, Fra-2/α-SMA positive and Fra-2/SM22α-positive blood vessels were counted. Blood vessel density was assessed exclusively in the dermis without the epidermal and the subdermal layers. The mean ± SEM number of vWF-positive as well as the mean ± SEM number of α-SMA/vWF-positive and SM22α/vWF-positive vessels was then calculated for each group using Graph Pad Prism software.

Dilatation as a sign of morphological changes was assessed by measuring the diameters of vWF-positive/SM22α-negative vessels (capillaries) in Fra-2 tg mice compared to wt mice. At x400 magnification pictures were taken from 3 randomly selected HPF. The median \((Q_{1}, Q_{3})\) diameter of vWF-positive/SM22α-negative vessels (capillaries) was then calculated using Graph Pad Prism software.

Proliferation of blood vessels was assessed by measuring the thickness of α-SMA and SM22α positive vessel walls. At x200 magnification pictures were taken from 3 randomly selected HPF. The median \((Q_{1}, Q_{3})\) thickness of walls of α-SMA-positive/SM22α-positive vessels (vessels with muscular wall) was then calculated using Graph Pad Prism software.

**Detection of apoptosis by TUNEL assay and caspase 3 staining**

For immunohistochemical detection and quantification of apoptotic cells, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) technology was applied using the In Situ Cell Death Detection Kit, POD (peroxidase) (Roche, Basel, Switzerland) according to the manufacturer’s protocol: The slides were deparaffinized and pre-treated with nuclease-free trypsin for permeabilization at
37°C for 30 min. After application of the TUNEL reaction mixture, the slides were incubated for 1 h in a humidified atmosphere in the dark, then treated with converter-POD, covered with parafilm and incubated for another 30 min. All steps were performed at room temperature. Staining was visualized with DAB, using a peroxidase substrate kit (Vector). Slides, treated only with the label solution, but not the enzyme solution, were used as negative controls. The analysis of apoptotic endothelial cells was done using sequential sections stained for vWF. To confirm our results with an additional technique, we employed a fluorescent apoptosis kit (ApopTag, Chemicon) according to the instructions of the manufacturer, and double labeling for vWF was performed on the same sections. Caspase 3 staining was performed according to standard immunohistochemical protocols using active+pro caspase 3 antibodies (abcam). Staining was visualized with DAB, using a peroxidase substrate kit (Vector). Irrelevant isotype-matched antibodies were used as negative controls.

For the semiquantitative analysis of apoptotic nuclei, pictures of three randomly chosen HPF/slide at x400 magnification were taken with a digital camera using AxioVision software. Next, all nuclei were counted manually, and the percentage of apoptotic nuclei was calculated. Nuclei were counted as apoptotic based on the intensity of the staining (light brown for normal nuclei, dark brown to black for apoptotic nuclei) and based on morphological criteria such as nuclear shrinkage. The median(Q1,Q3) percentage of apoptotic nuclei was then calculated for each group, using Graph Pad Prism software. For independent confirmation, TUNEL-positive nuclei were quantified by automated counting performed by image analysis software (Image J, NIH). The analysis included 16 Fra-2 tg and 17 wt mice.
Transfection with siRNAs against Fra-2

For in vitro experiments, human dermal microvascular endothelial cells (HMVEC-d adult, Cambrex, Rockland, USA) were cultured in 6-well plates in endothelial cell medium (EGM-2 MV, Lonza, Basel, Switzerland), and passages 3-8 were used for analysis.

HUMECs were transfected using the HMVEC-L Nucleofector Kit (Amaxa, Cologne, Germany) as previously described with 2 μM pre-designed small interfering RNA duplexes against Fra-2. Two different siRNA duplexes against Fra-2 were designed and used in combination for nucleofection. The sequences were as follows: Fra-2 siRNA duplex 1: sense 5´-CCUCCAUGUCAAACCAUA-3´, antisense 5´-UAUGGGUUGGACAUGGAGG-3´; Fra-2 siRNA duplex 2: sense 5´-CCUCGUUCUACCUAUCC-3´, antisense 5´-AGGAUAGGUGAAGACGACG-3´. HUMECS transfected with control siRNAs (scrambled siRNA, Ambion, Darmstadt, Germany) were used as negative controls. Twenty-four hours after siRNA transfection, HUMECS were collected for further analyses. Transfection efficiency was controlled by Western blot and FACS analysis showing a reduction of Fra-2 expression by 72 % compared to control siRNA 24 h after transfection. The cell viability after transfection was assessed by FACS using propidium iodide staining and found to be 58.5(53,70)% (n = 6).

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the instructions of the manufacturer and reverse transcribed into complementary DNA (cDNA) with random hexamers as previously described. Gene expression was quantified by SYBR Green Real-time PCR using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Rotkreuz,
Specific primer pairs for human Fra-2 were designed with Primer 3 software and synthesized from Microsynth (Balgach, Switzerland). SYBR Green primers were as follows: forward primer 5'-GCAGGAGGAGAGATGAGCAG-3', reverse primer 5'-CGACGCTTCTCCTCCTCTTC-3'. Samples without enzyme in the reverse transcription reaction were used as negative controls to exclude genomic contamination. Non-specific signals caused by primer dimers were excluded by dissociation curve analysis and use of no-template controls. To normalize for the amounts of loaded cDNA, a predeveloped 18S assay (PE Applied Biosystems) was used as an endogenous control. Differences were calculated with the threshold cycle (Ct) method and with the comparative delta Ct method for relative quantification.

**Western blot analysis for Fra-2**

Nuclear extracts were prepared according to the protocol of Andrews and Faller. The concentration of nuclear protein was measured with the BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois, USA) to normalize for the amounts of loaded protein. Eight µg of protein from each sample were separated by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Roth, Karsruhe, Germany) as previously described. After blocking with 2% BSA for 1 h, membranes were incubated with polyclonal rabbit-anti-human Fra-2 antibodies (abcam) overnight at 4°C. HRP-conjugated polyclonal goat anti-rabbit antibodies (DAKO, Hamburg, Germany) were used as secondary antibodies. Proteins were visualized with the ECL plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK) and exposed to high performance chemiluminescence film (Amersham Biosciences). For confirmation of equal loading of proteins, the amount of U2AF was determined using mouse anti-human U2AF antibodies (Sigma) and goat anti-mouse antibodies (Dako, Glostrup, Denmark).
Microtiter tetrazolium (MTT) assay

To assess cell proliferation, HUMECs transfected with Fra-2 or scrambled siRNA were cultured in 96-well plates and analyzed after 40 h. After removal of 100 μl of the growth medium, MTT was added at a final concentration of 1 mg/ml and the cells were incubated at 37°C for additional 4 h. After dilution with 100 μl of 0.04 N HCL in isopropanol, the wells were analyzed using an automatic ELISA plate reader (UV MAX, Molecular Devices, Sunnyvale, CA) at a test wavelength of 570 nm.

Scratch assay

HUMECs transfected with Fra-2 siRNA or scrambled siRNA were seeded on 6 well-plates (500 000 cells/well) with or without mitomycin C (Sigma) at a final concentration of 25 μg/ml to control for proliferation. After 24 h of incubation a single uniform scratch was made in each well using a 1 ml blue plastic pipet tip. The scratch resulted in a cell-free gap of approximately 1.0 mm between to adjacent areas of HUMECs. Each scratch was randomly photographed at 4 separate sites along the length of the scratch. For the analysis an inverted microscope (Axiovert25, Zeiss) was used. Photographs were taken at 0, 24 and 48 h. The distance of the wound edges was measured by two independent, blinded investigators as described recently.7

Chemotaxis-induced migration assay

Migration was analyzed using the QCMTM Chemotaxis 96-well cell migration assay (Chemicon) following the manufacturer’s protocol. To exclude misleading effects due to proliferation, the experiment was performed with or without mitomycin C (Sigma) as described above. Briefly, HUMECs transfected with Fra-2 or scrambled siRNA were harvested after 24 h of serum starvation. One-hundred-twenty-five μl of
serum-free medium with and without VEGF (20 ng/ml) as chemoattractant were added to the wells of the feeder tray. The cells were re-suspended in 100 µl medium without VEGF and placed into the migration chamber and incubated for 24 h at 37°C in a CO₂ incubator. Afterwards, cells/media were discarded from the top side of the insert, and the migration chamber plate was placed onto a new 96-well feeder tray containing 150 µl of pre-warmed cell detachment solution in the wells. After incubation for 30 min at 37°C, the cells were dislodged completely from underside. Then, 50 µl of lysis buffer/dye solution were added to each well of the feeder tray. After 15 min of incubation, 150 µl of the mixture were transferred to a new 96-well plate and analyzed with a fluorescence plate reader using the 480/520 nm filter set. Samples without cells, but containing cell detachment buffer, lysis buffer and dye were used as negative controls.

**Tube formation assay**

HUMECs transfected with Fra-2 siRNA were cultured in 6-well plates (Greiner) coated with 200 µl of Matrigel Basement Membrane Matrix (BD Biosciences). The angiogenic properties of cells transfected with Fra-2 siRNA or scrambled siRNA were quantified after 24 h by measuring the cumulative tube length and by assessment of the number of tubes in three randomly chosen microscopic fields as described previously. Two blinded investigators evaluated the results of the assay. For analysis, the Axiovert25 microscope (Zeiss) was used.

**Apoptosis assay**

For induction of apoptosis, HUMECs seeded on 6-well plates (500000 cells/well) were treated for 4, 6, and 8 hours with increasing concentrations of the proteine kinase inhibitor staurosporine (Sigma) at final concentrations of 1 µM, 2.5
µM and 5 µM. HUMECs transfected with scrambled siRNA and non-stimulated cells were used as controls. Untreated HUMECs transfected with either Fra-2 siRNA or scrambled siRNA were used as controls.

Apoptotic cells were detected by Annexin-V-Fluos/PI staining (Roche) using flow cytometry analysis (FACS) according to the instructions of the manufacturer. Briefly, HUMECs were washed with PBS, detached with trypsin/0.5% EDTA and centrifuged at 200 x g for 5 min. Afterwards cell pellets were resuspended in 100 µl of Annexin-V-Fluos labelling solution and incubated for 15 min at room temperature. Then, 200 µl of incubation buffer were added. For detection of necrotic cells, 10 µl of a 50 µg/ml propidium iodide solution (Sigma) were added. The analysis was performed using the FACScan flow cytometer (Becton Dickinson, Mansfield, MA), and the data were evaluated with CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA).

**Combined tube formation and apoptosis assay**

Apoptosis in tube forming HUMECs was analyzed by immunocytochemistry using Annexin-V-Fluos (Roche) staining for adherent cells according to the instructions of the manufacturer. After transfection with Fra-2 siRNA or scrambled siRNA, HUMECs were seeded on double chamber slides (75 000 cells/chamber) coated with Matrigel (50 µl/cm²). Twenty-four hours after transfection, tube formation was analyzed, and cells were treated with staurosporine at a final concentration of 0.5 µM. After 2 h, the medium was discharged and cells were incubated with Annexin-V-Fluos labelling solution for 10 min at RT. Afterwards, cells were stained with DAPI (Sigma) at a dilution of 1:200. After an additional incubation period of 10 min, the chambers and silicon borders were removed, and the slides were covered
with coverslips. For fluorescence analysis, an Imager1 microscope (Zeiss) and AxioVision software Release 4.6 were used. The percentage of apoptotic cells were calculated as rate of FITC-positive nuclei out of the total of DAPI stained nuclei/high power field. The experiments were performed in duplicates.
### Supplemental Tables

#### Supplementary Table 1: Patients’ characteristics

<table>
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<tr>
<th>SSc patients</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Sub-type of SSc</th>
<th>mRSS</th>
<th>Disease duration (years)</th>
<th>Antibodies</th>
<th>Disease symptoms</th>
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mRSS = modified Rodnan skin score; ANA = anti-nuclear antibodies; ACA = anti-cytoplasmatic antibodies; SCL70 = anti-topoisomerase-1 antibodies, PH = pulmonary hypertension. n.a. = not available
Supplemental figures

Supplementary Fig.1

Negative controls for Fra-2

A
healthy SSc wt tg

Negative controls for vWF, α-SMA, SM22α

B
SSc wt tg SSc tg

Negative controls for TUNEL (left) and caspase 3 (right)

C
wt tg wt tg
Supplementary Fig. 2

vWF- pos./SM22alpha- neg. vessels (capillaries)

Vessel diameter (μm)

week 9 wt, week 9 tg, week 12 wt, week 12 tg, week 16 wt, week 16 tg
Supplementary Fig. 4
Supplementary Fig. 5

A  untreated HUMECs

Fra-2  U2AF

B  Fra-2 siRNA  scrambled

Fra-2  U2AF

C  Fold change of Fra-2 protein

scrambled  Fra-2 siRNA
Supplemental table and figure legends

Supplementary Table 1: This table summarizes the characteristics of the enrolled patients (n=12). Organ involvement was defined according to the EUSTAR criteria\(^9\). Healthy age- and sex-matched volunteers were used as controls (n=5).

Supplementary Fig.1:

Fig. 1A: This panel shows the isotype controls of the Fra-2 staining in human and murine skin (magnification x100).

Fig. 1B: The first picture displays the isotype control of the double staining of vWF and α-SMA in the skin of SSc patients. The two following pictures represent the isotype controls of vWF staining in the skin of wt and tg mice (magnification x100). The last two pictures show the isotype controls of SM22α staining in the skin of SSc patients and Fra-2 tg mice (magnification x100).

Fig. 1C: The two pictures on the left show the negative controls of the TUNEL staining in wt and tg mice, and the two pictures on the right represent the negative controls of caspase 3 staining in wt and tg mice (magnification x100).

Supplementary Fig.2:

To assess morphological changes of vascular structures such as dilatation, the diameters of capillaries of Fra-2 tg mice compared to wt mice were analyzed at 9, 12 and 16 weeks. The semiquantitative analysis of vWF positive/SM22α negative blood vessel (capillaries) demonstrates that in Fra-2 tg mice the diameters of capillaries did not differ from those of wt littermates at weeks 9 and 12, but were reduced at 16 weeks of age. * indicates p<0.05.
Supplementary Fig.3:
Fig. 3A: Fra-2 staining (red) in representative lung sections of Fra-2 tg mice. Fra-2 protein was expressed ubiquitously; however, the expression was most prominent in small vessels (arrows) and proliferative vessels (thick arrows) (left picture). Fra-2 protein was present in the endothelial layer of proliferative vessels (upper arrow) as well as in bronchial epithelium (lower arrow) (right picture).

Fig. 3B shows representative skin sections of Fra-2 tg and wt mice which have been stained with α-SMA (blue). In contrast to the proliferative vascular changes in the lungs, in the skin of Fra-2 tg mice (left), there was no proliferative vasculopathy detectable. The right picture shows wt mice for comparison.

Supplementary Fig.4:
Fig. 4A: Representative HE stained skin section of a 9 week old Fra-2 tg mouse (magnification x100). The rectangles indicate areas of perivascular inflammation.

Fig. 4B: Magnification (x200) of the areas indicated in 3A. As assessed by HE staining, in Fra-2 tg mice, infiltrates (arrows) were present in the dermal layer in the vicinity of blood vessels (big arrows).

Fig. 4C: In the respective wt controls, local inflammation could not be observed (HE staining, magnification x100).

Supplementary Fig.5:
Fig. 5A: In HUMECs the basal expression of Fra-2 was confirmed by Western blot (n=2). To ensure loading of an equal amount of protein, U2AF$^{65}$ has been used as endogenous control.

Fig. 5B: The successful knock down of Fra-2 protein after transfection of HUMECs with Fra-2 siRNA was measured by Western blot.
Fig. 5C: After Fra-2 knock down the expression of Fra-2 protein in HUMECs decreased by 61 ± 0.7% (p=0.03).

Supplementary Fig.6:
Fra-2 knock down prevented HUMECs from staurosporine-induced apoptosis as analyzed by FACS. The effect was most pronounced at 6 h of treatment (n=5).
Supplemental References


