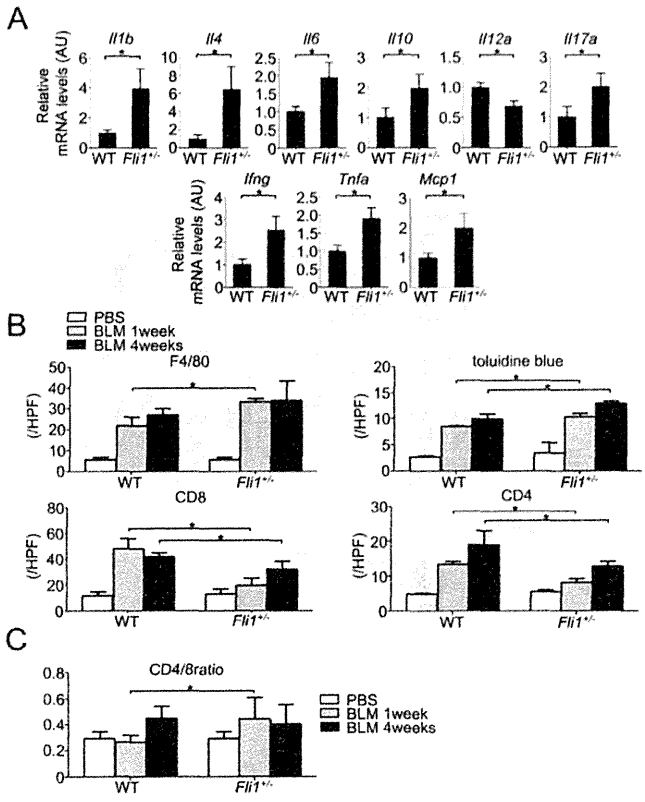

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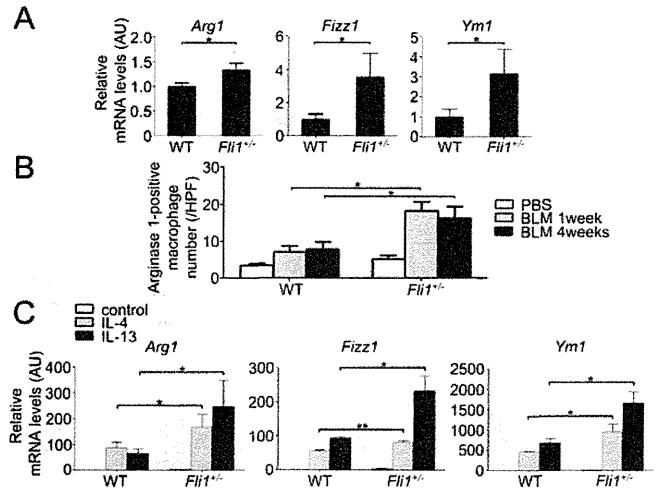

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A possible contribution of endothelial CCN1 downregulation due to Fli1 deficiency to the development of digital ulcers in systemic sclerosis

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A short title: The role of CCN1 in SSc vasculopathy

Abstract

CCN1 is a pleiotropic molecule involved in angiogenesis and postnatal vasculogenesis, both of which are impaired in systemic sclerosis (SSc). To elucidate the potential role of CCN1 in the development of SSc, we investigated CCN1 expression in the lesional skin of SSc patients and SSc animal models and the clinical correlation of serum CCN1 levels. CCN1

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expression was markedly decreased in dermal small blood vessels of SSc patients compared with those of healthy controls, while comparable between normal and SSc dermal fibroblasts. Transcription factor Fli1, whose deficiency due to epigenetic suppression is implicated in the pathogenesis of SSc, occupied the CCN1 promoter and gene silencing of Fli1 resulted in the reduction of CCN1 expression in human dermal microvascular endothelial cells. Consistently, CCN1 expression was suppressed uniformly and remarkably in dermal blood vessels of $Fli1^{+/-}$ mice and partially in those of endothelial cell-specific Fli1 knockout mice. Furthermore, serum CCN1 levels were significantly decreased in SSc patients with previous and current history of digital ulcers as compared to those without. Collectively, these results suggest that endothelial CCN1 downregulation at least partially due to Fli1 deficiency may contribute to the development of digital ulcers in SSc patients. This study further supports the idea that epigenetic downregulation of Fli1 is a potential predisposing factor in the pathogenesis of SSc.

Key words: systemic sclerosis, CCN1, angiogenesis, vasculogenesis, digital ulcers.

Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by vascular injury, autoimmunity, and fibrosis of the skin and various internal organs (1, 2). Although the detailed pathological process of SSc still remains elusive, aberrant vascular remodeling following autoimmunity-mediated vascular injury has been believed to contribute to the development of vasculopathy characteristic of this disease (3-8). It is generally accepted that aberrant vascular remodeling occurs as a result of impaired angiogenesis and vasculogenesis in SSc (9, 10). Serum levels of various angiogenic/angiostatic factors are largely altered and mostly suggest the constitutive pro-angiogenic status especially in the active stage of the disease. For instance, serum levels of vascular endothelial growth factor are elevated in SSc patients, especially in its earliest disease stage (11), and serum levels of angiopoietin 2, which reflect a pro-angiogenic status, positively correlate with disease activity and severity of SSc (12). On the other hand, circulating endothelial progenitor cells (CEPs), which play a role in

postnatal vasculogenesis, are decreased and functionally impaired in SSc (13-15), partially accounting for the loss of neovascularization following vascular injury in this disease.

CCN1 is a secreted cysteine-rich heparin-binding protein with diverse functions including the regulatory effect on angiogenesis. CCN1 belongs to CCN gene family together with CCN2 (connective tissue growth factor), CCN3 (nephroblastoma), and CCN4/5/6 (Wnt-inducible secreted protein-1, -2, and -3) (16). The role of CCN1 in embryonic neovascularization has been well studied in CCN1 null mice which indicates the highly impaired angiogenesis despite normal vasculogenesis (17). Supporting this, a line of evidence has demonstrated that CCN1 promotes angiogenesis through its direct binding to integrin $\alpha V\beta 3$ on endothelial cells and the induction of other angiogenic factors, such as vascular endothelial growth factor-A and -C, from dermal fibroblasts (18-20). In contrast to embryonic vasculogenesis, CCN1 contributes to postnatal vasculogenesis mediated by CEPs. CCN1 binds as a soluble factor to CEPs through integrin $\alpha V\beta 3$ and $\alpha M\beta 2$, which aids in the attachment and transmigration of CEPs into the area of neovascularization (21). Furthermore, CCN1 induces the release of various chemokines, cytokines, growth factors, and proteolytic enzymes from CEPs, leading to endothelial cell proliferation and angiogenesis (21). Thus, CCN1 is a novel molecule involved in both of angiogenesis and postnatal vasculogenesis.

These backgrounds encouraged us to investigate the potential role of CCN1 in the development of SSc. Therefore, in this study we investigated the expression levels of CCN1 in SSc lesional skin and the mechanism accounting for the altered expression of CCN1 utilizing *in vitro* cell culture system and *in vivo* animal models. Furthermore, we also examined the clinical correlation of serum CCN1 levels in SSc patients.

Methods

Ethics Statement

The study was performed according to the Declaration of Helsinki and approved by the ethical committee of The University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all of the patients and healthy controls.

Immunohistochemistry

Immunohistochemistry with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was performed on formalin-fixed, paraffin-embedded skin sections using anti-CCN1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Skin samples were obtained from forearms of 7 SSc patients and 7 healthy controls closely matched for age and gender and from the back skin of wild type and Fli1 muted mice which are previously described (22, 23).

Immunofluorescence

Immunofluorescence was carried out with human and murine skin sections, in which goat anti-platelet and endothelial cell adhesion molecule 1 (PECAM1) antibody (Santa Cruz Biotechnology) and rabbit anti-CCN1 antibody (Santa Cruz Biotechnology) were used as primary antibodies and FITC-conjugated donkey anti-rabbit IgG antibody (Santa Cruz Biotechnology) and Alexa Fluor donkey 555 anti-goat IgG antibody (Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies. Coverslips were mounted by using Vectashield with DAPI (Vector Laboratories), and staining was examined by using Bio Zero BZ-8000 (Keyence, Osaka, Japan) at 495 nm (green), 565 nm (red), and 400 nm (blue).

Cell cultures

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of 6 diffuse cutaneous SSc patients with less than 2 years of skin thickening and from the corresponding area of 6 closely matched healthy donors. Fibroblasts were cultured in Dulbecco's modified eagle medium with 10% fetal calf serum, 2 mM L-glutamine, and the antibiotic antimycotic solution. These cells were individually maintained as monolayers at 37°C in 95% air, 5% CO_2. Human dermal microvascular endothelial cells (HDMECs) were purchased from Takara Bio Inc. (Shiga, Japan) and cultured on collagen-coated tissue culture plates in EBM-2 medium supplemented with the EGM-2 Bullet Kit (Lonza, Walkersville, MD, USA). Experiments were conducted with dermal fibroblasts in passage 3 - 6 and with HDMECs in passage 3 - 5.

RNA isolation and reverse transcript (RT)-real time PCR in cultured cells and skin samples

Gene silencing of Fli1 in HDMECs, the generation of a BLM-induced murine SSc model, RNA isolation from those cells and skin tissue, and RT-real time PCR were carried out as described previously (24-26). The sequences of primers were as follows: CCN1-forward 5'-AAGAAACCCGGATTTGTGAG-3', CCN1-reverse 5'-GCTGCATTTCTTGCCCTTT-3'; Ccn1-forward 5'-TGACCTCCTCGGACTCGAT-3', Ccn1-reverse 5'-GGTTCGGTGCCAAAGACA-3'; FLI1-forward 5'-GGATGGCAAGGAACTGTGTAA-3', FLI1-reverse 5'-GGTTGTATAGGCCAGCAG-3'; GAPDH-forward 5'-ACCCACTCCTCCACCTTTGA-3', GAPDH-reverse 5'-CATACCAGGAAATGAGCTTGACAA-3'; Gapdh-forward 5'-CGTGTTCCTACCCCCAATGT-3', Gapdh-reverse 5'-TGTCATCATACTTGGCAGGTTTCT-3'.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, USA), as described previously (26). Putative Fli1 transcription factor binding site in the CCN1 promoter was predicted by Tfsitescan. The primers were as follows: CCN1/F-1082, 5'-ATGATTTCAGGCCACTCCAC-3'; CCN1/R-882, 5'-CTTTAGTTCCAGCCCACTGC-3'.

Patients enrolled in the measurement of serum CCN1 levels

Serum samples, frozen at -80°C until assayed, were obtained from 66 SSc patients (63 women, 3 men; age, median [25-75 percentile]: 59.5 years [48.8-68.0]; disease duration, 6.0 years [2.0-19.0]) and 20 healthy individuals (19 women, one man; age 54.5 years [45.3-71.5]). Patients having been treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by the LeRoy's classification system (27): 40 with diffuse cutaneous SSc (dcSSc) and 26 with limited cutaneous SSc (lcSSc). All patients fulfilled the criteria proposed by the American College of Rheumatology (28).

The measurement of serum CCN1 levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum CCN1 levels (R & D Systems, Minneapolis, MN, USA). Briefly, polystyrene cups coated with anti-CCN1 antibodies were incubated with 100 μl of assay diluent and 50 μl of serum at room temperature for 2 hours. Then, the cups were washed and incubated at room temperature for 2 hours with horseradish peroxidase conjugated anti-CCN1 antibodies. Next, the wells were washed again, added with tetramethylbenzidine, and incubated at room temperature for 30 minutes. Finally, sulfuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum CCN1 levels were calculated using standard curve.

Clinical assessment

The definition of disease onset and disease duration and the details of assessment for organ involvement were previously described.(29) Patients with digital ulcers were defined as patients with previous and current history of digital ulcers.

Statistical analysis

Statistical analysis was carried out with one-way ANOVA followed by Turkey *post hoc* test for multiple comparison, with Mann-Whitney U-test to compare the distributions of two unmatched groups, with a paired t-test for the comparison of paired data after confirming the normal distribution of the data, with Spearman's rank correlation coefficient to evaluate the correlation with clinical data, and with Shapiro-Wilk normality test to confirm a normal distribution. Statistical significance was defined as a P value of <0.05.

Results

CCN1 expression is decreased in dermal blood vessels of SSc patients.

We initially investigated the expression levels of CCN1 in normal and SSc skin sections by immunohistochemistry. Representative results are shown in Figure 1a and 1b. The results of 7 closely matched pairs are summarized in Table 1. In healthy controls, CCN1 expression was detected in epidermal keratinocytes, dermal fibroblasts, and dermal small blood vessels

and small arteries (Figure 1a). In SSc patients, CCN1 was expressed in epidermal keratinocytes and dermal fibroblasts at comparable levels to those seen in healthy controls though there was a small variation. The most striking difference was observed in dermal small blood vessels and small arteries. In 6 out of 7 pairs, CCN1 expression in vascular walls was markedly decreased in SSc skin sections compared with normal skin sections (Figure 1b). Importantly, co-localization of CCN1 with PECAM1, an established endothelial cell marker, was also confirmed by double immunofluorescence (Figure 1c). Consistent with this observation, the lesional skin of SSc patients exhibited mRNA levels of the *CCN1* gene significantly lower than the healthy control skin (Figure 1d), which seems to be at least partially attributable to CCN1 downregulation in SSc dermal small blood vessels. Since it is difficult to detect a small difference in the expression levels of target molecules by immunohistochemistry, we also determined the expression levels of CCN1 in cultured normal and SSc dermal fibroblasts by RT-real time PCR. As shown in Figure 1e, mRNA levels of the *CCN1* gene were comparable between normal and SSc dermal fibroblasts. Collectively, these results indicate that CCN1 expression is decreased in dermal small blood vessels, but not in dermal fibroblasts, of SSc patients.

CCN1 expression is decreased in dermal blood vessels of Fli1$^{+/-}$ mice and endothelial cell-specific Fli1 knockout mice.

We next investigated the potential mechanism by which CCN1 expression is suppressed in dermal blood vessels of SSc patients. Since Fli1 expression is markedly suppressed at least partially through an epigenetic mechanism in various cell types of SSc skin including dermal microvascular endothelial cells (26, 30, 31), which was also confirmed in skin samples used in the present study (Figure 1a, 1b and 1d), and endothelial Fli1 deficiency reproduces the histopathological and functional abnormalities characteristic of SSc vasculopathy in mice (22), we hypothesized that endothelial Fli1 deficiency inhibits the expression of CCN1 in dermal blood vessels of SSc patients. To address this issue, we examined the effect of Fli1 gene silencing on mRNA levels of the *CCN1* gene in HDMECs. As shown in Figure 2a, gene silencing of Fli1 resulted in a significant decrease in mRNA levels of the *CCN1* gene in

HDMECs (0.68 ± 0.14 fold increase, p = 0.0021). Furthermore, ChIP analysis revealed the binding of Fli1 to the CCN1 promoter in HDMECs (Figure 2b). These results indicate that Fli1 directly targets the *CCN1* promoter and is required for homeostatic CCN1 expression in endothelial cells. To further confirm if endothelial Fli1 deficiency results in the downregulation of CCN1 in dermal blood vessels *in vivo*, we carried out immunohistochemistry with the skin sections from Fli1 mutated mice. Notably, CCN1 expression was uniformly and totally undetectable in dermal blood vessels of $Fli1^{+/-}$ mice and only partially detectable in those of endothelial cell-specific Fli1 knockout (Fli1 ECKO) mice, while abundantly seen in those of wild type mice (Figure 2c, 2d, 2f, 2g, 2i, and 2j). As previously reported (22), Fli1 mRNA levels are decreased up to 20 – 50% of baseline in dermal microvascular endothelial cells isolated from Fli1 ECKO mice, suggesting that most of endothelial cells of Fli1 ECKO mice express Fli1 at lower levels than endothelial cells of $Fli1^{+/-}$ mice. Given the variable efficiency of the Cre enzyme in individual endothelial cells (22), in Fli1 ECKO mice Fli1 expression is not affected in some endothelial cells which theoretically produce CCN1 to a similar extent to wild type endothelial cells, as shown in Figure 2i and 2j. We also confirmed the co-localization of CCN1 with PECAM1 by double immunofluorescence in murine blood vessels (Figure 2e, 2h and 2k). These results indicate that endothelial Fli1 deficiency induces CCN1 downregulation in dermal blood vessels *in vivo*, suggesting that endothelial Fli1 deficiency contributes to CCN1 suppression in SSc dermal blood vessels.

The decrease in serum CCN1 levels correlates with vascular involvement in SSc patients.

We next evaluated the clinical correlation of serum CCN1 levels in SSc patients. As shown in Figure 3a, serum CCN1 levels were comparable among dcSSc, lcSSc, and healthy controls (median [25 - 75 percentiles]; 87.2 pg/ml [70.4 - 127.0], 117.1 pg/ml [95.4 - 140.2], and 106.0 pg/ml [80.1 - 166.3], respectively; p = 0.29, one-way ANOVA). We also examined the correlation of serum CCN1 levels with dermal and pulmonary fibrotic markers, including modified Rodnan total skin thickness score, the percentage of predicted vital capacity, and the

percentage of predicted diffusion lung capacity for carbon monoxide, but failed to detect any significant correlation (data not shown).

Since CCN1 is downregulated in dermal small blood vessels of SSc as described above, we further looked at the correlation of serum CCN1 levels with clinical symptoms relevant to SSc vasculopathy, such as Raynaud's phenomenon, nailfold bleeding, telangiectasia, digital ulcers, pulmonary arterial hypertension, and scleroderma renal crisis. To this end, serum CCN1 levels were compared between SSc patients with each symptom and those without. The presence of cutaneous vascular symptoms, including Raynaud's phenomenon, nailfold bleeding, and telangiectasia, did not affect serum CCN1 levels (patients with symptoms versus those without; 103.1 [80.7 - 134.6] pg/ml versus 91.3 [41.6 - 139.3] pg/ml [p = 0.35] for Raynaud's phenomenon, 101.3 [81.9 - 134.6] pg/ml versus 113.6 [74.8 - 155.6] pg/ml [p = 0.95] for nailfold bleeding, 87.8 [73.6 - 132.9] pg/ml versus 106.6 [78.9 - 154.1] pg/ml [p = 0.34] for telangiectasia). Regarding organ involvement associated with proliferative obliterative vasculopathy, SSc patients with digital ulcers had serum CCN1 levels significantly lower than those without (84.9 [69.8 - 121.5] pg/ml versus 114.2 [87.2 - 147.4] pg/ml [p = 0.019], Figure 3b), while the presence of elevated right ventricular systolic pressure or scleroderma renal crisis did not alter serum CCN1 levels in SSc patients (113.6 [72.5 - 134.6] pg/ml versus 99.5 [78.4 - 142.8] pg/ml [p = 0.73] for elevated right ventricular systolic pressure, 98.9 [39.6 - 144.2] pg/ml versus 101.3 [78.1 - 135.8] pg/ml [p = 0.62] for scleroderma renal crisis). Importantly, serum CCN1 levels were decreased in SSc patients with digital ulcers than in healthy controls (P = 0.045), while comparable between SSc patients without digital ulcers and healthy controls (P = 0.75). These results suggest that the decreased expression of CCN1 in dermal small blood vessels is linked to the development of digital ulcers in SSc patients.

Discussion

It is generally accepted that the impairment of angiogenesis in response to vascular injury is a potential mechanism leading to progressive vascular and fibrotic complications in SSc (9, 10, 13, 15, 32, 33). Therefore, this study was undertaken to investigate the potential role of CCN1 in the developmental process of SSc. In accordance with the critical role of CCN1 in regulating the behavior of endothelial cells during vascular remodeling (17-21), the altered expression of CCN1 was prominent in dermal small blood vessels and small arteries of SSc patients. As shown by the analysis of serum CCN1 levels, SSc patients with digital ulcers had serum CCN1 levels significantly lower than the other patients. Since soluble molecules released from endothelial cells are much more easily accessible to blood stream than those secreted from keratinocytes and fibroblasts, the decrease in serum CCN1 levels may be mainly attributable to the downregulation of CCN1 in vascular walls. Therefore, the close association of low serum CCN1 levels with current and previous history of digital ulcers suggests a possible association of endothelial CCN1 deficiency with the development of digital ulcers in SSc patients. Given that digital ulcers associated with SSc are largely attributable to aberrant vascular remodeling (9, 10), CCN1 may be a member of soluble factors involved in the pathological process underlying SSc vasculopathy.

The expression profile of pericyte markers in dermal small blood vessels represents the constitutive pro-angiogenic status of SSc lesional skin, namely, the increased expression of Rgs5, a marker of pericytes with pro-angiogenic phenotype, and the decreased expression of α-smooth muscle actin, a marker of pericytes with angiostatic phenotype (22, 34). However, various clinical data have revealed the complicated vascular events in SSc, which is characterized by altered serum levels of various angiogenic/angiostatic molecules. Most of the data imply the constitutive activation of angiogenesis, while the others suggest the defective angiogenesis (9, 10). Although the detailed molecular mechanisms accounting for the complicated vascular events in SSc still remain unknown, our previous studies have disclosed that Fli1 ECKO mice recapitulate the morphologically and functionally abnormal blood vessels characteristic of SSc vasculopathy, such as stenosis of arterioles, dilation of

capillaries, and increased vascular permeability (22). Furthermore, endothelial Fli1 deficiency contributes to the altered expression of angiogenesis-related molecules, such as VE-cadherin, PECAM1, platelet-derived growth factor-B, matrix metalloproteinase 9, cathepsin V, cathepsin B, and CXCL5, in SSc dermal microvascular endothelial cells (22, 26, 35, 36). These results suggest the critical contribution of endothelial Fli1 deficiency to the development of SSc vasculopathy. Based on this idea, we examined the effect of Fli1 deficiency on the expression levels of the *CCN1* gene *in vivo* and *in vitro*. Notably, gene silencing of Fli1 significantly suppressed mRNA levels of the *CCN1* gene and Fli1 occupied the CCN1 promoter. Furthermore, CCN1 expression was markedly reduced in dermal small blood vessels of Fli1 mutated mice. These results indicate that CCN1 is a member of angiogenesis-related molecules directly regulated by Fli1 and closely involved in the pathological angiogenesis of SSc.

The impairment of CEPs has been well studied in the research field of SSc by focusing on the number and the property of CEPs (13-15, 32, 33). On the other hand, the recruitment of CEPs to injured vascular areas is another important step in the regulation of postnatal vasculogenesis, in which endothelial cell-derived CCN1 is involved. Dual roles of CCN1 in angiogenesis and postnatal vasculogenesis suggest that this molecule coordinately regulates the complex interplay between the two processes during the physiological vascular remodeling. Therefore, the downregulation of endothelial CCN1 expression may be involved in the defective vasculogenesis of SSc. As far as we know, the mechanism regulating CEP recruitment to injured vessels has not been well examined in SSc. Further studies on CCN1 may provide us a new clue to understand the mechanism underlying defective vasculogenesis of SSc.

In summary, this is the first study evaluating the role of CCN1 in the pathological process of SSc. A series of clinical and laboratory data suggest that endothelial CCN1 downregulation at least partially due to Fli1 deficiency may contribute to the defective angiogenesis characteristically seen in SSc, leading to the development of digital ulcers. This

study further supports the idea that epigenetic downregulation of Fli1 is a potential predisposing factor in the pathogenesis of SSc (22, 31, 37, 38).

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Authors' contributions

Study conception and design: RS, YA. Acquisition of data: RS, T Taniguchi, TY, T Takahashi, YI, T Toyama, ZT. Analysis and interpretation of data: RS, YA, YT, MS, TK SS. Drafting and/or critical revision of the manuscript: RS, YA, YT, MS, TK, SS. All authors have read and approved the final manuscript.

Conflict of interests

The authors have declared no conflicts of interest.

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Table 1. CCN1 levels in skin sections from SSc patients and healthy controls

Samples	Age/sex	Duration (years)	dcSSc/lcSSc	Keratinocytes	Fibroblasts	Blood vessels
NS1	65F			++	++	+++
SSc1	60F	0.1	dcSSc	+++	+	+
NS2	42F			+++	++	+++
SSc2	48F	0.7	dcSSc	+++	+	++
NS3	65F			+++	+	+++
SSc3	68F	2	dcSSc	++	+	+
NS4	41F			+++	+	++
SSc4	43F	2.5	dcSSc	++	+	+
NS5	71F			+++	+	++
SSc5	70F	5	dcSSc	+++	+	++
NS6	31F			+++	+	+++
SSc6	32F	8	dcSSc	++	+	++
NS7	37F			+++	+	+++
SSc7	38F	15	lcSSc	+++	+	+

Disease duration means the interval between the onset defined as the first clinical event of SSc other than Raynaud's phenomenon and the time the blood samples were drawn. NS, normal skin; SSc, systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis. We used the following grading system: -, no staining; +, slight staining; ++, moderate staining; +++, strong staining.

Legends for illustrations

Figure 1. The expression levels of CCN1 in the lesional skin of SSc patients.

a, b. The expression of CCN1 and Fli1 was evaluated by immunohistochemistry in skin sections from healthy controls (HC; a) and SSc patients (b). Representative results are shown (original magnification, x400). Right upper panels of each picture group depict representative dermal small vessels shown with dotted squares in left panels. Right lower panels of each

picture group exhibit dermal small arteries. **c.** Immunofluorescence was carried out with DAPI (nuclei; blue) and antibodies against PECAM1 (red) and CCN1 (green) in skin sections from healthy controls (HC) and SSc patients. Representative results of dermal small vessels are shown. **d.** mRNA levels of the *CCN1* and *FLI1* genes were determined by RT-real time PCR in normal and SSc skin sections (n = 5 for healthy controls and n = 16 for SSc subjects). **e.** mRNA levels of the *CCN1* gene were examined by RT-real time PCR in normal and SSc dermal fibroblasts (n = 6 for both of healthy and control subjects). The relative value compared with controls is expressed as mean ± SEM.

Figure 2. The impact of endothelial Fli1 deficiency on the expression levels of CCN1 *in vivo* and *in vitro*.

a. mRNA levels of the *FLI1* and *CCN1* genes in HDMECs transfected with Fli1 siRNA or non-silencing scrambled RNA (SCR) were measured by RT-real time PCR and normalized to mRNA levels of the *GAPDH* gene. The relative values compared with the controls are expressed as mean ± SEM of 6 independent experiments. Statistical analysis was carried out with a 2-tailed paired t-test after confirming the normal distribution of the data. **b.** Chromatin was isolated from HDMECs and immunoprecipitation was conducted with rabbit anti-Fli1 antibody or rabbit IgG. PCR amplification was carried out using CCN1 promoter-specific primers. One representative of three independent experiments is shown. **c-k.** CCN1 expression in small blood vessels was determined by immunohistochemistry (c, d, f, g, i, j) and immunofluorescence (e, h, k) in the skin sections of 3 month-old wild type mice (c-e), $Fli1^{+/-}$ mice (f-h), and Fli1 ECKO mice (i-k). Epidermis, dermis, and subcutaneous fat tissue are shown in c, f, and i (original magnification × 200). Small blood vessels of c, f, and i are shown in d, g, and j with a higher magnification, respectively. Corresponding vessels are shown with arrows. Immunofluorescence was carried out with DAPI (nuclei; blue) and antibodies against PECAM1 (red) and CCN1 (green). Representative results of small vessels are shown. Representative results of 5 mice for each strain are shown. Similar staining levels were seen in all the other mice of each strain.