Endothelial CCR6 Expression Due to FLI1 Deficiency Contributes to Vasculopathy Associated with Systemic Sclerosis

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Abstract

Background: We have recently demonstrated that serum CCL20 levels positively correlate with mean pulmonary arterial pressure in patients with systemic sclerosis (SSc). Considering a proangiogenic effect of CCL20 on endothelial cells via CCR6, the CCL20/CCR6 axis may contribute to the development of SSc vasculopathy. Therefore, we explored this hypothesis using clinical samples, cultured cells and murine SSc models.

Methods: The expression levels of CCL20 and CCR6 in the skin, mRNA levels of target genes and the binding of transcription factor FLI1 to the target gene promoter were evaluated by immunostaining, quantitative reverse transcription PCR and chromatin immunoprecipitation, respectively. Vascular permeability was evaluated by Evans blue dye injection in bleomycin-treated mice. Angiogenic activity of endothelial cells was assessed by *in vitro* angiogenesis assay.

Results: CCL20 expression was significantly elevated in dermal fibroblasts of patients with early diffuse cutaneous SSc, while CCR6 was significantly up-regulated in dermal small vessels of SSc patients irrespective of disease subtypes and disease duration. In human dermal microvascular endothelial cells, *FLI1* siRNA induced the expression of CCR6, but not CCL20, and FLI1 bound to the *CCR6* promoter. Importantly, vascular permeability, a representative SSc-like vascular feature of bleomycin-treated mice, was attenuated by *Ccr6* siRNA treatment, and *CCR6* siRNA suppressed the angiogenic activity of human dermal microvascular endothelial cells assayed by *in vitro* tube formation.

Conclusions: The increased expression of endothelial CCR6 due to FLI1 deficiency may contribute to the development of SSc vasculopathy.

Background

Systemic sclerosis (SSc) is a multisystem autoimmune disease representing vasculopathy and tissue fibrosis of the skin and various internal organs [1]. Recent clinical studies have demonstrated the efficacy of several drugs for tissue fibrosis and vasculopathy associated with SSc. For instance, tocilizumab and bosentan prevent the decrease in the percentage of vital capacity and the onset of new digital ulcers, respectively [2, 3]. Also, several new therapeutic candidates are now under the clinical trials [4], but the identification of new therapeutic targets is quite important to further facilitate the development of new therapies against SSc.

Chemokines have attracted much attention as a potential therapeutic target family of molecules in the field of SSc based on the results of clinical studies and animal models [5]. With respect to CCL20, we have recently found that serum CCL20 levels correlate with mean pulmonary arterial pressure (mPAP) in SSc patients [6], suggesting that the CCL20/CCR6 axis underlies the developmental mechanism of SSc vasculopathy. According to previous studies, the CCL20/CCR6 axis promotes the chemotaxis of immature dendritic cells, T helper (Th) 17 cells, regulatory T cells and B cells under both homeostatic and inflammatory conditions [7], contributing to the maintenance of homeostatic immune balance and the development of pathologic inflammation, such as psoriasis [8, 9], atopic dermatitis [9], inflammatory bowel disease [10], systemic lupus erythematosus [11], dermatomyositis/polymyositis [12] and SSc [13]. On the other hand, an accumulating body of evidence indicates that the CCL20/CCR6 axis regulates endothelial behaviors related to tumor angiogenesis. For instance, Hippe et al. revealed the following findings; (i) CCL20 expression levels in tumors correlate with advanced tumor stage, increased lymph node metastasis and decreased survival, (ii) microvascular endothelial cells abundantly express CCR6, (iii) CCR6 signaling in endothelial cells induces angiogenesis, (iv) tumor growth and tumor-associated vascularization are decreased in CCR6-deficeint mice due to its deficiency in stromal cells, but not within the immune system [14]. Thus, the CCL20/CCR6 axis is involved in the development of inflammatory diseases and tumor angiogenesis, but its role remains unknown in vascular disorders, including SSc vasculopathy.

Friend leukemia virus integration 1 (FLI1) is a member of the ETS transcription factor family, the expression of which is broadly suppressed in various cell types in SSc lesional and non-lesional skin [15, 16]. Since FLI1 expression is genetically and epigenetically suppressed in SSc patients [17, 18], FLI1 deficiency likely serves as a predisposing factor in SSc development. Indeed, FLI1 deficiency induces SSc-like phenotypes in dermal fibroblasts, endothelial cells, macrophages, keratinocytes and dermal dendritic cells [16, 19–25]. Therefore, the molecular analysis based on FLI1 deficiency provides us with a useful clue to know the significance of target molecules in the development of SSc.

Based on these backgrounds, we investigated the potential role of CCL20/CCR6 in SSc vasculopathy and the contribution of FLI1 deficiency to this process by a series of experiments with clinical samples, cultured endothelial cells and animal models.

Methods

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded skin sections with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and antibodies against CCL20 (Thermo Fisher Scientific, Waltham, MA, USA), CCR6 (Abcam, Cambridge, UK) and a-smooth muscle actin (a-SMA) (Abcam). Antigen retrieval was performed using Dako Target Retrieval Solution pH 9 (Dako North America, Inc., Carpinteria, CA, USA). Skin samples were obtained from forearms of 6 diffuse cutaneous SSc (dcSSc) patients, 6 limited cutaneous SSc (lcSSc) patients and 5 healthy controls. Horseradish peroxidase activity was detected by 3, 3′-diaminobenzidine. Counterstaining was conducted with methyl green. Arterioles, capillaries and venules were distinguished based on their histological features. Arterioles 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 [26]. The determination of blood vessel type was performed by two independent dermatologists (T. Ikawa and Y. Asano).

Gene silencing of *FLI1*

Human dermal microvascular endothelial cells (HDMECs) (Lonza, Walkersville, MD, USA) were cultured on collagen-coated tissue culture plates in Endothelial Basal Medium-2 supplemented with the Endothelial Cell Growth Medium-2 Bullet Kit (Lonza). Shortly after seeded, HDMECs were transfected with *FLI1* siRNA or non-silencing scrambled RNA (SCR) (10 nM, both purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA) mixed with HiPerFect Transfection Reagent (Qiagen, Valencia, CA, USA) for 48 hours. Cells were then collected using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for RNA isolation.

RNA isolation and quantitative reverse transcription (qRT)-PCR

RNA isolation from HDMECs and qRT-PCR were conducted as described previously [27]. The sequences of primers were as follows: *FLI1*-forward 5'-GGATGGCAAGGAACTGTGTAA-3', *FLI1*-reverse 5'-GGTTGTATAGGCCAGCAG-3', *CCL20*-forward 5'- TTGTGCGTCTCCTCAGTAAAAA-3', *CCL20*-reverse 5'-GCAAGTGAAACCTCCAACCC-3'; *CCR6*-forward 5'- GGGGGCTGTCAGTCATCATC-3', *CCR6*-reverse 5'- CGTAGAGCACAGGGTTCAGG-3'; *GAPDH*-forward 5'-ACCCACTCCTCCACCTTTGA-3', *GAPDH*-reverse 5'-CATACCAGGAAATGAGCTTGACAA-3'.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, USA) as described previously [27]. Putative FLI1 binding site in the *CCR6* promoter was predicted using a web site, JASPAR. The primers that amplify fragments of the *CCR6* transcript variant 1 (-252 bp to -39 bp) and transcript variant 2 (-1395 bp to -1186 bp) were as follows: *CCR6* transcript variant 1/Forward, 5'- ACTGCCGTATCCCTTGTGC-3'; *CCR6* transcript variant 1/Reverse, 5'- TGGGAGAATGGACATTGTGACC-3', *CCR6* transcript variant 2/Forward, 5'- TTCTTTCCAGGCAGGCATTG-3'; *CCR6* transcript variant 2/Reverse, 5'-TCCTCCTCATTTCTACCATCGC-3'. The amplified DNA products were resolved by agarose gel electrophoresis.

In vivo local gene silencing of *Ccr6* with atelocollagen

Ccr6 siRNA was transfected to murine skin *in vivo* using atelocollagen (AteloGene® Local Use 'Quick Gelation', Koken, Tokyo, Japan). Ten micromolar of Silencer select *Ccr6* siRNA or SCR (both from Thermo Fisher scientific) was mixed with atelocollagen, 150 μL of which was subcutaneously given to shaved lower back of wild-type (WT) mice once a week. After the injection of siRNA, 200 mg of BLM was subcutaneously injected to the same place every day for a week or 4 weeks.

Vascular permeability assay

Evans blue dye (0.5%) (Sigma-Aldrich, St. Louis, MO, USA) in 200 μl of 0.9% saline was injected into the tail vein, and mice were sacrificed in half an hour. The presence of vascular leakage was macroscopically evaluated in the skin.

In vitro angiogenesis assay

HDMECs were treated with 20 nM of *CCR6* siRNA or SCR (both from Thermo Fisher Scientific) for 48 hours. Then, cells were treated with 10 μg/mL of mitomycin C (Sigma Aldrich) for 2 hours. A 24-well plate was coated with 250 μL of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA). After the gel was solidified, cells were trypsinized and seeded onto the Matrigel at 7×10^4 cells per well and incubated for 24 hours. Cells were treated with calcein AM before observation. Five photographs were taken randomly from each well. The numbers of meshes, tubes and intersections were counted manually.

Statistical analysis

Statistical analysis was conducted with Welch's t-test to compare two unpaired data. Statistical significance was defined as a P value of <0.05.

Results

The expression profiles of CCL20 and CCR6 in the involved skin of SSc patients.

As an initial experiment, we evaluated the expression of CCL20 and CCR6 in skin biopsy samples of SSc patients and healthy controls (Fig. 1 and Table 1). In healthy control skin CCL20 was abundantly expressed in keratinocytes, dermal small vessels and inflammatory cells, while marginally detected in dermal fibroblasts, as previously reported [28]. In the skin of SSc patients similar expression profiles to those of healthy controls were observed in keratinocytes, dermal small vessels and inflammatory cells, but an increased trend of CCL20 expression was evident in dermal fibroblasts relative to those cells of healthy control skin (the evaluation with grading scale; median [25–75 percentiles], 1.5 [0.25-3.0] versus 0 [0-1.5], $p = 0.078$). Importantly, CCL20 was significantly up-regulated in dermal fibroblasts of dcSSc patients (disease duration of < 2 years) compared with those cells of healthy controls (3 [0.75-3] versus 0 [0-1.5], $p = 0.046$), which is consistent with a previous finding that CCL20 expression is increased in dermal fibroblasts of early SSc patients [13]. With respect to CCR6, there were detectable signals in various cell types of healthy control skin, but dermal fibroblasts displayed a low expression level relative to the other cell types. In the skin of SSc patients, CCR6 expression was significantly increased in

dermal small vessels and keratinocytes as compared to those cells of healthy control skin (2 [2-2.8] versus 1 [0.5–1.5], p = 0.022; 2.0 [2.0–3.0] versus 1.0 [1.0–2.0], p = 0.0089; respectively), while comparable in dermal fibroblasts and inflammatory cells. Taken together, these results suggest the potential contribution of dermal fibroblasts of early SSc patients to recruiting Th17 cells to the affected skin lesion, as previously reported [13], and the activation of CCL20/CCR6 axis in endothelial cells and keratinocytes of SSc-involved skin. The role of CCL20/CCR6 axis has been well studied in endothelial cells, while the CCL20/CCR6 axis does not affect the migration and proliferation of keratinocytes [29]; therefore, we focused on endothelial cells in the following experiments.

Table 1
Expression profiles of CCL20 and CCR6 in skin sections of SSc patients and healthy controls.

				CCL20				CCR6			
Samples		Age/Sex	disease duration (year)	Keratinocytes	Fibroblasts	Blood vessels	Infiltrated Cells	Keratinocytes	Fibroblasts	Blood vessels	Infiltrated Cells
HC	1	38/F		++	−	++	+	−	−	+	+
	2	25/F		+	−	++	++	+	−	+	++
	3	21/M		+	−	++	++	++	−	++	++
	4	22/M		++	+	++	++	−	−	−	−
	5	30/F		+	++	++	++	++	+	+	+
SSc	dcSSc1	76/M	0.5	+	+++	+++	++	++	−	+	+
	dcSSc2	44/F	0.3	+	+	++	++	++	−	+	+
	dcSSc3	40/M	0.5	++	+++	++	++	++	++	++	++
	dcSSc4	59/F	0.3	++	+++	+++	+++	+++	−	++	++
	dcSSc5	74/F	1	++	+++	++	++	++	−	++	++
	dcSSc6	35/F	1.5	+	−	+	+	++	−	++	+
	lcSSc1	47/F	5	++	++	++	++	+	−	++	++
	lcSSc2	32/F	0.2	++	+	++	++	++	++	+++	+++
	lcSSc3	45/F	6	++	−	++	++	++	−	++	+
	lcSSc4	75/F	25	++	+	++	++	++	−	++	++
	lcSSc5	57/F	2	++	+++	++	++	+++	−	+++	+++
	lcSSc6	81/F	4	++	−	++	++	+++	++	+++	+++

We used the following grading system for the immunohistochemistry: −, no staining; +, slight staining; ++, moderate staining; +++, strong staining. HC, healthy control; lcSSc, limited cutaneous systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis.

FLI1 deficiency induces CCR6 expression in dermal microvascular endothelial cells.

Given that Fli1 deficiency reproduces SSc-like properties, including the expression profiles of chemokines, in endothelial cells [30, 31], we examined the effect of *FLI1* siRNA on the expression of CCL20 and CCR6 in HDMECs. As shown in Fig. 2A, *FLI1* siRNA enhanced CCR6 expression on the mRNA level, while not affecting CCL20 expression. Furthermore, chromatin immunoprecipitation revealed the binding of FLI1 to the *CCR6* promoters of 2 transcript variants (Transcript variant 1; NM_004367, Transcript variant 2; NM_031409) in HDMECs (Fig. 2B). These results suggest that FLI1 serves as a transcriptional repressor of the *CCR6* gene, and that FLI1 deficiency at least partially contributes to CCR6 up-regulation in endothelial cells.

Ccr6 **siRNA restores vascular hyperpermeability induced by BLM injection in mice.**

To further confirm if CCR6 upregulation is involved in the development of SSc vasculopathy, we evaluated the effect of *Ccr6* siRNA on the vascular aspect of BLM-treated mice, a widely recognized animal model of SSc. As a part of SSc-like vascular features induced by BLM injection, we focused on vascular permeability which can be evaluated by Evans blue dye injection [32]. In mice treated with SCR, 1-week BLM challenge increased the permeability of vasculature in the injected skin area relative to adjacent non-injected areas (a left panel of Fig. 3A). On the other hand, Evans blue dye extravasation was remarkably attenuated after 1-week BLM injection in mice treated with *Ccr6* siRNA (a right panel of Fig. 3A). These results indicate that CCR6 is required for vascular destabilization induced by BLM-dependent inflammation.

To further confirm this finding, we employed immunostaining for a-SMA, a marker of vascular stabilization. Generally, a-SMA is highly expressed in pericytes engaged in vascular stabilization, while being marginally found in those cells promoting angiogenesis [33, 34]. As shown in Fig. 3B, the

expression of a-SMA was increased in dermal small vessels (arterioles, capillaries and venules) of *Ccr6* siRNA-treated mice as compared to SCR-treated ones after 4-week BLM injection. These results indicate that *Ccr6* siRNA stabilizes dermal small vessels in BLM-treated mice.

Decreased angiogenic activity of CCR6 **siRNA-treated HDMECs.**

As described above, CCR6 downregulation was associated with vascular stabilization in BLM-treated mice. Generally, vascular stabilization is associated with reduced angiogenic activity, suggesting that CCR6 reduction suppresses the pro-angiogenic activity of endothelial cells. To address this issue, we employed *in vitro* angiogenesis assay with the Matrigel. As shown in Fig. 4A, SCR-treated HDMECs formed favorable tube networks, whereas *CCR6* siRNA-treated HDMECs showed relatively large tube networks under the same culture condition. To objectively evaluate the activity of angiogenesis, we looked at the numbers of meshes, tubes and intersections. Of note, the numbers of meshes (91 [87–92] versus 23 [21-29.5], $p < 0.001$), tubes (180 [160.5–198] versus 45 [38.5–57], $p < 0.001$) and intersections (124 [100.5-130.5] versus 29 [20.5–38.5], $p < 0.001$) were decreased significantly in *CCR6* siRNA-treated HDMECs compared with SCR-treated HDMECs (Fig. 4B). Taken together, CCR6 downregulation suppresses the pro-angiogenic activity of endothelial cells, which at least partially underlies vascular stabilization in *Ccr6* siRNA-treated BLM-injected mice.

Discussion

This study was undertaken to investigate the role of CCL20/CCR6 axis in the development of SSc vasculopathy because serum CCL20 levels correlate with mPAP values in SSc patients [6]. Given that mPAP is positively correlated with the severity of nailfold capillary changes [35], it was postulated that the activation of CCL20/CCR6 axis plays a part in the destabilization of dermal small vessels. Based on this idea, we evaluated the role of CCL20/CCR6 axis in the regulation of endothelial behaviors. Our initial study with immunochemistry demonstrated the up-regulated expression of CCR6 in dermal microvascular endothelial cells of SSc patients. Further cell culture studies revealed the downregulation of CCR6 by FLI1 deficiency, a critical predisposing factor of SSc, in HDMECs. More importantly, *Ccr6* siRNA restored BLM-induced vascular hyperpermeability in mice. Taken together with evidence that the CCL20/CCR6 axis functions as a potent pro-angiogenic regulator [36], CCR6 up-regulation may be involved in the development of SSc vasculopathy.

A previous study by Tao et al. [13] demonstrated the increased expression of CCL20 in dermal fibroblasts of early SSc patients relative to those cells of healthy control skin, suggesting that CCL20 produced by dermal fibroblasts contributes to Th17 infiltration into the involved skin of patients with early SSc. Supporting this previous finding, we found a significant elevation of CCL20 in dermal fibroblasts of dcSSc patients with disease duration of < 2 years. Under the physiological condition, CCL20 is abundantly expressed by keratinocytes and endothelial cells, but its expression is relatively low in dermal fibroblasts, as shown in the current and previous studies [28]. Therefore, CCL20 up-regulation seems to be a characteristic feature of SSc dermal fibroblasts. With respect to the expression levels of CCR6, our results were also consistent with the study by Tao et al. [13] in that the elevation of CCR6 was observed significantly in keratinocytes and slightly in dermal fibroblasts and infiltrated cells. In addition, our current study disclosed a significant elevation of CCR6 in dermal small vessels of SSc patients, especially in lcSSc patients, irrespective of disease duration. Taken together, these results suggest that CCL20 secreted from dermal fibroblasts plays a role in the early stage of dcSSc, while CCR6 up-regulation in endothelial cells contributes to the development of SSc vasculopathy throughout the whole disease course. This notion is likely plausible because SSc-related pulmonary arterial hypertension (PAH), which is linked to elevated serum CCL20 levels, is a complication frequently seen in lcSSc patients with a long disease history.

SSc vasculopathy is characterized by vascular structural changes, such as arteriolar stenosis, capillary dilation and capillary loss [37]. The chronological capillary changes are well documented in nailfold capillaries [38]. The initial changes are capillary dilation and bleeding, reflecting vascular destabilization. These changes are followed by capillary loss, finally leading to abnormal angiogenic changes, such as ramified capillary. These vascular alterations are caused by dysregulated angiogenesis and defective vasculogenesis. FLI1 deficiency is a key disease factor regulating a broad spectrum of endothelial behaviors and vascular remodeling associated with SSc vasculopathy, including angiogenesis and vasculogenesis [39]. FLI1 deficiency suppresses the expression of CD31, vascular endothelial cadherin, $S1P_1$ and platelet-derived growth factor-B in endothelial cells, while up-regulating matrix metalloproteinase-9, resulting in vascular destabilization [40]. The expression of CCN1, which regulates the recruitment of circulating endothelial progenitor cells, is decreased in FLI1-deficient endothelial cells [23]. In the current study, CCR6 was up-regulated in dermal microvascular endothelial cells of SSc-involved skin, and FLI1 deficiency increased CCR6 expression in HDMECs. Given that FLI1 bound the *CCR6* promoter in HDMECs, CCR6 is a member of molecules involved in the mechanism by which FLI1 deficiency promotes the development of SSc vasculopathy.

Chemokines are initially recognized as a family of proteins recruiting inflammatory cells to the specific tissues and organs. On the other hand, various chemokines have been shown to serve as regulators of angiogenesis associated with inflammation. For instance, CXC chemokines with glutamic acid-leucine-arginine (ELR) motif in their N terminus are potent promoters of angiogenesis, while those without ELR motif are potent inhibitors of angiogenesis [41]. Of note, the various CXC chemokines, such as CXCL4 (ELR-) [42], CXCL5 (ELR+) [30], CXCL6 (ELR+) [31], CXCL12 (ELR-) [43], CXCL13 (ELR-) [24], and CXCL14 (ELR-) [44], are thought to be associated with the development of SSc vasculopathy. With respect to CC chemokines, the broad-spectrum inhibitor of CC chemokines, 35K, suppresses inflammation-driven angiogenesis, whereas preserving physiological ischemia-mediated angiogenesis [45]. Thus, the inhibition of chemokines may be an alternative therapeutic strategy against disease-related inflammatory vascular changes without the undesirable effects on physiological angiogenesis. Considering this point, the blockade of CCR6 is likely a potential therapeutic strategy against SSc vasculopathy. Further studies are required to clarify this point in the future.

The limitation of this study is the lack of data on the global inhibition of CCR6 in BLM-treated mice. This is a critical point because CCR6 is a chemoattractant receptor of Th17 cells. In this study, we used the local injection of atelocollagen mixed with *Ccr6* siRNA in the back skin of BLM-treated

mice, in which CCR6 expression in circulating $CD4^+$ T cells were not altered (data not shown). To elucidate the effect of global CCR6 inhibition, we need to investigate whether anti-CCR6 neutralizing antibody affects skin fibrosis or other vascular changes, such as PAH, using BLM-treated mice and other SSc animal models recapitulating SSc-PAH.

Conclusion

This is the first report demonstrating a potential contribution of CCR6 to the development of SSc vasculopathy as an inflammation-associated angiogenic factor. These findings suggests that the modification of CCR6 expression can be an effective intervention for SSc vascular symptoms. Also, the induction of CCR6 expression in FLI1-deficient endothelial cells further supports the canonical idea that FLI1 deficiency is a critical disease factor of SSc.

Abbreviations

SSc, systemic sclerosis; mPAP, mean pulmonary artery pressure; Th, T helper; FLI1, friend leukemia virus integration 1; a-SMA, a-smooth muscle actin; dcSSc, diffuce cutaneous SSc; lcSSc, limited cutaneous SSc; HDMECs, human dermal microvascular endothelial cells; SCR, non-silencing scrambled RNA; qRT-PCR, quantitative reverse transcription-PCR; ChIP, chromatin immunoprecipitation; WT, wild-type; PAH, pulmonary arterial hypertension; ELR, glutamic acid-leucine-arginine; HC, healthy controls; BLM, bleomycin.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethical committee and the committee on animal experimentation of University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design, TI, YA and SS; Acquisition of data, TI, TM, YF, ST, JO, KA, YN, YW and AY; Analysis and interpretation of data, TI, YA and SS; Obtaining funding, YA and SS; Supervision, YA, AY and SS.

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Figures

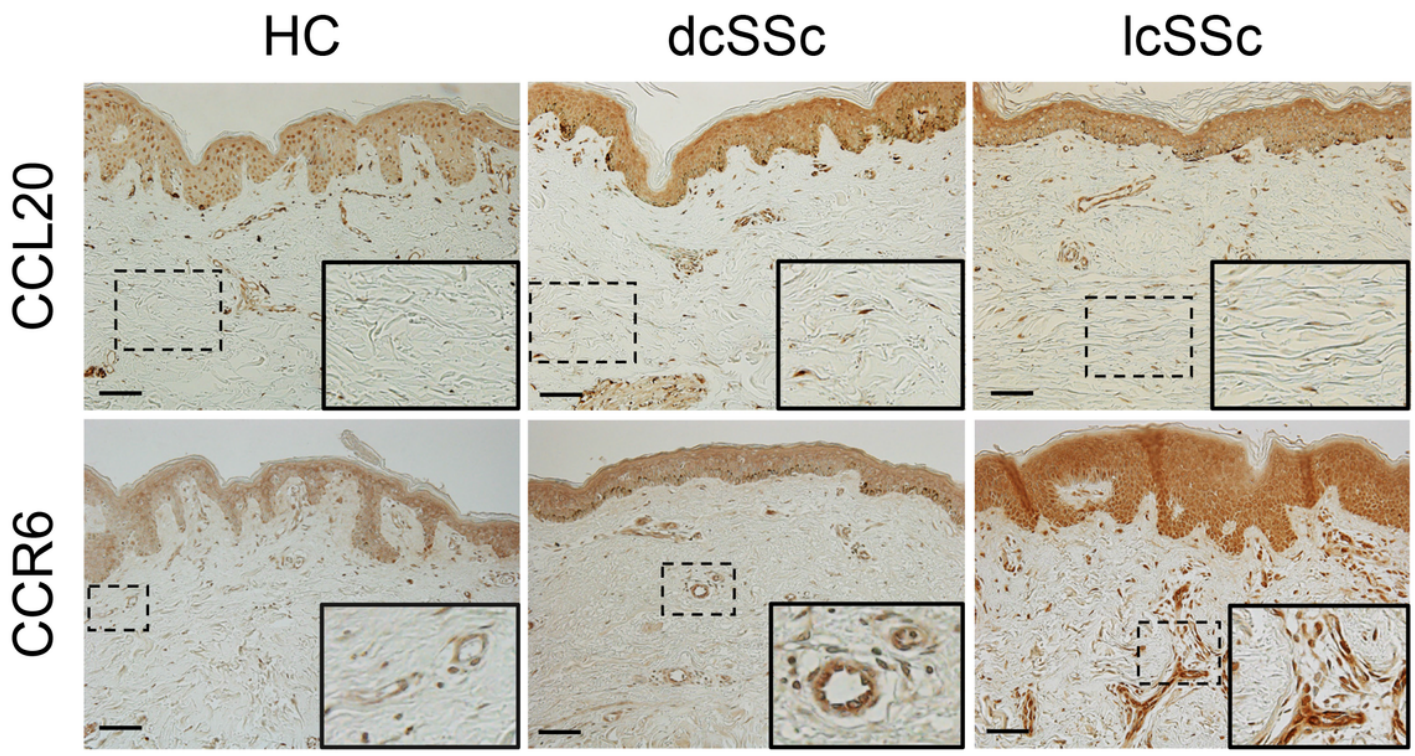

Figure 1

The expression profiles of CCL20 and CCR6 in SSc patients and healthy controls. The expression of CCL20 and CCR6 was evaluated by immunohistochemistry in the skin sections from diffuse cutaneous SSc (dcSSc) patients, limited cutaneous SSc (lcSSc) patients and healthy controls (HC). Representative results are shown. Lower panels of each picture exhibit dermal fibroblasts in CCL20 staining and dermal small vessels in CCR6 staining, which are shown with solid squares in upper panels. Horseradish peroxidase activity was detected by 3, 3'-diaminobenzidine. Counterstaining was carried out with methyl green. A scale bar is 100 μm.

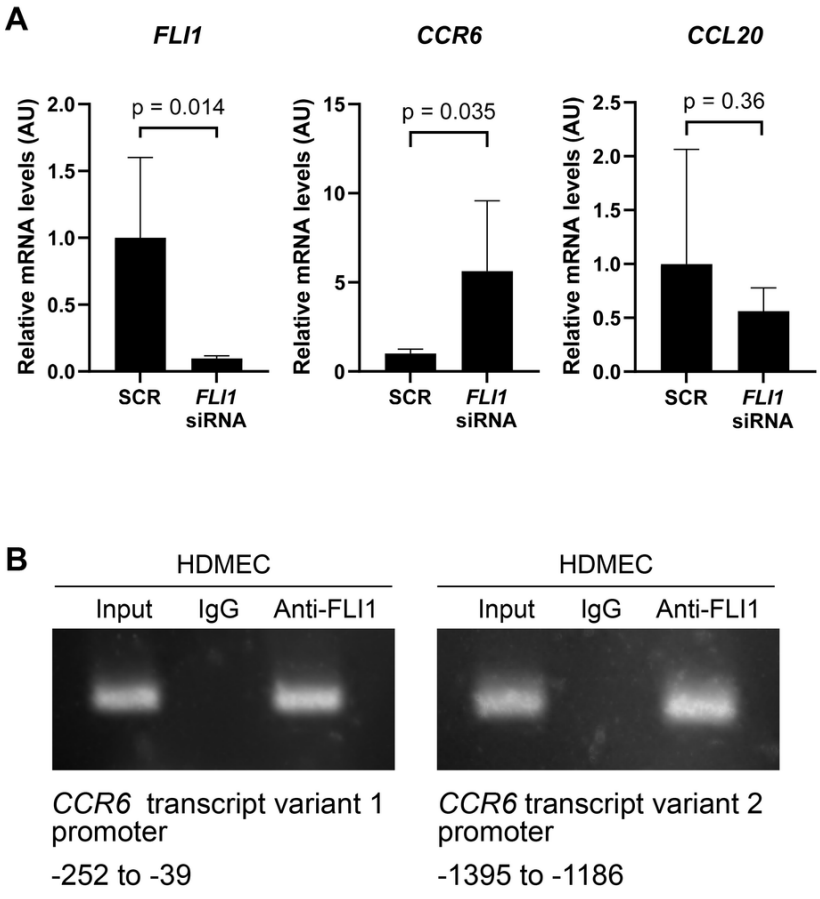

Figure 2

The contribution of Fli1 deficiency to the up-regulated expression of CCR6 in endothelial cells. A. mRNA levels of FLI1, CCL20 and CCR6 in human dermal microvascular endothelial cells (HDMECs) transfected with FLI1 siRNA or non-silencing scrambled RNA (SCR) were examined by qRT-PCR (n = 6 for each group). Results are expressed as means ± SEM. AU, arbitrary unit. B. Chromatin was isolated from HDMECs and immunoprecipitated using rabbit anti-FLI1 antibody or rabbit IgG. After isolation of bound DNA, PCR amplification was carried out using two sets of primers specific for the promotes of CCR6 transcript variant 1 (NM_004367) and CCR6 transcript variant 2 (NM_031409).

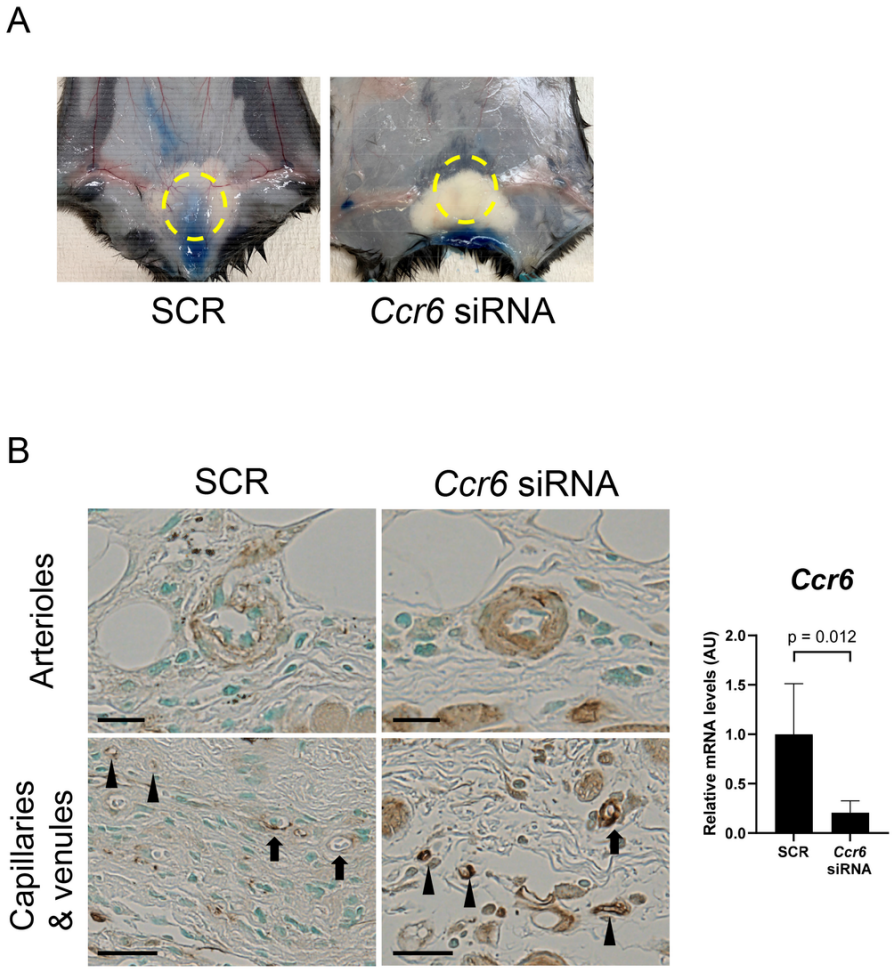

Figure 3

Ccr6 gene silencing restores BLM-induced vascular hyperpermeability in mice. Wild-type mice were injected with Ccr6 siRNA or scrambled non-silencing RNA (SCR), followed by 1-week and 4-week bleomycin (BLM) challenge. A. The leakage of Evans blue dye was macroscopically evaluated in the skin after 1-week BLM injection. B. After 4-week BLM injection, ±-smooth muscle actin (α-SMA) was visualized by immunohistochemistry. Representative results of 6 independent experiments were shown. Arrows and arrow heads show venules and capillaries, respectively. CCR6 knockdown was confirmed by qRT-PCT with the whole skin sections (a right panel). Each graph indicates mean ±SEM of the indicated parameters. A scale bar is 25μm. AU, arbitrary unit.

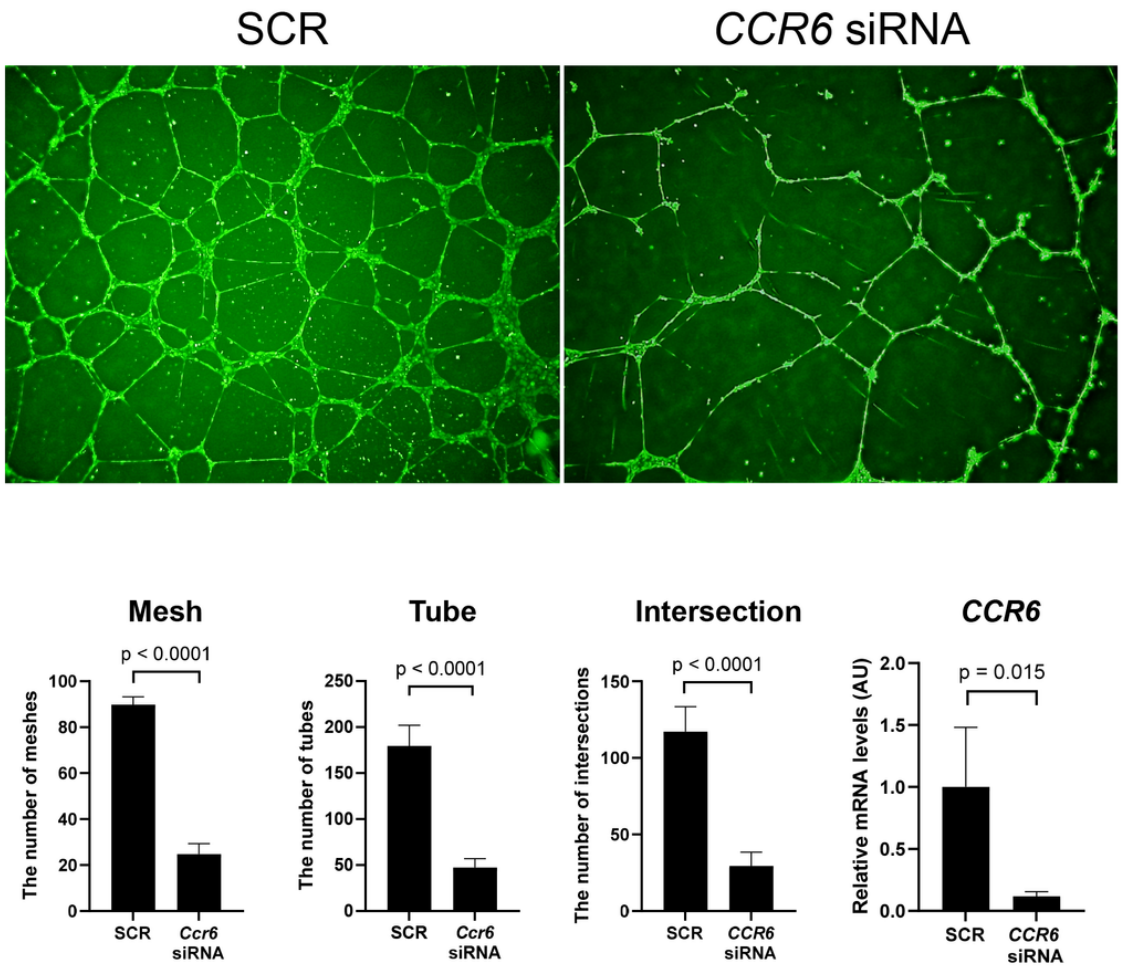

Figure 4

CCR6 siRNA suppresses tubulogenic activity of HDMECs. Tube formation assay was performed by applying HDMECs treated with Ccr6 siRNA or scrambled non-silencing RNA (SCR) onto the Matrigel and incubating for 24 hours. To eliminate the influence of proliferation, cells were treated with mitomycin C before the assay. Representative images are shown (n = 5 for each group). The numbers of meshes, tubes and intersections were counted manually. CCR6 mRNA levels were determined by qRT-PCR. Each graph represents mean ± SEM of the indicated parameters.