Journal of Dermatological Science 93 (2019) 24–32

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Original Article

Increased expression of aquaporin-1 in dermal fibroblasts and dermal microvascular endothelial cells possibly contributes to skin fibrosis and edema in patients with systemic sclerosis

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ARTICLE INFO

Article history:
Received 26 June 2018
Received in revised form 27 August 2018
Accepted 18 September 2018

Keywords:
Systemic sclerosis
Aquaporin 1
Fibroblasts
Endothelial cells
Migration

ABSTRACT

Background: Aquaporin-1 (AQP1), a water channel protein controlling the water contents of cells and tissues, exerts pleiotropic effects on various biological activities, including inflammation, angiogenesis, and extracellular matrix remodeling, by regulating cell behaviors and tissue water balance.
Objective: To investigate AQP1 roles in systemic sclerosis (SSc) which is characterized by autoimmune inflammation, vasculopathy, and tissue fibrosis.
Methods: AQP1 expression was evaluated by immunohistochemistry and quantitative reverse transcription PCR in skin samples from human and animal models and by immunoblotting in cultured cells. Fli1 binding to the *AQP1* promoter was evaluated by chromatin immunoprecipitation. Cell migration was assessed by scratch assay.
Results: Dermal fibroblasts and endothelial cells highly expressed AQP1 in SSc lesional skin, and AQP1 expression in dermal fibroblasts and endothelial cells positively correlated with the degrees of tissue fibrosis and edema, respectively. Consistently, SSc dermal fibroblasts up-regulated AQP1 compared with normal dermal fibroblasts *in vitro*. Furthermore, TGF-β stimulation induced AQP1 expression in normal dermal fibroblasts, while TGF-β1 antisense oligonucleotide suppressed AQP1 expression in SSc dermal fibroblasts. In endothelial cells, Fli1 deficiency resulted in AQP1 up-regulation *in vivo* and *in vitro* and Fli1 bound to the *AQP1* promoter. Importantly, SSc dermal fibroblasts and *FLI1* siRNA-treated endothelial cells had a pro-migratory property, which was remarkably diminished by gene silencing of *AQP1*.
Conclusion: AQP1 is up-regulated in SSc dermal fibroblasts and SSc endothelial cells at least partially due to autocrine TGF-β stimulation and Fli1 deficiency, respectively, possibly contributing to inflammation, vasculopathy, and tissue fibrosis by regulating tissue edema and cell migration.
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1. Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vasculopathy and fibrosis of the skin and various internal organs. Although the pathogenesis of SSc still remains unknown, its disease process is well organized as represented by sequential pathological events, namely, starting with initial vascular injury due to autoimmune attacks, followed by impaired vascular remodeling and aberrant inflammation, and eventually resulting in extensive tissue fibrosis [1]. Therefore, the identification of molecules involved in this sequential pathological cascade helps us to better understand the molecular mechanism underlying the complex pathology of SSc.

Aquaporins (AQPs) are water channel cell-membrane proteins ubiquitously expressed in almost all organs and tissues of mammals and plants as well as in bacteria [2,3]. AQPs regulate the water contents of cells, tissues, and organs through transmembrane water transport which was previously thought to occur only by free diffusion. So far, 13 kinds of AQPs (AQP0 to AQP12) have been identified. Several lines of evidence have demonstrated that AQPs behave more like multifunctional, highly adapted

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https://doi.org/10.1016/j.jdermsci.2018.09.007
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channels rather than simple water pores, such as regulating tissue edema and cell migration [4]. Given that there is a disease-specific expression profile of AQPs, it is assumed that these water channels may be involved in the development of various diseases [4]. For instance, the increased expression of AQP1 and the decreased expression of AQP5 are reported in the lung of bleomycin (BLM)-treated pulmonary fibrosis model mice [5], while the contrary expression pattern is characteristically seen in the acute phase of radiation-induced pulmonary fibrosis in rat [6]. In addition, AQP1 is up-regulated in endothelial cells at the site of angiogenesis in a murine model of liver cirrhosis and AQP1 knockout mice exhibit reduced liver fibrosis along with the suppression of angiogenesis [7]. Since AQP1 up-regulation is also evident in endothelial cells of human cirrhotic liver [8], AQP1 is likely to be involved in the development of liver fibrosis through the activation of endothelial cells. Thus, AQP1 seems to play a pivotal role in the development of fibrotic disorders. However, as far as we know, the contribution of AQP1 to skin fibrotic conditions has not been investigated.

Based on these backgrounds, we here examined the expression of AQP1 in the lesional skin of SSc patients and the molecular mechanisms by which AQP1 expression is regulated in dermal fibroblasts and dermal microvascular endothelial cells.

2. Materials and methods

2.1. Ethics statement

This 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. All animal protocols were approved by the Animal Care and Use Committee of University of Tokyo.

2.2. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded skin sections with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and antibodies against AQP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Skin samples were obtained from forearms of 10 patients with SSc and 5 healthy controls and from the back skin of mice. Patients were grouped by the LeRoy's classification system [9]: 5 with diffuse cutaneous SSc and 5 with limited cutaneous SSc. All patients fulfilled the new classification criteria of SSc [10]. BLM-treated mice and endothelial cell-specific *Fli1* knockout (*Fli1* ECKO) mice were generated as previously described [11,12]. Histological evaluation with grading scale was conducted independently by 3 investigators (T. Y, R. S and Y. A).

2.3. Cell cultures

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of 6 diffuse cutaneous SSc patients with <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). Murine dermal microvascular endothelial cells (MDMECs) were isolated from wild type (WT) and *Fli1* ECKO mice as described previously [13]. Endothelial cells were 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 cells in passage 3 - 5.

2.4. RNA isolation and quantitative reverse transcription PCR in cultured cells and skin samples

The treatment with transforming growth factor (TGF)-β1 antisense oligonucleotide and sense oligonucleotide in dermal fibroblasts, gene silencing of *FLI1* in HDMECs, and the generation of a BLM-induced murine SSc model were carried out as described previously [11,14–16]. Total RNA was isolated from the skin or cultured cells with RNeasy spin columns (Qiagen, Crawley, UK). One μg of total RNA from each sample was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Gene expression levels were determined by quantitative reverse transcription PCR (qRT-PCR) using Fast SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) on ABI prism 7000 (Applied Biosystems) in triplicates. mRNA levels of target genes were normalized to those of the *GAPDH* or *Gapdh* genes by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers were as follows: *AQP1*-forward: 5′-ATCCTCTCAGGCATCACCTC-3′, *AQP1*-reverse: 5′-GCCCGAGTTCACACCAT-3′;

FLI1-forward: 5′-GGATGGCAAGGAACTGTGTAA-3′, *FLI1*-reverse 5′-GGTTGTATAGGCCAGCAG-3′; *GAPDH*-forward 5′-ACCCACTCCTC-CACCTTTGA-3′, *GAPDH*-reverse 5′-CATACCAGGAAATGAGCTTGA-CAA-3′; *Aqp1*-forward: 5′-CCGAGACTTAGGTGGCTCAG-3′, *Aqp1*-reverse: 5′-TGATACCGCAGCCAGTGTAG-3′; *Fli1*-forward: 5′-ACTTGGCCAAATGGACGGGACTAT-3′, *Fli1*-reverse: 5′-CCCGTAGT-CAGGACTCCCG-3′; *Gapdh*-forward: 5′-CGTGTTCCTACCCCCAATGT-3′, *Gapdh*-reverse: 5′-TGTCATCATACTTGGCAGGTTTCT-3′.

2.5. Immunoblotting

Confluent quiescent fibroblasts were serum-starved for 48 h and harvested. In some experiments, cells were stimulated with TGF-β1 (R&D, systems, Minneapolis, MN, USA) for the last 24 h before harvested. Whole cell lysates prepared from dermal fibroblasts or MDMECs were subjected to sodium dodecyl sulfate-polyacrylamide gels electrophoresis and immunoblotting with antibodies against AQP1, Fli1, and β-actin (all from Santa Cruz Biotechnology). Bands were detected using enhanced chemiluminescent techniques (Thermo Scientific, Rockford, IL, USA).

2.6. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, USA), as described previously [17]. Putative Fli1 binding site in the *AQP1* promoter was predicted by Tfsitescan. Primer sequences for the *AQP1* gene were as follows: *AQP1*/F-260 5′-CCAAAGCCTATTAGAGCAACG-3′ and *AQP1*/R-95 5′- ACCACACA-GACCTCCTCTGG-3′. No non-specific amplification was detected in these experiments.

2.7. Scratch assay

Dermal fibroblasts and HDMECs were transfected with *AQP1* siRNA or non-silencing scrambled RNA in a 12-well plate. Each of HDMEC groups was simultaneously transfected with *FLI1* siRNA or non-silencing scrambled RNA. When cell culture became 80–90% confluent, cells were treated with 10 μg/ml of mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 2 h in order to remove the influence of cell proliferation. After refreshing culture media, cells were scratched with a pipette tip. Then, cell migration was monitored for 24 h by microscopy. Distances between one side of scratch and the other were measured at certain interval (μm) using Image J (http://rsb.info.nih.gov/ij/). The average distances were shown as straight lines in images.

2.8. Statistical analysis

Statistical analysis was done with Mann-Whitney *U* test to compare the distributions of two unmatched groups and with Spearman's rank correlation coefficient to assess the correlation of two parameters. Statistical significance was defined as a P value of <0.05.

3. Results

3.1. AQP1 expression is increased in dermal fibroblasts and dermal blood vessels of SSc patients

We initially investigated AQP1 expression in normal and SSc skin sections by immunohistochemistry (Fig. 1A). In healthy

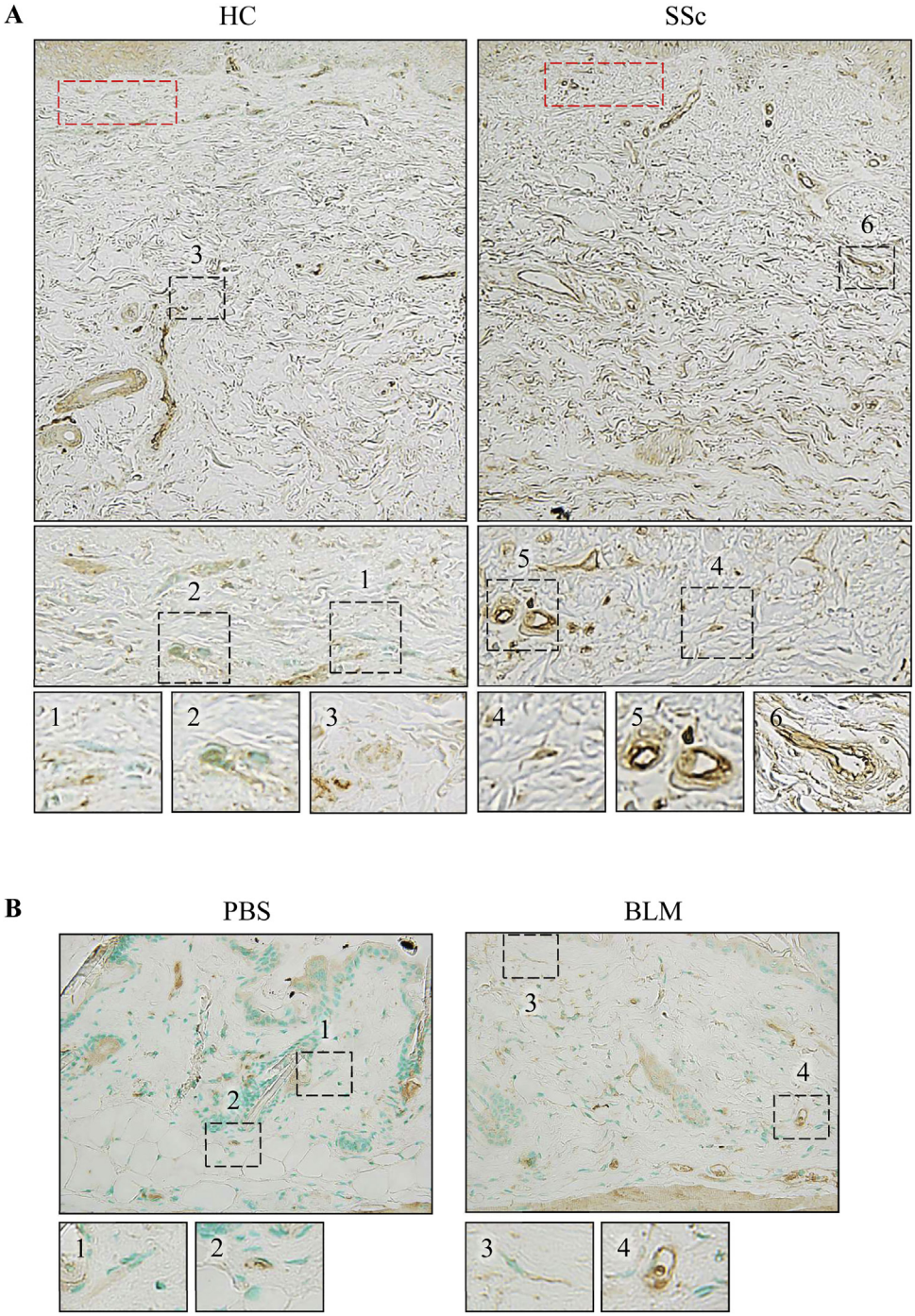

Fig. 1. The expression levels of AQP1 in the lesional skin of SSc patients and BLM-treated mice.
A. AQP1 expression was evaluated by immunohistochemistry in the skin of patients with systemic sclerosis (SSc) and healthy controls (HC). Representative results (HC2 and SSc8 in Table 1) are shown (original magnification, x 100). Middle panels represent higher magnification images of the areas surrounded with red dotted squares in top panels. Lower panels show higher magnification images of dermal fibroblasts (1 and 4) and blood vessels (2, 3, 5 and 6) corresponding to the areas surrounded with black dotted squares in top and middle panels. Tissue edema was evaluated by gaps between collagen bundles as shown in middle panels. **B**. AQP1 expression was evaluated in the skin of phosphate-buffered saline (PBS)- and bleomycin (BLM)-treated mice by immunohistochemistry. Lower panels show dermal fibroblasts (1 and 3) and dermal small vessels (2 and 4), corresponding to the areas shown with dotted squares in upper panels (original magnification, x 400).

controls, AQP1 expression was detectable in epidermal keratinocytes, dermal fibroblasts, and dermal small blood vessels, including capillaries, arterioles, venules, and small arteries. In SSc patients, AQP1 expression was observed in various types of cells as well. When the signal intensity was evaluated by a grading scale (Table 1), AQP1 expression in dermal fibroblasts and dermal blood vessels was much greater in SSc skin sections than in control skin sections (1.8 ± 0.79 versus 0.4 ± 0.55, $P = 0.0086$ for dermal fibroblasts, 2.5 ± 0.53 versus 1.6 ± 0.55, $P = 0.024$ for dermal blood vessels). Also, AQP1 expression was significantly higher in dermal fibroblasts of diffuse cutaneous SSc patients than in those of limited cutaneous SSc patients (2.4 ± 0.55 versus 1.2 ± 0.45, $P = 0.019$). Importantly, when the degrees of fibrosis and edema were quantified with mRSS and grading scale respectively (Table 1), the correlation of tissue edema with endothelial AQP1 expression and that of skin fibrosis with AQP1 expression in dermal fibroblasts were noted in SSc skin sections ($r = 0.82$, $p = 0.0058$ for tissue edema and endothelial AQP1 expression; $r = 0.74$, $p = 0.02$ for mRSS and AQP1 expression of dermal fibroblasts), suggesting the contribution of AQP1 to vascular and fibrotic processes of SSc.

In order to confirm if the elevation of AQP1 in dermal fibroblasts and dermal blood vessels is a biological process associated with SSc, we looked at the expression of AQP1 in the skin of BLM-treated mice, an established and well-studied animal model of SSc [18]. As shown in Fig. 1B, the expression of AQP1 in dermal fibroblasts and dermal blood vessels was much higher in BLM-treated mice than in PBS-treated mice. Taken together, these results suggest that AQP1 is up-regulated in dermal fibroblasts and dermal blood vessels as a part of the mechanism driving the disease process associated with SSc.

3.2. AQP1 expression is induced by autocrine TGF- β stimulation in SSc dermal fibroblasts

We next investigated the mechanism by which AQP1 is up-regulated in SSc dermal fibroblasts. First, we confirmed the up-regulated expression of AQP1 in cultured SSc dermal fibroblasts compared with cultured normal dermal fibroblasts by immunoblotting (Fig. 2A). Since TGF-β is a critical growth factor promoting the induction of an SSc-like phenotype in dermal fibroblasts, we next examined the effect of TGF-β1 stimulation on the expression levels of AQP1 in normal dermal fibroblasts. As shown in Fig. 2B and C, AQP1 expression was remarkably enhanced by 2 ng/ml of TGF-β1, which is sufficient to induce an SSc-like phenotype in normal dermal fibroblasts [19–21], at mRNA and protein levels in normal dermal fibroblasts. Taken together with the evidence that SSc dermal fibroblasts highly express integrin αVβ3, integrin αVβ5, and thrombospondin-1, which promote the release of active TGF-β from its latent form [22–24], it is suggested that autocrine TGF-β stimulation is a possible mechanism explaining the increased expression of AQP1 in SSc dermal fibroblasts. To further confirm this notion, we treated SSc dermal fibroblasts with TGF-β1 antisense oligonucleotide, an established method to suppress the production of TGF-β1 [14,15], and looked at the expression levels of AQP1. As shown in Fig. 2D, TGF-β1 antisense oligonucleotide significantly suppressed the expression of AQP1 in SSc dermal fibroblasts, while TGF-β1 sense oligonucleotide did not show any significant effect. These results indicate that AQP1 expression is at least partially regulated by autocrine TGF-β in SSc dermal fibroblasts.

3.3. AQP1 expression is regulated by Fli1 deficiency in dermal microvascular endothelial cells

We continuously reported that Fli1 deficiency induces an SSc-like phenotype in dermal microvascular endothelial cells [13,17,25–30]. To investigate if this theory is applicable to AQP1 expression in those cells, we looked at the expression levels of AQP1 in *Fli1* ECKO mice that lack Fli1 expression only in endothelial cells. As shown in Fig. 3A, AQP1 expression was remarkably increased in the innermost layer of dermal small vessels in *Fli1* ECKO mice compared with control littermates, suggesting that AQP1 expression is up-regulated in Fli1-deficient dermal microvascular endothelial cells. This finding was consistent with the results of qRT-PCR and immunoblotting with MDMECs isolated from mice, showing that AQP1 expression was elevated in MDMECs of *Fli1* ECKO mice compared with MDMECs of control mice (Fig. 3B and C). Furthermore, *FLI1* siRNA enhanced *AQP1* mRNA expression and Fli1 occupied the *AQP1* promoter in HDMECs (Fig. 3D and E). Taken together, these results indicate that *AQP1* gene expression is directly regulated by Fli1 in endothelial cells and Fli1 deficiency contributes to the induction of AQP1 in SSc endothelial cells.

Table 1
AQP1 levels in skin sections from SSc patients and healthy controls.

Samples	Age/Sex	Duration (year)	dcSSc /lcSSc	Keratinocytes	Fibroblasts	Blood vessels	Histological edema	mRSS
HC 1	69M			++	–	+	–	
HC 2	63F			++	+	+	–	
HC 3	71F			++	–	++	+	
HC 4	63M			++	–	++	–	
HC 5	57F			++	+	++	+	
SSc 1	75F	20	lcSSc	+	+	++	++	6
SSc 2	59F	1	lcSSc	++	++	+++	+++	6
SSc 3	65F	6	lcSSc	++	+	++	+	3
SSc 4	57F	2	lcSSc	++	+	++	++	2
SSc 5	37F	17	lcSSc	++	+	++	+	6
SSc 6	45F	1	dcSSc	++	++	+++	++	11
SSc 7	25M	1.5	dcSSc	+++	+++	+++	+++	24
SSc 8	59F	2	dcSSc	+	++	+++	+++	27
SSc 9	57F	7	dcSSc	+	++	++	++	32
SSc 10	61F	8	dcSSc	++	+++	+++	+++	20

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. HC, healthy control; SSc, systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; mRSS, modified Rodnan total skin thickness score. We used the following grading system: -, no stainig; +, slight staining; ++, moderate staining; +++, strong staining for keratinocytes, fibroblasts and blood vessels. We used the following grading system: -, no changes; +, mild spreading between collagenous fibers of the dermal papillary layer; ++, distinguished spreading between collagenous fibers of the dermal papillary layer; +++, spreading between collagenous fibers of the dermis reticulum for histological edema.

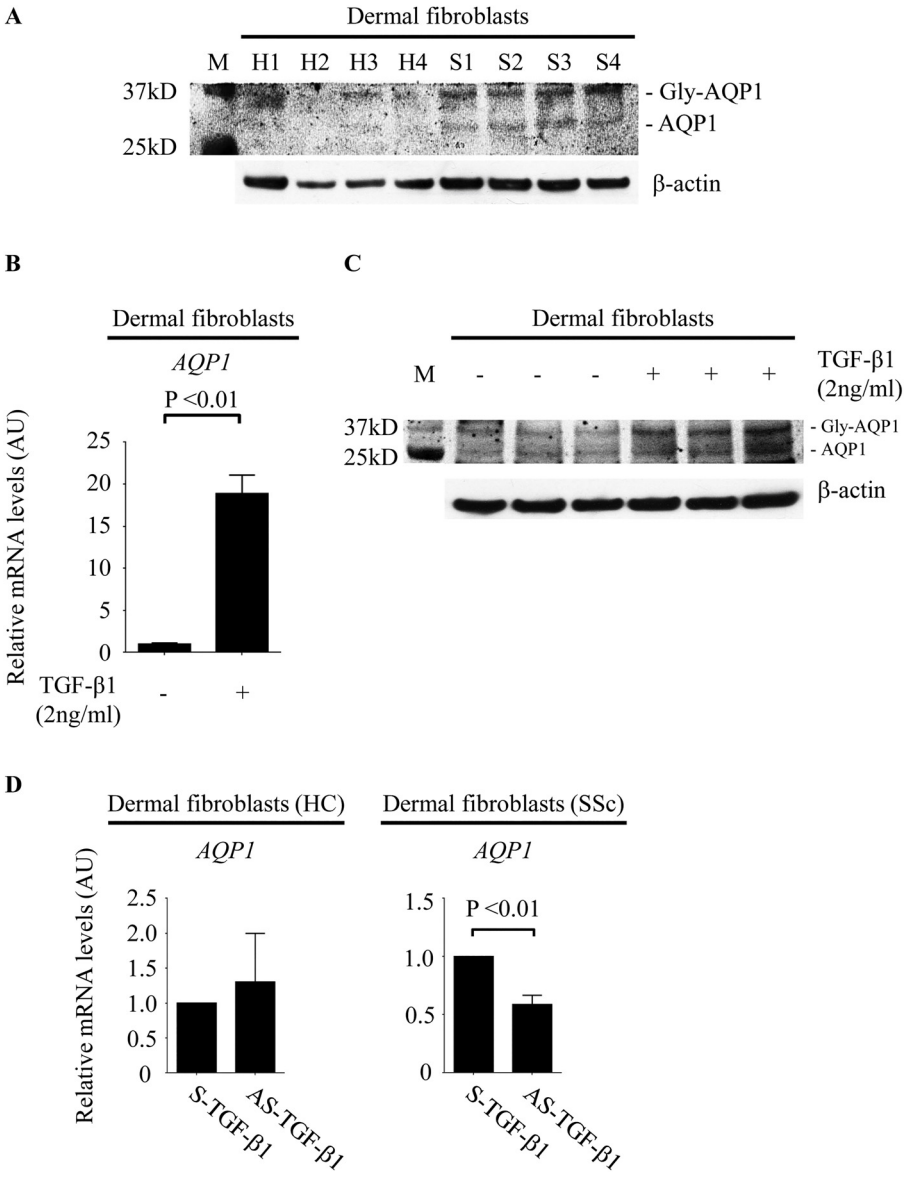

Fig. 2. Autocrine TGF- β stimulation induces AQP1 expression in SSc dermal fibroblast.
A. The expression level of AQP1 was determined in dermal fibroblasts from 4 healthy controls (H1 - 4) and 4 SSc patients (S1 - 4) by immunoblotting. **B**, **C**. Normal human dermal fibroblasts were stimulated with TGF-β1 (2 ng/ml) for 24 h. *AQP1* mRNA expression was determined by qRT-PCR (n = 9, B) and AQP1 protein expression was evaluated by immunoblotting (n = 3, C). **D**. *AQP1* mRNA levels were assessed by qRT-PCR in dermal fibroblasts from 5 SSc patients and 5 healthy controls (HC) treated with a TGF- β1 antisense oligonucleotide (AS-TGF- β1) or a TGF- β1 sense oligonucleotide (S-TGF- β1) for 48 h. In immunoblotting (A and C), upper and lower bands correspond to glycosylated AQP1 (Gly-AQP1) and unglycosylated AQP1, respectively. M means molecular weight markers. Representative blots of three independent experiments are shown. In the graphs, *AQP1* mRNA levels were normalized to those of the *GAPDH* gene. The relative value compared with controls is expressed as mean ± SEM. AU, arbitrary unit.

3.4. Silencing of the AQP1 *gene suppresses the migration of SSc dermal fibroblasts and* FLI1 *siRNA-treated endothelial cells*

A common biological feature of pro-fibrotic dermal fibroblasts and pro-angiogenic endothelial cells is enhanced migratory activity [31,32]. Since AQP1 regulates cell migration through water flux [33], we evaluated the effect of AQP1 loss on the migratory activity of SSc dermal fibroblasts and endothelial cells with an SSc-like phenotype. As described above, *FLI1* siRNA-treated HDMECs reproduce an SSc-like phenotype. Therefore, we carried out scratch assay with SSc dermal fibroblasts and *FLI1* siRNA-treated HDMECs in the presence of mitomycin C, an inhibitor of cell proliferation. As shown in Fig. 4A, the migratory activity was accelerated in SSc dermal fibroblasts compared with normal dermal fibroblasts, which is consistent with previous data [32]. Of note, *AQP1* siRNA reduced the migratory activity of SSc dermal fibroblasts to a similar level to that of *AQP1* siRNA-treated normal dermal fibroblasts. As well, *FLI1* siRNA accelerated the migration of HDMECs, and AQP1 siRNA canceled Fli1 deficiency-induced cell migration (Fig. 4B). These results indicate that AQP1 is related to the pro-migratory property of SSc dermal fibroblasts and endothelial cells with an SSc-like phenotype.

4. Discussion

In this study, we focused on the expression levels of AQP1 in SSc lesional skin because this molecule is potentially involved in the development of pathological tissue fibrosis, such as pulmonary fibrosis and liver cirrhosis [5–8]. A series of experimental data indicate that AQP1 is up-regulated in dermal fibroblasts and

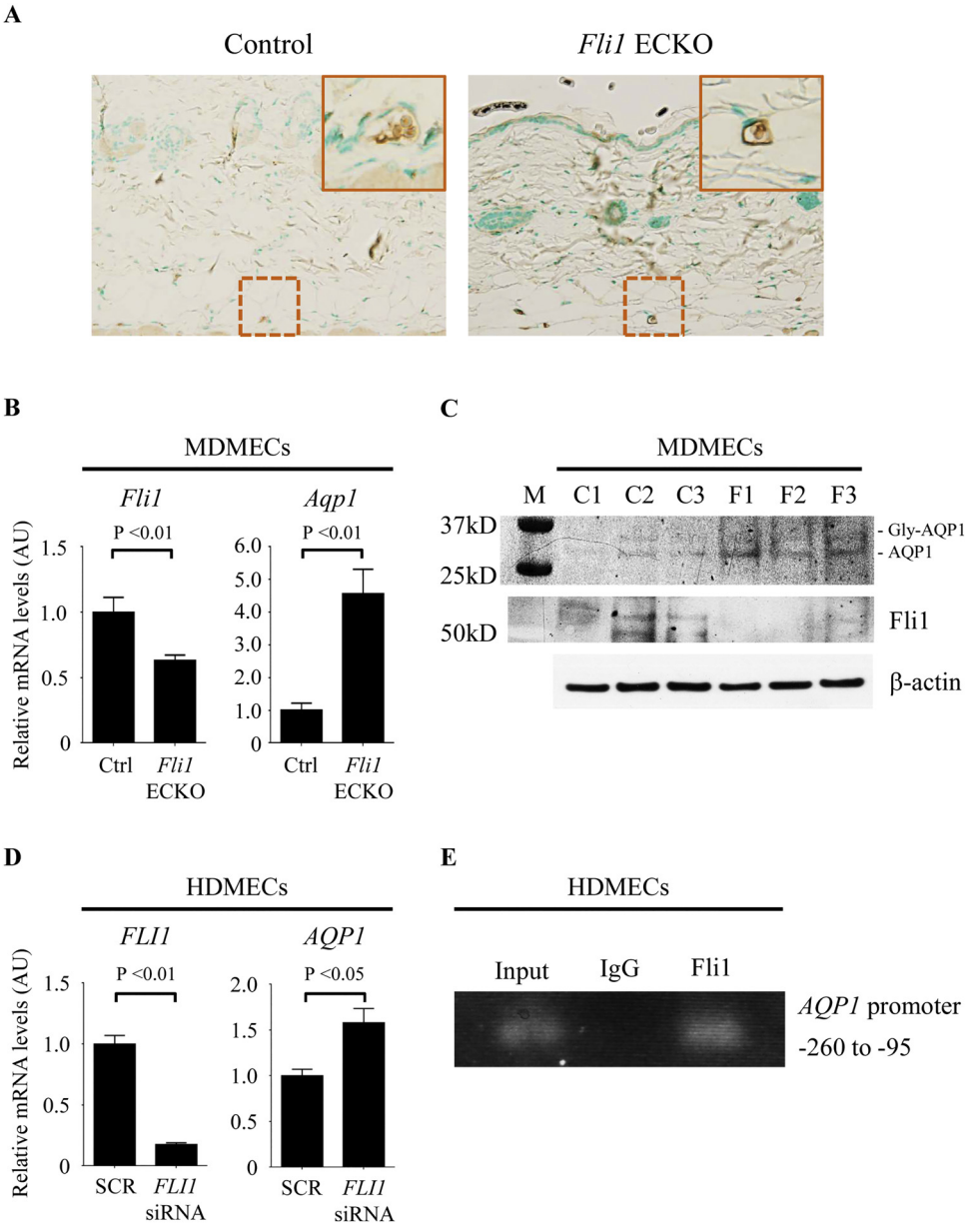

Fig. 3. Fli1 deficiency induces AQP-1 expression in endothelial cells *in vivo* and *in vitro*.
A. AQP1 expression was evaluated by immunohistochemistry in the skin of *Fli1* ECKO mice (*Fli1*$^{flox/flox}$;*Tie2*-Cre) and control littermates (*Fli1*$^{flox/flox}$). Representative results are shown (original magnification, x 200). Expansion panels of each picture group depict representative dermal small vessels. **B**, **C**. Murine dermal microvascular endothelial cells (MDMECs) were obtained from *Fli1* ECKO mice and control littermates (Ctrl). *Aqp1* mRNA expression was determined by qRT-PCR (n = 5 for control littermates and n = 6 for *Fli1* ECKO mice, B). AQP1 and Fli1 protein expression was evaluated by immunoblotting (control littermates: C1 - 3, *Fli1* ECKO mice: F1 - 3, M: molecular weight markers, C). In the blot of AQP1, upper and lower bands correspond to glycosylated AQP1 (Gly-AQP1) and unglycosylated AQP1, respectively. Representative blots of three independent experiments are shown. **D**. mRNA levels of the *FLI1* and *AQP1* genes in human dermal microvascular endothelial cells (HDMECs) transfected with non-silencing scrambled RNA (SCR) or *FLI1* siRNA were measured by qRT-PCR (n = 6). **E**. Chromatin was isolated from HDMECs and immunoprecipitation was conducted with rabbit anti-Fli1 antibody or rabbit IgG. PCR amplification was carried out using the *AQP1* promoter-specific primers. One representative of three independent experiments is shown. In the grahs, mRNA levels of the target genes were normalized to those of the *GAPDH* or *Gapdh* genes. The relative values compared with controls are expressed as mean ± SEM. AU, arbitrary unit.

dermal microvascular endothelial cells in SSc lesional skin. As a part of mechanism regulating AQP1 expression, we identified TGF-β1 and Fli1 deficiency as inducers in dermal fibroblasts and endothelial cells, respectively (no effect of TGF-β1 on AQP1 expression in endothelial cells [Supplementary Fig. 1A]; no effect of *Fli1* siRNA on AQP1 expression in dermal fibroblasts [Supplementary Fig. 1B]). Consistent with the notion that AQP1 promotes cell migration [33], SSc dermal fibroblasts and *FLI1* siRNA-treated HDMECs exhibited the accelerated healing of wound in cell culture in an AQP1-dependent manner. Taken together with the evidence that Fli1 deficiency is a key regulator for the induction of an SSc-like phenotype in endothelial cells [12], these results indicate that AQP1 may be involved in the disease process of SSc.

The contribution of AQP1 to angiogenesis and fibrosis has been reported in several animal models. For instance, in a murine model of liver cirrhosis generated by bile duct ligation, AQP1 is overexpressed by endothelial cells at the sites of robust angiogenesis in wild type mice, while angiogenesis is suppressed in *Aqp1*$^{-/-}$ mice in parallel with the attenuation of liver fibrosis and portal hypertension. Consistently, the abilities of invasion and proliferation are suppressed in *Aqp1*$^{-/-}$ endothelial cells *in vitro* [7]. Considering that anti-angiogenic therapies mostly attenuate liver

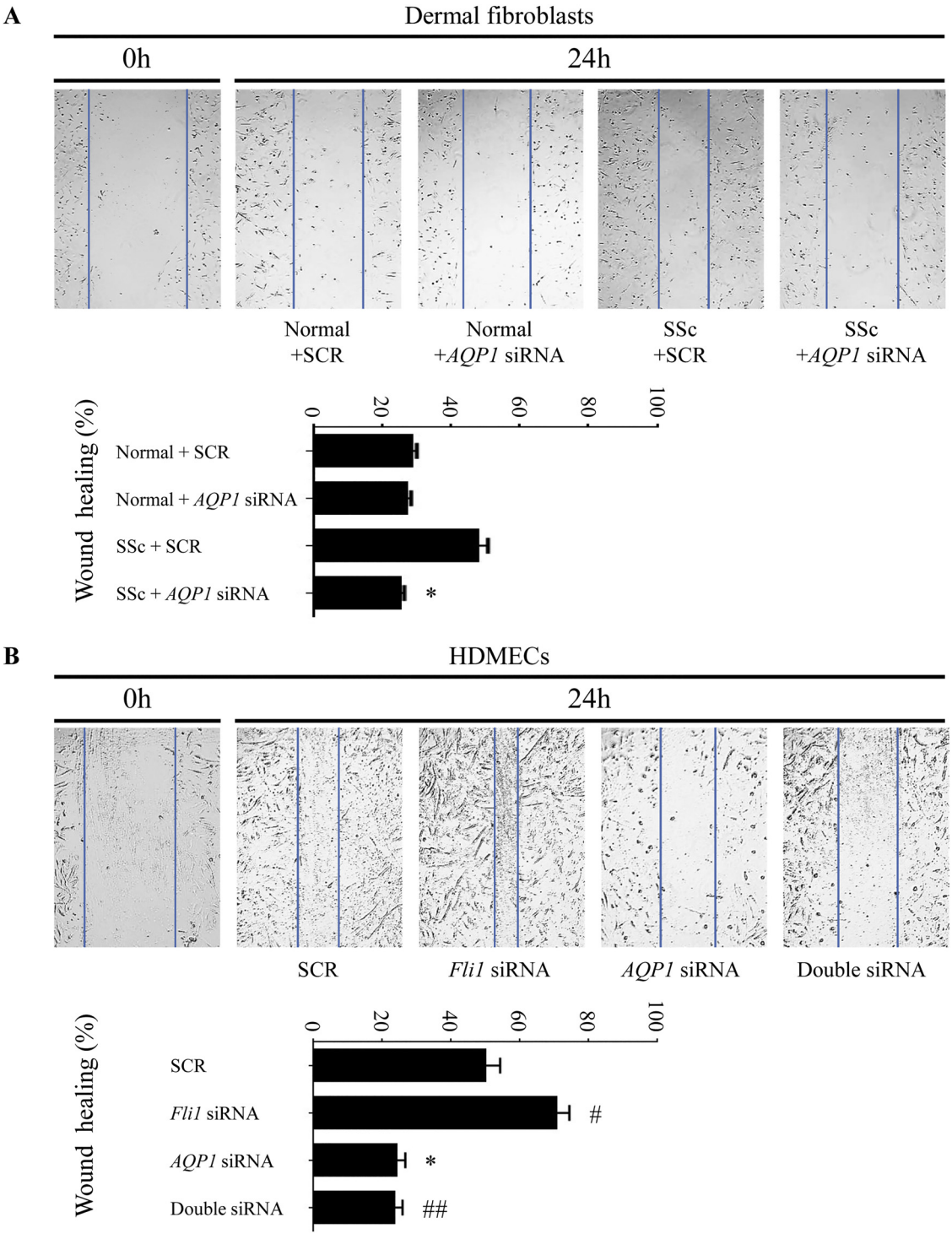

Fig. 4. AQP1 is involved in the pro-migratory property of SSc dermal fibroblasts and *FLI1* siRNA-treated endothelial cells.
A, **B**. Normal and SSc dermal fibroblasts (independent 6 stains for each group) were transfected with *AQP1* siRNA and non-silencing scrambled RNA (SCR) (A). Human dermal microvascular endothelial cells (HDMECs) were transfected with 10 nM of *FLI1* siRNA, *AQP1* siRNA, and/or SCR (B). After 34 h of siRNA treatments, 80–90% confluent cells in a 12-well plate were treated with 10 μg/ml mitomycin C (Sigma) for 2 h to remove the influence of cell proliferation. Cells were then scratched, and cell migration was monitored by microscopy for 24 h (n = 6 for A, n = 12 for B). The relative values compared with controls are expressed as mean ± SEM. * $P < 0.01$ compared with *AQP1* siRNA treated SSc dermal fibroblasts for A and compared with HDMECs transfected with SCR for B. # $P < 0.01$ compared with HDMECs transfected with SCR. ## $P < 0.01$ compared with HDMECs transfected with *FLI1* siRNA.

fibrosis in animal models [34], AQP1-dependent angiogenesis seems to contribute to the development of tissue fibrosis in the liver. Similarly, tissue fibrosis is usually preceded by vascular changes in SSc. SSc vasculopathy is pathologically characterized by accelerated angiogenesis and impaired vasculogenesis [35]. Numerous data on SSc animal models have demonstrated that endothelial Fli1 deficiency induces the activation of pro-angiogenic gene programs and eventually results in the development of vascular changes characteristic of SSc, such as capillary dilation and arteriolar stenosis [12,36]. Given the pro-angiogenic property

of AQP1 and the up-regulation of AQP1 by Fli1 deficiency in endothelial cells, AQP1 is likely to be involved in a member of Fli1 deficiency-induced pro-angiogenic gene program underlying the development of SSc vasculopathy.

Another potential role of AQP1 in endothelial cells of SSc lesional skin is the regulation of edema, which modulates inflammation and fibrosis in various organs, especially in the lung. In a murine model of pulmonary fibrosis induced by BLM, the degree of pulmonary edema positively correlates with the severity of fibrosis [5]. In addition, early removal of alveolar edema fluid is associated with a better clinical outcome, specifically with a lower mortality rate in patients with acute lung injury and the acute respiratory distress syndrome [37]. Although the relationship between edema and fibrosis is unknown in skin fibrotic conditions, edematous skin change usually precedes the development of skin sclerosis in SSc and puffy fingers are a pivotal sign for suspicion of this disease [38]. Therefore, AQP1 may amplify tissue fibrosis through the maintenance of edema in the skin as well as in the lung. Further studies with animal models are required to clarify this point in the future.

At this moment, the direct association of AQP1 with fibroblast activation is unknown. In the present study, we demonstrated the inhibitory effect of *AQP1* siRNA on the migration of SSc dermal fibroblasts. According to previous reports, the migration of fibroblasts into wounded areas is an initial step of tissue remodeling and persistent migration of myofibroblasts results in the development of tissue fibrosis [39]. Of note, some drugs inhibiting myofibroblast migration exerts an anti-fibrotic effect on BLM-induced skin fibrosis [32]. In endothelial cells, AQP1 regulates the migration through the formation of prominent membrane ruffles at the leading edge with polarization of AQP1 protein to lamellipodia [33], where raid water fluxes occur. Therefore, AQP1 may also regulate the migration of myofibroblasts through the rapid water fluxes. Supporting this notion, the migratory activity of SSc dermal fibroblast was remarkably blocked by silencing of the *AQP1* gene expression. Although further studies are required, the AQP1-dependent pro-migratory property may explain at least in part the mechanism underlying AQP1-dependent activation of dermal fibroblasts in SSc.

In summary, this is the first report documenting the up-regulated expression of AQP1 in dermal fibroblasts and dermal microvascular endothelial cells of SSc lesional skin. AQP1 contributes to the induction of a pro-migratory phenotype in dermal fibroblasts and endothelial cells and possibly to the maintenance of tissue edema, potentially leading to the development of tissue fibrosis. Fli1 deficiency-dependent AQP1 expression in endothelial cells strongly supports the notion that Fli1 deficiency is a critical predisposing factor of SSc.

Funding

This work was supported by a grant for Research on Intractable Diseases from the Ministry of Health, Labour, and Welfare of Japan. The funder is not involved in study design, data collection, data analysis, manuscript preparation and publication decisions.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgement

We thank T. Kaga, N. Toda and Y. Hasegawa for technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.09.007.

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