Systemic Sclerosis Dermal Fibroblasts Suppress Th1 Cytokine Production via Galectin-9 Overproduction due to Fli1 Deficiency

Ryosuke Saigusa, Yoshihide Asano, Kouki Nakamura, Megumi Hirabayashi, Shunsuke Miura, Takashi Yamashita, Takashi Taniguchi, Yohei Ichimura, Takehiro Takahashi, Ayumi Yoshizaki, Tomomitsu Miyagaki, Makoto Sugaya and Shinichi Sato

Dermal fibroblasts promote skin-localized transdifferentiation of regulatory T cells to T helper (Th) type 2-like cells in systemic sclerosis (SSc). However, the entire effect of SSc dermal fibroblasts on immune cells still remains unknown. Because galectin-9 induces Th2 cytokine-predominant immune imbalance by negatively regulating Th1/Th17 cells in inflammatory diseases, we investigated the contribution of galectin-9 to Th immune balance in SSc lesional skin. We used human clinical samples and Fli1<sup>−/−</sup> mice because Fli1 deficiency induces SSc-like phenotypes in various cell types. Galectin-9 was overexpressed in SSc dermal fibroblasts in vivo and in vitro. Serum galectin-9 levels were significantly elevated in SSc patients and positively correlated with skin score. Galectin-9 was up-regulated by autocrine endothelin stimulation and Fli1 deficiency, and Fli1 occupied the LGALS9 promoter in dermal fibroblasts. Co-culture of splenic CD4<sup>+</sup> T cells with Fli1<sup>−/−</sup> dermal fibroblasts significantly increased IL-4—producing cell proportion, and this effect was cancelled in parallel with the increased interferon-γ production when Fli1<sup>−/−</sup> dermal fibroblasts were transfected with Lgals9 small interfering RNA. Furthermore, Lgals9 small interfering RNA suppressed dermal collagen deposition by increasing interferon-γ production of skin-infiltrating CD4<sup>+</sup> T cells in bleomycin-treated mice. These results suggest that SSc dermal fibroblasts suppress interferon-γ expression of skin-infiltrating CD4<sup>+</sup> T cells through galectin-9 overproduction, promoting skin fibrosis development.


INTRODUCTION

Systemic sclerosis (SSc) is a multisystem autoimmune, vascular, and fibrotic disease in which a variety of cells interact with each other, eventually leading to the constitutive activation of interstitial fibroblasts (Asano and Sato, 2015; Denton, 2015). As a part of such interaction, SSc dermal fibroblasts induce skin-localized transdifferentiation of regulatory T cells into T helper (Th) type 2-like cells through IL-33 production. Subsequently, IL-13 produced by Th2-like regulatory T cells further activates SSc dermal fibroblasts (MacDonald et al., 2015). Given that SSc is characterized by Th2/Th17-skewed immune polarization (Murata et al., 2008; Matsushita et al., 2006), SSc dermal fibroblasts may much more broadly affect the immune response in the skin than previously thought.

Galectins are soluble members of the lectin superfamily that have a carbohydrate recognition domain with β-galactoside binding affinity. So far, 15 mammalian galectins have been identified (Arthur et al., 2015). Among them, galectin-9 has been well studied in terms of its immunomodulatory effect through the specific interaction with a unique glycoprotein ligand, TIM-3 (Wiersma et al., 2013). In humans, TIM-3 is expressed highly on Th1 cells and at lower levels on Th17 cells, but not on Th2 cells. TIM-3 signaling inhibition induces the expression of IFN-γ, IL-17, IL-2, and IL-6 but not IL-10, IL-4, or tumor necrosis factor-α in activated human CD4<sup>+</sup> T cells (Hastings et al., 2009). In addition, in acute rejection models of rat liver transplantation, the transfection of galectin-9—expressing adenovirus suppresses Tbet and Rorgt mRNA expression, but not Gata3 and Foxp3 mRNA expression, resulting in prolonged liver allograft survival (Liu et al., 2014). Furthermore, in a murine model of anti-glomerular basement membrane antibody-mediated glomerulonephritis, galectin-9 administration decreases Th1 and Th17 cell infiltration in the kidney by CXCL9, CXCL10, and CCL20 reduction (Zhang et al., 2014). Therefore, galectin-9 seems to suppress the development, cytokine production, and migration of Th1 and Th17 cells in a disease-specific manner. Regarding skin diseases, galectin-9 suppresses the inflammatory skin conditions in murine models of
hypersensitivity and IL-23—induced psoriasis-like dermatitis (Niwa et al., 2009). Furthermore, in atopic dermatitis that is characterized by Th2-predominant immune imbalance, galectin-9 is mainly expressed by epidermal keratinocytes and mast cells, and its serum levels correlate with the severity of skin symptoms (Nakajima et al., 2015). Therefore, galectin-9 is also involved in Th cell-mediated skin disease development.

Fli1 is a member of the Ets transcription factor family, and its expression is broadly suppressed in various cell types in involved and noninvolved skin of SSc patients (Kubo et al., 2003). Because Fli1 expression is epigenetically suppressed in the bulk skin and cultivated dermal fibroblasts from SSc patients (Wang et al., 2006), Fli1 deficiency potentially serves as a predisposing factor in SSc. Indeed, Fli1 haploinsufficiency enhances SSc-like phenotypes in dermal fibroblasts, endothelial cells, and macrophages in mice treated with bleomycin (BLM) (Taniguchi et al., 2015). Also, Fli1 deficiency regulates the expression of various disease-related molecules in SSc dermal fibroblasts, SSc endothelial cells, and SSc keratinocytes (Akamata et al., 2015; Ichimura et al., 2014, 2015; Noda et al., 2012, 2013; Saigusa et al., 2015, 2016; Takahashi et al., 2015, 2016, 2017; Yamashita et al., 2016). Therefore, the molecular analysis based on Fli1 deficiency provides us with a useful clue to elucidate the significance of target molecules in SSc pathogenesis.

Considering these backgrounds, we investigated the potential role of galectin-9 in SSc development, especially focusing on the interaction between dermal fibroblasts and CD4+ T cells. Also, the impact of Fli1 deficiency on galectin-9 expression was examined in dermal fibroblasts. In addition, the clinical association of serum galectin-9 levels was analyzed in SSc patients. Our results indicate a possible contribution of Fli1 deficiency-dependent galectin-9 up-regulation in dermal fibroblasts to the suppression of Th1 cytokine production in SSc lesional skin.

Figure 1. Galectin-9 is up-regulated in SSc dermal fibroblasts in vivo and in vitro. (a) Representative images of galectin-9 staining in human skin samples from healthy control (HC) and systemic sclerosis (SSc) subjects. Right panels (original magnification ×400, scale bar = 50 μm) show the images with higher magnification, corresponding to the areas shown with dotted squares in left panels (original magnification ×100, scale bar = 200 μm). (b, c) LGALS9 mRNA levels in (b) the bulk skin and (c) cultivated dermal fibroblasts from HC and SSc subjects were assessed by quantitative real-time reverse transcriptase—PCR (n = 5 for each group). In the graphs, the relative value compared with HC is expressed as mean ± standard error of the mean. AU, arbitrary unit.
RESULTS

Galectin-9 expression is increased in SSc dermal fibroblasts compared with normal dermal fibroblasts in vivo and in vitro

We initially compared galectin-9 expression in SSc lesional skin and healthy control skin (see Supplementary Table S1 online for a summary of all results). In normal skin (upper panels of Figure 1a), galectin-9 expression was abundant in epidermal keratinocytes, was weak in inflammatory infiltrates, and varied from none to moderate in dermal fibroblasts and endothelial cells. In SSc skin (lower panels of Figure 1a), galectin-9 expression was detected to a similar extent as in normal skin in epidermal keratinocytes, endothelial cells, and inflammatory cells. However, dermal fibroblasts in SSc lesional skin expressed galectin-9 to a greater extent than those in healthy control skin. Consistently, quantitative real-time reverse transcriptase–PCR using the bulk skin showed a significantly higher mRNA expression of the LGALS9 gene, which encodes human galectin-9, in SSc lesional skin than in healthy control skin (Figure 1b).

Furthermore, SSc dermal fibroblasts expressed LGALS9 mRNA at higher levels than normal dermal fibroblasts in cell culture (Figure 1c). Collectively, these results indicate that galectin-9 expression is selectively up-regulated in dermal fibroblasts among various cell types in SSc lesional skin.

Galectin-9 levels in the skin and/or sera correlate with the severity of skin sclerosis and the activity of alveolitis in SSc patients

We next assessed the clinical association of serum galectin-9 levels in SSc patients. In line with the enhanced expression of galectin-9 in SSc lesional skin, serum galectin-9 levels were significantly elevated in SSc patients compared with healthy control subjects (median [25–75 percentiles] = 3.3 ng/ml [1.8–5.9] versus 2.2 ng/ml [1.7–3.2], P = 0.037). When evaluated in diffuse cutaneous SSc and limited cutaneous SSc subtypes, circulating galectin-9 levels tended to be increased in patients with diffuse cutaneous SSc relative to those with limited cutaneous SSc (median [25–75 percentiles] = 4.4 ng/ml [2.0–7.1] versus 2.5 ng/ml
By Mann-Whitney U test; $P < 0.0167$ is statistically significant, and $0.0167 < P < 0.033$ represents a tendency of difference after Bonferroni correction. (Figure 2a) and were significantly elevated in diffuse cutaneous SSc patients compared with healthy control subjects ($P = 0.0004$). More importantly, there was a significant positive correlation between serum galectin-9 levels and modified Rodnan total skin thickness score (mRSS) in total SSc patients ($r = 0.36$, $P = 0.01$) (Figure 2b). Also, LGALS9 mRNA expression in the lesional skin significantly correlated with mRSS ($r = 0.43$, $P = 0.024$) (Figure 2c). Taken together, these results suggest that galectin-9 is involved in the dermal fibrotic process of SSc.

We also examined the association of serum galectin-9 levels with clinical symptoms other than skin sclerosis. As summarized in Supplementary Table S2 online, serum galectin-9 levels tended to be increased in SSc patients with interstitial lung disease (ILD) compared with those without, whereas the presence of other symptoms, including organ involvement (esophageal dysfunction, heart involvement, elevated right ventricular systolic pressure, and scleroderma renal crisis) and skin and vascular symptoms (Raynaud’s phenomenon, nailfold bleeding, pitting scars, digital ulcers, and telangiectasia) did not affect serum galectin-9 levels. Relevant to this finding, serum galectin-9 levels positively correlated with ground-glass opacity score ($r = 0.35$, $P = 0.0091$) (Figure 2d) but not with fibrosis score ($r = -0.17$, $P = 0.33$) in SSc patients with ILD. Because ground-glass opacity score reflects active alveolitis rather than established fibrosis, these results indicate that galectin-9 may be involved in alveolitis development in SSc-associated ILD and that serum galectin-9 levels may serve as a marker of the activity of this complication.

Galectin-9 expression is increased by endothelin stimulation in the lesional skin of BLM-treated mice and cultivated SSc dermal fibroblasts

To further assess the involvement of galectin-9 in pathological skin fibrosis, we evaluated galectin-9 expression in the lesional skin of BLM-treated mice, an established animal...
model of SSc. As shown in Figure 3a, a significant increase in mRNA levels of the Lgals9 gene, which encodes murine galectin-9, was evident in BLM-injected skin compared with phosphate buffered saline-injected skin. Similarly to SSc skin, galectin-9 was mainly produced by dermal fibroblasts in BLM-treated mice (Figure 3b).

To elucidate the molecular mechanism regulating galectin-9 expression in dermal fibroblasts, we looked at the effect of bosentan, a dual endothelin receptor antagonist, in BLM-treated mice because this drug suppresses skin fibrosis of BLM-treated mice by inhibiting endothelin-dependent activation of profibrotic gene programs (Akamata et al., 2014). As shown in Figure 3c, bosentan administration remarkably suppressed Lgals9 mRNA expression in the lesional skin of BLM-treated mice. This result was also confirmed at protein levels by immunostaining (Figure 3d). Therefore, galectin-9 expression is regulated by endothelin signaling in BLM-treated mice.

Because SSc dermal fibroblasts are constitutively activated at least partly by autocrine endothelin stimulation (Akamata et al., 2014), we further examined the effect of bosentan on galectin-9 expression in SSc dermal fibroblasts. As shown in Figure 3e, bosentan suppressed galectin-9 expression in SSc dermal fibroblasts in a dose-dependent manner. Therefore, galectin-9 is up-regulated in SSc dermal fibroblasts by autocrine endothelin stimulation.

Fli1 haploinsufficiency induces the expression of galectin-9 in dermal fibroblasts

In normal dermal fibroblasts, endothelin-1 reduces the expression of Fli1 protein by promoting its degradation (Akamata et al., 2014). Therefore, we examined whether Fli1 is involved in the regulation of galectin-9 expression in dermal fibroblasts. To this end, we looked at the effect of FLI1 small interfering RNA (siRNA) on LGALS9 mRNA expression in normal dermal fibroblasts and found a significant enhancement of LGALS9 mRNA expression in FLI1 siRNA-treated cells compared with scrambled nonsilencing RNA-treated cells (Figure 4a). Fli1 occupied the LGALS9 promoter in those cells (Figure 4b), suggesting that Fli1 directly serves as a potent repressor of the LGALS9 gene. We also confirmed the increased expression of galectin-9 in the skin of Fli1+/− mice at mRNA levels and in cultivated Fli1+/− murine dermal fibroblasts at mRNA and protein levels compared with each of the wild-type counterparts (Figure 4c, 4d). Taken together, these results indicate that transient and persistent down-regulation of Fli1 induces
galectin-9 expression in dermal fibroblasts in vivo and in vitro.

**Fli1-haploinsufficient dermal fibroblasts suppress Th1 cytokine production of CD4\(^+\) T cells through galectin-9 in an in vitro co-culture system**

According to previous data, galectin-9 suppresses Th1 and Th17 immune responses (Liu et al., 2014; Nakajima et al., 2015; Niwa et al., 2009; Zhang et al., 2014). Therefore, we further investigated whether Fli1-haploinsufficient dermal fibroblasts affect Th1/Th2/Th17 cytokine production of CD4\(^+\) T cells by in vitro co-culture assay with murine cells. As shown in Figure 5a, wild-type CD4\(^+\) splenic T cells co-cultured with *Fli1*\(^{+/−}\) dermal fibroblasts exhibited a higher proportion of IL-4—producing cells than those cells co-cultured with wild-type dermal fibroblasts. To assess if galectin-9 is involved in this process, we carried out the same experiments with *Fli1*\(^{+/−}\) dermal fibroblasts treated with *Lgals9* siRNA or scrambled nonsilencing RNA. The proportion of IFN-\(\gamma\)—producing CD4\(^+\) T cells was significantly increased, whereas that of IL-4—producing CD4\(^+\) T cells was significantly decreased when co-cultured with *Lgals9* siRNA-treated *Fli1*\(^{+/−}\) dermal fibroblasts (Figure 5b). On the other hand, the proportion of IL-17A—producing CD4\(^+\) T cells was not altered under the same condition. More importantly, the average of fluorescence intensity of IFN-\(\gamma\) in each of CD4\(^+\) T cells was significantly elevated by *Lgals9* siRNA, whereas that of IL-4 was not altered, suggesting that galectin-9 suppresses IFN-\(\gamma\) production without affecting IL-4 expression in individual CD4\(^+\) T cells (Figure 5c). In these experiments, more than 70% knockdown of *Lgals9* mRNA expression was confirmed in each condition (Figure 5d). These results indicate that *Fli1*\(^{+/−}\) dermal fibroblasts suppress Th1 cytokine production in CD4\(^+\) T cells at least partly via galectin-9 overproduction, which subsequently increases the proportion of Th2 cytokine-producing CD4\(^+\) T cells.

**Lgals9 siRNA attenuates dermal fibrosis and increases IFN-\(\gamma\) expression in skin-infiltrating CD4\(^+\) T cells in BLM-treated mice**

To further confirm if galectin-9 regulates the cytokine production of CD4\(^+\) T cells in vivo, we used an in vivo siRNA transfection technique. The pretreatment of *Lgals9* siRNA increased the expression of IFN-\(\gamma\) but did not affect IL-4 and IL-17A expression in skin-infiltrating CD4\(^+\) T cells of BLM-treated mice 1 week after injection. Furthermore, collagen deposition was partially but significantly attenuated by *Lgals9* siRNA in the lesional skin of BLM-treated mice 4 weeks after injection. These results suggest that galectin-9 is involved in the mechanism underlying BLM-induced skin fibrosis at least partially through the suppression of IFN-\(\gamma\) production in skin-infiltrating CD4\(^+\) T cells.

**Bosentan suppresses galectin-9 expression in SSc dermal fibroblasts in vitro**

A previous report showed that bosentan treatment significantly increases serum IL-12 levels in SSc patients (Hamaguchi et al., 2009), suggesting that bosentan enhances Th1 immune response. As described above, bosentan suppressed galectin-9 expression in BLM-treated mice in vivo and in SSc dermal fibroblasts in vitro. In addition, galectin-9 knockdown in *Fli1*\(^{+/−}\) dermal fibroblasts promoted Th1 cytokine production of wild-type splenic CD4\(^+\) T cells in an in vitro co-culture system. Therefore, we further evaluated whether bosentan suppresses the expression of galectin-9 in SSc dermal fibroblasts in vivo. To this end, we compared the galectin-9 expression in dermal fibroblasts by immunohistochemistry between three SSc patients administered bosentan and three closely matched SSc patients untreated with bosentan. There was a trend toward a decrease of galectin-9 expression in the dermal fibroblasts of SSc patients treated with bosentan compared with those cells of untreated patients (Figure 6a, Supplementary Table S1). To confirm this finding, we also looked at the effect of bosentan on serum galectin-9 levels in SSc patients. As shown in Figure 6b, when analyzed in six patients, serum galectin-9 levels were decreased after the treatment with bosentan. As a control, we assessed serum galectin-9 levels in seven SSc patients treated with intravenous cyclophosphamide pulse. In contrast to bosentan, intravenous cyclophosphamide pulse did not affect circulating galectin-9 levels (Figure 6c). These results indicate that galectin-9 suppression may contribute to the induction of Th1-predominant immune imbalance by bosentan together with IL-12 induction in SSc patients.

**DISCUSSION**

This study was undertaken to investigate the influence of dermal fibroblasts on the cytokine expression profile of CD4\(^+\) T cells in SSc lesional skin, especially focusing on galectin-9, a potential inhibitor of Th1/Th17 immune response. First, we found the increased expression of galectin-9 in SSc dermal fibroblasts in vivo and in vitro. Then, the contribution of autocrine endothelin signaling to galectin-9 up-regulation was shown in SSc dermal fibroblasts in cell culture. Also, *FlI1* was shown to serve as a potent repressor of galectin-9 expression in dermal fibroblasts in vivo and in vitro. More importantly, galectin-9 was involved in *Fli1*\(^{+/−}\) dermal fibroblast-mediated suppression of IFN-\(\gamma\) expression of CD4\(^+\) T cells, and galectin-9 loss resulted in a moderate but significant reduction of skin fibrosis at least partially due to IFN-\(\gamma\) induction in skin-infiltrating CD4\(^+\) T cells in BLM-treated mice. These results indicate that this molecule may play a part in Th2-predominant immune imbalance in SSc lesional skin.

Several lines of evidence suggest a critical role for the interaction between dermal fibroblasts and CD4\(^+\) T cells in SSc development. In the early stage of SSc, inflammatory cells, mainly composed of CD4\(^+\) T cells expressing the early activation marker CD69, are infiltrated in perivascular regions of the involved skin (Kalogerou et al., 2005; Mavalia et al., 1997). The effect of CD4\(^+\) T cells on SSc dermal fibroblasts has been well studied so far. SSc dermal fibroblasts are resistant to the antifibrotic effect of membrane-bound tumor necrosis factor-\(\alpha\) of Th2 cells (Chizzolini et al., 2003), which is at least in part attributable to the production of progranulin, an intrinsic inhibitor of tumor necrosis factor-\(\alpha\) signaling (Ichimura et al., 2015). Also, SSc dermal fibroblasts are hyporesponsive to the anti-fibrotic effect of IL-17A because of the decreased expression of IL-17 receptor type A (Nakashima et al., 2012). Thus, SSc dermal fibroblasts have an intrinsic mechanism accounting for the selective responsiveness to the profibrotic stimulation of Th2 cytokines, such as IL-4 and IL-13, leading to the efficient...
Figure 5. Galectin-9 suppresses IFN-γ production of CD4⁺ T cells in cell culture and BLM-treated mice. (a) Percentages of CD4⁺ T cells expressing IFN-γ, IL-4, and IL-17A co-cultured with dermal fibroblasts from wild-type (WT) and Flit1⁺/− mice (n = 6). (b) Percentages of IFN-γ, IL-4, and IL-17A-expressing CD4⁺ T cells co-cultured with dermal fibroblasts from Flit1⁺/− mice treated with scrambled nonsilencing RNA (SCR) or Lgals9 siRNA (n = 6). (c) Mean fluorescence intensity (MFI) of IFN-γ, IL-4, and IL-17A in CD4⁺ T cells co-cultured with dermal fibroblasts from Flit1⁺/− mice treated with SCR or Lgals9 siRNA (n = 6).
activation of SSc dermal fibroblasts by CD4\(^+\) T cells under the Th2/Th17-skewed immune balance (Murata et al., 2008; Matsushita et al., 2006). On the other hand, little is known about the impact of SSc dermal fibroblasts on CD4\(^+\) T cells. To address this issue, we focused on the impact of Fli1\(^++/++\) dermal fibroblasts on the cytokine expression profile of CD4\(^+\) T cells because Fli1\(^++/++\) dermal fibroblasts mimic a large part of the profibrotic phenotype of SSc dermal fibroblasts (Ichimura et al., 2015; Taniguchi et al., 2015). As represented by co-culture assay, Fli1\(^++/++\) dermal fibroblasts increased the proportion of IL-4-producing CD4\(^+\) T cells, suggesting that Fli1\(^++/++\) dermal fibroblasts produce some soluble factors promoting Th2 cytokine production and/or suppressing Th1 cytokine production. We identified galectin-9 as a potential soluble factor regulating Fli1\(^++/++\) dermal fibroblast-dependent suppression of Th1 cytokine production. Although further studies are still required to identify some factors promoting Th2 cytokine production, these results shed light on the role of dermal fibroblasts as a regulator of CD4\(^+\) effector T-cell activation in SSc.

A previous report showed that bosentan increases circulating IL-12 concentrations in SSc patients (Hamaguchi et al., 2009). This observation suggests that endothelin signaling results in the induction of Th1 immune response. Taken together with our current data showing the decreased expression of galectin-9 in dermal fibroblasts of SSc lesional skin after bosentan administration, bosentan seems to promote Th1 immune response through multiple pathways. In general, Th1-skewed immune polarization becomes predominant along with the resolution of skin sclerosis in SSc; therefore, it is assumed that bosentan has an anti-fibrotic effect on tissue fibrosis of SSc. Indeed, there have been an open-label prospective study and a retrospective cohort study showing mRSS improvement by 6 months of bosentan administration, although both are noncomparative studies (Giordano et al., 2010; Kuhn et al., 2010). On the other hand, endothelin receptor antagonists did not alter mRSS in the controlled, observational, real-life cohort study with a large sample size from the European League Against Rheumatism Scleroderma Trial and Research database (Vegfors et al., 2016). Given that average mRSS values generally improve after entry into the clinical trials (Amjadi et al., 2009) and that a placebo-controlled, randomized, double-blind, prospective trial has not been performed so far, the effect of bosentan on skin sclerosis is still questionable. However, bosentan seems to suppress at least partially the profibrotic gene program in the

![Image](https://example.com/image.png)
lesional skin of SSc patients, such as galectin-9 down-regulation. Therefore, the combination therapy of bosentan and other antifibrotic drugs would be a promising therapeutic strategy against skin sclerosis in SSc.

In this study, there was a positive correlation between circulating galectin-9 levels and ground-glass opacity score in patients with SSc-associated ILD. Relevant to this observation, a potential pathological role of galectin-9 in the lung is reported in patients with interstitial pneumonia. The levels of galectin-9 in bronchoalveolar lavage fluid are increased in patients with collagen vascular disease-associated interstitial pneumonia and patients with idiopathic interstitial pneumonia, especially in the former patient group. Furthermore, galectin-9 concentrations positively correlate with the total cell counts and the absolute number of lymphocytes in bronchoalveolar lavage fluid of those patients (Matsumoto et al., 2013). Thus, galectin-9 is likely to be involved in the inflammatory process of interstitial pneumonia. Given that ground-glass opacity reflects alveolitis rather than established fibrosis, galectin-9 is also likely involved in the inflammatory process of SSc-associated ILD. The bronchoalveolar lavage fluid levels of CXCL11, a Th1-associated chemokine, are elevated in the samples taken from SSc patients without ILD versus those with ILD (Srįsio et al., 2012), suggesting that Th1 immune response is protective to SSc-associated ILD. Therefore, galectin-9 seems to drive the pathological process of SSc-associated ILD by suppressing Th1 immune response. Further studies with animal models are required to clarify this point in the future.

In summary, we report a potential role of galectin-9 as a mediator of fibroblast-dependent suppression of Th1 cytokine production in SSc, previously unreported to our knowledge. A set of experiments with clinical samples suggests that the suppression of galectin-9 expression in dermal fibroblasts may underlie a potential disease-modifying effect of bosentan on SSc. Fli1 deficiency-dependent galectin-9 expression in dermal fibroblasts strongly supports the notion that Fli1 deficiency is a critical predisposing factor of SSc.

MATERIALS AND METHODS

Methods

This study was approved by the ethical committee and the Committee on Animal Experimentation of the University of Tokyo Graduate School of Medicine and was performed according to the Declaration of Helsinki. Written informed consent was obtained from all of the participants. 

**FLI1**+/+ mice were kindly provided by Dennis K. Watson (Spyropoulos et al., 2000). Gene silencing of FLI1 in dermal fibroblasts, quantitative real-time reverse transcriptase–PCR, chromatin immunoprecipitation, the measurement of serum galectin-9 levels, clinical assessments, co-culture experiments, in vivo transfection with siRNA, hydroxyproline assay, and statistical analysis are described in Supplementary Materials and Methods online.

Patients

Serum samples, frozen at −80 °C until assayed, were obtained from 53 SSc patients (50 women, 3 men; median age [25–75 percentile] = 63 years [51–70]; median disease duration [25–75 percentile] = 4 years [2–10]). Patients treated with corticosteroids or other immunosuppressants before their first visits were excluded. Patients were grouped by the LeRoy’s classification system: 30 patients with diffuse cutaneous SSc and 23 patients with limited cutaneous SSc, whose demographics are summarized in Supplementary Table S3 online. All patients fulfilled the new classification criteria of SSc (van den Hoogen et al., 2013). For the longitudinal analysis, six SSc patients treated with bosentan (median treatment period [25–75 percentile] = 20.5 months [16.2–26.2]) and seven SSc patients treated with intravenous cyclophosphamide pulse (monthly infusions, 500 mg/body surface area; median treatment period [25–75 percentile] = 6 months [5.0–9.5]) were enrolled.

CONFLICT OF INTEREST

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.04.035.

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