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Thy-1 plays a pathogenic role and is a potential biomarker for skin fibrosis in scleroderma

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Thy-1 (CD90) is a well-known marker of fibroblasts implicated in organ fibrosis, but its contribution to skin fibrosis remains unknown. We examined Thy-1 expression in scleroderma skin and its potential role as a biomarker and pathogenic factor in animal models of skin fibrosis. Skin from patients with systemic sclerosis demonstrates markedly elevated Thy-1 expression compared to controls, co-localizes with fibroblast activator protein (FAP) in the deep dermis, and is correlated with the severity of skin involvement (MRSS). Serial imaging of skin from Thy-1 YFP reporter mice by IVIS showed an increase in Thy-1 expression which correlated with onset and progression of fibrosis. In contrast to lung fibrosis, Thy-1 KO mice had attenuated skin fibrosis in both bleomycin and Tsk-1 murine models. Moreover, Thy-1 regulated key pathogenic pathways involved in fibrosis including inflammation, myofibroblast differentiation, apoptosis and multiple additional canonical fibrotic pathways. Therefore, while Thy-1 deficiency leads to exacerbated lung fibrosis, in skin it is protective. Moreover, Thy-1 may serve as a longitudinal marker to assess skin fibrosis.

Keywords: scleroderma, skin, fibrosis, Thy-1, fibroblasts
INTRODUCTION

Systemic sclerosis (scleroderma, SSc) is a prototypical multisystem fibrotic disease characterized by excessive matrix production by activated fibroblasts(1). The etiology of skin fibrosis, a hallmark of SSc, is multifactorial and marked by a complex interaction of cells and cytokines which promote profibrotic signaling pathways and resultant mechanical forces(2).

Thy-1 (CD90), a GPI-anchored glycoprotein highly expressed in neurons, T cells, and endothelial cells, has long been recognized as a fibroblast marker(3). Thy-1 has multiple physiologic functions including cell-cell signaling, mechanotransduction, and cellular differentiation(4). In fibroblasts, Thy-1 specifically regulates cell adhesion and myofibroblast differentiation through integrin signaling and is directly involved in FAS-mediated apoptosis(5, 6). Thy-1 also regulates the PPAR-γ pathway and orchestrates mesenchymal cell differentiation between adipogenic and osteogenic fates(7, 8). Lung fibroblast heterogeneity can be viewed on the basis of Thy-1 positivity(9), and likewise, synovial fibroblast subsets are defined anatomically and functionally by expression of Thy-1 and additional markers including cadherin-11, CD34, fibroblast activation protein (FAP) and MHC class II(10-12).

In skin, distinct fibroblast lineages are defined geographically based on their presence in the papillary or reticular dermis (13, 14). Of interest, these fibroblasts subtypes have previously been defined based on their expression of Thy-1 and FAP with Thy-1 positive cells residing primarily in the reticular dermis(13).

While Thy-1 is a marker of fibroblast subsets, the specific actions of this molecule in the process of fibrosis are less established. In animal models of fibrosis, Thy-1 modulates fibrotic pathways in the lung, liver, and orbit(9, 15, 16). In the lung, loss of Thy-1 exacerbates and slows the resolution of fibrosis, and these effects are reversed by pharmacologic treatment with Thy-1 replacement (6, 17). In the skin, fibrosis has not been investigated, but Thy-1 deficiency or silencing leads to impaired wound healing(18, 19) suggesting that either skin fibroblasts are functionally distinct from those that reside in the lung, or that Thy-1 has different effects in wound healing and fibrosis.

Thy-1 is over-expressed in SSc skin and serum(20, 21) but the function of Thy-1 in SSc or other human skin diseases is unknown. In this study, we assessed Thy-1 as a marker of skin fibrosis and looked to elucidate its potential pathologic role in scleroderma.

RESULTS

Thy-1 is increased in SSc skin fibroblasts and correlates with disease severity

To investigate Thy-1 expression in SSc, we performed immunofluorescence staining in skin biopsies from healthy controls and patients with SSc (Supplemental table 1). Remarkably, while there was almost no expression of Thy-1 in the reticular dermis from healthy control skin (9.3 ± 8.7 %), Thy-1 was expressed in SSc with increasing levels over time with early stage patients (disease duration < 3 years) demonstrating moderate...
expression (36.8 ± 11.1%) and later stage patients showing high expression (69.2 ± 8.9%) (figure 1A-C). It is known that FAP and Thy-1 expression status define subsets of fibroblasts in the skin with FAP+ cells localizing primarily to the papillary dermis and Thy-1+ fibroblasts to the reticular dermis with a small number of double positive cells in the transition zone(13). Therefore, to further characterize SSC fibroblasts, we assessed co-expression of Thy-1 with FAP (Figure 1A, Supplemental figure 1 for higher magnification) and observed a remarkable change to FAP+Thy-1+ cells in SSc (45.3 ± 6.9% Thy-1+ cells double positive, compared to 1.4 ± 1.1% in controls) but did not note substantial differences in double positive cells in early vs late SSc.

We next explored the gene expression of Thy-1 in skin from SSc patients in two publicly available microarray datasets (GSE58095 and GSE76886). We observed a significant increase in expression of Thy-1 in SSc across cohorts and elevated levels in both limited and diffuse cutaneous SSc (figure 1A, B, Supplemental figure 2). We also found a striking positive correlation between Thy-1 expression and severity of skin fibrosis measured by the modified Rodnan skin score (MRSS) (figure 1D, Spearman R = 0.63, p<0.0001).

We next assessed Thy-1 lung expression in a publicly available data set (GSE48149) and found that Thy-1 was significantly elevated in SSc lung explants (Supplemental figure 3). This dataset included SSc patients with either ILD or pulmonary hypertension, patients with idiopathic pulmonary hypertension, and patients with idiopathic pulmonary fibrosis (IPF). Notably, all SSc patients and IPF patients had significantly elevated Thy-1 with a trend toward the largest increase in patients with SSc-ILD. A second smaller study (GSE81292) did not show any significant difference in Thy-1 expression between SSc-ILD and controls.

To address whether Thy-1 expression was coming from fibroblasts or other skin cells, we correlated Thy-1 expression with genes specifically representing multiple skin cell types. We found strong correlations between Thy-1 expression and multiple fibroblast markers (PRSS23, FFRP2, FBN1, Col1α1, Col1α2, LUM, Spearman > 0.5) with no significant correlation in markers of most other cell types including keratinocytes, smooth muscle cells, T cells, melanocytes and endothelial cells (Figure 1F). A subset of myeloid genes (CD68 and FCER1) had strong correlation with Thy-1 while others (AIF1, LYZ) demonstrated a moderate correlation.

Thy-1 knockdown reduces fibrotic gene expression in SSc fibroblasts:

Dermal fibroblasts from healthy control (n=3) and SSc (n=3) were explanted; consistent with gene expression and immunostaining, SSc fibroblasts demonstrated increased Thy-1 gene expression (3.38 fold increase relative to control, p=0.1). After electroporation, cells were transfected with siNC or siThy-1 and treated with TGF-β (see methods for full details). TGF-β treated SSc fibroblasts demonstrated a significant reduction in TGF-β-induced aSMA expression (p=0.03) and showed a trend towards reduced Col1α1 expression whereas no reduction in fibrotic gene expression was observed in healthy control fibroblasts (Supplemental figure 4).
Thy-1 can serve as a surrogate marker of in vivo skin fibrosis

To interrogate whether Thy-1 can serve as a marker of fibrosis in vivo, we performed time-course experiments in Thy-1 YFP reporter mice (22) treated with bleomycin, and tracked expression of Thy-1 using YFP fluorescence intensity analyzed by IVIS imaging. Thy-1 expression increased over time in skin injected with bleomycin (Figure 2B,C) with maximal expression at days 21 and 28 (figure 2A-C), at the time of maximal fibrosis (figure 2D, I, G). Remarkably, Thy-1 fluorescence intensity strongly and positively correlated with multiple markers of fibrosis including dermal thickness (Spearman=0.76, p=0.006; figure 2F), fibrogenic gene expression (Spearman=0.88, p=0.0016; figure 2H) and procollagen 1+ cells (Spearman=0.82, p=0.009; figure 2K). These findings provide evidence that Thy-1 expression increases during fibrogenesis and can serve as a marker to non-invasively and longitudinally measure fibrosis in skin.

Thy-1 deficient mice have attenuated skin fibrosis

To investigate the overall effect of Thy-1 in skin fibrosis, we performed in vivo experiments using mice deficient for Thy-1 (Thy-1 KO) (23) in complementary models of fibrosis. Thy-1 KO mice that received subcutaneous injections of bleomycin demonstrated attenuation of dermal thickness compared to wildtype (WT) mice (figure 3A, C). Moreover, expression of fibrogenic gene expression including collagen (Col1a1 and Col5a2), ASMA, and PAI-1 measured by qPCR were significantly reduced in the Thy-1 KO mice (figure 3B).

To confirm the effect of Thy-1 in skin fibrosis in a complementary genetic model, Tsk-1 mice (24) were crossed to Thy-1 KO mice (23). By 3 months of age, male Tsk-1 mice develop significant fibrosis of the hypodermis (25). Consistent with the bleomycin model, Tsk-1 mice deficient in Thy-1 were significantly protected from cutaneous fibrosis as measured both by hypodermal thickness and fibrotic gene expression (figure 4A-C). Thy-1 has previously been shown to modulate adipogenesis (26) and Thy-1 KO mice fed a high fat diet-induced develop increased obesity and bone loss (8, 27, 28). To assess whether high fat diet (HFD) would lead to an augmented skin fibrosis phenotype, Thy-1 KO were fed HFD for 3 months and then treated with bleomycin. We found that in mice fed a HFD, Thy-1 KO mice were protected from skin fibrosis, but to the same extent as mice fed a chow diet (figure 3F-H). This suggests that unlike bone where HFD plays a large role in defining mesenchymal cell fate (28), other pathways are likely responsible for the protective effect of Thy-1 deficiency in skin fibrosis.

In direct contrast to the skin findings, Thy-1 deficiency has previously been shown to worsen lung fibrosis induced by intratracheal bleomycin (29). We confirmed these opposing findings in our subcutaneous bleomycin model: while Thy-1 KO mice had attenuated skin fibrosis, they demonstrated exacerbated lung fibrosis under chow conditions (figure 3D-E, supplemental figure 5).
Thy-1 regulates inflammation, myofibroblast differentiation, and apoptosis in skin fibrosis

It is well-established that bleomycin-induced skin fibrosis leads to a macrophage predominant inflammatory infiltrate (30), and we assessed if Thy-1 has an effect on these cells. We performed immunohistochemistry for F4/80 as a marker of macrophages and also investigated the expression of multiple inflammatory genes which we identified as significantly up-regulated in skin fibrosis by bleomycin (inflammatory genes with >10 fold increase at both day 7 and day 21 in the GEO dataset GSE132869). We observed a remarkable decrease in the F4/80+ cells in the absence of Thy-1 (p=0.002, figure 5A,B) and a significant down-regulation in the expression of inflammatory genes FCGR4, IFI44, CCR5, TLR13 (figure 5C) suggesting that Thy-1 regulates multiple different inflammatory pathways during fibrosis.

Bleomycin leads to myofibroblast differentiation, and Thy-1 deficient mice show impaired apoptosis of myofibroblasts leading to non-resolving lung fibrosis (17). To examine whether the protective actions of Thy-1 deficiency in skin fibrosis is related to decreased numbers of myofibroblasts and regulation of apoptosis, we performed immunohistochemistry staining for ASMA and FASL. We observed a significant decrease in the number of myofibroblasts (p=0.01, figure 5D,E) and less FASL+ cells (p=0.02, figure 5F,G) in the skin in the bleomycin treated Thy-1 KO mice compared to WT.

These findings support the concept that Thy-1 is engaged and functional in multiple established mechanisms of skin fibrosis including regulation of inflammation, differentiation of myofibroblasts, and regulation of apoptosis.

Thy-1 differentially modulates key fibrotic pathways

Based on the findings that Thy-1’s effects are multifactorial, we performed bulk RNA sequencing on skin tissue from PBS and bleomycin treated WT and Thy-1 KO mice harvested at day 21 to identify the molecular mechanisms that underlie the anti-fibrotic effects. Our findings show a clear distinction between WT and KO mice from a global expression standpoint, and while there were marked changes between WT PBS and bleomycin treated mice, KO PBS and bleomycin treated mice had many fewer differentially expressed genes consistent with the ameliorated fibrotic phenotype (figure 6A). Thy-1 KO skin also displayed a number of baseline changes compared to WT skin (supplemental figure6). Examination of KEGG pathways known to be relevant in fibrosis and regulation of Thy-1 revealed that TGF-β, WNT, integrin, hippo (YAP/TAZ), PPAR-γ, apoptosis, NF-κB, and chemokine pathways were all significantly up-regulated in WT bleomycin treated mice but not in Thy-1 KO mice. All of these pathways except NF-κB were down-regulated in Thy-1 KO bleomycin compared to WT bleomycin (figure 6B). Unsupervised pathway analysis of WT bleomycin treated mice showed over-expression of pathways consistent with fibrosis (ie. focal adhesion) and inflammation (leukocyte transendothelial migration, TLR signaling) with down-regulation of pathways related to skin cell homeostasis (cell cycle, basal cell carcinoma, melanogenesis) (figure 6C), while
Thy-1 KO mice treated with bleomycin differentially expressed metabolic pathways (upregulation of calcium and phosphatidylinositol signaling, downregulation of urea cycle, glycoxylate, and terpenoid metabolism) (figure 6D). Strikingly, when we compared the bleomycin treated mice across genotypes (WT vs Thy-1 KO), differential regulation of the same pathways seen in the WT PBS vs WT bleomycin comparison (over-expressed in WT relative to Thy-1 KO) was observed suggesting that the Thy-1 KO mice are protected from the fibrotic and inflammatory pathways induced by bleomycin (figure 6E).

**DISCUSSION**

In this study, we demonstrate that Thy-1 is aberrantly expressed in SSc fibroblasts and correlates with extent of fibrosis. We then demonstrated that Thy-1 is an *in vivo* marker of skin fibrosis and that loss of Thy-1 is protective in two complementary models of skin fibrosis. Thy-1 regulates a variety of relevant pathways including inflammation, myofibroblast activation, and apoptosis. Taken together, these findings indicate that Thy-1 serves as a marker of fibrosis and is involved in pathways that lead to skin fibrogenesis.

Thy-1 has long been considered a fibroblast marker (31). Thy-1 expression status can differentiate subtypes of fibroblasts, and in lung Thy-1⁺ and Thy-1⁻ fibroblasts are functionally distinct based on their ability to differentiate into myofibroblasts or lipofibroblasts (9). More recently, fibroblast subsets defined by Thy-1 have been further refined, particularly in the synovium where Thy-1 status along with the markers CD34 and cadherin 11 define lining and sublining synovial fibroblasts in human RA (11) while Thy-1 and FAP mark subsets of inflammatory and destructive phenotypes in murine arthritis (10).

The role of Thy-1 has been studied in lung fibrosis where it has been reported that Thy-1 plays an important role in lung fibrogenesis in IPF in which patients have been reported to have decreased Thy-1 expression (29). Moreover, treatment with soluble Thy-1 has shown to reduce bleomycin induced lung fibrosis severity (6). No specific report has been made in SSc-ILD, and we therefore assessed Thy-1 expression in publicly available datasets and found that SSc-ILD patients had significantly elevated Thy-1 expression in lung explants. Moreover, in a recent paper assessing SSc-ILD by single cell RNA sequencing, it appears that Thy-1 represents a general marker of fibroblasts in SSc-ILD rather than marking a specific subpopulation of fibroblasts (32) as has been suggested previously (33).

However, an opposite role has been reported for Thy-1 in cutaneous wound healing in which Thy-1 suppresses fibroblast proliferation and promotes apoptosis via β3 integrin signaling (18). In this context, blocking Thy-1 in wound beds reduces repair and hinders re-epithelialization (19). Given these opposing findings, it is of particular interest that we demonstrated opposite effects in skin and lung fibrosis in Thy-1 KO mice.

As our skin findings were divergent from those reported in lung fibrosis (29), we confirmed that Thy-1 KO mice had increased lung fibrosis suggesting that loss of Thy-1 truly has organ specific effects which are pro-fibrotic in lung and anti-fibrotic in skin. This may be related to Thy-1’s ability to regulation differentiation into myofibroblasts (6) in different
tissue contexts and that global Thy-1 knockout likely has pleiotropic and tissue-context specific effects. Creation of a cell-specific Thy-1 KO mouse would enable better characterization of the specific cells that may be driving these organ specific effects.

In skin, Thy-1 does not mark a single fibroblast sub-population and does not uniformly differentiate pathologic subtypes of fibroblasts because it also marks other mesenchymal cells(34). However, in recent studies, Thy-1 and FAP have been shown to delineate anatomically distinct subsets of fibroblasts with FAP+ cells primarily restricted to the papillary dermis and Thy-1+ cells to the reticular dermis(13). Moreover, similar to the lipofibroblast phenotype in lung, Thy-1+ but not FAP+ Thy-1- fibroblasts are capable of adipogenic differentiation (13). In our studies of human skin, we noted a remarkable transition from predominantly FAP+Thy-1- cells in healthy controls to FAP+Thy-1+ cells in SSc. The FAP+Thy-1+ population is not well defined in human skin, but given these cells' functional role as immune effector fibroblasts in the synovial sub-lining in mouse joints, they may serve as pro-inflammatory cells in SSc. Our correlational analysis of Thy-1 expression with fibroblast genes in public dataset interestingly demonstrated the highest correlation with two genes which have recently been identified in a subset of fibroblasts (SFRP2hi) which are uniquely overexpressed in SSc skin, specifically the subset marked by PRSS23(35). Correlation analysis demonstrated that no skin cells other than fibroblasts and myeloid cells correlated with Thy-1. Whether myeloid cells are expressing Thy-1 or simply increased in tissues with increased Thy-1 is difficult to ascertain in this analysis and further staining and/or single cell sequencing studies should be done to clarify this point. Moreover, single cell RNA sequencing may further refine SSc fibroblast populations defined by Thy-1, FAP, and other markers of interest.

Our findings that Thy-1 is up-regulated in SSc skin and dermal fibroblasts confirms previous reports (21, 36) and we also identified an important correlation between Thy-1 expression and the MRSS. This correlation was strong (Spearman R = 0.63 using publicly available transcriptional data, R = 0.66 in immunostaining). The publicly available data did not have information available regarding disease duration, and while late biopsies had a higher percentage of Thy-1+ cell numbers than early biopsies (Fig 1B), regression analysis showed that disease duration was not associated with the number of Thy-1 positive cells (R=0.08, N.S.) and was not a significant covariate in the MRSS correlation.

Using Thy-1 YFP reporter mice, which were originally described as a tool to track neurons(22) and also to mark inducible Thy-1 in cancer and would healing(37), we found an aberrant expression of Thy-1 in skin injected with bleomycin in a manner that recapitulated the peak of fibrosis seen with histologic and gene expression parameters. These findings indicate that Thy-1 can longitudinally assess fibrosis \textit{in vivo}. Taken together, this reporter mouse and the IVIS imaging system hold potential for inclusion in pharmacologic studies where non-invasive assessment of fibrosis could be evaluated over time.

While Thy-1’s role as a marker is of interest, its potential functional role is relevant to disease pathogenesis. Thy-1 deficiency in lung leads to worsening of fibrosis(6, 29) while
in skin, Thy-1 deficiency impairs wound healing(18, 19). The major finding of our study is that lack of Thy-1 leads to attenuation of skin fibrosis in both bleomycin and Tsk models. To confirm the functional effect of this finding in patient samples, we performed siRNA knockdown experiments in healthy and SSc fibroblasts and found that knockdown of Thy-1 in SSc specifically ameliorated aSMA expression suggesting that Thy-1 may play a pathogenic role. However, due to limited power in the setting of small sample size, further studies are needed to confirm these findings.

Thy-1 modulates adipogenesis in both skin and mesenchymal stem cells(26). Thy-1 KO mice are more susceptible to high fat diet-induced obesity(27, 28) and bone loss(8, 28). As our group has shown that adipose tissue plays a critical role in skin fibrosis(38-41), we investigated whether high fat diet may be modulating the anti-fibrotic effect seen in Thy-1 KO mice. Interestingly, while we continued to see a protective effect in the Thy-1 KO mice, a substantial change in the phenotype was not observed under high fat diet conditions. This finding suggests that metabolism and adipose tissue is not the key pathway by which Thy-1 regulates skin fibrosis. The interpretation of these findings, however, is challenging because it is possible that pro-inflammatory effects of the high fat diet(42) and lipolysis(43) may counterbalance some of the anti-fibrotic effects or that loss of Thy-1’s protective role prevents significant further protection in this setting. These effects should be further explored using adipose-specific knockout mice, different high fat diets with less pro-inflammatory properties, or pharmacologic modulation of fatty acid pathways.

Thy-1 is known to have pleiotropic effects including regulating pathways known to modulate fibrosis such as integrin-signaling, inflammation, myofibroblast differentiation, apoptosis and mechanotransduction(4, 26). We found that Thy-1 deficiency in bleomycin-treated skin involves regulation of multiple pathways. Thy-1 KO mice had decreased macrophage-driven inflammation and down-regulation of pro-inflammatory genes representing TLR, interferon, B cell, and macrophage pathways. Consistent with the anti-fibrotic phenotype and the reports from wound healing(18), we also found reduced numbers of myofibroblasts. Moreover, we observed reduced FASL in skin from bleomycin treated Thy-1 KO mice. Since lung fibrosis studies have shown that Thy-1 deficiency prevents resolution of fibrosis by regulating myofibroblast apoptosis(6), this raises the possibility that Thy-1 may play different functions in early and late stages of fibrosis.

To better characterize the multiple effects of Thy-1 in skin fibrosis, RNA sequencing was performed and revealed that multiple relevant pathways in skin fibrosis are regulated by Thy-1. Gene expression clearly differentiated bleomycin-treated Thy-1 KO mice from WT, and the pathways that were most differentially expressed are those critical for fibrosis including those linked to TGF-β, Wnt, focal adhesion, integrin and PPARγ signaling. Further studies are needed to define the cell-specific mechanism and cell-specific knockout mice or single cell RNA sequencing may clarify which cells are driving pathology.
In summary, we find that Thy-1 is over-expressed in SSc skin fibroblasts, correlates with severity of disease, and that deficiency of Thy-1 is anti-fibrotic in skin. The primary protective mechanism appears to be driven by decreased inflammation, myofibroblast differentiation, and apoptosis. It is intriguing that the effect of loss of Thy-1 in skin is opposite to that seen in lung, and that we were able to confirm that the effects are opposite in our systemic bleomycin model which confers fibrosis of both organs. These findings add both clarity and complexity to the actions exerted by Thy-1 in fibroblast, fibrosis, and skin biology. Given the increasingly important role of Thy-1 in defining functional subsets of fibroblasts across tissues, further elucidation of subsets of fibroblasts in SSc marked by Thy-1, FAP, and additional markers hold great promise to identify pathologic subsets of SSc fibroblasts.

MATERIAL AND METHODS

Human skin tissue. Paraffin embedded skin biopsy sections were examined from five patients with scleroderma and three healthy controls obtained from the Scleroderma Registry at the University of Rochester Medical Center or Northwestern University. All SSc patients fulfilled American College of Rheumatology (ACR) criteria(44).

Animals. Thy-1 knockout (Thy-1 KO) female mice (8–12 wk old)(23), Thy1-YFP mice(22) (Jackson Laboratories, Bar Harbor, ME, USA, #003709) both in the isogenic C57BL/6 background, and C57BL/6J (WT) were housed at constant temperature on a 12 h light/dark cycle, and given regular chow or high-fat diet (HFD), and water ad libitum. The HFD contained 60% fat (D12492; Research Diets Inc., New Brunswick, NJ, USA) and mice were fed for 3 months. All mice were genotyped using genomic DNA isolated from tail biopsies using real-time PCR (Transnetyx Inc., Cordova, TN). All control mice represent littermate controls. Both Thy-1 KO and Tsk-1 strains were backcrossed at least 10 generations with C57BL/6 before crosses were performed. The tight skin phenotype was assessed in Tsk-1 mice and double transgenic mice by scruffing the neck as previously described (25), and the Tsk -1 single transgenic mice uniformly demonstrated the tight skin phenotype.

Bleomycin-induced fibrosis. Mice were treated with bleomycin (1 mg/ml in PBS; 10 mg/kg/d) or phosphate buffered saline (PBS) by daily subcutaneous injections in two spots on the dorsal back skin for up to 14 days and harvested at various time-points as indicated. At the end of experiments, blood and tissues (skin and lung) were collected and processed for analysis. Each experimental group consisted of three to five mice, and experiments were repeated at least two times with consistent results.

Tight-skin-1 model of fibrosis. Male Tsk1/+ mice(24) (Jackson Laboratories, Bar Harbor, ME, USA, #014632) were crossed with female Thy-1 KO mice to generate Thy-1 deficient Tsk1/+ progeny. Male mice were aged to 3 months and full-thickness skin was harvested and processed for histology (Trichrome staining) and qPCR.

Evaluation of fibrosis. Harvested skin and lungs were fixed in 10% formalin, embedded in paraffin and 4 μm thick sections were stained with hematoxylin and eosin.
(H&E) or Masson’s Trichrome. Thickness of the dermis was determined at five randomly selected locations/slide using QuPath(45).

**Lung fibrosis scores.** H&E-stained lungs were assessed for histological features consistent for fibrosis using the modified Ashcroft score as previously described(46). Lung fibrosis scoring was performed in a blinded fashion in 10 high power fields (images at 20X), which included 8 subpleural and 2 central lung regions.

**qRT-PCR.** Upon harvest, tissues (skin and lung) were immersed in RNAlater (Qiagen, Valencia, CA, USA) and stored at −80 °C. The samples were homogenized with the Bullet Blender 24 Gold (Next Advance, Troy, NY, USA). RNA was isolated using the RNeasy Micro Kit or RNeasy Fibrous Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed using SYBR GreenER qPCR SuperMix (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and quantification of gene expression was performed as previously described(47). All samples were normalized to YWHAZ gene expression, and results are expressed as the fold change of Ct values (mean of 3 replicates) relative to controls, using the $2^{-\Delta \Delta Ct}$ formula.

**Immunofluorescence.** Four μm thick paraffin-embedded sections of mouse lesional skin were incubated with rat anti-procollagen I (1:100, EMD Millipore, Billerica, MA) primary antibodies, followed by species-appropriate secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA). Nuclei were detected using 4′,6-diamidino-2-phenylindole (DAPI). Healthy controls and SSc skin sections were incubated with sheep anti-FAP (1:100 dilution, AF3715, R&D systems) and rabbit anti-Thy-1 (1:50 dilution, ab92574, Abcam) primary antibodies, followed by species-appropriate secondary antibodies conjugated to Alexa Fluor 488 or 647 (Invitrogen, Carlsbad, CA). Nuclei were detected using Hoechst 33342 (NucBlue Live Ready Probes, Molecular Probes, Thermo Fisher Scientific). Slides were evaluated using a Nikon A1 laser scanning confocal microscope.

**Immunohistochemistry.** Four μm thick paraffin-embedded sections of mouse lesional skin were processed for immunohistochemistry using chromogenic enzyme substrate reactions with DAB (DAKO cat#K3468). Slides were deparaffinized on an automated platform (Leica Autostainer XL) and next treated with an antigen retrieval step using a sodium citrate solution at pH6, in a pressure cooker. Primary antibodies for FASL (1:500, ab5285, Abcam, Cambridge, MA), F4/80 (1:750, Cell Signaling Technology, Inc., Danvers, MA), and ASMA (1:3000, ab5285, Abcam) were incubated at 4C overnight in a humid chamber. Secondary antibody incubation and chromogenic reactions were performed using an automated system (Biocare intellipath), and specimens were counterstained with Hematoxylin. The stained sections were digitized using a whole slide imaging system (Olympus VS120) and quantitative analysis was performed using QuPath software5.
**IVIS.** Thy1-YFP mice \((n=18)\) were treated with PBS \((n=3)\) or Bleomycin \((n=15)\) and imaged serially using fluorescence imaging on an IVIS Spectrum animal fluorescence scanner (Perkin Elmer, Waltham, MA, USA). Fluorescence images were acquired with YFP filter settings \((\lambda \text{ ex max, } 465 \text{ nm and } \lambda \text{ em max, } 520 \text{ nm})\), and the minimum, mean, and maximum intensities \((\text{photons/s/cm}^2/\text{sr})\) were quantified by Living Imaging Acquisition and Analysis software.

**Thy-1 Skin Expression in SSc.** To interrogate expression of Thy-1 in SSc, two publicly available microarray datasets \((\text{GSE58095 and GSE76886})\) of skin biopsies from patients with SSc and healthy controls were assessed. Normalized expression of Thy-1 was first determined for each sample and compared across disease subsets and controls. Amongst SSc samples with available clinical data, Thy-1 expression was correlated with the modified Rodnan skin score (MRSS), a validated measure of skin fibrosis\((48)\). In lung tissue, Thy-1 expression in SSc and control lungs was assessed in two additional publicly available datasets \((\text{GSE48149 and GSE81292})\).

**Thy1 gene knockdown in primary skin fibroblasts**

Primary skin fibroblast cells were generated from biopsied skin samples collected from healthy subjects and scleroderma patients. For Thy-1 gene knockdown, cultured primary skin fibroblast cells \((\text{passage } 4)\) were harvested and transfected with siRNA targeting human Thy1 \((\text{Stealth RNAi™ siRNA targeting human Thy1, Assay ID HSS144275, Invitrogen})\) or scrambled RNA \((\text{Stealth RNAi™ siRNA Negative Control, Catalog number: 12935112, Invitrogen})\) using a Neon transfection system \((\text{Invitrogen})\). For this, 50 pmol siRNA were electroporated into skin fibroblast cells using the Neon transfection system in 10 μl tips at 1400 mV/20 ms/2 pulse, and cells were replated in 24-well plates. Eighteen hours after the transfection, cells were serum-starved for 8 hours and after that were cultured with or without 5ng/ml of TGFβ1 for 24 hours \((\text{for RNA})\) or 48 hours \((\text{for protein})\). After that, cells were washed twice with PBS and harvested for RNA or protein isolation and subsequent quantification of mRNA and protein level expression levels of Thy1 and fibrosis-related genes.

**RNA sequencing.** RNA was extracted from skin of PBS and bleomycin treated WT and Thy-1 KO mice using RNeasy Micro Kit or RNeasy Fibrous Mini Kit \((\text{Qiagen, Valencia, CA, USA})\). Next, a genomic mRNA library was generated using a TruSeq stranded library \((\text{Illumina, San Diego, CA})\). RNA sequencing was performed using the NextSeq platform \((\text{Illumina, San Diego, CA})\) at a density of 20 Million read pairs/library. DESeq2\((49)\) v1.22.1 within R-3.5.1 was used to perform data normalization and differential expression analysis with an adjusted p-value threshold of 0.05 on each set of raw expression measures. The 'lfcShrink' method was applied, which moderates log2 fold-changes for lowly expressed genes. Significantly up-regulated and down-regulated genes \((\text{based on } p\text{-adj }<0.05 \text{ and } \text{abs(log}_2\text{FoldChange}) > 0)\) were submitted to Enrichr (https://maayanlab.cloud/Enrichr/) to identify significantly enriched pathways dysregulated in each phenotype, and we assessed the top significantly enriched KEGG pathways based on pairwise comparison between up-regulated or down-regulated genes. RNA sequencing data has been deposited in the Gene Expression Omnibus \((\text{GEO})\) with accession number GSE211834.
Statistical analysis. Results are presented as the mean ± SD unless otherwise indicated. One way ANOVA with Tukey’s multiple comparison test was used when more than 2 values were directly compared with each other. For two group comparisons, a two-sided t-test was used. Generally, experiments included at least 3 independent values from 2 independent experiments. P values less than 0.05 were considered statistically significant. In pairwise comparisons, Spearman’s rank method was used to assess correlation. Statistical analyses and graphs were performed with GraphPad Prism (GraphPad Software version 9, La Jolla, CA, USA).

Study approval. Animal studies complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and all animal protocols were approved by the University Committee on Animal Resources of University of Rochester (#102056). For human studies, samples were de-identified and were obtained as part of an IRB approved protocol at the University of Rochester (study 1713). Written informed consent was obtained from all participants, and in accordance with the Helsinki Declaration.

Author Contributions
RGM, BDK designed the study. RGM, PD, AP, SD, MN, LS, BDK performed the experiments and analyzed the data. RNAseq analysis was performed by BDK. JV, AP, and CR gave conceptual advice and helped with the data interpretation and manuscript draft. RGM and BDK wrote the manuscript draft. All authors contributed to the draft review.

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References


A. Healthy control  Early SSc  Late SSc

FAP / Thy-1

B. Upper dermis

Thy-1+ cells (%) - Lower Dermis (IF)

Spearman R = 0.66
p = 0.03

HC  Early SSc  Late SSc

0.0 0.2 0.4 0.6 0.8

Relative Thy-1 Expression

MRSS

FAP+ Cells (%)

p < 0.0001

p < 0.0001

p = 0.04

C. Lower dermis

FAP+ Cells (%)

p < 0.0001

p < 0.0001

p < 0.0001

D. Thy1+/FAP+  (%)

p < 0.0001

E. Thy-1 + cells (%)

p < 0.0001

p < 0.0001

p < 0.0001

F. Correlation with Thy-1 Expression

Correlation with Thy-1 Expression

-0.2 0.0 0.2 0.4 0.6 0.8

Fibroblast  Myeloid  SMC  T-cell

Endothelial  Keratinocyte  LEC  Melanocyte
**FIGURE LEGENDS**

**Figure 1. Increased Thy-1 expression in SSc skin biopsies.**

(A) Immunofluorescence using antibodies to Thy-1 (magenta) and FAP (green); nuclei stained with Hoechst (blue). Skin biopsies from diffuse scleroderma patients with early (<3 years duration, n = 7), late (>3 years duration, n=4) disease and age-matched healthy controls (n = 6). Representative photomicrographs. Scale bars: 250 μm (upper panel) 100 μm (lower 2 panels). (B) Quantification of cells within the upper (top panel) and lower dermis (bottom panel) immunopositive for FAP (left), Thy-1 (middle) per total cells in 3 high-power fields per subject, and FAP+Thy-1+ cells per total Thy-1+ cells (right) in 3 high-power fields per subject. ANOVA with Tukey post-hoc test. Bars represent means with standard deviations. (C) Correlation between Thy-1+ cells in the reticular dermis with modified Rodnan skin score (MRSS) (D) Thy-1 gene expression was assessed in a SSc skin biopsy transcriptome dataset (GSE58095). Two-sided t test. n = 64 SSc and 38 controls. Data are presented as means with standard deviations. (E) Correlation between Thy-1 gene expression and MRSS. Spearman’s rank correlation test. n = 63. (F) Heatmap representing correlation between Thy-1 expression and expression of genes demonstrated to identify subpopulations of skin cells. Scale represents correlation (Spearman R) and genes are listed in order from highest to lowest correlation with colors on the right corresponding to cellular populations marked by each gene.
Figure 2. Thy-1 YFP can serve as an in vivo marker of skin fibrosis.

A) Representative images of YFP signal (radiant efficiency [p/sec/cm$^2$/sr]/[μW/cm$^2$]) measured at $\lambda$ ex max, 465 nm and $\lambda$ em max, 520 nm by IVIS Spectrum after bleomycin injection. (B) Average and (C) Maximal YFP fluorescent radiance measured over time. Data are shown as mean ± SD. Tukey’s multiple comparison test. *$P < 0.05$ relative to day 0. $n = 3$ per group. (D) Representative H&E-stained skin images showing temporal increase in dermal thickness induced by bleomycin. Scale bar: 100 μm. (E) Quantification of dermal thickness over time in bleomycin-treated mice, as determined by 5 high-power fields/mouse. Values are the mean± S.D. Two-sided $t$ test. *$P \leq 0.05$ versus day 0. $n = 3-4$ per group. (F) Correlation between dermal thickness and YFP maximal fluorescent intensity by IVIS. Spearman’s rank correlation. (G) Gene expression changes over time (Pai1, ASMA, Col1a1, Thy1) in skin treated with bleomycin analyzed by qPCR. The thicker black line represents the mean of all fibrotic genes. Data represents mean gene expression per time point. $n = 3-4$. (H) Correlation between the average of fibrotic gene expression and YFP maximal fluorescent intensity. Spearman’s rank correlation test. (I) Representative immunofluorescence imaging for antibodies against pro-collagen type 1 (red, white arrowheads) as well as endogenous YFP label (green). Scale bar: 100 μm. (J) Number of procollagen immunopositive cells per high-power field (hpf). Results are mean ± S.D. from three hpf/mouse. Two-sided $t$ test. *$P \leq 0.05$. $n = 3$ (K) Correlation between number of procollagen 1$^+$ cells and YFP maximal fluorescent intensity. Spearman’s rank correlation.
A. 

Chow

PBS | BLM

WT

Thy-1KO

B.

WT

Thy-1KO

C. 

Dermal thickness (µm)

p = 0.0003

D. 

Modified Ashcroft Score

p = 0.007

E. 

Col5a2 mRNA (-fold)

p = 0.002

PAI1 mRNA (-fold)

p < 0.0001

Col1a1 mRNA (-fold)

p < 0.0001

WT | Thy-1 KO

F. 

Dermal thickness (µm)

p = 0.05

G. 

Modified Ashcroft Score

n.s.

H. 

Col5a2 mRNA (-fold)

p = 0.0002

PAI1 mRNA (-fold)

p = 0.0003

Col1a1 mRNA (-fold)

p < 0.0001

WT | Thy-1 KO

PBS | BLM

HFD | BLM
Figure 3. Thy-1 KO mice have attenuated bleomycin-induced skin fibrosis.

(A) Representative images of H&E-stained skin after bleomycin or PBS injections in chow and high-fed diet (HFD)-fed mice. Scale bar: 100 μm. (B) Representative H&E-stained lungs after bleomycin or PBS injections in chow and high-fed diet (HFD)-fed mice. Scale bar: 100 μm. (C) Dermal thickness as determined by 5 high-power fields/hpf/mouse (n = 3-5 mice/group). ANOVA with Tukey post-hoc test. (D) Fibrosis scores (modified Ashcroft score). Results are mean ± S.D. from ten hpf per mouse (n = 3-5 mice/group chow diet, n = 5-10 mice/group HFD). Mann-Whitney U test. (E) Expression of Col1a1, PAI-1, ASMA, Col5a2 assessed by qPCR. Results were normalized to YWHAZ (n = 3-5 mice/group chow diet, n = 5-10 mice/group HFD). ANOVA with Tukey post-hoc test.
A. Control and Thy-1 KO

B. Hypodermal Thickness (µM)

C. Col5A2 mRNA (-fold)

D. Col1a1 mRNA (-fold)

E. PAI-1 mRNA (-fold)
Figure 4. Tsk-1 mice lacking Thy-1 have attenuated skin fibrosis

(A) Representative Masson’s trichrome-stained images. Dashed line represents hypodermis. Scale bar: 250 μm. (B) Quantification of hypodermal thickness as determined by 5 high-power fields/mouse. $n = 4-9$ mice/group. ANOVA with Tukey post-hoc test. (C) Expression of PAI-1, Col5a2 and Col1a1 assessed by qPCR. Results were normalized to YWHAZ ($n = 3-6$ mice/group). Results are mean ± S.D. ANOVA with Tukey post-hoc test.
Figure 5. Thy-1 KO mice display decreased inflammation, number of myofibroblasts and apoptosis during skin fibrosis

Representative immunohistochemistry stains against F4/80 (A), ASMA (D) and FASL (F). Scale bar: 50µm. Quantitative analysis of positive cells for F4/80 (B), ASMA (E) and FASL (G). Values are the mean ± S.D of 3 high power fields per mouse. ANOVA with Tukey post-hoc test (E). n = 2-5 mice/group; solid circles represent WT mice and open circles for Thy-1 KO mice. (C) Expression of FCGR4, IFI44 CCR5 and TLR13 assessed by qPCR. Results were normalized to YWHAZ (n = 4-5 mice/group). Results are mean ± S.D. ANOVA with Tukey post-hoc test.
### KEGG Pathways

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### Downregulated in KO-BLM

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Figure 6. Thy-1 differentially modulates fibrotic pathways
(A) Heatmap of differentially expressed genes visualized using hierarchical clustering. \( n \) = 2-3 per group. (B) Pairwise pathway analysis for KEGG pathways implicated in fibrosis. Data represents \( P \) values for Tukey’s multiple comparison test. Significant \( p \)-values are represented in bold. (C) Differentially expressed KEGG pathways identified by Enrichr comparing PBS- and bleomycin (BLM)-treated WT mice, (D) PBS- and BLM-treated Thy-1 KO mice, (E) BLM-treated WT and Thy-1 KO.