Integrin–modulating therapy prevents fibrosis and autoimmunity in mouse models of scleroderma

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In systemic sclerosis (SSc), a common and aetiologically mysterious form of scleroderma (defined as pathological fibrosis of the skin), previously healthy adults acquire fibrosis of the skin and visceras in association with autoantibodies1. Familial recurrence is extremely rare and causal genes have not been identified. Although the onset of fibrosis in SSc typically correlates with the production of autoantibodies, whether they contribute to disease pathogenesis or simply serve as a marker of disease remains controversial and the mechanism for their induction is largely unknown2. The study of SSc is hindered by a lack of animal models that recapitulate the aetiology of this complex disease. To gain a foothold in the pathogenesis of pathological skin fibrosis, we studied stiff skin syndrome (SSS), a rare but tractable Mendelian disorder leading to childhood onset of diffuse skin fibrosis with autosomal dominant inheritance and complete penetrance. We showed previously that SSS is caused by heterogeneous missense mutations in the gene (FBN1) encoding fibrillin-1, the main constituent of extracellular microfibrils3. SSS mutations all localize to the only domain in fibrillin-1 that harbours an Arg-Gly-Asp (RGD) motif needed to mediate cell–matrix interactions by binding to cell-surface integrins4. Here we show that mouse lines harbouring analogous amino acid substitutions in fibrillin-1 recapitulate aggressive skin fibrosis that is prevented by integrin-modulating therapies and reversed by antagonism of the pro-fibrotic cytokine transforming growth factor β (TGF-β). Mutant mice show skin infiltration of pro-inflammatory immune cells including plasmacytoid dendritic cells, T helper cells and plasma cells, and also autotaxin production; these findings are normalized by integrin-modulating therapies or TGF-β antagonism. These results show that alterations in cell–matrix interactions are sufficient to initiate and sustain inflammatory and pro-fibrotic programmes and highlight new therapeutic strategies.

Fibrillin-1 contributes to the regulation of TGF-β, a cytokine that has been descriptively linked to many fibrotic diseases, including both SSS and SSc4,5. TGF-β is secreted from the cell in the context of a large latent complex (LLC) that includes the active cytokine bound to a dimer of its processed amino-terminal propeptide, latency-associated peptide (LAP), which in turn binds to latent TGF-β-binding proteins (LTBPs)6. Studies in mouse models and in vitro have shown that fibrillin-1 interacts directly with LTBPs, allowing sequestration of the LLC by microfibrils7.

Mutations throughout the FBN1 gene also cause Marfan syndrome (MFS), a disorder characterized by bone overgrowth, ocular lens dislocation and aortic dilatation8. Failed matrix sequestration of the LLC in fibrillin-1-deficient patients and mice promotes increased activation of, and signalling by, TGF-β. SSS mutations are specifically localized to the fourth transforming growth factor-β-binding protein-like domain (TB4) of fibrillin-1, which encodes the RGD motif, through which fibrillin-1 binds integrins α5β1, α5β1 and α5β1 (refs 3, 5).

To determine whether failed interaction between integrins and fibrillin-1 is sufficient to initiate skin fibrosis, two Fbn1-targeted knock-in mouse models were generated: one with SSS-associated change W1572C (the mouse equivalent of human W1570C) and the other with an RGD to RGE substitution (D1545E) predicted to cause an obligate loss of integrin binding to fibrillin-1 (Supplementary Fig. 1). Mice heterozygous for either mutation phenocopy SSS with increased deposition of collagen by 1 month of age and a decrease in subcutaneous fat by 3 months of age (Fig. 1 and Supplementary Fig. 2a, b). Whereas homozygosity for D1545E causes embryonic lethality before embryonic day 10.5, mice homozygous for W1572C are viable and show accelerated skin fibrosis when compared with heterozygous littermates (Fig. 1 and Supplementary Fig. 2a, b). As seen in patients with SSS or SSc, mutant mice show disorganized and excessive microfibrillar aggregates in the dermis, with sparsely distributed elastin (Supplementary Fig. 2c). Freshly isolated cells from mutant dermis show increased surface levels of integrins α5β1 and α5β1 in its active conformation (as assessed using WOW-1 antibody) by flow cytometry (Fig. 2a). There was no corresponding increase in either total β1, integrin or integrin β1, a subtype that can cross-react with WOW-1 (Supplementary Fig. 3). Freshly isolated cells from mutant dermis show increased surface levels of integrins α5β1 and α5β1 in its active conformation (as assessed using WOW-1 antibody) by flow cytometry (Fig. 2a). There was no corresponding increase in either total β1, integrin or integrin β1, a subtype that can cross-react with WOW-1 (Supplementary Fig. 3).

To assess for a pathogenic contribution for TGF-β, SSS mice were treated for 12 weeks with a pan-specific TGF-β-neutralizing antibody (TGF-βNAb; 1D11) or isotype-matched control IgG after establishment of dense fibrosis at 12 weeks of age. Clinical (Fig. 3a) and histological (Fig. 3b) findings confirmed full reversal of skin stiffness and epidermal thickening (irrespective of Fbn1 genotype) reminiscent of the aberrant wound-healing described previously in β1 integrin-deficient mice (Supplementary Fig. 5c). To assess for a pathogenic contribution for TGF-β, SSS mice were treated for 12 weeks with a pan-specific TGF-β-neutralizing antibody (TGF-βNAb; 1D11) or isotype-matched control IgG after establishment of dense fibrosis at 12 weeks of age. Clinical (Fig. 3a) and histological (Fig. 3b) findings confirmed full reversal of skin stiffness and epidermal thickening (irrespective of Fbn1 genotype) reminiscent of the aberrant wound-healing described previously in β1 integrin-deficient mice (Supplementary Fig. 5c).

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analysis of skin in mutant mice did not reveal increased expression of free TGF-β1 (Supplementary Fig. 6b), which is known to be activated by integrins through interaction with the RGD sequence in its LAP (LAP1). There was an increase in total (free and active) TGF-β1 (Supplementary Fig. 6b), which has not been shown to be activated by integrins presumably as a result of the absence of an RGD sequence in LAP2). Furthermore, there was excessive concentration of both LAP1 and LAP2 in the dermis of mouse models of SSS, suggesting accumulation of the LLC for TGF-β1 and TGF-β2, respectively. Although we cannot exclude a contribution of integrin-mediated TGF-β activation, these data suggest that enhanced TGF-β bioavailability contributes predominantly to increased TGF-β activity in mutant mice.

As seen in SSc, SSS mouse models show circulating anti-nuclear and anti-topoisomerase I antibodies (Fig. 4a and Supplementary Fig. 7). The finding that the deep dermal fibrosis seen in early SSS (Fig. 1) co-localizes with high expression of active β3 integrin and accumulation of CD45+ marrow-derived cells (Supplementary Fig. 8a) prompted speculation that an infiltrating class of immune cells might contribute to disease progression. In keeping with this hypothesis, nearly all dermal cells express the pro-inflammatory cytokines interleukin (IL)-6 and interferon (IFN)-α (Fig. 4c and Supplementary Fig. 8e). There is also dermal polarization towards pro-inflammatory T helper (Th1) cell populations, including CD4+ IL-4+ T1/2, CD4+ IL-17+ T1/17 and CD4+ IL-9+ T1/9 cells (Supplementary Fig. 9a, b). In keeping with skewing towards Th1/2, Th1/9 and/or T1/17 cells, there was also an increased expression of IL-9, IL-13 and IL-22 by CD3+ dermal cells (Supplementary Fig. 9a–c). There was no corresponding increase in either IFN-γ+ CD4+ T1 or FoxP3+ CD4+ T regulatory (Treg) cells in mutant animals (Supplementary Fig. 9d). Last, the dermis of SSS mice also showed infiltration with B220+/CD19+ activated B cells and CD138+ B220+CD19+ plasma cells (Supplementary Fig. 10). These abnormalities, including circulating autoantibodies and immune cell infiltration and activation, were normalized by treatment of mutant mice with β3aAb (Fig. 4 and Supplementary Figs 9 and 10). A similar response was seen in association with reversal of skin fibrosis on treatment with TGF-β1Ab (Supplementary Fig. 11).

We speculated that altered presentation of the fibrillin-1 RGD sequence might directly influence the expression of integrins by, and the performance of, pDCs. In keeping with this hypothesis, we found that wild-type spleen-derived pDCs showed increased adherence and activation (expression of IFN-α and IL-6) when plated on the matrix expressed by SSS murine embryonic fibroblasts (MEFs) in comparison with control MEFs (Supplementary Fig. 12).

SSc fibroblasts showed increased cell-surface presentation of total β1 integrin (Supplementary Fig. 13) and active β1 integrin (as monitored by WOW-1 staining) in comparison with controls, whereas levels of total β3 and β6 integrins were normal (Supplementary Fig. 14a). Treatment with β3aAb TS2/16, which promotes and stabilizes integrin β1–ligand interactions, normalized cell-surface levels of active β3 integrin (Supplementary Fig. 14a). Treatment with β3 integrin-blocking antibody (β3aAb) did not significantly decrease the cell-surface presentation of total β1 integrin (Supplementary Fig. 13). Human SSc cells in culture showed decreased levels of microRNA-29 (miR-29) (Supplementary Fig. 14b), a small regulatory RNA that is repressed by TGF-β and is known to inhibit the expression of multiple matrix elements and to suppress fibrosis in selected disease states. Treatment with β3aAb normalized miR-29 expression and dose-dependently attenuated the expression of type I and type III collagen in SSc fibroblasts (Supplementary Fig. 14b). SD208, an antagonist of the kinase activity of the type I TGF-β receptor subunit, also normalized the expression of collagen and miR-29a (Supplementary Fig. 14c).

In addition to canonical (Smad-dependent) signalling, TGF-β can also initiate so-called non-canonical cascades, prominently including extracellular signal-regulated kinase (ERK1/2). SSc fibroblasts showed normal TGF-β1-dependent phosphorylation of Smad3 (pSmad3) that was not influenced by integrin-modulating therapies, but uniquely showed TGF-β1-dependent phosphorylation of ERK1/2 (pERK1/2) in contrast with control fibroblasts that was normalized on treatment with either β3aAb or β6aAb (Supplementary Fig. 14d, e). The activation of ERK1/2 in SSc fibroblasts was seen within 5 min of stimulation with TGF-β1 and was inhibited by pretreatment with SD208, suggesting a relatively direct response (Supplementary Fig. 14d, e). In keeping with a pathogenic contribution of pERK1/2, treatment of SSc fibroblasts with U0126, an inhibitor of the mitogen-activated protein kinase/ERK kinase (MEK), increased miR-29a levels and decreased collagen expression in SSc fibroblasts (Supplementary Fig. 14f). Both SSS mouse models showed excessive activation of ERK1/2 in CD31+ pDCs and other dermal cells (Supplementary Fig. 14g). Treatment of Fbn1+/W1572C/+ mice with the MEK inhibitor RDEA119 prevented skin stiffness, the accumulation of dermal collagen and the loss of subcutaneous fat (Supplementary Fig. 14h, i).

This study shows that point mutations specifically in the sole integrin-binding domain of fibrillin-1 are sufficient to recapitulate the SSS phenotype in mice and to initiate many findings reminiscent of SSc, including...
human correlates, and both the mechanism and pathogenic relevance reported autoantibodies and subdermal fibrosis in tight skin (Tsk) antibody) in mutant mice that is eliminated on treatment with Fbn1 isotype-matched control (IgG). Representative contour plots (left) and (right) are shown. An agonist and antagonist of β1 integrin activation were used to assess to the specificity of the WOW-1 antibody (Supplementary Fig. 16b). For isotype control-treated animals, n = 5 (Fbn11/1) and 7 (Fbn1D1545E/); for β1aAb-treated animals, n = 4 (Fbn11/1) and 7 (Fbn1D1545E/). b. Clinical assessment demonstrated that β1aAb prevented skin stiffness in mutant animals when compared with those treated with an isotype-matched control (IgG). c. Masson’s trichrome staining reveals decreased skin collagen and preservation of subcutaneous fat in β1aAb-treated mutants (left). Quantification of the thickness of the zones of dermal collagen and subcutaneous fat is shown (right). For isotype control-treated animals, n = 12 (Fbn11/1), 9 (Fbn1D1545E/1) and 8 (Fbn1W1572C/); for β1aAb-treated animals, n = 12 (Fbn11/1), 10 (Fbn1D1545E/1) and 10 (Fbn1W1572C/). Scale bars, 50 μm. **P < 0.01; ***P < 0.001; †P < 0.0001. The upper and lower margins of each box define the 75th and 25th centiles, respectively; the internal line defines the median, and the whiskers define the range. Values outside the interquartile range are shown as open circles.

Figure 2 | Integrin-modulating interventions prevent skin fibrosis. a. Flow cytometry of cells derived from the dermis reveals a unique population expressing both α5β1, and active β1 integrins (Supplementary Fig. 16a). d. In dermal cells from the Fbn1 D1545E mouse, a decreased concentration of abnormal microfibrillar aggregates and increased expression of integrin α5β1 are evident. e. Quantification of the thickness of zones of dermal collagen and subcutaneous fat is shown (right). Fbn1+/+ Fbn1D1545E/ and Fbn1+/+ Fbn1W1572C/ show marked differences in dermal collagen and subcutaneous fat thickness. Scale bars, 50 μm. **P < 0.01; ***P < 0.001; †P < 0.0001. The upper and lower margins of each box define the 75th and 25th centiles, respectively; the internal line defines the median, and the whiskers define the range. Values outside the interquartile range are shown as open circles.

Figure 3 | A pan-specific TGF-β-neutralizing antibody reverses established skin fibrosis. a. Clinical assessment showing that stiffness was fully normalized by treatment with TGF-βNAb, starting at 3 months of age and continuing for 12 weeks. b. Histological and morphometric analyses using Masson’s trichrome stain. For isotype control-treated animals, n = 14 (Fbn11/1), 9 (Fbn1D1545E/1) and 9 (Fbn1W1572C/); for TGF-βNAb-treated animals, n = 14 (Fbn11/1), 10 (Fbn1D1545E/1) and 8 (Fbn1W1572C/). Scale bars, 50 μm. **P < 0.01; ***P < 0.001; †P < 0.0001. The upper and lower margins of each box define the 75th and 25th centiles, respectively; the internal line defines the median, and the whiskers define the range. Values outside the interquartile range are shown as open circles.
differences in the regulation of microfibrillar assembly. The stiffened ECM in SSS could support mechanical traction-based activation of the excessive amounts of latent TGF-β in the dermis, a plausible feedforward mechanism for the observed fibrosis. Thus the level of TGF-β signalling in a given tissue may, at least in part, be determined by the integration of both positive and negative regulation by microfibrils.

Although the cell type that first detects and responds to aberrant presentation of the RGD sequence in fibrillin-1 remains unknown, it is useful to speculate on the involvement of pre-pDCs that normally perform a surveillance function for viral pathogens at low concentrations in the skin. Previous work has shown that αββ integrin influences the adhesion, migration and maturation of DCs and that migration is inhibited by β1αAb, at least in part through podosome disassembly. It is therefore evident that pre-pDCs are informed by and respond to their matrix environment, with fibrillin-1 potentially serving as a prominent informant. In keeping with this, our in vitro observations (Supplementary Fig. 12) suggest that an altered matrix environment, devoid of any systemic influence, is sufficient to promote pDC recruitment and activation. It remains to be determined whether this relates to the loss of a physiological inhibitory signal by normal microfibrils or to a pathogenic gain of function through a Smad-dependent mechanism. Whereas the altered matrix environment in SSS probably contributes to excessive TGF-β activity early in the course of disease, TGF-β induces its own production and activation by pDCs, as well as IL-6 secretion (known prerequisites for T112 polarization). pDCs can also induce either T11 or T117 skewing through IL-6/IFN-β-dependent or OX40 ligation (Ox40L)/IL-4-dependent mechanisms, respectively (Supplementary Fig. 15). pDCs in a T112 environment become activated and show enhanced IL-4 secretion, constituting a potential feedforward mechanism for maintenance of a T112 response. In the context of high TGF-β signalling, this might also allow for T119 skewing. Cytokines related to IL-4/IL-13, IL-17 and IFN-β, have been prominently implicated in the fibrotic response in various disease states, including SSc and other fibrotic disease.

Although many studies have highlighted the contribution of integrins to fibrotic disease, their focus has been on the ability of certain integrins to release (activate) TGF-β1 or TGF-β3 from the LLC through a direct interaction with RGD sequences in LAP1 and LAP3 (ref. 8). Multiple observations in this study suggest that enhanced bioavailability of TGF-β, rather than its activation, may be the primary determinant of increased TGF-β activity in SSS and perhaps SSc. Our in vitro data in SSc fibroblasts suggest that cell-surface integrins can influence the inherent signalling properties of the TGF-β receptor complex in response to free and active TGF-β. Although the initiating pathogenic event in SSc remains unknown, this study provides evidence for a cell autonomous signalling defect that is maintained in culture. In theory, this could relate to primary but poorly penetrant genetic alterations or fixed epigenetic modifications, both of which may require a large environmental trigger.
Activation of ERK1/2 has previously been implicated in the TGF-β-mediated fibrotic response in general, and specifically in SSc fibroblasts.

It was previously observed that constitutive ERK1/2 signalling in SSc fibroblasts drives the expression of integrin αβ. Both αβ and TGF-β were required for excessive collagen production. Despite overlapping observations and the common conclusion that αβ represents an attractive therapeutic target, this study places ERK1/2 activation downstream of both TGF-β and enhanced active αβ expression in SSc fibroblasts and shows phenotypic rescue in response to ERK antagonism in an in vivo model of scleroderma. Furthermore, we show prominent ERK1/2 signalling in pDCs in SSc mice, a described prerequisite for the stabilization, nuclear export and translation of IFN-α messenger RNA and for Toll-like receptor-mediated expression of inflammatory cytokines. Whereas previous work associated low levels of miR-29, a negative regulator of collagen expression, with fibrotic-mediated fibrotic response in general, and specifically in SSc fibroblasts during viral infections, autoimmunity, and tolerance. D. Rev. Clin. Pract. 2, 679–685 (2006).

METHODS SUMMARY

Subjects. Patients were recruited from the Scleroderma Center and Connective Tissue Clinic at Johns Hopkins Hospital. All skin biopsies and protocols were performed in compliance with the Johns Hopkins School of Medicine Institutional Review Board after informed consent.

Mice. All mice were cared for in strict compliance with the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Fbn1+/- mice were purchased through Jackson Laboratories as heterozygotes. Skin biopsy, and drug treatments and dosing, as well as meth-

Statistics and graphs. All quantitative data are shown as standard boxplots produced in R statistical software. Statistical analysis was performed with a two-tailed t-test, assuming equal variance between the compared groups.

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Supplementary Information is available in the online version of the paper.

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Author Contributions E.E.G., E.M.G. and H.C.D. designed experiments and interpreted the data. E.M.G. performed enzyme-linked immunosorbent assays. F.M.W. obtained skin samples from patients (the Scleroderma Center of Johns Hopkins University School of Medicine) and provided guidance and clinical expertise. S.C.F. assisted in drug trials in vivo and in the collection of mouse sera. E.C.D. performed electron microscopy. E.E.G. generated mouse models and performed all other experiments. D.L.H. aided in complete blood count analysis, mouse surgery, and histopathology. E.E.G. and H.C.D. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence should be addressed to H.C.D. (hdietz@jhmi.edu).
METHODS

Participants. Patients were recruited from the Scleroderma Center and Connective Tissue Clinic at Johns Hopkins Hospital (F.M.W. and H.C.D.). All skin biopsies and research protocols were performed in compliance with the Johns Hopkins School of Medicine Institutional Review Board and after informed consent.

Mice. All mice were cared for in strict compliance with the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Fbn1\textsuperscript{D1545E/+} and Fbn1\textsuperscript{W1572C+/-} mice were generated by homologous recombination (Supplementary Fig. 1a). A 10-kilobase Fbn1 fragment was generated by PCR from mouse genomic tail DNA, digested with Ace65 and Nhel restriction enzymes (NEB), and ligated into pSL301 (Invitrogen Corp.). Site-directed mutagenesis was performed with the QuickChange mutagenesis kit (Strategene Inc.), creating either the D1545E or W1572C mutation. The targeting vector was assessed by sequence analysis. Site-directed mutagenesis was again performed to remove the AatII restriction site from pSL301. The NeoR cassette was amplified from pEGFP-C1 (Invitrogen Corp.) and the amplicon was subcloned into pCR2.1–TOPo (Invitrogen Corp.). A fragment containing the AatII restriction site and NeoR, with flanking loxp sequences, was subcloned into a unique AatII site in the Fbn1 intron before exon 38. The sequences of the loxp sites and site-directed mutagenesis-created mutations were confirmed by direct sequencing. The vector was linearized using a unique (NsiI) site and electroporated into R1 embryonic stem cells. Positive clones were identified by Southern blot analysis (Supplementary Fig. 1b) as described previously.\(^4\) Positive clones were injected into 129/SvEv blastocysts at embryonic day 3.5 and transferred into pseudo-pregnant females (C57BL/6, B6) at gestational day 1.5. The pups were genotyped on the basis of creation of a new AciI site (W1572C) or destruction of a BamAI site (D1545E) in correctly targeted mice (Supplementary Fig. 1c). Primers used for amplification were 5’-GATCCACACACTGGATGTC-3’ (sense) and 5’-CATGTTTTCACAGAAGGACAC-3’ (antisense). The loxp-flanked NeoR was removed by breeding Fbn1\textsuperscript{D1545E/+} and Fbn1\textsuperscript{W1572C+/-} mice with transgenic mice that ubiquitously expressed Cre recombinase using an EIIa promoter, purchased through Jackson Laboratories. More than 85 embryos were genotyped at embryonic day 10.5 for Fbn1\textsuperscript{D1545E/+} homozygosity.

In vivo drug treatment. All antibodies used to treat mice or cells were azide-free. Male mice were treated with \(\beta\)-integrin-activating antibody (\(\beta\)-aAb, Rat Clone 9EG7; special-ordered more than 98% pure and azide-free from BD Biosciences) or an isotype-matched control (Rat IgG2a, \(\kappa\); special-ordered more than 98% pure and azide-free from BD Biosciences) by intraperitoneal injection at 2 mg kg\(^{-1}\) every 5 days for 12 weeks, beginning at 1 month of age. Complete bleed blood cell counts were performed to exclude pancytopenia in \(\beta\)-aAb-treated animals (Supplementary Fig. 16a). For the TGF-\(\beta\)-neutralizing trial, 3-month-old male mice were treated with pan-specific TGF-\(\beta\)-neutralizing antibody (Mouse Clone 1D11, catalogue no. MAB1835; R&D) or an isotype control (Mouse IgG1, clone 11711, catalogue no. MAB1835; R&D) or an isotype control (Mouse IgG1, clone 11711, catalogue no. MAB1835; R&D). For all experiments, all mice were assessed in random order. A score of 1 was assigned to mice with overt skin disease or to mice in which one of the treated skin sites was clearly more affected than the untreated skin site. A score of 2 was assigned to mice in which 10%–20% of the skin surface was affected.

Stiffness scoring. A clinical stiffness score was assigned by five observers blinded to genotype and treatment status. Mice were assessed in random order. A score of 1 indicates no stiffness (that is, identical to wild-type mice). A score of 4 indicates extreme stiffness based on previous experience with untreated SSS mice; 2 and 3 indicate a subjective assessment of an intermediate level of stiffness. Early in the course of studies, the same mice were assessed by the same observer on a different day. This pilot demonstrated excellent intra-observer concordance. To measure stretched skin area and total surface area, mice were anaesthetized with isoflurane, and the back skin was shaved and treated briefly with Nair cream. Area measurements were performed with NIH image J software (National Institutes of Health). Mice were then suspended briefly with forceps secured to the back skin by a clamp and photographed in profile in a uniform manner (Supplementary Fig. 4a, b). The Flim software was used to calculate the area of the skin using NIH image J software (National Institutes of Health). Area measurements were performed with NIH image J software (National Institutes of Health). Mice were then suspended briefly with forceps secured to the back skin by a clamp and photographed. No differences in body weight between all experimental groups (Supplementary Fig. 4c).

Histology. For tissue analysis, animals were killed with inhalational halothane (Sigma) or anaesthetized with isoflurane. Back skin was shaved and treated briefly with Nair cream before biopsy. Fixed skin was paraffin-embbed, sectioned, and stained with a standard Mason's trichrome stain. Dermal and subcutaneous fat thicknesses were measured under high-power fields as described previously.\(^5\) Immunofluorescent staining was performed on frozen sections as described previously.\(^6\) Active \(\alpha\)\(\text{v}\beta\)\(\text{5}\) was detected with the WOW-1 antibody (gift from S. Shattil) and an anti-mouse Alexa Fluor 594 F(ab\(^{\prime}\))\_2 fragment secondary antibody (catalogue no. A11020, Invitrogen). Other antibodies used included anti-CD45 antibody (catalogue no. 556539, BD), anti-Siglec H (catalogue no. 14-0333-81, ebiosciences) and antibodies against LAP1 (catalogue no. 141402, BioLegend), LAP2 (catalogue no. LS-C137100, Lıbassion Sciences), active TGF-\(\beta\)1 (Clone LC1 (-30), gift from K. Flanders), and total TGF-\(\beta\)2 (catalogue no. ab66045, abcam). With the exception of WOW-1, all other antibodies were conjugated by means of an amine-based Alexa Fluor antibody labelling kit (catalogue nos A-20181, A20187, A-20185 and A-20186, Invitrogen).

Electron microscopy. Electron microscopy was performed as described previously.\(^7\) Enzyme-linked immunosorbent assay. Mouse sera were collected and enzyme-linked immunosorbent assays (ELISAs) were performed with the Mouse Anti-Nuclear Antibodies and Mouse Anti-Scl70 kits (catalogue nos 5210 and 6110, AlphaDiagnostic) in accordance with the manufacturer's instructions.

Cell culture. Primary human dermal fibroblasts were derived from skin biopsies from five patients with active diffuse systemic sclerosis and from six healthy controls. Biopsies were taken from the forearm and cultured as described previously.\(^8\) All experiments were performed in cell lines at low (less than 5) passage. Primary MEFs were derived from embryonic day 13.5 embryos as described previously.\(^8\) Murine pDCs were isolated from the spleens of wild-type C57Bl6/J mice by using the Plasmacytoid Dendritic Cell Isolation Kit II (catalogue no. 130-092-786, Miltenyi Biotec) and a midiMACS Separator (catalogue no. 130-042-302, Miltenyi Biotec) in accordance with the manufacturer's instructions. The pDC-containing cell suspensions routinely had more than 95% purity, as detected by flow cytometry. For MEF/pDC co-culture experiments, MEFs were cultured to complete confluence in culture medium containing RPMI 1640, 100 μg ml\(^{-1}\) streptomycin, 100 U ml\(^{-1}\) penicillin, 2 mM L-glutamine (Gibco) and 10% heat-inactivated fetal calf serum. At 72 h after confluence, 5 \(\times\) 10\(^{5}\) murine splenic pDCs ml\(^{-1}\) were plated onto mono-layers. After 72 h of co-culture, both adherent and non-adherent cellular fractions were harvested, counted, and analysed by flow cytometry.

Flow cytometric analysis. Mouse skin was digested for flow cytometric analysis as described previously.\(^8\) On average, 4 \(\times\) 10\(^{5}\) cells were obtained from a 1 \(\times\) 2 cm piece of skin for wild-type mice, and 8 \(\times\) 10\(^{5}\) cells were obtained from either SSS mouse model. Murine Fc receptors were blocked using antibodies against mouse CD16/32 antibodies (catalogue no. 553141, BD Biosciences). Murine plasmacytoid dendritic cells were isolated as reported previously.\(^8\) All isolated cells (including murine dermal cells, cultured MEFs, splenic murine pDCs or human dermal fibroblasts) were stained and fixed using the BD Cytofix/Cytoperm system (catalogue no. 554722, BD Biosciences). Data were acquired using CellQuest-Pro software on a FACSCalibur flow cytometer or BD FACSuite software on a FACSVerse flow cytometer (BD Biosciences). Data were analysed and all flow cytometry plots were generated using FlowJo software (TreeStar). For histograms, FlowJo software divides all events into 256 'bins', which are numerical ranges for the parameter on the x axis. The percentage of maximum (y axis) is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells. Gating for live cells was based on staining with the LIVE/DEAD Fixable Dead Cell Stain Kit (catalogue no. L34955, Invitrogen). All staining was performed with fluorochrome-conjugated primary and isotype control antibodies. All antibodies were either purchased as fluorochrome conjugates or conjugated by means of amine-based Alexa Fluor antibody labelling kits (catalogue nos A-20181, A20187, A-20185 and A-20186, Invitrogen). A subtype specific to the WOW-1 antibody (gift from S. Shattil), EDTA (10 mM) and 2 mM MnCl\(_2\) were used as negative and positive controls, respectively, for \(\alpha\)\(\text{v}\)\(\beta\)5 activation in flow cytometry experiments (Supplementary Fig. 16b).\(^9\) Integrin \(\alpha\)\(\text{v}\)\(\beta\)5, a subtype known to react with the WOW-1 antibody,\(^9\) was monitored in mouse and human
cells with a specific antibody (mouse catalogue no. LS-C36943, Lifespan Biosciences). Other antibodies used on mouse cells were: integrin β1 (Clone eBioHMb1-1, catalogue no. 17-0291-80, eBiosciences), integrin β3 (Clone C9G3, catalogue no. 12-0611, eBiosciences), integrin α2 (catalogue no. 11-0049-83, eBiosciences), integrin β6 (catalogue no. LS-C152915, Lifespan Biosciences), integrin β8 (Clone H-160, catalogue no. sc-25714, Santa Cruz Biotechnology) and pERK1/2 (catalogue no. 4370, Cell Signaling). Antibodies used for the immunological characterization of mouse cells included (from ebiosciences) IL-13 (catalogue no. 53-7133-82) and IL-22 (catalogue no. 12-7221-82); (from BD Biosciences) Ly6C (catalogue no. 17-0619-42, eBiosciences), IL-10 (catalogue no. 16-0721-82); (from Biolegend) CD3 (catalogue no. 100227) and Siglec H (catalogue no. 129611). The antibody against IFN-γ (catalogue no. 560469), CD19 (catalogue no. 550992), CD138 (catalogue no. 560593), CD11b (catalogue no. 562127), CD4 (catalogue no. 561226), and IL-22 (catalogue no. 12-7221-82); (from BD Biosciences) Ly6C (catalogue no. 12-0611, eBiosciences), integrin αvβ3 (Clone eBioHMb1-1, catalogue no. 16-4714-81, eBiosciences), IgG2a (0.2 mg ml⁻¹, and integrin αVβ1 (Clone eBioHMb1-1, catalogue no. LS-C152915, Lifespan Biosciences), integrin αvβ6 (catalogue no. 16-4724, eBiosciences), αvβ3-blocking (30 µg ml⁻¹, Clone LM609, catalogue no. MAB1976Z, Millipore), β1-activating (7 µg ml⁻¹, Clone TS2/16, catalogue no. 14-0299, eBiosciences) and β1-blocking (0.2 mg ml⁻¹, Clone P4C10, catalogue no. MAB1987Z, Millipore) antibodies.

**Western blotting.** Before lysate harvest, cells were washed with prewarmed (42°C) 1× PBS (Gibco). Total protein was isolated from cells with ice-cold RIPA buffer (25 mM Tris/HCl pH 7.6, 150 mM NaCl, 1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS) with phosphatase and protease inhibitors (catalogue nos 04906837001 and 11836170001, Roche). Western blotting was performed using the Bio-Rad and LiCor Odyssey detection systems as described previously. The relative intensities were measured using LiCor Odyssey software. The following antibodies were used: phosphorylated and total ERK (Clone D13.14.4E, catalogue no. 4370, and Clone 3A7, catalogue no. 9107, Cell Signaling), vinculin (Clone hVIN-1, catalogue no. V9131, Sigma), and phosphorylated and total Smad3 (catalogue nos 1880-1 and 1735-1, Epitomics).

**RNA isolation and qPCR.** Total RNA was isolated from cultured cells or tissue by using Trizol (Invitrogen) in accordance with the manufacturer’s protocol. Quantitative PCR for miR-29a and 18S ribosomal RNA was performed with pre-designed Taqman primers and probes (ABI) in accordance with the manufacturer’s instructions. Relative quantification for each transcript was obtained by normalizing against 18S transcript abundance according to the formula 2⁻ΔΔCt. The 49/50th and 25/75th centiles, respectively; the internal line defines the median, and the whiskers define the range. Statistical analysis was performed with a two-tailed t-test, assuming equal variance between the compared groups (asterisk, P < 0.05; two asterisks, P < 0.01; dagger, P < 0.001; double dagger, P < 0.0001). Values outside the interquartile range are shown as open circles (R software default), but were not excluded from or treated differently in statistical analyses.

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