Targeted Disruption of TGF- β /Smad3 Signaling Modulates Skin Fibrosis in a Mouse Model of Scleroderma

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Transforming growth factor- β (TGF- β) is a potent stimulus of connective tissue accumulation, and is implicated in the pathogenesis of scleroderma and other fibrotic disorders. Smad3 functions as a key intracellular signal transducer for profibrotic TGF- β responses in normal skin fibroblasts. The potential role of Smad3 in the pathogenesis of scleroderma was investigated in Smad3-null (Smad3^{-/-}) mice using a model of skin fibrosis induced by subcutaneous injections of bleomycin. At early time points, bleomycin-induced macrophage infiltration in the dermis and local TGF- β production were similar in Smad3^{-/-} and wild-type mice. In contrast, at day 28, lesional skin from Smad3^{-/-} mice showed attenuated fibrosis, lower synthesis and accumulation of collagen, and reduced collagen gene transcription *in situ*, compared to wild-type mice. Connective tissue growth factor and α -smooth muscle actin expression in lesional skin were also significantly attenuated. Electron microscopy revealed an absence of small diameter collagen fibrils in the dermis from bleomycin-treated Smad3^{-/-} mice. Compared to fibroblasts derived from wild-type mice, Smad3^{-/-} fibroblasts showed reduced *in vitro* proliferative and profibrotic responses elicited by TGF- β . Together, these results indicate that ablation of Smad3 is associated with markedly altered fibroblast regulation *in vivo* and *in vitro*, and confers partial protection from bleomycin-induced scleroderma in mice. Reduced fibrosis is due to deregulated fibroblast function, as the inflammatory response induced by bleomycin was similar in wild-type and Smad3^{-/-} mice. (*Am J Pathol* 2004, 165:203–217)

Scleroderma is a chronic autoimmune disease of unknown etiology characterized by early inflammation and vascular injury, followed by progressive fibrosis of the skin and other organs.¹ The pathological hallmark of scleroderma is excessive accumulation of highly cross-linked type I collagen and other extracellular matrix (ECM) components in the affected organs. Lesional fibroblasts display an activated phenotype, and are largely responsible for exaggerated matrix synthesis and tissue deposition. Smooth muscle cell-like myofibroblasts with strongly profibrotic potential are present in lesional tissue.² The identity of the signals responsible for initiating the activation of fibroblasts and accumulation of myofibroblasts, and for sustaining and amplifying this process over time, are still controversial.

Much attention has focused on the role of transforming growth factor- β (TGF- β) in the pathogenesis of fibrosis in scleroderma. Transforming growth factor- β is a prototypic member of a large superfamily of cytokines that induce pleiotropic effects in different cell types. TGF- β is involved in autocrine and paracrine regulation of embryogenesis and cellular differentiation, immune and inflammatory responses, cell proliferation, migration and apoptosis, and matrix remodeling.³ In fibroblasts, TGF- β is a potent stimulus for the synthesis of ECM proteins, including collagens, fibronectin, tenascin, tissue inhibitor of metalloproteinase-1 (TIMP-1), and plasminogen activator inhibitor-1 (PAI-1), as well as secretion of the cysteine-rich fibrogenic peptide connective tissue growth factor (CTGF), and TGF- β itself (autoinduction). Furthermore, TGF- β enhances the migration and proliferation of fibroblasts, prevents their apoptosis and induces their transformation into myofibroblasts, stimulates collagen contraction, and promotes collagen maturation into a highly cross-linked dense matrix. In light of its protean effects on connective tissue homeostasis, it is not surprising that

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altered TGF- β activity or signaling is implicated in the pathogenesis of fibrotic disorders.⁴

Recent studies shed light on the intracellular signaling mechanisms that mediate cellular TGF- β responses. Binding of TGF- β to the type II TGF- β receptor (T β RII), a transmembrane serine/threonine kinase, triggers its heterodimerization with and activation of T β RI. The signal is then propagated downstream through Smads, a family of recently characterized intracellular proteins that convey information from the cell membrane to the nucleus.⁵ In vertebrates, Smads involved in TGF- β signaling segregate into three functionally distinct groups: Smad2 and Smad3 are highly homologous direct substrates for activated T β RI; Smad4 is the common signaling partner; and Smad7 blocks ligand-induced Smad2/3 activation. In contrast to Smads2–4, which are expressed in all cell types, expression of inhibitory Smad7 is highly regulated by extracellular signals. On their phosphorylation by T β RI kinase at the cell membrane, Smad2 and Smad3 are released from the receptor complex and associate with Smad4. The heteromeric Smad complex is then transported into the nucleus, where, in cooperation with other DNA-binding factors or with transcriptional cofactors, it activates or represses target gene transcription. While Smad2 and Smad3 work synergistically and in tandem with Smad4 in transducing TGF- β signals, Smad7 serves critical negative feedback function to limit the amplitude and duration of cellular responses to TGF- β .

Targeted deletion of Smad2 or Smad3 genes in mice has revealed distinct developmental roles for these closely related Smads. Homozygous loss of Smad2 resulted in embryonic lethality due to defective gastrulation and mesoderm formation.⁶ In contrast, mice with homozygous deletions of the Smad3 gene were viable and survive into adulthood, but showed impaired mucosal immunity, defective neutrophil chemotaxis, and abnormal receptor-induced activation of thymocytes and peripheral T cells.^{7,8} When challenged with bleomycin, Smad3-deficient mice displayed attenuated pulmonary fibrosis despite a robust early inflammatory response and macrophage accumulation in the lungs.⁹ In contrast, cutaneous wound healing was found to be accelerated in the absence of Smad3, presumably as a consequence of enhanced epithelialization and reduced local inflammatory response in the skin.¹⁰ Studies using fibroblasts derived from embryos null for either Smad3 or Smad2 have revealed a complex picture of transcriptional regulation, indicating that distinct TGF- β -regulated genes show dependence on Smad2, Smad3, or both.¹¹ Furthermore, there is growing evidence that other signaling pathways besides Smads are also activated downstream from TGF- β receptors.¹² These non-Smad pathways may mediate biological responses elicited by TGF- β in a Smad-dependent or -independent manner, but their physiological roles remain poorly characterized.

To understand the mechanisms underlying fibrosis and the role of TGF- β /Smad pathways in this process at the cellular and molecular levels, animal models are useful. Subcutaneous injection of bleomycin induces chronic scleroderma-like skin fibrosis in mice.^{13,14} The process was associated with early inflammation and TGF- β ex-

pression in the skin, and neutralizing anti-TGF- β antibody prevented the development of dermal fibrosis.¹⁵ Moreover, we previously showed that bleomycin-induced murine scleroderma was associated with sustained activation of intracellular TGF- β /Smad signaling in the skin. In particular, strong nuclear accumulation and phosphorylation of Smad2/3 was observed in resident skin fibroblasts even following resolution of local inflammation.¹⁶ Because Smad3 is a key mediator for regulation of collagen synthesis and other profibrotic responses to TGF- β , we examined the hypothesis that loss of Smad3 may result in attenuation of dermal fibrosis in the bleomycin-induced murine model of scleroderma.

Materials and Methods

Animals and Experimental Protocol

Smad3 null mutant mice were generated by intercrossing Black Swiss $\times$ 129SVJ mice heterozygous for targeted disruption of exon 8 in the Smad3 gene.⁸ Wild-type control mice generated from heterozygous crosses were maintained under identical conditions as Smad3^{-/-} mice to ensure minimal variability in genetic background and environment between transgenic and control animals. The protocols in this study were institutionally approved, and were in accordance with the animal welfare guidelines of the NIH/Association for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained with Rodent Diet (Harlan, Madison, WI) and water *ad libitum*. Genotype was determined by PCR analysis of tail DNA obtained from 2-week old mice, using Primer 1 (5'-CCACTTCATTGCCATATGCCCTG-3') and Primer 2 (5'-CCGAACAGTTGGATTACACA-3') of exon 8 of the Smad3 gene to identify wild-type and heterozygous mice, and Primer 1 and Primer 3 (5'-CCAGACTGCCTTGGAAAAGC-3') to identify Smad3^{-/-} mice.⁸

Skin fibrosis was induced by injection of bleomycin. Groups of 6-week-old male and female Smad3^{-/-} mice (average weight 15 g), and wild-type littermates (average weight 20 g) were studied in parallel. Bleomycin (Nihon Kayaku, Tokyo, Japan) or phosphate-buffered saline (PBS) as vehicle was administered by subcutaneous injections as described previously.¹⁴ At 7 or 28 days after initiation of daily injections, mice were sacrificed by CO₂ asphyxiation and cervical dislocation. The body weight after 4 weeks of treatment increased slightly in both wild-type and Smad3^{-/-} mice. The injected skin was removed and processed for histological analysis, determination of hydroxyproline content, and other studies. Each study group contained at least five mice for each time point. Three separate experiments were performed with similar results.

Histochemical and Electronmicroscopic Studies

Lesional skin tissue from age-matched wild-type and Smad3^{-/-} mice were fixed overnight in 10% formalin, dehydrated, and embedded in paraffin using standard techniques. Consecutive 5- μ m serial sections were cut

and stained with hematoxylin and eosin. To determine the collagen content and organization of the lesional skin, deparaffinized sections were processed for Masson's trichrome stain or Picrosirius red stain, which was viewed under polarized light. Dermal thickness was determined in hematoxylin and eosin-stained sections viewed under $\times 100$ microscopic magnification by measuring the distance between the epidermal-dermal junction to the dermal-fat junction at five randomly selected sites/fields from two or more skin samples in each animal.¹⁶

For EM, samples from wild-type and Smad3^{-/-} mice was immersed in 4% glutaraldehyde and rinsed in 0.1 mol/L sodium cacodylate buffer. The samples were post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Spurr's epoxy resin (Electron Microscopy Sciences; Fort Washington, PA). Ultrathin sections (80 nm) were collected on grids, stained with uracyl acetate and lead citrate and examined with a transmission electron microscope (Hitachi H-600, 75kv). EM images taken at $\times 30,000$ magnification were scanned into Adobe Photoshop (Fremont, WA) on an Epson flat bed scanner. Fibril diameters were determined by an observer blinded to the experimental conditions by manual measurement of photographic negatives with a calibrated final magnification of $\times 30,000$, and histograms were generated. Three wild-type and three Smad3^{-/-} mice were used for quantification, with a minimum contribution of 200 collagen fibrils from each sample. For each mouse, four micrographs representing cross sections of collagen fibrils were used for quantitative studies.

Immunohistochemistry

Five- μ m sections from lesional skin samples were deparaffinized, re-hydrated in descending alcohol dilutions, and immersed in TBS-T buffer (Tris-buffered saline-Tween 20). The sections were treated with target retrieval solution (DAKO Corporation, Carpinteria, CA) at 95°C for 10 minutes, then with serum-free blocking agent (DAKO) for 15 minutes, followed by incubation with primary antibodies at room temperature for 2 hours. The following antibodies were used: anti-Mac-3 (BD PharMingen, San Diego, CA) to identify macrophages, anti- α -smooth muscle actin (Sigma-Aldrich, St. Louis, MO) to identify myofibroblasts, anti-proliferating cell nuclear antigen (PCNA) (Zymed Laboratories, San Francisco, CA) to identify proliferating cells, anti-CTGF (from L. Lau, University of Illinois, Chicago, IL), anti-TGF- β (LC1-30-1 from K. Flanders, NIH, Bethesda, MD), anti-Hsp47 (Stressgen Biotechnologies, Victoria, Canada), anti-Smad7 (from P. ten Dijke, The Netherlands Cancer Institute, Amsterdam, Netherlands), and anti-phospho-Smad2 (Cell Signaling Technology, Beverly, MA).

For detection of bound antibodies Histomouse (Zymed Laboratories), Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) or DAKO Envision + System were used according to the manufacturers' instructions. Substitution of the primary antibody with isotype-matched irrelevant IgG at the same concentration served as negative controls. After counterstaining with hematoxylin, sections were mounted with Permount (Fisher Scientific,

Pittsburgh, PA). Stained sections were viewed under an Olympus BH-2 microscope, and images were obtained by digital capture. Fibroblasts were identified by their characteristic spindle-shaped morphology. The number of positive fibroblasts in the dermis was determined semi-quantitatively by examining a minimum of 30 cells/field at $\times 400$ magnification in each slide. To prevent observer bias, all histological specimens were coded and examined without knowledge of experimental conditions. The ratio of positive cells to total number of cells counted in each field was then calculated.

Determination of Skin Hydroxyproline Content

The collagen content of lesional skin was determined by measuring the concentration of hydroxyproline. Samples of lesional skin from bleomycin-treated wild-type or Smad3^{-/-} mice at day 28 were taken with a 6-mm punch biopsy tool. A minimum of three mice of each genotype contributed to each time point. Hydroxyproline content was determined by phenyltiocarbamyl derivatization and isocratic reverse-phase high-performance liquid chromatography, as described previously.¹⁷ Briefly, samples were digested with papain, hydrolyzed in 6N HCl for 16 hours at 120°C, evaporated to dryness, and the residue dissolved in methanol:water:triethanolamine (2:2:1) and re-dried twice. After derivatization by phenylisothiocyanate, samples were separated isocratically using a reverse phase C18 octadecylsilane column and monitored on an absorbance detector at 254 nm. The mobile phase was composed of acetonitrile:water:140 mmol/L CH₃COONa buffer (6:4:90, v/v/v), pH 6.4 with 0.5 mL/L triethylamine. Purified hydroxyproline was used to establish a standard curve. The results are expressed as μ g of hydroxyproline per biopsy specimen.¹⁷

In Situ Hybridization

To detect the synthesis of collagen by dermal fibroblasts, *in situ* hybridization analysis was performed as described previously.¹⁶ Briefly, samples from lesional skin from PBS or bleomycin-treated wild-type or Smad3^{-/-} mice were fixed in 10% buffered formalin phosphate, embedded in paraffin and sectioned to 6 μ m on ProbeOn Plus slides (Fisher Scientific). The slides were treated with 100% xylene $\times 3$ for 2 minutes to remove paraffin from the sections. Antisense riboprobes specific for the mouse COL1A1 3' untranslated region were labeled with digoxigenin-11-UTP. A riboprobe transcribed in the sense orientation was used as a control. Hybridizations were carried out overnight at 60°C in fresh hybridization buffer containing antisense or sense probes (1 μ g/ml). Immunological detection of the hybridized probes was performed with an anti-digoxigenin antibody conjugated with alkaline phosphatase, and developed with nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) color substrates (Roche Diagnostics, Mannheim, Germany).

Fibroblast Cultures

Primary fibroblast cultures were established from dorsal skin of newborn *Smad3*^{-/-} mice or wild-type littermates as described.¹⁶ Fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% vitamins, 100 U/ml penicillin/streptomycin and 2 mmol/L L-glutamine at 37°C in 5% CO₂ atmosphere, and studied at early passage in parallel. Media were obtained from BioWhittaker (Walkersville, MD), and all other tissue culture reagents from Gibco BRL (Grand Island, NY). For all experiments, fibroblasts established from several wild-type or *Smad3*^{-/-} mice were examined separately, and yielded consistent results. Proliferation of wild-type or *Smad3*^{-/-} fibroblasts *in vitro* was determined by the EZ4U cell proliferation assay (Biomedica, Wien, Austria) according to the manufacturer's instructions. Briefly, 10⁴ cells/well were seeded into 96-well plates in DMEM containing 10% FBS. After allowing fibroblasts to attach overnight, cultures were incubated with 10 ng/ml TGF- β 2 (Genzyme, Framingham, MA). Following incubation of the samples with the chromogenic substance for 2 hours, absorbance at 450 nm was measured in triplicate samples at 0 hours and at various periods for up to 48 hours after addition of TGF- β 2. Results are expressed as means $\pm$ SD of -fold increase in absorbance compared to 0 hours.

The *in vitro* synthesis of collagen and fibronectin was determined by metabolic labeling studies with fibroblasts from wild-type and *Smad3*^{-/-} mice, as described.¹⁸ Briefly, confluent fibroblasts were incubated with 1 or 10 ng/ml TGF- β 2 in DMEM containing 1% FBS, L-ascorbic acid (50 μ g/ml) and β -aminopropionitrile (100 μ g/ml). Twenty-four hours later, 2.5 μ Ci/ml [¹⁴C]-proline (Amersham Pharmacia, Buckinghamshire, UK) was added to the media, and cultures were harvested after a further 24-hour incubation. Supernatants were extensively dialyzed, and labeled proteins secreted into the media were analyzed by electrophoresis in 4 to 20% SDS-polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA) under reducing conditions. After electrophoresis, gels were processed for fluorography and visualized by autoradiography.

Northern and Western Blot Analysis

The expression of mRNA in dermal fibroblasts derived from wild-type and *Smad3*^{-/-} mice was determined by Northern analysis, as described.¹⁹ Confluent fibroblasts were incubated in media with or without TGF- β 2 (10 ng/ml) for up to 48 hours, and total RNA was prepared using RNeasy Mini Kit (Qiagen, Valencia, CA). In selected experiments, 10 μ mol/L of the highly specific ALK5 inhibitor SB431542 or DMSO vehicle was added to cultures for 30 minutes before TGF- β . Toxicity was evaluated by the MTT cell viability assay using the TOX-1 kit (Sigma), according to manufacturer's instructions. Mouse COL1A1, PAI-1, CTGF, *Smad7*, and α -smooth muscle actin cDNA probes labeled with [α -³²P]dCTP using Ready-to-Go DNA Labeling Beads (Amersham Bio-

sciences) were used for hybridizations. Densitometric scans were quantitated with Molecular Analyst software, and results were normalized to the levels of 18S RNA in each sample.

Western analysis of whole cell lysates prepared from confluent fibroblasts was performed as previously described.¹⁹ For immunoblotting, antibodies against α -smooth muscle actin (Sigma), phospho-*Smad2* (Cell Signaling Technology), *Smad2* and *Smad3* (Zymed Laboratories), PAI-1 (Santa Cruz Biotechnology), type I collagen (Southern Biotechnology, Birmingham, AL), or actin (Santa Cruz) were used. Antibody specificities were confirmed with blocking peptides supplied by the manufacturers. Antigenic proteins were detected by enhanced chemiluminescence with Western Blotting Detection Reagent (Amersham Pharmacia Biotech). The relative signal intensities of the bands were quantitated using Molecular Analyst software, and the results were normalized by levels of actin in each sample. In selected experiments, the secretion of TGF- β 1 into the media was determined by ELISA (R & D Systems, Minneapolis, MN) after incubation of confluent cultures of wild-type and *Smad3*^{-/-} fibroblasts with TGF- β 2 (10 ng/ml) for up to 72 hours.

Flow Cytometry of Cultured Fibroblasts

Confluent cultures of dermal fibroblasts from wild-type or *Smad3*^{-/-} mice were trypsinized, washed in flow cytometry buffer, and placed on ice. Fibroblasts were fixed with 1% paraformaldehyde for 20 minutes and permeabilized with 0.2% saponin (Sigma) before staining with specific antibodies against type I collagen (mouse IgG1, Sigma) or Hsp47 (mouse IgG2b, Stressgen Biotechnologies). Bound antibodies were detected with mouse IgG1-PE or mouse IgG2a/2b-FITC (both from BD Biosciences) antibodies using the corresponding isotype controls. Following staining, fibroblasts were washed and fixed with 1% paraformaldehyde. Events were collected on a FACS-Calibur (Becton Dickinson) and data were analyzed using CellQuest software.

Statistical Analysis

Experiments were repeated at least three times. Results are expressed as the means $\pm$ SEM. Mann-Whitney's *U*-test (*in vivo* studies) or a Student's *t*-test (*in vitro* studies) was used for comparison of statistical significance between any two groups.

Results

Bleomycin-Induced Dermal Fibrosis and Collagen Synthesis Is Significantly Reduced in *Smad3*^{-/-} Mice

Because previous studies demonstrated a key role of *Smad3* in mediating TGF- β -induced fibrotic responses *in vitro*, we examined the consequences of *Smad3* ablation

on the development of TGF- β -mediated fibrosis *in vivo*. As we had described earlier in C3H mice,¹⁶ injections of bleomycin for 28 days resulted in dermal fibrosis in young adult wild-type Black Swiss $\times$ 129SVJ mice. Histological examination of lesional skin demonstrated a considerable increase in dermal thickness, with striking accumulation of densely packed parallel collagen bundles and replacement of subcutaneous fat by connective tissue (Figure 1A). In contrast, Smad3^{-/-} mice injected with bleomycin for the same period developed significantly less dermal fibrosis with loosely packed randomly oriented collagen bundles, and the subcutaneous adipose layer was frequently partially preserved. Quantitative analysis showed that at day 28 the thickness of the dermis in bleomycin-injected skin was more than twofold greater than in PBS-injected skin in wild-type mice, while it was only 1.3-fold greater in Smad3^{-/-} mice (Figure 1B).

Masson's trichrome stains collagen fibers blue. As shown in Figure 1C, the dermis in bleomycin-treated mice at day 28 showed dense staining of collagen replacing the normal dermal architecture. In contrast, there was less collagen deposition in Smad3^{-/-} mice treated with bleomycin and fibers appeared to be more loosely arranged (Figure 1C). The accumulation and organization of collagen in the dermis was further examined with Picrosirius red. This stain gives highly cross-linked "mature" collagen fibers in the dermis a strong red birefringence when viewed under polarized light, whereas less cross-linked fibers have a weakly birefringent yellow/orange appearance.²⁰ In wild-type mice the dermis showed weak orange birefringence, and bleomycin treatment for 28 days induced dense accumulation of collagen fibers with strong red birefringence. In contrast, in Smad3^{-/-} mice the appearance of the dermis remained essentially unchanged (Figure 1D). These findings suggest that bleomycin-induced accumulation of a highly cross-linked collagenous matrix in the skin was attenuated in the absence of Smad3.

Next, EM was used to examine the effect of bleomycin on the ultrastructural organization of collagen in the dermis. As shown in Figure 2A (top panels), in both wild-type and Smad3^{-/-} mice treated with vehicle, collagen in the dermis showed a highly uniform pattern of fibril diameter and organization. The development of dermal fibrosis at day 28 was associated with striking alterations in dermal collagen organization in wild-type mice, with accumulation of irregular fibrils of smaller diameter (Figure 2A, bottom panels). A similar preponderance of small diameter (45 to 75 nm) collagen fibrils in the dermis, thought to represent newly synthesized collagen, has been described previously in association with skin fibrosis in Tsk mice²¹ and in bleomycin-treated rats.¹³ In contrast, the dermis from Smad3^{-/-} mice treated with bleomycin showed larger and more uniform distribution of fibril diameter than corresponding fibrils from the dermis of wild-type mice. The size distribution profiles of collagen fibrils from Smad3^{-/-} and wild-type dermis were quantitated by EM. The results are shown in Figure 2B. The average dermal collagen fibril diameter was 89.4 ± 19.8 nm for PBS-treated, and 56.0 ± 29.3 nm for bleomycin-treated wild-type mice; in Smad3^{-/-} mice the cor-

responding values were 75.7 ± 15.4 nm for PBS, and 77.9 ± 19.4 nm for bleomycin. Collagen fibrils smaller than 75 nm in diameter were rare in Smad3^{-/-} dermis. These results indicate that in the absence of Smad3, bleomycin-induced alterations in dermal collagen ultrastructure characteristic of fibrotic skin were largely prevented.

As a quantitative measure of collagen accumulation, the hydroxyproline content of the dermis was determined. The results indicated that in wild-type mice, histological evidence of dermal fibrosis at day 28 was associated with a greater than twofold increase in skin collagen content compared to PBS-injected skin (Figure 3). In contrast, the increase in dermal collagen accumulation induced by bleomycin was only 1.35-fold in Smad3^{-/-} mice. The molecular chaperone Hsp47 is closely linked to collagen synthesis and intracellular processing in fibroblasts, and Hsp47 expression identifies collagen-producing cells.²² Flow cytometric analysis indicated that the intracellular levels of type I collagen correlated with Hsp47 levels in both wild-type and Smad3^{-/-} mice (see below, Figure 7B). Examination of Hsp47 expression by immunohistochemistry showed that in parallel with increased accumulation of collagen, the number of Hsp47-positive fibroblasts in the lesional dermis was markedly increased in bleomycin-treated wild-type mice. In contrast, the induction of Hsp47 expression was attenuated in Smad3^{-/-} mice (Figure 4A).

To compare directly the stimulation of type I collagen synthesis in dermal fibroblasts induced by bleomycin *in vivo*, COL1A1 mRNA expression was examined by *in situ* hybridization, using digoxigenin-11-UTP-labeled riboprobes specific for mouse COL1A1. The results indicated that while the dermis of vehicle-treated mice contained only a small number of widely dispersed fibroblasts positive for COL1A1 mRNA, the numbers were markedly elevated in bleomycin-injected lesional skin (Figure 4B). However, the increased accumulation of COL1A1-positive fibroblasts elicited by bleomycin was lower in the dermis of Smad3^{-/-} mice compared to wild-type mice. To quantitate the results from ISH assays, the proportion of fibroblasts that were COL1A1-positive was calculated. The results showed that bleomycin injection induced a comparable 30% increase in the numbers of fibroblasts in lesional dermis from both Smad3^{-/-} and wild-type mice. The percentage of COL1A1-positive fibroblasts was increased threefold in the wild-type mice but only 59% in the Smad3^{-/-} mice (Figure 4B, bottom panel).

Myofibroblasts play key roles in the pathogenesis of fibrosis, and their numbers are increased in the lesional dermis in patients with scleroderma and in bleomycin-induced dermal fibrosis in mice.^{23,24} To compare the accumulation of myofibroblasts in the skin in mice with the two genotypes, the expression of α -smooth muscle actin, a marker of the myofibroblast phenotype, was determined by immunohistochemistry. The results showed that at day 28 of bleomycin injections, α -smooth muscle actin expression in spindle-shaped fibroblastic cells in the dermis was substantially lower in Smad3^{-/-} mice compared to wild-type controls (Figure 4C). Quantitation of the results revealed that in bleomycin-injected wild-

Figure 1. Loss of *Smad3* reduces bleomycin-induced dermal fibrosis. Wild-type mice (**a–c**) and *Smad3*^{-/-} mice (**d–f**) received daily subcutaneous injections of PBS (**a** and **d**) or bleomycin (**b**, **c**, **e**, **f**). **A:** Lesional skin at day 28 was examined by hematoxylin and eosin stain. Bars in top and middle panels, 200 μ m; bottom panels, 20 μ m. **B:** Dermal thickness at day 7 or day 28 was determined at five randomly selected sites/field. Each treatment group includes at least eight mice. Results are expressed as the means $\pm$ SEM. *, $P < 0.01$. **C:** Skin sections were stained by Masson's trichrome; or **D,** Picrosirius red, and examined under polarized light. Bars in top and middle panels, 200 μ m; bottom panel, 50 μ m.

Figure 2. Altered dermal collagen fibril ultrastructure in Smad3^{-/-} mice. **A:** Lesional skin from wild-type mice (**a** and **b**) and Smad3^{-/-} mice (**c** and **d**) treated for 28 days with PBS (**a** and **c**) or bleomycin (**b** and **d**) were examined in transverse sections by electronmicroscopy. Note the presence of irregularly shaped small diameter fibrils in **b**. Bars, 250 nm. **B:** Quantification and distribution of individual collagen fibril diameters. The frequencies of collagen fibrils with a given diameter are shown in these histograms. Diameters of at least 200 fibrils were measured in eight different fields in each sample from three mice for each treatment group. **Left**, wild-type mice; **right**, Smad3^{-/-} mice.

type mice, 59% of the fibroblasts were positive for α -smooth muscle actin, compared to 23% in the Smad3^{-/-} mice ($P < 0.001$). These findings raised the possibility that bleomycin-induced differentiation of dermal fibroblasts into myofibroblasts, or migration of myofibroblasts into the lesional dermis, was dependent on Smad3-mediated mechanisms.

Smad3 has been implicated in the regulation of cell-cycle progression induced by TGF- β .⁷ Therefore, we examined whether reduced proliferation of dermal fibroblasts *in vivo* contributed to the reduction in skin fibrotic response observed in the Smad3^{-/-} mice. For this purpose, sections of lesional tissue were stained with antibody to PCNA, a marker for mitotically active cells in tissue sections. The results showed that there was a very low rate of proliferation of spindle-shaped fibroblastic cells in the dermis, and the number of PCNA-positive cells at 4 weeks of bleomycin injections was not significantly different in wild-type and Smad3^{-/-} mice (Table 1). Taken together, these results indicated that whereas bleomycin induced the development of dermal fibrosis in both wild-type and Smad3^{-/-} mice, the relative increase in dermal thickness, collagen accumulation and deposition, and type I collagen mRNA expression by lesional fibroblasts *in situ*, as well as accumulation of α -smooth

Figure 3. Reduced dermal collagen accumulation in Smad3^{-/-} mice. Smad3^{-/-} mice and wild-type littermates received subcutaneous injection of PBS or bleomycin for 28 days. Hydroxyproline content in 6-mm punch biopsy specimens from lesional skin was determined. Results represent the means $\pm$ SEM from three to four mice of each genotype, expressed as μ g hydroxyproline/sample. *, $P < 0.05$; **, $P < 0.01$.

muscle actin-positive myofibroblasts, were substantially attenuated in the absence of Smad3.

Bleomycin-Induced Dermal Inflammation in Smad3^{-/-} Mice

We have shown previously that injections of bleomycin induced an early and transient inflammatory response in the dermis.¹⁶ The dermal infiltrate, composed predominantly of macrophages, precedes, and is likely to play an important role in, the development of fibrosis in this model. Because the observed attenuation of the bleomycin-induced fibrotic response in Smad3^{-/-} mice may be due to reduced intensity or altered cellular composition of the infiltrate, the inflammatory response in the lesional dermis was examined at early time points. The results showed that at day 7, the number of infiltrating round cells/unit area in the dermis was increased by bleomycin to a comparable degree (fourfold) in both wild-type and Smad3^{-/-} mice (Figure 5A). Furthermore, no consistent differences in the numbers of Mac3-positive macrophages could be found by immunohistochemistry in lesional dermis (Figure 5B, and data not shown). These results indicate that bleomycin-induced dermal inflammatory response was similar in wild-type and Smad3^{-/-} mice. The observed attenuation of skin fibrosis in Smad3^{-/-} mice was therefore unlikely to be due to reduced inflammation in the skin.

Both TGF- β and CTGF induce profibrotic responses *in vivo* and *in vitro*, and their tissue levels are markedly

Table 1. Fibroblast Proliferation in Lesional Dermis

Experimental group		Number of proliferating fibroblasts
Wild-type mice	PBS	0.20 ± 0.15
	bleomycin	2.40 ± 0.30
Smad3 ^{-/-} mice	PBS	0.20 ± 0.15
	bleomycin	2.80 ± 0.28

Sections from each experimental group at 4 weeks were stained with antibody to PCNA, and five fields were examined to quantify proliferating spindle-shaped fibroblastic cells in the lesional dermis. Results are means ± S.D.

elevated in fibrosis. While normally macrophages are a predominant source of TGF- β , in fibrotic tissues resident fibroblasts also contribute. The principal stimulus for the production of TGF- β and CTGF in normal fibroblasts is TGF- β , and these responses *in vitro* were reported to be Smad3-dependent.^{11,25} Therefore, we used immunohistochemistry to examine whether loss of Smad3 modulated the expression of the fibrogenic cytokines in lesional skin *in vivo*. The results showed that both CTGF and TGF- β were highly up-regulated in the skin from bleomycin-treated wild-type mice, consistent with earlier reports.¹⁴ Elevated expression of TGF- β was early and transient (data not shown), whereas CTGF persisted for at least 4 weeks. However, in contrast to TGF- β , whose expression in the lesional dermis at day 7 was comparable in both wild-type and Smad3^{-/-} mice (data not shown), CTGF expression at day 28 appeared to be considerably attenuated in Smad3^{-/-} mice (Figure 5C).

Fibroblast from Smad3^{-/-} Mice Show Reduced Fibrotic Responses in Vitro

To investigate the mechanisms that may be responsible for attenuation of bleomycin-induced fibrosis in the presence of disrupted TGF- β /Smad3 signaling, primary dermal fibroblasts derived from newborn wild-type and Smad3^{-/-} mice were studied *in vitro*. First, early passage fibroblasts were seeded at low density in media containing 10% FBS in the absence or presence of TGF- β 2, and proliferation rates were determined at various time points, as described under Materials and Methods. The results of triplicate determinations showed that the number of wild-type fibroblasts increased throughout the incubation period, and TGF- β significantly enhanced their rate of proliferation (Figure 6A, and data not shown). In contrasts, fibroblasts from Smad3^{-/-} mice showed delayed

Figure 4. *In situ* COL1A1 mRNA expression in fibrotic skin. **A:** Expression of Hsp47 intracellular collagen chaperone in lesional dermis from bleomycin-treated wild-type (**a**) or Smad3^{-/-} mice (**b**) examined by immunohistochemistry. **Bar**, 25 μ m. **B:** Sections from wild-type (**a** and **b**) or Smad3^{-/-} mice (**c** and **d**) injected with PBS (**a** and **c**) or bleomycin (**b** and **d**) for 28 days were hybridized with antisense COL1A1 probes. **Top** panels show representative photomicrographs. **Arrows** indicate COL1A1 mRNA-positive fibroblasts. **Bars**, 50 μ m. In the **bottom** panel, the proportion of COL1A1-positive fibroblasts was quantitated by counting at least five fields from three mice in each group. The results indicate the means ± SEM. **C:** Expression of the myofibroblast marker α -smooth muscle actin in PBS-treated (**a** and **c**) or bleomycin-treated (**b** and **d**) lesional dermis from wild-type (**a** and **b**) or Smad3^{-/-} mice (**c** and **d**) examined by immunohistochemistry. **Bars**, 50 μ m; **inset**, 25 μ m.

Figure 5. Bleomycin-induced inflammation in wild-type and Smad3^{-/-} mice. **A:** Skin samples from bleomycin-treated wild-type mice (**a**) and Smad3^{-/-} mice (**b**) were harvested at day 7. Sections were stained with hematoxylin and eosin. **Bars**, 20 μ m. **B:** Sections from wild-type (**a** and **b**) or Smad3^{-/-} mice (**c** and **d**) injected with PBS (**a** and **c**) or bleomycin (**b** and **d**) for 7 days were incubated with antibody against Mac-3 to identify macrophages. **C:** Sections from wild-type (**a** and **b**) or Smad3^{-/-} mice (**c** and **d**) injected with PBS (**a** and **c**) or bleomycin (**b** and **d**) for 28 days were incubated against CTGF (day 28). **Bars**, 50 μ m; **inset**, 25 μ m.

proliferation, and TGF- β failed to stimulate proliferation at any time point. These results suggest that endogenous TGF- β may play a role in driving basal proliferation in normal fibroblasts, and loss of Smad3 disrupts both autocrine growth stimulation and responses to exogenous TGF- β .

Figure 6. Reduced TGF- β responses in fibroblasts from Smad3^{-/-} mice. Cultures of dermal fibroblasts established from newborn wild-type and Smad3^{-/-} mice were maintained in parallel. **A:** Fibroblasts (10^4 cells/well) were seeded in media with (**closed bars**) or without (**open bars**) TGF- β 2 (10 ng/ml). Proliferation was determined by EZ4U assay, and results are expressed as -fold increase in absorbance at 48 hours compared to time 0. **Bars** represent the means $\pm$ SD of triplicate determinations. *, $P < 0.0001$. **B:** Confluent fibroblasts were incubated with TGF- β 2, followed by metabolic labeling. Labeled proteins secreted into the media were analyzed in triplicate samples by SDS-PAGE. A representative autoradiogram is shown.

Metabolic labeling was used to assess the effect of TGF- β on the synthesis of collagen and fibronectin. For this purpose, wild-type and Smad3^{-/-} fibroblasts at confluence were incubated with TGF- β 2, and labeled with 14 C-proline for the last 24 hours. Media were then harvested, and non-dialyzable macromolecules in equal aliquots of supernatants were analyzed by SDS-PAGE. The results showed that TGF- β induced a dose-dependent increase in the levels of type I collagen and fibronectin secreted into the media (Figure 6B). Quantitative analysis revealed that TGF- β (10 ng/ml) induced a 2.1-fold increase in collagen production in wild-type fibroblasts, but only a $\sim$ 1.6-fold increase in Smad3^{-/-} fibroblasts; and a corresponding 5.6-fold *versus* 2.4-fold increase in fibronectin synthesis. Western blot analysis of whole cell lysates from wild-type and Smad3^{-/-} fibroblasts were used to further evaluate the effect of TGF- β on the levels of selected proteins. As shown in a representative immunoblot in Figure 7A, the increase in levels of cellular and secreted type I collagen induced by TGF- β at 48 hours (1.85-fold and 1.7-fold, respectively), and 1.7-fold induction of cellular PAI-1, were almost completely abrogated in the Smad3^{-/-} fibroblasts; in contrast, the 1.75-fold induction of α -smooth muscle actin was only minimally and inconsistently altered.

The regulation of Hsp47 expression was examined by flow cytometry. The results, representing the mean fluorescence intensities, indicated that while TGF- β induced 1.8-fold increase in Hsp47 protein levels in wild-type fibroblasts, in Smad3^{-/-} fibroblasts there was no stimu-

Figure 7. Attenuated TGF- β responses in Smad3^{-/-} fibroblasts. Confluent dermal fibroblasts from Smad3^{-/-} mice and wild-type littermates were incubated with TGF- β 2 for up to 48 hours. At the end of the indicated incubation periods, cultures were harvested. **A:** Whole cell lysates were subjected to Western blot analysis (left). Type I collagen levels in cell lysates and culture media were quantitated at 24 hours (right). Representative Western blots are shown. **B:** For flow cytometry, fibroblasts from wild-type mice (left) and Smad3^{-/-} mice (right) were stained with antibodies against Hsp47, or double-stained with antibodies against Hsp47 and type I collagen. Dotted lines, isotype control; continuous lines, untreated; dashed lines, TGF- β -treated fibroblasts. **C:** Total RNA isolated at indicated time points was examined by Northern blot analysis. Membranes were re-hybridized with the indicated mouse cDNA probes. A representative autoradiogram is shown. **D:** Following the indicated periods of incubation with TGF- β 2, secreted TGF- β 1 in the media was quantitated by ELISA. Results from a representative experiment are shown. Bars represent the means from triplicate determinations.

lation (Figure 7B, top panels). Double-staining revealed a close correlation between type I collagen and Hsp47 expression levels in both wild-type and Smad3^{-/-} fibroblasts, as indicated by the positions of the cells on the dot plots (Figure 7B, bottom panels). The regulation of ECM gene expression by TGF- β was further examined by Northern blot analysis. The results showed that COL1A1, PAI-1 and CTGF mRNA expression induced by TGF- β in wild-type fibroblasts was markedly attenuated in Smad3^{-/-} fibroblasts, and α -smooth muscle actin mRNA stimulation was moderately reduced (Figure 7C).

The inhibitory member of the Smad family, Smad7 blocks Smad2 and Smad3-mediated TGF- β responses by recruiting Smurfs to the type I TGF- β receptor, resulting in receptor ubiquitination and degradation.²⁶ In normal human skin fibroblasts, Smad7 behaves as an early

response gene whose expression is rapidly and transiently induced by TGF- β .²⁷ The present results demonstrated that Smad7 mRNA levels were rapidly induced by TGF- β in both wild-type and Smad3^{-/-} fibroblasts (Figure 7C). The induction of Smad7 mRNA was transient in wild-type fibroblasts, but appeared to persist for at least 24 hours in Smad3^{-/-} fibroblasts. TGF- β is known to induce its own gene expression.²⁸ To compare TGF- β autoinduction, the concentration of TGF- β 1 in the culture media from wild-type and Smad3^{-/-} fibroblasts was examined in parallel by ELISA. The results showed that whereas TGF- β 2 induced a time-dependent stimulation of TGF- β 1 secretion, basal levels of secreted TGF- β 1 were 50% lower, and TGF- β 2-induced stimulation at 72 hours was markedly abrogated (3.0-fold versus 8.5-fold), in Smad3^{-/-} fibroblasts (Figure 7D).

Activation of Smad2 in Smad3^{-/-} Fibroblasts

The observation that TGF- β retained its ability to stimulate *in vitro* gene expression in Smad3^{-/-} fibroblasts, albeit with a substantially reduced magnitude, suggested that intracellular signal transduction mechanisms distinct from Smad3 may be involved in mediating some of these responses. Despite their striking amino acid conservation, Smad2 and Smad3 have largely non-overlapping biological functions. Nevertheless, under some circumstances, Smad2 may possibly compensate for lack of Smad3. To investigate the regulation of Smad2 and its potential role in dermal fibrosis in the Smad3^{-/-} mice, the activation of Smad2 in lesional skin was examined by immunohistochemistry using an antibody specifically recognizing the phosphorylated form of Smad2. We and others have shown previously that distribution of phospho-Smad2 reliably identifies TGF- β target tissues in the mouse.^{16,29} The results indicated that bleomycin-induced fibrosis at 28 days was associated with a marked increase in phospho-Smad2 levels in dermal fibroblasts in both wild-type and Smad3^{-/-} mice (Figure 8A). The tissue levels of total Smad2 were similar in wild-type and Smad3^{-/-} mice, and inhibitory Smad7 was almost undetectable in dermal cells; these levels were not altered with bleomycin treatment (data not shown).

To compare Smad2 activation, TGF- β -induced phosphorylation of cellular Smad2 *in vitro* was examined in fibroblasts from wild-type or Smad3^{-/-} mice by immunoblot analysis of whole cell lysates. As shown in Figure 8B, neither full-length nor truncated Smad3 protein could be detected in Smad3^{-/-} fibroblasts using an antibody raised against the Smad3 linker region. The results confirmed the specificity of the anti-phospho-Smad2 antibody for the phosphorylated form of Smad2, and indicated that Smad2 levels and TGF- β -induced rapid Smad2 phosphorylation were similar in wild-type and Smad3^{-/-} fibroblasts (Figure 8B).

To further investigate the potential involvement of Smad2 in mediating TGF- β responses in Smad3^{-/-} fibroblasts, we took advantage of a novel inhibitor of ALK5-mediated TGF- β signaling.³⁰ In normal fibroblasts, SB431542 prevents TGF- β -induced phosphorylation and nuclear import of endogenous Smad2/3 by selectively blocking ALK5 activation (Y. Mori, I. Wataru, J. Varya, manuscript submitted). Confluent cultures were pre-incubated with SB431542 (10 μ M/L) for 30 minutes, followed by addition of TGF- β 2. The results showed that pre-incubation with SB431542 completely abrogated TGF- β -induced expression of PAI-1 and CTGF mRNAs transcripts. Importantly, the inhibitory effect of SB431542 was identical in wild-type and Smad3^{-/-} fibroblasts (Figure 8C). Importantly, the induction of α -smooth muscle actin mRNA expression was also prevented by the ALK5 inhibitor in fibroblasts of both genotypes. No evidence of cell toxicity due to SB431542 was detected. These findings suggested that the residual transcriptional responses elicited by TGF- β in Smad3^{-/-} fibroblasts involved ALK5, and were therefore possibly mediated through activation of Smad2.

Figure 8. Smad2 activation in Smad3^{-/-} dermal fibroblasts. **A:** Confluent fibroblasts from Smad3^{-/-} mice and wild-type littermates were incubated with TGF- β 2 (10 ng/ml) for the indicated periods. Whole cell lysates were subjected to Western blot analysis. **B:** Sections of lesional skin from wild-type mice (**a** and **b**) and Smad3^{-/-} mice (**c** and **d**) treated in parallel with PBS (**a** and **c**) or bleomycin (**b** and **d**) for 28 days were examined by immunohistochemistry using antibody against phospho-Smad2. Bars, 50 μ m; inset, 25 μ m. **C:** Confluent fibroblasts were pre-incubated with the ALK5 inhibitor SB431542 (10 μ M/L) for 30 minutes, followed by TGF- β 2 for 24 hours. Expression of mRNA was determined by Northern analysis. Representative autoradiogram is shown.

Discussion

The cellular and molecular mechanisms underlying the development of fibrosis in scleroderma remain poorly understood.³¹ While multiple cytokines have been implicated in the process, TGF- β is considered to play a pivotal role. The expression of TGF- β , and of its cellular receptors, is elevated in lesional scleroderma tissue and in cultured scleroderma fibroblasts.³² Animal models of scleroderma provide additional compelling support for the fundamental role of TGF- β in initiating and sustaining the fibrotic response.^{14,33,34} The phenotypic alterations characteristic of scleroderma fibroblasts correspond closely to the changes induced in normal fibroblasts activated by TGF- β .³⁵ In particular, TGF- β provides a strong stimulus for overexpression of collagen, the predominant structural component of skin, and other components of the ECM; as well as for the synthesis of CTGF and autoinduction of TGF- β . Each of these TGF- β responses contributes to the development of the fibrotic scar in target organs.

Extracellular blockade of the TGF- β signaling axis, involving interference at the level of the ligand or of its receptor, represents a potential molecular strategy to ameliorate the fibrotic process in scleroderma. The feasibility of this approach is amply supported by experimental results from animal models of scleroderma and other forms of fibrosis.³⁶ An alternate strategy for disrupting TGF- β -mediated fibrotic processes involves inhibitors specifically targeting downstream TGF- β signal transduction pathways. Targeting of individual intracellular mediators could permit selective blockade of pathological TGF- β responses such as fibrosis, without compromising physiologically important TGF- β responses. A multiplicity of downstream pathways have been shown to be activated by TGF- β , and they form a complex signaling network with extensive cross-talk. The specific roles of individual pathways, their overlapping functions and interdependence, and their relative significance in physiological TGF- β responses remain poorly understood.¹²

The Smad signal transduction pathways are crucial in mediating several TGF- β responses in fibroblasts. Important Smad-dependent profibrotic activities of TGF- β include stimulation of collagens,^{26,37,38} α -smooth muscle actin,^{39,40} TIMP-1,⁴¹ CTGF,²⁴ and PAI-1.⁴² These transcriptional responses appear to be mediated predominantly through Smad3, whereas the role of the closely related Smad2 remains less well-defined.^{11,40,41} Recent studies have revealed evidence of abnormal Smad activation and regulation in murine models of scleroderma,¹⁶ and in scleroderma fibroblasts.^{19,43} Together with similar findings from animal models of renal, hepatic, and lung fibrosis,⁴⁴ these observations highlight the significant role of Smad signaling in the pathogenesis of the fibrosis in scleroderma.

TGF- β is a potent chemoattractant for monocytes.⁴⁵ Previous reports indicated that compared to wild-type mice, injury in Smad3^{-/-} mice was associated with reduced mononuclear cell infiltration and local TGF- β expression in target tissues, implicating Smad3 in the influx of inflammatory cells. Inflammation in these studies was

induced by skin wounding,¹⁰ radiation,⁴⁶ hyperglycemia,⁴⁷ or unilateral ureteral obstruction.⁴⁸ Interestingly, whereas macrophage migration into wound beds was markedly reduced in Smad3^{-/-} mice,⁴⁶ no differences were found in similar wounds made in irradiated skin.⁴⁹ The present results indicate that bleomycin injections in the skin elicited a comparable degree of intense macrophage accumulation and local TGF- β production in wild-type and Smad3^{-/-} mice. Similarly, bleomycin-induced lung fibrosis was also preceded by robust monocytic inflammation in Smad3^{-/-} mice.⁹ These observations indicate that bleomycin- (or radiation) induced tissue injury elicits a local inflammatory response in the skin and the lungs that is largely independent of Smad3-mediated TGF- β signaling. The inflammatory response may instead involve the production of signals other than TGF- β that can recruit macrophages. As discussed below, a potential candidate responsible for mediating this response is MCP-1, a C-C chemokine that is directly induced by bleomycin and acts as a potent macrophage chemoattractant. Together, these findings suggest that attenuation of the bleomycin-induced fibrotic process in Smad3^{-/-} mice results not from lack of inflammation, but rather, the resistance of Smad3-deficient lesional fibroblasts to the stimulatory activities of TGF- β .

The present results showed that Smad3^{-/-} mice survived into adulthood, and although at 6 weeks of age were smaller than their wild-type littermates, they generally showed no sign of wasting syndrome during these experiments. Furthermore, in contrast to TGF- β 1^{-/-} mice that developed severe multi-focal inflammatory disease with progressive lymphocyte infiltration in most organs, no spontaneous inflammation was observed. The loss of Smad3 was associated with attenuated skin fibrosis induced by bleomycin, with reduced thickness of the dermis and collagen accumulation in the lesional skin, and reduced transcription of collagen gene in dermal fibroblasts *in vivo*. Furthermore, in cultured Smad3^{-/-} dermal fibroblasts, TGF- β stimulation of collagen gene expression, as well as that of PAI-1 and Hsp47, was markedly reduced *in vitro*. These observations are consistent with dependence of these responses on cellular Smad3, as shown previously in adult skin fibroblasts using an antisense oligonucleotide approach.²⁷ Whereas TGF- β -induced modest stimulation of dermal fibroblast proliferation was attenuated in cultured Smad3^{-/-} fibroblasts, after 4 weeks of bleomycin injections the number of proliferating fibroblastic cells in the lesional dermis did not appear to differ significantly in wild-type and Smad3^{-/-} mice, as determined by PCNA immunostaining. Picrosirius red staining of the skin revealed altered collagen matrix architecture in Smad3^{-/-} mice, suggesting decreased formation of fibril cross-links induced by bleomycin. Because fibrosis of the skin is characterized not only by accumulation of fibrillar collagen, but also its cross-linking and organization, the alterations noted in Smad3^{-/-} mice may have significant effects on the mechanical and elastic properties of fibrotic skin.^{50,51}

Expression of TGF- β in the lesional skin, a relatively early event in bleomycin-induced scleroderma¹⁴ was reduced in Smad3^{-/-} mice, despite a comparable macro-

phage response. The synthesis of TGF- β is induced by TGF- β (autoinduction), as well as by MCP-1, in resident skin fibroblasts, and endogenous TGF- β plays an important role in sustaining fibrotic signaling. The results from *in vitro* studies demonstrated that TGF- β autoinduction was markedly reduced in Smad3^{-/-} fibroblasts *in vitro*, consistent with previous observations.¹¹ Together, these results implicate Smad3 in autoregulation of TGF- β 1 in fibroblasts. Like TGF- β , CTGF is also expressed in fibrotic tissue, and is involved in amplifying TGF- β -induced fibrotic responses. The present results indicated that bleomycin-induced CTGF expression was lower in Smad3^{-/-} mice, and stimulation of CTGF mRNA by TGF- β was reduced in Smad3^{-/-} fibroblasts *in vitro*. Diminished levels of CTGF may account in part for attenuation of bleomycin-induced fibrosis in Smad3^{-/-} mice. These findings are consistent with the notion that stimulation of CTGF by TGF- β , its most potent and physiologically relevant inducer, is Smad3-dependent.²⁵

Accumulation and persistence of myofibroblasts in lesional tissues is a consistent finding in pathological fibrosis, and is thought to contribute to pathogenesis.⁵² By inducing the expression of α -smooth muscle actin and its incorporation into cellular stress fibers, TGF- β enhances the transdifferentiation of fibroblasts into myofibroblasts *in vitro*.⁴⁰ A recent study demonstrated that overexpression of Smad2, but not Smad3, in normal fibroblasts resulted in TGF- β -independent myofibroblast differentiation *in vitro*.⁴⁰ The present results indicate that whereas accumulation of α -smooth muscle actin-positive fibroblasts *in vivo* was clearly reduced in Smad3^{-/-} mice, TGF- β stimulation of α -smooth muscle actin expression was intact in cultured Smad3^{-/-} fibroblasts. Supporting the notion that α -smooth muscle actin expression is a Smad3-independent response are recent reports indicating that in embryonic or primary dermal fibroblasts or hepatic stellate cells, Smad3 may be sufficient, but is not necessary for this process.^{46,53-55} Together, these observations suggest that the relative paucity of lesional myofibroblasts noted in Smad3^{-/-} mice may have resulted from their reduced chemotaxis, rather than altered transdifferentiation.

Smad7 is an early response gene whose expression is rapidly and transiently stimulated by TGF- β . In cultured cells, the regulation of Smad7 was shown to be mediated through Smad3, as well as through MAP kinase pathways.⁵⁶ Previous studies using fibroblasts derived from Smad3^{-/-} and Smad2^{-/-} mouse embryos found that TGF- β -induced Smad7 mRNA expression was either attenuated in the absence of Smad3,¹¹ or was intact.⁵⁷ The present results with dermal fibroblasts from newborn mice indicated a comparable 2- to 3-fold early increase in Smad7 mRNA levels in wild-type and Smad3^{-/-} cells.

The ability of TGF- β to stimulate gene expression *in vitro* in Smad3^{-/-} fibroblasts suggested a role for Smad3-independent signal transduction mechanisms mediating these responses. In particular, Smad2 is a receptor-regulated Smad expressed in dermal fibroblasts that binds directly to the ALK5 type I TGF- β receptor and is activated by TGF- β with kinetics similar to Smad3. Although these two proteins are 91% identical in

their amino acid sequence and are expressed ubiquitously, they show largely non-overlapping functions, with Smad2 most strongly identified with regulation of developmental responses. An insertion of 30 amino acids in its MH1 domain prevents Smad2 from directly binding to DNA.⁵⁸ An alternately spliced variant of Smad2 that lacks exon 3 can bind to DNA, and function as a signal transducer. This short-form Smad2 variant may possibly compensate for the loss of Smad3 in mediating selected TGF- β responses in Smad3^{-/-} fibroblasts.¹¹

In addition to TGF- β , multiple growth factors, cytokines and chemokines, have also been implicated in the pathogenesis of fibrosis and scleroderma.³² The MCP chemokines are potent chemoattractants for monocytes, and have a profibrotic effect by directly stimulating collagen synthesis and endogenous TGF- β production.⁵⁹ The expression of MCP-1 and MCP-3, and of their receptors, was up-regulated in lesional dermis in patients with scleroderma, as well as in mice with spontaneous or bleomycin-induced scleroderma.⁶⁰⁻⁶² We have previously reported that mice with targeted deletion of MCP-1 show impaired wound healing and reduced collagen synthesis in the skin.⁶³ Thus, MCP-1 may be one of the Smad3-independent factors accounting for dermal inflammation, as well as some of the subsequent profibrotic responses, in Smad3^{-/-} mice. Activin A, a member of the TGF- β superfamily, activates Smad2 and Smad3 through the ALK4 receptor. Activin A is up-regulated in wound healing, and its overexpression in the skin in transgenic mice results in dermal fibrosis.⁶⁴ In light of these observations, MCP-1 and Activin A may be involved in mediating the modest fibrotic process elicited by bleomycin in Smad3^{-/-} mice. Studies to address these possibilities are in progress.

Together, the present results demonstrate the bleomycin-induced scleroderma was significantly ameliorated in mice with targeted deletion of Smad3. In the absence of Smad3, the severity of key parameters of fibrosis, including collagen accumulation and deposition and myofibroblast accumulation in the dermis was reduced, despite a robust early inflammation elicited by bleomycin. Reduced dermal fibrosis could not be attributed to reduced proliferation of lesional fibroblasts *in vivo*. *In vitro* studies with fibroblasts derived from Smad3^{-/-} mice established that TGF- β responses important in the fibrogenic process, including stimulation of collagen, PAI-1 and Hsp47 gene expression, and induction of CTGF and autoinduction, were impaired in these cells. Thus, despite the multiplicity of cellular signaling events activated by TGF- β , disruption of a single pathway had a marked effect on the fibrotic process. Because bleomycin-induced scleroderma in the mouse shares key cellular and biochemical features of human scleroderma, including the fundamental role of TGF- β , the present results raise the possibility that selective targeting of Smad3 may provide a novel approach to the treatment of skin fibrosis.

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