Smad3 Signaling Critically Regulates Fibroblast Phenotype and Function in Healing Myocardial Infarction

Marcin Dobaczewski, Marcin Bujak, Na Li, Carlos Gonzalez-Quesada, Leonardo H. Mendoza, Xiao-Fan Wang, Nikolaos G. Frangogiannis

Rationale: Cardiac fibroblasts are key effector cells in the pathogenesis of cardiac fibrosis. Transforming growth factor (TGF)-β/Smad3 signaling is activated in the border zone of healing infarcts and induces fibrotic remodeling of the infarcted ventricle contributing to the development of diastolic dysfunction.

Objective: The present study explores the mechanisms responsible for the fibrogenic effects of Smad3 by dissecting its role in modulating cardiac fibroblast phenotype and function.

Methods and Results: Smad3 null mice and corresponding wild-type controls underwent reperfused myocardial infarction protocols. Surprisingly, reduced collagen deposition in Smad3−/− infarcts was associated with increased infiltration with myofibroblasts. In vitro studies demonstrated that TGF-β1 inhibited murine cardiac fibroblast proliferation; these antiproliferative effects were mediated via Smad3. Smad3−/− fibroblasts were functionally defective, exhibiting impaired collagen lattice contraction when compared with wild-type cells. Decreased contractile function was associated with attenuated TGF-β–induced expression of α-smooth muscle actin. In addition, Smad3−/− fibroblasts had decreased migratory activity on stimulation with serum, and exhibited attenuated TGF-β1–induced upregulation of extracellular matrix protein synthesis. Upregulation of connective tissue growth factor, an essential downstream mediator in TGF-β–induced fibrosis, was in part dependent on Smad3. Connective tissue growth factor stimulation enhanced extracellular matrix protein expression by cardiac fibroblasts in a Smad3-independent manner.

Conclusions: Disruption of Smad3 results in infiltration of the infarct with abundant hypofunctional fibroblasts that exhibit impaired myofibroblast transdifferentiation, reduced migratory potential, and suppressed expression of fibrosis-associated genes. (Circ Res. 2010;107:418-428.)

Key Words: fibroblasts • myocardial infarction • transforming growth factor-β, growth factors • fibrosis

Activated fibroblasts are critically involved in both reparative and fibrotic processes. In normal tissues, resident fibroblasts are quiescent, producing limited amounts of extracellular matrix proteins and exhibiting few actin-associated cell-matrix and cell-cell contacts.1 Tissue injury triggers alterations in the mechanical microenvironment and activates cytokine and growth factor–mediated pathways resulting in differentiation of fibroblasts into myofibroblasts.2,3 Phenotypically modulated cells characterized by the presence of a microfilamentous contractile apparatus enriched with α-smooth muscle actin (α-SMA). In the healing wound, activated myofibroblasts are the main source of extracellular matrix proteins.4,5 Release of biologically active transforming growth factor (TGF)-β1 plays an important role in activation of fibroblasts in wound repair, both by promoting myofibroblast transdifferentiation and by enhancing synthesis of extracellular matrix proteins.6,7 TGF-β1 exerts many of its effects through a cascade of intracellular effectors, the Smads.8 Extensive evidence suggests that activation of the Smad2/3 cascade plays an essential role in extracellular matrix protein gene expression9 and regulates fibrous tissue deposition in a variety of experimental models.10,11 Smad3 deficiency attenuates collagen deposition in a model of bleomycin-induced pulmonary fibrosis12 and protects from the development of renal fibrosis after ureteral obstruction.13 Experiments from our laboratory have demonstrated a critical role for Smad3 signaling in fibrotic remodeling of the infarcted heart.14 Smad3 null mice had significantly reduced collagen deposition in the scar and in the remodeling noninfarcted ventricle and exhibited reduced dilative remodeling and attenuated diastolic dysfunction after myocardial infarction.15 Although Smad3 is essential for the development of cardiac fibrosis,
the mechanisms responsible for its profibrotic actions have not been systematically investigated.

Our current study examines the effects of Smad3 gene disruption on phenotype and function of cardiac fibroblasts. We found that Smad3 absence results in infiltration of the infarcted heart with a large number of cells that exhibit impaired functional properties and defective matrix synthetic capability. Smad3 null fibroblasts showed impaired myofibroblast transdifferentiation, reduced migratory potential, and reduced capacity to contract collagen pads on TGF-β1 stimulation. Upregulation of the essential TGF-β-induced fibrogenic mediator connective tissue growth factor (CTGF) in the infarcted myocardium was in part dependent on Smad3. Our findings suggest that Smad3 gene disruption abrogates the antiproliferative effects of TGF-β1 in the infarcted heart and results in accumulation of abundant but functionally defective fibroblasts attenuating adverse fibrotic remodeling of the infarcted ventricle.

Methods

A detailed description of the methods is provided in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

All animal studies were approved by the animal protocol review committee at Baylor College of Medicine. Smad3+/− mice16,17 and wild-type (WT) C57/BL/6 controls underwent reperfused myocardial infarction experiments using a closed-chest model of coronary occlusion/reperfusion.18

Sections from paraffin-embedded hearts were immunolabeled with anti–Ki-67 and anti–α-SMA antibodies to allow identification of proliferating myofibroblasts. Identification of apoptotic cells was performed using fluorescent In situ Cell Death Detection Kit (Roche).19 The collagen content in infarcted hearts network was assessed using picrosirius red staining and a hydroxyproline assay. Flow cytometry using single-cell suspension of proliferating myofibroblasts was comparable between Smad3 null and WT mice (Figure 2H). However, when normalized to the total number of myofibroblasts present in the infarcted heart, the percentage of proliferating myofibroblasts was reduced in the absence of Smad3.

To investigate the role of Smad3 signaling in fibroblast phenotype and function, mouse fibroblasts from WT and Smad3+/− hearts were isolated from the infarcted heart with a large number of cells that exhibit impaired functional properties and defective matrix synthetic capability. Histochemical sirius red staining (Figure 1G) demonstrated that the density of apoptotic cells (Figure 2I). TGF-β1 stimulation reduced proliferation of α-SMA+ collagen I+ myofibroblasts (Figure 1F). Assessment of mean fluorescent intensity for collagen I in infarct myofibroblasts demonstrated that cells isolated from Smad3 null infarcts had lower collagen content than WT cells (Figure 1J and 1K).

The Role of Smad3 in Cardiac Fibroblast Proliferation

To examine whether the increased myofibroblast density in Smad3 null infarcts is due to enhanced proliferative activity, we identified proliferating myofibroblasts using dual immunohistochemical staining for α-SMA and ki-67 (Figure 2A through 2F). Quantitative analysis showed that the density of proliferating myofibroblasts in the infarcted heart peaked after 72 hours of reperfusion (Figure 2G). Smad3 null mice had a significantly higher number of proliferating myofibroblasts per area of infarcted heart when compared with WT animals. However, when normalized to the total number of myofibroblasts present in the infarcted heart, the percentage of proliferating myofibroblasts was not significantly different between Smad3 null and WT mice (Figure 2H).

Antiproliferative Actions of TGF-β1 on Isolated Cardiac Fibroblasts Are Dependent on Smad3

Serum-deprived Smad3+/− cardiac fibroblasts exhibited higher basal proliferative activity than corresponding WT cells (Figure 2I). TGF-β1 stimulation reduced proliferation of serum-deprived WT murine cardiac fibroblasts in a dose-dependent manner (Figure 2I). In contrast, TGF-β1 did not significantly affect proliferation of Smad3 null fibroblasts (P=NS), indicating that the antiproliferative effects of TGF-β1 are reduced in the absence of Smad3.

Results

Smad3 Null Infarcts Exhibit Increased Myofibroblast Infiltration But Attenuated Collagen Deposition

α-SMA immunofluorescence was used to identify infarct myofibroblasts as spindle-shaped immunoreactive cells located outside the vascular media (Figure 1A and 1B). Smad3 absence resulted in formation of a highly cellular wound with reduced deposition of collagen. Quantitative analysis showed that Smad3 null mice had significantly increased cellular content and higher myofibroblast density in the infarcted heart (Figure 1C through 1E). Histochemical sirius red staining (Figure 1G and 1H) and a biochemical hydroxyproline assay (Figure 1I) demonstrated that the enhanced accumulation of myofibroblasts, collagen deposition was significantly reduced in Smad3 null infarcts. Flow cytometry using single-cell suspensions harvested from infarcted hearts after 72 hours of reperfu-
myofibroblasts in the infarct may reflect loss of contractile protein content as these cells become apoptotic or may be due to their rapid clearance from the healing infarct.

TGF-β1–Mediated Contraction of Fibroblast-Populated Collagen Lattices Is Partially Dependent on Smad3

To examine the effects of Smad3 absence on fibroblast function, we assessed the ability of Smad3 null cells to elicit wound contraction using an assay of fibroblast-populated collagen lattices (Figure 4). In the absence of serum, WT fibroblasts induced more extensive lattice contraction than Smad3 null fibroblasts (Figure 4A, 4D, and 4G). Stimulation with 5% serum induced marked lattice contraction in gels populated with WT or Smad3 null fibroblasts resulting in comparable changes in size between groups (Figure 4B, 4E, and 4G). Although TGF-β1 stimulation enhanced collagen contraction in both WT and Smad3 null fibroblasts; contraction was markedly attenuated in lattices populated with Smad3 null cells (Figure 4C, 4F, and 4G).
Smad3 Null Cardiac Fibroblasts Exhibit Reduced α-SMA Expression on Stimulation With TGF-β1

To examine the role of Smad3 on phenotypic modulation of cardiac fibroblasts into myofibroblasts, we compared α-SMA expression in WT and Smad3 null cardiac fibroblasts on stimulation with TGF-β1. WT cardiac fibroblasts showed robust upregulation of α-SMA mRNA after 24 hours of stimulation (Figure 5A); α-SMA mRNA synthesis was significantly attenuated in Smad3 null cells. Flow cytometric analysis (Figure 5B and 5C) and Western blotting (Figure 5D and 5E) confirmed the essential role of Smad3 in TGF-β1–mediated α-SMA upregulation. Baseline mean fluorescent intensity was comparable between WT and Smad3 null cardiac fibroblasts (Figure 5B). TGF-β1 stimulation for 24 hours significantly increased the mean intensity of α-SMA expression in WT but not in Smad3 null cardiac fibroblasts.
Western blotting demonstrated that TGF-β1–induced α-SMA protein upregulation was abrogated in Smad3 null cells (Figure 5D and 5E). Moreover, dual fluorescent staining for α-SMA and Alexa Fluor 694-labeled phalloidin showed that Smad3 absence results in impaired organization of the polymerized actin cytoskeleton and reduced incorporation of α-SMA-positive filaments (Figure 5F through 5K) in TGF-β1–stimulated fibroblasts.

In vivo studies using flow cytometric analysis demonstrated that Smad3 null infarct myofibroblasts had significantly lower α-SMA expression than WT cells (Online Supplement).

Smad3 Signaling Mediates Cardiac Fibroblast Migration

Incubation with 1% serum significantly increased cardiac fibroblast migration (Figure 6A, 6B, and 6E). On stimulation with serum, Smad3 null cardiac fibroblasts had significantly diminished migratory activity when compared with WT cells (Figure 6). TGF-β1 exerted a modest effect on migration of WT fibroblasts that did not reach statistical significance and had no effect on Smad3 null cells (Figure 6E).

TGF-β1–Mediated Induction of Extracellular Matrix Protein Synthesis in Cardiac Fibroblasts Is Dependent on Smad3

TGF-β1 induced marked upregulation of type I collagen (Figure 7A), type III collagen (Figure 7B), and fibronectin (Figure 7C) mRNA in WT cardiac fibroblasts that peaked after 4 hours of stimulation. TGF-β1–induced extracellular matrix protein synthesis was significantly impaired in Smad3 null cardiac fibroblasts (Figure 7A through 7C).

Late But Not Early CTGF Upregulation in the Infarcted Heart Is Smad3-Dependent

Because CTGF is an important downstream effector of the profibrotic actions of TGF-β, we examined whether Smad3 loss results in reduced CTGF upregulation in healing myo-
cardiac infarcts. In WT animals, reperfused infarction resulted in early upregulation of CTGF mRNA after 6 hours of reperfusion (Figure 7D). CTGF mRNA levels remained elevated after 24 to 72 hours of reperfusion. Although early CTGF upregulation was not affected by the absence of Smad3, CTGF expression after 24 to 72 hours of reperfusion was significantly reduced in Smad3 null infarcts (Figure 7D). Thus, sustained induction of CTGF expression in the infarcted heart was Smad3-dependent.

TGF-β–Induced CTGF Upregulation in Cardiac Fibroblasts Is in Part Dependent on Smad3

TGF-β1 upregulated CTGF synthesis by WT fibroblasts, peaking after 4 hours of stimulation (Figure 7E). Smad3 null fibroblasts had significantly attenuated CTGF induction on stimulation with TGF-β1 (Figure 7E), indicating that TGF-β1–mediated CTGF upregulation is, at least in part, Smad3-dependent.

CTGF Stimulates Synthesis of Fibrosis-Associated Genes by Cardiac Fibroblasts in a Smad3-Independent Manner

We next examined the effects of CTGF on cardiac fibroblast gene expression. Incubation with CTGF significantly enhanced type I collagen (Figure 7F), type III collagen (Figure 7G), and fibronectin (Figure 7H) mRNA synthesis by fibroblasts peaking after 4 hours of stimulation. Costimulation of fibroblasts with both CTGF and TGF-β1 induced significantly higher extracellular matrix protein expression than each one of the mediators alone. CTGF induced comparable upregulation of collagen and fibronectin mRNA in WT and Smad3−/− cells, suggesting that its effects on cardiac fibroblasts are independent of Smad3 (Figure 7F through 7H). In contrast, the stimulatory effects of CTGF and TGF-β1 coinubcation were significantly attenuated in Smad3 null cells.

Discussion

After myocardial infarction, cardiac fibroblasts migrate into the infarct border zone, where they proliferate and undergo myofibroblast transdifferentiation promoting contraction of the scar. Activated myofibroblasts are the main source of extracellular matrix proteins in the infarcted and remodeling heart.6 We have previously demonstrated that selective activation of the TGF-β/Smad3 pathway in the infarct border zone is critically involved in the pathogenesis of fibrotic cardiac remodeling and contributes to the development of diastolic dysfunction following reperfused infarction by enhancing collagen deposition in the remodeling myocardium. We now report that several essential phenotypic and functional alterations of cardiac fibroblasts with a key role in development of fibrotic remodeling are dependent on Smad3. In the absence of Smad3, the healing infarct is infiltrated by abundant but defective fibroblasts that exhibit disrupted myofibroblast transdifferentiation, diminished migratory capacity, and impaired contractile and matrix-synthetic function. The functional impairment of Smad3 null fibroblasts results in attenuated interstitial fibrosis of the remodeling ventricle.
Figure 5. TGF-β1–induced α-SMA upregulation in cardiac fibroblasts is dependent on Smad3. A, qPCR demonstrated that TGF-β1 stimulation for 24-hour induced marked upregulation of α-SMA mRNA in WT cardiac fibroblasts. α-SMA induction was abrogated in Smad3 null cells (**P<0.01 vs corresponding WT; ###P<0.01 vs WT control). B and C, Flow cytometry confirmed the essential role of Smad3 in TGF-β1–mediated α-SMA upregulation. Baseline mean fluorescent intensity was comparable between WT (white area) and Smad3 null (black area) cardiac fibroblasts (B). TGF-β1 stimulation for 72 hours resulted in significantly higher α-SMA expression in WT cells (white) in comparison to Smad3 null (black) fibroblasts. D and E, Western blotting demonstrated that TGF-β1–mediated α-SMA upregulation was abrogated in Smad3 null cardiac fibroblasts (*P<0.05 vs corresponding WT cells). To visualize α-SMA incorporation into the cytoskeleton WT (F through H) and Smad3−/− (I through K) fibroblasts were stimulated with 25 ng/mL TGF-β1 for 3 days and stained with Alexa Fluor 694 labeled phalloidin (which labels the actin filaments) (F through I) and FITC-conjugated anti-α-SMA antibody (G and J). Note the impaired formation of cytoskeletal fibers and reduced incorporation of α-SMA in Smad3 null myofibroblasts (I through K).

Surprisingly, reduced collagen deposition in remodeling infarcted Smad3 null hearts was associated with significantly increased peak myofibroblast density (Figure 1). Expansion of the myofibroblast population in Smad3 null infarcts may be due to loss of the antiproliferative effects of TGF-β. Although extensive evidence suggests that the TGF-β/Smad3 pathway exerts growth-inhibitory effects on a variety of cell types, its role in fibroblast proliferation appears to be dependent on the context and on the unique characteristics of fibroblasts populating various sites of injury. Thus, TGF-β induced proliferation in dermal fibroblasts but attenuated the proliferative response in oral fibroblasts; both responses were dependent on Smad3.25,26 In the mouse heart transgenic overexpression of a mutant human TGF-β1 that prevents tethering of the latent complex to the
extracellular matrix leading to enhanced TGF-β activity resulted in reduced fibroblast proliferation in the absence of injury, but markedly enhanced proliferative activity in the injured heart.27 Our experiments demonstrated antiproliferative effects of TGF-β/Smad3 on cardiac fibroblasts in vitro; these actions were abrogated in the absence of Smad3 (Figure 3). However, in vivo the proliferation index was comparable between Smad3 null and WT infarcts, suggesting that in the complex and dynamic environment of the healing infarct contextual factors may modulate the effects of TGF-β/Smad3 signaling.

Because, as the wound matures, mesenchymal cell populations in the healing infarct are cleared through apoptosis, we examined the effects of Smad3 deficiency on apoptosis of granulation tissue cells. TUNEL staining demonstrated that the density of apoptotic cells was comparable between Smad3 null and WT animals; whereas the percentage of infarct cells exhibiting apoptosis was somewhat lower in Smad3 null infarcts after 72 hour of reperfusion (Figure 4). Apoptotic myofibroblasts were rarely found in the infarcted heart, reflecting their rapid clearance by professional phagocytes or indicating loss of myofibroblast phenotype as the cells undergo apoptosis (Figure 3).

Beyond its involvement in regulating the proliferative activity of fibroblasts in the healing infarct, Smad3 signaling also played an important role in their functional activation. The abundant myofibroblasts accumulating in Smad3 null infarcts showed marked impairment of key functional responses. In a wound contraction assay, collagen lattices populated with Smad3 null cardiac fibroblasts exhibited markedly attenuated gel contraction when compared with pads populated with WT cells (Figure 4). Defective contractile function of Smad3−/− cardiac fibroblasts was associated with reduced expression of α-SMA mRNA and protein and with disturbed formation of contractile microfilaments on stimulation with TGF-β, indicating impaired myofibroblast transdifferentiation (Figure 5). The role of the Smad3 pathway in α-SMA transcription has been previously demonstrated in fibroblast populations from noncardiac tissues.28 Experiments using embryonal mouse fibroblasts showed that receptor-regulated Smads, and in particular Smad3, are rate limiting for α-SMA enhancer activation.29 In addition, transfection with a Smad3-expressing plasmid markedly increased α-SMA expression, whereas transfection with an antisense Smad3 plasmid attenuated α-SMA synthesis in rat pulmonary fibroblasts.30 Thus, the Smad3 pathway appears to govern acquisition of the myofibroblast phenotype by fibroblasts in both reparative and fibrotic responses, and mediates TGF-β-induced contraction of collagen gels.31

Smad3 signaling is also involved in fibroblast migration. Using a transwell assay, we found that serum stimulation induced a robust migratory response in isolated cardiac fibroblasts, whereas the effects of TGF-β1 were modest and statistically insignificant (Figure 6). Smad3 absence attenuated serum-mediated fibroblast migration. The findings indicate that the highly proliferative Smad3 null cardiac fibroblasts infiltrating...
the infarcted myocardium exhibit impaired migratory capacity. Previous studies have suggested that signaling flux through Smad3 is critically involved in TGF-β-mediated chemotaxis in cutaneous wound healing and in fibrotic processes.32,33 Smad3 absence also markedly reduced the matrix-synthetic ability of cardiac fibroblasts. Fibronectin and type I and type III collagen transcription was markedly upregulated in Smad3 null fibroblasts on stimulation with TGF-β1; this effect was markedly attenuated in the absence of Smad3 (Figure 7A and 7C). These findings may explain the marked reduction in collagen deposition observed in infarcted Smad3 null hearts, despite the presence of abundant myofibroblasts (Figure 1). Previous studies have identified several extracellular matrix proteins as Smad-dependent TGF-β targets in human dermal and in mouse embryonal fibroblasts.10

Beyond the attenuation of direct TGF-β-mediated fibroblast responses, Smad3 gene disruption may reduce fibrosis by decreasing expression of CTGF, a TGF-β–inducible fibrogenic mediator with a central role in the pathogenesis of fibrosis.7 TGF-β–induced induction of CTGF is dependent on Smad3 in cutaneous fibroblasts,34 and lesional skin from Smad3−/− mice showed reduced CTGF expression.28 Our experiments showed that CTGF mRNA levels were markedly increased in the infarcted murine myocardium after 6 to 72 hours of reperfusion. Although the early peak of CTGF expression was not dependent on Smad3, CTGF levels during the proliferative phase of healing (after 24 to 72 hours of reperfusion) were markedly reduced in Smad3 null infarcts (Figure 7). This observation may reflect different stimulatory signals responsible for CTGF induction or may indicate distinct cellular sources of CTGF at various stages of healing. The effects of late attenuation of CTGF induction in Smad3 null infarcts may be particularly important for the fibrotic response, diminishing the activity of infarct myofibroblasts that accumulate in the murine myocardium after 3 to 5 days of reperfusion.18 Our in vitro studies demonstrated that CTGF stimulation induced intense upregulation of collagen and fibronectin synthesis by cardiac fibroblasts in a Smad3–independent manner (Figure 7). Costimulation of WT cardiac fibroblasts with TGF-β and CTGF had additive effects on extracellular matrix protein mRNA expression. In contrast, collagen and fibronectin mRNA synthesis in Smad3 null cells on costimulation with CTGF and TGF-β was comparable with the levels of expression observed in CTGF-stimulated cells (P<0.01; *P<0.05 vs WT control; ##P<0.01 vs KO control; P<0.05 vs corresponding WT).
has not been identified, CTGF functions are independent of the TGF-β/Smad3 pathway and appear to be mediated through integrins, proteoglycans and the low density lipoprotein receptor-related protein.\(^{2,35}\) Whereas TGF-β causes the induction of skin fibrosis, CTGF is required for persistent fibrous tissue formation.\(^{36}\) Moreover, CTGF is a necessary cofactor for activation of TGF-β-mediated adhesive cascades in embryonic fibroblasts\(^ {37}\) and activates TGF-β signals by direct binding in the extracellular space.\(^ {38}\)

Our findings contribute new information essential for understanding the role of the TGF-β/Smad3 pathway in fibrotic cardiac remodeling. Despite its antiproliferative effects, Smad3 signaling promotes fibrogenic actions, essential in the pathogenesis of cardiac fibrosis mediating myofibroblast transdifferentiation and wound contraction, inducing fibroblast migration and upregulating synthesis of extracellular matrix proteins. In addition, activation of the Smad3 pathway results in upregulation of CTGF that further enhances the fibrogenic properties of infarct fibroblasts. Because Smad3 activation is primarily localized in the infarct border zone and in the adjacent remodeling myocardium,\(^ {16,39}\) Smad3-mediated actions result in expansion of the fibrotic process into noninfarcted areas and accentuate diastolic dysfunction. Thus, Smad3 inhibition may be a promising therapeutic approach in conditions associated with fibrotic cardiac remodeling.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is Known?

- Transforming growth factor (TGF)-β1 participates in the pathogenesis of cardiac remodeling by activating cardiac fibroblasts; promoting myofibroblast transdifferentiation, and by enhancing extracellular matrix protein synthesis.
- The profibrotic actions of TGF-β may be mediated through a cascade of intracellular effectors, the Smads, or through Smad-independent pathways.
- TGF-β/Smad3 signaling is involved in tissue fibrosis; however, the mechanisms responsible for the profibrotic actions of the Smad3 pathway in the infarcted myocardium remain unknown.

What New Information Does This Article Contribute?

- Despite a reduction in the amount of collagen in the infarcted heart, Smad3-null mice exhibit increased infiltration of the infarct with fibroblasts, possibly due to loss of the Smad3-dependent antiproliferative effects of TGF-β on cardiac fibroblasts.
- Smad3-null fibroblasts are functionally impaired, exhibiting reduced α-smooth muscle actin expression and defective myofibroblast transdifferentiation, reduced migratory potential, diminished capacity to contract collagen pads on TGF-β1 stimulation, and attenuated matrix-synthetic capacity.
- Connective tissue growth factor (CTGF) induction in the infarcted heart and in stimulated cardiac fibroblasts is in part dependent on Smad3 and exerts profibrotic actions through Smad3-independent pathways.

Activation of TGF-β/Smad3 signaling in the infarct border zone is involved in the pathogenesis of postinfarction cardiac remodeling. Because the Smad3 pathway may promote fibrous tissue deposition in the remodeling ventricle, we studied the effects of Smad3 loss on murine cardiac fibroblast phenotype and function in vivo and in vitro. Our study demonstrates that Smad3 disruption results in infiltration of the infarct with abundant but dysfunctional fibroblasts that exhibit impaired myofibroblast transdifferentiation, decreased capacity to contract collagen pads, reduced migratory potential, and suppressed expression of fibrosis-associated genes. The increased myofibroblast density in Smad3-null infarcts may be due to loss of the antiproliferative actions of TGF-β. Moreover, upregulation of CTGF, an essential downstream mediator of TGF-β-induced fibrosis, was in part dependent on Smad3; however, CTGF-mediated fibrogenic actions were independent of Smad3 signaling. Thus, activation of the Smad3 pathway in the injured myocardium induces fibrosis by promoting myofibroblast transdifferentiation, recruitment and activation, and by enhancing synthesis of other fibrogenic signals, such as CTGF, while preventing uncontrolled cellular proliferation. Smad3 inhibition may be a promising therapeutic strategy in conditions associated with cardiac fibrosis.
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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS:

1. Murine ischemia/reperfusion protocols.

All animal studies were approved by the animal protocol review committee at Baylor College of Medicine. Smad3 -/- mice 1,2 in a C57/BL/6 background and wildtype (WT) C57/BL/6 controls (both from our own colony) were used for myocardial infarction experiments. 8-12 week-old mice (18.0-22.0 g body weight) were anesthetized by an intraperitoneal injection of sodium pentobarbital (60 µg/g). A closed-chest mouse model of reperfused myocardial infarction was utilized in order to avoid the confounding effects of surgical trauma and inflammation, which may influence the baseline levels of chemokines and cytokines 3. The left anterior descending coronary artery was occluded for 1h then reperfused for 24h to 7 days. At the end of the experiment, the chest was opened and the heart was immediately excised, fixed in zinc-formalin, and embedded in paraffin for histological studies, or snap frozen and stored at – 80°C for RNA isolation. Sham animals were prepared identically without undergoing coronary occlusion/reperfusion. Animals used for histology underwent 24 h, 72 h and 7 day reperfusion protocols (8 animals per group). Mice used for RNA extraction underwent 6 h, 24 h, 72 h and 7 days of reperfusion (8 animals per group). Additional WT (n=4 per group) and KO (n=5 per group) animals were used for isolation of protein and single cell suspensions from infarcted myocardium after 72 h of reperfusion.

2. Hydroxyproline assay

Protein extracts from infarcted WT and Smad3 null hearts were hydrolyzed in 6 N HCl at 110°C for 12 h. Subsequently, samples were desiccated in Speed-Vac centrifuge (Savant) and were oxidized using 1.27% chloramine T (Sigma, St. Louis, MO), 10% n-propanol, 0.2 M sodium citrate, and 0.5 M sodium acetate, 0.7 M sodium hydroxide at pH 6.5. After 20 min of incubation at room temperature, Erlich's solution (1 M p-dimethylaminobenzaldehyde [Sigma] in 70% n-propanol, 20% perchloric acid) was added and a 15 min incubation at 65°C performed. Absorbance was measured at 550 nm and the amount of hydroxyproline was determined against a standard curve. Collagen concentration was normalized to protein concentration in sample.

3. Immunohistochemistry and quantitative histology

Murine hearts were fixed in zinc-formalin (Z-fix; Anatech, Battle Creek, MI), and embedded in paraffin. Sections were cut at 5 µm and immunolabeled with the anti-Ki-67 and anti-α-SMA antibodies to allow identification of proliferating myofibroblasts as previously described. 4 Briefly, sections were stained with anti-α-SMA antibody using alkaline phosphatase-based technique and developed with Red substrate kit (Vector Labs). Subsequently, sections were incubated for 20 min in 95°C non-boiling citrate buffer (Lab Vision) to allow antigen retrieval. To avoid nonspecific binding of the avidin-peroxidase complex in the second step of labeling, sections were pretreated with avidin and biotin blocking kit (Vector Labs). Finally, proliferating nuclei were stained with rat monoclonal TEC-3 anti –mouse Ki67 antibody (Dako) using a peroxidase-based technique and developed with diaminobenzidine + nickel (Vector Labs). Stained sections were scanned using a Zeiss Axioskop microscope equipped with a Zeiss digital camera. Quantitative assessment of proliferating myofibroblast density (cells/unit area) was performed by counting the number of α-SMA immunoreactive cells with Ki-67 positive nuclei in the infarcted area. In addition the percentage of proliferating myofibroblasts was calculated. The
collagen network was identified using picrosirius red staining. The percentage of the collagen-stained area was assessed in the infarcted myocardium using ImagePro software. Ten distinct fields from two different sections were used for quantitative analysis of collagen content in each area.

4. TUNEL staining and immunofluorescence

Identification of apoptotic cells in myocardial infarction was performed using fluorescent In situ Cell Death Detection Kit (Roche)\(^4\). Briefly, slides were deparaffinized, rehydrated in graded alcohols and permeabilized using 0.1% solution of Triton X in 0.1% sodium citrate. Consequently, slides were incubated with TUNEL reaction mixture for 1h at 37 °C. To allow detection of apoptotic myofibroblasts, sections were stained with mouse anti-α-SMA antibody followed by biotinylated anti-mouse IgG secondary antibody (Vector Labs) and streptavidin-Alexa Fluor 594 complex (Invitrogen). Nuclei were stained with DAPI (Invitrogen). The density of TUNEL+ and TUNEL+/α-SMA+ cells was quantitatively assessed in the infarct and in the remote remodeling myocardium and was expressed as cells/mm\(^2\). In addition, the apoptotic index was assessed as the percentage of apoptotic TUNEL+ cells to the total number of nuclei.

5. Cardiac fibroblast isolation and stimulation

Mouse fibroblasts from WT and Smad3 -/- hearts were isolated by enzymatic digestion with a collagenase buffer as previously described.\(^4,5\) Cells at passage 3 were seeded in chambers of Culture Slides (BD Falcon) and allowed to attach overnight. Cells were serum-starved for 24h and subsequently stimulated with 25 ng/ml of TGF-β1 (R&D Systems) for 4 and 24 hours. Cells were harvested and total RNA extracted using TRIzol (Invitrogen). In additional experiments serum-deprived cardiac fibroblasts were stimulated with 5 ng/ml and 25 ng/ml TGF-β1 for 3 days. Cells were harvested and protein extracted using RIPA buffer (Pierce). Finally, quiescent cells were incubated with 25 ng/ml TGF-β1 and/or 250 ng/ml CTGF (Invitrogen) for 24 hours. The recombinant human CTGF used for stimulation is a 11.2 kDa isoform containing the C-terminal portion of the full-length CTGF protein (including the heparin-binding domain). Harvested cells were used for total RNA extraction.

6. Immunofluorescence

Cells at passage 3 were seeded in chambers of Culture Slides (BD Falcon) and allowed to attach overnight. Cells were serum-starved for 24h and subsequently stimulated with 25 ng/ml of TGF-β1 for 3 days. After incubation fibroblasts were fixed for 5 min in 3.7% solution of paraformaldehyde (Sigma) in PBS and permeabilized using 0.1% Triton-X (Sigma) in PBS. Subsequently, slides were stained with FITC-labeled anti-α-SMA antibody (Sigma). Finally to visualize the polymerized actin cytoskeleton, slides were incubated in 0.5 μM solution of Alexa Fluor 594-conjugated phalloidin (Molecular Probes) in PBS for 20 min. Nuclei were stained with DAPI (Invitrogen).

7. Fibroblast flow cytometry

Serum-starved cardiac WT and Smad3 -/- fibroblasts from passage 3 were incubated for 3 days in serum free medium enriched with either 25 ng/ml TGF-β1 or vehicle. Cells were harvested, counted and reconstituted in staining buffer (BD Biosciences) to concentration of 3x10⁶ cells/ml. 100 μl of cell suspension was incubated with anti-FcγIII/II (clone 2.4G2) antibody (BD Pharamingen), fixed and permeabilized using fixation/permabilization kit (BD
Biosciences). Subsequently, cells were incubated with FITC conjugated anti-α-SMA (Sigma, Clone 1A4). Finally cells were washed twice, resuspended in staining buffer and immediately analyzed with a Cell Lab Quanta SC flow cytometer (Beckman Coulter). Cell permeable DRAQ5 dye (Alexis Biochemicals) was used to define the gate for nucleated cells. Data analysis was performed using FlowJo (Tree Star, Inc).

8. Transwell migration assay

WT and Smad3 -/- cardiac fibroblast migration was studied using the colorimetric transwell system QCM™ (Millipore Corporation, Billerica, MA), which allows cells to migrate through an 8-µm pore size polycarbonate membrane. Fibroblasts used in the assay were serum starved for 24h prior to the experiment. WT and KO cells were harvested using TrypLe express (GIBCO Invitrogen Corporation, Carlsbad, CA), counted and reconstituted with serum free DMEM/F12 (GIBCO Invitrogen Corporation, Carlsbad, CA) to bring the cells to concentration of 5*10^5 cells/ml. WT and KO cell suspensions were stimulated with vehicle or recombinant mouse TGF-β1 (R&D systems, Mineapolis, MN) (25 ng/ml) and incubated for 1h. Subsequently, 300 µl of control or TGF-β1-treated WT and KO cell suspensions were added to the upper chamber of each insert. 500 µl of serum free DMEM/F12, 1% Fetal Calf Serum (FCS) DMEM/F12 or 25 ng/ml TGF-β1 DMEM/F12 were added to the lower chamber and incubated for 4 h at 37ºC in 5% CO2. After incubation remaining cell suspensions from upper wells were removed and inserts were placed in cell stain solution for 20 min at room temperature. Subsequently, inserts were collected and rinsed several times in distilled water. While the insert were still moist, cotton swabs were used to remove adherent non-migratory cells from the upper chamber. Migrated cells were visualized and photographed using an inverted microscope. Dry inserts were transferred to extraction buffer and placed on rocking platform for 15 min. Optical density of dye extract was measured at 560 nm using NanoDrop spectrophotometer. Cell migration in each individual experiment was normalized to mean migration in chamber without chemoattractant (control).

9. Collagen lattice contraction assay

Fibroblasts from WT and Smad3 null hearts were passaged 3 times and prior experiment rendered quiescent in serum-free medium. Cells were harvested and reconstituted with serum free DMEM (GIBCO Invitrogen Corporation, Carlsbad, CA) to achieve the concentration of 2*10^6 cells/ml. Collagen matrix was prepared on ice by diluting a stock solution of rat 3,48 mg/ml collagen I (GIBCO Invitrogen Corporation, Carlsbad, CA) with 2x DMEM and distilled water for a final concentration of 1 mg/ml collagen. WT and KO cell suspensions in 1x DMEM were mixed with collagen solution to achieve the final 3*10^5 cells/ml concentration. Subsequently, 500 µl of this suspension was aliquoted to a 24-well culture plate (BD Falcon, San Jose, CA) and allowed to polymerize at 37ºC for 15 min. Following polymerization pads were released from wells, transferred to 6-well culture plate (BD Falcon, San Jose, CA) and cultured in 0% FCS DMEM, 5% FCS DMEM or 25 ng/ml TGF-β1 DMEM. At 0 h and after 24 h the pictures of the plates were taken in flatbed scanner and the area of each pad was measured using Image Pro software.

10. Cell proliferation assay

Proliferation of WT and Smad3 null cardiac fibroblasts was assessed using a colorimetric BrdU Cell Proliferation ELISA kit (Roche Applied Biosciences, Indianapolis, IN) as previously
described. Briefly, cells at passage 3 were harvested, counted and reconstituted in 10% FBS DMEM/F12 at density of 10^5 cells/ml. 100 μl of cell suspension was transferred to 96-well culture plates (BD Falcon, San Jose, CA) and incubated for 24 h to allow the fibroblasts to attach. Prior to experiment cells were rendered quiescent by overnight incubation in serum deprived medium. The following conditions were studied to examine the effect of Smad3 loss on fibroblast proliferation; 5% FCS, 1% FCS with either vehicle or range of TGF-β1 concentrations from 10 pg/ml to 100 ng/ml in decimal increments. After 24 h of stimulation, each well was enriched with BrdU labeling solution at final concentration of 10 μM. Cells were allowed to proliferate in presence of growth factors and incorporate BrdU for another 24 h. Subsequently, cells were fixed, immunolabeled with peroxidase conjugated anti-BrdU antibody for 2h and washed several times with PBS. Finally, peroxidase substrate solution was added to enable spectrophotometric detection of product at 370 nm. Blank samples without cells were analyzed to subtract absorbance resulting from the non-specific binding of BrdU and anti-BrdU antibody to the surface of microplate. Cell proliferation in each individual experiment was normalized to mean proliferation in presence of 5% FBS.

11. Flow cytometry of infarct single cell suspensions

Single cell suspensions were prepared from infarcted WT (n=4) and KO (n=5) hearts after 72 hours of reperfusion. Briefly, hearts were rapidly excised and placed in ice cold Krebs-Henseleit (KH) buffer containing (in g/L) 2 glucose, 0.141 MgSO4, 0.16 NaH2PO4, 0.35 KCl, 6.9 NaCl, 2.1 NaHCO3, 0.373 CaCl2, 1 NaN3 at pH 7.4. Subsequently, the aorta was cannulated with a 22-gauge tubing adaptor and flushed with ice cold KH buffer to remove residual cells in the coronary vasculature. Two hearts per experiment were minced with fine scissors and placed into a cocktail of 0.25 mg/ml Liberase Blendzyme 3 (Roche Applied Science), 20 U/ml DNase I (Sigma Aldrich), 10 mM HEPES (Invitrogen), 0.1% Sodium Azide in HBSS with Ca2+ and Mg2+ (Invitrogen) and shaken at 37°C for 40 min. Cells were then passed through a 40 μm nylon mesh (BD Falcon), centrifuged (10 min, 500 g, 4C) and reconstituted with staining buffer (dPBS without Ca2+ and Mg2+, 2% FBS, 0.1% sodium azide). 5×10^5 cells were incubated with anti-FcγIII/II (clone 2.4G2) antibody (BD Pharmingen), fixed and permeabilized for 45 min at 4°C with fixation/permeabilization kit (eBioscience). Subsequently, cells were incubated with FITC conjugated anti-α-SMA (Sigma, Clone 1A4) and purified rabbit anti-collagen I (Rockland Inc.) antibodies for 30 min at 4°C. Cells were washed twice and incubated for 30 min at 4°C with PE conjugated anti-rabbit IgG (BD Pharmingen). Finally cells were washed twice, resuspended in staining buffer and immediately analyzed with a Cell Lab Quanta SC flow cytometer (Beckman Coulter). Data analysis was performed using FlowJo (Tree Star, Inc).

12. Real time PCR

Isolated total RNA from the hearts and cultured fibroblasts was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Quantitative PCR was performed using the SYBR green (Bio-Rad) method on the iQ™5 Real-Time PCR Detection System (Bio-Rad). Primers were synthesized at the Baylor College of Medicine Child Health Research Center core facility. The following sets of primers were used in the study: α-SMA (forward) GCTGGACTCTGGAGATGG, (reverse) GCAGTAGTCACGAAAGGAA TAG; CTGF (forward) CTGCGAGGAGTGGGTGTG, (reverse) ATGTGTCTTCCAGTCCGATGG; collagen I pro-alpha I chain (forward)
GTATGCTTGATCTGTATCTG, (reverse) CGACTCCTACATCTTTCTG; collagen III pro-alpha I chain (forward) CCTTGGTCAGTCCTATGAG, (reverse) CAGGAGCAGGTGTAG AAG; fibronectin (forward) AGGCAATGGACGCATCAC, (reverse) TTCCTCGGTTGTCC TTCTTG; 18S RNA (forward) ACCGCAGCTAGGAATAATGGA, (reverse) GCCTCAGTT CCGAAAACC. Each sample was run in triplicate. The 2^{ΔΔC_T} method using 18S RNA and sham/control as the reference and calibrator respectively, was employed for relative quantification of gene expression.

13. Western blotting
Protein concentration in samples extracted from infarcts and cultured cardiac fibroblasts was quantified using the BCA protein assay (Pierce). Forty micrograms of total cellular proteins suspended in SDS sample buffer containing 15 mM dithiothreitol were separated via 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blots were probed overnight with 1:500 dilution of polyclonal rabbit anti-α-SMA (Novus) in 3% milk/Tween-Tris buffered saline. After incubation with the secondary antibody, anti-rabbit IgG-horseradish peroxidase (Cell signaling), α-smooth muscle actin levels were assessed using enhanced chemiluminescence (Amersham Pharmacia). Protein expression was normalized to GAPDH.
Online Figure I
Online Figure I: Smad3 null mice exhibit attenuated expression of α-SMA in infarct myofibroblasts. A: Quantitative analysis of western blotting experiments studying α-SMA expression in infarcted hearts. WT and Smad3 -/- (KO) infarcts had comparable α-SMA levels after 72h of reperfusion. B. Representative western blotting experiments are shown (upper band: α-SMA; lower band: GAPDH). C-D: In order to specifically compare α-SMA expression in infarct myofibroblasts isolated single cell suspensions were obtained from animals undergoing 1h occlusion/72h reperfusion protocols. Myofibroblasts were identified as α-SMA+/collagen I + cells. Representative experiments are shown demonstrating that infarct myofibroblasts harvested from Smad3 null infarcts (black curve) had lower α-SMA expression than WT cells (white curve). Quantitative analysis of mean fluorescent intensity (MFI) demonstrated that Smad3 null (KO) cells had significantly lower α-SMA content when compared with WT cells (*p<0.05 vs. WT). The absence of a difference in total α-SMA expression between WT and Smad3 null infarcts despite the reduced α-SMA content in myofibroblast likely reflects the higher number of myofibroblasts in Smad3 KO infarcts or indicates a significant contribution of smooth muscle cells.
SUPPLEMENTAL REFERENCES:


