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Opposing Actions of Fibroblast and Cardiomyocyte Smad3 Signaling in the Infarcted Myocardium

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BACKGROUND: Transforming growth factor–βs regulate a wide range of cellular responses by activating Smad-dependent and Smad-independent cascades. In the infarcted heart, Smad3 signaling is activated in both cardiomyocytes and interstitial cells. We hypothesized that cell-specific actions of Smad3 regulate repair and remodeling in the infarcted myocardium.

METHODS: To dissect cell-specific Smad3 actions in myocardial infarction, we generated mice with Smad3 loss in activated fibroblasts or cardiomyocytes. Cardiac function was assessed after reperfused or nonreperfused infarction using echocardiography. The effects of cell-specific Smad3 loss on the infarcted heart were studied using histological studies, assessment of protein, and gene expression levels. In vitro, we studied Smad-dependent and Smad-independent actions in isolated cardiac fibroblasts.

RESULTS: Mice with fibroblast-specific Smad3 loss had accentuated adverse remodeling after reperfused infarction and exhibited an increased incidence of late rupture after nonreperfused infarction. The consequences of fibroblast-specific Smad3 loss were not a result of effects on acute infarct size but were associated with unrestrained fibroblast proliferation, impaired scar remodeling, reduced fibroblast-derived collagen synthesis, and perturbed alignment of myofibroblast arrays in the infarct. Polarized light microscopy in Sirius red–stained sections demonstrated that the changes in fibroblast morphology were associated with perturbed organization of the collagenous matrix in the infarcted area. In contrast, α-smooth muscle actin expression by infarct myofibroblasts was not affected by Smad3 loss. Smad3 critically regulated fibroblast function, activating integrin-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–2 (NOX-2) expression. Smad3 loss in cardiomyocytes attenuated remodeling and dysfunction after infarction. Cardiomyocyte-specific Smad3 loss did not affect acute infarct size but was associated with attenuated cardiomyocyte apoptosis in the remodeling myocardium, accompanied by decreased myocardial NOX-2 levels, reduced nitrosative stress, and lower matrix metalloproteinase–2 expression.

CONCLUSIONS: In healing myocardial infarction, myofibroblast- and cardiomyocyte-specific activation of Smad3 has contrasting functional outcomes that may involve activation of an integrin/reactive oxygen axis.

*Drs Kong and Shinde contributed equally.

Key Words: cardiomyocyte ■ fibroblast ■ heart failure ■ remodeling ■ SMAD

Sources of Funding, see page 723

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Clinical Perspective

What Is New?

- Our study demonstrates for the first time cell-specific effects of Smad3 signaling in the infarcted myocardium.
- Smad3 activation in myofibroblasts plays a critical role in repair after myocardial infarction, restraining fibroblast proliferation and contributing to scar organization by stimulating integrin synthesis.
- Smad3 signaling in cardiomyocytes does not affect acute ischemic injury but triggers nitrosative stress and induces matrix metalloproteinase expression in the remodeling myocardium, promoting cardiomyocyte death and contributing to systolic dysfunction.

What Are the Clinical Implications?

- Implementation of therapeutic strategies targeting transforming growth factor–β cascades in patients with myocardial infarction has been hampered by the pleiotropic and context-dependent actions of the cytokine.
- Using genetic tools with in vivo and in vitro experiments, we demonstrated the cellular specificity of transforming growth factor–β/Smad3—dependent actions that stimulate distinct cellular responses in fibroblasts and cardiomyocytes.
- Therapeutic targeting of transforming growth factor–β in pathological conditions is likely to interfere with both detrimental and beneficial actions.
- Design of interventions with specific cellular targets is needed for the development of safe and effective therapies.

Transforming growth factor (TGF)–βs are highly pleiotropic mediators with critical roles in regulating cellular phenotype and function in embryonic development, tissue homeostasis, and disease. Normal tissues contain stores of latent TGF-β bound to the extracellular matrix through its association with a large binding protein, the latent TGF-β binding protein. Tissue injury is associated with marked induction of TGF-β isoforms and activation of TGF-β signaling cascades. Parenchymal cells, extravasated leukocytes, and platelets synthesize and release large amounts of TGF-β in the injury site. Reactive oxygen species, proteases, matricellular proteins, and integrins cooperate to trigger the release of bioactive TGF-β from the latent stores. Subsequent binding of the active TGF-β dimer to the type II TGF-β receptor, followed by transphosphorylation of the type I receptor, triggers the TGF-β signaling response. The cellular effects of TGF-β are mediated through a canonical pathway involving a series of intracellular effectors, the Smads, or through activation of noncanonical signaling cascades. Activation of TGF-β signaling induces phosphorylation of the receptor-activated Smads, Smad2 and Smad3, which can form heteromeric complexes with the common Smad, Smad4. These complexes are transported to the nucleus, where they regulate gene transcription. TGF–β receptors and Smads are ubiquitously expressed by all cell types. Thus, all cells are responsive to the actions of TGF-β.

Cardiac injury is associated with the marked induction of TGF-β and activation of TGF-β cascades.[1,2] Our laboratory and other investigators have documented activation of Smad2 and Smad3 signaling in the infarcted myocardium,[3,4] localized in both cardiomyocytes and interstitial cells.[5] In isolated cardiac fibroblasts, Smad3 signaling accentuates myofibroblast transdifferentiation and stimulates a matrix-preserving program.[5] In a model of reperfused infarction, global loss of Smad3 attenuated remodeling after infarction.[4] However, considering the ubiquitous expression of Smad3 in all cell types, the cell biological basis for the actions of Smad3 in the infarcted heart remains unknown.

Our study dissects the cell-specific actions of Smad3 signaling in the infarcted myocardium by developing and studying mice with cell-specific loss of Smad3 in activated fibroblasts and cardiomyocytes. It is surprising that fibroblast-specific loss of Smad3 worsened remodeling after infarction, resulting in accentuated chamber dilation. The deleterious consequences of fibroblast-specific Smad3 loss reflected unrestrained fibroblast proliferation, defective scar remodeling, and perturbed organization of myofibroblast arrays in the border zone. Smad3 signaling regulated fibroblast function, activating integrin-mediated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)–2 expression. In contrast, cardiomyocyte-specific loss of Smad3 protected the infarcted heart from dysfunction after infarction. The protective effects of cardiomyocyte-specific Smad3 loss were associated with attenuated cardiomyocyte apoptosis in remodeling myocardium and accompanied by decreased NOX2 levels, reduced nitrosative stress, and decreased matrix metalloproteinase (MMP)–2 expression.

METHODS

Detailed methods are described in the online-only Data Supplement

Relevant data, methods, and study materials will be made available on request.

Generation of Mice With Myofibroblast and Cardiomyocyte-Specific Smad3 Loss

We generated mice with Smad3 loss in activated infarct myofibroblasts (FS3KO) by breeding Smad3$^{fl/fl}$ mice[6] with transgenic mice in which Cre recombinase is driven by a 3.9-kb mouse *Postn* promoter.[7] Endogenous periostin, which is encoded by

Postn, is not expressed in cardiomyocytes, vascular cells, hematopoietic cells, or quiescent cardiac fibroblasts[8] but is upregulated in injury site fibroblasts in infarcted hearts.[9] To generate mice with cardiomyocyte-specific loss of Smad3 (CMS3KO), Smad3$^{fl/fl}$ mice were crossed with α-myosin heavy chain (MHC) MHC-Cre-transgenic animals.[10]

Mouse Models of Myocardial Infarction

Animal studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine and conform with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. A closed-chest model of reperfused myocardial infarction and a model of nonreperfused infarction were used, as previously described.[11] Female and male mice, 2 to 4 months of age, were anesthetized using inhaled isoflurane; 75 Smad3$^{fl/fl}$ mice, 73 FS3KO mice, and 36 CMS3KO mice underwent in vivo experimentation.

Acute Infarct Size Assessment

Infarct size was assessed using Evans blue–triphenyltetrazolium chloride staining.

Echocardiography

Echocardiography was performed before instrumentation and after 7 days and 28 days of reperfusion using the Vevo 2100 system (VisualSonics).

Immunohistochemistry and Histology

For histopathologic analysis, murine hearts were fixed in zinc-formalin and embedded in paraffin.

Isolation and Culture of Cardiac Fibroblasts

Fibroblasts were isolated from normal wild-type (WT) and Smad3 knockout (KO) mouse hearts as previously described.[4]

Isolation of Fibroblasts and Myeloid Cells From Infarcted Hearts

Macrophages and fibroblasts were isolated from infarcted hearts for RNA extraction as previously described.[9]

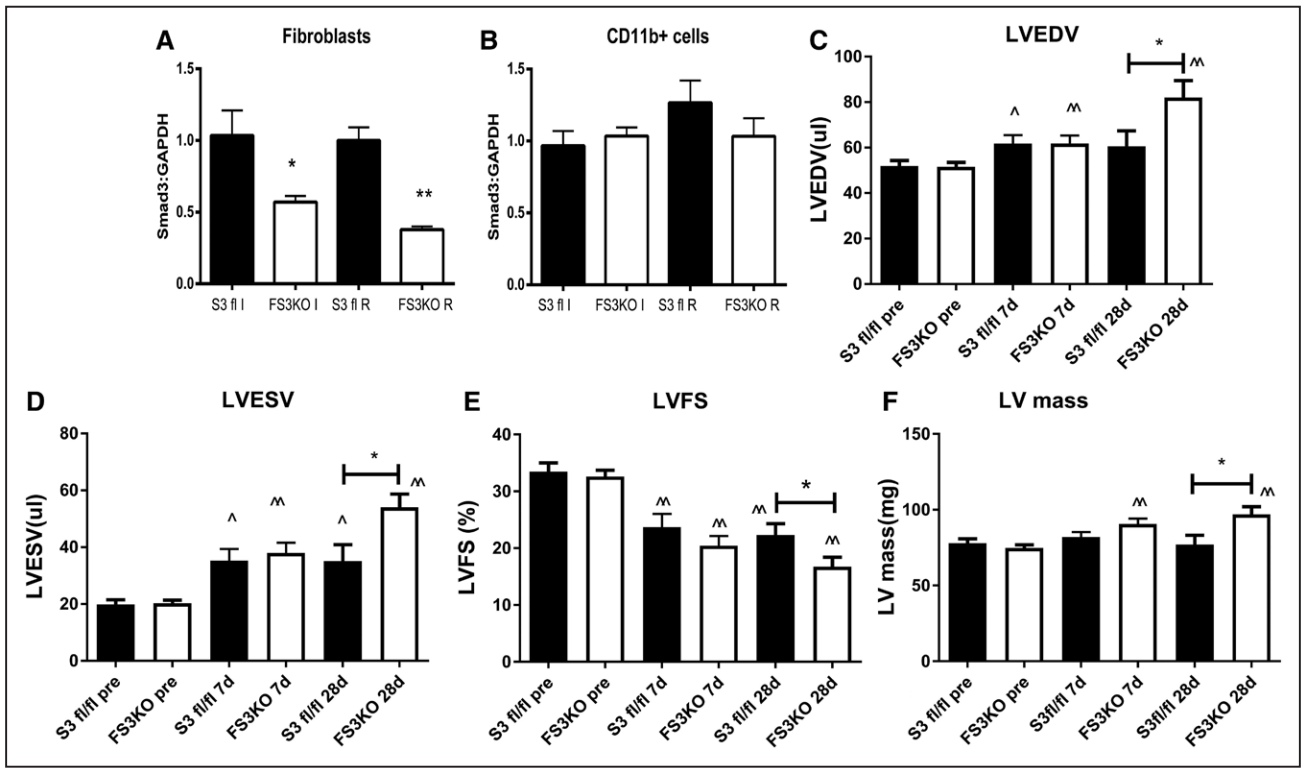

Figure 1. Fibroblast-specific Smad3 disruption accentuates adverse remodeling following reperfused myocardial infarction.

A and B, Smad3$^{fl/fl}$Postn-Cre mice (FS3KO) exhibited reduced Smad3 expression in fibroblasts (**A**) but not in CD11b^{+} myeloid cells (**B**) harvested from the infarcted (I) or remodeling myocardium (R) after 1 hour of ischemia and 7 days of reperfusion. (*P<0.05, **P<0.01 versus corresponding Smad3$^{fl/fl;}$ n=5/group). **C**–**F,** Fibroblast-specific Smad3 loss increased adverse remodeling and worsened systolic dysfunction after 1 hour of ischemia and 28 days of reperfusion. In the model of reperfused myocardial infarction, FS3KO mice exhibited increased LVEDV (**C**) and LVESV (**D**), reduced LV fractional shortening (**E**), and increased LV mass (**F**) in comparison with Smad3$^{fl/fl}$ controls after 28 days of reperfusion (*P<0.05, ^P<0.05, ^^P<0.01 versus corresponding baseline values; n=11/group). FS3KO indicates fibroblast-specific Smad3 knockout mice; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LV mass, left ventricular mass; and S3, Smad3.

Collagen Pad Contraction Assay

A collagen pad contraction assay was performed as previously described.[11]

RNA Extraction and Quantitative Polymerase Chain Reaction

Gene expression was assessed using quantitative polymerase chain reaction.

Protein Extraction and Western Blotting

Western blotting using protein extracted from hearts or cardiac fibroblasts was performed.

Statistical Analysis

Data are presented as mean±SEM. For comparisons of 2 groups, the unpaired, 2-tailed Student's *t* test using (when appropriate) Welch's correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way analysis of variance was performed, followed by Tukey's multiple comparison test. The Kruskal-Wallis test, followed by Dunn's multiple comparison posttest, was used when ≥1 groups did not show Gaussian distribution. The paired *t* test was used for comparisons of functional data within the same group. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

RESULTS

FS3KO Mice Have Normal Baseline Cardiac Geometry and Function

To generate mice lacking Smad3 in infarct myofibroblasts, we used a transgenic mouse line in which Cre is driven by the mouse *Postn* promoter.[7] Periostin is not expressed by quiescent fibroblasts in the adult mouse heart but is markedly and specifically upregulated in infarct myofibroblasts. Postn-Cre mice have been used for fibroblast-specific targeting in models of cardiac injury and remodeling.[12,13] Smad3$^{fl/fl}$Postn-Cre mice (FS3KO) had normal baseline cardiac geometry and function. At 4 months of age, FS3KO animals and Smad3$^{fl/fl}$ controls had comparable left ventricular volume, ejection fraction, wall thickness, and left ventricular mass (Figure I in the online-only Data Supplement). To examine the efficacy of Cre-mediated deletion in infarct fibroblasts, we assessed Smad3 levels in fibroblasts and CD11b$^+$ myeloid cells harvested immediately from the infarcted and noninfarcted myocardium to avoid effects of culture conditions on cellular phenotype. FS3KO mice had markedly reduced Smad3 expression in fibroblasts isolated from the infarct and the remodeling noninfarcted myocardium (Figure 1A). FS3KO and Smad3$^{fl/fl}$ mice exhibited comparable Smad3 expression in CD11b$^+$ myeloid cells harvested from the infarct and noninfarcted remodeling heart (Figure 1B), indicating fibroblast-specific Smad3 targeting.

Smad3 Loss in Activated Fibroblasts Accentuates Dilative Remodeling in Both Reperfused and Nonreperfused Myocardial Infarction

Both FS3KO and Smad3$^{fl/fl}$ mice subjected to reperfused infarction had low mortality; however, FS3KO mice exhibited worse remodeling. After 28 days of reperfusion, FS3KO animals had increased left ventricular end-diastolic volume and left ventricular end-systolic volume (Figure 1C and 1D), suggesting accentuated dilative remodeling. Moreover, fibroblast-specific Smad3 loss worsened systolic dysfunction (Figure 1E) and augmented hypertrophy after reperfused infarction (Figure 1F and Figure II in the online-only Data Supplement).

In contrast to the negligible mortality associated with reperfused infarction, mice undergoing nonreperfused infarction protocols exhibit ≈50% mortality during the first week after coronary occlusion primarily because of cardiac rupture. Statistical analysis showed that FS3KO mice had a trend toward higher mortality (percent survival at 28 days: Smad3$^{fl/fl}$, 37.0% versus FS3KO, 18.7%, P=0.16, n=42–46). Although all deaths in Smad3$^{fl/fl}$ mice occurred during the first 5 days after coronary occlusion, FS3KO animals continued to exhibit mortality after the first 5 days. Mortality analysis after day 6 after occlusion showed that FS3KO mice had significantly increased late mortality (P=0.007), reflecting a high incidence of late cardiac rupture (Figure 2A and 2B). FS3KO animals surviving nonreperfused infarction exhibited trends toward increased dilative remodeling and worse systolic dysfunction (Figure 2C through 2F).

Fibroblast-Specific Smad3 Loss Does Not Affect Acute Infarct Size But Is Associated With Larger Scars, Increased Myofibroblast Density, and Accentuated Myofibroblast Proliferation

Next, we examined whether worse remodeling after infarction in FS3KO mice was caused by accentuated acute cardiomyocyte injury. Fibroblast-specific Smad3 loss did not affect acute infarct size (Figure 3A through 3C). Despite comparable acute injury, FS3KO mice had significantly larger scars after 7 to 28 days of reperfusion (Figure 3D through 3H). Increased scar size in FS3KO mice was associated with higher myofibroblast density in the infarcted region (Figure 3I through 3M). We have previously demonstrated that in vitro, Smad3 mediates the antiproliferative effects

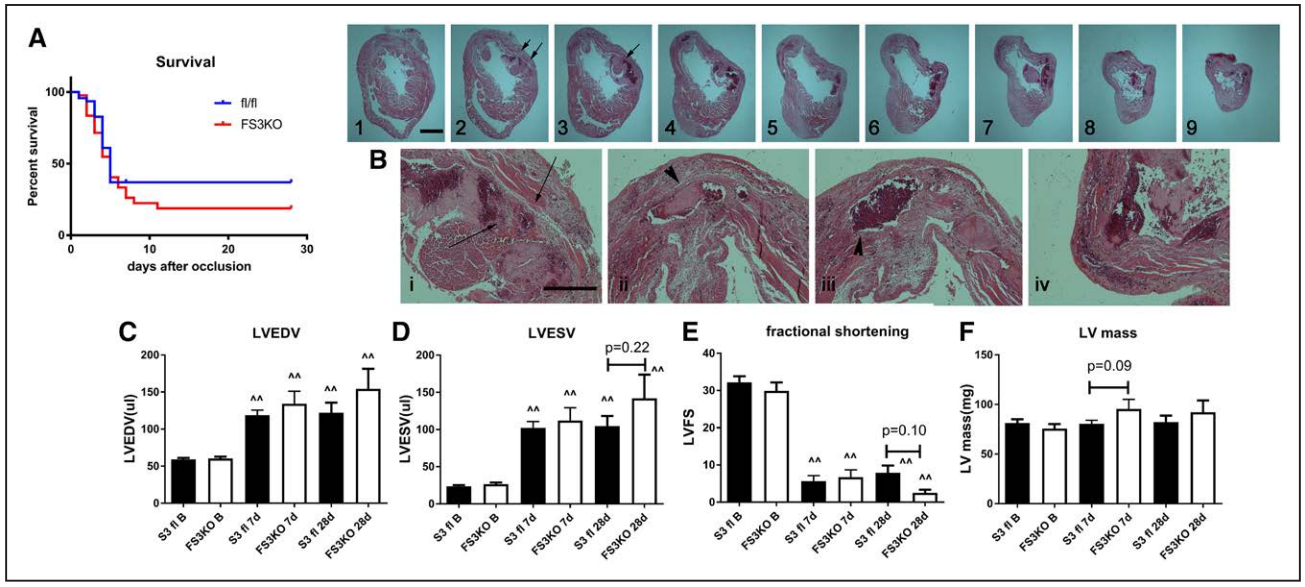

Figure 2. Smad3$^{fl/fl}$Postn-Cre (FS3KO) mice exhibit increased late rupture-related mortality after nonreperfused myocardial infarction.

A, In contrast to mice undergoing reperfused infarction protocols (that exhibit low mortality rates), nonreperfused infarction is associated with a ≈50% mortality in WT mice predominantly because of cardiac rupture. Control Smad3$^{fl/fl}$ mice had a 37.0% survival rate compared with 18.7% for FS3KO animals (P=0.16, n=42–46 mice). In Smad3$^{fl/fl}$ animals, all deaths occurred during the first 5 days after coronary occlusion. Comparison of survival curves for late deaths (after the sixth day) suggested that FS3KO mice had increased late mortality (P=0.007). **B,** Systematic histological analysis of the heart of an FS3KO mouse that died 11 days after coronary occlusion shows H&E staining performed at 9 different levels, sectioned at 250-μm partitions (1–9). The site of rupture is shown (arrows), filled with clot (scale bar=0.5 mm). Higher magnification images (Bi-iv) identify the site of rupture (arrows). Please note the presence of a dilated vascular structure within the healing scar (arrowhead) (scale bar=200 μm). **C**–**F,** Echocardiographic analysis showed that surviving FS3KO mice had comparable LVEDV (**C**) and trends toward higher LVESV (**D**), lower fractional shortening (**E**), and increased LV mass (**F**) (Smad3$^{fl/fl}$: 7 d, n=16; 28 d, n=9; FS3KO: 7 d, n=10; 28 d, n=4; ^^P<0.01 versus corresponding baseline values [**B**]). FS3KO indicates fibroblast-specific Smad3 knockout mice; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LV mass, left ventricular mass; and S3, Smad3.

of TGF-β in cardiac fibroblasts.[5] Accordingly, we examined whether increased myofibroblast density in FS3KO mice is a result of increased proliferative capacity of infiltrating fibroblasts. Dual immunofluorescence for α-smooth muscle actin (SMA) and ki-67 showed that FS3KO mice had a trend toward increased density of proliferating cells and significantly higher numbers of proliferating myofibroblasts (Figure III in the online-only Data Supplement). Taken together, the findings suggest that fibroblast-specific Smad3 loss may accentuate fibroblast proliferation while perturbing contraction and remodeling of the healing scar.

Effects of Fibroblast-Specific Smad3 Loss on Collagen Deposition in the Infarcted and Remodeling Myocardium

Sirius red staining followed by polarized light microscopy was used to investigate the effects of fibroblast-specific Smad3 loss on collagen deposition in the infarcted and remodeling myocardium. When visualized under polarized microscopy, thicker cross-linked fibers show orange or red birefringence, whereas thinner fibers appear green (Figure IVA through IVP in the online-only Data Supplement). Quantitative analysis showed no significant effects of fibroblast-specific Smad3 loss on collagen deposition in the infarct and remote remodeling myocardium after 7 days of reperfusion. However, after 28 days of reperfusion, FS3KO infarcts had lower levels of thinner green fibers in the infarct zone and higher amounts of green fibers in the remote remodeling myocardium. These findings may reflect the accentuated adverse remodeling in FS3KO animals (Figure IV in the online-only Data Supplement).

Smad3 Loss Impairs Contraction of Fibroblast-Populated Collagen Pads

In healing wounds, transdifferentiated myofibroblasts mediate scar contraction.[14] To explore the role of fibroblast Smad3 in fibroblast-mediated collagen contraction, we used an in vitro assay, in which fibroblasts harvested from the mouse heart populate and contract free-floating collagen pads.[5] First, we examined the effects of

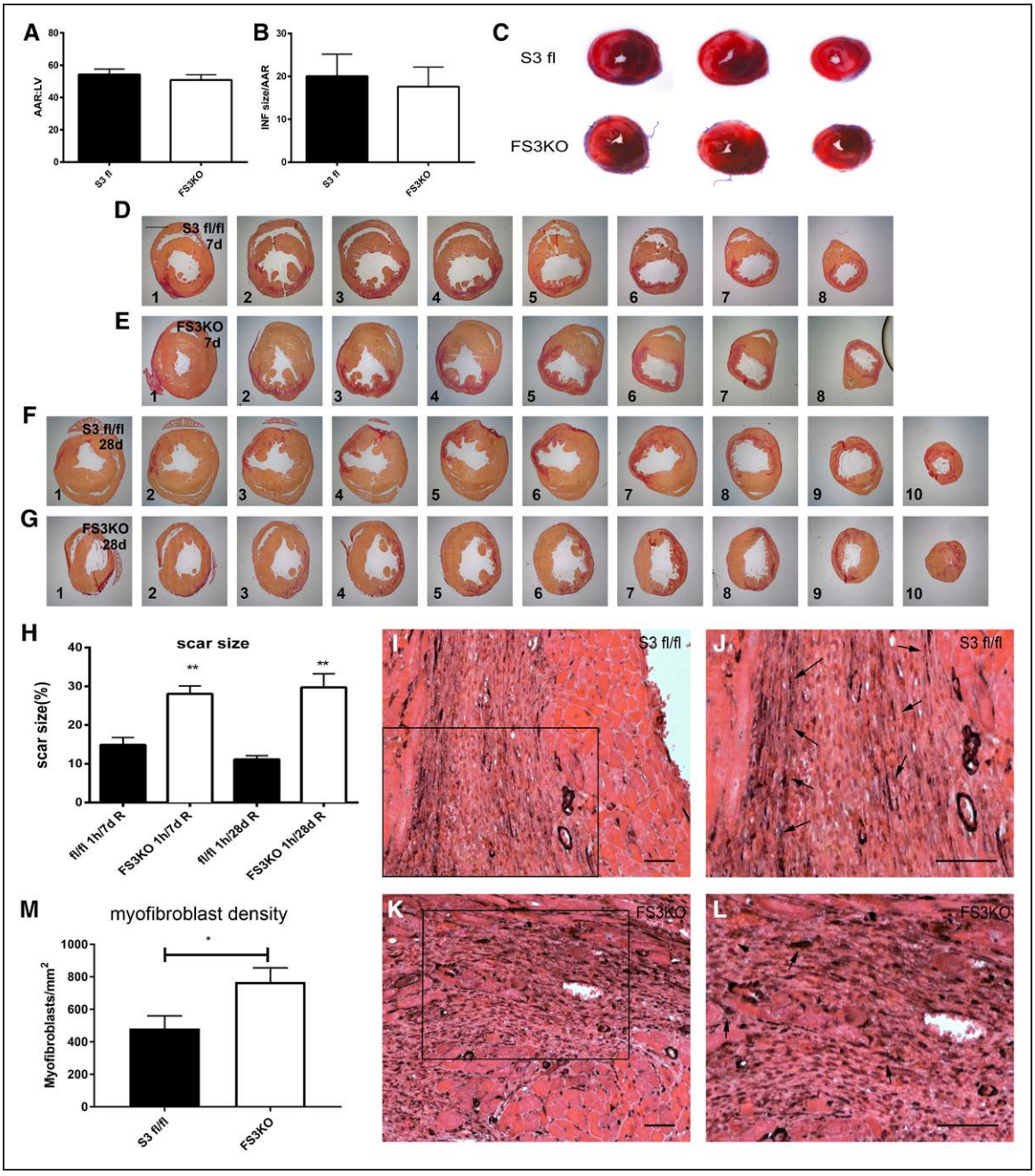

Figure 3. Fibroblast-specific loss of Smad3 does not affect acute infarct size but is associated with larger scars and increased myofibroblast density.

A–**C,** Smad3$^{fl/fl}$Postn-Cre (FS3KO) and Smad3$^{fl/fl}$ mice had comparable area at risk (AAR) (**A**) and infarct size (IS):AAR (**B**) (*P*=NS, n=10–11/group). **D**–**H,** However, despite comparable acute cardiomyocyte injury, FS3KO animals had significantly larger scars after 28 days of reperfusion. Scar size was assessed by sectioning the entire heart from base to apex and by staining for Sirius red the first section from each 300-μm partition. **D**–**G,** Representative Sirius red–stained sections from Smad3$^{fl/fl}$ mouse and an FS3KO animal after 7 days of reperfusion (**D** and **E**, respectively) and after 28 days of reperfusion (**F** and **G**, respectively) (scale bar=1 mm). **H,** Despite similar segmental distribution of the infarct, the size of the collagenous scar was larger in FS3KO animals after 7 and 28 days of reperfusion (***P*<0.01 versus Smad3$^{fl/fl}$, n=7–10/group). Fibroblast-specific Smad3 loss impaired contraction and remodeling of the scar. **I**–**L,** α-SMA immunohistochemistry was used to identify myofibroblasts in the infarct border zone after 7 days of reperfusion as α-SMA$^+$ cells located outside the vascular media (arrows) (scale bar=60 μm). **M,** Quantitative analysis showed that FS3KO mice had higher myofibroblast density (**P*<0.05, n=9/group).

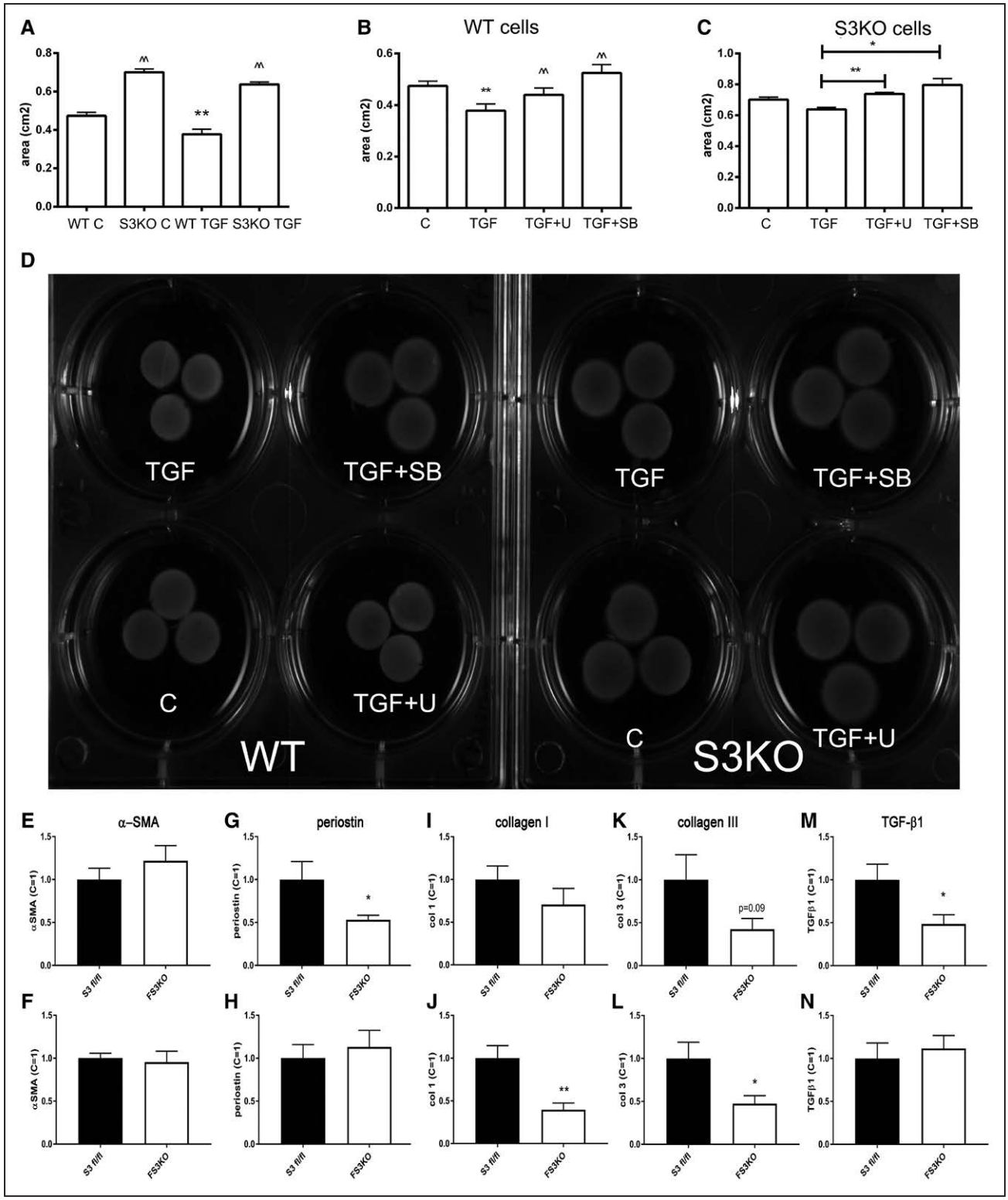

Figure 4. Smad-dependent and Smad-independent pathways mediate fibroblast-induced collagen pad contraction. **A,** Smad3 knockout (KO) fibroblasts exhibited impaired capacity to contract collagen pads (^^*P*<0.01 versus wild-type [WT], n=6). Transforming growth factor (TGF)–β1 increases contraction in WT cells (**P*<0.01, n=6) but has no significant effects on Smad3 KO cells. **B,** Both Erk inhibition (with U0126) and p38 mitogen-activated protein kinase (MAPK) inhibition (with SB203580) attenuated TGF-β–induced pad contraction (^^*P*<0.01 versus TGF, n=6), **C,** Incubation with U0126 or SB203580 reduced pad contraction in TGF-β–stimulated Smad3 KO cells, suggesting that Erk and p38 MAPK actions are independent of Smad3 (**P*<0.05, ***P*<0.01 versus TGF; n=6). **D,** Representative experiments illustrate the findings. **E**–**N,** Comparison of gene expression in infarct fibroblasts harvested from Smad3$^{fl/fl}$ and Smad3$^{fl/fl}$Postn-Cre (FS3KO) mice after 3 days (*Continued*)

known Smad3 activators on fibroblast-induced pad contraction. Only TGF-β1, TGF-β2, and TGF-β3 increased fibroblast-mediated pad contraction. In contrast, activins A and B, myostatin, and growth differentiation factor–11 had no effects (Figure V in the online-only Data Supplement). Then we examined the role of Smad3 in contraction of fibroblast-populated collagen pads. We obtained Smad3 KO (S3KO) cardiac fibroblasts from mice with global Smad3 loss.[5] S3KO cells had impaired capacity to contract collagen pads at baseline and on stimulation with TGF-β1 (Figure 4A and 4D). To investigate the potential role of Smad-independent pathways, we performed Erk and p38 mitogen-activated protein kinase (MAPK) inhibition experiments. Coincubation with the Erk inhibitor U0126 or the p38 MAPK inhibitor SB203580 significantly attenuated TGF-β1–mediated pad contraction in both WT and S3KO cells (Figure 4B and 4C), suggesting that gel contraction involved both Smad3-dependent and Smad-independent actions.

Effects of Fibroblast-Specific Smad3 Loss on Synthesis of α-SMA and Extracellular Matrix Proteins

Because α-SMA induction and incorporation in the cytoskeleton are implicated in contractile function of myofibroblasts,[15] we hypothesized that perturbed collagen pad contraction by S3KO fibroblasts in vitro and impaired scar remodeling in vivo may be a result of reduced α-SMA expression levels. To examine whether impaired scar contraction in FS3KO mice was a result of attenuated myofibroblast conversion, we compared α-SMA expression levels in infarct myofibroblasts harvested from $S3^{fl/fl}$ and FS3KO mice after 3 and 7 days of reperfusion. $S3^{fl/fl}$ and FS3KO fibroblasts showed comparable levels of α-SMA expression after 3 days (Figure 4E) and 7 days (Figure 4F) of reperfusion. Comparison of extracellular matrix protein expression levels showed that FS3KO cells had lower periostin expression after 3 days of reperfusion and reduced collagen I and III synthesis after 7 days of reperfusion (Figure 4G through 4L). Expression levels of TGF-β1 were lower in infarct fibroblasts harvested at the 3-day time point (Figure 4M and 4N).

Because ED-A fibronectin is critically involved in myofibroblast conversion and activation in response to TGF-β, we examined whether fibroblast-specific Smad3 loss affects fibronectin transcription and localization in the healing infarct. Quantitative polymerase chain reaction analysis showed that fibroblasts harvested from infarcted FS3KO mice after 7 days of reperfusion had significantly lower fibronectin transcription. Moreover, dual immunofluorescence studies showed that FS3KO mice had attenuated ED-A fibronectin immunoreactivity in areas exhibiting myofibroblast infiltration (Figure VI in the online-only Data Supplement).

Next, we examined the effects of Smad3 loss on α-SMA expression by activated cardiac fibroblasts in vitro using 2 different models: cardiac fibroblasts cultured in the high-tension environment of the culture plate and cells cultured in the low-tension environment of the pad. As we have recently demonstrated,[16] high-tension fibroblasts expressed 70- to 80-fold higher α-SMA mRNA levels than low-tension pad fibroblasts (Figure VII in the online-only Data Supplement). Immunofluorescence showed that although high-tension fibroblasts exhibit a myofibroblast phenotype, associated with intense cytoskeletal α-SMA staining, low-tension pad fibroblasts have a dendritic morphology and express low levels of punctate α-SMA staining (Figure VIIB in the online-only Data Supplement). In contrast to the reduction in α-SMA levels noted in high-tension Smad3 KO fibroblasts, Smad3 loss did not affect the low levels of α-SMA expressed by low-tension pad cells (Figure VIIA through VIIC in the online-only Data Supplement). Thus, impaired collagen contraction in pads populated with S3KO cells and perturbed scar contraction in FS3KO mice (Figures 3 and 4) cannot be attributed to attenuated α-SMA expression.

Fibroblast-Specific Smad3 Loss Perturbs Scar Organization

To identify fundamental alterations responsible for defective contractile activity in the absence of Smad3, we compared the morphological characteristics of infarct myofibroblasts between $Smad3^{fl/fl}$ and FS3KO animals. In the infarcted myocardium, myofibroblasts are organized in arrays and exhibit alignment along the direction of the ventricular wall. Fibroblast-specific Smad3 loss resulted in defective organization of the healing scar, associated with perturbed alignment of infarct myofibroblasts that exhibited a more rounded shape and chaotic orientation (Figure 5A through 5F). Quantitative analysis demonstrated that infarct myofibroblasts in FS3KO mice had a comparable mean cell area (Figure 5G) but a smaller perimeter (Figure 5H), lower perimeter/area ratio (Figure 5I), and reduced long axis/short axis ratio (Figure 5J). To compare the alignment of fibroblasts in the healing infarct, we measured the angle between the long axis of the fibroblast and the tangent of the ventricular wall. In FS3KO infarcts, the mean angle

Figure 4 Continued. (**E**, **G**, **I**, **K**, **M**) and 7 days (**F**, **H**, **J**, **L**, **N**) of reperfusion. Fibroblast-specific Smad3 loss did not affect α-SMA mRNA expression (**E** and **F**). FS3KO fibroblasts exhibited lower periostin (**G**) and TGF-β1 (**M**) expression after 3 days of reperfusion and lower levels of collagen I and III mRNA (**J** and **L**) after 7 days of reperfusion (**P*<0.05, ***P*<0.01 versus corresponding $Smad3^{fl/fl}$; n=5–6).

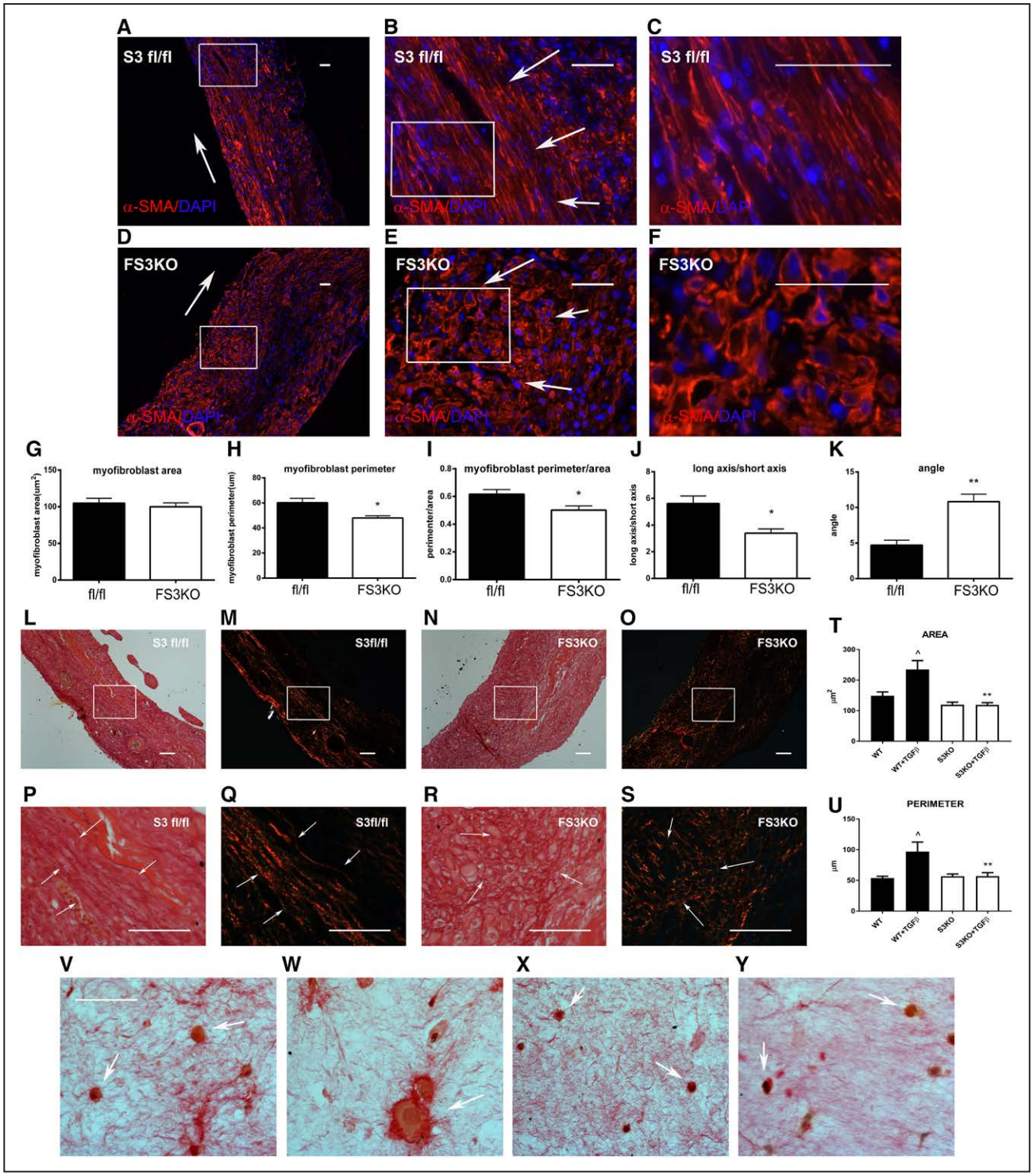

Figure 5. Fibroblast-specific Smad3 loss perturbs scar organization and attenuates transforming growth factor (TGF)–β—mediated changes in cell geometry.

A–**C,** α-SMA staining of healing myocardial infarcts (7 days) shows that, in Smad3$^{fl/fl}$ mice, α-SMA^{+} myofibroblasts are spindle-shaped cells, localized in highly organized arrays (arrows, **B** and **C**). **D**–**F,** In contrast, distribution of α-SMA^{+} myofibroblasts in Smad3$^{fl/fl}$Postn-Cre (FS3KO) mouse infarcts is disorganized and chaotic because the cells have a rounded morphology and are misaligned (arrows, **E** and **F**) (scale bar=30 μm). Although the mean area of the myofibroblasts was comparable between groups (**G**), the cell perimeter (**H**), perimeter/area ratio (**I**), and long axis/short axis ratio (**J**) were lower in FS3KO animals (*P<0.05, n=5), reflecting the altered geometry of the cells. **K,** To quantitatively assess cell alignment in the healing infarct, we measured the angle between the long axis of the cell and the tangent of the ventricular wall (indicated by an arrow in **A** and **D**). The alignment angle was markedly higher in FS3KO mice, reflecting cellular malalignment in the absence (*Continued*)

was significantly higher than in Smad3$^{fl/fl}$ animals (Figure 5K), indicating that fibroblast-specific Smad3 loss resulted in the misalignment of infarct myofibroblasts. Polarized light microscopy of Sirius red–stained sections showed that FS3KO mice also exhibited perturbations in the alignment of collagen fibers (Figure 5L through 5S). In vitro experiments showed that Smad3 loss abrogated the increase in cell area and perimeter induced by TGF-β1 stimulation in cardiac fibroblasts cultured in collagen pads (Figure 5T through 5Y).

Smad3 Mediates α2, α5, and β3 Integrin Expression in Cardiac Fibroblasts

Misalignment of myofibroblasts in FS3KO infarcts suggests impaired interactions between cells and the extracellular matrix. We hypothesized that Smad3 loss may perturb scar organization by altering the expression of integrins, surface proteins that mediate cell–matrix interactions. Accordingly, we examined the effects of Smad3 loss on integrin expression in pad fibroblasts. S3KO fibroblasts had significantly lower baseline expression of α2, α5, and β3 integrin (Figure 6A through 6C). Moreover, TGF-β1 stimulation markedly upregulated α2, α5, and β3 integrin expression in WT cells but not in S3KO fibroblasts (Figure 6A through 6C). In contrast, Smad3 loss did not affect fibroblast α1 and β1 integrin expression (Figure 6D and 6E). To investigate the role of Smad-independent pathways in the regulation of integrin synthesis, we examined the effects of Erk and p38 MAPK inhibition (Figure VIII in the online-only Data Supplement). Erk inhibition attenuated TGF-β1–mediated β3, α2, and α5 integrin mRNA expression but had no effects on β1 and α1 integrin synthesis. p38 MAPK inhibition attenuated α5 integrin expression without affecting synthesis of the other integrins. Erk and p38 MAPK inhibition did not affect integrin expression in Smad3 KO cells, suggesting that the effects of Erk and p38 in WT cells are not independent of Smad3.

α2 and α5 Integrin Mediate Contraction in Fibroblast-Populated Pads

Next we examined whether integrin blockade affects fibroblast function. In the collagen pad assay, α2 and α5 integrin blockade markedly impaired collagen contraction mediated by both WT and S3KO cells (Figure 6F), demonstrating the critical role of integrin signaling in pad contraction.

Smad3-Mediated Integrin Activation Induces NOX2 Transcription in Cardiac Fibroblasts

Reactive oxygen species generation is critical for fibroblast function;[17] activation of integrin signaling stimulates an oxidative response.[18] Accordingly, we examined the role of integrin signaling in the expression of genes associated with reactive oxygen generation and scavenging in cardiac fibroblasts. In WT cells, α5 but not α2 blockade markedly reduced NOX2 expression levels (Figure 6G) but had no effects on superoxide dismutase (SOD)–1, SOD2, and glutathione reductase levels (Figure 6H through 6J). S3KO cells exhibited markedly lower baseline expression of NOX2 (Figure 6G) and significantly higher expression of SOD1 (Figure 6H) but comparable SOD2 and glutathione reductase levels. In contrast to its effects on WT cells, α5 blockade did not affect NOX2 expression in S3KO cells (Figure 6G), suggesting that NOX2 transcription in cardiac fibroblasts is dependent on Smad3-mediated α5 integrin activation.

Integrin α5 Is Not Sufficient for Contraction of Fibroblast-Populated Collagen Pads

Next we performed overexpression experiments to investigate whether defective function of Smad3-null fibroblasts is caused by attenuated α5 integrin synthesis (Figure 6K). Although α5 integrin overexpression increased pad contraction in WT cells, no significant effects were noted in pads populated with Smad3 KO cells, indicating that α5 expression is not sufficient to restore defective function in Smad3 KO fibroblasts (Figure 6L).

Cardiomyocyte-Specific Smad3 Loss Attenuates Adverse Remodeling After Myocardial Infarction

In contrast to the detrimental effects of fibroblast-specific loss of Smad3 (Figure 1), mice with global loss of

Figure 5 Continued. of Smad3 (**P<0.01 versus *fl/fl*, n=5). Perturbed cellular alignment would be expected to reduce the tractional forces exerted by the cells, resulting in impaired scar contraction. **L–S,** Both light microscopy (**L**, **N**, **P**, **R**) and polarized light microscopy (**M**, **O**, **Q**, **S**) in Sirius red–stained sections showed that although Smad3$^{fl/fl}$ animals had aligned collagen fibers (**P**–**Q**, arrows), FS3KO animals exhibited areas of matrix disorganization (**R** and **S**, arrows) (scale bar=40 μm). **T–Y,** Smad3 loss abrogated the effects of TGF-β1 on fibroblast shape. Fibroblasts from wild-type (WT) (**V** and **W**) and Smad3 knockout (KO) mice (S3KO, **X** and **Y**) were cultured in collagen pads in the presence (**W** and **Y**) or absence (**V** and **X**) of TGF-β1. Sections of fibroblast-populated collagen pads were stained with Sirius red/hematoxylin to identify fibroblasts (arrows) and the extracellular matrix (scale bar=25 μm). TGF-β1 stimulation increased cell area and perimeter in WT but not in S3KO fibroblasts (**T** and **U**) (^P<0.05 versus WT, **P<0.01 versus corresponding WT, n=6–7).

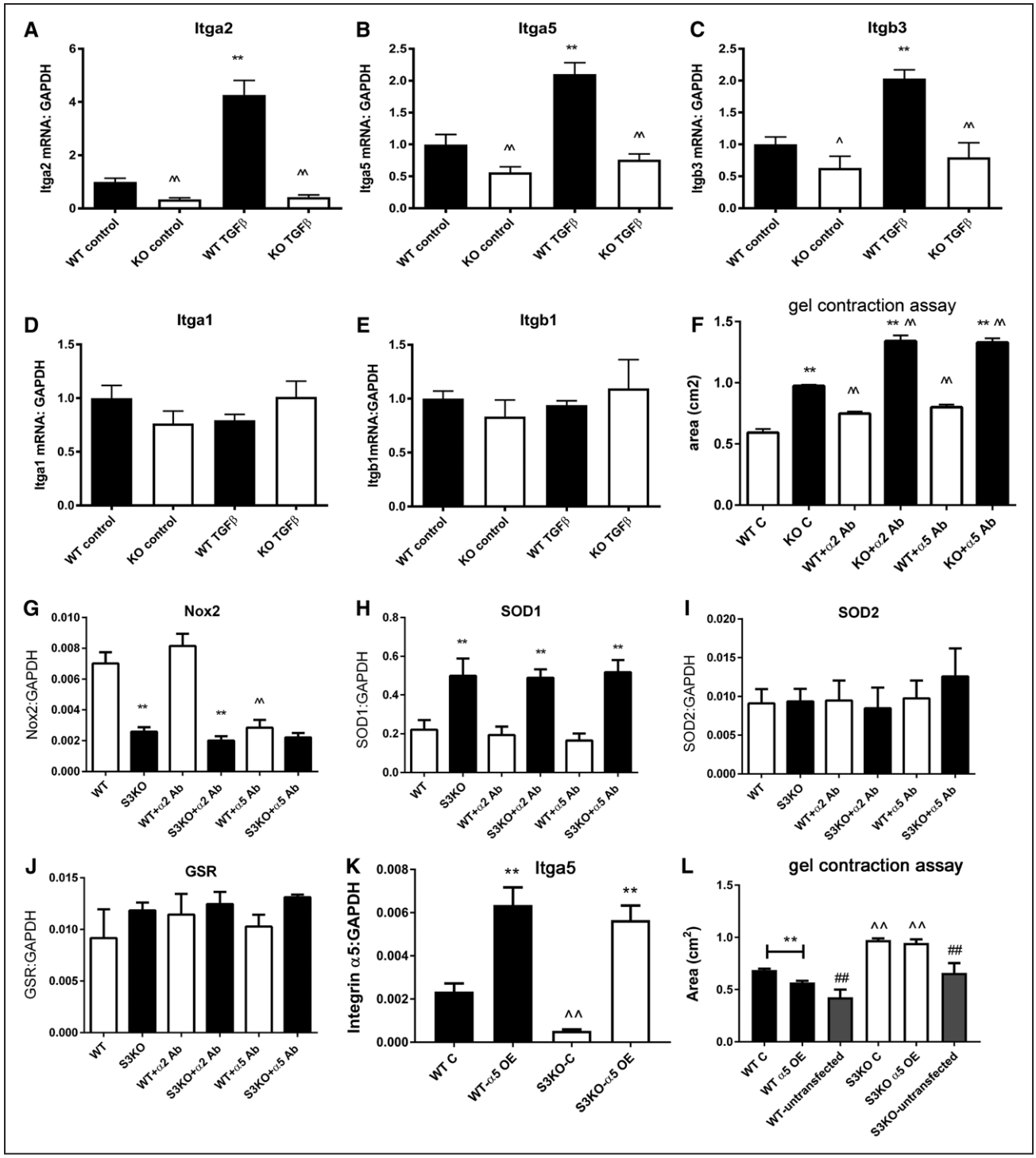

Figure 6. Smad3 loss markedly reduces expression of α2, α5, and β3 integrins in cardiac fibroblasts.
A–**C,** In comparison with wild-type (WT) cells, Smad3 knockout (KO) cardiac fibroblasts had significantly lower baseline expression of α2 (**A**, Itga2), α5 (**B**, Itga5), and β3 (**C**, Itgb3) mRNA. Transforming growth factor (TGF)–β1 stimulation markedly induced α2, α5, and β3 expression in cardiac fibroblasts in WT cells (***P*<0.01 versus WT control, n=6) but not in Smad3 KO cells. α2, α5, and β3 integrin expression levels were significantly lower in Smad3 KO cells in the presence or absence of TGF-β1 (^*P*<0.05, ^^*P*<0.01, n=6). **D** and **E,** In contrast, Smad3 loss did not affect α1 (**D**, Itga1) and β1 (**E**, Itgb1) integrin expression (expression normalized to **C**; WT=1). **F**–**J,** Integrins mediate fibroblast activation, inducing expression of NOX2. **F**, α2 and α5 integrin mediate pad contraction in both WT and Smad3 KO cells. α2 or α5 integrin blockade reduces contraction of collagen pads populated with WT or Smad3 KO fibroblasts (^^*P*<0.01 versus corresponding control, n=6). KO cells exhibit impaired capacity to contract gels in comparison with WT cells (***P*<0.01). **G**, α5 blockade attenuates NOX2 mRNA synthesis in WT fibroblasts (^^*P*<0.01 versus WT control, n=3). In contrast, α2 blockade does not affect NOX2 expression. (*Continued*)

Smad3 exhibit attenuated remodeling.[4] Thus, Smad3 signaling in other cell types involved in cardiac injury and repair may exert detrimental actions on the infarcted heart. Because TGF-β signaling and the Smad3 pathway have been implicated in the regulation of cardiomyocyte function and survival in vitro,[19] we examined the in vivo role of cardiomyocyte-specific Smad3 signaling after myocardial infarction. Smad3$^{fl/fl}$α-myosin heavy chain (MHC)-Cre (CMS3KO) mice were healthy and exhibited normal baseline cardiac geometry and function at 4 months of age (Figure I in the online-only Data Supplement). mRNA analysis showed that when compared with Smad3$^{fl/fl}$ animals, CMS3KO mice had significantly lower Smad3 levels (Figure IXA in the online-only Data Supplement). Dual fluorescence combining Smad3 immunohistochemistry and wheat germ agglutinin lectin histochemistry (to outline cardiomyocytes) showed that CMS3KO mice had reduced Smad3 immunoreactivity in cardiomyocytes (Figure IXB in the online-only Data Supplement). Echocardiographic analysis showed that, when compared with Smad3$^{fl/fl}$ controls, CMS3KO mice had reduced left ventricular dilation, attenuated systolic dysfunction, and decreased hypertrophic remodeling (Figure 7 and Figure X in the online-only Data Supplement) after reperfused infarction.

The Protective Effects of Cardiomyocyte-Specific Smad3 Loss Are Not Caused by a Reduction in Acute Infarct Size

Because attenuated remodeling in the model of reperfused infarction may reflect reduced acute cardiomyocyte injury, we examined the effects of cardiomyocyte-specific Smad3 loss on the size of the infarct. CMS3KO and Smad3$^{fl/fl}$ controls had a comparable area at risk and infarct size/area at risk ratio (Figure XIA through XIC in the online-only Data Supplement). Moreover, cardiomyocyte-specific loss of Smad3 did not significantly affect scar size after 7 to 28 days of reperfusion (Figure XID in the online-only Data Supplement). Quantitative analysis of Sirius red–stained sections visualized under polarized light microscopy demonstrated that CMS3KO mice had reduced deposition of thinner green collagen fibers in the remote remodeling myocardium after 7 days of reperfusion. After 28 days of reperfusion, CMS3KO mice had significantly increased deposition of thicker red collagen fibers in the infarct zone and marked reductions in deposition of orange and green collagen fibers in the remote remodeling myocardium (Figure XIE through XIX in the online-only Data Supplement).

Effects of Cardiomyocyte-Specific Smad3 Loss on Cardiomyocyte Apoptosis After Myocardial Infarction

Next we examined whether protection from adverse remodeling in cardiomyocyte-specific Smad3 KO mice is caused by attenuated cardiomyocyte apoptosis. Dual labeling for terminal deoxynucleotidyl transferase dUTP nick end labeling and wheat germ agglutinin was performed at 2 different time points after 2 and 7 days of reperfusion. No significant difference occurred in the density of apoptotic cardiomyocytes in the infarcted area after 48 hours of reperfusion. After 7 days of reperfusion, cardiomyocyte-specific loss of Smad3 was associated with a modest but significant attenuation in the number of apoptotic cardiomyocytes in the viable remodeling myocardium (Figure 8A and 8B).

CMS3KO Mice Have Reduced NOX2 Expression and Attenuated Nitrosative Stress in the Remodeling Myocardium

Oxidative and nitrosative stress are implicated in cardiomyocyte apoptosis.[20] Because our findings in fibroblasts suggested that Smad3 may regulate fibroblast function stimulating NOX2 expression, we hypothesize that the proapoptotic effects of cardiomyocyte Smad3 may also involve modulation of genes involved in reactive oxygen species generation and scavenging. Quantitative polymerase chain reaction analysis demonstrated that after 7 days of reperfusion, CMS3KO mice had significantly reduced NOX2 levels in the noninfarcted remodeling myocardium (Figure 8C) but comparable NOX4 and SOD1 expression (Figure 8D and 8E). No significant differences were noted in the NOX2, NOX4, and SOD1 levels between infarcted segments. Moreover, cardiomyocyte-specific Smad3 loss also reduced nitrosative

Figure 6 Continued. Smad3 KO cells exhibited markedly lower expression of NOX2 (**P<0.01 versus WT). In contrast to its effects on WT cells, α5 blockade did not affect NOX2 expression in Smad3 KO cells, suggesting that NOX2 transcription in cardiac fibroblasts is dependent on Smad3-mediated α5 integrin activation. **H,** Smad3 KO cells also had a higher expression of superoxide dismutase (SOD)–1 (**P<0.01 versus corresponding WT). However, SOD1 levels were not affected by integrin inhibition. **I** and **J**, SOD2 and GSR expression was not affected by Smad3 loss or integrin blockade. **K** and **L**, α5 integrin overexpression does not reverse the contraction defect in Smad3 KO cells. **K**, α5 integrin overexpression in WT and Smad3 KO cells resulted in markedly increased α5 integrin expression (**P<0.01 versus corresponding controls, ^^P<0.01 versus WT C, n=6). **L**, Although α5 integrin overexpression (OE) increased contraction in WT cells, no effects on pad contraction were noted in Smad3 KO cells, indicating that α5 integrin is not sufficient to correct the contraction defect. Please note that untransfected cells exhibit increased contraction, suggesting that transfection modestly but significantly reduces contraction in fibroblast-populated pads (##P<0.01 versus corresponding cells transfected with control plasmid).

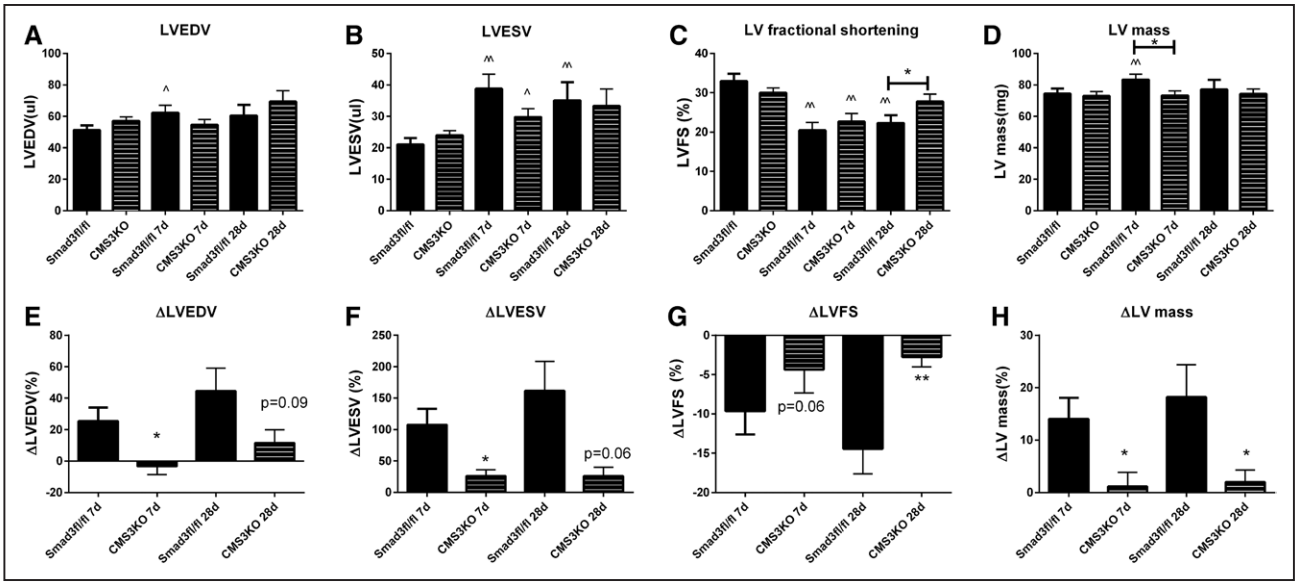

Figure 7. Cardiomyocyte-specific Smad3 loss attenuates systolic dysfunction after infarction and reduces hypertrophic remodeling.

A–C, Although LVEDV (**A**) and LVESV (**B**) were comparable between CMS3KO and Smad3$^{fl/fl}$ animals, cardiomyocyte-specific Smad3 loss attenuated systolic dysfunction (28 days) (**C**). **D,** LV mass was lower in CMS3KO mice after 7 days of reperfusion (*P<0.05, n=10–18/group; ^P<0.05, ^^P<0.01 versus corresponding baseline values). **E** and **F,** CMS3KO mice exhibited significantly lower ΔLVEDV and ΔLVESV after 7 days and trends toward reduced ΔLVEDV and ΔLVESV after 28 days of reperfusion, reflecting attenuated dilative remodeling. **G,** ΔLVFS was significantly lower in CMS3KO animals after 28 days, reflecting decreased systolic dysfunction (**P<0.01). **H,** ΔLVmass was markedly attenuated at both time points (*P<0.05).

stress after myocardial infarction. Western blotting demonstrated that CMS3KO animals had markedly reduced 3-nitrotyrosine levels in the noninfarcted remodeling myocardium and the infarcted area (Figure 8F and 8G).

CMS3KO Mice Have Reduced MMP2 Levels in the Infarct and in the Noninfarcted Remodeling Myocardium

Induction and activation of MMPs play important roles in adverse remodeling of the infarcted heart.[21,22] Because TGF-β signaling pathways are involved in the regulation of MMPs,[23,24] we examined whether cardiomyocyte-specific Smad3 loss affects MMP2 expression. CMS3KO mice had markedly lower levels of latent and active (cleaved) MMP2 in the infarcted segment and in the noninfarcted remodeling myocardium (Figure 8H through 8J).

DISCUSSION

We report for the first time that cell-specific activation of Smad3 signaling in cardiomyocytes and cardiac myofibroblasts differentially regulates repair and remodeling of the infarcted heart. Using cell-specific Smad3 KO mice and in vitro studies, we demonstrate that fibroblast-specific Smad3 signaling is crucial for scar organization, mediating the formation of aligned myofibroblast arrays in the infarct border zone. Smad3 restrains fibroblast proliferation and stimulates integrin-dependent fibroblast activation and NOX2 transcription. In contrast, Smad3 actions in cardiomyocytes are detrimental, accentuating adverse remodeling and worsening systolic dysfunction after myocardial infarction. Cardiomyocyte-specific Smad3 does not act by increasing acute infarct size but accentuates NOX2 transcription, augments nitrosative stress, and increases MMP2 expression in noninfarcted remodeling myocardial segments. These actions may stimulate cardiomyocyte apoptosis and trigger MMP-dependent degradation of contractile proteins, thus promoting dysfunction. Our findings dissect the cell-specific actions of TGF-β–mediated Smad signaling in injury and repair and highlight the crucial role of activated cardiac myofibroblasts in protecting the infarcted heart (Figure 9).

Myofibroblast Activation in Repair and Remodeling of the Infarcted Heart

The adult mammalian heart has negligible endogenous regenerative capacity. After myocardial infarction, sudden death of a large number of cardiomyocytes triggers an inflammatory response that clears the wound of dead cells and activates fibroblasts and vascular cells, resulting in the formation of granulation tissue. During the early hours after reperfused myocardial infarction, the abundant cardiac fibroblasts that reside in the ischemic myocardium respond to the proinflammatory environment of

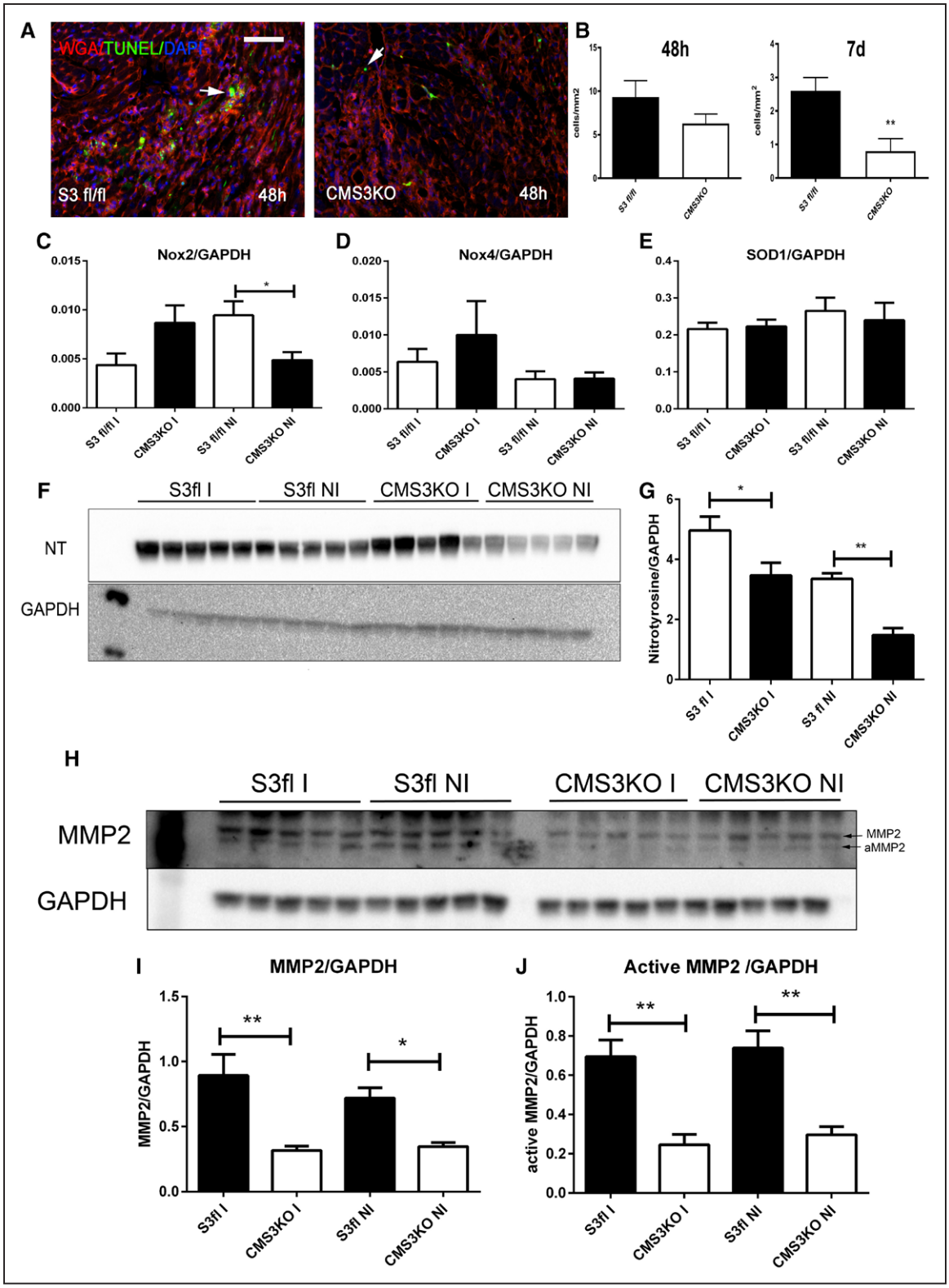

Figure 8. Cardiomyocyte-specific Smad3 loss attenuated cardiomyocyte apoptosis in the viable remodeling myocardium, reduced NOX2 expression, and decreased nitrosative stress and matrix metalloproteinase–2 (MMP2) expression.

A and **B**, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)/Wheat Germ Agglutinin (WGA) dual staining was used to identify apoptotic cardiomyocytes in the infarcted myocardium after 48 hours of reperfusion and in the viable remodeling myocardium after 7 days of reperfusion (scale bar=50 μm). No statistically significant difference in the density of apoptotic cardiomyocytes was noted after 48 hours of reperfusion (*P*=0.21, n=6–7/group). After 7 days of (*Continued*)

the infarct by releasing cytokines and chemokines and by secreting MMPs.[25] Induction of interleukin-1β in the infarct zone inhibits myofibroblast conversion,[25] preventing premature matrix deposition until the wound is cleared of dead cells and matrix debris. Debridement of the wound and phagocytosis of apoptotic cells by professional macrophages are associated with activation of inhibitory cascades that suppress inflammation while stimulating the reparative properties of fibroblasts. During the proliferative phase of infarct healing, the cardiac fibroblast population is enriched through the proliferation of resident fibroblasts via endothelial to mesenchymal transition and through recruitment of circulating fibroblast progenitors.[26,27] Fibroblasts in the healing infarct acquire a matrix-synthetic myofibroblast phenotype[28] and form highly organized arrays in the infarct border zone.[29] Experimental studies have suggested that activated cardiac myofibroblasts may serve reparative functions[30] but may also promote diastolic dysfunction by secreting extracellular matrix proteins in the cardiac interstitium[4] and stimulating cardiomyocyte hypertrophy.[31] Whether reparative and profibrotic fibroblast functions are mediated through distinct molecular pathways remains unknown.

Fibroblast-Specific Smad3 Signaling Protects the Infarcted Heart From Adverse Remodeling, Playing a Critical Role in Activation and Topographical Organization of the Fibroblast-Based Scar

TGF-β is induced and activated in the healing infarct[1,32] and is ideally suited to act as a key regulator of the myofibroblast phenotype. In vitro, TGF-β activates myofibroblast conversion and promotes a matrix-synthetic phenotype,[33] exerting actions mediated through Smad-dependent and Smad-independent pathways.[5,34,35] Our findings demonstrate for the first time that cell-specific activation of Smad3 signaling in infarct myofibroblasts is of critical significance for cardiac repair. Myofibroblast-specific loss of Smad3 accentuates adverse remodeling and dysfunction in a model of reperfused myocardial infarction and increases mortality, causing late rupture in a model of nonreperfused infarction (Figures 1 through 3). The deleterious effects of myofibroblast-specific Smad3 loss are not caused by effects on the size of the acute infarct but involve disruption of key reparative functions that regulate scar organization and remodeling.

Our in vivo and in vitro findings suggest that fibroblast Smad3 signaling mediates formation of an organized scar by restraining fibroblast proliferation and stimulating integrin-dependent fibroblast activation, fibroblast-derived collagen synthesis, and subsequent formation of well-aligned arrays of myofibroblasts in the infarct border zone (Figure 9). The phenotypic and functional alterations of Smad3-null infarct myofibroblasts are associated with perturbed organization of the collagen fibers in the healing scar (Figure 5). Smad3 loss in fibroblasts does not affect the expression of α-SMA by activated infarct myofibroblasts in vivo (Figure 4) but is associated with a hyperproliferative fibroblast phenotype and perturbed contraction of the collagenous matrix both in vitro and in vivo (Figures 3 and 4). Defective contraction of the scar in mice with fibroblast-specific loss of Smad3 may accentuate adverse remodeling by increasing the length of the infarcted segment, thus promoting chamber dilation. Optimal force transmission in the healing infarct is dependent on the presence of well-aligned arrays of fibroblasts in the infarct border zone. In contrast, chaotic orientation of infarct myofibroblasts in the absence of Smad3 may result in perturbed wound contraction and adverse ventricular remodeling.

Smad3 is critically involved in the regulation of fibroblast–matrix interactions by inducing the expression of integrins, the molecular anchors that bridge the cells to the extracellular matrix.[18,36,37] Smad3 loss in fibroblasts markedly reduces the synthesis of α2, α5, and β3 integrins (Figure 6), disrupting interactions between cells and the extracellular matrix, which are critical for fibroblast function and wound contraction. The role of integrin-dependent actions in mediating Smad-dependent profibrotic actions has been recently suggested in a model of renal fibrosis.[38] Our findings suggest that the loss of α2 or α5 integrin–dependent signaling inhibits fibroblast-mediated contraction of the collagenous matrix (Figure 6F) and perturbs fibroblast organization in the healing scar. Integrin-mediated adhesion triggers an oxidative response (Figure 6G) and may regulate actin polymerization[18,39] and extracellular matrix remodeling[40] in cardiac fibroblasts. However, α5 integrin overexpression in Smad3 KO fibroblasts was not sufficient to induce contraction in pads populated with Smad3 KO cells (Figure 6L), suggesting that restoration of fibroblast function may require cooperation of additional molecular signals. A growing body of evidence suggests that Smads interact with other intracellular effectors, such

Figure 8 Continued. reperfusion, cardiomyocyte-specific Smad3 loss attenuated cardiomyocyte apoptosis in the viable remodeling myocardium (**P*<0.01 versus S3$^{fl/fl}$, n=6). **C**, CMS3KO mice had reduced NOX2 mRNA expression in the noninfarcted remodeling myocardium (NI) after 7 days of reperfusion (*P*<0.05, n=5–9/group). **D** and **E,** NOX4 (**D**) and superoxide dismutase (SOD)–1 (**E**) levels were comparable between CMS3KO and Smad3$^{fl/fl}$. NOX2, NOX4, and SOD1 expression in infarcted segments (I) was comparable between groups (**C**–**E**). **F** and **G,** CMS3KO mice exhibited reduced 3-nitrotyrosine levels in infarcted (I) and noninfarcted (NI) areas, reflecting attenuated nitrosative stress (*P*<0.05, **P*<0.01, n=5/group). **H**–**J,** Moreover, CMS3KO animals had decreased levels of total and active MMP2 (aMMP2) (*P*<0.05, **P*<0.01, n=8/group).

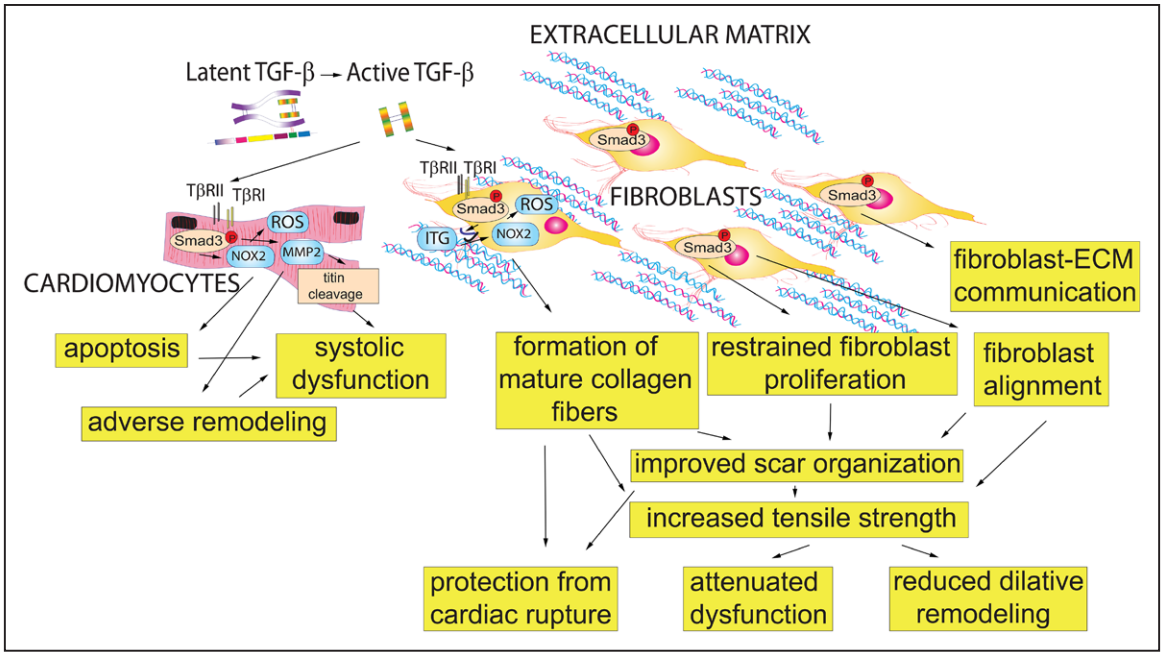

Figure 9. Schematic illustration of the novel findings of the study.
Generation of active transforming growth factor (TGF)–β in the infarcted myocardium triggers Smad3 activation in fibroblasts and cardiomyocytes. Our study investigates for the first time the role of fibroblast and cardiomyocyte-specific Smad3 signaling in the infarcted myocardium. Smad3 activation in cardiac fibroblasts restrains cell proliferation and controls alignment of fibroblasts in the infarct and the formation of an organized collagen-based scar, preventing late cardiac rupture and adverse dilative remodeling. Our findings suggest that Smad3-dependent activation of a novel integrin (ITG)-NOX2 axis may stimulate extracellular matrix (ECM) protein deposition and organization in the infarcted heart. In contrast, activation of Smad3 signaling in cardiomyocytes has deleterious effects, promoting cardiomyocyte apoptosis and enhancing adverse remodeling and dysfunction. The effects of Smad3 on cardiomyocytes may also involve activation of a NOX2-mediated reactive oxygen species (ROS)-dependent axis. However, in cardiomyocytes, oxidative stress may promote cell death and accentuate MMP2 expression. Overactive MMP2 may cause adverse remodeling through its effects on the ECM and may exacerbate systolic dysfunction by targeting proteins involved in sarcomere function, such as titin.

as signal transducer and activator of transcription 3, to modulate the fibroblast phenotype.[41]

Cardiomyocyte Smad3 Signaling Accentuates Cardiac Remodeling

Cardiomyocytes in the infarct border zone and remodeling myocardium also exhibit activation of TGF-β/Smad-dependent signaling[4] and are highly responsive to the effects of TGF-β. In vitro experiments have suggested both proapoptotic and antiapoptotic effects of TGF-β on cardiomyocytes,[42,43] highlighting the context-dependent actions of the cytokine. In vivo studies using conditionally targeted mice suggested that, in the infarcted myocardium, cardiomyocyte-specific TGF-β signaling suppresses the expression of protective cytokines, enhancing inflammatory leukocyte infiltration and causing cardiac rupture.[44] Our study demonstrates that, in contrast to the critical reparative role of fibroblast Smad3, cardiomyocyte-specific Smad3 signaling is implicated in chronic adverse remodeling of the ventricle. Cardiomyocyte-specific loss of Smad3 had no effect on baseline cardiac geometry and function but attenuated adverse remodeling and dysfunction after reperfused myocardial infarction (Figure 7). The protective actions of cardiomyocyte Smad3 disruption were not caused by effects on the size of the infarct but reflected attenuated cardiomyocyte apoptosis in the noninfarcted remodeling segments, associated with reduced NOX2 expression levels, decreased nitrosative stress, and significantly lower MMP2 levels (Figure 8). Attenuated MMP2 expression in CMS3KO infarcts is associated with the formation of a scar rich in thick collagen fibers that may increase tensile strength, preventing dilation and adverse remodeling of the ventricle. However, the effects of MMP2 may not be limited to remodeling of the extracellular matrix network. In cardiomyocytes, increased oxidative and nitrosative stress mediates cardiomyocyte apoptosis[45] and may also augment MMP expression.[46] Myocardial MMP2 expression after ischemic injury is known to induce cardiac dysfunction through effects independent of extracellular matrix proteolysis.[47] In the ischemic and reperfused myocardium, MMP2 cleaves titin, promoting systolic dysfunction.[48] Thus, the distinct effects of fibroblast- and cardiomyocyte-specific Smad3 responses on the infarcted heart may reflect the consequences of

cell-specific activation of a Smad-dependent oxidative response. In fibroblasts, integrin-mediated oxidative activity may be important for cardiac repair. In contrast, in cardiomyocytes, Smad-dependent oxidative stress may induce dysfunction by stimulating MMP-mediated degradation of contractile proteins (Figure 9).

Targeting the TGF-β/Smad System in Cardiac Remodeling

Despite its critical involvement in the pathogenesis of cardiac remodeling,[44,49] TGF-β remains a challenging therapeutic target. A recently published study showed that in the pressure-overloaded heart, fibroblast-specific Smad3 signaling critically contributes to the fibrotic process,[50] suggesting that interventions targeting Smad-dependent signaling may be effective in patients with chronic heart failure by attenuating fibrosis. In contrast, our findings highlight the reparative function of fibroblasts in infarctive myocardial injury. Because the adult mammalian heart lacks regenerative capacity, after myocardial infarction, activated fibroblasts serve a critical reparative role, preserving the structural integrity of the ventricle and protecting from adverse remodeling. Moreover, the contrasting functional consequences of fibroblast and cardiomyocyte-specific Smad3 loss in the infarcted heart illustrate the cellular specificity of TGF-β/Smad3–dependent actions. Because most cell types are highly responsive to the effects of TGF-β family members, therapeutic targeting of TGF-β in pathological conditions is likely to interfere with both detrimental and beneficial actions. The complexity of TGF-β signaling, involving both Smad-dependent and Smad-independent actions, further complicates the design of therapeutic interventions. Dissection of cell-specific actions in vivo using genetic tools and design of interventions with specific cellular targets are needed for the development of safe and effective therapies.

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Disclosures

None.

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