

Cardiac Fibroblast Glycogen Synthase Kinase-3 β Regulates Ventricular Remodeling and Dysfunction in Ischemic Heart

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Background—Myocardial infarction-induced remodeling includes chamber dilatation, contractile dysfunction, and fibrosis. Of these, fibrosis is the least understood. After myocardial infarction, activated cardiac fibroblasts deposit extracellular matrix. Current therapies to prevent fibrosis are inadequate, and new molecular targets are needed.

Methods and Results—Herein we report that glycogen synthase kinase-3 β (GSK-3 β) is phosphorylated (inhibited) in fibrotic tissues from ischemic human and mouse heart. Using 2 fibroblast-specific GSK-3 β knockout mouse models, we show that deletion of GSK-3 β in cardiac fibroblasts leads to fibrogenesis, left ventricular dysfunction, and excessive scarring in the ischemic heart. Deletion of GSK-3 β induces a profibrotic myofibroblast phenotype in isolated cardiac fibroblasts, in post-myocardial infarction hearts, and in mouse embryonic fibroblasts deleted for GSK-3 β . Mechanistically, GSK-3 β inhibits profibrotic transforming growth factor- β 1/SMAD-3 signaling via interactions with SMAD-3. Moreover, deletion of GSK-3 β resulted in the significant increase of SMAD-3 transcriptional activity. This pathway is central to the pathology because a small-molecule inhibitor of SMAD-3 largely prevented fibrosis and limited left ventricular remodeling.

Conclusions—These studies support targeting GSK-3 β in myocardial fibrotic disorders and establish critical roles of cardiac fibroblasts in remodeling and ventricular dysfunction. (*Circulation*. 2014;130:419-430.)

Key Words: fibroblasts ■ fibrosis ■ glycogen synthase kinase 3 beta ■ hypertrophy ■ myocardial infarction

Post-myocardial infarction (MI) remodeling is a major cause of heart failure worldwide, but despite more aggressive approaches to prevent remodeling, these strategies often fail. MI and most other cardiac diseases are associated with myocardial fibrosis, which is characterized by excess deposition of extracellular matrix (ECM) and accumulation of cardiac fibroblasts (CFs). Fibroblasts are the predominant cell type in the adult heart, are the principal producers of ECM, and contribute significantly to myocardial fibrosis. Virtually every form of heart disease is associated with expansion and activation of the CF compartment.¹ The plasma levels of collagen markers, which correlate with ongoing cardiac fibrosis, are emerging as predictive markers for heart failure in humans.² However, fibroblasts are still considered to play a secondary role in adverse cardiac remodeling and heart failure. Furthermore, most of the existing literature on CF biology has been generated either from in vitro culture models or from a mouse model in which genetic manipulation has been targeted to cardiomyocytes only.

CFs are critically involved in both reparative and detrimental fibrotic responses after MI. In the healthy heart, resident fibroblasts are quiescent and produce limited amounts of ECM proteins.³ In response to the loss of a large number of cardiomyocytes in the ischemic heart because of necrotic cell death, CFs, together with inflammatory cells, infiltrate to the ischemic area to initiate healing and scar formation, thereby maintaining the structural integrity of the myocardium.⁴ In addition, during acute tissue injury, mesenchymal and inflammatory cells secrete transforming growth factor (TGF)- β 1 to induce fibroblast to myofibroblast transformation. Myofibroblasts are 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 ECM and play a critical role in both wound healing and tissue remodeling. Myofibroblasts are not present in the healthy myocardium.⁵ Although required for the reparative response and scar formation, persistent myofibroblast activity can lead to excessive scarring, loss of tissue compliance, and an extensive fibrotic response that is the basis for fibrotic disorders in numerous organs.^{4,6,7}

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TGF- β 1 signals through at least 2 independent routes: (1) primarily through the SMAD-dependent canonical pathway and (2) through the SMAD-independent or noncanonical pathway. In the canonical pathway, activation of TGF- β type 2 receptor (TGFBR2) activates TGF- β type I receptor (TBRI; also known as TGFBR1 or ALK5), and then the TBRI phosphorylates the transcription factors SMAD-2 and SMAD-3 (receptor SMADs; R-SMAD). On phosphorylation, R-SMADs, together with the common mediator SMAD-4 (CO-SMAD), translocate to the nucleus to regulate transcriptional responses. SMAD-6 and SMAD-7 are inhibitory SMADs (I-SMAD).^{7–9} TGF- β 1 can also signal through noncanonical SMAD-independent pathways that include mitogen-activated protein kinases, TNF receptor-associated factor 4 (TRAF4), TRAF6, TGF- β -activated kinase 1 (TAK1), RHO, PI3K, AKT, nuclear factor- κ B, and TRPC6.⁷

The roles of glycogen synthase kinase-3 β (GSK-3 β) in cardiac myocyte biology and disease have been studied extensively.^{10–13} However, the role of GSK-3 β in CF activation and fibrotic remodeling after MI is not known. In the present study, we achieve CF-specific deletion of GSK-3 β by using Cre recombinase driven by *Postn* (periostin) promoter in *GSK-3 β ^{fl/fl}* mice (Per-KO). In addition to Per-KO mice, we also used tamoxifen-inducible *Col1a2-cre* mice (Col-KO) to obtain conditional fibroblast-specific GSK-3 β knockout (KO) mice. We report that deletion of GSK-3 β leads to hyperactivation of profibrotic TGF- β 1/SMAD-3 signaling, which results in excessive fibrosis and adverse ventricular remodeling, after MI. Furthermore, using SIS3, a small-molecule SMAD-3 inhibitor, we implicate unrestrained SMAD-3 activity as the key factor driving the detrimental phenotype in GSK-3 β KO hearts. To our knowledge, these studies are the first to demonstrate a surprising effect of CF-specific gene targeting on global cardiac function and adverse remodeling after MI.

Methods

Please see the online-only Data Supplement for detailed methods. A detailed list of antibodies used in the present study is supplied in Table I in the online-only Data Supplement.

Fibroblast-Specific Deletion of GSK-3 β

All studies involving the use of animals were approved by the Institutional Animal Care and Use Committee of the Temple University School of Medicine. Generation and characterization of fibroblast-specific GSK-3 β KO mouse models are described in Results. At 12 weeks of age, Col-KO mice were placed on a tamoxifen chow diet (400 mg/kg) for 28 days followed by regular chow for an additional 15 days (to allow the clearance of tamoxifen from the mice). Mice *GSK-3 β ^{fl/fl}/Cre^{Tam}* were conditional knockout (Col-KO), whereas littermates *GSK-3 β ^{fl/fl}/Tam* represented controls (wild-type [WT]).

Statistical Analysis

Differences between data groups were evaluated for significance with the use of the nonparametric Mann-Whitney test or 1-way ANOVA, as appropriate, and Bonferroni posttest (GraphPad Prism Software Inc, San Diego, CA). Repeated-measures ANOVA was used to evaluate the statistical significance of data acquired from same animals over multiple time points. Survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the log-rank (Mantel-Cox) test. Data are expressed as mean $\pm$ SEM, unless noted otherwise. For all tests, a *P* value ≤ 0.05 was considered to denote statistical significance.

Results

Activation of GSK-3 β in Mice and Human Ischemic/Fibrotic Heart

To test our hypothesis that GSK-3 β critically regulates post-MI fibrosis and fibroblast activation, we first determined the effect of MI on GSK-3 β activity (phosphorylation). Two-month-old WT mice were subjected to MI surgery, and left ventricular (LV) lysates from border and remote zones were analyzed for GSK-3 β phosphorylation at various time points, as indicated (Figure 1A through 1C). In the border zone, MI induced maximal GSK-3 β phosphorylation at day 3 after MI, which slowly declined at later time points (days 7 and 21; Figure 1B). In contrast, in the remote zone, GSK-3 β phosphorylation was first observed at day 7 and continued to increase until the termination of the study (day 21; Figure 1C). Thus, phosphorylation of GSK-3 β is associated with pronounced fibrogenesis, initially in the border zone and later in the remote zone. To confirm the localization of the observed MI-induced GSK-3 β phosphorylation to CFs, cells were isolated from sham- versus MI-operated hearts at 3 weeks after MI, and degree of phosphorylation of GSK-3 β was determined. As hypothesized, GSK-3 β phosphorylation (ie, inhibition) was significantly increased in the fibroblasts isolated from ischemic hearts (Figure 1D and 1E). MI-induced GSK-3 β inhibition was further confirmed by analyzing the GSK-3 β upstream effector P-AKT (P-AKT473, P-AKT308) and the downstream targets (Cyclin D1, c-Myc) 3 weeks after MI (Figure 1A through 1E in the online-only Data Supplement). To determine the potential importance of this finding in the failing human heart, we examined GSK-3 β phosphorylation in human heart tissues from patients with end-stage ischemic cardiomyopathy. Indeed, phosphorylation of GSK-3 β was significantly increased in the ischemic hearts compared with control hearts, consistent with the inhibition of GSK-3 β in our ischemic mouse hearts (Figure 1F and 1G).

Generation and Characterization of Fibroblast-Specific GSK-3 β KO Mice

To evaluate the role of fibroblast GSK-3 β in the regulation of MI-induced fibrotic remodeling and heart failure, we generated a mouse model in which GSK-3 β was conditionally deleted in fibroblasts. We crossed tamoxifen-inducible *Col1a2-cre* mice with *GSK-3 β ^{fl/fl}* mice to obtain conditional fibroblast-specific GSK-3 β KO mice (Col-KO). Tamoxifen treatment led to an $\approx 60\%$ reduction of GSK-3 β protein in the CFs from KO mice compared with littermate controls. However, GSK-3 β levels in the cardiomyocytes were unchanged (Figure IIA through IIC in the online-only Data Supplement). In addition to Col-KO, which targets all fibroblasts, we also generated a mouse model in which GSK-3 β could be specifically deleted in CFs. In this model, Cre recombinase is driven by a 3.9-kb mouse *Postn* promoter (provided by Dr Simon J. Conway, Indiana University, School of Medicine).¹⁴ A recent study¹⁵ further confirmed that periostin expression in the heart is restricted to CFs and is not expressed in cardiomyocytes, endothelial cells, or vascular smooth muscle cells in normal or injured hearts (ie, subjected to MI or to pressure overload). In the healthy adult heart, periostin expression is very low to absent,

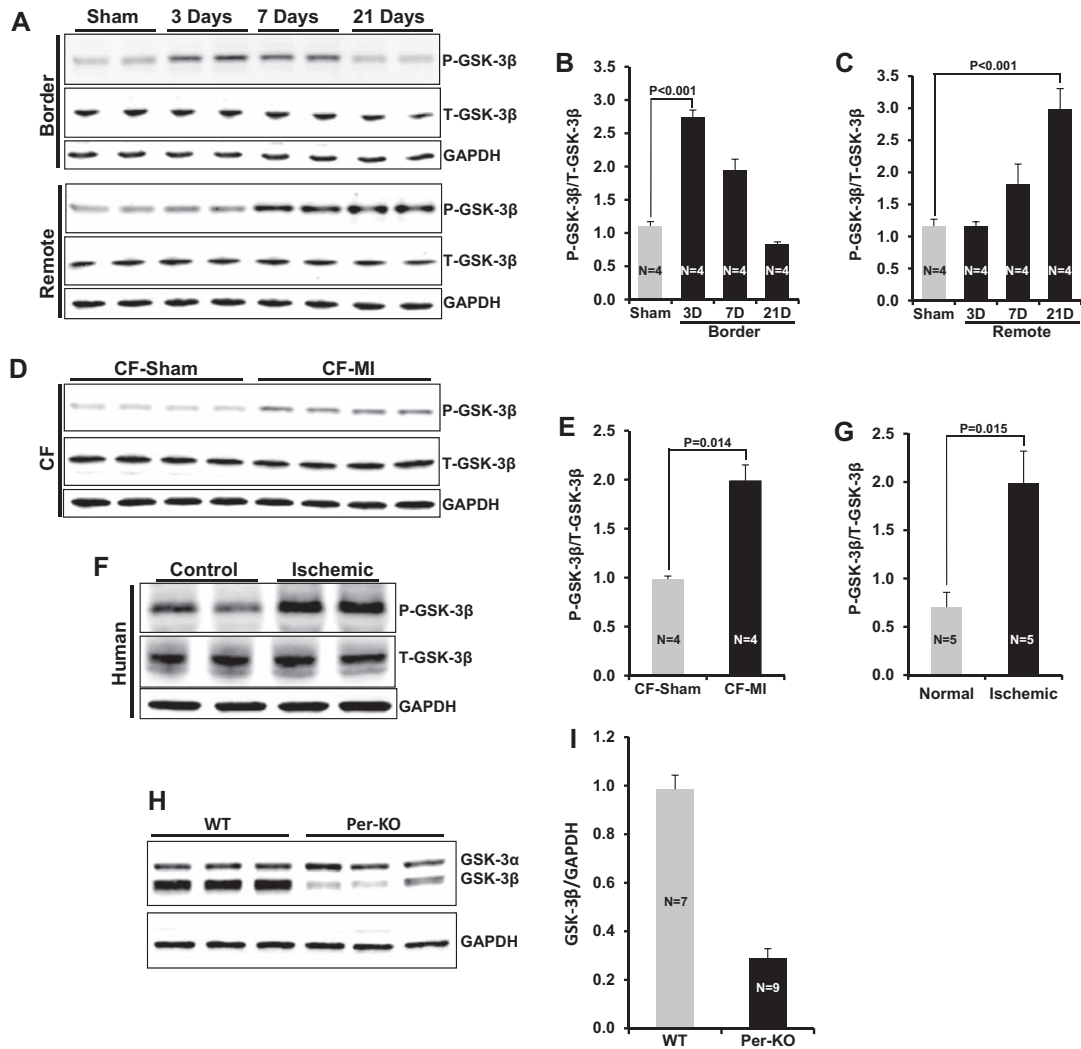


Figure 1. Activation of glycogen synthase kinase-3 β (GSK-3 β) in ischemic heart and cardiac fibroblast (CF)-specific deletion of GSK-3 β . **A**, Wild-type (WT) mice were subjected to myocardial infarction (MI) surgery at 2 months of age. Three weeks after MI, Western blotting was performed on the heart lysates from border and remote zones. **B** and **C**, Quantification of GSK-3 β phosphorylation in border vs remote zone. **D**, CFs were isolated from sham- and MI-operated hearts at 3 weeks after MI, and Western blot analysis was performed. **E**, Quantification of P-GSK-3 β in CFs from sham and MI hearts. **F**, Representative immunoblot showing significantly increased phosphorylation of GSK-3 β in the ischemic human heart vs control heart. **G**, Quantification of GSK-3 β phosphorylation in failing vs control human heart. **H**, GSK-3 $\beta^{fl/fl}$ mice bred with Postn-Cre mice to generate CF-specific GSK3 β knockout (Per-KO; GSK-3 $\beta^{fl/fl}$ Cre⁺) and WT (GSK-3 $\beta^{fl/fl}$ Cre⁻) mice were subjected to MI surgery at 2 months of age. Two weeks after MI, CFs were isolated from the area of injury (left ventricle), and Western blotting was performed. Representative immunoblot demonstrates 65% deletion of GSK-3 β in knockout mice. **I**, Quantification of GSK-3 β expression in GSK-3 β knockout fibroblasts vs WT fibroblasts.

but it increases dramatically, and specifically, in the fibroblast lineage after injury.^{14–17} In normal isolated fibroblasts, periostin is expressed in low amounts in CFs, but expression dramatically increases after passaging during active myofibroblast transformation. Periostin expression decreases again when, presumably, transformation is nearing completion (Figure IIIA and IIIB in the online-only Data Supplement). To generate injury-inducible CF-specific KO mice, GSK-3 $\beta^{fl/fl}$ mice were bred with Postn-Cre mice to generate CF-specific GSK3 β KO mice (Per-KO). Both Col-KO and Per-KO progeny were viable, fertile, reproduced at expected Mendelian ratios, and showed no overt pathological phenotypes. To determine the extent of CF-specific GSK-3 β deletion that was induced after injury, WT and Per-KO mice were subjected to MI at 2 months of age. CFs were isolated from the injury area

at 2 weeks after MI, and levels of GSK-3 β were determined by immunoblotting. GSK-3 β protein level was reduced by $\approx 65\%$ in comparison to littermate controls (Figure 1H and 1I). These complementary models have key advantages: Per-KO is injury inducible, allowing deletion of GSK-3 β specifically in CFs after injury, whereas Col-KO mice allowed us to delete the gene at desired time points (before or after injury).

Deletion of GSK-3 β in CFs Leads to Cardiac Dysfunction and Dilatative Remodeling After MI

To determine the effect of GSK-3 β deletion on infarct size, WT and Col-KO mice were subjected to MI at 4 months of age. Infarct size was determined on day 2 after MI with the use of triphenyl tetrazolium chloride-stained heart sections. We found that infarct size was comparable in WT and

Col-KO hearts (Figure IVA and IVB in the online-only Data Supplement). To determine the role of GSK-3 β in myocardial remodeling, WT and Col-KO mice were subjected to MI at 4 months of age. Before MI, WT and KO hearts had comparable chamber dimensions and ventricular function (Figure VA through VD in the online-only Data Supplement), but as early as 2 weeks after MI, KO animals had a greater increase in end-diastolic and end-systolic dimensions in comparison to WT animals, reflecting accelerated dilatative remodeling in the KO animals (Figure VIA and VIB in the online-only Data Supplement). This was associated with marked LV dysfunction, as reflected by reduced LV ejection fraction and LV fractional shortening (Figure VIC and VID in the online-only Data Supplement). LV dilatation and dysfunction remained worse in the KO animals throughout the duration study.

Because the Col-KO targets to all fibroblasts, we next examined this surprising phenotype in Per-KO mice, which

specifically target to CFs. Indeed, Per-KO animals showed accelerated cardiac dysfunction and ventricular chamber dilation after MI (Figure 2A through 2E). At termination, both heart weight/tibial length and lung weight/tibial length ratios were significantly increased in Per-KO mice, confirming increased post-MI hypertrophy and heart failure in GSK-3 β -deficient hearts (Figure 2F and 2G). Consistently, a pattern of increased mortality was also observed in the KO mice after MI; however, this did not reach statistical significance (Figure VII in the online-only Data Supplement).

CF-Specific Deletion of GSK-3 β Increases Post-MI Scar Circumference and Fibrosis

To determine the role of CF GSK-3 β in myocardial remodeling, WT and Per-KO mice were subjected to MI. At 6 weeks after MI, hearts were excised, and Masson trichrome staining was performed. The percent circumference of the LV that was

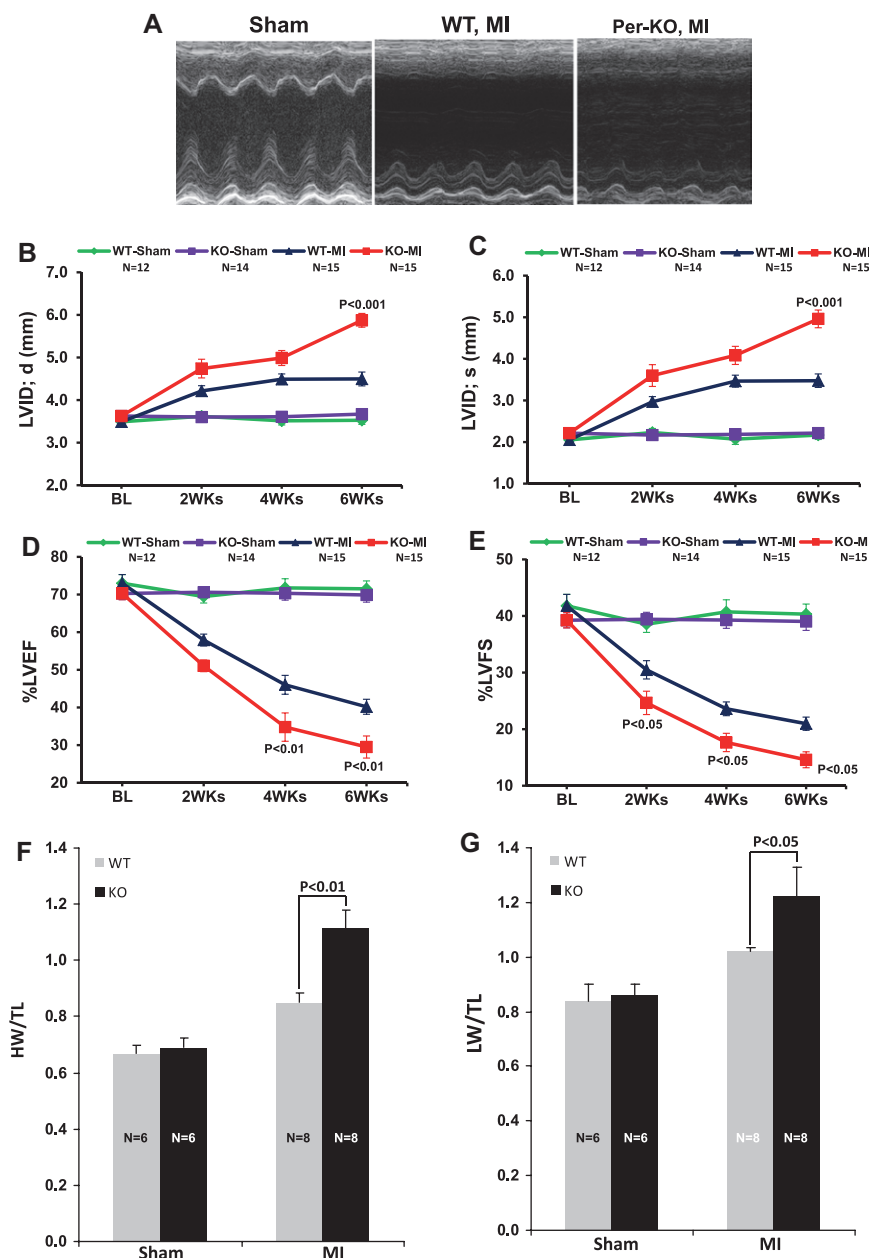


Figure 2. Cardiac fibroblast-specific deletion of glycogen synthase kinase-3 β (GSK-3 β) leads to cardiac dysfunction and dilatative remodeling after myocardial infarction (MI). Two-month-old wild-type (WT) and GSK-3 β ^{fl/fl} mice bred with Postn-Cre mice to generate CF-specific GSK3 β knockout (Per-KO) mice underwent baseline (BL) transthoracic echocardiographic examination. Twenty-four hours later, they were subjected to occlusion of the proximal left anterior descending coronary artery. Mice were then followed with serial echocardiography at the time points shown. **A**, Representative M-mode images from 6 weeks after MI are shown. **B**, Left ventricular internal dimension at end-diastole (LVID;d). **C**, LVID at end-systole (LVID;s). **D**, Left ventricular ejection fraction (LVEF). **E**, Left ventricular fractional shortening (LVFS). **F**, Increased hypertrophy in the Per-KO mice subjected to coronary artery ligation as shown by heart weight/tibia length (HW/TL) ratio. **G**, Increased heart failure in the Per-KO mice. The ratio of lung weight to tibia length (LW/TL, a measure of heart failure) was significantly increased in the knockout (KO) mice.

composed of scar tissue was determined as described.^{18,19} Scar tissue percent circumference was significantly increased in Per-KO hearts (Figure 3A and 3B). Fibrosis was also significantly increased in the border zone of Per-KO hearts (Figure 3C and 3D). Consistently, scar tissue percent circumference was significantly increased in the Col-KO hearts at 6 weeks after MI (Figure VIIIA and VIIIB in the online-only Data Supplement). Transformation of fibroblasts to myofibroblasts, characterized by expression of α -SMA and production of ECM components, is a key event in fibrotic remodeling. In agreement with previous reports, we did not observe α -SMA-positive cells in the sham-operated heart.^{20,21} However, consistent with the increased fibrosis and scar expansion, we also observed a significant increase in the number of α -SMA-positive cells (myofibroblasts) in the infarct region of KO hearts (Figure 3E and 3F).

Deletion of GSK-3 β Induces Myofibroblast Transformation

Previous studies have shown that fibroblasts rapidly differentiate into myofibroblasts in in vitro culture conditions, as

indicated by increased α -SMA expression. We found that untreated CFs spontaneously undergo this differentiation under normal culture conditions even in early passage (Figure 4A), emphasizing the importance of using low-passage fibroblasts when conducting studies on effects of exogenous agents on myofibroblast transformation. For this reason, all of our studies were conducted in serum-free conditions with the use of passage 1 cells. We asked whether spontaneous expression of α -SMA (ie, myofibroblast differentiation) is associated with GSK-3 β activity. To test this hypothesis, isolated CFs were cultured up to 3 passages (all other experiments were done with the use of passage 1 CFs), and myofibroblast differentiation (α -SMA expression) and GSK-3 β serine 9 phosphorylation (inhibition) were examined at every passage through Western blot analysis. Spontaneous expression of α -SMA was found to strongly correlate with inhibitory phosphorylation of GSK-3 β (Figure 4A and 4B). Next we tested the hypothesis that inhibition of GSK-3 β is required for spontaneous α -SMA expression in cultured CFs. To test this hypothesis, CFs were transfected with an adenovirus expressing a mutant form of

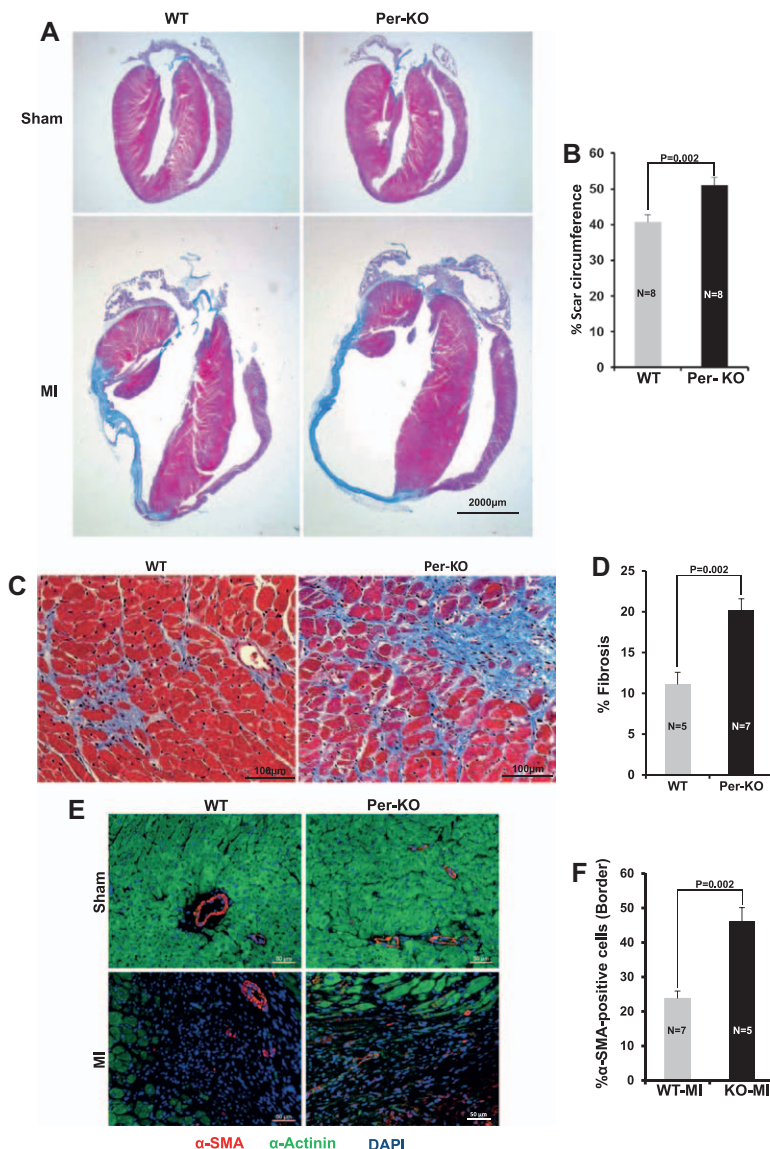


Figure 3. Cardiac fibroblast-specific deletion of glycogen synthase kinase-3 β (GSK-3 β) promotes post-myocardial infarction (MI) scar expansion and fibrosis. Two-month-old wild type (WT) and GSK-3 β ^{fl/fl} mice bred with Postn-Cre mice to generate CF-specific GSK3 β knockout (Per-KO) mice were subjected to MI surgery for 6 weeks, as described in Methods. **A**, Representative images of heart sections stained with Masson trichrome at 6 weeks after MI vs sham surgery. **B**, Scar circumference was measured and expressed as a percentage of total area of left ventricular myocardium. **C**, Representative images of left ventricular border zone showing increased fibrosis in the knockout (KO) mouse heart. **D**, Bar graph showing fold changes in fibrosis in the border zone of WT and KO hearts 6 weeks after MI. **E**, At 6 weeks after MI, immunofluorescence staining was performed to visualize α -smooth muscle actin (α -SMA)-positive cells (red). Sarcomeric α -actinin (α -SA) (green), and DAPI (blue) were used to label myocytes and nuclei, respectively. Representative merge images are shown. **F**, Quantification of α -SMA-positive cells is expressed as a percentage of total cells counted.

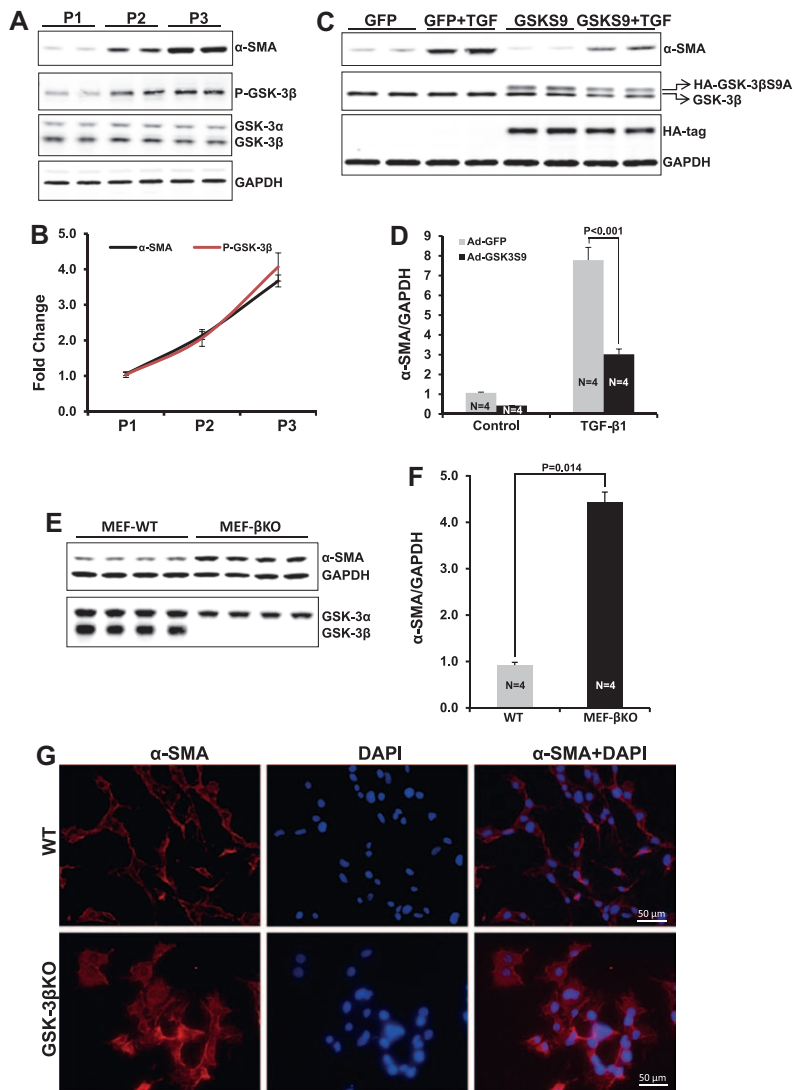


Figure 4. Glycogen synthase kinase-3 β (GSK-3 β) regulates fibroblast to myofibroblast transformation in cardiac fibroblasts. **A**, Cardiac fibroblasts were isolated from 1- to 3-day-old neonatal rat pups and were cultured up to 3 passages. Western blot analysis was performed to determine the expression of α -smooth muscle actin (α -SMA) and phosphorylation of GSK-3 β . **B**, Line graph shows quantification of fold changes in α -SMA expression and GSK-3 β phosphorylation. **C**, Neonatal cardiac fibroblasts were transfected with adenoviruses expressing constitutively active GSK-3 β (Ad-GSK-3 β S9) or GFP (Ad-GFP). After 24 hours of transfection, viral medium was replaced with fresh serum-free medium, and cells were treated with transforming growth factor- β 1 (10 ng/mL) for 48 hours before harvesting. Western blot analysis was performed to determine the expression of α -SMA, endogenous and mutant GSK-3 β , and HA-tag. **D**, Bar graphs show fold changes in α -SMA expression. **E**, Mouse embryonic fibroblast (MEF) cells were cultured in complete Dulbecco's modified Eagle's medium (10% fetal bovine serum and 1% antibiotics) to a confluence of $\approx$ 75% and then were switched to fresh serum-free medium for overnight starvation before harvesting. After starvation, cells were harvested and lysates were analyzed by immunoblotting with α -SMA and GSK-3 α/β antibodies, as indicated. **F**, Bar graphs show fold changes in α -SMA expression in GSK-3 β knockout (KO) MEFs compared with wild-type (WT) cells. **G**, WT and GSK-3 β KO MEFs were cultured on chamber slides, and immunofluorescence staining was performed. GSKS9 indicates GSK-3 β S9; and TGF- β 1, transforming growth factor β 1.

GSK-3 β (serine S9A), which cannot be phosphorylated and therefore is constitutive active. Western blot analysis revealed that Ad-GSK-3 β -S9A significantly decreases α -SMA expression in the presence of TGF- β 1 (Figure 4C and 4D).

To further confirm the role of GSK-3 β in myofibroblast transformation, loss of function studies were performed in WT and GSK-3 β KO mouse embryonic fibroblasts and isolated adult CFs. Western blot analysis revealed that expression of α -SMA was several-fold upregulated in GSK-3 β KO mouse embryonic fibroblasts at basal conditions in comparison to WT mouse embryonic fibroblasts (Figure 4E and 4F). Furthermore, GSK-3 β KO mouse embryonic fibroblasts showed a typical myofibroblast-like phenotype at basal conditions, as revealed by immunofluorescence analysis (Figure 4G). Next, adult CFs were isolated from *GSK-3 β ^{fl/fl}* mouse hearts, and GSK-3 β was deleted by adenovirus-mediated Cre expression. In this model, expression of Cre led to $\approx$ 90% deletion of the target gene and, as anticipated, led to significantly enhanced expression of α -SMA (Figure 5A and 5B). To rule out the possibility that Cre might play a role in myofibroblast transformation, we further confirmed these results by utilizing a short interfering RNA approach. Indeed, short interfering RNA-mediated

knockdown of GSK-3 β was sufficient to significantly increase α -SMA expression (Figure 5C, 5D, and 5E). Taken together, these data suggest that GSK-3 β negatively regulates CF to myofibroblast transformation, and its deletion/inhibition is sufficient to induce myofibroblast transformation.

Mechanism of Increased Fibrosis and Myofibroblast Transformation in GSK-3 β KO Mice

We next examined the possible mechanisms responsible for the increased myofibroblast formation and fibrotic remodeling in GSK-3 β KO mice. TGF- β 1 is the most potent inducer of ECM production characterized to date and promotes fibroblast to myofibroblast differentiation. TGF- β 1 is known to activate SMAD-2 and SMAD-3; however, the profibrotic effects of TGF- β 1 signaling have been attributed largely to SMAD-3-mediated signaling.^{6,22} To determine the role of GSK-3 β in TGF- β 1 signaling, WT and GSK-3 β KO mouse embryonic fibroblasts were treated with TGF- β 1 (10 ng/mL) for 1 hour, and phosphorylation of SMAD-3 at the C-terminus (Ser423/25) was determined. Indeed, on TGF- β treatment, the C-terminal phosphorylation of SMAD-3 was significantly increased in GSK-3 β -null cells compared with WT cells (Figure 6A and

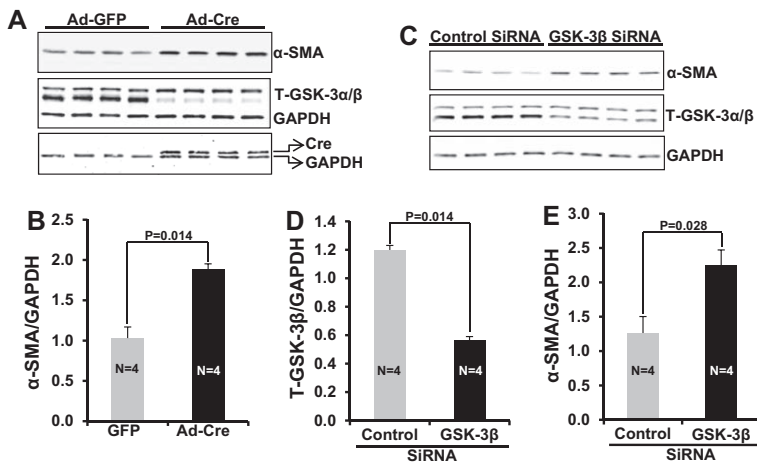


Figure 5. Glycogen synthase kinase-3 β (GSK-3 β) regulates fibroblast to myofibroblast transformation in adult cardiac fibroblasts. **A**, Adult cardiac fibroblasts were isolated from GSK-3 $\beta^{fl/fl}$ mice, and Cre was driven by adenoviral transfection to delete GSK-3 β . After 24 hours of transfection, viral medium was replaced with fresh serum-free medium, and cells were further maintained for 48 hours before harvesting. Western blot analysis was performed to determine the expression of α -smooth muscle actin (α -SMA), Cre, and T-GSK-3 α/β . **B**, Bar graphs show fold changes in α -SMA expression. **C**, Cardiac fibroblasts were isolated from wild-type mice and were transfected with control and GSK-3 β -targeted short interfering RNA (siRNA). After 24 hours of transfection, medium was replaced with fresh serum-free medium, and cells were further maintained for 48 hours before harvesting. **D** and **E**, Bar graphs show fold changes in T-GSK-3 β and α -SMA expression.

6B). To determine the role of GSK-3 β on TGF- β 1 signaling in CFs, neonatal CFs were isolated from 1- to 3-day-old rat pups. Cells were treated with TGF- β 1 (10 ng/mL, 1 hour) in the presence or absence of GSK-3 inhibitor SB216763 (10 μ mol/L, 30-minute pretreatment). As hypothesized, inhibition of GSK-3 β led to a significant increase in the phosphorylation of SMAD-3 at Ser 423/25 (Figure 6D and 6E).

To date, most studies on TGF- β signaling pathways have focused on TGF- β receptors directly phosphorylating and activating SMAD transcription factors within the C-terminal domain.^{8,9} However, there is increasing interest in alternate serine and threonine phosphorylation sites within the linker region of SMADs, which control a number of cellular responses including epithelial-mesenchymal transition and SMAD-3 transcriptional activity.^{23–25} In contrast to C-terminal domain phosphorylation (which leads to activation), linker region phosphorylation leads to inhibition of SMAD transcriptional activity.^{23,26} We asked whether GSK-3 β might also regulate SMAD-3 activity by modulating SMAD-3 phosphorylation in its linker region in fibroblasts. WT and GSK-3 β KO mouse embryonic fibroblasts were treated with TGF- β 1 for 1 hour, and phosphorylation of SMAD-3 at Ser204 was determined. Indeed, TGF- β 1-induced phosphorylation of SMAD-3 at Ser204 was significantly decreased in GSK-3 β KO cells (Figure 6A and 6C). Similar results were observed in neonatal CFs in the presence of GSK-3 inhibitor SB216763 (10 μ mol/L, 30-minute pretreatment; Figure 6D and 6F). These results indicate that GSK-3 β exerts dual control on TGF- β 1/SMAD-3 signaling, specifically by regulating both the C-terminal domain as well as the linker region. To investigate the role of GSK-3 β in the regulation of SMAD-3 transcriptional responses, SMAD-3 reporter assays were performed, as described in Methods. Indeed, TGF- β 1-induced transcriptional activity of SMAD-3 was significantly increased in the GSK-3 β KO mouse embryonic fibroblasts and GSK-3 β -deleted adult CFs (Figure 6G and 6H). These results indicate that GSK-3 β is a critical regulator of canonical TGF- β 1 signaling, and its deletion, or inhibition, leads to aberrant hyperactivation of profibrotic TGF- β 1/SMAD-3 signaling.

Nucleocytoplasmic trafficking of SMAD-3 is a rate-limiting step in TGF- β signaling and is important for determining the strength and duration of the signal and biological response.^{27–29}

To further dissect the role of GSK-3 β in restricting TGF- β /SMAD-3 activity, we examined the intracellular distribution of SMAD-3 in WT and GSK-3 β KO mouse embryonic fibroblasts and in CFs. After subfractionating cells into cytoplasmic and nuclear fractions, we analyzed the content of GSK-3 β and SMAD-3 in these 2 fractions by Western blotting. GSK-3 β was primarily found in the nuclear fraction in both mouse embryonic fibroblasts and CFs (Figure 7A through 7D). As expected, TGF- β treatment (10 ng/mL, 1 hour) significantly increased nuclear content of total SMAD-3 in mouse embryonic fibroblasts and CFs. Of note, TGF- β -induced nuclear translocation of SMAD-3 was independent of GSK-3 β in both mouse embryonic fibroblasts and CFs. These findings indicate that GSK-3 β -mediated regulation of TGF- β /SMAD-3 signaling is independent of nuclear cytoplasmic trafficking of SMAD-3.

To further dissect the mechanism by which GSK-3 β regulates SMAD-3 signaling, we examined whether these proteins might physically interact. Endogenous SMAD-3 in mouse embryonic fibroblasts and CFs was found to coimmunoprecipitate with GSK-3 β (Figure 7E and 7F). As an alternative approach to probe for a possible interaction between SMAD-3 and GSK-3 β , we tested whether immunoprecipitation of SMAD-3 also pulled down GSK-3 β in mouse embryonic fibroblasts and CFs. Indeed, a coimmunoprecipitation experiment with SMAD-3 antibody further suggests the interaction of SMAD-3 with GSK-3 β both in both mouse embryonic fibroblasts and CFs (Figure 7E and 7F). Furthermore, we demonstrated that GSK-3 β and SMAD-3 also interact in the human heart, as revealed by a coimmunoprecipitation experiment with human heart lysates (Figure 7G). To determine the effect of MI on GSK-3 β and SMAD-3 interaction, coimmunoprecipitation studies were performed with lysates from sham- and MI-operated hearts at 6 weeks after MI. MI leads to a decrease in the interaction of SMAD-3 and GSK-3 β (Figure 7H). Taken together, these studies suggest that GSK-3 β interacts with, and thereby maintains, the low level of activity of SMAD-3 in the normal healthy heart.

SMAD-3 Inhibitor Rescues the Detrimental Phenotype of GSK-3 β KO Mice After MI

Finally, we wanted to determine the molecular mechanism of the observed detrimental phenotype in the GSK-3 β KO mice,

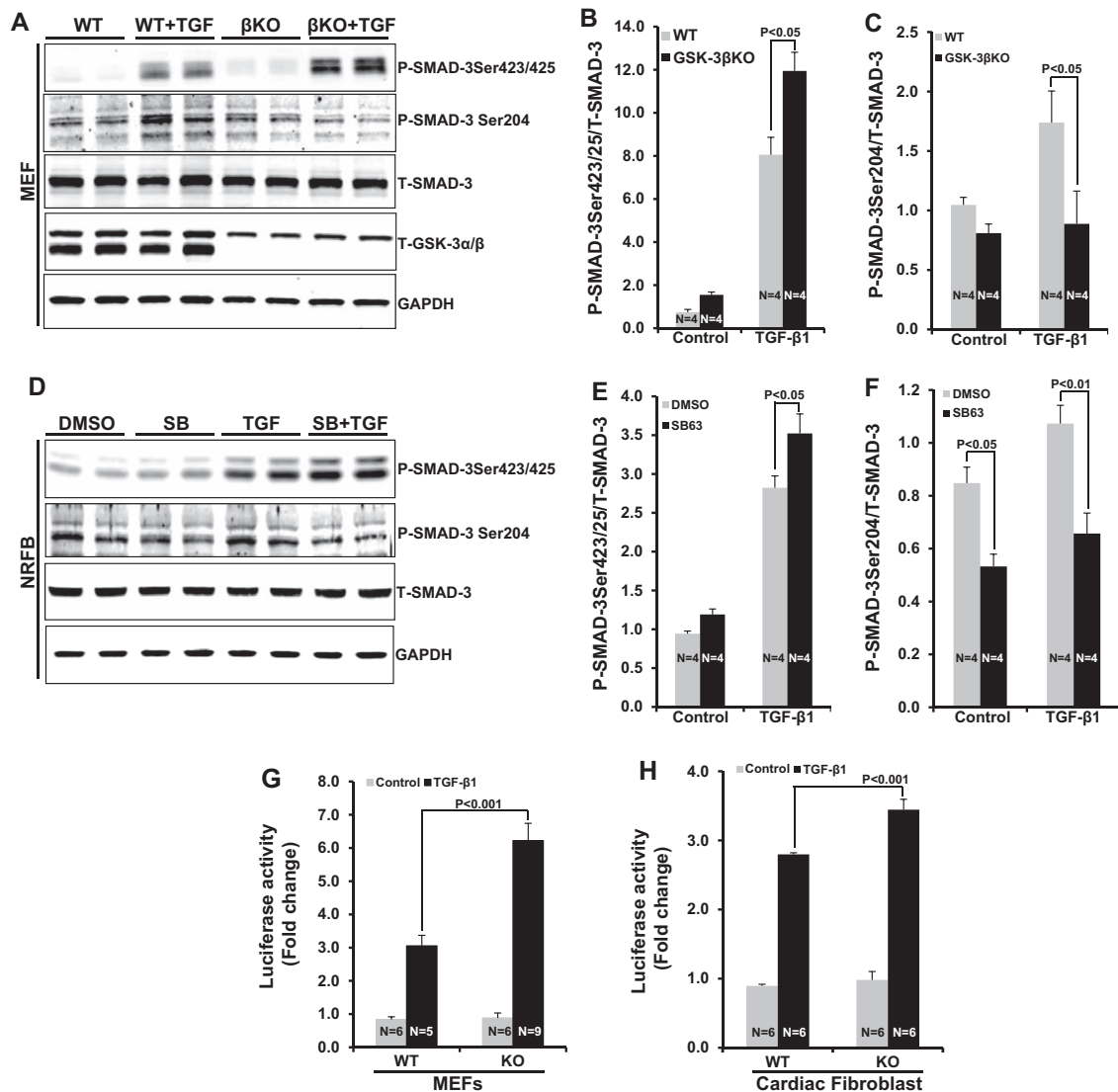


Figure 6. Deletion/inhibition of glycogen synthase kinase-3 β (GSK-3 β) in fibroblasts leads to hyperactivation of transforming growth factor (TGF)- β 1/SMAD-3 signaling. **A**, Wild-type (WT) and GSK-3 β knockout (KO) mouse embryonic fibroblasts (MEFs) were serum-starved overnight before receiving TGF- β 1 for 1 hour (10 ng/mL). Western blot analysis was performed to analyze the phosphorylation of SMAD-3 at Ser204 and Ser423/425. **B**, Bar graphs show fold changes in phosphorylation of SMAD-3 at Ser423/425 in GSK-3 β KO MEFs compared with WT cells in the presence or absence of TGF- β 1. **C**, Bar graphs show fold changes in phosphorylation of SMAD-3 at Ser204. **D**, Neonatal rat cardiac fibroblasts (NRFB) were serum starved overnight before receiving GSK-3 inhibitor SB216763 (SB; 10 μ M) for 30 minutes and an additional 1 hour of TGF- β 1 stimulation. Western blot analysis was performed to analyze the phosphorylation of SMAD-3 at Ser204 and Ser423/425. **E**, Bar graphs show fold changes in phosphorylation of SMAD-3 at Ser423/425. **F**, Bar graphs show fold changes in phosphorylation of SMAD-3 at Ser204. **G** and **H**, Cells were transfected with SMAD-3 Cignal Lenti Reporter virus. Forty-eight hours after transfection, cells were treated with 5 ng/mL TGF- β 1 for 24 hours, and the luciferase activity was quantified. DMSO indicates dimethyl sulfoxide.

with our hypothesis being that hyperactivation of SMAD-3 in the KO hearts is the cause, rather than the consequence, of excessive remodeling. Therefore, we asked whether a small-molecule inhibitor of SMAD-3 could rescue the observed detrimental phenotype in GSK-3 β KO mice. SIS3 (1.25 mg·kg⁻¹·d⁻¹) or vehicle was administered to WT and Per-KO mice by osmotic mini-pumps. Taking into consideration the injury-inducible model (ie, deletion starts with injury) and the requirement of fibrosis in the early healing process and scar maturation, we implanted the pumps 1 week after MI surgery to focus on the post-MI remodeling phase. Mice were followed with serial transthoracic echocardiography. The efficacy of SIS3 treatment in the applied experimental condition

was confirmed by Western blot analysis of P-SMAD-3 at Ser423/25 in the LV lysates (Figure IXA and IXB in the online-only Data Supplement). To our surprise, SIS3 administration nearly abolished the detrimental phenotype of GSK-3 β deletion, as evidenced by restored ventricular function and chamber dimensions (Figure 8A through 8D). SIS3 also significantly blunted scar expansion in Per-KO hearts compared with Per-KO treated with vehicle. Protective effects of SIS3 were also observed in heart weight/tibia length ratios, in lung weight/tibia length ratios, and in limiting scar expansion (Figure 8E through 8H). Of note, SIS3-mediated protection was seen much earlier in the KO hearts than in WT littermates (Figure XA and XB in the online-only Data Supplement). We

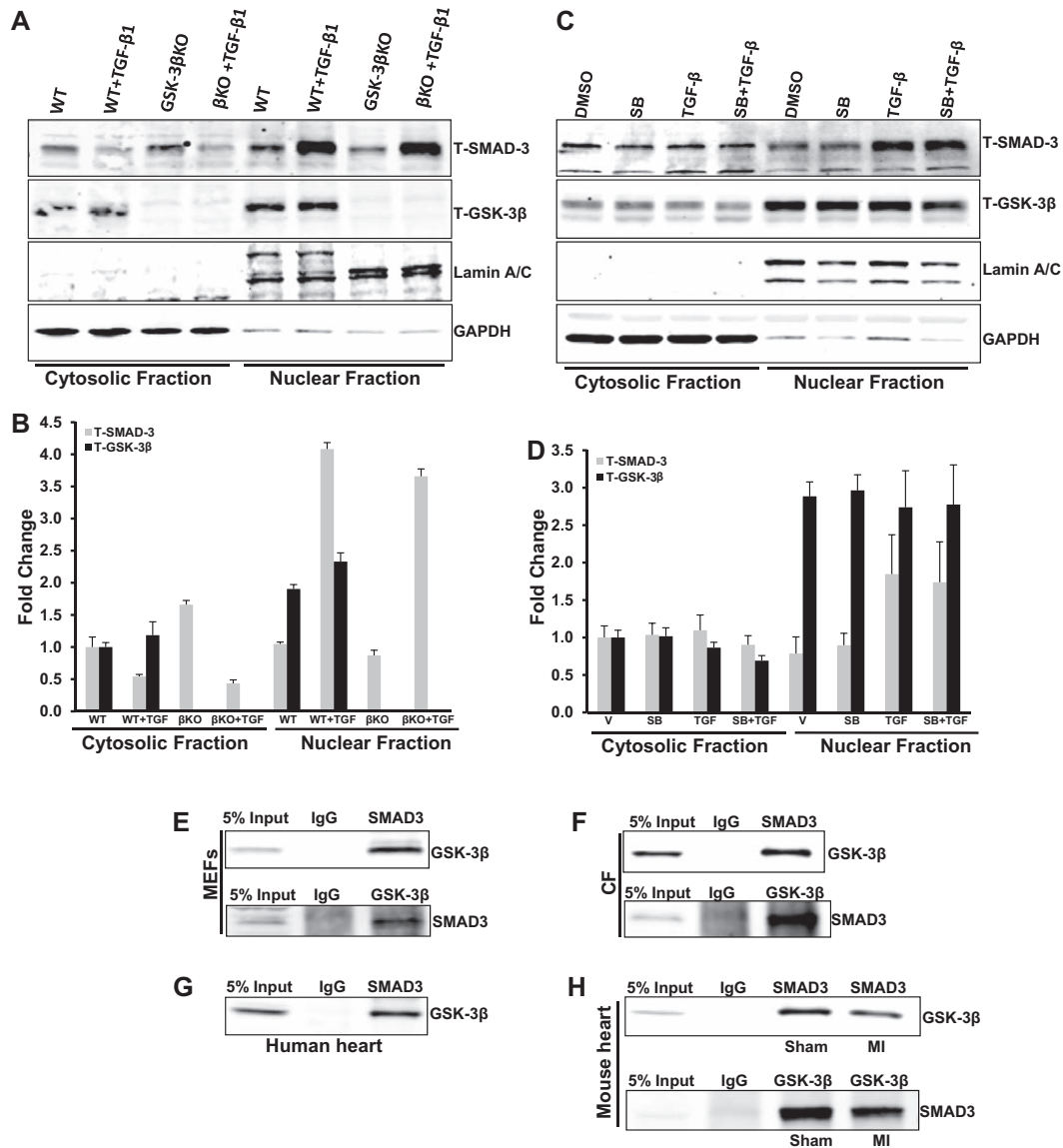


Figure 7. Glycogen synthase kinase-3 β (GSK-3 β) directly interacts with SMAD-3 but does not affect its nuclear accumulation after transforming growth factor (TGF)- β 1 stimulation. **A**, Nuclear and cytoplasmic fractionation was performed with wild-type (WT) and GSK-3 β knockout (KO) mouse embryonic fibroblast (MEF) cells treated with TGF- β 1 (10 ng/mL) for 1 hour. The content of GSK-3 β , SMAD-3, nuclear marker Lamin A/C, and cytosolic marker GAPDH was determined. **B**, Bar graphs show fold changes in SMAD-3 and GSK-3 β translocation to the nucleus after TGF- β 1 stimulation. **C**, Neonatal cardiac fibroblasts were serum starved overnight before receiving GSK-3 inhibitor SB415286 (SB; 10 μ M) for 30 minutes and an additional 1 hour of TGF- β 1 stimulation. Nuclear and cytoplasmic fractionation was performed, and content of GSK-3 β , SMAD-3, nuclear marker Lamin A/C, and cytosolic marker GAPDH was determined. **D**, Bar graphs show fold changes in SMAD-3 and GSK-3 β translocation to nucleus after TGF- β 1 stimulation. **E**, MEFs were lysed for endogenous coimmunoprecipitation assay with the use of either IgG or monoclonal antibodies, as indicated, and followed by immunoblotting. **F** and **G**, Physical interaction between SMAD-3 and GSK-3 β was also examined in lysates from cardiac fibroblasts (CF) and human heart. **H**, Coimmunoprecipitation assay was performed with lysates from sham- and myocardial infarction (MI)-operated hearts at 6 weeks after MI, with the use of either IgG or monoclonal antibodies, as indicated, and followed by immunoblotting. V indicates vehicle.

believe that this was due to the extent of aberrant hyperactivation of SMAD-3 in the KO versus WT animals. Together, these findings provide strong evidence that hyperactivation of SMAD-3 is largely responsible for the detrimental phenotype after selective inhibition or deletion of GSK-3 β in CFs.

Discussion

It is generally accepted that fibroblasts are critically involved in both the reparative response and the pathogenesis of cardiac remodeling after MI.^{1,30} Despite this, direct evidence for a role

of CFs in MI-induced remodeling is lacking, largely because of the lack of genetic tools for specifically manipulating the CF in vivo. Herein, we used 2 different fibroblast-specific GSK-3 β KO mice to demonstrate that GSK-3 β negatively regulates fibrotic remodeling in the ischemic heart. Specifically, we found that GSK-3 β modulates canonical TGF- β 1 signaling through direct interactions with SMAD-3. Furthermore, we show that GSK-3 β -mediated negative regulation of fibrosis is essential to limit the adverse ventricular remodeling in the ischemic heart. When GSK-3 β is deleted in CFs, SMAD-3

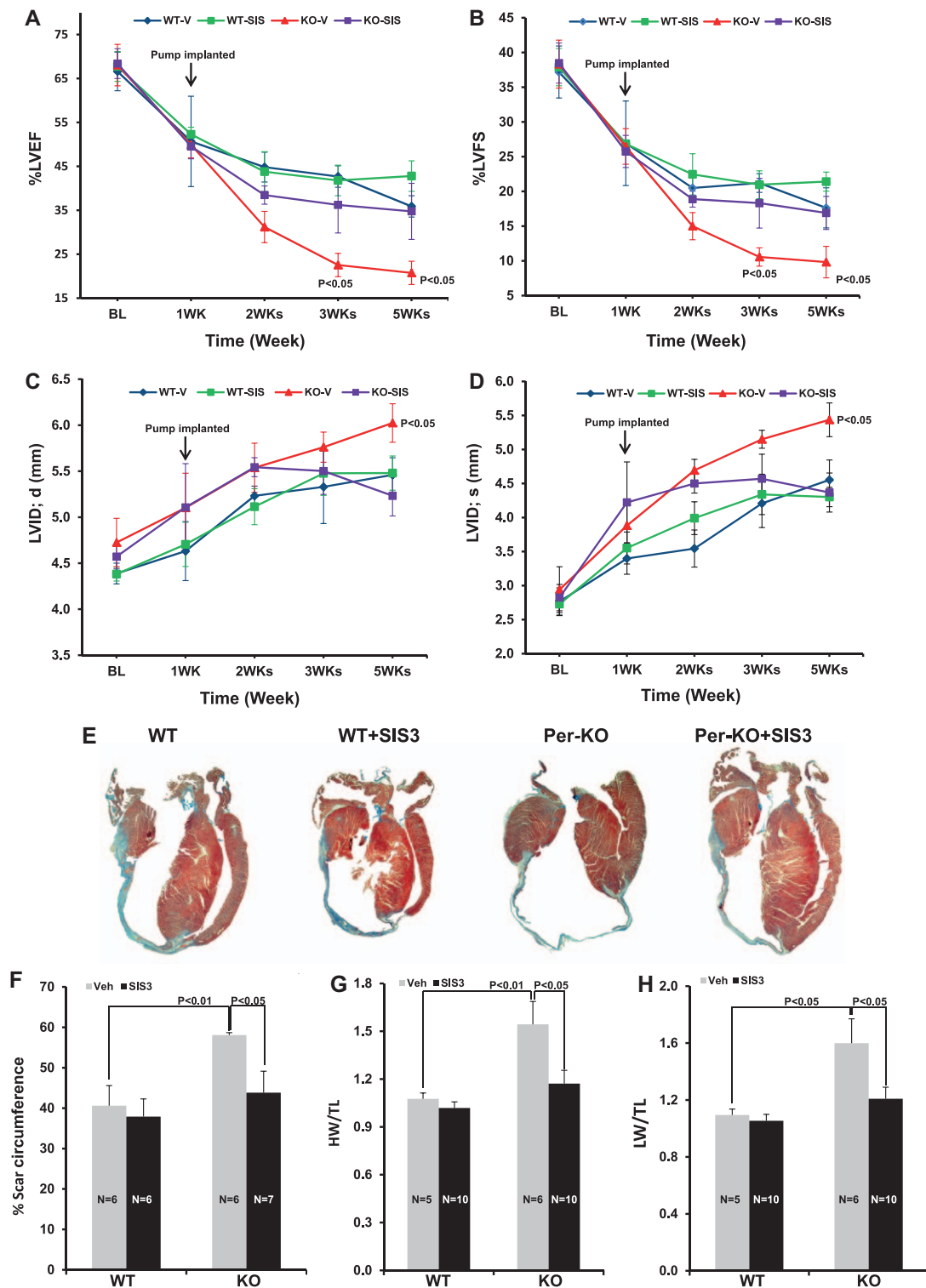


Figure 8. Pharmacological inhibition of SMAD-3 attenuated the cardiac dysfunction, dilative remodeling, and scar expansion in GSK-3 $\beta^{fl/fl}$ mice bred with Postn-Cre mice to generate CF-specific GSK3 β knockout (Per-KO) hearts after myocardial infarction (MI). Wild-type (WT) and Per-KO mice underwent baseline (BL) transthoracic echocardiographic examination. Twenty-four hours later, they were subjected to occlusion of the proximal left anterior descending coronary artery. Mice were then followed with serial echocardiography at the time points shown. Osmotic pumps were implanted 1 week after MI surgery. **A**, Left ventricular ejection fraction (LVEF). **B**, Left ventricular fractional shortening (LVFS). **C**, Left ventricular internal dimension at end-diastole (LVID;d). **D**, LVID at end-systole (LVID;s). **E**, Representative images of heart sections stained with Masson trichrome 5 weeks after MI. **F**, Scar circumference was measured and expressed as a percentage of total area of left ventricular myocardium. **G**, SIS3 rescues increased hypertrophy in the knockout (KO) mice subjected to coronary artery ligation as shown by heart weight/tibia length (HW/TL) ratio. **H**, SIS3 rescued the failing heart phenotype in the KO mice. The ratio of lung weight to tibia length (LW/TL, a measure of heart failure) was significantly attenuated by SIS3 treatment in the KO mice. SIS indicates SMAD-3 inhibitor SIS3; and Veh, vehicle.

is hyperactivated, resulting in excessive fibrosis that leads to rapid adverse ventricular remodeling. Reestablishing SMAD-3 inhibition by the small-molecule inhibitor SIS3 attenuates MI-induced ventricular dilatation and contractile dysfunction.

The literature examining the role of fibrotic remodeling *in vivo* is confusing, to say the least. Only 2 studies have attempted to address this issue with any success.^{14,31} The first, by Takeda et al,¹⁴ used deletion of Kruppel-like factor 5 (KLF5) using the periostin Cre model. They used thoracic aortic constriction (TAC) at 2 levels of severity. With low-intensity TAC, they found that deletion was protective with less fibrosis and less hypertrophy. On the basis of studies in conditioned media and using microarrays, they concluded that the mechanism of less hypertrophy was not via an effect on fibroblasts but was due to less secretion of insulin growth factor-1. Strikingly, with high-intensity TAC, the phenotype was the opposite: increased mortality and LV dysfunction. Mechanisms of these opposing effects remain unclear. The second attempt to discern mechanisms of fibrotic remodeling used a Col-Cre strategy to delete β -catenin.³¹ The conclusion drawn was that deletion of β -catenin led to defective fibrosis and cardiac dysfunction. However, there were virtually no mechanistic data to explain the phenotype other than possibly fibroblast proliferation. In striking contrast, we deleted GSK-3 β , an antifibrotic factor, and this led to markedly increased fibrosis and increased scar expansion.

To our knowledge, this is the first report describing a direct role of CFs in MI-induced fibrotic remodeling with the use of a CF-specific mouse model. We have reported previously that conditional deletion of GSK-3 β , specifically in cardiomyocytes, leads to cardiomyocyte proliferation but does not have any effect on fibrosis after pressure overload or MI.¹⁰ Taken together, these results suggest that the functional consequence of loss of GSK-3 β is cell type dependent in the heart and that deletion/inhibition of GSK-3 β in fibroblasts is detrimental. It is generally accepted that persistent myofibroblast activation, as well as the resultant increase in fibrous tissue it produces, causes progressive adverse myocardial remodeling, a pathological hallmark of the failing heart, irrespective of its etiologic origin. However, the responsible molecular mechanism(s) is complex and not well understood.^{4,32} Consistent with increased fibrosis, fibroblast to myofibroblast transformation was significantly increased in KO hearts. Moreover, by using a combination of genetic and pharmacological tools, we demonstrate that inhibition of GSK-3 β is essential to induce fibroblast to myofibroblast transformation. Importantly, inhibiting myofibroblast transformation after cardiac injury has been shown to decrease fibrosis.³³ Matsuda et al¹³ used a constitutively active global knock-in mouse model and showed that TAC-induced fibrosis was abolished in the heart of GSK-3 β knock-in mice, suggesting that inhibition of GSK-3 β is required for TAC-induced fibrosis. Our findings are consistent with this observation and show the robust increase in the fibrotic response in GSK-3 β KO hearts after MI. These data suggest that GSK-3 β is an essential regulator of myocardial fibrosis in the injured heart, and strategies to maintain GSK-3 β in an active state, especially in the remodeling phase after MI, may provide a therapeutic option to inhibit fibrosis and thus limit maladaptive remodeling.

To investigate the mechanisms by which CF GSK-3 β regulates fibrotic remodeling, we identified SMAD-3 as a key target of GSK-3 β in CFs. Inhibition or deletion of GSK-3 β in CFs leads to hyperactivation of SMAD-3, as evidenced by increased phosphorylation of SMAD-3 within the C-terminal domain (S423/425) and decreased phosphorylation in the linker region (S204). Both of these posttranslational modifications are known to increase transcriptional activity of SMAD-3. To our knowledge, this is the first report showing dual control of SMAD-3 activation by GSK-3 β . This occurs by modulating both the carboxyl terminal domain (S423/425) and linker (S204) phosphorylation sites. Our data also show that GSK-3 β and SMAD-3 directly interact. However, it is unclear precisely how deletion/inhibition of GSK-3 β might lead to increased phosphorylation of SMAD-3 at S423/425, and determining the mechanism is beyond the scope of this work.

Given the critical role played by SMAD-3 in tissue fibrosis, SMAD-3 modulation is likely a key mechanism by which GSK-3 β regulates fibrotic remodeling in the ischemic heart. Indeed, our studies clearly suggest that activation of TGF- β 1/SMAD-3 signaling after deletion of GSK-3 β in CFs is the primary mechanism leading to adverse fibrotic remodeling and ventricular dysfunction. This conclusion is strongly supported by our studies with the small-molecule SMAD-3 inhibitor SIS3, which rescued the detrimental phenotypes observed in GSK-3 β KO hearts. These results are consistent with the observations in *SMAD-3* KO and *SMAD-3*^{+/-} mice, which suggest that loss of SMAD-3 prevents fibrotic remodeling and attenuates adverse remodeling in both ischemic and pressure-overloaded hearts.^{33,34}

In summary, we believe that we have identified a novel and central role of GSK-3 β in regulating myocardial fibrotic remodeling in the infarcted heart. GSK-3 β exerts these effects via direct regulation of the canonical TGF- β 1/SMAD-3 signaling pathway. Clinically, pharmacological inhibitors targeting the GSK-3 family of kinases have been proposed for several select diseases. Given the profound findings of the present study, we are concerned that adverse fibrotic remodeling with any such inhibitory agents should raise caution, especially in the setting of long-term use after MI.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Fibrotic remodeling is a major driver of pathology after myocardial infarction. Unfortunately, there are no viable strategies to prevent, or even to importantly limit, the progression of fibrotic disease and its consequences. Herein, we report that deletion of the protein kinase glycogen synthase kinase-3 β (GSK-3 β), specifically in cardiac fibroblasts, promotes fibrotic remodeling. Surprisingly, this targeting of cardiac fibroblasts also leads to marked contractile dysfunction of the left ventricle. The transforming growth factor- β 1/SMAD-3 signaling pathway is a key mediator of fibroblast activation and fibrogenesis. Here we identify novel cross talk between GSK-3 β and the SMAD-3 pathway. We demonstrate, via multiple approaches, that GSK-3 β negatively regulates the profibrotic transforming growth factor- β 1/SMAD-3 pathway in cardiac fibroblasts. Thus, we identified the profibrotic cytokine SMAD-3 as a key mediator of injury downstream of GSK- β , and a small-molecule inhibitor of SMAD-3, SIS3, largely prevented the fibrosis and limited left ventricular remodeling after MI. Our findings suggest that strategies to maintain GSK-3 β in an active state or to inhibit SMAD-3 could be a novel approach to limit transforming growth factor- β 1 signaling in myocardial fibrotic disorders. We believe that these findings could radically change the approach to pathological fibrosis in the injured heart.