Glycogen Synthase Kinase-3β Regulates Post–Myocardial Infarction Remodeling and Stress-Induced Cardiomyocyte Proliferation In Vivo

Kathleen C. Woulfe, Erhe Gao, Hind Lal, David Harris, Qian Fan, Ronald Vagnozzi, Morgan DeCaul, Xiying Shang, Satish Patel, James R. Woodgett, Thomas Force, Jibin Zhou

Rationale: Numerous studies have proposed that glycogen synthase kinase (GSK)-3β is a central regulator of the hypertrophic response of cardiomyocytes. However, all of this work has relied on overexpression of GSK-3β, expression of constitutively active mutants, or small molecule inhibitors with documented off-target effects. Genetic loss of function approaches have not been used in the adult mouse because germ-line deletion of GSK-3β is embryonic-lethal.

Objective: This study was designed to define the role played by GSK-3β in pressure overload (PO)-induced hypertrophy and remodeling following myocardial infarction (MI).

Methods and Results: We used a mouse model that allows inducible, cardiomyocyte-specific deletion of GSK-3β in the adult knockout. Surprisingly, we find that knockout mice exposed to PO induced by thoracic aortic constriction exhibit a normal hypertrophic response. Thus, in contrast to virtually all prior published studies, GSK-3β appears to play at most a minor role in the hypertrophic response to PO stress. However, GSK-3β does regulate post-MI remodeling because the GSK-3β knockouts had less left ventricular dilatation and better-preserved left ventricular function at up to 8 weeks post-MI despite demonstrating significantly more hypertrophy in the remote myocardium. Deletion of GSK-3β also led to increased cardiomyocyte proliferation following PO and MI.

Conclusions: Deletion of GSK-3β protects against post-MI remodeling and promotes stress-induced cardiomyocyte proliferation in the adult heart. These studies suggest that inhibition of GSK-3β could be a strategy to both prevent remodeling and to promote cardiac regeneration in pathological states. (Circ Res. 2010;106:1635-1645.)

Key Words: cardiac hypertrophy □ myocardial infarction □ heart failure □ myocardial regeneration □ GSK-3

Cardiomyocytes are terminally differentiated cells that respond to different stresses such as pressure stress (e.g., hypertension or aortic valvular disease) and cardiac injury leading to myocyte loss (e.g., myocardial infarction) by undergoing hypertrophy. As cardiomyocytes hypertrophy, they go through specific characteristic changes regardless of pathological stimulus. These alterations typically include conversion to a fetal gene expression profile, increased protein synthesis, and changes in metabolism. Over time, hypertrophy can progress to heart failure. Heart failure is characterized by progressive cell loss, extracellular matrix remodeling and contractile dysfunction. It is unclear how (or if) hypertrophy contributes to these phenotypic manifestations of heart failure. Although the hypertrophic response normalizes cardiac wall stress, it seems that this response is not necessary to maintain contractile function. Thus, targeting hypertrophic signaling pathways could possibly prevent or delay pathological remodeling and heart failure progression, irrespective of the inciting stimulus.

Glycogen synthase kinase (GSK)-3β is thought to be a key integrator of many of the pathways activated by hypertrophic stimuli. Although GSK-3 has 2 isoforms, GSK-3α and GSK-3β, the majority of studies have focused on GSK-3β. The near exclusive focus on GSK-3β as the predominant isoform may have originated in two landmark studies in Drosophila that established GSK-3β as the dominant isoform for body patterning. Both isoforms are ubiquitously expressed and share 97% homology within their catalytic domains but differ at their N and C-terminal regions. The two isoforms of GSK-3 have both distinct and redundant functions in the cell, but in cardiac development, GSK-3β is dominant. GSK-3α knockout mice develop normally, consistent with full compensation by GSK-3β for loss of GSK-3α in cardiac development. In contrast, germ-line de-
letion of GSK-3β is embryonic lethal, because of hyperproliferation of cardiomyoblasts that largely obliterate the right ventricular and left ventricular (LV) cavities.14

The bias toward GSK-3β as the dominant isoform also applies to studies in the adult heart which have focused on GSK-3β as the key negative regulator of cardiac hypertrophy. GSK-3β is active in unstimulated cells. When GSK-3β phosphorylates its substrates, it typically leads to their inactivation. Multiple substrates have been identified, and several of these are proteins that appear to play central roles in regulating pathological cardiac hypertrophy and/or proliferation, including, eukaryotic protein synthesis initiation factor 2B (eIF2B),15 β-catenin,16,17 c-myc,18 nuclear factor of activated T-cells (NF-AT),19 mammalian target of rapamycin complex 1 (mTORC1),20,21 and cyclin D1.22 Phosphorylation of these and other substrates by active GSK-3β is believed to blunt hypertrophy. On activation of hypertrophic signaling pathways such as PI3K/Akt, PKC, and PKA, GSK-3 is phosphorylated and inactivated14 and this inactivation releases the substrates from inhibition.

In reality, however, it is unclear whether the two isoforms share redundant or distinct functions with regard to hypertrophy and remodeling in the adult heart. Studies to date in the adult heart of adult mice in vivo remain to be defined. Based on the majority of the published data discussed above, we expected that there would be exaggerated hypertrophy in response to thoracic aortic constriction (TAC) and, possibly, in response to myocardial infarction (MI). Herein, we demonstrate that GSK-3β, per se, plays at most a minor role in regulating pathological cardiac hypertrophy induced by pressure overload. In contrast, GSK-3β is central to the hypertrophic response following MI. More importantly, despite the exaggerated hypertrophic response to MI in the remote myocardium, we find that deletion of GSK-3β protects against the LV dilatation and dysfunction that follows a large MI. Finally, we find that cardiomyocyte-specific deletion of GSK-3β identifies a key role for the kinase in regulating cardiomyocyte proliferation in the setting of stress.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

C57Bl6 mice with lox-p sites flanking exon 2 of the gsk-3β gene12 were crossed with mice carrying the Mer-Cre-Mer transgene driven by the α-myosin heavy chain promoter (gift from Dr J. Molkentin, Cincinnati Children’s Hospital, Cincinnati, Ohio). For TAC studies, at 5 weeks of age, the mice were treated with tamoxifen (20 mg/kg IP [Sigma-Aldrich] dissolved at a final concentration of 4 mg/mL in 30% ethanol in sterile PBS) or vehicle (30% ethanol in PBS) for 5 days to induce excision of exon 2. At 7 weeks of age, mice underwent baseline echo followed by TAC. In contrast to a prior report,32 baseline LV function with this protocol was not compromised in the conditional KO (Online Table I and data not shown). For MI studies, we wanted to avoid impacting on infarct size so we administered tamoxifen for 5 days, beginning 3 days after occlusion of the proximal left anterior descending coronary artery.

Results

Deletion of GSK-3β in the Conditional Knockout Mouse

To clarify the role of GSK-3β in pressure overload-induced hypertrophy and post-MI remodeling, we first examined the degree of deletion of GSK-3β in our model. The GSK-3β(fl/fl)/Cre mice treated with vehicle (flox/Cre/V) demonstrated levels of GSK-3β protein expression that were comparable to

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the GSK-3β/fl/fl mice lacking mer-cre-mer treated with tamoxifen (referred to as flox/−/T). This confirms that there was no significant “leaky” excision of exon 2 in the absence of tamoxifen in our model.

The GSK-3β/fl/fl/Cre+/−/TAC (KO) mice (referred to as flox/Cre/T or KO) had a range of reduction of GSK-3β protein expression in their left ventricles (LV) (Figure 1). Residual GSK-3β protein ranged from 8% to 30% of control (Figure 1).

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### Table. Morphometric Measurements in the Various Groups Three Weeks After TAC or Sham TAC

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>Heart Weight (mg)</th>
<th>Body Weight (g)</th>
<th>LV Mass (mg)</th>
<th>HW/BW (mg/g)</th>
<th>LVM/BW (mg/g)</th>
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<tr>
<td>Flox/−/T + TAC</td>
<td>26</td>
<td>114 ± 11</td>
<td>23.2 ± 1.3</td>
<td>68 ± 17</td>
<td>5.1 ± 0.7</td>
<td>3.0 ± 0.7</td>
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<tr>
<td>Flox/Cre/+/−/TAC (KO)</td>
<td>26</td>
<td>120 ± 23</td>
<td>22.0 ± 2.0</td>
<td>70 ± 19</td>
<td>4.4 ± 1.0</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>Flox/+/−/T + Sham</td>
<td>11</td>
<td>115 ± 17</td>
<td>23.1 ± 2.8</td>
<td>52 ± 6.1</td>
<td>4.6 ± 1.0</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Flox/Cre/+/−/T + Sham (KO)</td>
<td>6</td>
<td>100 ± 16</td>
<td>21.9 ± 1.0</td>
<td>54 ± 7.8</td>
<td>4.5 ± 0.5</td>
<td>2.7 ± 0.4</td>
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All morphometric measurements (HW, BW, LVM, HW/BW, LVM/BW) from TAC groups were significantly different from sham groups (P<0.05).

Figure 2. Deletion of GSK-3β does not alter the hypertrophic response to TAC. A, Relationship between LV systolic pressure (mmHg) and HW/BW in flox/Cre/T mice and flox/−/T mice subjected to TAC. At 3 weeks after TAC, mice underwent invasive hemodynamic assessment to determine LVSP. The regression lines relating LVSP to HW/BW were not significantly different between the groups, suggesting deletion of GSK-3β did not affect the hypertrophic response. For flox/Cre/T (KO) mice, r=0.373; for flox/−/T mice, r=0.386. n=26 mice in each group. B, Hypertrophy in mice with mild, moderate, or severe TAC. Mice were subjected to TAC and HW/BW was determined at euthanasia. There is no difference in hypertrophy between KO and controls at any LVSP. Mean LVSPs for the groups are shown below the figure. C, Representative hematoxylin/eosin-stained LV sections from mice with systolic pressures between 150 and 180 mmHg, 3 weeks after TAC. Quantification of myocyte cross-sectional area is shown in the graph below. Cardiomyocyte size was not significantly different between the two control groups (flox/−/T mice and flox/Cre/V mice); therefore, these data were grouped together. n=200 cardiomyocytes/mouse with 8 mice as controls and 6 conditional KO mice.
40% of control GSK-3β expression in our model and the average residual protein was 23% (n=114 mice). The residual GSK-3β protein expression is attributable to incomplete recombination and the presence of nonmyocytes in the hearts. Mice that had less than 30% residual GSK-3β protein expression by western blot were included in the study.

Effect of Deletion of GSK-3β on Pressure-Overload Hypertrophy
The mice then underwent TAC. Three weeks after TAC, mice were analyzed for cardiac function and morphometrics. Mice were catheterized with a high fidelity pressure transducer to determine the LV systolic pressure (LVSP) (Online Table I). LVSP was comparable among the three groups, suggesting that the degree of TAC was also comparable in the groups. Heart weight (HW), left ventricle mass (LVM), and body weight (BW) were determined, and calculated HW/BW and left ventricle mass/BW (the primary morphometric measures of hypertrophy) were calculated. There were no significant differences in any of these parameters among the three groups that underwent TAC (Table). We then analyzed HW/BW ratio in relation to LVSP (Figure 2A). The regression lines relating LVSP to HW/BW were virtually identical, consistent with deletion of GSK-3β having little or no effect on the hypertrophic response. Next, we compared hypertrophy in mice with lower versus higher LVSPs to see whether deletion of GSK-3β might modulate hypertrophy differently in these groups (Figure 2B). There was no significant difference in hypertrophy in the KO mice in any of the ranges of LVSP. To examine hypertrophy at the cellular level, we measured cross-sectional area of hematoxylin/eosin-stained LV sections and found no significant increase in cardiomyocyte size in the conditional KO (Figure 2C). We also measured the surface area of isolated cardiomyocytes and found no difference between the conditional KO and the control groups (Online Figure I). Finally, we examined the fetal gene program and found that expression of atrial natriuretic peptide and β-myosin heavy chain were approximately 5-fold higher in the control compared to the KO (Online Figure II, A). These findings, which are contrary to numerous studies using transgenesis, suggest GSK-3β is not a central regulator of the hypertrophic response to pressure overload, whether the pressure load is mild, moderate or severe.

GSK-3β Deletion Does Not Affect Cardiac Function or Extracellular Matrix Remodeling After Pressure Overload Stress
We also assessed cardiac function by echocardiography both before and 3 weeks after TAC (Online Table II). As noted, there was no significant difference in the ejection fraction (EF) between the groups before TAC. Furthermore, there was no difference in EF between groups of mice exposed to more severe pressure overload (LVSP 150 to 190 mm Hg) (Online Figure III, A) or in mice with less severe pressure overload (LVSP=120 to 150 mm Hg) (Online Figure III, B). Analysis of contractility also failed to detect any differences between the groups in +dP/dt (a more sensitive measure of contractility) and −dP/dt (a sensitive measure of relaxation) (Online Table I).

Studies have suggested that GSK-3β is antifibrotic. However, we found no difference in the percent area of fibrosis between the treatment groups in LVSP-matched hearts (150 to 180 mm Hg) at 3 weeks after TAC (Online Figure IV). Therefore, deletion of GSK-3β in cardiomyocytes does not appear to affect fibrosis, at least in the early phases of the response to pressure overload in the heart.
Deletion Affects Several Signaling Pathways

Taken together, the data presented above suggest two possibilities: 1) the small amount of residual expression of GSK-3β in the conditional KO is sufficient to maintain normal functions of GSK-3β, or 2) GSK-3β plays at most a minor role in the response to pressure overload. Therefore we examined signaling pathways regulated by GSK-3β reasoning that if possibility 1 were the case, signaling pathways would not be significantly dysregulated in the KO. We first analyzed phosphorylation of glycogen synthase at serine 641 and found a striking reduction in the KO (Figure 3). This finding identifies GSK-3β as the dominant isoform regulating glycogen synthesis in the heart, but more importantly demonstrates that the magnitude of reduction in GSK-3β expression levels is sufficient to lead to dysregulation of signaling pathways.

To explore this further, we examined activity status of GSK-3α and downstream targets of GSK-3β known to play a role in the hypertrophic response. We found that GSK-3α protein levels and activity level (the latter as determined by phosphorylation at serine 21) were not different between GSK-3β KO and WT hearts, confirming no compensation (data not shown). Further, we examined the status of several GSK-3β targets that are known regulators of hypertrophy. We found a statistically significant decrease in phosphorylation of eIF2B (a direct target of GSK-3β), and an increase in c-myc expression (which is normally downregulated by GSK-3β-triggered proteasomal degradation) in LV lysates obtained 24 hours post-

Figure 4. Post-MI remodeling in the GSK-3β conditional KO. A, Representative images of sagittal sections of Control (flox/−/T) and KO hearts at 8 weeks post-MI. B, Echocardiographic findings in the post-MI heart. KO and the various controls underwent baseline echo examination and then were subjected to occlusion of the proximal LAD. Follow-up echo examinations were done at the times shown and ejection fraction and LVEDD were determined at each time-point. There were no significant differences in either parameter for the three control groups. Probability values reflect the following comparisons: P1: KO vs flox/Cre/V; P2: KO vs flox/−/V; P3: KO vs flox/−/T. There were n=32 KOs; 32 flox/Cre/V; 31 flox/−/V; 31 flox/−/T.
TAC (Online Figure V, A and B). Thus, the decreased levels of GSK-3β were impacting on target activity, but this was not sufficient to lead to enhanced hypertrophy. One reason for this may be that deletion of GSK-3β did not lead to activation of mTORC1, a central regulator of the hypertrophic response (data not shown). In summary, we conclude that GSK-3β is not a central regulator of pressure overload (PO)-induced hypertrophy.

GSK-3β Regulates Post-MI Remodeling

We next asked whether GSK-3β might regulate the other major form of remodeling in the heart: post-MI remodeling. We subjected KO mice and controls to permanent ligation of the proximal LAD. Because GSK-3β has been implicated in regulating acute ischemic injury (reviewed elsewhere), and we specifically wanted to examine post-MI remodeling, tamoxifen (or vehicle) injections were begun 3 days after MI, at a time when the infarct was completed. We followed the mice for 8 weeks with periodic echocardiography. The infarcts produced were quite extensive in size (Figure 4A). Of note, the absolute length of the scar was not different between the KO and various controls, suggesting infarct size was comparable among the groups (KO: 1226±64 pixels, n=7 mice; Control: 1257±88 pixels, n=12 mice; p=NS). Also supporting this conclusion, ejection fraction was virtually identical among the groups at one week post-MI (Figure 4B). In fact, at one week post-MI, LV end diastolic diameter (LVEDD) was greater in the KO, suggesting infarct size might have been slightly larger in the KO. However, beginning at 2 weeks post-MI, LVEF tended to be greater, and LVEDD tended to be smaller in the KO (Figure 4B). At 4 weeks post-MI and after, LVEF was better maintained in the KO, and at ≥6 weeks, LV dilatation (LVEDD) was significantly less. Thus deletion of GSK-3β protects against post-MI remodeling.

We wanted to examine contractile function more closely, so we performed invasive hemodynamic testing at 8 weeks post-MI. For these studies we present the various controls as one group because there were no significant differences between them in any parameter. We found no significant differences between KO and controls in LVSP or LVEDP, but +dP/dt and –dP/dt were significantly increased in the KO (Figure 5A through 5D). Thus LV function, both systolic and diastolic, are better maintained in the KO.

We next wanted to understand potential mechanisms of this protective effect of deletion of GSK-3β. Because smaller infarcts did not seem to account for the differences, we focused on the noninfarcted remote myocardium. Surprisingly, we found that overall HW/BW was significantly increased in the KO, suggesting that in the setting of post-MI remodeling, as opposed to TAC, GSK-3β is indeed antihypertrophic (Figure 6A). This increased hypertrophy was due, in large part, to cardiomyocyte hypertrophy because cross-sectional area of the KO myocytes was significantly increased (Figure 6B). Despite the increased hypertrophy in the KO, fibrosis was not increased (data not shown) and apoptosis was significantly reduced (Figure 6C). As for the fetal gene program, β-myosin heavy chain was increased in the KO, consistent with the increased hypertrophy, but atrial natriuretic peptide and brain natriuretic peptide, which are also markers of hemodynamic stress, were not increased in the KO (Online Figure II, B). The above data, taken together, suggest that the hypertrophy seen in the remote myocardium of the KO is more consistent with physiological, as opposed to pathological, hypertrophy.
myocytes (ARVMs) in culture, the only in vivo study to date, which used expression of a CA mutant, reported that GSK-3β did not regulate cardiomyocyte proliferation in the adult heart. To examine this further, we analyzed cardiomyocyte proliferation in LV heart sections both after TAC and post-MI by injecting mice with bromodeoxyuridine (BrdUrd) and then immuno-staining sections for BrdUrd. In the KO mice subjected to TAC, we found a significant increase in cells that were positive for BrdUrd (Figure 7A). All of the BrdUrd-positive nuclei overlapped with DRAQ5, a nuclear stain. Confocal analysis confirmed that the majority of the proliferating cells were from a cardiomyocyte lineage, based on positive staining for troponin (TnI) (Figure 7B). The increased cardiomyocyte proliferation in the KO was confirmed with staining for the M-phase marker, phosphorylated histone H3. Rates of positivity with this technique were 0.63±0.11% for KO (n=7 mice); 0.07±0.03% for flox/−/T (n=5 mice); and 0.05±0.03 for flox/Cre/V (n=3 mice) P<0.001 for KO versus both controls. We also examined cardiomyocyte proliferation in the KO hearts post-MI (at 4 weeks) and found a similar increase in BrdUrd-positive/TnI-positive cells compared to controls (Figure 7C). Thus, GSK-3β inhibits cardiomyocyte proliferation in the stressed adult heart.

Discussion

Our results indicate that GSK-3β does not play a central role in regulating the hypertrophic response to pressure overload but does inhibit hypertrophy following MI. Following deletion of GSK-3β, despite the fact that hypertrophy is increased in the remote myocardium, apoptosis is reduced and cardiomyocyte proliferation is enhanced. At the organ level, LV dilatation is reduced and LV function is better-preserved.

Previous studies point to GSK-3β as a critical regulator of cardiac hypertrophy, and reviewed elsewhere, but all of the in vivo models used to reach this conclusion involved overexpression of WT, CA, or dominant negative mutants or knock-in of CA mutants. Transgenic models can lead to nonphysiologic protein-protein interactions, aberrant subcellular localization, and phosphorylation of protein substrates that are not physiological substrates. In addition, overexpressed GSK-3β can subsume the physiological roles of GSK-3α, making it impossible to decipher isoform-specific functions. Finally, the CA GSK-3β mutant (with a Ser 9 to Ala mutation) can still be inhibited by the canonical Wnt pathway and by a novel p38-MAPK mediated mechanism, clouding interpretation of findings.

To attempt to clarify distinct GSK-3β isoform functions in pathological hypertrophy, we used a cardiac-specific, inducible GSK-3β knockout. Our model allows temporal control of GSK-3β deletion so we can specifically focus on the role of GSK-3β in the adult. We restricted our studies to mice with greater than 70% knockdown of GSK-3β protein expression in the heart. Average residual GSK-3β expression in these mice was ~20%. Considering that nonmyocytes account for roughly 10% of heart mass, and GSK-3β is not deleted in

GSK-3β Deletion Increases Cardiomyocyte Proliferation

Deletion of GSK-3β blocks differentiation and promotes proliferation in mouse embryonic stem cells in culture, although a small molecule inhibitor of GSK-3 has been reported to lead to increased proliferation of neonatal rat ventricular myocytes (NRVMs) and adult rat ventricular
these cells, residual GSK-3β expression in cardiomyocytes is more likely ≈10% to 15% of WT.

In the conditional KO, we did not see the expected exaggerated PO-induced hypertrophic phenotype. This is likely not attributable to inadequate deletion of GSK-3β because this level of deletion of GSK-3β did lead to increased hypertrophy in the post-MI model. This dichotomy between hypertrophic responses following TAC versus MI clearly suggests a complex pattern of isoform specificity versus redundancy for GSK-3α and GSK-3β in regulating these processes. In the case of PO-induced hypertrophy, GSK-3β is not a central regulator.

In distinct contrast, GSK-3β appears to be the dominant isoform regulating post-MI hypertrophy and remodeling. Although infarct sizes were virtually identical in WT versus KO, hypertrophy was increased in the remote myocardium of the KO. The underlying mechanisms by which GSK-3β mediates post-MI hypertrophy but not PO hypertrophy are...
not clear but the data point to a very different milieu of cytokines, signaling factors, etc., recruited by the different hypertrophic stimuli. Of note, given the reduction in LV dilatation, better-preserved EF, no increase in fibrosis, and no increase in atrial natriuretic peptide or brain natriuretic peptide in the KO, this hypertrophy may be viewed as more “physiological” than “pathological.”

The third key finding of this study is that GSK-3β regulates cardiomyocyte proliferation. One prior study concluded that GSK-3β regulates cardiomyocyte proliferation; however, this study was done in vitro and used a commercially available small molecule inhibitor (BIO). This agent has a poorly-defined selectivity profile and also inhibits both isoforms of GSK-3, making it impossible to decipher isoform-specific effects.

Herein, we found that deletion of GSK-3β in cardiomyocytes in vivo in the setting of pressure overload led to a 3- to 4-fold increase in BrdUrd-positive cardiomyocytes compared to control mice subjected to TAC. In the post-MI KO hearts, absolute rates of BrdUrd positivity were higher compared to TAC, but the relative fold increase in KO compared to controls was similar. It is impossible at this time to determine what, if any, role this enhanced proliferation had on the phenotypes of the KOs, but it is clear that GSK-3β deletion increases cardiomyocyte proliferation in the setting of pressure overload and MI just as it does in embryonic stem cells and in cardiomyocytes in the developing heart. We did not examine proliferation in progenitor cell populations committed to the cardiomyocyte lineage, but it seems likely that cell cycling in these populations would be increased as well and would contribute to the BrdUrd+/TnI+ population we measured.

Our findings may have potential implications for treatment of patients post-MI and for regenerative therapies. Traditional therapeutics to prevent post-MI remodeling following a large MI (eg, angiotensin-converting enzyme inhibitors and angiotensin receptor antagonists) are effective to some degree but progression to congestive heart failure or death, despite tensin receptor antagonists) are effective to some degree but increases in the heart in response to stress (reviewed elsewhere). Cardiac progenitor cells and/or small immature cardiomyocytes proliferate following pressure overload and myocardial infarction. The present beliefs held in the field are that although these cells proliferate and progenitor cells do differentiate into cardiomyocytes, this occurs at too slow a rate to keep up with the rate of cell death so that the net decrease of cells plays a role in the progression of heart failure. Strategies to manipulate progenitor cell behavior in situ by modulating activity of various kinases are being explored. GSK-3β inhibition could increase cardiomyocyte proliferation and perhaps keep pace with cell death rates, thereby preventing progression to heart failure. In addition, based on our earlier findings which show that inhibition of GSK-3 promotes embryonic stem cell proliferation whereas active GSK-3β promotes differentiation into the cardiomyocyte lineage, periodic withdrawal of a GSK-3β inhibitor could help drive progenitor differentiation into cardiomyocytes. That said, targeting both GSK-3α and GSK-3β long-term with small molecule inhibitors raises concerns over promoting cancer by enhancing β-catenin signaling. Selective targeting of individual isoforms (ie, GSK-3β) could, however, maintain normal β-catenin signaling while allowing increased cardiomyocyte proliferation.

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Disclosures
None.

References


Novelty and Significance

What Is Known?

- Large myocardial infarctions typically progress to remodeling of the ventricle and, in many cases, to heart failure.
- Molecular mechanisms underlying these processes are only beginning to be identified.
- Current therapeutic options for the sub-acute to chronic phases of MI are limited.

What New Information Does This Article Contribute?

- We identify glycogen synthase kinase (GSK)-3β as one key factor that drives the post-MI remodeling process; we find that deleting GSK-3β preserves LV structure and function.
- We also find that deletion of GSK-3β leads to proliferation of cardiomyocytes in situ.
- Contrary to numerous prior publications, we find that GSK-3β does not appear to importantly modulate pressure overload hypertrophy.

The molecular mechanisms that regulate the adverse remodeling that occurs following an MI are not known. Identification of these mechanisms could lead to novel therapeutic strategies to limit remodeling and progression to heart failure. In the first studies to examine the role of GSK-3β in the heart using gene targeting, we report that deletion of GSK-3β specifically in cardiomyocytes limits adverse remodeling and leads to cardiomyocyte proliferation post-MI. In contrast, despite numerous publications that have used transgenesis to suggest that GSK-3β regulates the hypertrophic response to pressure overload, we find that GSK-3β plays at most a minor role in this process. Thus we have identified a unique role of the β isoform of GSK-3 in the heart, which could have direct implications for patient care. Specifically, small molecule inhibitors of GSK-3 could be used in the post-infarct period to reduce remodeling and possibly to induce reparative cardiomyocyte proliferation. Given the limited treatment options currently available for patients who have experienced a large MI, and the dearth of new classes of drugs to treat these patients beyond the acute phase, GSK-3β inhibition could offer a novel strategy to prevent remodeling and heart failure.
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Online Methods

Echocardiography

Transthoracic two-dimensional echocardiography-guided M mode was performed with a 12-mHz probe (Visualsonics) on mice anesthetized by inhalation of isoflurane (1-1.5%). M mode interrogation was performed in the parasternal short-axis view at the level of the greatest LV end-diastolic dimension (LVEDD). LVEDD, left ventricular end-systolic dimension (LVESD), and septal and LV posterior wall thicknesses were determined and used to calculate percent fractional shortening (FS), ejection fraction (EF), and LV mass (LVM). FS was calculated with the formula: \((\text{LVEDD} - \text{LVESD}) / \text{LVEDD}) \times 100\). EF was calculated with the formula: \(((\text{LVEDV} - \text{LVESV}) / \text{LVEDV}) \times 100\) where EDV is end-diastolic volume and ESV is end-systolic volume. LVM was calculated with the formula: \(((2 \times \text{PWT}) + \text{EDD})^3 - \text{EDD}^3\) where PWT is posterior wall thickness.

Thoracic Aortic Constriction (TAC)

TAC was performed as described. Briefly, male mice were anesthetized with ketamine (100mg/kg IP) plus xylazine (5mg/kg IP) then were intubated with a 20-gauge endotracheal tube. Mice were ventilated at 120 breaths/min (Harvard Apparatus Rodent Ventilator). A 3mm left-sided thoracotomy was performed, a 27-gauge needle was placed adjacent to the thoracic aorta, and then a suture ligature was tied around the vessel and needle. The needle was removed leaving a 65-70% stenosis between the innominate and left common carotid arteries. Perioperative mortality was twenty percent. After post-operative day one, mortality was less than one percent. At 3 weeks post-TAC, mice underwent echocardiography, invasive hemodynamic measurement, and then were sacrificed for determination of morphometrics and the various studies outlined below.

Myocardial Infarction

Following baseline echocardiography, permanent occlusion of the proximal left anterior descending coronary artery was performed exactly as described in Gao et al. Echocardiography was repeated at 1, 2, 4, 6, and 8 weeks post MI. Following the 8 week echo examination, mice underwent invasive hemodynamic measurement, and then were sacrificed for studies outlined below.

Hemodynamic Measurements

Three weeks post-TAC or 8 weeks post MI, mice were anesthetized by inhalation of isoflurane (2%) and a 1.4F catheter with a high fidelity pressure transducer (Millar Instruments) was inserted into the right carotid and advanced to the LV. The hemodynamic measurements obtained included heart rate, LV end-diastolic pressure, LV systolic pressure, +dP/dt, and –dP/dt. Data were analyzed using LabChart 6 Reader (AD Instruments).

Bromo-deoxyuridine (BrdU) protocol

Mice were treated with tamoxifen or vehicle and underwent TAC, MI, or sham surgeries as described above. At 2 weeks post-TAC or sham surgery, or 4 weeks post-MI or sham surgery, the mice were injected with BrdU (50mg/kg IP; Sigma-Aldrich) twice a day for seven days, and then were sacrificed for immunocytochemistry studies (see below).

Isolation of Cardiomyocytes

Cardiomyocytes were isolated as described with some modifications. Briefly, the mice were anesthetized with ketamine and the heart was excised. The aorta was cannulated with a 20-gauge needle then mounted on the perfusion apparatus. The perfusion solution was composed of Tyrodes buffer [150 mM NaCl, 10 mM dextrose, 5.4 mM KCl, 1.2 mM MgCl2, 2 mM Na-pyruvate, and 5 mM Hepes] adding 5 mM taurine. The aorta was perfused for 2–3 min, then 50 mg of type-II collagenase (Worthington) and 6 mg of trypsin were added and perfused for 13 minutes. The temperature of perfusate was maintained at 34°C and all solutions were continuously bubbled with 95% O2, 5% CO2. LV tissue was separated from the great vessels, atria and right ventricle, and minced. The digested heart was filtered through 200 µm nylon mesh,
placed in a conical tube, and spun at 100 rpm to allow viable myocytes to settle. The supernatant was removed and 4% paraformaldehyde solution was added to the cells. After 10 minutes, the cells were centrifuged for 5 minutes at 100 x g. The supernatant was removed and PBS with 0.01% sodium azide was added.

**Protein analysis**

Snap-frozen LV sections were homogenized in cell lysis buffer: (Tris (20mM pH 7.5), NaCl (150mM), EDTA (1mM), EGTA (1mM), Triton-X (1%), sodium pyrophosphatase (2.5mM), β-glycerolphosphate (1mM), Na3VO4 (1mM)), and containing phosphatase and protease inhibitors (Sigma-Aldrich). The lysates were centrifuged at 14,000rpm for ten minutes and the protein concentration was quantified by BioRad DC Protein Assay (BioRad). Equal amounts of protein were heated at 95°C for five minutes in 5x SDS sample buffer, and then were electrophoresed through a 4-20% polyacrylamide gel (Invitrogen), and transferred to nitrocellulose membrane (Schleichert and Schuell). The membranes were blocked in Odyssey blocking buffer (Li-COR) and incubated in primary antibody at 4°C overnight. After three washes in Tris-buffered saline with Tween-20, the membranes were incubated at room temperature in the appropriate secondary antibodies (Alexa Fluor 680; Invitrogen). Proteins were visualized with LI-COR infrared imager and quantitative densitometric analysis was done using Odyssey version 1.2 software (Li-COR).

Primary antibodies used include: GSK-3β, p-GSK-3β (Ser9), GSK-3α, p-GSK-3α/β (Ser21/9), glycogen synthase, p-glycogen synthase (Ser641), p-Histone H3(Ser10), cardiac troponin I (TnI), β-catenin, DRAQ5 (from Cell Signaling); p-eIF2B (Ser 540; BD Bioscience); c-myc, cyclin D1 (Santa Cruz); BrdU (Roche); 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich); and GAPDH (Research Diagnostics, Inc). All antibodies were used at dilution of 1:1000 except for GAPDH which was used at 1:1,000,000 for Western blot. For immunostaining p-Histone H3 (Ser10) and TnI were used at 1:400, BrdU was used at 1:10 dilution, and DRAQ5 was used at 1:1000 dilution.

**Histological analysis**

LV tissue was fixed with 4% paraformaldehyde for 24 hours, dehydrated through increasing concentrations of ethanol, and then embedded in paraffin. LV sections (5µm) were stained with hematoxylin-eosin (H&E; Sigma-Aldrich) or Masson trichrome (Sigma-Aldrich). A Nikon Eclipse 80i microscope was used to visualize the cells and NIS Elements software was used to record images. Cross-sectional area of H&E stained cardiomyocytes was assessed using NIS Elements software. Further, NIS Elements software was used to analyze the total area of fibrosis on trichrome-stained sections. All analyses were performed by an observer blinded to the conditions.

**Immunocytochemistry**

LV sections were immunostained with anti-BrdU and TnI, and were counterstained with DRAQ5 or DAPI, (nuclear markers). Briefly, slides were deparaffinized in xylenes and rehydrated through decreasing concentrations of ethanol. Then sections were heated in sodium citrate buffer for ten minutes and cooled. Primary antibodies were added individually and incubated at 37°C for one hour. After washing the slides in PBS, the appropriate secondary was added for one hour at 37°C. Following this, the second primary antibody was added at 37°C for one hour. The slides were then washed in PBS and the other secondary antibody was added as above. After washing the final secondary with PBS, DRAQ5 (or DAPI) was added and the slides incubated at room temperature for ten minutes. Following two more washes in PBS, the slides were covered in Immuno-mount (Thermo Scientific) and a coverslip was applied. An Olympus Fluoview confocal microscope was used to visualize the cells and Olympus Fluoview software was used to record images. Only cells that were TnI positive and BrdU positive with BrdU stain co-localizing with DAPI were counted as proliferating cardiomyocytes.

**Statistics.**
Data were analyzed using Student’s T-test for all except the linear regression data. For this determination, a single linear regression model of 2 sets of data was completed using an indicator variable to distinguish experimental sets. The initial model allowed both intercept and slope to differ by experiment. Computation was completed in SAS version 9.1. Significance was set at p < 0.05 and all data presented are means ± standard error of the mean.

References
Online Supplement Tables

<table>
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<tr>
<th></th>
<th>n</th>
<th>HR (bpm)</th>
<th>LVSP (mmHg)</th>
<th>LVEDP (mmHg)</th>
<th>+dP/dt</th>
<th>-dP/dt</th>
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<td>Flox/-/T + TAC</td>
<td>26</td>
<td>411 ± 24</td>
<td>135 ± 27</td>
<td>9.0 ± 5.5</td>
<td>6286 ± 1680</td>
<td>-6056 ± 1854</td>
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<td>421 ± 30</td>
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<td>7418 ± 1594</td>
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<td>5987 ± 902</td>
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**Online Table I:** Heart rate and hemodynamic parameters in the various groups 3 weeks post TAC or sham TAC. HR, heart rate; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt, rate of rise of LVSP; -dP/dt, rate of fall of LVEDP.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>EDD (mm)</th>
<th>EF (%)</th>
<th>FS (%)</th>
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<td>Flox/-/T Baseline</td>
<td>26</td>
<td>3.66 ± 0.35</td>
<td>61.0 ± 7.2</td>
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<td>Flox/Cre/T Baseline (KO)</td>
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<td>3.68 ± 0.37</td>
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<td>Flox/Cre/V Baseline</td>
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<td>3.95 ± 0.40</td>
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<td>Flox/-/T + TAC</td>
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<td>3.30 ± 0.45</td>
<td>66.1 ± 8.3</td>
<td>37.0 ± 9.1</td>
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<td>Flox/Cre/T + TAC (KO)</td>
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<td>3.35 ± 0.32</td>
<td>63.3 ± 9.0</td>
<td>34.3 ± 6.5</td>
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<td>Flox/Cre/V + TAC</td>
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<td>3.30 ± 0.60</td>
<td>58.1 ± 14</td>
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**Online Table II:** Baseline and 3 weeks post TAC echocardiographic measurements in the various groups. EDD, end-diastolic dimension; EF, ejection fraction; FS, fraction shortening.
Online Figure I. Representative images of isolated cardiomyocytes 3 weeks post TAC. Quantification of myocyte area is shown below. Cardiomyocyte size was not significantly different between flox/-/T mice and flox/Cre/V mice; therefore, these controls were grouped together in the graph. n = 200 cardiomyocytes/mouse with 4 mice as controls and 4 conditional KO mice.
Online Figure II. Fetal gene expression in GSK-3β KO mice post TAC and post MI.

Transcripts for markers of hypertrophy in hearts from control and GSK-3β KO mice were detected by quantitative PCR 3 weeks post TAC (IIA) and 8 weeks post MI (IIB). Values indicate relative expression levels (± SEM). ANP (atrial natriuretic peptide); BNP (brain natriuretic peptide); β-MHC (β-Myosin heavy chain; Myh7). Differences in gene expression between groups were analyzed by t-test.
Online Figure III

**III A**
LVSP = 150-180 mmHg

**III B**
LVSP = 120-150 mmHg

**Online Figure III.** GSK-3β deletion does not affect cardiac function post TAC.

III A/B. EF was determined by echocardiography at baseline and again at 3 weeks post TAC. Data in panel III A are from mice with systolic pressures between 150mmHg -180mmHg post TAC, and in panel III B from mice with systolic pressures between 120mmHg – 150mmHg post TAC. There was no difference in EF between the conditional KO vs. control mice either at baseline or post TAC, in either the higher or lower LVSP groups.

In IIIA, n = 3 for flox/Cre/V; n = 8 for flox/-/T; n = 12 for flox/Cre/T (KO).
In IIIB, n = 3 for flox/Cre/V; n = 14 for flox/-/T; n = 12 for flox/Cre/T (KO).
Online Figure IV. GSK-3β deletion does not alter extracellular matrix remodeling post TAC. Three weeks post TAC, LV sections from mice with systolic pressures between 150-180mmHg were stained with Masson trichrome stain. Representative images are shown. Total fibrotic area as a percent of total area was determined as described in Methods. While there was an increase in fibrosis post TAC in all groups, fibrosis was not different between the groups.
Online Figure V. Activity of hypertrophic signaling pathways.

Immunoblot (VA) and quantification (VB) of eIF2B phosphorylation and c-myc expression 24 hours post TAC. p-eIF2B is significantly decreased and c-myc levels are significantly increased in conditional KO hearts.