The GSK-3 Family as Therapeutic Target for Myocardial Diseases

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Abstract: Glycogen synthase kinase-3 (GSK-3) is one of the few signaling molecules that regulate a truly astonishing number of critical intracellular signaling pathways. It has been implicated in several diseases including heart failure, bipolar disorder, diabetes mellitus, Alzheimer disease, aging, inflammation, and cancer. Furthermore, a recent clinical trial has validated the feasibility of targeting GSK-3 with small molecule inhibitors for human diseases. In the current review, we will focus on its expanding role in the heart, concentrating primarily on recent studies that have used cardiomyocyte- and fibroblast-specific conditional gene deletion in mouse models. We will highlight the role of the GSK-3 isoforms in various pathological conditions including myocardial aging, ischemic injury, myocardial fibrosis, and cardiomyocyte proliferation. We will discuss our recent findings that deletion of GSK-3α specifically in cardiomyocytes attenuates ventricular remodeling and cardiac dysfunction after myocardial infarction by limiting scar expansion and promoting cardiomyocyte proliferation. The recent emergence of GSK-3β as a regulator of myocardial fibrosis will also be discussed. We will review our recent findings that specific deletion of GSK-3β in cardiac fibroblasts leads to fibrogenesis, left ventricular dysfunction, and excessive scarring in the ischemic heart. Finally, we will examine the underlying mechanisms that drive the aberrant myocardial fibrosis in the models in which GSK-3β is specifically deleted in cardiac fibroblasts. We will summarize these recent results and offer explanations, whenever possible, and hypotheses when not. For these studies we will rely heavily on our models and those of others to reconcile some of the apparent inconsistencies in the literature. (Circ Res. 2015;116:138-149. DOI: 10.1161/CIRCRESAHA.116.303613.)

Key Words: fibrosis ■ glycogen synthase kinase-3 ■ heart failure ■ myocardial infarction

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed, serine/threonine kinase that was identified in 1980 for its role in regulating glycogen synthase, the rate-limiting enzyme in glycogen synthesis. GSK-3 was first cloned in 1990, based on partial peptide sequencing. GSK-3 is a highly conserved protein kinase as isoenzymes from species as distant as flies and humans display >90% sequence homology within the protein kinase domain. The GSK-3 family consists of 2 isoforms, α and β, which are 98% identical within their kinase domains but differ substantially in their N- and C-terminal sequences, which accounts for an overall sequence homology of 85%. This exceptionally high homology in the kinase domain makes the prospect of development of isoform-specific small molecule inhibitors daunting (although still possible). Although the GSK-3 isoforms have similar structures and overlapping functions, they also have some unique properties: (1) only the β-isoform is phosphorylated at Ser389/Thr390; (2) β-isoform has a neuronal-specific splice-site inserted at AA-13; and (3) some substrates are isoform specific. Furthermore, the phenotype of global deletion of both isoforms is different, as global deletion of GSK-3β is embryonic lethal but GSK-3α-deficient mice born are normal and can survive up to several years. Both isoforms also have some unique regulatory signaling mechanism as cells lacking GSK-3β prevent proper activation of nuclear factor κB (although the precise mechanism is unclear), but α-null cells show no effect. Another example of isoform-specific regulation is contraction of Sma and Mad (mothers against decapentaplegic) (SMAD-3), which is strongly regulated by GSK-3β in a GSK-3α independent manner.

Unlike most protein kinases, GSK-3 is typically active in unstimulated cells and is inhibited in response to a variety of stimuli. Although GSK-3 is one of the few protein kinases that can be inactivated by phosphorylation, the molecular mechanism of GSK-3 regulation is complex and not fully understood. GSK-3s are negatively regulated by N-terminal phosphorylation of serine residues of the enzyme (Ser21 for GSK-3α and Ser9 for GSK-3β), herein after referred to as S21 and S9, respectively. In addition to negative regulation by N-terminal phosphorylation, the activity of GSK-3 is positively regulated...
by tyrosine phosphorylation at Tyr279 for GSK-3α and Tyr216 for GSK-3β.3–4 p38 MAPK (mitogen-activated protein kinase) can also inactivate GSK-3β via phosphorylation within its C-terminal region at Ser389 and Thr390.5 The role of S21 and Tyr216 phosphorylation in myocardial pathophysiology has been studied extensively6–10; however, the physiological significance of tyrosine phosphorylation and p38-mediated C-terminal phosphorylation is not yet clear. The basic biology and molecular mechanisms of GSK-3 signaling have been reviewed previously and the readers are referred to a recent review for details.11

The initial in vitro studies examining the role of GSK-3 in cardiac disease processes were first published a decade ago and identified GSK-3β as a negative regulator of the hypertrophic response in cardiomyocytes.12,13 Haq et al.12 demonstrated that adenovirus-mediated gene transfer of GSK-3β with a Ser9 to Ala mutation (a mutant that cannot be inhibited by Akt) led to a reduced hypertrophic response of cardiomyocytes after stimulation with hypertrophic agonists. This study suggested that inactivation of GSK-3β was required for cardiomyocytes to recruit hypertrophic response.12 Since then, numerous in vivo studies, using a variety of genetically modified mouse models have been published and suggest an essential role of GSK-3α/β in several important aspects of cardiac biology.6,14–18

The Table summarizes a list of studies with genetically modified mouse models, suggesting crucial roles of GSK-3α/β in regulating cardiac homeostasis and responses to stresses in vivo.6,7,10,15–28 Previously, most of the attention on the GSK-3 family has centered on GSK-3β. However, based on our recent studies in isofrom-specific conditional knockouts, both isoforms seem to play overlapping, unique, and even opposing functions in the heart. Herein, we will focus on recent data derived from cardiomyocyte- and fibroblast-specific conditional mouse models. We will discuss our recent findings that deletion of GSK-3α, specifically in cardiomyocytes, is protective in the setting of myocardial infarction (MI), suggesting that specifically targeting GSK-3α could be a novel strategy to limit adverse remodeling in ischemic hearts. These findings are in stark contrast with the studies of germline global deletion of GSK-3α, which suggest that loss of GSK-3α is detrimental, leads to spontaneous hypertrophy by 6 months of age,19 aggravates MI-induced remodeling,20 and shows accelerated aging-related pathologies.21 We will also discuss our recent findings that GSK-3β negatively regulates fibrotic remodeling in the ischemic heart by modulating canonical transforming growth factor β1 (TGF-β1) signaling through direct interactions with SMAD-3, suggesting that GSK-3β-mediated negative regulation of fibrosis is essential to limit the adverse ventricular remodeling in the ischemic heart.17 Figure 1 highlights some of the important upstream activators and downstream targets of GSK-3 that have been implicated in cardiac pathology.

### Role of GSK-3α in Cardiac Hypertrophy and Fibrosis

As terminally differentiated cells, cardiomyocytes respond to various physiological (eg, exercise) and pathological (eg, hypertension) stresses by undergoing cellular and subsequently organ level hypertrophy. It is well established that physiological hypertrophy enhances cardiac performance via increasing stroke volume and oxygen consumption.32,33 However, pathological hypertrophy is typically associated with re-expression of the fetal gene program, increased fibrosis, cell death, and cardiac dysfunction and may lead to heart failure. Henceforth, we will use the term cardiac hypertrophy to mean pathological cardiac hypertrophy.

We used a global loss of function model to demonstrate that GSK-3α is a negative regulator of cardiac hypertrophy and fibrosis at baseline and in the stressed heart.14,20 Mechanistically, mammalian target of rapamycin 1 (mTORC1) has been identified as a key target of GSK-3α to regulate cardiac hypertrophy. We found that mTORC1 was profoundly dysregulated as evidenced by significantly increased phosphorylation of mTORC1 targets including eIF4E binding protein and ribosomal S6 kinase in the GSK-3α KO.15 Transgenic overexpression of GSK-3α in mice led to smaller hearts with increases in apoptosis and fibrosis but with preserved cardiac function at baseline. However, in response to PO, these mice showed attenuated cardiac hypertrophy, enhanced apoptosis and fibrosis, and significantly reduced ventricular function.19 Mechanistically, GSK-3α seems to inhibit physiological cardiac growth through inhibition of extracellular signal–regulated kinase activation.19 Maejima et al.21 used a constitutively active MAPK/Erk kinase 1/GSK-3α bigenic mouse model and successfully rescued most of the detrimental phenotype of the GSK-3α-tg mice. Surprisingly, increased cardiac fibrosis was not rescued. Consistently, GSK-3α global knockin led to markedly increased pathological cardiac hypertrophy, fibrosis, and

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ventricular dysfunction in response to PO.5 We used a cardiac-specific conditional GSK-3α KO (GSK-3αc KO) mouse and challenged them with MI. Consistently, GSK-3αc KO mitigates post-MI remodeling, contractile dysfunction, and heart failure.26 However, our findings suggest that GSK-3α has no direct role in MI-mediated cardiac hypertrophy and fibrosis.

**Deletion of GSK-3α Accelerates Aging in Mice**

Aging is a naturally occurring complex biological process, which is characterized by deterioration in physiological integrity that leads to progressive functional impairment and increased mortality.24 The deterioration in cellular physiology promotes pathological conditions, such as cardiovascular...
disorders, cancer, and neurodegenerative diseases. Genetic, epigenetic, and external factors such as dietary intake and intrinsic stresses such as reactive oxygen species are known key drivers of aging. Understanding of the aging process has been hampered by several issues such as gradual and heterogeneous inception in the body, diverse phenotypes, and most importantly lack of adequate molecular biomarkers to quantify the degree of aging. Recently, we reported that GSK-3α seems to be one of the key regulators of aging. We found that GSK-3α actively suppresses the aging process, and global deletion of this kinase accelerates age-related pathologies, thereby reducing life span. GSK-3α KO mice showed profound aging phenotypes in most of the organs systems including the heart, gut, liver, and bone. Further studies in the heart revealed vacuolar degeneration consistent with marked sarcopenia as well as significant myocyte dropout with increased fibrosis. Mechanistically, we found that insulin receptor substrate-1 expression, a direct target of GSK-3, was upregulated and mTORC1 function was dysregulated as evidenced from increased activity of downstream targets, that is, eIF4E binding protein, S6 kinase, and ribosomal S6 protein in the aging mice. mTORC1 dysregulation in KO hearts led to impaired autophagy as reflected by decreased microtubule-associated protein light chain 3 (LC3-II to LC3-I) ratio and increased p62 expression in the GSK-3α KOs. To confirm that the observed aging phenotypes in the GSK-3α-deficient mice were because of dysregulation of mTORC1, we used a second generation inhibitor of mTORC1, everolimus, and indeed, the pathologies observed in aged KO mice were partially rescued. Thus, GSK-3α is a critical regulator of mTORC1, autophagy, and aging and its deletion leads to accelerated aging in multiple tissues. This study suggests that strategies to maintain GSK-3α activity in the elderly could retard the appearance of age-related pathologies. One major limitation of this study is the exclusive dependence on an embryonic global KO model, which has well-known limitations of developmental and compensatory effects. Further studies with inducible tissue-specific loss of

Figure 1. Signaling cascades regulated by glycogen synthase kinase-3 (GSK-3) in heart. In response to growth factor binding to their receptors, the phosphoinositide 3 kinase/AKT8 virus oncogene cellular homolog (PI3K/Akt) pathway is activated, leading to inhibition of GSK-3. GSK-3 negatively regulates a host of factors downstream of growth factor signaling, so the consequences of GSK-3 inhibition are activation of these factors including (1) NF-AT-hypertrophy regulator, (2) glycogen synthase-glycogen synthesis regulator, (3) mammalian target of rapamycin (mTORC1) autophagy and metabolism regulator, (4) D- and E-type cyclin cell cycle regulator, and (5) Myc-metabolism and proliferation regulator. An alternative mechanism to inhibit GSK-3 is mediated by p38. GSK-3β directly binds with contraction of Smad and Mad (mothers against decapentaplegic) (SMAD-3) to negatively regulate the profibrotic transforming growth factor β1 (TGF-β1) signaling. GSK-3 is well known to regulate the canonical Wnt signaling. Phosphorylation of β-catenin by GSK-3 leads to the ubiquitination and degradation of β-catenin by the proteasome, preventing gene expression. In the absence of GSK-3, β-catenin is stabilized, and then translocates to the nucleus leading to gene expression. β-catenin regulates a host of processes from fibrosis to cardiac hypertrophy. GSK-3α regulates AMP-activated protein kinase (AMPK) and mTOR, the master regulators of autophagy and metabolism. In the absence of GSK-3α, mTOR is dysregulated leading to impaired autophagy, which accelerates the progression of aging. GSK-3α also regulates the β-adrenergic receptor responsiveness and cAMP production via unknown mechanisms. GSK-3α directly interacts and phosphorylates cyclin E1 in cardiomyocytes and its deletion promotes E2F-1 and cyclin E1 recruitment and induces the re-entry of adult cardiomyocytes into the cell cycle. 4EBP1 indicates eIF4E binding protein; APC, adenomatous polyposis coli; Dvl, disheveled; ECM, extracellular matrix; elf, eukaryotic initiation factor; EMAT, epithelial-to-mesenchymal transition; FAK, focal adhesion kinase; GPCR, G-protein coupled receptor; GS, glycogen synthase; ILK, integrin-linked kinase; IRS, insulin receptor substrate; MPTP, mitochondrial permeability transition pore; NFAT, nuclear factor of activated T cells; PDK1, 3-phosphoinositide-dependent protein kinase 1; PLB, phospholamban; RhoA, Ras homolog gene family, member A; RTK, receptor tyrosine kinase; and TjR, TGF-β1 receptor (Illustration credit: Ben Smith).
function model will be useful to better understand the role of GSK-3α in aging process.

**GSK-3α Regulates Cardiomyocyte Proliferation in the Adult Injured Heart**

The neonatal heart has the capability of regeneration through cardiomyocyte proliferation. However, the mammalian adult heart has limited regenerative capacity and has been considered generally as a terminally differentiated postmitotic organ. Various stressors, most importantly ischemic injury, can lead to progressive cardiomyocyte loss. The loss of cardiomyocytes after an MI in humans results in scar formation, loss of contractile capacity, and reduced cardiac function. Cardiomyocyte proliferation occurs at a low level throughout life, but increases modestly in the heart in response to stress. The endogenous regenerative capacity of the heart seems insufficient to replenish lost myocytes, so even relatively low levels of apoptosis can have profound effects on cardiac function. This has necessitated employment of strategies that are required for activation of endogenous repair mechanisms of the heart.

GSK-3β is a potent regulator of cell proliferation during development (see below). How ever, the role of GSK-3α isoforms in cardiomyocyte proliferation has not been studied adequately. Persistent activation of GSK-3α is detrimental and abrogates cardiomyocyte proliferation. Moreover, this study indicated that genes involved in the cell cycle and cell proliferation were significantly downregulated in the GSK-3α global knockout hearts after PO. Mechanistically, this study reported that persistent activation of GSK-3α suppresses the E2F transcription factor, likely through D-type cyclins in cardiac myocytes. Consistently, GSK-3α KO is protective in the setting of MI. Moreover, GSK-3α KO hearts were associated with robust cardiomyocyte proliferation and protection against scar expansion and thinning in the ischemic heart. Mechanistically, GSK-3α deletion induces cardiomyocyte proliferation in the injured adult heart as evidenced by increased number of Ki67 and BrdU (bromodeoxyuridine-[5-bromo-2’-deoxyuridine]-positive cardiomyocytes. Deletion of GSK-3α increases E2F-1 and cyclin E1 recruitment in the heart after MI. Further ex vivo studies show that GSK-3α regulates cyclin E1 levels in cardiomyocytes through phosphorylation. The elevated levels of E2F-1 and cyclin E1 in the GSK-3α- KO hearts seem to be the central mechanism of cardiomyocyte proliferation in vivo. These findings suggest that GSK-3α is a key regulator of cell cycle activators in the cardiomyocyte and strategies to inhibit GSK-3α could potentially be used in cardiac regeneration in patients with chronic MI. Taken together, these findings suggest that inhibition of GSK-3α limits ventricular remodeling and preserves cardiac function, after MI. Thus, specifically targeting GSK-3α could be a novel strategy to limit adverse remodeling and heart failure.

**Role of GSK-3β in Ischemic Injury**

Numerous studies support the notion that phosphorylation (inhibition) of GSK-3β at Ser9 is required for the cardioprotection mediated by ischemic preconditioning. Juhaszova et al. reported that inhibition of GSK-3β delays the opening of the mitochondrial permeability transition pore, which is largely responsible for the cardioprotection. Using RNA interference, Juhaszova et al. also showed that protective signaling is specifically mediated via the GSK-3β isoform, in a GSK-3α independent manner. Gomez et al. used transgenic GSK-3β-S9A mice to demonstrate that serine 9 phosphorylation of GSK-3β is required for cardioprotection from ischemic postconditioning and likely acts by inhibiting opening of the mitochondrial permeability transition pore in a cyclophilin D-independent mechanism. It has also been reported that GSK-3β interacts with adenine nucleotide translocase at the inner mitochondrial membrane. However, the exact permeability transition pore–regulatory target(s) of GSK-3β is not known.

Interesting twists in the story began to appear when investigators used knockins of the inhibition-resistant form of GSK-3β in ischemic preconditioning in which the phosphorylation sites on GSK-3α(Ser21) and GSK-3β(S9) are mutated to alanine. These studies questioned the obligatory role of GSK-3 isoforms in cardiac protection and suggested that the inhibition of GSK-3α/β is unlikely to be the key determinant of cardioprotective signaling. Thus, the role of GSK-3β in ischemic preconditioning is not clear and requires additional studies with conditional loss of function mouse models and isoform-specific pharmacological inhibitors.

We used inducible cardiomyocyte-specific GSK-3β KO mice to demonstrate that the deletion of GSK-3β specifically in cardiomyocytes is protective in the setting of permanent MI. GSK-3β knockouts (GSK-3β KO) displayed reduced left ventricular (LV) remodeling, better-preserved LV function, and less dilatation after MI. Importantly, this protection was not because of reduced infarct size in the GSK-3α KO because we used a permanent occlusion MI model and we did not delete the gene until 5 days after MI, at a time when the infarct was completed. Surprisingly, the role of cardiomyocyte GSK-3β in heart is stress dependent because GSK-3β KO develops hypertrophy in response to MI but not in response to PO stress (discussed below). However, the observed hypertrophy seen in the remote myocardium of the GSK-3α KO after MI seems consistent with physiological, as opposed to pathological hypertrophy because these mice display better-preserved heart function, reduced LV remodeling, and apoptosis with significantly increased cardiomyocyte proliferation.

Webb et al. used double KI mice with constitutive activation of both GSK-3α and GSK-3β and demonstrated that constitutive GSK-3α/β activity has no effect on chronic stress remodeling after permanent MI, suggesting that LV remodeling after regional infarction is independent of GSK-3. These results contradict our findings in the conditional GSK-3β KO mice and are difficult to reconcile. Recently, Sadoshima and colleagues investigated the role of GSK-3β in the heart subjected to either prolonged ischemia alone or a short period of ischemia followed by reperfusion, using genetically engineered mouse models of GSK-3β inhibition (transgenic dominant negative GSK-3β and GSK-3β-ko) and activation (transgenic GSK-3β knockin). This study suggested that inhibition of GSK-3β exacerbates ischemic injury but
Role of GSK-3β Signaling in Cardiac Hypertrophy

The role of GSK-3β in cardiac hypertrophy has been studied extensively, in both in vitro culture models and in vivo with gain and loss of function mouse models.6,7,12,13,15,24,25,49–53 Haq et al12 and Morisco et al13 were the first to discover that GSK-3β is a negative regulator of cardiac hypertrophy. Adenoviral gene transfer of GSK-3β-S9A in cardiomyocytes was sufficient to block hypertrophic responses to α-adrenergic, β-adrenergic, and endothelin-1 stimulation. Considering the importance of this discovery, after these initial cell culture–based reports, several groups created genetically modified animal models to confirm these findings in vivo.7 To determine whether activated GSK-3 can act as an antagonist of hypertrophic signaling in the adult heart in vivo, Olson and colleagues7 generated transgenic mice that express a constitutively active form of GSK-3β under the control of a cardiac-specific promoter. Cardiac-specific expression of activated GSK-3β diminished hypertrophy in response to chronic β-adrenergic stimulation and PO. These findings confirmed the role for GSK-3β as a negative regulator of hypertrophic signaling in vivo and suggest that elevation of cardiac GSK-3β activity may provide clinical benefit in the treatment of pathological hypertrophy.

Using a gain of function approach, we observed that transgenic expression of GSK-3β in the mouse heart leads to dramatic impairment of normal postnatal cardiomyocyte growth and results in small (hypotrophic) hearts associated with markedly abnormal cardiac contractile function because of impaired calcium handling.24 Thus, these studies confirmed that GSK-3β regulates both normal and pathological cardiac growth. To examine the effect of persistent inactivation of GSK-3β on cardiac phenotypes at baseline and in response to PO, transgenic mice expressing a kinase inactive (dominant negative) form of GSK-3β were used.15 Interestingly, inhibition of GSK-3β at baseline induces well-compensated cardiac hypertrophy similar to physiological hypertrophy and stimulates cardiac function. This report further suggests that inhibition of GSK-3β during PO stress plays a protective role by inhibiting apoptosis and fibrosis, thereby preventing cardiac decompensation. Matsuda et al16 used GSK-3β KI mice and demonstrated that the expression of GSK-3β-S9A prevents cardiac hypertrophy and dysfunction in response to PO stress.

We used a cardiomyocyte-specific, conditional knockout model to define the role of GSK-3β in pathological hypertrophy.25 Based on the majority of the published data (discussed above), we expected that there would be exaggerated hypertrophy in GSK-3βc KO in response to PO. However, to our surprise, we found that GSK-3β has no role in basal or transverse aortic constriction–induced pathological cardiac hypertrophy.26 Furthermore, we did not find any differences in the cardiac function of wild-type and GSK-3βc KO at baseline and in response to transverse aortic constriction. In distinct contrast, GSK-3β seems to be the dominant isoform regulating hypertrophy in the ischemic heart, which seems to be physiological hypertrophy (discussed in detail in Ischemic Injury section of this article). This study suggests that in the case of transverse aortic constriction–induced hypertrophy, GSK-3β is not a central regulator.

GSK-3β, but Not GSK-3α, Regulates Cardiac Development and Cardiomyocyte Proliferation and GSK-3α Is Dispensable

The first set of questions to address that allowed us to begin to understand what roles were being played by the 2 isoforms were studies ex vivo. In these, we used embryonic stem (ES) cells to create embryoid bodies (EBs) from wild-type, GSK-3α KO, or GSK-3β KO. We found that GSK-3α KO cardiomyocytes had somewhat impaired differentiation compared with wild-type EBs. Most striking, however, were the GSK-3β KO EBs. These EBs exhibited marked increases in size and numbers of EBs that were proliferating based on phosphohistone H3 staining. Thus, terminal cardiomyocyte differentiation was substantially blunted in GSK-3β KO EBs. It also became apparent that abnormalities were associated with factors typically involved in promoting cell proliferation (GATA, cyclin D1, and c-Myc). Importantly, β-catenin/Wnt pathway was not affected in GSK-3β KO EBs. Similarly, GATA4 and Nkx2.5 were not substantially altered in GSK-3β KO versus wild type.16

Differences in GSK-3α and -3β signaling in cardiomyocyte differentiation were even more apparent in the heart development. Although the GSK-3α KO embryos were born at the expected frequency and had no cardiac developmental defects, live births were not observed in GSK-3β KO mice. Most striking was the profound hypertrophic myopathy that developed, with near obliteration of the ventricular cavities because of marked myocyte proliferation. Of note, congenital heart abnormalities, including double outlet right ventricle and ventricular septal defects, were observed but were not common, and these certainly did not account for the marked thickening of the ventricular walls in the GSK-3β KO. Equally striking, impaired cardiomyocyte differentiation in ES cells and pronounced hyperplasia of cardiomyocytes during embryonic development were blatantly obvious.

To determine the mechanism, we examined rates of proliferation of the myocytes and found highly significant increases in myocytes that were positive for phosphohistone H3, a marker of proliferation. Thus, it was abundantly clear that the hypertrophy was virtually completely due to myocyte proliferation rather than true hypertrophy. Thus, GSK-3β, but not
GSK-3α, regulates cardiac development and partners with GATA4, cyclin D1, and c-Myc, which seem to play key roles in the developing embryonic heart. In summary, GSK-3β is a central regulator of embryonic cardiomyocyte proliferation and differentiation, as well as outflow tract development. These findings raise the possibility that mutations in GSK-3β may contribute to cases of double outlet right ventricle and ventricular septal defects and possibly even some cases of idiopathic LV hypertrophy. The role of GSK-3β in congenital heart disease is still unclear to our knowledge.

Emerging Role of GSK-3β Signaling Cascades in Cardiac Fibrosis

Fibrosis affects nearly every tissue in the body. It is a leading cause of morbidity and mortality, as highly aggressive fibrotic processes eventually lead to organ malfunction and death.54 In reference to myocardial diseases, virtually every form of heart disease is associated with expansion and activation of the cardiac fibroblast compartment, the primary source of ECM production and fibrosis.55 Despite its enormous impact on human health, there are currently no approved treatments that specifically target fibrosis. Although the direct evidence on the role of cardiac fibroblasts in normal cardiac function is lacking, it is thought that cardiac fibroblasts play a central role in the maintenance of ECM in the normal heart. Recent studies with fibroblast-targeted mouse models clearly demonstrate that cardiac fibroblasts could play a driving role in myocardial diseases processes, for example, PO-induced hypertrophy and MI-induced remodeling and heart failure.17,56,57 In an elegant study, Takeda et al,56 for the first time, used mouse lines in which Cre recombinase was driven by the periostin (Postn) promoter (cardiac fibroblast–specific gene targeting) to clearly demonstrate that cardiac fibroblasts are essential for the adaptive response of the heart to PO. Importantly, cardiomyocyte-specific deletion of the same target did not lead to any phenotype. This pioneering work proposed the concept that cardiac fibroblasts are not mere bystanders, acting only in fibrosis but are crucial mediators of myocardial hypertrophy and adaptive responses in the heart.96 Duan et al37 used tamoxifen-inducible Col1a2Cre(T) to delete β-catenin specifically in fibroblasts (global) and demonstrated the critical requirement of cardiac fibroblasts for preserving cardiac function after acute ischemic cardiac injury.

Several studies using a variety of models have supported the roles of GSK-3β in cardiac myocyte biology and disease.6,12,16,24,25,50 However, until recently the role of GSK-3β in myocardial fibrosis was virtually unknown. Therefore, we used both Postn and Col1a2Cre to target GSK-3β specifically in fibroblasts and demonstrated that deletion of GSK-3β leads to hyperactivation of profibrotic TGF-β1-SMAD-3 signaling which results in excessive fibrosis and adverse ventricular remodeling after MI. The GSK-3β pathway has been implicated in regulating the transition of fibroblasts into myofibroblast and fibrotic signaling.96,97 Recently, Bergmann et al98 reported that inhibition of GSK-3β induces dermal fibrosis by activation of the canonical Wnt pathway. Incubation of cultured dermal fibroblasts with specific GSK-3 inhibitor SB216763 led to increased expression and release of col1a1. Furthermore, in vivo administration of SB216763 alone was sufficient to induce progressive dermal fibrosis.58 The major caveat of the above-referenced study is that the conclusion exclusively relies on non–isoform-specific GSK-3 inhibitors and systemic administration. Kapoor et al99 used fibroblast-specific knockouts of GSK-3β KO mice to investigate the function of GSK-3β in fibrogenic responses and wound healing in a skin injury model. Global fibroblast-specific GSK-3β KO mice exhibited increased fibrogenesis, accelerated wound closure, and excessive scarring compared with control mice. In addition, knockouts showed elevated collagen production, increased fibroblast proliferation, and myofibroblast formation during wound healing. Thus, this study was limited to skin tissue and the major criticism was the exclusive dependence on either a global fibroblast mouse model or non–isoform–selective pharmacological inhibitors.

In our recent report,17 we demonstrated that GSK-3β is phosphorylated (inhibited) in fibrotic tissues from ischemic human and mouse hearts. Using 2 different fibroblast-specific GSK-3β KO mouse models, we demonstrated that deletion of GSK-3β, specifically in cardiac fibroblasts, leads to fibrogenesis and profound scarring in the postischemic heart. Deletion of GSK-3β also induces a profibrotic, myofibroblast phenotype in isolated cardiac fibroblasts, in mouse embryonic fibroblasts and in post-MI hearts deleted for GSK-3β. This report was the first to study MI-induced fibrotic remodeling using cardiac fibroblast–specific gene targeting. Furthermore, this study is the first to demonstrate the effect of cardiac fibroblast–specific gene targeting on global cardiac function and adverse remodeling after MI.

Molecular Mechanism of GSK-3β–Mediated Fibrosis

In the healthy heart, GSK-3β actively protects from fibrosis, and injury leads to its inhibition (phosphorylation), which allows fibrotic genes to be expressed. Two different mechanisms have been proposed by which GSK-3β exerts its inhibitory effect on fibroblast activation and fibrotic gene expression: β-catenin–dependent mechanism and TGF-β1-SMAD-3–dependent mechanism.

β-Catenin–Dependent Mechanism

It is well established that GSK-3β is a central component of the Wnt/frizzled signal transduction pathways that use β-catenin as a second messenger.61–63 The components and molecular mechanisms of Wnt signaling have been studied extensively and the readers are referred to a recent review for details.64 In brief, β-catenin levels are normally kept low by a phosphorylation event that is mediated by GSK-3β, which targets β-catenin for ubiquitination and proteasomal degradation. Because GSK-3β is an essential component of this degradation complex, its deletion leads to β-catenin accumulation in the cytoplasm and enters the nucleus, where it coactivates transcription of target genes.

There is growing evidence suggesting that this accumulated β-catenin leads to fibroblast activation and fibrogenesis in multiple organ systems.65–67 The role of Wnt/β-catenin signaling in fibroproliferative disorders was initially supported by
strong correlations between β-catenin mutations and aggressive fibromatosis in human desmoids tumors.68,69 Consistently, forced activation of β-catenin using a mutant that resists ubiquitination-dependent degradation is sufficient to drive exuberant collagen synthesis and fibrosis.70 In a dermal fibroblast model, Bergmann et al56 applied a small interfering RNA approach and demonstrated that deletion of β-catenin is sufficient to abolish the profibrotic effect observed on inactivation of GSK-3β. These data suggest that inhibition of GSK-3 induces the fibrotic phenotype by activating the canonical Wnt pathway. Subsequently it was shown that β-catenin is a central mediator of profibrotic signaling in systemic sclerosis.67,71 Using a tamoxifen-inducible model, Duan et al37 demonstrated that deletion of β-catenin in fibroblasts leads to impaired fibrogenic responses and decreased cardiac performance in an I/R model. This study suggests that a profibrotic β-catenin injury response is critically required for healing and thus preserving cardiac function after acute I/R injury. Taken together these data suggest that inhibition/deletion of GSK-3 induces a fibrotic phenotype by activating the canonical Wnt-β-catenin pathway.

TGF-β1-SMAD-3–Dependent Mechanism

TGF-β1 is the most potent profibrotic cytokine identified to date, is a key mediator of fibroblast activation, and drives the aberrant synthesis of ECM in fibrotic diseases. The detailed components and mechanisms of TGF-β1 signaling have been reviewed elsewhere.72–74 In brief, TGF-β1 signals through ≥2 independent routes: primarily through the SMAD-dependent canonical pathway and the SMAD-independent or noncanonical pathways. In the SMAD-dependent pathway, activation of TGF-β type 2 receptor activates TGF-β type I receptor and then type I receptor phosphorylates the transcription factor SMAD2/3 on the 2 serine residues at their C termini (Ser423/25). Phosphorylated SMAD2/3 collaborates with the common mediator SMAD-4 (CO-SMAD) for cytosol to nucleus translocation and subsequent transcriptional regulation.

Most of the literature on TGF-β signaling pathways have predominantly focused on TGF-β receptors directly phosphorylating and activating SMAD transcription factors within the C-terminal domain.72,73 However, recently there is increasing interest in alternate serine and threonine phosphorylation sites within the linker region of SMADs, which control several cellular responses including epithelial–mesenchymal transition and SMAD-3 transcriptional activity.75–77 The linker region is defined as the domain which lies between the Mad homology (MH) domains 1 and 2 of a SMAD protein.78 In contrast to C-terminal domain phosphorylation (which leads to activation), linker region phosphorylation leads to inhibition of SMAD transcriptional activity.75,76

Phosphorylation and nuclear translocation of SMAD2/3 is a rate-limiting step in TGF-β signaling and is important for determining the strength and duration of the signal and biological response.80,81 Emerging evidence suggests that GSK-3β exerts its antifibrotic effect by negatively regulating SMAD-3 activity (Figure 2). Guo et al82 were the first to demonstrate that GSK-3β controls SMAD-3 protein stability to modulate TGF-β1 signaling. This study demonstrates that reduction in the expression or activity of GSK-3β leads to increased SMAD-3 stability and transcriptional activity without affecting TGF-β receptors or SMAD-2. Conversely, overexpression of GSK-3β promotes SMAD-3 basal degradation and desensitizes cells to TGF-β.82 Subsequently, it was shown that GSK-3β phosphorylates SMAD-3 within the linker region at Ser204 to negatively regulate its activity.79,83 However, all of this work was done either in immortalized cell lines or in isolated primary cells. Thus, until recently the in vivo role and physiological significance of this pathway were virtually unknown. We administered a small molecule inhibitor of SMAD-3 in vivo to test whether hyperactivation of SMAD-3 is really responsible for excessive fibrotic responses observed in cardiac fibroblast–specific GSK-3β KO mice.17 Small molecule inhibitor of SMAD-3 treatment significantly blunted scar expansion in KOs hearts compared with KO treated with vehicle. Moreover, small molecule inhibitor of SMAD-3 administration nearly abolished the detrimental phenotype of cardiac fibroblast–specific GSK-3β deletion as evidenced by restored ventricular function and chamber dimensions. 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-3β in cardiac fibroblasts. Furthermore, we demonstrated that GSK-3β directly interacts with SMAD-3 in human heart and cardiac fibroblasts. Inhibition/deletion of GSK-3β leads to increased phosphorylation of SMAD-3 at the carboxyl terminus (S423/425) and decreased phosphorylation at the N terminus (S204). Thus, our study suggests that GSK-3β exerts its effect on TGF-β1-SMAD-3 signaling specifically by regulating both the C-terminal domain and the linker region (Figure 2). Taken together the available evidence suggests that inhibition/deletion of GSK-3β induces the fibrotic phenotype by activating the SMAD-3 pathway.

Perspectives and Future Directions

The pivotal functions of GSK-3α/β in cardiac repair and, to a significant but lesser degree, regeneration, combined with the fibrosis and degeneration seen, are fascinating issues in cardiac biology that clearly deserve much more attention. These dichotomy’s of GSK-3 signaling raise serious problems in terms of pharmacological targeting of GSK-3s. It is therefore essential to acquire much more knowledge on isoform-specific functions in various cardiac model systems. This should allow better prediction as to preclinical outcomes. Recent findings that cardiomyocyte-specific deletion of GSK-3α is protective in the ischemic heart place GSK-3α as a prime candidate to focus efforts on patients with MI, particularly those with incomplete revascularization or larger infarcts. Given the fact that small molecule inhibitors targeting GSK-3 are available and have been used in clinical trials in patients, one could envision going forward into large animal models with the possibility of eventually going into the clinic. The caveat is that all GSK-3–targeted drugs to date target both isoforms of GSK-3 and as discussed above, long-term, non–isoform-specific inhibition of GSK-3α/β could be detrimental. However, it is reasonable to hypothesize
that partial or short-term inhibition could be beneficial in the setting of I/R injury. Furthermore, as pharmacological strategies are enhanced, and are not so reliant on standard approaches for drug discovery, it is clearly feasible that specific compounds could be generated that would specifically target GSK-3α, thereby leading to better cardioprotection in the setting of MI. To achieve isoform-specific selectivity, it is possible that small molecule inhibitors could be generated that are not targeted to the active site (which is highly conserved). There are specific regions in α and β that could be targeted. Alternatively, RNAi approaches or monoclonal antibodies could be used in some circumstances. Furthermore, finding the appropriate delivery route and timing of the treatment will be key determinants to achieve the therapeutic outcome while avoiding or limiting any possible adverse effects. As discussed, most of the knowledge about the role of GSK-3 isoforms in failing heart has been generated using various genetic mouse models. Although these mouse models are excellent tools to understand the basic biology of the targeted molecule, they all have some limitations. In fact, degree of inhibition achieved by pharmacological agents and a complete loss of function by genetic knockout cannot be directly compared. We think that more preclinical studies with small molecule inhibitors will be warranted to take the GSK-3 inhibitors into the clinic for the management of cardiovascular diseases. Indeed, in searching Clinicaltrials.gov, we could not find any registered clinical trials in cardiovascular disease with GSK-3 inhibitors. However, there is 1 trial using an agent other than the traditional inhibitors of GSK-3 (lithium and valproic acid) for Alzheimer disease (ClinicalTrials.gov Identifier: NCT00948259). This pilot, double-blind, placebo-controlled, randomized, escalating-dose trial explored the safety and efficacy of NP031112 (tideglusib; Noscira SA; an inhibitor of glycogen synthase kinase-3), in patients with Alzheimer disease. Treatment was reasonably well tolerated except for some moderate increases of serum transaminases, which were fully reversible. This small pilot study provides valuable safety data for the use of GSK-3 inhibitors. Importantly, there was no indication of any adverse consequences in the heart. Furthermore, many patients have been treated with the GSK-3 inhibitor lithium for years without any noticeable side effect on the cardiac system further validates the feasibility of targeting GSK-3 for cardiac diseases. In any case, we need isoform-selective small molecule inhibitors as long-term non–isoform-selective inhibition could lead to myocardial fibrotic remodeling. Furthermore, our findings in the developing heart raise concerns about the long-term use of more potent GSK-3 inhibitors in women of child-baring potential (these subjects were excluded from the Noscira trials). We think that the greatest promise for drug development may lie in the development of isoform-selective small molecule GSK-3 inhibitors. We think that such precision drugs, while much harder to develop, will allow chronic treatment with GSK-3 inhibitors in various disorders.

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References


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