Published in final edited form as:

Circ Res. 2016 April 15; 118(8): 1208–1222. doi:10.1161/CIRCRESAHA.116.308544.

Loss of Adult Cardiac Myocyte GSK-3 Leads to Mitotic Catastrophe Resulting in Fatal Dilated Cardiomyopathy

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Abstract

Rationale—Cardiac myocyte-specific deletion of either *Glycogen Synthase Kinase (GSK)3A* or *GSK3B* leads to cardiac protection following myocardial infarction, suggesting that deletion of both isoforms may provide synergistic protection. This is an important consideration due to the fact that all GSK-3-targeted drugs including the drugs already in clinical trial target both isoforms of GSK-3 and none are isoform specific.

Objective—To identify the consequences of combined deletion of cardiac myocyte *GSK3A* and *GSK3B* in heart function.

Methods and Results—We generated tamoxifen-inducible cardiac myocyte-specific mice lacking both GSK-3 isoforms (double knockout, DKO). We unexpectedly found that cardiac myocyte GSK-3 is essential for cardiac homeostasis and overall survival. Serial echocardiographic analysis reveals that within 2 weeks of tamoxifen treatment, DKO hearts leads to excessive dilatative remodeling and ventricular dysfunction. Further experimentation with isolated adult cardiac myocytes and fibroblasts from DKO implicated cardiac myocytes intrinsic factors responsible for observed phenotype. Mechanistically, loss of GSK-3 in adult cardiac myocytes resulted in induction of mitotic catastrophe, a previously unreported event in cardiac myocytes. DKO cardiac myocytes showed cell cycle progression resulting in increased DNA content and multi-nucleation. However, increased cell cycle activity was rivaled by marked activation of DNA damage, cell cycle checkpoint activation, and mitotic catastrophe induced apoptotic cell death. Importantly, mitotic catastrophe was also confirmed in isolated adult cardiac myocytes.

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Conclusion—Together, our findings suggest that cardiac myocyte GSK-3 is required to maintain normal cardiac homeostasis and its loss is incompatible with life due to cell cycle dysregulation that ultimately results in a severe fatal dilated cardiomyopathy.

Keywords

GSK-3; cell cycle; mitotic catastrophe; dilated cardiomyopathy; heart failure; cardiovascular pathophysiology

INTRODUCTION

Heart failure is a complex and debilitating clinical syndrome with an estimated economic burden of \$32 billion a year in the United States.^{1,2} Despite advances in treatment and increased availability of heart transplants, approximately half of the patients that develop heart failure die within 5 years of diagnosis.^{1,2} Dilated cardiomyopathy (DCM), the most common cause of heart failure, manifests itself with structural thinning and expansion of cardiac chambers with a progressive and sharp decline in cardiac function. Genetic studies have identified a number of genes that contribute to isolated cases of familial DCM. However, as DCM is the result of multiple etiologies (both genetic and injury induced), the common underlying molecular mechanisms remain poorly defined.

Glycogen synthase kinase-3 (GSK-3) is a highly conserved, integral regulator of numerous cellular processes including cell proliferation, metabolism, and cell death.^{3–6} GSK-3 consists of GSK-3 α and GSK-3 β isoforms, which our laboratory and others have shown to have both distinct and overlapping functions in the heart.^{7–15} Germline homozygous deletion of *GSK3B* results in embryonic lethality due to the development of hypertrophic cardiomyopathy.¹⁶ This was the result of a hyper-proliferation of cardiac myocytes that obliterated the ventricular cavity. In contrast, mice with germline homozygous deletion of GSK-3 α are viable but develop cardiac hypertrophy with progressively deteriorating cardiac function in the non-stressed heart.^{17,18} These studies show that GSK-3 is a critical regulator of the cardiac myocyte cell cycle during embryogenesis. To better understand the mechanisms by which GSK-3 protects against cardiomyopathy, we generated mice that allow for conditional deletion of GSK-3 isoforms specifically in cardiac myocytes. Surprisingly, adult mice with cardiac myocyte-specific deletion of either *GSK3A* or *GSK3B* demonstrate preserved cardiac function and reduced progression to heart failure following myocardial infarction.^{7,14} However, it is unknown whether deletion of both isoforms may provide synergistic protection for cardiac function, and thereby reduce heart failure progression. Indeed, genetic studies assessing GSK-3 function in the heart to date have focused on isoform-specific models and none have explored the consequences of combined targeting of GSK-3 isoforms. Moreover, there are multiple clinical trials targeting GSK-3 isoforms for treatment of severe neurological diseases that would benefit from a clearer understanding of the cardiac effects of chronic GSK-3 inhibition.^{19–21}

Herein, we report that mice with adult cardiac myocyte-specific deletion of both isoforms of GSK-3 (DKO) rapidly succumb to death. Microarray analysis of DKO hearts identified GSK-3 regulated transcriptional changes that provide insight on adult cardiac myocyte cell

cycle activation. DKO adult cardiac myocytes exhibited cell cycle re-entry resulting in increased DNA content and multi-nucleation. However, instead of successful completion of cell cycle, cardiac myocytes accumulated severe DNA damage, activated cell cycle checkpoints, and culminated in mitotic catastrophe. The loss of cardiac myocytes severely impaired cardiac function and ultimately caused DCM and heart failure. These findings are the first to provide evidence for mitotic catastrophe as a cell death mechanism for adult cardiac myocytes. Thus, cardiac myocyte GSK-3 is required to maintain cardiac homeostasis and overall survival.

METHODS

An expanded Materials and Methods section is available in the Online Data Supplement.

Mice

The *GSK3A^{flox/flox}*, *GSK3B^{flox/flo}*, and α -MHC *Mer-Cre-Mer* (MCM) mice have been previously described.^{7,14} Cardiac myocyte-specific conditional GSK-3 double knockout mice (*GSK3A^{flox/flox}*; *GSK3B^{flox/flox}*; α -MHC MCM) were generated through several rounds of mating the above strains. All strains were maintained on the C57BL/6 background. At 12 weeks of age male mice began tamoxifen (tam) chow treatment (400 mg/kg, TD.130860 Harlan) for 14 days followed by standard rodent diet (5001* LabDiet).

Statistics

Differences between data groups were evaluated for significance using unpaired two-tailed Student's t-test or one-way analysis of variance (ANOVA), as appropriate and Bonferroni post-test (GraphPad Prism Software Inc., San Diego, CA). Survival analysis was performed by the Kaplan-Meier method, and between-group differences in survival were tested by the Gehan-Breslow-Wilcoxon test. For statistical assessment of double-positive pH3 and TUNEL cells, Chi-Square analysis was used. Data are expressed as mean $\pm$ SEM, unless noted otherwise. For all tests, a *P*-value of < 0.05 was considered statistically significant.

RESULTS

Cardiac myocyte-specific deletion of GSK3 causes dilated cardiomyopathy and death

To determine the effect of combined cardiac myocyte GSK-3 α and GSK-3 β deletion, we generated cardiac myocyte-specific knockout mice lacking both isoforms. This model allows for conditional deletion of all four *GSK3* alleles (2 α and 2 β) using a tamoxifen-inducible mER-Cre-mER system (herein referred to as DKO). Tamoxifen was administered using a well-established oral dosing regimen.⁷ Throughout this study we refer to the tamoxifen-timeline (tam-timeline) to indicate the relative duration of tamoxifen-chow administration and age of mouse (Fig. 1A). All results are reported as day (d) post-onset of tamoxifen administration. Following administration of tamoxifen, Western blot analysis was utilized to evaluate efficiency of Cre-mediated gene excision. Results demonstrate a significant decline in GSK-3 α (85.54%) and GSK-3 β (66.84%) total protein levels within 2 weeks of tamoxifen administration (Fig. 1B).

DKO mice demonstrated increased mortality versus controls with 100% death by d42 (Fig. 1C). The majority of DKO mice were found dead between days 35–40. Furthermore, at d15 of the tamoxifen timeline, DKO mice demonstrated visible symptoms of heart failure as evidenced by tachypnea, labored breathing, and peripheral edema. At autopsy, the DKO mouse hearts exhibited severe DCM with multi-chamber enlargement. On gross examination, ascites, pleural effusions, and pericardial effusions were found. To assess the cardiac function in DKO and littermate controls, we performed serial M-mode echocardiography. At baseline, WT and DKO hearts had comparable chamber dimensions and ventricular function, but as early as 2 weeks of tamoxifen timeline, DKO animals had a substantial increase in end-diastolic and end-systolic dimensions in comparison to littermate controls, reflecting accelerated dilatative remodeling (Fig. 1D–E). This was associated with marked left ventricular (LV) dysfunction as reflected by significant decline in LV ejection fraction (EF) and fractional shortening (FS) (Fig. 1F–G). LV dilatation and dysfunction remained worse in the DKO throughout the study duration.

DKO leads to pathological hypertrophy, accelerated fibrosis and heart failure

Histological analysis of trichrome-stained cardiac sections at 0, 2, 3 and 4 wk of tamoxifen timeline revealed myocardial thinning with enlarged atrial and ventricular chambers, consistent with DCM (Fig. 2A). Cardiac myocytes were both elongated and widened in DKO heart sections compared to control (Fig. 2B). For further assessment of cardiac hypertrophy, heart weight to tibia length (HW/TL) ratios were compared. Hearts from DKO mice displayed 120.19% increase in HW/TL ratio compared to controls (Fig 2C). Quantification of Trichrome stained heart sections demonstrated enhanced fibrosis in the DKO hearts starting at 3 wk time point (Fig. 2D–E). Importantly, fibrosis was comparable between groups at 2 wk, a critical time point at which morphological and functional changes were already evident suggesting fibrosis as a consequence not the cause of observed phenotype. Histological assessment of H&E-stained pulmonary sections from DKO lungs demonstrated marked thickening of the alveolar interstitium which is consistent with congestive heart failure (Fig. 2F). DKO lungs demonstrated characteristic hemosiderin-laden macrophages, enhanced interstitial infiltrates, and marked thickening of the alveolar interstitium, all of which were consistent with congestive heart failure (Fig. 2F). Overall, these data are the first to characterize the complete loss of cardiac myocyte-specific GSK-3 in the adult heart and reveal that cardiac myocyte GSK-3 is critical for organism survival. Loss of GSK-3 in the adult cardiac myocyte results in severe dilated cardiomyopathy and death.

Cardiac myocyte nuclear enlargement and ultra-structural defects in DKO hearts

Examination of H&E-stained cardiac sections from DKO mice indicated cardiac myocyte enlargement with expanded interstitium in all four chambers (Fig. 3A). Interestingly, nuclear enlargement was clearly evident in all cardiac chambers in the DKO heart on H&E stained cardiac sections. Nuclei in the DKO heart were irregular in size and shape, with variably distributed central and peripheral basophilic aggregates.

To further characterize the abnormalities in cardiac myocyte morphology found on H&E, cardiac sections from the DKO were evaluated using electron microscopy (EM).

Interrogation of overall cardiac myocyte structure revealed architectural abnormalities with loss of structural integrity at the sarcomeric Z-line, reduced sarcomere protein content, and disordered mitochondrial and sarcomeric organization (Fig. 3B). Although mitochondria did appear altered, their morphology was preserved when compared to mitochondria of previously reported GSK-3 α homozygous knockout animals which revealed severe mitochondrial swelling with disrupted cristae.^{17,18} Furthermore, mitochondria numbers were comparable between DKO and littermate controls.

Due to the prominent nuclear enlargement observed on H&E, we focused our EM analyses on the structural changes in the nucleus (Fig. 3C–D). DKO hearts confirmed both nuclear enlargements as well as revealed alterations in nuclear composition and shape. The shape of the nuclear membrane was clearly different from control nuclei as evidenced by the extensive nuclear membrane invaginations. The nuclear membrane was thickened with increased space between membrane and cytoskeleton. Compositions of nuclei were variable and markedly different from control nuclei. DKO nuclei contained enlarged nucleoli and enhanced peripheral and central electron dense clumping indicative of the extent of heterochromatin aggregation (Fig. 3C–D). Taken together, these results demonstrate the severity of structural derangements in the DKO hearts as evidenced by cardiac myocytes with both gross and ultra-structural morphological abnormalities.

Microarray analysis reveals alterations in cell cycle and suggests G2/M blockade in cardiac myocytes/

To gain better insight into the processes that might contribute to the unexpected fatalities in DKO mice, a microarray analysis was performed at d21 of the tam-timeline (Online Fig. IA–C). Bioinformatics analyses revealed 419 coding transcripts that were up-regulated and 204 transcripts that were down-regulated by a greater than- or lesser than- 1.9 fold at a statistical significance of $P < 0.05$ (Online Table IA). Furthermore, we compared our microarray data set with numerous other publically available data sets of dilated cardiomyopathy and other heart failure models.^{22,23} Indeed, along with the markers of heart failures, we uniquely observed an enrichment of differentially expressed genes related to cell cycle and checkpoint activation. Analysis of microarray data through Ingenuity Pathway Analysis (IPA) indicated enrichment of differentially expressed genes related to fibrosis and cell cycle pathways (Online Table IB). At 2 wk of tamoxifen timeline, adult cardiac myocyte and fibroblast were isolated from DKO and littermate controls to delineate their specific role in observed phenotype. Western blot analysis reveals activation of cell cycle pathways and apoptosis specifically in cardiac myocytes only (Fig. 4A–E). Importantly, markers of cell cycle, apoptosis and myofibroblast activation were comparable in isolated adult fibroblast from DKO and littermate controls (Fig. 4A–E). Taken together, at 2 wk of tamoxifen timeline, fibrosis in DKO hearts and fibroblast activation in an *in vitro* setting were comparable in DKO and controls. These data exclude fibroblast or fibrosis as the primary mechanism responsible for the observed phenotype. Hence, we focus on identifying the dysregulation of cell cycle pathway and associated mechanism. Specifically, assessment of cell cycle related genes showed transcriptional changes in genes associated with G2/M (Table 1). Overall, these data revealed that adult cardiac myocyte *GSK3* deletion results in severe alterations in

cell cycle control with subsequent activation of cell cycle checkpoints, thus providing insight into potential mechanisms by which this severe phenotype may be occurring.

GSK3 deletion induces cell cycle re-entry with polyploidization and multinucleation

The combined findings of nuclear abnormalities on histological analysis and cell cycle related gene expression differences in the DKO prompted further investigation of the consequence of GSK-3 deletion on the cell cycle. Detailed histological analysis of cardiac myocyte nuclei in H&E stained DKO heart sections revealed the severity of nuclear enlargement and alterations in nuclear shape (Fig 5A). Although both GSK-3 α and GSK-3 β cardiac myocyte-specific conditional knockouts heart displayed increased DNA synthesis upon injury, this was not found in respective unstressed hearts compared to controls.^{7,14} In addition, such striking nuclear morphological abnormalities were not detected in isoform specific conditional knockout hearts as well.

We performed bromodeoxyuridine (BrdU)-based assessment of DNA synthesis on DKO heart sections to provide insight on the observed nuclear abnormalities. Results showed increased DNA synthesis in the DKO cardiac myocytes compared to controls indicating that loss of both GSK-3 isoforms stimulates S-phase entry at baseline (Fig 5B and Online Fig. II). To assess the degree of DNA synthesis per nuclei, we utilized pericentriolar material 1 (PCM1) for flow cytometric analysis of nuclei extracted from DKO and control hearts. PCM1 is a centrosomal protein which accumulates at the nuclear surface of mature cardiac myocytes and has frequently been utilized for quantification of DNA content.²⁴ In corroboration with reports in the literature, control cardiac myocyte nuclei were in a predominantly 2N state with a minimal number of nuclei with 4N content.^{25,26} However, DKO hearts revealed a reduction in nuclei with 2N and an increase in nuclei with 4N (Fig. 5C). Although these results indicate an increase in overall cellular DNA content, they are not informative of the distribution of nuclear changes within the cardiac myocyte. To address this, we completed manual quantitation of the number of DAPI+ nuclei per isolated adult cardiac myocyte using confocal microscopy (Fig 5D). We found ~50% of the cardiac myocytes isolated from DKO hearts contained greater than 2 nuclei with up to 8 nuclei in several cells (Fig. 5E). Interestingly, the nuclei in the DKO did not all appear to have completed karyokinesis, suggesting failed mitosis. These results demonstrate enhanced G1/S phase transition, with polyploidy, and multinucleation in DKO hearts.

DKO cardiac myocytes show mitotic entry, DNA damage, and apoptotic cell death

With evidence of cell cycle progression with polyploidy, we were interested in characterizing the molecular mechanisms associated with these changes and characterizing later stages of the cell cycle. We completed western blot analysis of representative markers of key modulators of the cell cycle. First we examined CDK1-CyclinB1, a critical regulatory complex for cellular commitment towards entering mitosis.²⁷ We find that CDK1 and its respective binding partner Cyclin B1 were significantly up-regulated suggesting a cardiac myocyte mitotic entry (Fig. 6A). Normally, adult cardiac myocytes do not express CDK1-Cyclin B1 and forced expression results in increased proliferative capacity.²⁸ However, at the end of G2 phase, CDC25 phosphatases activate Cyclin B1-CDK1 for nuclear entry. CDC25C phosphatase is a rate limiting inducer of mitosis and its loss results in mitotic

blockade.²⁹ As the DKO heart has a significant increase in Cyclin B1 accompanied with polyploidization (indicating inhibited mitosis), we assessed CDC25C activity. Western blot analysis revealed significant inactivation of CDC25C and thus provides evidence for impaired mitotic progression in the DKO cardiac myocyte (Fig. 6A&B).

The increase in CDC25C-pSer216 suggested increased DNA damage in the DKO cardiac myocytes. To examine this, we assessed phosphorylation of H2AX (γ H2A.X Ser139), which occurs upon detection of DNA damage and is not normally found at baseline in hearts.^{30, 31} Histological assessment of γ H2A.X in isolated adult cardiac myocytes from DKO mice demonstrated significant activation of DNA damage pathways (Fig. 6C). In addition, Western blot analysis showed significant induction of activated Checkpoint kinase 2 (CHK2) and γ H2A.X confirming double-stranded DNA damage (Fig. 6D–E). We also assessed other DNA damage activated cell cycle inhibitors, p21 and p27^{kip1}.^{32,33} Western blot analysis showed a significant increase in p21 and p27^{kip1} (Fig 6D–E). Together, these findings implicate an ongoing requirement for both GSK-3 α and GSK-3 β in the adult cardiac myocyte which, when perturbed, results in DNA damage and cell cycle checkpoint activation.

As our results provide evidence for impaired mitotic entry, we measured various parameters associated with mitosis. Phosphorylation of Histone H3-Ser10 is a common marker for assessing mitotic progression and is related to chromosome condensation.^{34,35} Histological assessment of DKO hearts showed increased number of pH3-Ser10 positive cardiac myocytes indicating mitotic entry (Fig. 6F–G). Quantification of cardiac myocytes dual positive for pH3-Ser10/ α -actinin revealed ~12% detection in DKO compared to negligible detection in controls (Fig. 6G). Furthermore, microarray data provides evidence for upregulation of multiple markers of mitosis including: Plk1, AurkB, Kif4, Anln, and Cenpa (Online Table IA).^{36,37} These results demonstrate the presence of a large number of cardiac myocytes with condensed chromatin and are indicative of mitotic entry. However, the increased accumulation of pH3-Ser10+ cardiac myocytes in the presence of cell cycle checkpoint activation is highly suggestive of incomplete or delayed mitosis.

DKO cardiac myocytes leads to death by mitotic catastrophe

As our findings provide evidence of cardiac myocyte mitosis in the DKO, we sought to identify the final fate of these cells. Although, as it was clear that the DKO hearts were in heart failure, we first characterized cardiac myocyte fate by assessing cellular death. Quantitative analysis of TUNEL positive nuclei in cardiac myocytes revealed increased apoptotic cell death in the DKO hearts (Fig. 7A). Western blot analysis of pro-apoptotic markers BAX and p53 indicated a significant induction of apoptotic cell death pathways (Fig. 7B). The ratio of anti-apoptotic BCL-2 to pro-apoptotic BAX member proteins is a major checkpoint in the common pathway of apoptotic cell death.^{38,39} Quantitation of the BCL-2/BAX ratio in the DKO indicates a reversal from high to low ratio, supporting the activation of an apoptotic cellular death (Fig. 7C). Of more interest, we identified many cells that were positive for both pH3-Ser10 as well as TUNEL stain in DKO hearts versus none in the controls (Fig. 7D–E). Quantification of histological findings show that the majority of TUNEL+ cells are indeed also p-H3+. These findings provide strong evidence for the

presence of apoptotic death in mitotically active cells. The combined morphological findings, and apoptotic cellular death in the presence of mitosis indicates the occurrence of mitotic catastrophe. Mitotic catastrophe is a mechanism for eliminating mitosis incompetent cells and occurs in the presence of inappropriate entrance into mitosis.^{40–42} A major classification of mitotic catastrophe indicates activation of cellular death in the presence of elevated cyclin B1.^{40,43,44} Our findings, which include striking nuclear aberrations on morphological assessment, elevated cyclin B1, and mitotic entry in the presence of cellular death, indicate the occurrence of mitotic catastrophe in cardiac myocytes.

Taken together, we conclude that GSK-3 in the adult cardiac myocyte is a critical suppressor of cell cycle induction and its loss leads to improper cell cycle re-entry, culminating in mitotic catastrophe. This associated loss of functional cardiac myocytes then results in impaired cardiac function with rapid onset of congestive heart failure and death in the DKO mice (Fig. 8).

DISCUSSION

In the DKO, we find that adult cardiac myocytes re-enter the cell cycle in the absence of cardiac stress. Although we previously demonstrated that cell cycle re-entry occurred in GSK-3 isoform-specific conditional knockout mice after cardiac injury, these mice were protected compared to control littermates and exhibited a reduction in apoptosis.^{7,14} Strikingly, in the DKO hearts, the induction of cell cycle corresponded to a severe hypertrophic response with apoptotic cell death in the absence of injury. Cardiac myocytes were able to transit through G1/S and as well as partially through G2/M, however did not complete cellular division due to mitotic catastrophe resulting in the ultimate development of fatal heart failure. This mode of cellular death has not yet been described in the setting of cardiomyopathy or connected to GSK-3 in the cardiac setting. The implications of these findings are broad including the potential consequence of chronic administration of GSK-3 inhibitors and our understanding of prolonged cardiac myocyte cell cycle re-activation in heart.

Currently, there is a major research focus on identifying pathways for activation of cardiac myocyte cell cycle re-entry. Many investigators have demonstrated successful cardiac myocyte cell cycle re-entry; however the final fate of these cardiac myocytes is variable.^{45,46} Conditional adult cardiac myocyte-specific knockout mouse models have also successfully activated cardiac myocyte cell cycle re-entry, and some studies including ours have even demonstrated preserved cardiac function following injury.^{7,47–49} Although successful completion of adult cardiac myocyte cell cycle has been demonstrated,⁵⁰ the final outcome may include multi-nucleation and polyploidy.^{46,51,52} Furthermore, a majority of cardiac myocytes in congestive heart failure and cardiac hypertrophy demonstrate polyploidy.^{53–56} The cell's ability to bypass mitotic completion and proceed through several rounds of DNA replication may be beneficial as this allows for increased transcription; however the true relevance is unknown.⁵⁷ Thus far the number of molecules known to regulate cardiac myocyte polyploidy is limited and we demonstrate GSK-3 is a critical component of the cardiac myocyte polyploidization response. The mechanism by which GSK-3 loss results in polyploidy is unknown, but studies in pancreatic beta cells have implicated p27

accumulation to be primarily responsible for polyploidy in that model.^{58,59} We speculate that the observed accumulation of p27 in the DKO cardiac myocytes may also play a role in polyploidization in the DKO cardiac myocyte. However, polyploidy in the DKO cardiac myocytes is accompanied by mitotic catastrophe; suggesting that the degree of cell cycle re-entry induced was detrimental and that therapeutic chronic activation of cardiac myocyte cell cycle could be maladaptive.

There is a limited body of literature that reports multinucleation and polyploidy similar to what is observed in the DKO in the absence of injury.^{46,47,60} Of most interest, none of these models report associated cellular death. Furthermore, cardiac restricted Cyclin D1 overexpression induced G1/S transit, polyploidy, multinucleation, but demonstrated a downregulation of mitotic markers including auroraB, pH3-Ser10, Plk1, and Mad2.⁴⁶ In contrast, DKO hearts showed increased auroraB, pH3-Ser10, and Plk1 thus indicating increased mitotic activity in the DKO. Cell death in the presence of mitotic entry is a defining characteristic indicative of mitotic catastrophe.

Mitotic catastrophe as a potential outcome for cardiac myocyte cell cycle re-entry is novel and not yet observed in the heart. A recent report linked GSK-3 β inhibition to mitotic catastrophe in cancer cells, further providing evidence for the occurrence of this phenomenon in pathology.⁶¹ This form of cellular death is defined to precede cellular death pathways and has been suggested to act as an onco-suppressive mechanism to suppress genomic instability.^{40,62} Mitotic catastrophe is a mechanism for eliminating mitosis incompetent cells and is triggered as a result of abnormal or inappropriate induction of mitosis. In stark contrast to activation of G2/M checkpoint induced cell death, mitotic catastrophe requires mitotic entrance. In the DKO, we provide sufficient evidence for activation of cell cycle checkpoints as well as the presence of mitotic entry in cardiac myocytes. Although, the morphological definition of mitotic catastrophe is incomplete, common findings have been suggested to be critical for assessing mitotic catastrophe.⁴² These include mitosis, polyploidy, and apoptotic cell death. Functionally, mitotic catastrophe has been described to include the activation of cell death machinery in the presence of elevated cyclin B1.⁴⁰ Our results provide strong evidence of cyclin B1 accumulation and induction of apoptotic cell death. Although the presence of mitotic catastrophe is indicated in the DKO, the reason for mitotic failure is not understood. It is currently believed that all types of mitotic catastrophe have a common underlying perturbation in the mitotic apparatus in the processes required for proper chromosome segregation.⁴⁰ Evidence supporting the role of GSK-3 in regulation of chromosome segregation is multifold in the literature.^{63–65} At the heart of chromosome segregation is the spindle assembly checkpoint (SAC), which when prolonged is offered as a common mechanism by which cells trigger mitotic catastrophe.^{66,67} Microarray data from the DKO provides support of SAC dysregulation as indicated by significant up-regulation of: Mad2l1, Bub1, Aurkb, Bub1b and Spc25. This is supportive of the notion that mitotic catastrophe in the DKO is associated with prolonged SAC activation. Overall, our data provides the first evidence for GSK-3 mediated regulation of cardiac myocyte polyploidy and mitotic catastrophe. Further studies are required to establish the mechanism by which GSK-3 deletion in the cardiac myocyte results in mitotic catastrophe.

Two previous reports with conditional deletion of both isoforms of GSK-3 demonstrate significant alterations in cell cycle but do not result in cellular death.^{68,69} In both studies, mice survived and there were no reported reductions in lifespan. Kim et al,⁶⁸ investigated the consequences of combined GSK-3 α and GSK-3 β deletion in neural progenitor cells. These mice showed massive increases in neural progenitor cell proliferation without enhanced apoptosis when compared to controls. Jung et al,⁶⁹ generated a conditional knockout mouse, in which GSK-3 α and GSK-3 β were deleted in astrocytes. Astrocyte specific GSK-3-DKO mice exhibited anxiety and altered social behavior with no effect on the lifespan. However, to date, only adult cardiac myocyte-specific deletion of both GSK-3 isoforms results in fatality, indicating that cardiac myocyte GSK-3 is essential for normal cardiac homeostasis and survival. The molecular mechanisms associated with the disparate fates for cells with cell cycle re-entry in these various GSK-3 knockout models are unknown and further studies are required to address this question.

The differences in tissue-specific consequences of GSK-3 deletion demonstrate the importance of GSK-3 in the cardiac myocyte as well as confer it as a preferential target during long term GSK-3 inhibition. Indeed, optimism for developing GSK-3 inhibitors for clinical use remains high for treatment of a variety of severe pathological conditions including progressive central nervous system disorders, cancer, metabolic disorders and even ischemic cardiac injury.^{5,19–21} Current clinical trials are testing GSK-3 inhibitors for chronic treatment of patients with Alzheimer's disease and supranuclear palsy.^{19,20} Although safety was achieved in two Phase-1 clinical trials, our current data provides a cautionary note for the potential consequences of chronic pharmacological GSK-3 inhibition in the heart.

Herein we have found that simultaneous deletion of both GSK-3 isoforms in cardiac myocytes results in increased cell cycle re-entry and progression but does not result in cardiac myocyte replication. The deletion of GSK-3 isoforms results in activation of cell cycle checkpoints with severe DNA damage and resultant mitotic catastrophe and cellular death. The subsequent excessive loss of cardiac myocytes impairs cardiac function and heart failure. In conclusion, loss of GSK-3 in the adult cardiac myocyte is incompatible with life due to cell cycle dysregulation that ultimately results in a severe fatal dilated cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by grants from the NHLBI to T.F. (HL061688, HL091799, HL119234) CIHR operating grant (FRN 12858) to J.W. and American Heart Association Scientist Development Grant (13SDG16930103) to H.L. Funding support for S.P. includes: Pharmacology Training Grant (T32 GM07628) and the NIGMS Vanderbilt Medical Scientist Training Program (T32 GM07347).

DISCLOSURES

Dr. Force received research funding, as well as consultancy fees from GlaxoSmithKline.

Nonstandard Abbreviations and Acronyms

Anln	anillin actin binding protein
AurkB	Aurora B kinase
BAX	BCL2-Associated X Protein
BCL-2	B-cell lymphoma 2
BrdU	bromodeoxyuridine
Bub1	budding uninhibited by benzimidazoles 1
CDC25	Cell division cycle 25
CDK1	Cyclin-dependent kinase 1
Cenpa	centromere protein A
CHK2	Checkpoint kinase 2
DAPI	4',6-diamidino-2-phenylindole
DCM	dilated cardiomyopathy
DKO	double knockout
GSK-3	Glycogen synthase kinase 3
H2AX	H2A histone family, member X
HW	heart weight
Kif4	kinesin family member 4
Mad2	mitotic arrest deficient 2
PCM-1	pericentriolar material 1
Plk1	polo-like kinase 1
SAC	spindle assembly checkpoint
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Novelty and Significance

What Is Known?

- Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed, serine/threonine kinase. In mammals, the GSK-3 family is encoded by two genes, GSK-3 alpha (*GSK3A*) and GSK-3 beta (*GSK3B*).
- Isoform-specific deletion of either *GSK-3α* or *GSK-3β* specifically in cardiac myocytes leads to profound cardiac protection, post-MI. The consequence of combined targeting of both GSK-3 isoforms in the hearts is unknown and it is expected that targeting of both isoforms may provide synergistic cardiac protection.

What New Information Does This Article Contribute?

- GSK-3 is essential for cardiac myocyte cellular homeostasis and overall survival.
- GSK-3 in the adult cardiac myocyte is a critical suppressor of cell cycle induction as its loss leads to cell cycle re-entry, enhanced G1/S and S/G2 phase transition, activated cell cycle checkpoints, polyploidy and multinucleation.
- Aberrant cell cycle re-entry of GSK-3 deficient cardiac myocytes culminates to mitotic catastrophe, leading to severe fatal dilated cardiomyopathy.

Studies with isoform-specific mouse models have implicated the GSK-3 family as an attractive therapeutic target for management of cardiac diseases. However, the effect of complete loss of GSK-3 in cardiac myocytes is unknown. This is critical considering the fact that all GSK-3-targeted drugs including drugs already in clinical trials target both GSK-3 isoforms, and none are isoform specific. To determine the effect of complete loss of GSK-3α/β, we generated cardiac myocyte-specific knockout mice lacking both GSK-3 isoforms (double knockout, DKO). To our surprise, we found that cardiac myocyte-specific deletion of *GSK3* causes rapid heart failure and death. DKO cardiac myocytes leads to abnormal cell cycle re-entry, increased DNA content, multi-nucleation leading to mitotic catastrophe. Mitotic catastrophe as a potential outcome for cardiac myocyte cell cycle re-entry is novel and previously unreported event in cardiac myocytes. In summary, GSK-3 is required for normal homeostasis of adult cardiac myocytes and its deletion leads to cell cycle dysregulation resulting in a severe fatal dilated cardiomyopathy. These findings have important clinical implications and raise serious concerns over chronic administration of GSK-3 inhibitors.

Figure 1. Cardiac myocyte-specific deletion of *GSK3* leads to severe cardiac dysfunction and death

A, Diagrammatic representation of the timeline for tamoxifen (tam)-induced Cre-recombinase mediated deletion of *GSK3*. **B**, Representative Western blot showing tamoxifen-induced deletion of GSK-3α/β (n=4). **C**, Kaplan-Meier survival curves for DKO mice versus controls indicate a significant reduction in lifespan within 40 days of tam-treatment. **D–G**, WT and DKO mice underwent baseline transthoracic echocardiographic examination and subjected to tamoxifen protocol. Mice were then followed with serial echocardiography at the time points shown. **D**, Left ventricular internal dimension at end-diastole (LVID;d). **E**, LVID at end-systole (LVID;s). **F**, left ventricular ejection fraction (LVEF). **G**, LV fractional shortening (LVFS). * $P < 0.05$; *** $P < 0.005$. Tam, tamoxifen.

Figure 2. Cardiac myocyte-specific deletion of *GSK3* leads to dilated cardiomyopathy, cardiac myocyte enlargement, accelerated fibrosis and heart failure

A, Gross morphology of hearts from DKO versus control demonstrates multi-chamber enlargement. Representative images displaying morphological changes in a temporal manner. **B**, Cardiac myocyte cross-sectional area was significantly increased in the DKO hearts at d21 of tamoxifen timeline (n=4). **C**, Morphometric analysis of cardiac hypertrophy in d25 animals using heart weight/tibia length (HW/TL) ratio indicates significant increases in HW/TL ratio in DKO mice (n=17) compared to controls (n=21). **D**, Representative Trichrome stained heart sections **E**, quantification of fibrosis demonstrating increased

fibrosis in DKO hearts starting from day 21 at tamoxifen timeline. **F**, Representative H&E stained lung sections demonstrate thickening of alveolar interstitium (arrow). *** $P<0.005$.

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Figure 3. DKO mice demonstrate ultra-structural defects and enlarged cardiac myocyte nuclei
A, Representative H&E stained heart sections from DKO versus control mice on d35 of the tam-timeline showing enlarged nuclei in multiple chambers (Arrows indicate enlarged cardiac myocyte nuclei, B.C indicates blood clot). **B**, Representative transmission electron micrographs of d24 mouse hearts on tam-timeline demonstrate widened sarcomere z-line (arrow) and disrupted mitochondrial morphology (solid triangle). **C&D**, Representative transmission electron micrographs of d24 mouse hearts on tam-timeline demonstrate enlarged nuclei (Nu) with nuclear aggregates (NO), abnormal sarcomeres (mf), and nuclear membrane invaginations.

Figure 4. DKO leads to activation of cell cycle and apoptosis in cardiac myocytes but does not in fibroblasts

A, At 2 wk of tamoxifen timeline, cardiac myocytes and fibroblasts were isolated from DKO and littermate controls hearts and lysates were analyzed by western blotting. **B**, Quantification of α -smooth muscle actin (α -SMA) shows unaltered myofibroblast activation. **C–E**, Western blot analysis reveals activation of cell cycle pathways and apoptosis specifically in cardiac myocytes only. ** $P < 0.005$.

Figure 5. GSK3 deletion induces cell cycle re-entry with polyploidization and multinucleation
A, Representative H&E images of DKO left ventricle from d35 mice on the tam-timeline reveal the presence of multi-lobulated (box) and multinucleated cardiac myocytes (arrow). **B**, Quantitation of BrdU+ α -actinin+ cardiac myocytes from d24 mice on the tam-timeline reveals statistically significant increases in DNA synthesis in DKO (n=3) cardiac myocytes compared to control (n=3). **C**, Flow cytometry analysis of PCM-1+ nuclei from isolated adult d21 cardiac myocytes on the tam-timeline. Results indicate a statistically significant increase in nuclei with $>4N$ in the DKO (n=3). **D**, Representative immunofluorescence

images of adult d21 cardiac myocytes mice on the tam-timeline stained with α -actinin and DAPI revealed cardiac myocytes with up to 4 nuclei in karyokinesis in the DKO compared to control. **E**, Quantitation of number of DAPI+ nuclei in isolated adult cardiac myocytes from d24 mice on the tam-timeline reveals a statistically significant reduction in binucleated cardiac myocytes and increase in cardiac myocytes with greater than 4 nuclei in DKO (n= 493 cells from 5 mice) versus controls (n = 386 cells from 3 mice).

Figure 6. DKO cardiac myocytes show mitotic entry and DNA damage

A, Representative Western blot analyses of various markers of the cell cycle. Results indicate increased protein expression of CDK1, Cyclin B1, and CDC25C pSer-216 in DKO hearts compared to controls. **B**, Graph showing folds changes in CDK1, Cyclin B1 and CDC25C (pSer-216). **C**, Representative Western blots of DNA damage and cell cycle checkpoint markers γ -H2A.X, CHK2 (pThr68), p21 and p27. **D**, Graph showing fold changes in γ -H2A.X and -CHK2 (pThr68), p21 and p27 in the DKO hearts compared to controls. **E**, Representative immunofluorescence staining for γ -H2A.X in isolated adult cardiac myocytes from d25 mice on the tam-timeline shows predominant detection of γ -

H2A.X+ nuclei in DKO cardiac myocytes (n=3). **F**, Representative immunofluorescence staining for pH3-Ser10 in cardiac sections from d21 mice on the tam-timeline shows predominant detection in α -actinin/ pH3-Ser10 dual positive nuclei in DKO cardiac myocytes. **G**, Quantitation of pH3-Ser10 (red) in α -actinin (green)/DAPI+ (blue) nuclei shows statistically significant increase in DKO cardiac myocytes (n=3029 nuclei from 3 mice) compared to controls (n=3680 nuclei from 3 mice). * $P<0.05$; *** $P<0.005$.

Figure 7. Mitotic entry of DKO cardiac myocytes leads to death by mitotic catastrophe

A, Quantitative TUNEL analysis on cardiac sections from d35 mice on the tam-timeline show a significant increase in TUNEL-positive cardiac myocytes in the DKO (n= 6513 cells from 6 mice) compared to controls (n= 5211 cells from 5 mice). **B**, Representative Western blots of apoptotic markers indicate a steady increase in p53 and an decrease in BCL-2/BAX ratio after tam-administration in DKO hearts compared to controls (n=4). **C**, Graphical representation of the ratio of Bcl-2/Bax in DKO hearts to control hearts during the Tam-timeline. Results indicate a significant reduction in the anti-apoptotic Bcl-2 to pro-apoptotic Bax protein ratio (n=4). * $P < 0.05$; *** $P < 0.005$. **D**, Representative immunofluorescence staining for pH3-Ser10/TUNEL dual positive nuclei from cardiac sections at d21 mice on the tam-timeline shows predominant detection of dual positive nuclei in DKO hearts. **E**, Quantitation of the distribution of pH3+/TUNEL+/DAPI+ nuclei in the DKO versus control heart sections. Results indicate a significant proportion of triple+ nuclei in the DKO versus controls. P value $< .05$, Chi-Square test.

Figure 8. Schematic representation of the effects of *GSK3* deletion on cardiac myocyte cell cycle During normal cardiac development cardiac myocytes retain the ability to proliferate. These cells are mono-nucleated during early development to birth but progress to a predominantly post-mitotic, bi-nucleated state by maturity. Upon deletion of *GSK3*, adult cardiac myocytes re-enter cell cycle. Cell cycle re-entry is opposed by cell cycle checkpoint activation and DNA damage. Although it is clear that DKO cardiac myocytes are able to bypass critical checkpoints to complete karyokinesis, these cells have impaired mitotic capacity and do not progress to cytokinesis. Instead, abnormal mitosis within these cells induces mitotic catastrophe resulting in a loss of functional cardiac myocytes. A possible alternative hypothesis showing loss of cardiac myocyte *GSK-3* may lead to apoptotic cell death without cell cycle reentry has been shown by dotted line. Mice develop dilated cardiomyopathy as a result of cardiac myocyte loss and ultimately succumb to death.

Genes that are involved in cell cycle with differential expression in the heart of DKO versus Ctrl

Table 1

Gene	log (ratio)	P	FDR	FC	GO Classification
Cks2	3.1766	0.0004	0.0197	9.0	cell cycle, cell division
Ube2c	2.6760	2.87E-07	0.0014	6.4	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process, cell cycle
Knstm	2.4585	1.22E-05	0.0044	5.5	cell cycle, cell division
Cdkn3	2.4525	0.0004	0.0214	5.5	cell cycle, cell cycle arrest
Esc2	2.3776	0.0005	0.0236	5.2	cell cycle, chromosome segregation
Spe25	2.3373	1.27E-05	0.0044	5.1	cell cycle, cell division
Ccnb1	2.3128	2.05E-05	0.0051	5.0	cell cycle, cell division
Ckap2	2.2990	0.0001	0.0090	4.9	apoptotic process, cell cycle
Aurka	2.2086	2.76E-05	0.0060	4.6	anterior/posterior axis specification, cell cycle
Plk1	2.1487	4.09E-06	0.0028	4.4	activation of mitotic anaphase-promoting complex activity, cell cycle
Cdk1	2.1427	1.90E-06	0.0024	4.4	apoptotic process, cell aging
Anlh	2.1270	0.0007	0.0277	4.4	cell cycle, cell division
Cenpk	2.1182	0.0014	0.0388	4.3	positive regulation of transcription from RNA polymerase II promoter
Fam83d	2.0174	1.31E-05	0.0044	4.0	cell cycle, cell division
Cenpi	1.9967	0.0014	0.0380	4.0	centromere complex assembly
Ccna2	1.9860	0.0003	0.0188	4.0	cell cycle, cell division
Birc5	1.9349	1.98E-05	0.0051	3.8	apoptotic process, cell cycle
Bub1	1.9272	0.0006	0.0248	3.8	apoptotic process, cell cycle
Ccnb2	1.9143	1.10E-05	0.0043	3.8	cell cycle, cell division
Oip5	1.9031	2.62E-06	0.0024	3.7	cell cycle, cell division
Dlgap5	1.8876	0.0001	0.0112	3.7	cell-cell signaling, cell cycle
Ndc80	1.8717	1.70E-05	0.0049	3.7	attachment of spindle microtubules to kinetochore, cell cycle
Nuf2	1.8590	4.07E-05	0.0074	3.6	attachment of spindle microtubules to kinetochore, cell cycle
Cenpn	1.8564	6.26E-06	0.0033	3.6	centromere complex assembly, chromosome segregation
Spag5	1.8483	2.77E-05	0.0060	3.6	cell cycle, cell division
Cdc25c	1.8178	0.0008	0.0289	3.5	cell cycle, cell division
Mis18bp1	1.7971	0.0045	0.0685	3.5	cell cycle, cell division
Kif11	1.7171	0.0003	0.0165	3.3	cell cycle, cell division
Cdc43	1.6944	5.15E-06	0.0032	3.2	cell cycle, cell division

Gene	log (ratio)	P	FDR	FC	GO Classification
Pcl1	1.6129	0.0004	0.0209	3.1	cell cycle, cell division
Racgap1	1.5941	2.20E-05	0.0052	3.0	actomyosin contractile ring assembly, cell cycle
Cenpe	1.5798	0.0001	0.0091	3.0	attachment of spindle microtubules to kinetochore, cell cycle
Cdc20	1.5736	2.08E-07	0.0014	3.0	activation of anaphase-promoting complex activity, anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process
1500015O10Rik	1.5627	0.0008	0.0292	3.0	cellular senescence, cyclin catabolic process
Nusap1	1.5373	0.0009	0.0305	2.9	cell cycle, cell division
Casc5	1.5355	0.0004	0.0219	2.9	attachment of spindle microtubules to kinetochore, cell cycle
Mad2l1	1.5198	0.0004	0.0209	2.9	cell cycle, cell division
Mki67	1.5100	0.0033	0.0587	2.8	cell proliferation, meiotic nuclear division
H2afx	1.4946	0.0005	0.0233	2.8	cell cycle, cellular response to DNA damage stimulus
4632434I11Rik	1.4844	0.0001	0.0115	2.8	apoptotic process, cell cycle
Cenpp	1.4657	0.0003	0.0192	2.8	CENP-A containing nucleosome assembly at centromere
Ncapg	1.4575	2.94E-06	0.0025	2.7	cell cycle, cell division
Cdca8	1.4234	0.0003	0.0188	2.7	cell cycle, cell division
2810417H13Rik	1.4151	0.0004	0.0213	2.7	cellular response to DNA damage stimulus, centrosome organization
Cene2	1.3900	0.0175	0.1432	2.6	cell cycle, cell division
Ska3	1.3789	0.0080	0.0943	2.6	cell cycle, cell division
Cenpw	1.3730	0.0003	0.0191	2.6	cell cycle, cell division
Mifl	-1.3126	0.0009	0.0302	2.5	cell cycle, cell cycle arrest
Cep55	1.3094	0.0005	0.0233	2.5	cell cycle, cell division
Mastl	1.2973	0.0017	0.0418	2.5	cell cycle, cell division
Ube2t	1.2835	0.0019	0.0450	2.4	cellular response to DNA damage stimulus, DNA repair
Bora	1.2738	0.0002	0.0154	2.4	cell cycle, cell division
Aspm	1.2712	0.0001	0.0091	2.4	brain development, cell cycle
Aurkb	1.2658	0.0012	0.0361	2.4	cell cycle, cell division
Cenpn	1.2653	0.0003	0.0194	2.4	biological_process
Anapc1	1.2542	0.0237	0.1682	2.4	cell cycle, cell division
Foxm1	1.2525	0.0000	0.0075	2.4	cell cycle, cellular response to DNA damage stimulus
E2f1	1.2197	0.0031	0.0184	2.3	anoikis, regulation of cell cycle
Gas2l3	1.2293	0.0003	0.0191	2.3	actin cytoskeleton organization, cell cycle arrest
Ncapg	1.2231	0.0011	0.0339	2.3	mitotic chromosome condensation

Gene	log (ratio)	P	FDR	FC	GO Classification
Zwisch	1.1995	0.0009	0.0313	2.3	cell cycle, cell division
Bub1b	1.1990	0.0001	0.0111	2.3	apoptotic process, cell cycle
Dsn1	1.1961	0.0017	0.0416	2.3	cell cycle, cell division
Smc4	1.1627	0.0005	0.0235	2.2	cell cycle, cell division
Mem5	1.1357	0.0021	0.0476	2.2	cell cycle, cell division
Dbf4	1.1328	0.0016	0.0400	2.2	cell cycle, DNA replication
Ccn1	1.1311	0.0004	0.0203	2.2	cell cycle, cell division
Clasp1	-1.1253	1.35E-05	0.0044	2.2	cell cycle, cell division
Cenpa	1.0933	0.0013	0.0368	2.1	establishment of mitotic spindle orientation, kinetochore assembly
Cdc45	1.0927	0.0002	0.0164	2.1	cell cycle, cell division
Cep72	1.0822	0.0022	0.0478	2.1	gamma-tubulin complex localization, spindle organization
Cdkn1a	1.0781	0.0073	0.0898	2.1	cell cycle, cell cycle arrest
Fancd2	1.0642	0.0003	0.0180	2.1	cell cycle, cellular response to DNA damage stimulus
Mem7	1.0618	0.0005	0.0233	2.1	cell cycle, cell proliferation
Smc2	1.0586	0.0038	0.0630	2.1	cell cycle, cell division
Nek2	1.0441	1.75E-05	0.0049	2.1	blastocyst development, cell cycle
Kif2c	1.0435	0.0012	0.0346	2.1	cell cycle, cell division
Mem6	1.0267	0.0009	0.0300	2.0	cell cycle, DNA replication
Cdc42	0.9984	0.0004	0.0215	2.0	cell cycle, cell division
E2f8	0.9885	0.0214	0.1595	2.0	cell cycle, cell cycle comprising mitosis without cytokinesis
Ncapg2	0.9673	0.0029	0.0554	2.0	cell cycle, cell division
Fam64a	0.9578	0.0015	0.0390	1.9	cell cycle, cell division
Kif18b	0.9458	0.0015	0.0390	1.9	cell cycle, cell division
Skil	0.9437	0.0003	0.0165	1.9	blastocyst formation, cell cycle arrest

Note: ratio, mean from DKO mice to Ctrl ratio (n = 4 biological duplicates);

FC, fold change; FDR, false discovery ratio; GO, gene ontology.