ESC European Society of Cardiology

Cardiovascular Research (2019) **115**, 20–30
doi:10.1093/cvr/cvy255

REVIEW

Inhibition of GSK-3 to induce cardiomyocyte proliferation: a recipe for *in situ* cardiac regeneration

Anand Prakash Singh[1], Prachi Umbarkar[1], Yuanjun Guo[1,2], Thomas Force[1], Manisha Gupte[1], and Hind Lal[1]*

[1]Division of Cardiovascular Medicine, Vanderbilt University Medical Center, 2220 Pierce Ave, Suite PRB#348A, Nashville, TN 37232, USA; and [2]Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA

Received 24 April 2018; revised 23 August 2018; editorial decision 17 September 2018; accepted 9 October 2018; online publish-ahead-of-print 15 October 2018

Abstract

With an estimated 38 million current patients, heart failure (HF) is a leading cause of morbidity and mortality worldwide. Although the aetiology differs, HF is largely a disease of cardiomyocyte (CM) death or dysfunction. Due to the famously limited amount of regenerative capacity of the myocardium, the only viable option for advanced HF patients is cardiac transplantation; however, donor's hearts are in very short supply. Thus, novel regenerative strategies are urgently needed to reconstitute the injured hearts. Emerging data from our lab and others have elucidated that CM-specific deletion of glycogen synthase kinase (GSK)-3 family of kinases induces CM proliferation, and the degree of proliferation is amplified in the setting of cardiac stress. If this proliferation is sufficiently robust, one could induce meaningful regeneration without the need for delivering exogenous cells to the injured myocardium (i.e. cardiac regeneration *in situ*). Herein, we will discuss the emerging role of the GSK-3s in CM proliferation and differentiation, including their potential implications in cardiac regeneration. The underlying molecular interactions and cross-talk among signalling pathways will be discussed. We will also review the specificity and limitations of the available small molecule inhibitors targeting GSK-3 and their potential applications to stimulate the endogenous cardiac regenerative responses to repair the injured heart.

Keyword Glycogen synthase kinase • Cardiomyocyte • Proliferation • Heart failure • Signalling mechanism

1. Introduction

Heart failure (HF) is a chronic, progressive condition in which heart is unable to pump enough blood to fulfil the body's need. With an alarming statistics of 38 million patients currently suffering from HF, it is a major and growing public health problem worldwide. More than 700 000 new patients are diagnosed with HF in the USA annually; about half of these patients dying within 5 years of diagnosis.[1] Furthermore, HF restricts physical activity and negatively influences the quality of life. The annual financial burden of HF management is over $30.7 billion in the USA alone and is projected to increase to almost $70 billion by 2030.[2,3] Despite significant improvement in the current therapies, patients with advanced HF have very few options other than cardiac transplantation. This is primarily due to extremely limited regenerative potential of the adult mammalian hearts. Due to this poor regenerative capacity, the injured heart has no alternate to adapt and compensate (adverse ventricular remodelling) to maintain the pump function for the time being. Unfortunately, adverse remodelling leads to a significantly scarred hearts, which are destined to fail over the time. Thus, novel approaches to regenerate the damaged heart are urgently needed. Therefore, it is not surprising that cardiac regeneration is at the centre of the myocardial research from the past several decades. Although there are plenty of discrepancies, disagreement, and even controversies, the leaders of cardiac regeneration field have recently published a consensus statement to endorse a general agreement.[4] They agreed that cardiomyocyte (CM) in rodent hearts continue to proliferate in the early postnatal period. A cardiac injury during this early phase of life is fully replaced by newly generated CMs validating the robust cardiac regeneration capacity. Due to the loss of this CM proliferation capacity, a cardiac injury after this early phase of life leads to a scarred heart with compromised function. Thus, there is a consensus that the renewal rate of CMs in the adult mammalian heart is very low (0.5–2% per year). The exact molecular mechanism of this shift from proliferating CMs to terminally differentiated one is not clearly known and currently a matter of a great scientific interest, as elucidating these mechanisms could provide novel strategies to unlock this terminally differentiated status to resume

* Corresponding author. Tel: +1 615 936 5289; fax: +1 615 936 3486, E-mail: hind.lal@vanderbilt.edu

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author(s) 2018. For permissions, please email: journals.permissions@oup.com.

the proliferative potential. However, achieving this goal is still far from reality. The fact that a myocardial injury leads to increased CM proliferation provides some hope that stimulation of latent endogenous CM proliferation is conceivable. Indeed, studies with genetic lineage tracing tools suggest that the newly generated CMs in a healthy or injured heart primarily comes from the division of pre-existing CMs.[5,6]

In contrast to the minimal regenerative capacity of the mammalian hearts, some of the Teleost's like zebrafish have shown tremendous cardiac regenerative capacity.[7] In fact, adult zebrafish are able to fully regenerate the heart within 2 months of 20% ventricular resection. Consistent with the mammalian hearts, this efficient regeneration in zebrafish is primarily facilitated through the robust proliferation of pre-existing CMs localized at injury area.[7] With this remarkable regenerative capacity, the zebrafish heart efficiently overcomes the injury-induced scarring, allowing robust cardiac regeneration. Given this robust regenerative capacity and the availability of tools for genetic manipulation, at present, zebrafish is one of the best models to dissect the molecular mechanism of CM proliferation and cardiac regeneration.[8]

As discussed above, adult mammalian hearts possess none to minimal regenerative potential. However, analysis of human postmortem histological specimens has shown significant cardiac regenerative potential in early human life.[9,10] Though, the precise time window of the human heart regenerative potential and its loss with the age is not clear. The premise of a regenerative window in the neonatal mammalian heart is strongly supported by experimental studies with neonatal rodent's hearts with multiple cardiac injury models.[11–13] Taken together, the available evidence supports the notion that irrespective of the model organism or injury type, proliferation of pre-existing CMs is the primary mechanism to facilitate the cardiac regeneration.[5,6] Although multiple strategies have been proposed to achieve the cardiac regeneration, as discussed above, approaches to induce the proliferation of pre-existing CMs is the most promising avenue. Emerging data from our lab and others have shown that CM-specific deletion of glycogen synthase kinase (GSK)-3 family of kinases induces CM proliferation in the injured heart.[14–16] This level of robust CM proliferation could be exploited to achieve meaningful cardiac regeneration without the need for exogenous cell delivery to the injured myocardium. In this review, we will focus on the endogenous mechanisms for CM proliferation and cardiac regeneration. The implicated signalling pathways will be discussed with special emphasis on the role of the GSK-3 pathway in CM proliferation and differentiation.

2. Approaches to cardiac regeneration

Extensive research in the past several decades have suggested multiple approaches to achieve the cardiac regeneration, these include (i) strategies to enhance the proliferation of pre-existing CMs, (ii) activation and/or transplantation of progenitor populations, (iii) *in vivo* direct cardiac

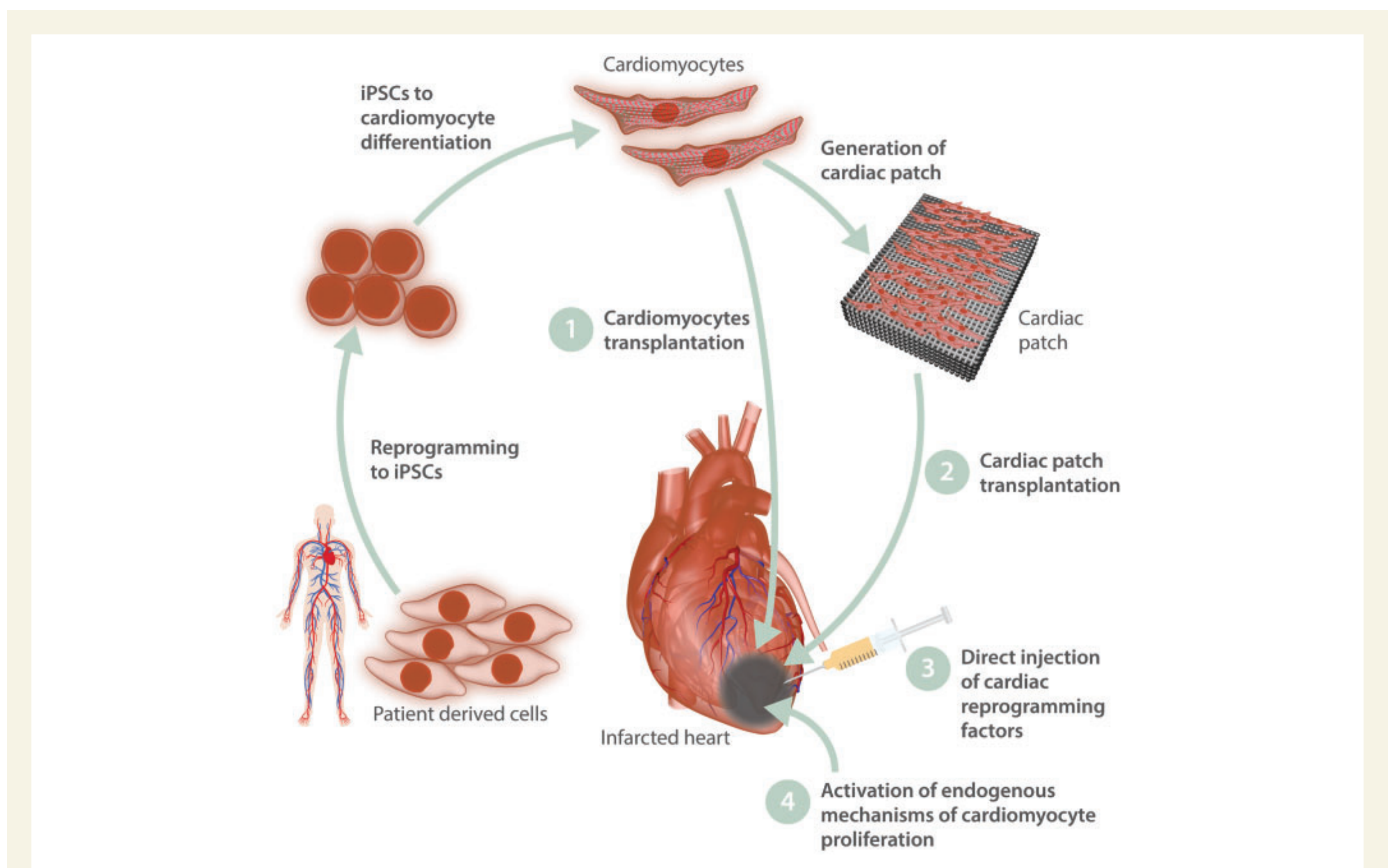

Figure 1 Current strategies for cardiac regeneration. *In vitro* production and expansion of patient-derived iPSC-derived CMs (iPSC-CMs) for delivery into the injured heart either by direct implantation (1) or by implanting cardiac patch (2). Direct cardiac programming (3) involves combinations of known factors, small molecules or drugs that can be injected directly into the infarct in attempts to reprogramme resident cardiac fibroblasts in CM-like cells. Activation of endogenous mechanisms (4) of CM proliferation like NRG1-ErbB signalling, cell cycle regulators etc. can be harness to achieve cardiac regeneration. CMs, cardiomyocytes; iPSC, induced pluripotent stem cell; NRG1, neuregulin-1.

reprogramming, and (iv) exogenous CM replacement (*Figure 1*). The advantages and the limitations of these regenerative strategies have been recently reviewed in detail,[12,17] herein; we will focus our discussion on the strategies to stimulate the proliferation of endogenous CMs.

3. Signalling pathways critical to CM proliferation

Extensive research in past several decades in cardiac regeneration field is making to believe that the goal of cardiac regeneration is achievable. As discussed above, robust cardiac regeneration in neonatal mice heart and adult zebrafish heart is primarily mediated by the proliferation of pre-existing CMs. In addition to these findings with the rodents and fish model, there is evidence for low rate renewal of new CMs in adult humans.[18] Furthermore, recent studies also suggest that progenitors contribute minimally to none to the generation of new CMs in adult mammalian hearts.[5,6] Thus, even though mammalian CMs are notably non-proliferative, at present, the proliferation of pre-existing CMs is the most accepted source for newly generated CMs in the injured myocardium. Therefore, strategies to stimulate the proliferation of pre-existing CMs are the most feasible approach to repair the injured heart. To achieve this, the research has focused towards identifying the growth factors and signalling pathways capable of augmenting endogenous cardiac regenerative potential. Several key pathways have been identified that regain the CMs proliferative capacity, these include (i) direct activation of cell cycle regulators, (ii) inhibition of HIPPO pathway, (iii) activations of Neuregulin (NRG)-1/ErbB Signalling, and (iv) inhibition of GSK-3 signalling (*Figure 2*). Herein, we will briefly introduce these pathways and move to the detail discussion on the role of GSK-3 family of kinases in CM proliferation and cardiac regeneration.

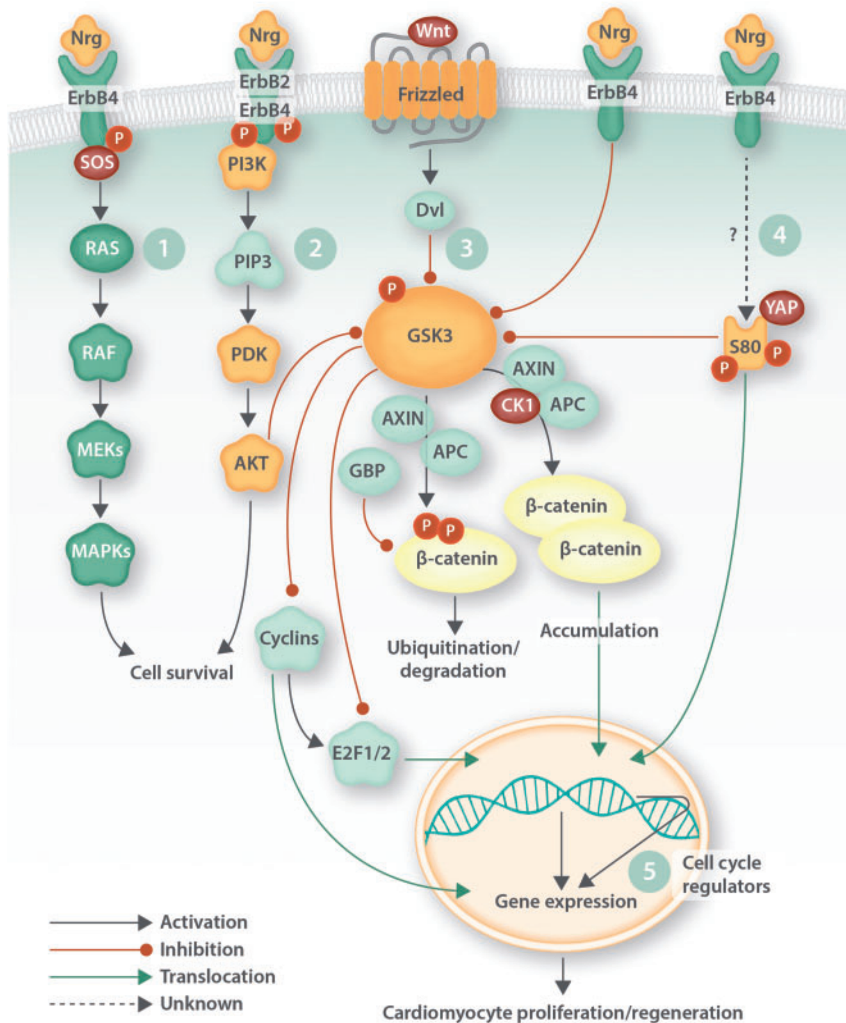

Figure 2 Signalling pathways critical to CM proliferation. Pathways that promote CM proliferation can be used for improving heart regeneration. Studies suggested that NRG1-ErbB signalling (1), AKT signalling (2), GSK-3 signalling (3) Hippo-YAP signalling (4), and direct cell cycle regulators (5) are essential pathways which can be modulated to achieve robust cardiac regeneration *in vivo*. Dvl, disheveled; PDK, 3-phosphoinositide-dependent protein kinase.

4. Direct cell cycle regulators and CM proliferation

Initial strategies to reactivate CM proliferation were primarily focused on directly modulating the cell cycle machinery itself.[19–22] Overexpression of the cyclin B1-CDC2 (cell division cycle 2 kinase) re-initiated the cell division in adult CMs, suggesting that strategies targeting the cyclin B1-CDC2 complex might re-initiate cell division in mature CMs and facilitate myocardial regeneration.[19] Similarly, therapeutic delivery of cyclin A2 induced myocardial regeneration and preserved cardiac function in ischaemic HF.[20,21] Additionally, targeted expression of cyclin D2 in CMs induced DNA synthesis and infarct size regression in the transgenic mice.[23] Consistently, knockdown of cyclin-dependent kinase inhibitor induced CM re-entry in the cell cycle.[22] Taken together, the direct modulation of cell cycle regulators produced a relatively modest response to cell cycle entry. Importantly, the primary readout in most of these studies was the DNA synthesis and cell cycle entry, not the generation of new functional CMs.

5. HIPPO pathway and CM proliferation

The Hippo signalling pathway, also known as the Hippo/YAP pathway (mammalian nomenclature), is critical to control the organ size via regulating cell survival and death. The pathway was named based on the Hippo (Hpo), a key protein kinase component of the pathway. Most of the core components of the Hippo pathway were initially identified in Drosophila and the corresponding mammalian homolog genes were identified later, suggesting that this signalling cascade is evolutionarily conserved.[24] Hippo cascade is best known for its anti-growth characteristic, meaning its inhibition leads to massive tissue overgrowth.[24–26] In fact, the *hpo* gene was named after its mutant adult head phenotype resemblance with the head of the '*hippo*potamus'. The Hippo signalling network is complex and comprises more than 30 components. In an overly naive view, the mammalian Ste20-like kinases 1/2 (MST1/2; homologs of Drosophila Hippo [Hpo]) phosphorylate and activate large tumour suppressor 1/2 (LATS1/2; homologs of Drosophila Warts [Wts]) to eventually limit the activities of two transcriptional coactivators, Yes-associated protein (YAP) and PDZ-binding motif (TAZ; two homologs of Drosophila Yorkie [Yki]). In their active stage, YAP and TAZ translocate into the nucleus to bind with TEA domain (TEAD) family of transcription factors (homologs of Drosophila Scalloped [Sd]) and induces the expression of multiple genes critical to cell survival and proliferation.[24,25]

A critical role of Hippo pathway in CM proliferation and cardiac regeneration is now demonstrated by numerous studies from multiple groups.[27–34] Heallen *et al.*[27] were the first to discover that Hippo pathway inhibits Wnt signalling to restrain CM proliferation and heart size. Subsequently, Xin *et al.*[28] showed that hippo effector Yap is necessary and sufficient for embryonic cardiac growth in mice. Deletion of Yap in the embryonic mouse heart hampered CM proliferation leading to myocardial hypoplasia and lethality at embryonic stage 10.5. Conversely, transgenic expression of a constitutively active form of Yap in the embryonic heart increased CM number and heart size.[28] von Gise *et al.*[29] employed both the gain and loss of function approaches to investigate the role of hippo effector Yap1 in postnatal CM proliferation and cardiac growth. In the gain of function approach, YAP1 activation was sufficient to induce CM proliferation, both in culture and *in vivo*. Conversely, a dominant negative peptide that blocked YAP1 binding to TEAD transcription factors inhibited YAP1 proliferative activity, suggested the requirement of YAP1-TEAD interaction for the proliferative effect.[29] Xin *et al.*[30] employed the myocardial injury model to demonstrate that forced expression of active YAP in the adult ischaemic hearts improves contractility via cardiac regeneration. Further studies with various models have demonstrated the key role of miR302-367, phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K-AKT) pathway, and actin cytoskeletal remodelling in Hippo mediated regulation of CM proliferation.[31,35,36] Morikawa *et al.*[33] discovered that Dystrophin-glycoprotein complex sequesters Yap to inhibit CM proliferation. Recently, the same research group demonstrated that Hippo pathway deficiency reverses systolic HF after infarction.[34] Taken together, the Hippo pathway is a powerful suppressor of the CM proliferation and its inhibition induces robust CM proliferation. Thus, the strategies to inhibit the hippo signalling could be employed to repair the injured heart through cardiac regeneration.

6. NRG-1/ErbB signalling and CM proliferation

Multiple high profile studies have implicated the Neuregulin/ErbB signalling in CM proliferation and cardiac regeneration.[37–41] NRG family contains four structurally related proteins (NRG1 to 4) and belongs to epidermal growth factor (EGF) family. NRGs transmit their signals through transmembrane tyrosine kinase receptors of the ErbB family. The ErbB receptor family contains four structurally related members, ErbB1 (Her1, EGFR), ErbB2 (Her2, Neu), ErbB3 (Her3), and ErbB4 (Her4). The cardiac NRG1 receptors, ErbB2 and ErbB4 express highly throughout the embryonic development until early neonatal age, thereafter significantly down-regulated in postnatal and adult hearts. This specific expression pattern correlates nicely with the robust regenerative capacity of embryonic and neonatal heart vs. minimal regeneration potential of adult hearts. Germline deletion of NRG1, ErbB2, or ErbB4 genes leads to a phenotype of compromised CM generation, thinner myocardium and embryonic death at midgestation. Conversely, postnatal induction of a constitutively active ErbB2 exhibited a phenotype of pronounced cardiomegaly characterized by extensive CM hypertrophy, dedifferentiation, and proliferation.[41] Furthermore, expression of activated ErbB2 in adult animals induces CM proliferation and cardiac regeneration of the injured heart. Consistently, treatment of NRG1 improves cardiac function of failing hearts in preclinical animal models[37,40–43] and in HF patients.[40,44–46] In an elegant study, Bersell *et al.*[37] demonstrated that the NRG1 can induce proliferation of mononucleated CMs and promote myocardial regeneration of the ischaemic heart. Taken together, augmentation of NRG1/ErbB signalling is a very promising avenue to induce the dormant CMs to dedifferentiate, proliferate and regenerate the damaged heart.

7. GSK-3 signalling

GSK-3 is a serine/threonine kinase, expressed ubiquitously and highly conserved throughout the evolution. This family of kinase was first discovered in 1980 as a regulatory kinase for its namesake, Glycogen synthase. In mammals, GSK-3 is encoded by two known genes, *GSK3A* (GSK-3α) and *GSK3B* (GSK-3β). Both GSK-3 isoforms share high homology in the kinase domains (98%). However due to significant variation in

N- and C-terminals, the overall sequence homology of both isoforms is limited to 85%. Unlike to most other kinases, GSK-3s are constitutively active in the resting cells and various cellular stimuli lead to their phosphorylation and inhibition.[47] The phosphorylation-mediated inhibition of GSK-3s is primarily regulated by N-terminal Ser21 for GSK-3α and Ser9 for GSK-3β. Additionally, the activity of GSK-3 is positively regulated by tyrosine phosphorylation at Tyr 279 for GSK-3α and Tyr216 for GSK-3β. However, the physiological relevance of these Tyr regulatory sites is not well characterized as the Ser21 and Ser9 phosphorylation of GSK-3α and GSK-3β, respectively.[47] GSK-3 mediated phosphorylation of its downstream targets usually inhibits their activity. GSK-3 signalling has been implicated in a number of biological processes including proliferation, metabolism, apoptosis, migration, and autophagy (*Figure 3*). From a clinical standpoint, GSK-3 has been associated with numerous diseases.[48–51] GSK-3 inhibitors are currently being tested for therapeutic use in Alzheimer's disease, Type 2 diabetes mellitus, some forms of cancer. In fact, Lithium, a modest inhibitor of GSK-3 is in the clinic from past many years to treat bipolar disorder. GSK-3 family of kinases are critical for cardiac and global metabolism.[52–58] In fact, GSK-3 was originally identified and named based on its ability to regulate the glycogen metabolism.[59] GSK-3 phosphorylates and inactivates glycogen synthase (GS), the rate-limiting enzyme of glycogen synthesis. Specifically, GSK-3 phosphorylates four GS regulatory serine residues (Ser641, Ser645, Ser649, and Ser653), critical to inhibiting GS activity and hence glycogen synthesis.[60] Thus, inhibition/deletion of GSK-3 results in activation of GS.[61] Importantly, glycogen serves as a primary energy source for the foetal heart and occupies more than 30% of the cell volume in foetal CM.[62] Glycogen synthesis in cardiac muscle is critical for normal heart development and its impairment leads to congenital heart defects and death.[63] However, in postnatal hearts, there is a metabolic shift from glucose and lactate oxidation to fatty acid oxidation as the energy source. This energy metabolic switch is paralleled by changes in the expression and activity of the enzymes involved in the respective metabolic pathways.[62] With that said, the comparative expression or activity of GSK-3 isoforms at various developmental stages is not well established and warrant further investigation.

In addition to its critical role in regulating glycogen synthase, GSK-3 has been implicated in several other aspects of glucose homeostasis, including gluconeogenesis and phosphorylation of insulin receptor IRS.[60] Furthermore, it is now well established that GSK-3 is crucial for the

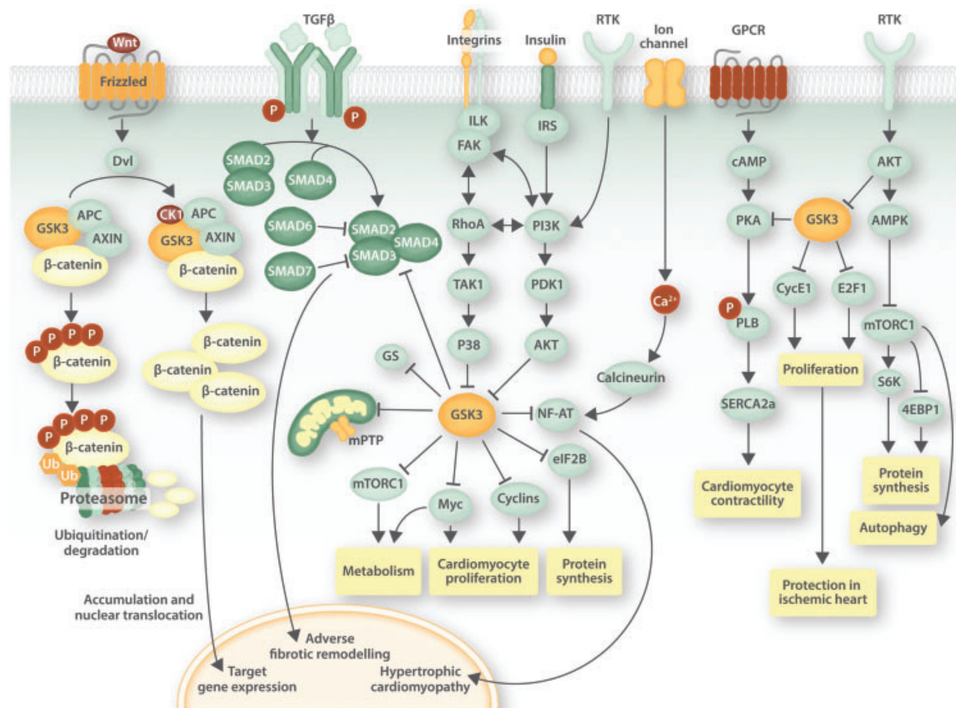

Figure 3 GSK-3 signalling in heart. The salient features of cardiac GSK3 signalling are illustrated in this schematics. The binding of growth factors to their receptors (e.g. Insulin) leads to activation of PI3K/Akt pathway. AKT subsequently phosphorylates GSK-3 and inhibits its activities. GSK-3 plays a key regulatory role in the highly conserved canonical Wnt signalling pathway. GSK-3 mediated phosphorylation of β-catenin leads to its ubiquitination and degradation by the proteasome, preventing target gene expression. In the absence of GSK-3, stabilized β-catenin accumulates and translocates to the nucleus. GSK-3 negatively regulates plethora of signalling molecules, so the consequences of GSK-3 inhibition are activation of these factors, including: (i) TGF-β1-SMAD-3 signalling, (ii) Mitochondrial mPTP pore opening, (iii) NF-AT, hypertrophy regulator, (iv) glycogen synthase, glycogen synthesis regulator, (v) mTORC1, autophagy, and metabolism regulator, (vi) D- and E-type cyclins, cell cycle regulator, (vii) Myc, metabolism, and proliferation regulator. p38 inhibit GSK-3 through an alternative mechanism. GSK-3α regulates AMPK and mTOR, the master regulators of autophagy and metabolism. In the absence of GSK-3α, mTOR is dysregulated leading to impaired autophagy. GSK-3α also regulates the β-adrenergic receptor responsiveness and cAMP production via unknown mechanisms. In addition, GSK-3α directly interacts and phosphorylates cyclin E1 in CMs and its deletion promotes E2F-1 and cyclin E1 recruitment and CM proliferation.

regulation of many other aspects of cardiac biology, including the cardiac homeostasis.[15,58,64–68] Several studies with CM-specific GSK-3β KOs have established the concept that inhibition of GSK-3β during HF is protective.[15,69] Furthermore, our findings with CMs-specific GSK-3α KO suggest that inhibition of GSK-3α protects from myocardial infarction (MI)-induced adverse cardiac remodelling.[14] These findings with CM-specific GSK-3α KOs are in stark contrast with the studies of germ-line global deletion of GSK-3α, which suggest that loss of GSK-3α is detrimental, leads to spontaneous hypertrophy,[68] exacerbates MI-induced remodelling[67] and shows accelerated aging.[70] Studies with GSK-3α-S21A and GSK-3β S9A knock-in mouse model have revealed distinct roles of GSK-3 isoforms in pressure overload-induced pathological cardiac hypertrophy.[16] We have recently demonstrated that CMs GSK-3 is required to maintain normal cardiac homeostasis, and its loss is incompatible with life because of cell cycle dysregulation that ultimately results in a severe dilated cardiomyopathy and death.[65] Thus, the role of GSK-3s in myocardial biology has been extensively studied and we have recently reviewed the literature regarding GSK-3 and myocardial biology in general,[50] herein, in the following sections, we will focus on the isoform-specific role of GSK-3α and GSK-3β in CM proliferation and cardiac regeneration.

8. GSK-3 mediated regulation of CMs proliferation; lesson learned from GSK-3-specific pharmacological agents

Numerous studies have suggested the crucial role of GSK-3 signalling in CM proliferation (*Table 1*). Tseng *et al.*[71] were the first to demonstrate that pharmacological inhibition of GSK-3 signalling by small molecule 6-bromoindirubin-3′-oxime (BIO) promotes proliferation in mammalian CMs. In the isolated neonatal CM model, BIO-mediated inhibition of GSK-3 induced up-regulation of cell cycle regulators and cell division. In addition to multiple markers of cell cycle entry, authors utilized Aurora B staining to successfully demonstrate the completion of cytokinesis.[72] Consistently, BIO treatment induced dedifferentiation, cell cycle progression and increased mitosis in adult rat CMs. Although mechanistic detail was not investigated, authors proposed that BIO-induced activation of canonical Wnt pathway is primarily responsible for the proliferative signal. The ability of pharmacological inhibition of GSK-3 to induce CM proliferation was also recognized in chemical library screen designed to identify the compounds with abilities to promote CM proliferation.[72] In this unbiased chemical library screening, GSK-3 inhibitors were ranked highest for their potential to induce CM proliferation. Recently, Wang *et al.*[73] extended these findings to human CMs by showing that GSK-3β inhibitor CHIR-99021 promotes human CM proliferation through canonical Wnt signalling. As a significant step forward, Fang *et al.*[74] employed a novel hybrid hydrogel system for sustained co-delivery of BIO and insulin growth factor 1 (IGF-1) to repair the infarcted rat hearts. To achieve this, both BIO and IGF-1 were first encapsulated in gelatin nanoparticles and thereafter cross-linked with the oxidized alginate to form a novel hybrid hydrogel system. The sustained delivery of BIO and IGF-1 enhanced the proliferation of CMs, promoted revascularization at the injury site and improved cardiac function. These experiments were the first to demonstrate the usefulness of pharmacological GSK-3 inhibition to achieve endogenous CM proliferation and cardiac regeneration. Subsequently, it was demonstrated that GSK-3 inhibitor BIO treatment favours the reprogramming of skin fibroblasts to generate cardiospheres for myocardial regeneration.[75] A recent study [76] suggests that the cardiac benefit of GSK-3 inhibitor BIO is not limited to its direct effects on CM proliferation, instead, BIO modulates cardiac recovery via unique modulation of the cardiac microenvironment, these includes effects on other cardiac cells, namely fibroblasts and macrophages. Specifically, BIO induced CMs proliferation, inhibited fibroblast proliferation, and suppressed the expression of pro-inflammatory gene in macrophages. Furthermore, BIO modulated the cellular crosstalk between cardiac fibroblasts and differentiating macrophages to induce polarization of the anti-inflammatory M2 phenotype. In the zebrafish HF model, BIO-induced efficient CM proliferation and completely recovered survival rate.[76] In summary, it is reasonable to conclude that pharmacological inhibition of GSK-3 is sufficient to induce CM cell cycle re-entry, DNA synthesis, and likely proliferation. However, all GSK-3 targeted drugs to date are non-isoform specific. This is a major concern since long-term, non-isoform specific inhibition of GSK-3α/β could be detrimental.[65]

9. GSK-3α negatively regulates CMs proliferation

Traditionally, GSK-3β has been considered the dominant isoform of the GSK-3 family, though this bias goes back many years to studies with Drosophila in which mammalian GSK-3β was comparatively better rescued the frizzled phenotype in GSK-3 mutants (Zw3/shaggy).[80,81] Even after many years of these early reports, the bias towards GSK-3β isoforms is very apparent by the fact that many more studies are consistently coming with a primary focus on GSK-3β than on GSK-3α. In contrast to this traditional belief, now numerous studies have shown that both GSK-3 isoforms are critical but entirely redundant in regulating the canonical Wnt pathway.[82,83] Furthermore, it is also now clear that GSK-3s are not entirely redundant in all function.[50,68,77] The best example of their isoform-specific function can be explained by the fact that while germline GSK-3α knockout mice survive several years, the GSK-3β knockout is embryonic lethal.[67,68,77] Although GSK-3α germline knockout survives, they develop spontaneous cardiac hypertrophy at two months of age and displayed an aging phenotype later.[68,70]

In stark contrast to findings with GSK-3α global knockout, studies with more specific approaches, like site-specific GSK-3α knock-in mice and inducible CM-specific knock-out mice have supported the concept that inhibition of GSK-3α in heart induces CM proliferation and protects from myocardial injuries.[14,16] Matsuda *et al.*[16] created GSK-3α S21A knock-in (αKI) mice to investigate the role of GSK-3α in CM proliferation and pressure overload induced pathological hypertrophy. Constitutive activation of GSK-3α (S21 knock-in) suppressed the stress-induced CM proliferation and worsens cardiac function. Importantly, in comparison to their littermate controls, GSK-3α knock-in hearts had significantly less number of CMs. Thus, this elegant study established that persistent activation of GSK-3α attenuates stress-induced CM proliferation and proposed the hypothesis that strategies to specifically inhibit GSK-3α in injured heart should promote CM proliferation and regeneration. To test this hypothesis, we employed inducible CM-specific GSK-3α KO mice in an ischaemic injury model.[14] Indeed, CM-specific conditional GSK-3α deletion induced significant CM proliferation to achieve a marked protection from post-MI adverse remodelling and cardiac dysfunction.[14] Furthermore, the ischaemic area in the GSK-3α KO hearts contained significantly higher numbers of viable CMs than littermate controls. The CM proliferation in the GSK-3α KO hearts was also evident by

Table 1 GSK-3 signalling and CM proliferation

SN	Nature of manipulation (pharmacological/genetic)	Model	Phenotype and mechanism	References
1	Pharmacological [6-bromoindirubin-3′-oxime (BIO)]	Neonatal and adult rat CMs	This was the first report demonstrating that GSK-3 inhibition by small molecule BIO can induce the proliferative ability of mammalian CMs	71
2	Germline GSK-3β knockout mice	Spontaneous phenotype	GSK-3β knockout embryos developed lethal hypertrophic myopathy caused by CM hyperproliferation. The hyperproliferative phenotype was associated with increased expression and nuclear localization of three regulators of proliferation—GATA4, cyclin D1, and c-Myc	77
3	Isoform-specific knock-in mice (GSK-3α S21A and GSK-3β S9A)	Pressure overload	These studies with isoform-specific knock-in mice demonstrated differential roles of GSK-3α and GSK-3β in CM proliferation. Specifically, activation of GSK-3α, but not GSK-3β, inhibits CM proliferation in response to pressure overload	16
4	A combination of genetic and pharmacological	Differentiation of murine bone marrow-derived mesenchymal stem cells (MSCs) to CM	This report demonstrates the distinct role of GSK-3α and GSK-3β in CM differentiation of bone marrow-derived MSCs. GSK-3β, primarily localizes in the cytosol and induces CM differentiation of MSCs via down-regulation of β-catenin. In contrast, GSK-3α in the nucleus inhibits CM differentiation through down-regulation of c-Jun	18
5	Conditional GSK-3β KO	Pressure overload and MI	Tamoxifen induced CM-specific GSK-3β deletion led to increased CM proliferation in the pressure overloaded and ischaemic hearts	15
6	GSK-3β-overexpressing bone marrow-derived MSCs	MI	GSK-3β increases the survival of MSCs and induces CM differentiation in the ischaemic heart	78
7	β-catenin inhibitor (indirect implication)	Embryonic heart growth	This important study identified a cross-talk of the Hippo signalling pathway and GSK-3β to regulate the CM proliferation and heart size. Mechanistically, Hippo effector Yap increases the abundance of IGF1 receptor, resulting in activation of downstream effectors that inactivate GSK-3, leading to stabilization and increased abundance of β-catenin, a positive regulator of CM proliferation	28
8	Pharmacological (BIO and CHIR-99021)	Chemical Library Screening for Cardiomyocyte Proliferation	GSK-3 inhibitors (BIO and CHIR-99021) were identified as a most potent inducer of CM proliferation in an unbiased screening of small molecule chemical library. GSK-3 inhibitors substantially induced re-entry and progression of cell cycle in neonatal as well as in adult CMs	72
9	Pharmacological	Mouse ventricular CMs	This is a key paper describing the lab protocol for expansion of mouse ventricular CMs through GSK-3 inhibition. The reproducibility of this method provides a strong rationale for the critical role of GSK-3 signalling in CM proliferation or differentiation	79
10	Conditional GSK-3α KO	MI	In ischaemic hearts, CM-specific inducible deletion of GSK-3α promotes E2F-1 and cyclin E1 recruitment and induces the CM proliferation	14
11	Sustained co-delivery of BIO and IGF-1	MI	Sustained co-delivery of BIO and IGF-1 by a novel hydrogel system induces CM proliferation and protects against MI induced cardiac dysfunction and adverse remodelling	74

Continued

Table 1 *Continued*

SN	Nature of manipulation (pharmacological/genetic)	Model	Phenotype and mechanism	References
12	Pharmacological (BIO)	Cell culture and MI	In an experimental setting of reprogramming fibroblasts to functional CMs, GSK-3 inhibitor BIO, and Oncostatin promoted the generation of induced cardiospheres (iCS). Furthermore, transplantation of these iCS improved the cardiac function of the ischaemic heart	75
13	Pharmacological (BIO)	Cell culture, rat MI model and zebrafish	BIO selectively indices CM proliferation, recovery after MI in rat model, and protects CM loss in zebrafish model of heart failure	76
14	GSK-3β inhibitor (CHIR-99021)	Human atrial CMs	Inhibition of GSK-3β increased EdU-positive and Ki67-positive CMs suggesting that GSK-3 inhibition can promote human atrial CM proliferation	76
15	CM-specific GSK-3α/β DKO mice	Spontaneous phenotype	Double-knockout cardiac myocytes showed cell cycle progression resulting in increased DNA content, multinucleation and mitotic catastrophe-induced apoptotic cell death	65

increased Ki67, BrdU, and phospho-Histone-3 positive CMs. In summary, both, the gain of function (GSK-3α KI) and loss of function approaches (CM-GSK-3α KO) consistently support the idea that GSK-3α could be targeted to achieve robust CM proliferation and cardiac regeneration.

At the molecular level, GSK-3α controls CM proliferation by directly regulating the cell cycle machinery.[14,16] Persistent activation of GSK-3α negatively regulates the E2F-mediated transcription in CMs.[16] In a luciferase assay with isolated CMs, GSK-3α (S21A) co-transfection inhibited the E2F-Luc activity. Further mechanistic studies reveal that GSK-3α inhibits E2F through suppression of D-type cyclins.[16] Specifically, TAC-induced activation of cyclin D1 and D2 was markedly attenuated in GSK-3α-KI hearts.[16] A rescue experiment with Cyclin D1 strongly supported the critical role of cyclins in GSK-3α mediated CM proliferation, as overexpression of cyclin D1 largely rescued the detrimental cardiac phenotype of GSK-3α-KI heart.[16] Consistently, the studies with CM-GSK-3α KO hearts also support the central role of GSK-3α in directly regulating the cell cycle machinery to induce CM proliferation.[14] The protein levels of transcription factor E2F1, cyclin E1, and cyclin E1-positive CMs, all were significantly increased in the ischaemic CM-GSK-3α KO hearts. The co-immunoprecipitation studies confirmed a direct interaction of E2F1 and cyclin E1 with GSK-3α. In summary, GSK-3α is a potent negative regulator of CMs cell cycle machinery and approaches to inhibit GSK-3α in diseased heart could potentially be used to promote CM proliferation and cardiac regeneration.

10. GSK-3β is critical to CMs proliferation and differentiation

The early studies investigating the role of GSK-3β in CM differentiation were done using WT and GSK-3β KO ES cell lines. In these studies, ES cells were induced to create CM embryoid bodies (EBs).[77] The number of spontaneously contracting EBs was significantly less in the GSK-3β KOs compared with WT controls suggesting that GSK-3β is necessary for CM differentiation. Importantly, the effect of GSK-3α deletion on CM differentiation was comparatively minor.[77] These findings are consistent with the fact that GSK-3α null mice develop normally, however, GSK-3β nulls are embryonic lethal.[77] The ventricle walls of the GSK-3β KO embryos were strikingly thickened, and the ventricular cavities were packed with cells, with little or no apparent ventricular cavity left, resulting in HF and death.[77] Further analysis of the GSK-3β KO embryos reveals that hyperproliferation of CMs accounts for the hyper grown cells in the ventricular cavities and thickened myocardium at late gestation. Thus, the inability of GSK-3β KO cardiomyoblasts to fully differentiate appears to allow continued proliferation, leading to a hypertrophic myopathy. At the molecular level, dysregulation of GATA4, cyclin D1, and c-Myc mediated the hyperproliferation phenotype of GSK-3β deficient CMs. As expected, double knockout (DKO) of GSK-3α/β EBs also failed to produce any contracting CMs. Consistently, conditional deletion of both isoform of GSK-3 (DKO) in adult hearts leads to a lethal phenotype associated with mitotic catastrophe.[65] In stark contrast, isoform-specific deletion of GSK-3α or GSK-3β in the adult heart does not lead to any baseline phenotype and displayed striking protection against ischaemic injury.[14,15] Conditional deletion of GSK-3β from fully mature adult heart induced CM proliferation to protect against infarction induced adverse remodelling.[15] This line of evidence suggested that inhibition of GSK-3β in fully mature hearts could be a strategy to promote cardiac regeneration and prevent adverse remodelling in pathological states. Further

studies are needed to fully delineate the specific role of GSK-3β in CM proliferation, differentiation and formulate the strategies to achieve the clinical benefit of modulating GSK-3β in the injured hearts.

11. A signalling network of NRG-1/ErbB, HIPPO and GSK-3 pathways to regulate CM proliferation

As discussed above, GSK-3 can regulate CM proliferation through a direct effect on cell cycle machinery and associated transcription factors. In this section, we will be focusing on the role of GSK-3 signalling circuits and cross-talk's with other implicated pathways to coordinate the cardiac regeneration. It is not surprising that most of the pathways implicated in endogenous CM proliferation operate via multiple crosstalks at several levels to synchronize the cardiac regeneration process.[28,41] It has been demonstrated in multiple systems that activations of NRG-1/ErbB signalling leads to AKT mediated phosphorylation of GSK-3 (inhibition), resulting in β-catenin accumulation.[84,85] D'Uva *et al.*[41] employed CM-specific, constitutively active ERBB2 caERBB2 expressing mice to directly investigate the crosstalk of ErbB and GSK-3β signalling in the heart. caERBB2 mice displayed an increased β-catenin accumulation in CMs and heart lysates. As discussed above, GSK-3 is an essential component of the β-catenin destruction complex and its inhibition or deletion leads to β-catenin accumulation. Thus, activation of CMs ErbB signalling in the heart leads to GSK-3 inhibition which may account for some of its CM proliferative potentials. GSK-3 also interacts with HIPPO pathway to coordinate the CM proliferation and cardiac regeneration.[28] Xin *et al.* employed CM-specific transgenic YapS112A mice (constitutively active) to directly investigate the HIPPO and GSK-3 crosstalk with respect to CM proliferation and cardiac regeneration. YapS112A-expressing CMs showed an increased GSK-3β^{S9} phosphorylation, the inactive form of GSK-3β. Thus, the Hippo pathway may facilitate some of its CM proliferation signal via inhibiting GSK-3β. Further analysis of YapS112A transgenic hearts reveals that Yap mediated inhibition of GSK-3β is primarily facilitated via the PI3K-AKT pathway. As discussed above, GSK-3 signalling is critical for β-catenin phosphorylation and subsequent ubiquitination. As expected, YapS112A-expressing CMs were associated with an increased abundance of the active nonphosphorylated form of β-catenin and total β-catenin. Increased β-catenin activity in expressing YapS112A CMs was also evident by increased TOPflash luciferase reporter, readout of β-catenin transcriptional activity. Pharmacological inhibition or knocked down of β-catenin in YapS112A expressing CMs reduced the number of proliferating cells. Thus, β-catenin is essential for Yap induced CM proliferation. In summary, GSK-3 facilitates its key role in CM proliferation by multiple mechanisms including direct regulation of cell cycle machinery and also via novel signalling networks/crosstalk involving ErbB/NRG-1 and Hippo signalling pathways.

12. Conclusion, limitations and future perspective

The concept of myocardial regeneration/proliferation by cell transplantation and tissue or genetic engineering promises a revolutionary approach for myocardial healing after heart damage. These strategies, however, still face significant challenges before they can develop into clinically relevant therapeutic tools.[41,86–88] A recent study with a comparatively large number of nonhuman primates have shown lack of remuscularization following transplantation of human embryonic stem cell-derived cardiovascular progenitor cells into infarcted myocardium.[88] Further, studies have shown a proarrhythmic risk of embryonic stem cell-derived CM transplantation in infarcted myocardium.[89] Considering these adverse outcomes with exogenous cell transplantation, it is reasonable to conclude that augmenting the endogenous CM regeneration is probably the most natural and safe strategy to promotes cardiac repair. If this proliferation is sufficiently robust, one could induce meaningful regeneration without the need for transplantation. It is important to recognize that the master of all the 'nature', must have its reason to wire the adult mammalian CMs as a non-dividing cell. Given this fact, it is tempting to speculate that long-term chronic activation of CM proliferation may lead to adverse consequences. However, activating endogenous pathways to promote CM proliferation just for the right moment could be beneficial. As of now, optimizing this 'just the right moment' seems hard to achieve. The only way forward is to better understand the mechanisms responsible to regulate endogenous CM proliferation and cardiac regeneration. In this regard, a considerable amount of progress has been made over the past few years, as discussed above, key pathways essential to regulate endogenous CM proliferation have been identified. At this end, our lab focuses on the GSK-3 family mediated regulation of endogenous CM proliferation in the ischaemic hearts. However, it is clear that the regulation of endogenous CM proliferation is not linear and as discussed above involves multiple signalling crosstalks. A better understanding of these signalling circuits will be critical to precisely control the signalling strength and duration to achieve the 'just the right moment' for inducing the CM proliferation. One critical limitation of the GSK-3 studies in the context of CM proliferation is the reliance on readouts other than the creation of functional new CMs. These readouts includes, phospho-histone H3, Ki67 positivity. As pointed out in the recent consensus statement,[4] these readouts do not necessarily provide evidence for new functional CMs and may result into polyploidy or multinucleated cells. Further studies with reporter mice lines to clearly mark the newly generated CMs are needed to validate the creation of new CMs via GSK-3 inhibition. Furthermore, since all the available small molecule GSK-3 inhibitors are non-isoform selective, it is critical to define the isoform-specific function and develop the small molecules with isoform selectivity. With that said, a recent clinical trial with a GSK-3 inhibitor (Tideglusib), have validated the feasibility of targeting GSK-3 in human diseases (ClinicalTrials.gov Identifier: NCT00948259). The drug was well tolerated, except the transient increase in the serum transaminases. Importantly, the increase in the serum transaminases was fully reversible. Furthermore, a modest GSK-3 inhibitor, Lithium, has been used in the clinic for many years to treat bipolar disorder without any significant adverse consequences specific to the heart. Thus, there is sufficient evidence for the feasibility of therapeutic targeting of GSK-3, at least acutely. In summary, a better understanding of signalling network responsible to regulate endogenous CM proliferation may provide a promising new therapeutic avenue to rejuvenate the injured heart.

Conflict of interest: none declared.

Funding

This work was supported by the NHLBI (R01HL133290, R01HL119234 to H.L.) and American Heart Association (13SDG16930103).

References

1. Writing Group Members, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S,

Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics-2016 update: a report from the American Heart Association. *Circulation* 2016;**133**:e38–e360.

2. Heidenreich PA, Albert NM, Allen LA, Bluemke DA, Butler J, Fonarow GC, Ikonomidis JS, Khavjou O, Konstam MA, Maddox TM, Nichol G, Pham M, Pina IL, Trogdon JG; American Heart Association Advocacy Coordinating Committee; Council on Arteriosclerosis, Thrombosis and Vascular Biology; Council on Cardiovascular Radiology and Intervention; Council on Clinical Cardiology; Council on Epidemiology and Prevention; Stroke Council. Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association. *Circ Heart Fail* 2013;**6**:606–619.
3. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD, Finkelstein EA, Hong Y, Johnston SC, Khera A, Lloyd-Jones DM, Nelson SA, Nichol G, Orenstein D, Wilson PW, Woo YJ; American Heart Association Advocacy Coordinating Committee; Stroke Council; Council on Cardiovascular Radiology and Intervention; Council on Clinical Cardiology; Council on Epidemiology and Prevention; Council on Arteriosclerosis; Thrombosis and Vascular Biology; Council on Cardiopulmonary; Critical Care; Perioperative and Resuscitation; Council on Cardiovascular Nursing; Council on the Kidney in Cardiovascular Disease; Council on Cardiovascular Surgery and Anesthesia, and Interdisciplinary Council on Quality of Care and Outcomes Research. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation* 2011;**123**:933–944.
4. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisen J, Giacca M, Hare JM, Houser S, Lee RT, Marban E, Martin JF, Molkentin JD, Murry CE, Riley PR, Ruiz-Lozano P, Sadek HA, Sussman MA, Hill JA. Cardiomyocyte regeneration: a consensus statement. *Circulation* 2017;**136**:680–686.
5. van Berlo JH, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marban E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature* 2014;**509**:337–341.
6. Vagnozzi RJ, Molkentin JD, Houser SR. New myocyte formation in the adult heart: endogenous sources and therapeutic implications. *Circ Res* 2018;**123**:159–176.
7. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science* 2002;**298**: 2188–2190.
8. Major RJ, Poss KD. Zebrafish heart regeneration as a model for cardiac tissue repair. *Drug Discov Today Dis Models* 2007;**4**:219–225.
9. Macmahon HE. Hyperplasia and regeneration of the myocardium in infants and in children. *Am J Pathol* 1937;**13**:845.
10. Haubner BJ, Schneider J, Schweigmann U, Schuetz T, Dichtl W, Velik-Salchner C, Stein JI, Penninger JM. Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ Res* 2016;**118**:216–221.
11. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. *Science* 2011;**331**: 1078–1080.
12. Uygur A, Lee RT. Mechanisms of cardiac regeneration. *Dev Cell* 2016;**36**:362–374.
13. Bryant DM, O'Meara CC, Ho NN, Gannon J, Cai L, Lee RT. A systematic analysis of neonatal mouse heart regeneration after apical resection. *J Mol Cell Cardiol* 2015;**79**: 315–318.
14. Ahmad F, Lal H, Zhou J, Vagnozzi RJ, Yu JE, Shang X, Woodgett JR, Gao E, Force T. Cardiomyocyte-specific deletion of Gsk3alpha mitigates post-myocardial infarction remodeling, contractile dysfunction, and heart failure. *J Am Coll Cardiol* 2014;**64**: 696–706.
15. Woulfe KC, Gao E, Lal H, Harris D, Fan Q, Vagnozzi R, DeCaul M, Shang X, Patel S, Woodgett JR, Force T, Zhou J. Glycogen synthase kinase-3beta regulates post-myocardial infarction remodeling and stress-induced cardiomyocyte proliferation *in vivo*. *Circ Res* 2010;**106**:1635–1645.
16. Matsuda T, Zhai P, Maejima Y, Hong C, Gao S, Tian B, Goto K, Takagi H, Tamamori-Adachi M, Kitajima S, Sadoshima J. Distinct roles of GSK-3alpha and GSK-3beta phosphorylation in the heart under pressure overload. *Proc Natl Acad Sci USA* 2008;**105**: 20900–20905.
17. Tzahor E, Poss KD. Cardiac regeneration strategies: staying young at heart. *Science* 2017;**356**:1035–1039.
18. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science* 2009;**324**:98–102.
19. Bicknell KA, Coxon CH, Brooks G. Forced expression of the cyclin B1-CDC2 complex induces proliferation in adult rat cardiomyocytes. *Biochem J* 2004;**382**:411–416.
20. Chaudhry HW, Dashoush NH, Tang H, Zhang L, Wang X, Wu EX, Wolgemuth DJ. Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. *J Biol Chem* 2004;**279**:35858–35866.
21. Woo YJ, Panlilio CM, Cheng RK, Liao GP, Atluri P, Hsu VM, Cohen JE, Chaudhry HW. Therapeutic delivery of cyclin A2 induces myocardial regeneration and enhances cardiac function in ischemic heart failure. *Circulation* 2006;**114**:I206–I213.
22. Di Stefano V, Giacca M, Capogrossi MC, Crescenzi M, Martelli F. Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. *J Biol Chem* 2011;**286**:8644–8654.
23. Pasumarthi KB, Nakajima H, Nakajima HO, Soonpaa MH, Field LJ. Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circ Res* 2005;**96**:110–118.
24. Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. *Genes Dev* 2016;**30**:1–17.
25. Plouffe SW, Hong AW, Guan KL. Disease implications of the Hippo/YAP pathway. *Trends Mol Med* 2015;**21**:212–222.
26. Zhao B, Tumaneng K, Guan KL. The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nat Cell Biol* 2011;**13**:877–883.
27. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, Martin JF. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* 2011;**332**:458–461.
28. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, Richardson JA, Bassel-Duby R, Olson EN. Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal* 2011;**4**:ra70.
29. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, Ma Q, Ishiwata T, Zhou B, Camargo FD, Pu WT. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad Sci USA* 2012;**109**:2394–2399.
30. Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM, Richardson JA, Sadek HA, Bassel-Duby R, Olson EN. Hippo pathway effector Yap promotes cardiac regeneration. *Proc Natl Acad Sci USA* 2013; **110**:13839–13844.
31. Lin Z, Zhou P, von Gise A, Gu F, Ma Q, Chen J, Guo H, van Gorp PR, Wang DZ, Pu WT. Pi3kcb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival. *Circ Res* 2015;**116**:35–45.
32. Matthews RW, Bhoola KD, Rasker JJ, Jayson MI. Salivary secretion and connective tissue disease in man. *Ann Rheum Dis* 1985;**44**:20–26.
33. Morikawa Y, Heallen T, Leach J, Xiao Y, Martin JF. Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature* 2017;**547**:227–231.
34. Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, Hill MC, Segura A, Willerson JT, Martin JF. Hippo pathway deficiency reverses systolic heart failure after infarction. *Nature* 2017;**550**:260–264.
35. Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N, Zhou S, Lu M, Gao E, Koch WJ, Stewart KM, Morrisey EE. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci Transl Med* 2015;**7**:279ra238.
36. Morikawa Y, Zhang M, Heallen T, Leach J, Tao G, Xiao Y, Bai Y, Li W, Willerson JT, Martin JF. Actin cytoskeletal remodeling with protrusion formation is essential for heart regeneration in Hippo-deficient mice. *Sci Signal* 2015;**8**:ra41.
37. Bersell K, Arab S, Haring B, Kuhn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 2009;**138**:257–270.
38. Ky B, Kimmel SE, Safa RN, Putt ME, Sweitzer NK, Fang JC, Sawyer DB, Cappola TP. Neuregulin-1 beta is associated with disease severity and adverse outcomes in chronic heart failure. *Circulation* 2009;**120**:310–317.
39. Hedhli N, Huang Q, Kalinowski A, Palmeri M, Hu X, Russell RR, Russell KS. Endothelium-derived neuregulin protects the heart against ischemic injury. *Circulation* 2011;**123**:2254–2262.
40. Polizzotti BD, Ganapathy B, Walsh S, Choudhury S, Ammanamanchi N, Bennett DG, dos Remedios CG, Haubner BJ, Penninger JM, Kuhn B. Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. *Sci Transl Med* 2015;**7**:281ra245.
41. D'Uva G, Aharonov A, Lauriola M, Kain D, Yahalom-Ronen Y, Carvalho S, Weisinger K, Bassat E, Rajchman D, Yifa O, Lysenko M, Konfino T, Hegesh J, Brenner O, Neeman M, Yarden Y, Leor J, Sarig R, Harvey RP, Tzahor E. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat Cell Biol* 2015;**17**:627–638.
42. Gupte M, Lal H, Ahmad F, Sawyer DB, Hill MF. Chronic neuregulin-1beta treatment mitigates the progression of postmyocardial infarction heart failure in the setting of type 1 diabetes mellitus by suppressing myocardial apoptosis, fibrosis, and key oxidant-producing enzymes. *J Card Fail* 2017;**23**:887–899.
43. Mahmoud AI, O'Meara CC, Gemberling M, Zhao L, Bryant DM, Zheng R, Gannon JB, Cai L, Choi W-Y, Egnaczyk GF, Burns CE, Burns CG, MacRae CA, Poss KD, Lee RT. Nerves regulate cardiomyocyte proliferation and heart regeneration. *Dev Cell* 2015; **34**:387–399.
44. Gao R, Zhang J, Cheng L, Wu X, Dong W, Yang X, Li T, Liu X, Xu Y, Li X, Zhou M. A Phase II, randomized, double-blind, multicenter, based on standard therapy, placebo-controlled study of the efficacy and safety of recombinant human neuregulin-1 in patients with chronic heart failure. *J Am Coll Cardiol* 2010;**55**: 1907–1914.
45. Odiete O, Hill MF, Sawyer DB. Neuregulin in cardiovascular development and disease. *Circ Res* 2012;**111**:1376–1385.
46. Jabbour A, Hayward CS, Keogh AM, Kotlyar E, McCrohon JA, England JF, Amor R, Liu X, Li XY, Zhou MD, Graham RM, Macdonald PS. Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *Eur J Heart Fail* 2011;**13**: 83–92.
47. Patel P, Woodgett JR. Glycogen synthase kinase 3: a kinase for all pathways? *Curr Top Dev Biol* 2017;**123**:277–302.

48. Zhang Y, Huang NQ, Yan F, Jin H, Zhou SY, Shi JS, Jin F. Diabetes mellitus and Alzheimer's disease: GSK-3beta as a potential link. *Behav Brain Res* 2018;**339**:57–65.
49. Golpich M, Amini E, Hemmati F, Ibrahim NM, Rahmani B, Mohamed Z, Raymond AA, Dargahi L, Ghasemi R, Ahmadiani A. Glycogen synthase kinase-3 beta (GSK-3beta) signaling: implications for Parkinson's disease. *Pharmacol Res* 2015;**97**:16–26.
50. Lal H, Ahmad F, Woodgett J, Force T. The GSK-3 family as therapeutic target for myocardial diseases. *Circ Res* 2015;**116**:138–149.
51. O'Leary O, Nolan Y. Glycogen synthase kinase-3 as a therapeutic target for cognitive dysfunction in neuropsychiatric disorders. *CNS Drugs* 2015;**29**:1–15.
52. Kaidanovich-Beilin O, Eldar-Finkelman H. Long-term treatment with novel glycogen synthase kinase-3 inhibitor improves glucose homeostasis in ob/ob mice: molecular characterization in liver and muscle. *J Pharmacol Exp Ther* 2006;**316**:17–24.
53. MacAulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, Nagy A, Woodgett JR. Glycogen synthase kinase 3alpha-specific regulation of murine hepatic glycogen metabolism. *Cell Metab* 2007;**6**:329–337.
54. Bouskila M, Hirshman MF, Jensen J, Goodyear LJ, Sakamoto K. Insulin promotes glycogen synthesis in the absence of GSK3 phosphorylation in skeletal muscle. *Am J Physiol Endocrinol Metab* 2008;**294**:E28–E35.
55. Liu Z, Tanabe K, Bernal-Mizrachi E, Permutt MA. Mice with beta cell overexpression of glycogen synthase kinase-3beta have reduced beta cell mass and proliferation. *Diabetologia* 2008;**51**:623–631.
56. Patel S, Doble BW, MacAulay K, Sinclair EM, Drucker DJ, Woodgett JR. Tissue-specific role of glycogen synthase kinase 3beta in glucose homeostasis and insulin action. *Mol Cell Biol* 2008;**28**:6314–6328.
57. Liu Y, Tanabe K, Baronnier D, Patel S, Woodgett J, Cras-Méneur C, Permutt MA. Conditional ablation of Gsk-3beta in islet beta cells results in expanded mass and resistance to fat feeding-induced diabetes in mice. *Diabetologia* 2010;**53**:2600–2610.
58. Gupte M, Tumuluru S, Sui JY, Singh AP, Umbarkar P, Parikh SS, Ahmad F, Zhang Q, Force T, Lal H. Cardiomyocyte-specific deletion of GSK-3beta leads to cardiac dysfunction in a diet induced obesity model. *Int J Cardiol* 2018;**259**:145–152.
59. Woodgett JR. Judging a protein by more than its name: GSK-3. *Sci STKE* 2001;**2001**: re12.
60. MacAulay K, Woodgett JR. Targeting glycogen synthase kinase-3 (GSK-3) in the treatment of Type 2 diabetes. *Expert Opin Ther Targets* 2008;**12**:1265–1274.
61. Woodgett JR. Physiological roles of glycogen synthase kinase-3: potential as a therapeutic target for diabetes and other disorders. *Curr Drug Targets Immune Endocr Metabol Disord* 2003;**3**:281–290.
62. Kassiotis C, Rajabi M, Taegtmeyer H. Metabolic reserve of the heart: the forgotten link between contraction and coronary flow. *Prog Cardiovasc Dis* 2008;**51**:74–88.
63. Pederson BA, Chen H, Schroeder JM, Shou W, DePaoli-Roach AA, Roach PJ. Abnormal cardiac development in the absence of heart glycogen. *Mol Cell Biol* 2004; **24**:7179–7187.
64. Guo Y, Gupte M, Umbarkar P, Singh AP, Sui JY, Force T, Lal H. Entanglement of GSK-3beta, beta-catenin and TGF-beta1 signaling network to regulate myocardial fibrosis. *J Mol Cell Cardiol* 2017;**110**:109–120.
65. Zhou J, Ahmad F, Parikh S, Hoffman NE, Rajan S, Verma VK, Song J, Yuan A, Shanmughapriya S, Guo Y, Gao E, Koch W, Woodgett JR, Madesh M, Kishore R, Lal H, Force T. Loss of adult cardiac myocyte GSK-3 leads to mitotic catastrophe resulting in fatal dilated cardiomyopathy. *Circ Res* 2016;**118**:1208–1222.
66. Lal H, Ahmad F, Zhou J, Yu JE, Vagnozzi RJ, Guo Y, Yu D, Tsai EJ, Woodgett J, Gao E, Force T. Cardiac fibroblast glycogen synthase kinase-3beta regulates ventricular remodeling and dysfunction in ischemic heart. *Circulation* 2014;**130**:419–430.
67. Lal H, Zhou J, Ahmad F, Zaka R, Vagnozzi RJ, Decaul M, Woodgett J, Gao E, Force T. Glycogen synthase kinase-3alpha limits ischemic injury, cardiac rupture, post-myocardial infarction remodeling and death. *Circulation* 2012;**125**:65–75.
68. Zhou J, Lal H, Chen X, Shang X, Song J, Li Y, Kerkela R, Doble BW, MacAulay K, DeCaul M, Koch WJ, Farber J, Woodgett J, Gao E, Force T. GSK-3alpha directly regulates beta-adrenergic signaling and the response of the heart to hemodynamic stress in mice. *J Clin Invest* 2010;**120**:2280–2291.
69. Hirotani S, Zhai P, Tomita H, Galeotti J, Marquez JP, Gao S, Hong C, Yatani A, Avila J, Sadoshima J. Inhibition of glycogen synthase kinase 3beta during heart failure is protective. *Circ Res* 2007;**101**:1164–1174.
70. Zhou J, Freeman TA, Ahmad F, Shang X, Mangano E, Gao E, Farber J, Wang Y, Ma XL, Woodgett J, Vagnozzi RJ, Lal H, Force T. GSK-3alpha is a central regulator of age-related pathologies in mice. *J Clin Invest* 2013;**123**:1821–1832.
71. Tseng AS, Engel FB, Keating MT. The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. *Chem Biol* 2006;**13**:957–963.
72. Uosaki H, Magadum A, Seo K, Fukushima H, Takeuchi A, Nakagawa Y, Moyes KW, Narazaki G, Kuwahara K, Laflamme M, Matsuoka S, Nakatsuji N, Nakao K, Kwon C, Kass DA, Engel FB, Yamashita JK. Identification of chemicals inducing cardiomyocyte proliferation in developmental stage-specific manner with pluripotent stem cells. *Circ Cardiovasc Genet* 2013;**6**:624–633.
73. Wang S, Ye L, Li M, Liu J, Jiang C, Hong H, Zhu H, Sun Y. GSK-3beta inhibitor CHIR-99021 promotes proliferation through upregulating beta-catenin in neonatal atrial human cardiomyocytes. *J Cardiovasc Pharmacol* 2016;**68**:425–432.
74. Fang R, Qiao S, Liu Y, Meng Q, Chen X, Song B, Hou X, Tian W. Sustained co-delivery of BIO and IGF-1 by a novel hybrid hydrogel system to stimulate endogenous cardiac repair in myocardial infarcted rat hearts. *Int J Nanomed* 2015;**10**: 4691–4703.
75. Xu JY, Lee YK, Ran X, Liao SY, Yang J, Au KW, Lai WH, Esteban MA, Tse HF. Generation of induced cardiospheres via reprogramming of skin fibroblasts for myocardial regeneration. *Stem Cells* 2016;**34**:2693–2706.
76. Kim YS, Jeong HY, Kim AR, Kim WH, Cho H, Um J, Seo Y, Kang WS, Jin SW, Kim MC, Kim YC, Jung DW, Williams DR, Ahn Y. Natural product derivative BIO promotes recovery after myocardial infarction via unique modulation of the cardiac microenvironment. *Sci Rep* 2016;**6**:30726.
77. Kerkela R, Kockeritz L, Macaulay K, Zhou J, Doble BW, Beahm C, Greytak S, Woulfe K, Trivedi CM, Woodgett JR, Epstein JA, Force T, Huggins GS. Deletion of GSK-3beta in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. *J Clin Invest* 2008;**118**:3609–3618.
78. Cho J, Zhai P, Maejima Y, Sadoshima J. Myocardial injection with GSK-3beta-overexpressing bone marrow-derived mesenchymal stem cells attenuates cardiac dysfunction after myocardial infarction. *Circ Res* 2011;**108**:478–489.
79. Buikema JW, Zwetsloot PP, Doevendans PA, Sluijter JP, Domian IJ. Expanding mouse ventricular cardiomyocytes through GSK-3 inhibition. *Curr Protoc Cell Biol* 2013;**61**: 23.9.1–23.9.10.
80. Ruel L, Bourouis M, Heitzler P, Pantesco V, Simpson P. Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. *Nature* 1993;**362**:557–560.
81. Siegfried E, Chou TB, Perrimon N. wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. *Cell* 1992;**71**:1167–1179.
82. Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev Cell* 2007;**12**:957–971.
83. Force T, Woodgett JR. Unique and overlapping functions of GSK-3 isoforms in cell differentiation and proliferation and cardiovascular development. *J Biol Chem* 2009; **284**:9643–9647.
84. Schade B, Lesurf R, Sanguin-Gendreau V, Bui T, Deblois G, O'Toole SA, Millar EK, Zardawi SJ, Lopez-Knowles E, Sutherland RL, Giguere V, Kahn M, Hallett M, Muller WJ. β-Catenin signaling is a critical event in ErbB2-mediated mammary tumor progression. *Cancer Res* 2013;**73**:4474–4487.
85. Wen Y, Planel E, Herman M, Figueroa HY, Wang L, Liu L, Lau LF, Yu WH, Duff KE. Interplay between cyclin-dependent kinase 5 and glycogen synthase kinase 3 beta mediated by neuregulin signaling leads to differential effects on tau phosphorylation and amyloid precursor protein processing. *J Neurosci* 2008;**28**:2624–2632.
86. Cahill TJ, Choudhury RP, Riley PR. Heart regeneration and repair after myocardial infarction: translational opportunities for novel therapeutics. *Nat Rev Drug Discov* 2017; **16**:699–717.
87. Cai CL, Molkentin JD. The elusive progenitor cell in cardiac regeneration: slip Slidin' away. *Circ Res* 2017;**120**:400–406.
88. Zhu K, Wu Q, Ni C, Zhang P, Zhong Z, Wu Y, Wang Y, Xu Y, Kong M, Cheng H, Tao Z, Yang Q, Liang H, Jiang Y, Li Q, Zhao J, Huang J, Zhang F, Chen Q, Li Y, Chen J, Zhu W, Yu H, Zhang J, Yang HT, Hu X, Wang J. Lack of remuscularization following transplantation of human embryonic stem cell-derived cardiovascular progenitor cells in infarcted nonhuman primates. *Circ Res* 2018;**122**:958–969.
89. Liao SY, Liu Y, Siu CW, Zhang Y, Lai WH, Au KW, Lee YK, Chan YC, Yip PM, Wu EX, Wu Y, Lau CP, Li RA, Tse HF. Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium. *Heart Rhythm* 2010; **7**:1852–1859.