Turning back the clock: A concise viewpoint of cardiomyocyte cell cycle activation for myocardial regeneration and repair

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Abstract

Patients with acute myocardial infarction (MI) could progress to end-stage congestive heart failure, which is one of the most significant problems in public health. From the molecular and cellular perspective, heart failure often results from the loss of cardiomyocytes—the fundamental contractile unit of the heart—and the damage caused by myocardial injury in adult mammals cannot be repaired, in part because mammalian cardiomyocytes undergo cell-cycle arrest during the early perinatal period. However, recent studies in the hearts of neonatal small and large mammals suggest that the onset of cardiomyocyte cell-cycle arrest can be reversed, which may lead to the development of entirely new strategies for the treatment of heart failure. In this Viewpoint, we summarize these and other provocative findings about the cellular and molecular mechanisms that regulate cardiomyocyte proliferation and how they may be targeted to turn back the clock of cardiomyocyte cell-cycle arrest and improve recovery from cardiac injury and disease.

1. Introduction

Despite improvements in the effectiveness of conventional treatment regimens for heart disease, such as catheterization and maximal medical therapy, some patients with acute myocardial infarction (AMI) progress to end-stage congestive heart failure, which remains one of the most significant problems in public health [1,2]. At the most fundamental level, heart failure is caused by the loss of cardiomyocytes—the contractile units of the heart—and because mammalian cardiomyocytes exit the cell cycle shortly after birth, myocardial tissues damaged by injury or disease cannot be adequately replaced via

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Conflict of interest

The authors declared that they have no conflict of interest.
the proliferation of endogenous cardiomyocytes. Thus, although a number of promising strategies (e.g., cell- and tissue-based therapies, the transdifferentiation of non-myocytes into cardiomyocyte-like cells) for promoting cardiac repair are currently being developed, emerging evidence that proliferation can be re-activated in the cardiomyocytes of postnatal mammals [3-5], which launches an entirely new strategy for the treatment of cardiac disease. This Viewpoint summarizes these recent provocative findings and their implications for the remuscularization of infarcted hearts (Fig. 1).

2. The onset of cardiomyocyte cell cycle arrest in late fetal and early neonatal mammals

Cardiomyocytes in mammalian hearts rapidly divide during the weeks before birth but begin to transition from hyperplastic growth (i.e., increases in cardiomyocyte number) to hypertrophic growth (increasing cardiomyocyte size) between the late fetal and very early neonatal periods. Single-nucleated cardiomyocytes predominate during hyperplasia; then, the cells undergo one final phase of DNA synthesis without subsequent cytokinesis, producing hypertrophic cardiomyocytes with multiple nuclei in non-primate species or single nuclei with polyploid DNA in primates [6-8]. Mean cardiomyocyte cell volume increases from 1000 to 1500 μm$^3$ on postnatal day (P) 1 to 25,000–35,000 μm$^3$ in adult animals [9-15], and although mononucleated fetal and early neonatal cardiomyocytes typically lack components of the M-line, T-tubules, sarcoplasmic reticulum, and the extracellular interstitial matrix, immunohistochemical studies have identified precursors for these structural proteins [16,17] that begin to mature as the cells enter the hypertrophic phase.

The timing of the hyperplastic/hypertrophic transition varies widely among species, beginning at P10–12 in dogs [11], P4–7 in rodents [10,12], and during the last stages of fetal growth in pigs [18], sheep [13-15], and primates [19]. Notably, when embryonic day 12 rat hearts were implanted on the irises of adult rats [20], the cardiomyocytes continued to replicate with no change in myocyte diameter or nuclear/cytoplasmic ratio from 1 to 3 weeks after implantation, binucleated cells appeared by week 3, and the amount of cytoplasm per nucleus increased by four-fold between weeks 3 and 5.

The hyperplastic/hypertrophic transition is also accompanied by a series of changes in subcellular structure that likely need to be reversed for cardiomyocytes to re-enter the cell cycle. For example, nuclear division is driven by the centrosomes, which are no longer present after the transition to hypertrophic growth, and mitosis in early neonatal cardiomyocytes requires disassembly of the contractile apparatus, which progresses (in order) via the dissociation of proteins in the Z-band (sarcomeric α-actinin, titin, and cardiac α-actin), the A-band (myosin and actin), and then the M-band (myomesin) [16]. Furthermore, the tubular structures of the mitotic spindle relocate to the nuclear envelope as cardiomyocytes become hypertrophic [21,22], and the intercalated discs enlarge and migrate to the ends of the cell, while sarcomeres are brought into alignment by the formation of Z-Z connections. Hypertrophic cardiomyocytes are also stabilized by the growth of T-tubules and the sarcoplasmic reticulum, and the dense network of collagen and elastic fibrils that develop
in the interstitial space during the early hypertrophic growth phase [23] not only contributes to cardiomyocyte stability, but also participates in cell cycle inhibition by activating the Hippo-yes-associated-protein (Hippo-YAP) pathway [13], which impedes cardiomyocyte proliferation in adult mammals [24].

3. Endogenous cardiac regenerative capacity in mammals

In contrast to lower vertebrates [25-31], cardiomyogenesis in adult mammalian heart following injury is very limited [32-36] and is insufficient to restore normal cardiac function. Studies in the late 90s elegantly mapped the DNA synthesis and cell cycle dynamics of the mammalian heart during development and following birth [9,12,37], where they showed that DNA synthesis drops significantly around birth with low level of DNA synthesis few days after birth. Recent reports examined the cardiac regeneration potential in large mammal neonatal hearts. The results from these studies demonstrated that the neonatal porcine heart is capable or regeneration following AMI only for the first 2 days of life. This phenomenon is associated with induction of cardiomyocyte proliferation, and is lost when cardiomyocytes exit cell cycle shortly after birth. The findings of this study and the recent case report from a clinical observation of one neonatal patient [40] suggest a significant myocardial regeneration window in the early postnatal stage of large mammalian hearts, which is highly impactful, as it provides insight into the regenerative properties and potential of human hearts.

3.1. Impactful of cell cycle on heart failure management

Anecdotal reports over the past several decades suggest that newborn humans can recover left ventricular function following various degrees of myocardial infarction [41-43]. Therefore, identifying a regenerative window in mammals is a crucial step towards understanding the human heart regenerative window, and may serve as a platform for future clinical studies in human infants afflicted by the devastating heart conditions. This can have numerous important implications, from establishing new guidelines for timing of pediatric heart surgery that benefit from the regenerative potential of the newborn human heart, to designing novel surgical techniques for pediatric heart surgery, and developing new therapeutic modalities to enhance or prolong this regenerative window. Importantly, once we have a better understanding of the regulators underpinning the drastic changes in neonatal cardiac cardiomyocyte proliferation during the first week of life, we may be able to manipulate the mechanisms to promote myocardial regeneration in injured hearts not only in pediatric but also in adult patients.

3.2. Regenerative capacity of neonatal mouse hearts

In 2011, Sadek lab published the first report of spontaneous heart regeneration in small mammals [3], where apical resection (AR) of 15% of the left ventricle shortly after birth resulted in induction of cardiomyocyte proliferation with regeneration of the lost myocardium within 21 days. This remarkable regenerative response however is lost by 7 days postnatally, coinciding with cell cycle exit of cardiomyocytes [3]. Sadek lab has previously outlined the regenerative capacity of the newborn mammalian heart and defined several mechanisms that regulate this process [3,44-48]. Specifically, it has been
demonstrated that the endogenous regenerative capacity of the newborn mouse heart is mediated by proliferation of preexisting cardiomyocytes and is lost when cardiomyocytes exit cell cycle within 7 days after birth. In addition, recent reports indicate that the slow turnover of cardiomyocytes (~0.76% per year) that occurs in the adult mouse heart is mediated by proliferation of preexisting cardiomyocytes [49]. Therefore, current evidence suggests that both the regenerative ability of the early postnatal heart, and cardiomyocyte turnover in the adult heart are mediated by proliferative competency of pre-existing cardiomyocytes. However, mechanisms of regulation of mammalian cardiomyocyte cell cycle arrest shortly after birth remain poorly understood.

3.3. Oxygen metabolism and heart regeneration in mice

A unique character shared by organisms that are capable of heart regeneration is the low oxygenation state [50,51]. The zebrafish circulatory system is relatively hypoxemic, as it has a primitive two-chambers heart, which results in mixing of arterial and venous blood. Similarly, the mammalian fetal circulation is shunt-dependent with significant mixing of arterial and venous blood. Although blood in the umbilical vein going to the fetus is 80%-90% saturated with a PaO$_2$ of 32-35 mmHg, the saturation of the blood ejected from the left ventricle is only 65% saturated with a PaO$_2$ of 25-28 mmHg [52], which is quite hypoxemic compared to the postnatal circulation with a saturation above 95% and a PaO$_2$ of 100 mmHg. Therefore, both zebrafish and mammalian fetal hearts reside in relatively hypoxic environments. However, the transition from embryonic- to postnatal-circulation soon after birth drastically changes the oxygenation state of mammalian cardiomyocytes [53]. In parallel to the oxygenation state, energy metabolism of the embryonic and adult hearts is quite distinct. During embryonic development, when cardiomyocytes rapidly proliferate, the relatively hypoxic embryonic heart utilizes anaerobic glycolysis as a main source of energy [54,55], whereas adult cardiomyocytes utilize the oxygen-dependent mitochondrial oxidative phosphorylation as an energy source [56,57]. Given these initial observations, future studies are warranted to examine whether environmental oxygen changes can metabolically reprogram adult cardiomyocytes, and modulate DNA damage and cell cycle activity [48,58].

3.4. Regenerative capacity of neonatal large mammalian hearts

Myocardial regeneration has also been observed in neonatal large mammals (pigs) after experimentally induced MI [4,5] (Fig. 2). When MI was induced on P1, cardiac contractile function on Day 30 was largely restored with little evidence of scar formation or wall-thinning, and the proportion of cardiomyocytes that expressed markers for cell cycle activity or proliferation (e.g., Ki67, phosphorylated histone 3 [PH3], Aurora B) on P7 was significantly greater in the hearts of P1-injured animals than in noninfarcted hearts. Cardiomyocytes also proliferated in response to MI induction on P2, but not enough to preserve normal cardiac function, and the regenerative response to MI induction at later time points was minimal, which suggests that the time window for myocardial regeneration in large mammals normally closes less than three days after birth. However, pigs that underwent AR surgery on P1 (AR$_{P1}$) recovered completely from MI-induction surgery on P28 (MI$_{P28}$) with no decline in contractile performance and no residual infarcted tissue at the site of MI induction [59], and whereas the expression of proliferation markers declined to nearly undetectable levels by P28 and P56 in the hearts of animals that did not undergo
ARP1 or MI, which is consistent with the postnatal onset of cell cycle arrest, markers for proliferation were ~10-fold greater in the hearts of age-matched animals that underwent both procedures. Thus, AR surgery on P1 appeared to prolong the window for myocardial regeneration by preserving the cell cycle machinery in cardiomyocytes, which enabled the cells to proliferate in response to MI on P28 [59]. Collectively, the results from these studies [3-5,59,60] suggest that strategies for enhancing cardiomyocyte cell cycle re-activation could substantially remuscularize infarcted hearts.

4. Promoting cardiomyocyte cell cycle activity and proliferation in the hearts of adult mammals

The meager amount of endogenous cardiomyocyte turnover in the hearts of adult mammals appears to be driven primarily by the proliferation of pre-existing cardiomyocytes, rather than via the differentiation of progenitor cells or other mechanisms [49], which indicates that adult cardiomyocytes at least occasionally re-enter the cell cycle. Cell cycle progression is governed by checkpoints at the G1/S and G2/M phase transitions, and analyses of the transcriptomes of hearts harvested from fetal (embryonic day 10.5), newborn (P1), and adult (8-week-old) mice identified 15 differentially expressed genes [61], including cyclins B1 and D1 (CycB1 and CycD1), as well as cyclin-dependent kinases 1 and 4 (Cdk1 and Cdk4), which form complexes with CycB1 and CycD1, respectively, to regulate the G1/S (Cdk4/CycD1) and G2/M (Cdk1/CycB1) phase transitions [62-64]. Simultaneous overexpression of all four factors promoted proliferation in cardiomyocytes obtained from mice at P7 and from adult (4-month-old) rats, as well as in post-mitotic [65] (60-day-old) cardiomyocytes that had been differentiated from human induced pluripotent stem cells (hiPSCs). When adenoviruses coding for all four factors were injected into the hearts of mice one week after MI induction, the treatment was associated with significant improvements in cardiac function and a 50% reduction in scar size [61]. Furthermore, constitutive overexpression of cyclin D1 (CycD1), D2 (CycD2), or D3 (CycD3) from the cardiomyocyte-specific myosin heavy chain (MHC) promoter activates ventricular DNA synthesis in the uninjured hearts of adult mice [66-70]. Although myocardial injury attenuated ventricular DNA synthesis in MHC-CycD1 and -CycD3 transgenic mice, MHC-CycD2 expression both increased DNA synthesis and reduced fibrosis in infarcted mouse hearts [67]. MHC-CycD2 transgenic mice also displayed high levels of left-atrial DNA synthesis and cytokinesis after isoproterenol infusion, and whereas cyclin D1 and D3 tended to accumulate in the cytosol of cardiomyocytes after myocardial injury, the distribution of CycD2 in nuclei was unchanged. Collectively, these observations confirm that the D-type cyclins are not functionally redundant in injured hearts, and that CycD2 overexpression promotes myocardial repair.

Recent studies have identified a number of molecules and pathways, e.g., Erb-B2 [71,72], the calcineurin–Hoxb13 axis [73], and hypoxic signaling [48] that regulate the conversion from hyperplastic to hypertrophic growth and, consequently, may be viable targets for reactivating the cardiomyocyte cell cycle in adult hearts. Genetic knockdown of the Hippo pathway gene Salvador (Sav) in border-zone cardiomyocytes appeared to promote cell division and improve measures of cardiac function and infarct size in a pig MI model [24,74-76]; however, proliferating cardiomyocytes remained rare (<5%), which suggests that
other mechanisms, such as declines in cardiomyocyte apoptosis or increases in vascularity, may have contributed to the benefits associated with Hippo-YAP inactivation [24]. Using the reprogramming factors Oct4, Sox2, Klf4, and c-Myc (OSKM), Chen et al. demonstrated the overexpression of OSKM in cardiomyocytes can reactivate CMs cell cycle, which in turn, is accompanied by significant myocardial regeneration and improved cardiac function in a mouse model of MI [77]. Gene activity can also be knocked down via the administration of microRNAs (miRNAs or miRs)—highly conserved small non-coding RNAs that regulate gene expression by binding to partially complementary sequences in messenger RNAs. Early studies indicated that miR-1, −133, and −15 [78-80] inhibited cardiomyocyte proliferation, and the cardiac-specific deletion of miR-128 in mice promoted cardiomyocyte cell cycle re-entry and improved measures of fibrosis and cardiac function after MI [81]. Furthermore, the miR-17-92 cluster appears to be both necessary and sufficient for inducing cardiomyocyte proliferation in embryonic and postnatal hearts [82], and high-throughput screening of 875 miRNA mimics identified 204 miRNAs that increased the proliferation of neonatal rat cardiomyocytes by at least two-fold. Two of the miRNAs (miR-590 and miR-199a) also promoted cardiomyocyte proliferation and myocardial regeneration when being administered to the infarcted hearts of adult mice [83].

5. Targeting the cardiomyocyte cell cycle to enhance the regenerative potency of cell-based myocardial therapy

Numerous studies in rodents, pigs, and nonhuman primates have shown that cells differentiated from hiPSCs can improve recovery from myocardial injury [84-87]. However, the proportion of transplanted cells that are retained and survive at the site of administration (i.e., the engraftment rate) is exceedingly small [88,89], which suggests that the beneficial effects evolve primarily via paracrine mechanisms that are activated by the transplanted cells [90,91], rather than via the replacement of myocardial scar tissue with functional cardiac muscle [92]. Thus, observations that MHC-CycD2 overexpression promoted cardiomyocyte proliferation and reduced infarct sizes in a mouse MI model [67] led researchers to transfect hiPSCs with a lentivirus coding for the MHC-driven expression of human CycD2 (CCND2) before differentiating the cells into cardiomyocytes (CCND2^OE^hCMs) [93]. The CCND2^OE^hCMs were more proliferative than cardiomyocytes differentiated from hiPSCs with wild-type levels of CCND2 expression (CCND2^WT^hCMs), and engrafted CCND2^OE^hCMs were three-fold more prevalent than CCND2^WT^hCMs four weeks after administration to infarcted mouse hearts. The number of engrafted CCND2^OE^hCMs also increased from one to four weeks after administration, which suggests that even an initially small number of engrafted CCND2^OE^hCMs could proliferate and substantially remuscularize the myocardial scar.

Measures of cardiac function and infarct size were also significantly better in mice treated with CCND2^OE^hCMs than in CCND2^WT^hCM-treated animals at Week 4 [93], and optical mapping assessments conducted six months after cell administration, when engrafted CCND2^OE^hCMs occupied more than 50% of the myocardial scar and exceeded the number of engrafted CCND2^WT^hCMs by 8-fold [94], confirmed that the engrafted cells were electrically coupled with each other and the native myocardium. AP durations were ~4-fold
longer (80–160 ms) for the engrafted (human) CCND2OEhCMs than for native (mouse) cardiomyocytes, which is consistent with previous reports [95], and when paced at 70 ms, the upstroke for each of the longer human cardiomyocyte AP traces was followed by two or three small spikes, which corresponded to the upstrokes of the shorter mouse cardiomyocyte AP traces [94]. Thus, the engrafted CCND2OEhCMs may contributed to the improvements in cardiac function by direct electromechanical coupling, though the enhanced engraftment may also have led to an increase in the cells’ paracrine activity.

Combined treatment with fibroblast growth factor 1 (FGF1) and CHIR99021 (a pharmacological inhibitor of glycogen synthase kinase 3) promoted the cell cycle activity and proliferation of hiPSC-derived cardiomyocytes (hiPSC-CMs) both in culture and when nanoparticles carrying FGF1 and CHIR99021 were incorporated with hiPSC-CMs into a human cardiac-muscle patch (hCMP). CHIR99021- and FGF1-containing nanoparticles also promoted hiPSC-CM cell cycle activity and proliferation four weeks after hCMP administration to infarcted mouse hearts, and measurements of cardiac performance, infarct size, and angiogenesis were significantly better in animals treated with the FGF1/CHIR99021-containing hCMP than in animals treated with hCMPs containing empty nanoparticles [96]. Notably, because FGF1 and CHIR99021 are chemical agents, this method for promoting hiPSC-CM proliferation may be more easily translated to the clinic than genetic approaches such as MHC-CCND2 overexpression or therapeutic genome editing with techniques such as clustered regulatory interspaced short palindromic repeats (CRISPR) [97]. CRISPR technology may also be less effective for cardiac applications in adults, because homology-directed repair tends to be inefficient in nonproliferating cells such as cardiomyocytes [97,98].

6. Future directions and clinical implications

New approaches for heart remuscularization can be stratified according the mechanistic targets and time frame. The first strategy entails the use of pluripotent (PSC) derived cardiac cells aiming at repopulating the heart with new muscle cells that could engraft and improve its contractile function. Enhancing the maturity of PSC-derived cardiomyocytes before transplantation and gene-editing PSC lines to make them immune-evasive would be critical. The recognition that the molecular and cellular basis for progressive heart failure is the result of the inability of damaged and apoptotic cardiomyocytes to be replaced, the second strategy entails approaches targeting at a more direct remuscularization of the injured left ventricle by “turning back the clock” of cardiomyocyte cell cycle.

Because hiPSCs can be reprogrammed from the cells of each individual patient, the proportion of autologous hiPSC-CMs that are rejected by the patient’s immune system is likely to be minimal. However, the time required to reprogram and differentiate the cells precludes their use for treatment of acute conditions (e.g., AMI); thus, researchers have attempted to generate a line of “universal donor” hiPSCs by manipulating the expression of human leukocyte antigens and other immunomodulatory factors [99]. Furthermore, one of the primary concerns associated with cardiomyocyte transplantation is whether the cells adequately couple with the endogenous myocardial tissue to prevent arrhythmia. Strategies based on the proliferation of endogenous cardiomyocytes may partially alleviate...
this concern, at least in theory, because coupling seems likely to be more extensive between daughter cardiomyocytes generated via the division of a parent cell than between endogenous and transplanted cardiomyocytes. However, although viral-mediated miR-199a overexpression promoted myocardial recovery and regeneration in infarcted pig hearts, most of the animals subsequently died of arrhythmia, perhaps because persistent and uncontrolled overexpression of miR-199a led to the appearance of proliferating cells with an incomplete cardiomyocyte phenotype [100]. Whether arrhythmogenic complications are specifically associated with miR-199a, or could be mitigated by reducing the magnitude of miR-199a overexpression, has yet to be determined. Nevertheless, these observations demonstrate that the duration of therapeutically induced cardiomyocyte proliferation must be tightly controlled, and that techniques for improving the development and maturation of newly generated cardiomyocytes may be necessary to ensure patient safety.

Viral-mediated gene delivery is perhaps the most straightforward method for manipulating the expression of molecules that regulate cardiomyocyte proliferation, but it is also associated with concerns of genomic integration, so mRNA transfection is generally considered a safer alternative. However, mRNA is both unstable, because it is cleaved by RNase, and potentially immunogenic, because it can be recognized by Toll-like receptors (TLR). Thus, studies with synthetically modified RNA (modRNA), in which the uridine residues are replaced by pseudouridine, are becoming increasingly common, because modRNA remains transcriptionally active and resists both RNase degradation and TLR recognition [101]. Notably, the cardiomyocyte-specific delivery of modRNA coding for the glycolytic enzyme pyruvate kinase muscle isoenzyme 2 (Pkm2) appeared to activate the cell cycle in cardiomyocytes by interacting with β-catenin, which subsequently induced CycD1 and C-Myc expression, and by upregulating the pentose phosphate pathway, which reduced the production of reactive oxygen species and DNA-damage–induced cell cycle arrest [102]. Taken together, data from these preclinical studies suggested that reactivation of cardiomyocyte cell cycle via gene therapy or pharmacological treatment is promising for myocardial repair post injuries. Developing new cardiac specific drug/gene delivery system is warranted in the future to enhance the efficacy and reduce the off-target effects of these treatments.

7. Summary

The proliferative capacity of cardiomyocytes in adult mammalian hearts is far too low to replace the cells lost to cardiac injury or disease. However, a series of recent studies have shown that cell cycle activity persists in cardiomyocytes for a short period after birth, and that the time window for cardiac regeneration increases when myocardial injury occurs on P1. Collectively, these observations suggest that adult cardiomyocytes may retain some latent proliferative capacity that could be re-activated to promote the growth of endogenous contractile tissue. Ongoing investigations of the mechanisms that drive the injury-induced preservation of cardiomyocyte cell cycle activity could identify new therapeutic targets for improving the effectiveness of regenerative myocardial therapy.
Funding

This work is supported by NIH grants R01s HL114120, HL131017, HL149137, U01HL134763, T32 EB023872; and R01HL142627, AHA TPA Award 20TPA35490001 to WZ.

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Fig. 1. Approaches to remuscularize the myocardial infarct.
1) The left ventricular (LV) injuries such as apical resection on postnatal day 1 (P1) mammals result in activation of CM cell cycle; 2) using small molecules to target CM cell cycle regulators for promoting cell proliferation; and 3) Using MHC-driven CCND2 overexpression to promote proliferation of engrafted CMs.
Fig. 2. Left ventricular (LV) Injury on postnatal day 1 (P1) pig heart prolongs the window for CMs cell-cycle, which in turn, results in remuscularization of LV infarcts. Apical resection performed on P1 piglet results in disruption of the cardiomyocytes exit cell cycle, which in turn, enables the remuscularization of acute myocardial infarction secondary to left anterior descending artery (LAD) ligation on P28.