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Cardiac regeneration based on mechanisms of cardiomyocyte proliferation and differentiation

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Abstract

Cardiomyocyte proliferation and progenitor differentiation are endogenous mechanisms of myocardial development. Cardiomyocytes continue to proliferate in mammals for part of postnatal development. In adult mammals under homeostatic conditions, cardiomyocytes proliferate at an extremely low rate. Because the mechanisms of cardiomyocyte generation provide potential targets for stimulating myocardial regeneration, a deep understanding is required for developing such strategies. We will discuss approaches for examining cardiomyocyte regeneration, review the specific advantages, challenges, controversies, and recommend approaches for interpretation of results. We will also draw parallels between developmental and regenerative principles of these mechanisms and how they could be targeted for treating heart failure.

Introduction

Cardiomyopathies constitute a group of heart muscle diseases of varied etiology that are treatable with medical therapies, but cardiac function generally declines despite therapy. Most cardiomyopathies at the stage of heart failure exhibit cardiomyocyte loss. Adult mammals do not sufficiently regenerate cardiomyocytes to compensate for lost cardiomyocytes. Cardiac transplantation is the only proven method to replace lost cardiomyocytes. In light of insufficient numbers of donor organs, stimulating myocardial regeneration offers a potential strategy for treating heart failure.

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In contrast to adult mammals, amphibians, reptiles, and zebrafish regenerate cardiomyocytes after myocardial injury (1, 2). The research community has directed attention to two principal strategies to regenerate myocardium: Use of stem and progenitor cells to repair damaged myocardium and enhancement of endogenous regenerative mechanisms (3). This review focuses on endogenous regeneration mechanisms by cardiomyocyte proliferation.

Historical and current perspectives in cardiac regeneration

Anecdotal reports over the past 100 years have shown mitoses of cardiomyocyte nuclei, however, evidence for cardiomyocyte division remained elusive. The first systematic and quantitative examinations of the cellular mechanisms of human heart growth were published in the 1950s (4, 5). One study examined the increase of cardiomyocyte cross-sectional area in the left ventricular papillary muscle in humans and concluded that cardiomyocyte enlargement could fully account for physiologic myocardial growth between birth and adulthood (4, 5). Because examining orthogonally sectioned papillary muscles is associated with biases, the validity of extrapolating these results to the entire heart is limited. Still, historically, the mammalian heart has been viewed as a post-mitotic organ in which the primary parenchymal cells, cardiomyocytes, do not increase in number after birth (6).(4)

Additional classical studies of cardiomyocyte proliferation used microscopy to visualize mitotic figures. However, these approaches probably did not have the precision necessary for definitive visualization of cardiomyocyte cytokinesis nor the throughput for quantifying rare events. Although not conclusive, these studies led to two paradigms: First, unlike skeletal muscle, the adult heart does not have progenitors supporting the generation of new cardiomyocytes. The second paradigm argued that there is a single cellular mechanism of post-natal developmental and pathological heart growth: cardiomyocyte enlargement (7, 8). In the latter-20th century, technical advances, including use of confocal microscopy to visualize cell cycle events, automated analyses of large cell populations, and genetic and metabolic labeling for cellular fate mapping, have provided new data. This has advanced a new cellular paradigm, which includes adult cardiomyocyte renewal, while incorporating the significant role that cardiomyocyte enlargement plays in physiologic and pathologic heart growth.

The recognition of endogenous cardiomyocytes as a source for new cardiomyocytes raises the possibility of stimulating this process for myocardial repair. By understanding the mechanisms limiting the regenerative response of mammalian myocardium, therapeutic strategies could be developed. To realize this therapeutic potential, the scientific community needs to address questions involving the precise cellular source for new cardiomyocytes, the regulating signaling pathways, the underlying cellular mechanisms, and the genesis rates and dynamics.

Mechanisms of cardiomyocyte regeneration pose challenges for investigation

Cardiomyocyte generation in the adult mammalian heart is a slow process compared with the blood, skin, and the digestive system, which makes it difficult to characterize turnover

dynamics. *In situ* time lapse imaging of the beating mammalian heart at cellular resolution is technically challenging (9, 10). Myocardial biopsies from patients provide only a small amount of tissue for analysis. The cellular heterogeneity and close spatial packing of cells in the myocardium can obscure the identification of cardiomyocyte nuclei from neighboring non-cardiomyocytes (11, 12). Perhaps the most challenging aspect is that cardiomyocytes exhibit non-proliferative cell cycles that increase the DNA content without cell division. Despite these intrinsic biological features that complicate studying regeneration, several new methods have enabled the advance of our understanding of cardiomyocyte proliferation.

Classical and new methods have advanced cardiac regeneration research

Labeled thymidine and thymidine analogs are stably incorporated into the genome during DNA synthesis and can be used to record cell cycle events. The use of tritiated thymidine is limited by potential radioactive toxicity due to long-term administration and by lower spatial resolution of visualization by autoradiography, compared with confocal microscopy (11). The early manual analysis of autoradiographs of tritiated thymidine in isolated cardiomyocytes fits with recent studies almost 2 decades later (11, 13–15). Thymidine analogs such as bromo-deoxyuridine (BrdU) have also been administered for up to several weeks for detecting cardiomyocyte cell cycle activity. Though high concentrations of BrdU can affect cell proliferation and differentiation, adverse effects were not reported with long-term labeling (16, 17). Similar to *Soonpaa et al* with a tritium label, *Li et al*, using a single BrdU injection, observed progressive loss of cardiomyocyte-DNA synthesis in rats within ten days of birth (13, 15). However, it should be noted that using Ki67 as a marker, Walsh et al. detected cell cycle activity in cardiomyocyte nuclei in mice up to 21 days of age (18). Naqvi et al. detected BrdU uptake and markers of mitosis and cytokinesis in cardiomyocytes in 13–15 day-old mice (19).

In the adult heart, *Soonpaa et al* examined mice 2 hours after administration of tritiated thymidine (3 injections, 12-hour intervals) and determined a 0.0005% labeling frequency of cardiomyocytes expressing a transgenic marker from the α-MHC-promoter (11). More recently, the use of long term BrdU labeling demonstrated a low basal activity of cardiomyocyte proliferation though an order higher than previously reported (13, 17).

Antibodies raised against markers of the cell cycle can be used for visualization by microscopy (e.g. Ki67 for G1, S, G2 and M-phase, phospho-histone 3 for M-phase, Aurora B kinase for cytokinesis) (14, 17, 20, 21). Antibody-based detection has the advantage of broad applicability, but is potentially susceptible to false-positives and –negatives. These issues can be addressed with appropriate negative controls. It should be noted that Ki-67 is present in G1-, S-, G2-, and M-phase of the cell cycle. Thus, for mechanistic studies, Ki-67 assays should be combined with other, more specific cell cycle markers.

Automated systems provide an unbiased approach for sampling large numbers of cardiomyocytes, but so far, they require dispersed cells. Flow cytometry (FACS) and laser scanning cytometry (LSC) offer high throughput methods to detect and quantify antibody labeling of isolated cardiomyocytes or nuclei (17, 18, 21). Recently, we examined cardiomyocyte cycling in humans using the M-phase marker phospho-histone 3 and used

LSC for read-out. This demonstrated a decrease in cardiomyocyte proliferation between birth and 20 years of life in humans (21). We compared the LSC results with visual quantification of H3P-positive cardiomyocyte nuclei on stained cryosections, which reproduced the pattern of decreasing H3P-activity between birth and 20 years (21). LSC in humans and FACS in mice showed agreement in that the frequency of cardiomyocyte cell cycle activity decreases after birth (18, 21).

Carbon-14 birth dating is an approach devised by the Frisen group to monitor cellular turnover in tissue (22). This strategy makes use of the transient increase of carbon-14 (^{14}C) in the biosphere due to a period of above ground nuclear testing that ended in the 1960s. The carbon-14 was taken up by humans through the diet and incorporated into the genomic DNA, which can be used as a time-stamp to calculate the mean birth date of a stable cell population (22). The carbon-14 content in cardiomyocyte nuclear DNA is quantified using accelerator mass spectrometry (AMS, which has a lower detection limit of 10^{-21} moles). The mean age of cardiomyocytes is then compared with the age of the source individual. This indicated generation of new cardiomyocytes at a rate of ~1% after 25 years of age. The fidelity of this approach depends on the quality of cardiomyocyte nuclei isolated from post-mortem myocardium. AMS provides a tissue-average, which poses a challenge since in cardiomyocytes, S-phase can be connected with cell division and differentiation. Hence, the analysis relied on additional assumptions that had to be made for mathematical modeling of the data (22), including assumptions about potential changes of the number of cardiomyocytes after birth, rates of cardiomyocyte cell death, and the extent of non-proliferative cell cycles (multinucleation and ploidy) with age (23).

Fate mapping with genetic tags has been used to label distinct cell populations to determine the source of new cells with time or intervention. Multiple groups have used the α-MHC promoter driven Cre model crossed with reporter lines to assay the role of progenitor cells in the generation of new cardiomyocytes after injury (14, 17, 20). After tamoxifen induction, a majority of differentiated cardiomyocytes is labeled with a reporter such as green fluorescent protein (GFP) or LacZ (14, 24). Using this technique, we have demonstrated that during normal aging in mice, the percent of preexisting cardiomyocytes, indicated by the percentage of GFP-positive cardiomyocytes, remains unchanged (25). Influx of cardiomyocytes generated from undifferentiated progenitor cells should result in a decrease of the percentage of GFP-labeled (preexisting) cardiomyocytes. This “dilution” was the case after experimental myocardial infarction, leading to the conclusion that myocardial injury induced an influx of progenitor cell-derived cardiomyocytes (25). However, multiple other studies showed very little or no contribution of progenitor cells to generating cardiomyocytes in adult mammals (14, 17, 20, 24, 26). Considering one specific progenitor type, *Ellison et al* reported a 0.15% generation rate for c-kit progenitor-derived cardiomyocytes in a 4-week period of normal aging (20). This contrasts with results from using a knock-in of an inducible Cre into the c-kit gene locus (27). This more direct approach demonstrated that c-kit positive cells do not generate cardiomyocytes in mice after birth to a significant degree (27). In this context, it is important to note that c-kit positive cells isolated from neonatal mice can generate cardiomyocytes (28). Systematic molecular

characterization of myocardial c-kit cells and comparison with the cardiomyocyte lineage should help resolve this controversy.

Potential explanations of our discrepant results with the genetic α-MHC-MerCreMer/ZEG fate mapping techniques include cardiomyocyte toxicity due to high expression levels of Cre (29, 30). However, we did not observe these effects in the 14-day induction protocol with 5-hydroxytamoxifen (26, 29–31). A concern of genetic tags expressed under control of cell-specific promoters is the dependence on the fidelity of transcriptional activity. In addition, it may be possible that the labeled cell population is phenotypically heterogenous. This possibility must be considered when the genetic labeling technique does not label the entire cell population, as is the case with the α-MHCMerCreMer mice (32). Other genetic approaches such as retrospective clonal assays utilize random labeling of precursor cells and have been used to address lineage in early cardiac development (33, 34). This system selectively labels daughter cells of mononucleated cells in divergent fluorescent tags so that one cell is labeled with red and the other with green. Using two transgenic models for a cardiomyocyte specific source and a non-discriminate source, respectively, *Ali et al* demonstrated that very few new mononucleated cardiomyocytes are generated primarily from pre-existing cardiomyocytes (33). Genetic labeling remains one of the most powerful approaches to monitor cell population dynamics, if applied appropriately.

Stable isotopes can be used to mark biological molecules for monitoring cell cycle activity over long periods. Stable isotope-labeled biomolecules are integrated into cells without disrupting biological functions. The safety of stable isotope labels allows long-term labeling strategies to both characterize events over extended periods and record rare events. The read-out utilizes a high-resolution analytical imaging system called Multi-Isotope Imaging Mass Spectrometry (MIMS). The MIMS platform was developed by Dr. Claude Lechene at the National Resource for Imaging Mass Spectrometry as a quantitative approach to image biological processes (35). MIMS generates detailed quantitative images based on the isotope composition of the sample surface. The spatial resolution of MIMS is similar to electron microscopy and can be used for analyzing sub-cellular structures, including chromatin, cytoplasmic proteins, and organelles. Thymidine marked with nitrogen-15 (^{15}N, the rare stable isotope of the abundant nitrogen-14) can be introduced into cell culture media or animals and is incorporated into the genome to make an *in situ* record of DNA synthesis. We have used this approach for quantifying several successive cell divisions in the intestine (36). In *Senyo et al*, we employed this approach to study cardiomyocyte turnover (14).

Cardiomyocyte cell cycle activity decreases during post-natal development

After birth, most mammalian cardiomyocytes transition from proliferation to terminal differentiation. The terminally differentiated phenotype is commonly characterized as having no proliferative potential. Cardiomyocyte DNA-synthesis activity decreases during the first 1–2 week of life in rodents (13, 15). However, flow cytometry analysis has detected cardiomyocytes positive for the cell cycle marker Ki-67 in mice up to 2–3 weeks of age (18, 37). We have detected cardiomyocytes positive for the mitosis marker H3P in humans in the first 20 years of adult life, after which the activity was very low (21). In mice, we administered isotope-labeled thymidine for 8 weeks to increase our sensitivity of detecting

cardiomyocyte cell cycle activity. By MIMS analysis, we measured a 56% labeling index of cardiomyocytes with at least one cell cycle event in mice in the first 2 months of life. Using the same labeling protocol, adult mice exhibited a significant decrease of cardiomyocytes with history of cell cycle activity (0.84% over 2 months; 0.015%/day). Due to the 8 week-long duration of labeling, we could not determine the specific time pattern of cell cycle withdrawal during the neonatal period. In summary, the postnatal period during which cardiomyocyte cell cycle activity decreases is much longer in humans, as compared with mice and rats. All three species show extremely low cardiomyocyte cell cycle activity as adults.

Post-natal cardiomyocyte cell cycle activity may be associated with proliferation, resulting in a change of the number of cardiomyocytes. A study using biochemical quantification of cardiac DNA content reported an increase in cardiomyocyte number in humans after birth (38). Our direct stereologic quantifications of cardiomyocyte numbers in humans demonstrated an increase from 1.1 to 3.7 billion cardiomyocytes from birth to age 20 years (21). A recent study showed an increase of the number of cardiomyocytes in pre-adolescent mice by 40% (19). Manual counting of isolated cardiomyocytes in rats showed an increase from 13.6 to 22.9 billion from birth to day 3 (13). In summary, post-natal cardiomyocyte proliferation has been documented in mice, rats, and humans. However, the time period during which this happens varies widely.

After birth, rodent cardiomyocytes transition from cell division to incomplete cell cycles, resulting in approximately 80–90% binucleated cardiomyocytes in adult mice and rats. Humans are borne with approximately 30% of cardiomyocytes being binucleated, and this proportion does not change significantly after birth (21). However, in humans the ploidy of cardiomyocyte nuclei increases between birth and adulthood (21, 38).

Molecular changes associated with cell cycle withdrawal have been described, but molecular mechanisms of terminal differentiation remain largely uncharacterized

The transition to the terminally differentiated phenotype is accompanied by down-regulation of cell cycle factors and up-regulation of cell cycle inhibitors (15, 21, 38–40). Cell cycle activators such as the Cdk/cyclin complex, Myc, E2F transcription factors were demonstrated to be repressed. Negative cell cycle regulators are increased, such as p21, p27, retinoblastoma protein, and cyclin-dependent kinase inhibitors (41–43). However, the functional relevance of these associations has not been fully characterized. Decreased cardiomyocyte cell cycle activity was shown to be associated with the increase of the tissue oxygen tension upon birth, leading to increased cardiomyocyte DNA damage (44).

Under homeostatic conditions, cardiomyocyte turnover in the mammalian heart is very low

Turnover is the dropout and generation of new cells without a change of the total number of cells in the organ, *i.e.* during homeostasis. The number of cardiomyocytes per heart is

important when considering the rate of cardiomyocyte turnover and regeneration. Few studies have quantified the number of cardiomyocytes in human hearts (25) (45). Using stereology, we quantified the number of cardiomyocytes in adult humans to be approximately 3.7 billion (n = 7), corresponding to 5.6 billion cardiomyocyte nuclei(21). Our results are between the results from another stereology study that quantified the number of cardiomyocyte nuclei in adult humans to be 9.5 billion (n = 6 hearts, ref. (46)), and a study using biochemical techniques, which showed 2 billion cardiomyocytes (n = 30, ref. (38)).

Quantification of cardiomyocyte loss is challenging. Apoptosis markers are reported to have variable sensitivity. DNA repair and postmortem enzymatic degradation may yield false-positive results with the terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay (45, 47). The irreversible progression of cell death in cells expressing late stage apoptosis markers has also been called into question in several organs, including the heart (48). Thus, there is a lack of consensus for the frequency of cardiomyocyte apoptosis in normal hearts, with estimates varying by more than an order of magnitude (45, 47, 49–51).

Cardiomyocyte turnover was quantified with different techniques. The carbon-14 birth dating strategy showed that adult humans turn over approximately 0.5% of cardiomyocytes per year (22). Our MIMS approach showed a turnover rate of approximately 1% in adult mice (14). Several groups found multinucleation and polyploidy to account for a majority of cell cycle events after the first two weeks of life in murines (14, 17, 18). Using immunofluorescence microscopy, we have not been able to detect cardiomyocytes in cytokinesis in adult humans (21). Overall, reported rates of cardiomyocyte turnover in adult mice and humans are converging at approximately 1% or less per year (14, 15, 21, 22).

We have utilized the α-MHC-MerCreMer; Z/EG mice for cell lineage tracing in concert with stable isotope-labeled thymidine to detect cardiomyocytes with a history of cell cycle activity (14). Approximately 80% of preexisting cardiomyocytes were genetically labeled with GFP by injection of 4-hydroxy-tamoxifen. We have labeled with isotope-labeled thymidine for four to ten weeks at different points after birth. We used MIMS analysis of histological sections to visualize isotope-labeled thymidine localization. We identified cardiomyocyte nuclei by staining for cardiac markers (sarcomeric actin) and nuclei (DAPI or PAS histological staining). To analyze multi-nucleated cardiomyocytes, we used fiduciary marks on sections to retrieve corresponding nuclei on adjacent sections. Fluorescent in situ hybridization in at least two sections on either side of the MIMS section was used to determine ploidy. Using this method, we quantified that < 1% mononucleated diploid cardiomyocytes per year in adult mice were generated.

The source of cardiomyocytes generated during turnover remains an active area of research. Data support a role for both progenitor differentiation and cardiomyocyte proliferation. A more expansive discussion of cardiogenic progenitor potential in the adult heart is described in other articles in this series of reviews. In short, several classes of progenitor cells both in development and in the adult myocardium have been reported to contribute to the generation of cardiomyocytes.

Cardiomyocyte cycling increases after myocardial injury, but does not lead to effective regeneration

In adult mice after experimental myocardial infarction, the border zone shows a 10-fold increase of cardiomyocyte cell cycle activity. As described above, the α-MHC-MerCreMer mouse in combination with Cre-reporter lines has the potential to distinguish progenitor and cardiomyocyte contributions to cardiomyocyte generation (32). Injection of tamoxifen prior to inducing injury triggers GFP expression in cardiomyocytes. After injury, an increase of the GFP-negative percentage is interpreted as generation of cardiomyocytes derived from a progenitor pool. Using this system, *Malliaras et al* demonstrated a role for proliferative cardiomyocytes in adult mouse hearts, but a larger role for progenitor cells (17).

Combining the Cre model with stable isotope mapping on the MIMS platform, we have found that cell cycle activity occurs primarily in preexisting cardiomyocytes (14). While this study replicated our privious finding of the dilution of GFP+ cardiomyocytes, indicative of progenitor differentiation (25, 26), we observed cell cycle activity by stable isotope labeling in preexisting cardiomyocytes in the injury border (14). This contrasts with our previous reports that demonstrated generation of cardiomyocytes from undifferentiated progenitor cells (25, 26). These differences may be due to different long-term viability of GFP− and GFP+ cardiomyocytes, although our evaluation of short-term proliferation and apoptosis rates did not show differences.

Examining the mechanisms of cardiac regeneration when cardiomyocyte cell cycling and proliferation are active may provide new insight. Such studies were enabled by emulating the zebrafish model of excising 10–20% of the ventricular myocardium (52). This was achieved by the Sadek group in 2011 (53, 54). The apical resection model was reported to increase cardiomyocytes in M-phase and cytokinesis in neonatal mice (53). The authors demonstrated scarless myocardial repair and proliferation of preexisting cardiomyocytes using a Cre model combined with BrdU labeling. The aspects of scarless repair and increased cardiomyocyte cell cycle activity after apical amputation in neonatal mice have recently been challenged (55, 56). The difference in terms of scarless repair may be due to our inability to demarcate the resection level. Given the challenges of apical resection, the myocardial infarction (MI) approach by ligation of the left anterior descending coronary artery (LAD) in the neonatal heart may provide an alternative (57, 58). This model represents a more relevant model for inducing an injury and offers the advantage of using a defined landmark for inducing injury. However, the challenge of this technique is the need for microsurgical skills for identifying the LAD. Mastering these neonatal mouse models is required for advancing our understanding of post-natal cardiomyocyte proliferation in the setting of injury.

There is anecdotal clinical evidence that the human heart has regenerative potential. For example, an ectopic heart transplant recipient recovered normal function of the original heart, thus allowing the donor heart to be removed (59). Examinations of the mechanisms and degree of cardiac regeneration are technically difficult in humans for ethical reasons because they require pieces of myocardium for analysis.

Mechanisms of cycling and division of differentiated cardiomyocytes

The term ‘terminally differentiated cardiomyocyte’ in the context of cardiac regeneration is meant to describe the cardiomyocyte phenotype that lacks proliferative capacity, that is, these cardiomyocytes do not actively cycle nor can they be stimulated to re-enter the cell cycle. In the adult mammalian heart, terminally differentiated cardiomyocytes represent the vast majority. Different explanations for the lack of proliferative capacity have been proposed: the presence of the differentiated sarcomeric cytoskeleton may prohibit cell division and the binucleated and polyploid phenotype may prevent cell cycle entry. However, cardiomyocyte mitotic figures have been reported in adult zebrafish and mice (1, 53, 60, 61), suggesting that the presence of sarcomeres may not be prohibitive to cardiomyocyte division. The mechanisms of cell cycle entry and division of adult cardiomyocytes may involve cellular dedifferentiation. Sarcomere disassembly has been reported in proliferating zebrafish and neonatal mouse and rat cardiomyocytes and correlated with distinct changes in gene expression (13, 62–64). Oncostatin M, p130, and retinoblastoma protein regulate dedifferentiation in culture (64, 65). Reversal of epigenetic gene silencing mechanisms may be involved with cardiomyocyte cell cycle re-entry (59, 65, 66).

We have demonstrated that neuregulin-induced proliferation of differentiated adult mouse and rat cardiomyocytes occurs primarily in the mononucleated portion (60). Most adult zebrafish cardiomyocytes are mononucleated, which is consistent with their significant proliferative activity during regeneration (67). Newt cardiomyocytes are 98% mononucleated *in vivo*, but in culture ~30% acquire a binucleated phenotype by way of incomplete cytokinesis (68). Of these bi- and multi-nucleated newt cardiomyocytes, ~20% re-entered the cell cycle and some divided. It is important to note that bi- and multi-nucleated cardiomyocytes are exceedingly rare in newts *in vivo* (68). In human infants and children, cardiomyocyte cell cycle activity occurs predominantly in the mononucleated fraction (21). A recent report proposed division of binucleated cardiomyocytes in mice during the pre-adolescent growth spurt (19). In summary, some evidence suggests that the morphologic phenotype of cardiomyocytes with respect to mono-/binucleation may be associated with cell cycle capacity, although this association may not be strict.

What is the evidence for a distinct phenotype of differentiated cardiomyocytes that can re-enter the cell cycle? In animal models where cardiomyocyte cycling has been induced, cardiomyocytes have been described as smaller in size (69–72). In the border region of ischemic injury, actively cycling cardiomyocytes have been measured to be smaller in dimensions (21, 73). In *Mollova et al*., we observed cycling human cardiomyocytes in vivo to be mononucleated (21). In mice, some mononucleated cardiomyocytes have the ability to undergo multiple rounds of division *in vivo* as determined by sequential labeling schemes and clustering of cardiomyocytes with a record of cell cycle (60). We found with MIMS analysis that most cardiomyocytes with evidence for a history of division were mononucleated and diploid (14). In summary, independent reports indicate a higher cell cycle activity in mononucleated as compared with binucleated cardiomyocytes (14). It remains to be determined whether a proliferation-competent cardiomyocyte phenotype can be defined based on molecular characteristics.

Cultured cardiomyocytes are a useful tool for mechanistic studies. In culture, isolated adult cardiomyocytes progressively change their rod-like morphology to a flat shape coinciding with cytoskeleton reorganization (17, 64, 74–76). This morphologic change *in vitro* may facilitate the ability of mitogenic stimuli to induce cell division in cardiomyocytes that in vivo may not proliferate and must be considered when interpreting the results (60, 64, 74, 75).

Stimulating cardiac regeneration by inducing cardiomyocyte proliferation

Fetal and 1-day neonatal mice exhibit regenerative capacity in response to genetically induced disease and surgical damage, respectively (53, 77). The capacity for scarless repair appears to be present in mice until the neonatal period (53, 58, 77). Within the first two weeks of life in mice (15, 18) and approximately 10–20 years in humans (21, 22), cardiomyocytes transition from cell cycle activity to a non-cycling state. The association between cardiomyocyte cell cycle activity and regenerative potential has raised interest in modulating cardiomyocyte proliferation after the end of the regenerative period.

To regain proliferative capacity in adult cardiomyocytes, cell cycle checkpoints have been modulated with molecular interventions. Forced expression of cyclin B1-CDC2, and knockdown of p21/p27 have been shown to increase cell cycle activity of cardiomyocytes *in vitro* (78, 79). However, cell cycle factors do not universally activate cell division as over-expression of cyclin D1 in adult cardiomyocytes promotes DNA synthesis and multinucleation without proliferation (80). In vivo, cyclin D2, but not cyclin D1 and D3, resulted in persistent cardiomyocyte DNA synthesis and reduced scarring after experimental myocardial infarction in mice (81). Similarly, transgenic expression of cyclin A2 increased cardiomyocyte cycling and myocardial regeneration (82, 83). Overexpression of the adenoviral oncogene E1A induced cardiomyocyte cycling, followed by apoptosis (84). Overexpression of the transcription factor E2F-1 increased cardiomyocyte apoptosis, whereas overexpression of E2F4 stimulated cardiomyocyte cycling *in vivo* (85–87). Knock-out of the regulators of the E2F transcription factors, the pocket proteins Rb and p107, increased cardiomyocyte cycling (65, 88). Chemical inhibition and genetic knockout of GSK3β led to increased cardiomyocyte cycling (89, 90), which indicates the Wnt signaling pathway as a potential target for stimulating cardiac regeneration.

Recapitulating developmental signaling pathways in differentiated cardiomyocytes has the potential to stimulate proliferation. Under this premise, developmental signaling pathways should be present in differentiated cardiomyocytes and available for activation. For example, developmentally expressed cell surface receptors could be stimulated with extracellular factors. Neuregulin and its receptors on cardiomyocytes, the receptor tyrosine kinases ErbB2 and ErbB4 regulate developmental cardiomyocyte proliferation and differentiation (91). Administration of neuregulin and oncostatin M, an extracellular factor activating cytokine receptors has been shown to stimulate cardiomyocyte proliferation (60, 64). In addition, intracellular components of developmental signaling pathways can be targeted. For example, modifications of positive and negative regulators of the Hippo pathway have been shown to enhance neonatal and adult mouse cardiomyocyte cycling (54, 92, 93).

Delivery of neuregulin1 (Nrg1), fibroblast growth factor (FGF1) with pharmacologic p38 MAP kinase blockade, and periostin peptide has been demonstrated to promote myocardial repair (60, 94, 95). We have shown that administration of recombinant NRG1 induces cell cycle and division markers in cardiomyocytes, resulting in generation of new cardiomyocytes and improved function (60). We have also demonstrated that local delivery of a peptide of periostin in rats immediately after MI increased cardiomyocyte proliferation, reduced scar formation, and improved myocardial function (95). Mice with a germline knockout of the periostin gene showed a very mild general phenotype and were viable (96). Other periostin germline knockouts did not reduce cardiomyocyte cycling or myocardial growth, but showed reduced scar formation and impaired repair after myocardial infarction (97, 98). Overexpression of the full-length periostin gene under control of the α-MHC promoter in differentiated cardiomyocytes did not increase cardiomyocyte cell cycle activity (98). In this context, is important to note that normally, cardiomyocytes do not express the periostin gene. In addition, there is evidence for functional significance of a periostin splice variant, but a definitive structure-function relationship has not yet been achieved (99). We have subsequently reported periostin peptide-stimulated cardiomyocyte mitosis in large animals (100). This was recapitulated in an independent analysis, which also showed a reduction of scar formation, but increased fibrosis on microscopic analysis (101). In summary, the apparent discrepancy between administration of periostin peptide and manipulation of the full-length gene with respect to cardiomyocyte cell cycling may be associated with fundamental differences of the activated mechanisms, possibly due to specifics of the molecular strategy (truncated peptide vs. full-length gene). These differences can also be due to timing (acute peptide therapy vs. chronic overexpression by transgene) and heterologous expression in cardiomyocytes. This comparison indicates that specifics of molecular interventions matter and should ideally be addressed with side-by-side experimentation.

Discussion

Multiple studies have demonstrated that adult mammals renew cardiomyocytes at a very low rate from a cellular source of pre-existing cardiomyocytes (14, 17, 20, 21). Endogenous progenitor cells may play a role in therapeutic approaches. The 2012 POSEIDON clinical trial shows some potential for autologous mesenchymal cells in patients with ischemic cardiomyopathy (102, 103). The release of cell cycle blocks in adult cardiomyocytes and progenitor cell activation can facilitate an increase in cell cycle activity (17, 21, 26, 70). An emerging line of research seeks to define endogenous mechanisms of cardiomyocyte proliferation in the context of post-natal development and in response to neonatal injury (3).

Establishing standards for defining cardiac regeneration would be useful for comparing different studies and ultimately for advancing the field. These standards should be high and broadly accepted. When using metabolic labels (for example BrdU and labeled thymidine preparations), the labeling strategy should be clearly described, and the labeling kinetics should be measured (blood concentrations, effectiveness of cellular labeling). Assay sensitivities should be quantified and reported. Cardiomyocyte cell cycle activity should be measured with multiple, direct, and independent methods on the same sample. A conclusion that cardiomyocyte proliferation occurred should be supported by direct quantitative

evidence. Stereology is considered by many as the gold standard for quantifying cardiomyocytes in intact hearts (104). Cell cycle outcomes should be determined, i.e. multinucleation, ploidy, and division. Cell cycle activity should be examined and reported separately in diploid-mononucleated and bi-/multinucleated cardiomyocytes, whenever possible. Given the plasticity of cardiomyocytes in culture, timelines of in vitro experiments have to be reported. Karyokinesis and cytokinesis of cultured cardiomyocytes can be documented by video microscopy.

Regenerative cardiac medicine may be able to harness the growing understanding of endogenous cardiomyocyte renewal, stimulated cardiomyocyte proliferation, and progenitor differentiation (3, 105). To address these processes in the tissue context, heterocellular interactions have to be addressed. The extracellular matrix may have a potential function. The injury border region represents a transient space for cellular proliferation. Cardiac stromal cells, including fibroblasts, are known to communicate directly with cardiomyocytes via paracrine factors and cell-to-cell junctions, which may contribute to cell cycle signaling in cardiomyocytes (106).

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Highlights

- Studies should resolve cardiomyocyte proliferation and differentiation.
- Very sensitive techniques should be used for quantitative analyses.
- Cardiomyocyte generation decreases with age.
- Molecular interventions can stimulated cardiomyocyte cycling and division