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Mechanisms of Cardiomyocyte Proliferation and Differentiation in Development and Regeneration

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Abstract

a) Purpose of review—Congenital heart disease is the most common birth defect and acquired heart disease is the leading cause of death in adults. Understanding the mechanisms that drive cardiomyocyte proliferation and differentiation has the potential to advance the understanding and potentially the treatment of different cardiac pathologies, ranging from myopathies, heart failure, to myocardial infarction. This review focuses on studies aimed at elucidating signal transduction pathways and molecular mechanisms that promote proliferation, differentiation, and regeneration of differentiated heart muscle cells, cardiomyocytes.

b) Recent findings—There is now significant evidence that demonstrates cardiomyocytes continue to proliferate into adulthood. Potential regulators have been identified, including cell cycle regulators, extracellular ligands such as neuregulin, epigenetic targets, reactive oxygen species, and micro RNA.

c) Summary—The necessary steps should involve validating and applying the new knowledge about cardiomyocyte regeneration towards the development of therapeutic targets for patients. This will be facilitated by the application of standardized pre-clinical models to study cardiomyocyte regeneration.

Keywords

cardiomyocyte; proliferation; differentiation; regeneration; cell cycle

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Conflict of Interest

Jessie Wettig Yester and Bernhard Kühn declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction: The Premise for Research on Cardiomyocyte Proliferation

It is generally accepted that mammalian cardiomyocytes proliferate during development. Exactly when and by what mechanisms they stop proliferating is a matter of great interest as unlocking the underlying mechanisms could provide new strategies for stimulating cardiac regeneration (1, 2). Adult mammalian hearts do not regenerate cardiomyocytes after injury. Instead, dying cardiomyocytes are replaced by fibrosis and scar, which places the heart at further risk for continued injury in the future. While medications can improve heart function, the only way to replace lost cardiomyocytes is cardiac transplantation. However, there are limitations on the number of donors, and not all patients are good transplant recipients. Cardiovascular disease currently accounts for over 17 millions deaths globally each year it is the greatest cause of morbidity and mortality worldwide (3). Congenital heart disease, a failure of morphogenesis, accounts for nearly one-third of all major congenital anomalies worldwide, and is the most common congenital disorder in newborns (4, 5). Research in the area of cardiomyocyte regeneration and proliferation has the potential to provide new treatment strategies for many patients.

To what extent cardiomyocytes are capable of regeneration after birth has been a highly researched topic in cardiology for the past 100 years (6–8). Currently, two models describe the progression from active cardiomyocyte division in human infants and children to relative stagnation in adults (9, 10). Both models were based on the analysis of the same donor hearts(11). The models agree in that human infants and children generate new cardiomyocytes. Evidence from our lab demonstrates that humans generate new cardiomyocytes between birth and 20 years of life, *i.e.* until the heart has reached adult size. The rate of cardiomyocyte cell cycle activity was highest in infants and declines to very low, but measurable, levels in adults. This indicates that cardiomyocyte generation is highest in infants. We have detected evidence for cardiomyocyte cytokinesis up to 20 years after birth, but not in adults. As such, the cell cycle activity that we have measured in adults should be interpreted as evidence for cell cycles that do not lead to cytokinesis, *i.e.* multinucleation and endoreduplication. Direct quantification by stereology showed that the number of cardiomyocytes in the first year of life is approximately 1 billion and in adults 4 billion, which corresponds to the number of cardiomyocytes that was predicted to be generated from cell divisions.

The Frisen group has presented a model of cardiomyocyte turnover in humans (12, 13). This model was developed by carbon-dating of homogenized cardiomyocyte nuclei. Population modeling was used to infer cardiomyocyte birth and death kinetics. In this model cardiomyocyte generation is highest in infants and declines to very low levels in adults. The mathematical fit of this data showed that 3.1 billion cardiomyocytes were present at birth and the same number in adults. The constant number of cardiomyocytes after birth assumes that the significantly higher generation of cardiomyocytes in infants must be matched by a corresponding activity of cardiomyocyte death. However, by examining the same hearts, we have not found evidence for cardiomyocyte cell death (apoptosis) in human infants, children, or adolescents (10). By measuring the volume of individual cardiomyocytes, the Frisen group showed that cardiomyocyte hypertrophy was not detectable until 2 years after birth ($n = 6$ hearts below 2 years of age), which raises the question of how the heart grows below 2

years of age if not by an increase of the number or volume of cardiomyocytes. In summary, the models proposed by Bergmann et al and Mollova et al both show that human infants and children generate new cardiomyocytes and that in adult humans this activity is extremely low or undetectable; however, the base support regarding the growth mechanism differs (12, 13).

Post-natal cardiomyocyte proliferation in mice was not predicted from original cell cycle studies, although similar studies in rats showed an increase of the number of cardiomyocytes in the first 10 days of life (14, 15). This matter was recently re-evaluated. Naqvi et al. identified two distinct periods of cardiomyocyte proliferation: one in the first week and one in the second week of life (16). While the first proliferative period was also detected by stereology methods, the second one was not (17). Indirect evidence for cell cycle activity supporting the second proliferative burst was not present in the original or in recent reports (17). In conclusion, mice and rats show evidence for transient post-natal cardiomyocyte proliferation. The ability of mouse hearts to regenerate in the corresponding period of time has been proposed to be causatively related (18).

While the proliferation of cardiomyocytes in adult mammals is minimal, other species can regenerate cardiomyocytes throughout their lifespan. Amphibians, reptiles, and zebrafish regenerate cardiomyocytes after myocardial injury (19–22). To date, three main strategies for cardiomyocyte replacement in mammals have been pursued: transplantation of stem and progenitor cells, transdifferentiation of resident cardiac fibroblasts, and the reactivation of endogenous regenerative mechanisms (23). This review focuses on the endogenous mechanisms for cardiomyocyte regeneration and proliferation.

Cardiomyocyte proliferation is a key component of the morphogenesis of the developing heart (24, 25). Cardiomyocytes transition from proliferative to hypertrophic growth, which is usually considered as terminal differentiation. During this transition, in most mammals, the portion of binucleated cardiomyocytes increases (7, 14, 15) and the excitation-contraction properties change. The timing of this transition is usually placed after birth. However, human neonates have binucleated cardiomyocytes, indicating that in some mammals cardiomyocytes begin to undergo terminal differentiation before birth (9, 10).

Cell Cycle Regulators

In general, positive cell cycle regulators such as cyclins, cyclin-dependent kinases, and proto-oncogenes are highly expressed in the fetal and neonatal heart, whereas the expression is down-regulated in adult hearts. Conversely, negative regulators of the cell cycle, such as cyclin-dependent kinase inhibitors are up-regulated in adult hearts.

Overexpression of cyclin D2, a positive regulator of the G1/S transition, induced DNA synthesis and proliferation in mammalian cardiomyocytes (26, 27). Cyclin D2 activated CDK4/6 and led to subsequent phosphorylation of retinoblastoma protein (Rb) and downregulation of the cell cycle inhibitors p21Waf1/Cip1 and p27Kip1 (26). Cyclin D2 is required for c-Myc-induced cardiomyocyte cycle reentry as determined by BrdU staining.

Additionally, cyclin D2 enzymatic activity was required for DNA synthesis, but not required for hypertrophy (28).

Cyclin A2, which promotes the G1/S and G2/M transitions, has also been implicated in cardiomyocyte proliferation. α -MHC-cyclin A2 transgenic mice showed increased postnatal cardiomyocyte mitosis (29). Overexpression of cyclin A2 improved cardiac function after ischemic injury in mice, and also induced DNA synthesis and mitotic indices (30). Subsequent studies have shown that adenoviral delivery of cyclin A2 to ischemic porcine hearts promoted recovery after ischemic injury, as well as induced cytokinesis with preservation of the sarcomere structure (31).

The E2F family of transcription factors has DNA-binding domains and binds to the pocket domain of retinoblastoma protein, pRB, p107, and p140 (32). E2Fs can mediate both cell proliferation and p53-dependent/independent apoptosis. When transiently over-expressed with E1B in mouse cardiomyocytes, E2F-1 induced DNA synthesis and accumulation of cardiomyocytes in G2/M. However, there was no increase in cell number after 24 hours. Transfection with E1B and E2F-1 inhibited cardiac and skeletal α -actin promoters (33). Expression of E1A in rat cardiomyocytes stimulated DNA synthesis (34). Adenoviral transfection of E2F2 expression in mouse cardiomyocytes caused a strong induction of cyclin A and E, without affecting the expression of cyclin-dependent kinase inhibitors such as p21, resulting in proliferation of adult cardiomyocyte (35).

Signal Transduction Pathways

Identification of potential endogenous ligands and their receptors regulating cardiomyocyte proliferation and regeneration has been an important area of research. Some of these ligands can be administered exogenously and signal through cell surface receptors to promote cardiomyocyte proliferation. Many target molecules such as FGF-1, FGF-2, IGF, EGF, NRG1, IF-1 β , and EPO, CT-1 have been identified (36, 37).

One of the key pathways responsible for cell fate specification and tissue patterning in the embryo and in the adult is the Notch signaling pathway. The Notch signaling pathway is an evolutionarily conserved pathway that regulates genes important for proliferation, differentiation, and apoptosis via cell transactivation, in which a ligand-expressing cell transactivates a neighboring cell. Activation of the Notch pathway reinforces ligand expression in the signaling cell and receptor expression in the receiving cell (38). Notch signaling is important for the developing heart. Humans with inactivating mutations in the Notch ligand, Jagged1, suffer from Alagille syndrome, a condition characterized by several cardiac defects including Tetralogy of Fallot, ventricular septal defects, atrial septal defects, aortic stenosis, and aortic coarctation (39, 40).

Notch activation is essential to sustain proliferation at birth as nearly half of the proliferating cardiomyocytes contain activated Notch1 in the nuclei (41). Transfection of the intracellular domain of Notch1 prolonged the proliferative potential of neonatal rat cardiomyocytes, characterized by the persistence of histone 3 lysine 4 trimethylation. Unfortunately, activation of the Notch pathway was ineffective in adult hearts as Notch responsive

promoters were buried in closed chromatin, characterized by the presence of repressive marks, including histone 3 lysine 27 trimethylation and the polycomb group complex repressive methylase enhancer of zeste homolog 2 (42). This demonstrates the effectiveness of epigenetic regulation, but also the potential for modification. Removing the repressive marks may activate Notch-mediated proliferation pathways. Additional mechanisms and examples of epigenetic regulation of cardiomyocyte proliferation will be discussed below.

Our lab has focused on the modulation of neuregulin-1 (NRG1) in order to promote or induce cardiomyocyte proliferation (43–47). NRG1 acts through the receptor tyrosine kinases ErbB2, ErbB3, and ErbB4 (48). ErbB2 over-expression is implicated in breast cancer, and ErbB2 inhibition by Herceptin (trastuzumab), used in the treatment of breast cancer, has cardiotoxicity (49). NRG1 and its co-receptors ErbB2 and ErbB4 are required for mouse cardiac development (50–52). The lethal ErbB4^{-/-} phenotype could be rescued with transgenic expression of α -MHC-ErbB4, indicating that deletion of ErbB4 specifically in cardiomyocytes is required for the phenotype (53). NRG1-induced cardiomyocyte proliferation diminished after birth due to a reduction in ErbB2 expression; induction of constitutively active ErbB2 in neonatal, juvenile, and adult cardiomyocytes resulted in proliferation. Additionally, transient expression of ErbB2 resulted in significantly improved recovery of cardiac performance after myocardial ischemic injury induced by permanent ligation of the left anterior descending coronary artery, as a result of cardiomyocyte dedifferentiation and proliferation followed by redifferentiation and tissue replacement (54). In ErbB2-over-expressing transgenic mice pro-survival pathways and hypertrophic cardiomyopathy were activated (55).

NRG1 signaling stimulated DNA synthesis, karyokinesis, and cytokinesis in zebrafish heart and in cultured adult rat ventricular cardiomyocytes (56, 57). Administration of recombinant NRG1 induced the proliferation of mononucleated mouse cardiomyocytes (43). Recombinant NRG1 also induced cardiomyocyte proliferation in myocardial explants from human infants (44). The NRG1 gene is activated by myocardial injury in zebrafish and required for heart regeneration (58). While these studies have identified NRG1 as a key regulator of cardiomyocyte proliferation and pharmaceutical trials are underway to bring recombinant NRG1 to bedside for patients, upstream regulators and downstream effectors of NRG1 remain largely elusive (59, 60).

One possible upstream regulator of NRG1 is direct innervation of the myocardium (61). NRG1 is able to rescue impairment of heart regeneration mitigated by direct mechanical denervation in mouse and zebrafish (62). Additionally, inhibition of cholinergic nerve function reduced cardiomyocyte proliferation. Transcriptional analysis of denervated hearts suggested that the inflammatory and immune system responses were decreased following cardiac injury (62). This data suggested that nerve function is required for both zebrafish and mouse heart regeneration. NRG-1/ErbB signaling may also modulate cardiac structure and function indirectly via the central nervous system, as intracisternal administration of NRG-1 attenuated LV hypertrophy and improved LV function in a pressure overload-induced heart failure model in rats (63).

Inflammatory and immune-mediated signaling pathways may regulate cardiomyocyte proliferation. One proinflammatory cytokine that has been shown to be up-regulated in human patients and in transgenic mouse models of dilated cardiomyopathy is oncostatin M (OSM). OSM was positively correlated with dedifferentiation in both dilated cardiomyopathy and myocardial infarction. In a mouse model of myocardial infarction intraperitoneal injections of recombinant OSM were protective and deletion of OSM receptor worsened cardiac function after myocardial infarction (64). The importance of the immune system is further highlighted by the requirement of macrophages for cardiac regeneration in neonatal and adult mice (65, 66).

Two additional extracellular regulators of myocardial cell cycle entry are follistatin-like 1 (Fstl1) and insulin-like-growth factor2 (IGF2). Both of these growth factors are produced by the epicardium (67–69). Application of recombinant Fstl1 via an epicardial patch stimulated cell cycle entry and promoted myocardial division in rodent and pig models (67). Interesting, the function of Fstl1 is site specific, as following myocardial infarction epicardial Fstl1 expression declines and is replaced by myocardial expression, which does not promote regeneration. In mouse hearts, IGF2 is expressed during midgestation heart development and is the primary mitogenic factor made by embryonic epicardial cells (68). Inhibition of this pathway prevented cardiomyocyte proliferation (68). Regulation of IGF2 is biphasic, first induced by erythropoietin and then regulated by oxygen and glucose with onset placental function (69).

How these multiple extracellular factors are integrated by signal transduction cascades to control cardiomyocyte proliferation and cardiac regeneration is not yet fully elucidated. Research in both cancer and cardiac models has identified multiple downstream effectors of NRG1/ErbB including PI3K/Akt, MAPK/ErK, and FAK (70). NRG-1 β signaling activates the ErbB2-ERK-SIRT1 signaling pathway in a model of radiation-induced heart disease and is a protective mechanism (71). Additional downstream mediators of ErbB signaling pathways include the Rac-PAK intracellular kinases (72, 73). In a breast cancer model HER2, which utilizes the receptor ErbB2, induces Rac and Pak activation (72, 73). In breast cancer, Rac/Pak is downstream of PI3K (74). The Rac1-PAK2 pathway is essential for zebrafish heart regeneration (75). In a mouse model of transient ErbB2 overexpression, the ERK, AKT, and GSK3 β / β -catenin pathways were activated in order to promote the dedifferentiation and proliferation of cardiomyocytes induced by the activation of the NRG1/ErbB2 signaling pathway (54).

In addition to positive regulators of cardiomyocyte proliferation, negative regulators have also been identified. The signaling molecule p38 mitogen-activated protein (MAP) kinase (p38) induced cell cycle exit and differentiation of many cell types, including the differentiation of embryonic carcinoma cells to cardiomyocytes (76). Activated p38 phosphorylates downstream signaling molecules important for cardiomyocyte differentiation and hypertrophy (77). Overexpression of p38 blocks fetal rat cardiomyocyte proliferation. Inhibition of p38 utilizing conditional p38 knock-out mice and pharmacologic inhibition with SB203580 promoted cardiomyocyte proliferation (56).

Another signal transduction pathway regulating proliferation is the Hippo signaling pathway. The Hippo pathway is evolutionarily conserved, initially discovered in *Drosophila melanogaster* (78). The terminal effector is the transcriptional co-activator Yes-associated protein (YAP). Activation of the Hippo pathway phosphorylates YAP and prevents its entry into the nucleus (79). Inactivation of YAP has a role in maintaining organ size during development. Enlarged hearts resulting from alterations in the Hippo pathway are attributed to proliferation and YAP is necessary and sufficient to regulate cardiomyocyte proliferation (80). Using gene expression profiling and chromatin immunoprecipitation, it was determined that Hippo signaling negatively regulates a subset of Wnt target genes to restrict cardiomyocyte proliferation and control heart size. Mouse embryos with inactivation of Hippo signaling had overgrown hearts with elevated cardiomyocyte proliferation (81). In mice, microRNA302–367 repressed the Hippo signal transduction pathway, which led to increased cardiomyocyte proliferation (82). While the identification of signal transduction pathways that promote cardiomyocyte proliferation and regeneration has potential, a better understanding of how these pathways are regulated will be instrumental for the development of clinically relevant strategies. One possible way that these pathways could be regulated on a broad scale is through development is via epigenetic modification.

Epigenetic Regulation

The next step after the identification of pathway responsible for cardiac regeneration and proliferation is to understand how the activity of these pathways is regulated at the molecular-genetic level. The epigenetic regulation of critical genes in signaling pathways is one possible change that happens during the transition of cardiomyocytes from a state of high replication in neonates to a state of quiescence in adults. As such, the identification of key regions and modifications of chromatin, and methodologies to prevent or reverse the permanent inactivation of regions corresponding to cardiac regeneration and proliferation would be one possible avenue for exploration. For genes to be transcribed they must be in accessible areas of chromatin, so that transcription factors can recruit RNA polymerase. Certain histone modifications enable areas of chromatin to be maintained in an accessible state, conversely other histone modifications promote tighter coiling and can contribute to the silencing of gene expression. Enhancer elements are areas of accessible chromatin, facilitated by histones possessing various modifications, such as acetylated lysine 27 of histone H3 (H3K27ac). These are areas primed to be occupied by transcription factors in order to up-regulate associated genes (83, 84).

Epigenetic modification of DNA has been recognized as a key mediator of development and disease in a variety of cell types, including cardiomyocytes. Embryonic development of the heart is a complex processes requiring temporal and spacial regulation of gene expression that is regulated in part by distinct chromatin patterns (85, 86). Pathologic cardiac hypertrophy and heart failure are characterized by a transcriptional reprogramming of gene expression. This process is also coordinated by chromatin remodeling. Brg1, a chromatin-remodelling protein, promotes embryonic myocyte proliferation; it also preserves embryonic α -myosin heavy chain expression. After cardiac stress, Brg1, which is typically inactivated in adults, is reactivated and forms complexes with its embryonic partners (87).

Bromodomain proteins associate with acetylated chromatin and facilitate transcription activation by the recruitment of coregulatory complexes. Bromodomain inhibition with JQ1 blocked phenylephrine-mediated cellular hypertrophy in vitro and in vivo (88). 5-azacytidine, a DNA methyl transferase inhibitor, promotes the differentiation of mouse embryonic carcinoma cells into cardiomyocytes. Differentiation was associated with increased expressions of cardiogenesis associated genes, accompanied by their hypomethylation status, increased histone 3 acetylation and activation of ERK signaling (89). A better understanding of epigenetic regulation of cardiogenesis and cardiac pathologies will be an important area for further research.

A recently identified regeneration-specific enhancer element LEN was identified as a regulator of *Leptin b* (*lepb*). *Lepb*, a secreted regulator of energy homeostasis, is upregulated by local injury of the cardiac ventricle (90–92). The upregulation of *lepb* after injury is mediated by an upstream enhancer element, LEN (90). This enhancer element was used to upregulate *nrg1* in transgenic zebrafish lines LENP2:*nrg1* after resection of the ventricular apex, which promoted cardiac regeneration (90). It is possible that such enhancers are also important in the regulation of mammalian NRG1 expression.

Down-regulation and inhibition of TERT and the resulting loss of telomerase activity is another mechanism that prevents the proliferation of cells (93, 94). It has previously been shown that TERT and telomerase activity are down-regulated in adult mouse myocardium (95). hTERT expression synergistically increased both neonatal cardiomyocyte S-phase entry and proliferation induced by cyclin-dependent kinase inhibitor (CKI) knock-down (p21, p27, and p57) (96). CKI knockdown cardiomyocytes assumed a stellate morphology, resembling that of neonatal cardiomyocytes (96). High levels of CKI expression have been observed in cardiomyocytes, and they have been hypothesized to play a role in maintaining the withdrawal from the cell cycle in both neonatal and adult cardiomyocyte. This effect can be rescued by the triple knock-down on p21, p27, p57, which promoted both neonatal and adult cardiomyocyte entrance into S-phase, and increased the nuclei/cardiomyocyte ratio (96).

Mitochondrial pathways and ROS

A potential regulator of cardiomyocyte proliferation is the intracellular activity of reactive oxygen species (ROS). One potential trigger for the transition from proliferative state to quiescence could be the transition to an oxygen-rich post-natal environment. Neonatal mice increase the amount of mitochondrial DNA, number of mitochondrial cristae, and ROS production as they develop (97). Oxidative metabolism is an essential source of energy for the adult cardiomyocyte, however it is also associated with an increased production of reactive oxygen species (ROS). Cardiomyocyte mitosis, assayed by phosphorylated histone 3 Ser10 (pH3), a marker of G2-M progression, was increased in mouse cardiomyocytes maintained in hypoxic conditions (97). The treatment with ROS reduced the mitosis of cardiomyocytes as determined by pH3 staining, without a significant increase in apoptosis, and scavenging ROS suppressed the postnatal cardiomyocyte cell cycle arrest (97). Using a tamoxifen-inducible Cre system that was regulated by hypoxia-inducible factor 1 α (HIF1 α), a rare population of cardiomyocytes that displayed characteristics similar to proliferative

neonatal cardiomyocytes was identified. This subpopulation of cells was more likely to be mononucleated, smaller, and surrounded by fewer capillaries. There was an increased number of the labeled cells 1 month after tamoxifen treatment. These labeled cells also had less oxidative DNA damage, determined by decreased expression of 8-oxoguanine, an enzyme that repairs DNA damage caused by ROS (98). The predictions of the hyperoxia/ROS-model could be tested in human infants with cyanotic congenital heart disease. This population would be anticipated to have fewer terminally differentiated cardiomyocytes. Conversely, prematurely borne babies, who are exposed to ambient oxygen tension, should show evidence for accelerated or increased cardiomyocyte terminal differentiation.

Dexrazoxane is an FDA approved ROS scavenger for the prevention of anthracycline-induced cardiotoxicity in adult and pediatric oncology patients (99, 100). Doxorubicin-induced cardiotoxicity is thought to be due to iron-dependent oxidative stress (101). The mechanism of dexrazoxane-mediated cardioprotection is incompletely understood. Some studies have indicated that dexrazoxane minimize oxidative stress via iron chelation (102). Additional studies have observed depletion of the anthracycline target, topoisomerase II, after treatment with dexrazoxane suggesting that anthracycline cardiotoxicity is at least partly mediated by topoisomerase (103, 104). Beyond their use in anthracycline-induced cardiotoxicity, there is currently a phase 1 clinical trial evaluating the safety of Dexrazoxane in reducing ROS-mediated damage for congenital heart disease repair ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02519335) number: NCT02519335). Future studies aimed at determining if there is increased cardiomyocyte proliferation after dexrazoxane, in addition to its ability to decrease damage caused by anthracycline, would lend further evidence of the role of ROS in proliferation and regeneration.

Micro RNA

An exciting advance in the post-transcriptional regulation of gene expression is the discovery of microRNAs (miRNA). miRNA functions by base pairing to a complementary sequence on a target mRNA, typically leading to repression (105). Impairment in the miRNA pathways in cardiac muscle has been implicated in heart failure and cardiomyopathy (106, 107). There is a growing body of evidence implicating altered miRNA in cardiac disease (108–110). miRNA have been shown to both inhibit and stimulate cardiomyocyte proliferation (82, 111–115). Proliferation was induced in both neonatal and adult mice, and was mediated by the partial dedifferentiation of adult cardiomyocytes and subsequent proliferation. Simultaneous, local administration of two miRNA (has-miR-590 and has-miR-199a) preserved the function and minimized the infarct size after permanent left anterior descending coronary artery ligation in mice (111). Potential downstream mediators of these two microRNA include *Homer1*, involved in the regulation of calcium signaling in cardiac cells, *Hopx* a regulator suppressing embryonic cardiomyocyte proliferation, and *Clc5* a chloride intracellular ion channel. However, individual knock-down of these genes with siRNA did not induce an increase in cardiomyocyte proliferation, suggesting that the ability of has-miR-590 and has-miR-199a is due to a yet to be identified target, or more likely the result of a synergistic effect of multiple cellular mRNA targets. A third microRNA, miRNA-204 has been shown to promote both adult and neonatal cardiomyocyte proliferation in vitro. miRNA-204 subsequently increases the expression of multiple cell

cycle regulators, and directly targets Jarid2. Jarid2 is a repressor protein that interacts with Polycomb repressive complex 2 (PRC2), which is important in the regulation of gene expression during development. Knock-down of Jarid2, had similar effects of miRNA-204, and promoted the proliferation of cardiomyocytes (116).

Summary and Future Directions

The field of cardiomyocyte proliferation and differentiation has progressed significantly in the past 50 years. It is now generally accepted that cardiomyocytes proliferate to some degree after birth. Studies aimed at determining the mechanisms of cardiomyocyte cell cycle regulation have identified multiple potential targets. The validation of these targets and translation of this information into clinically relevant pharmacologic strategies should be a priority for future research. Advance in this direction is challenging because of controversial results with neonatal mouse myocardial injury models (117–120). Three recently published protocols for inducing myocardial injury in neonatal mice (surgical amputation, cryoinjury, and ligation of the left anterior descending artery) should provide the tools to resolve the controversy (121–123). With greater consistency and further study, mechanisms will continue to be elucidated and from this data there is the potential to improve treatment of a variety of cardiologic pathologies.

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••Of major importance

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Annotated references (MARDI WILL ADD THESE IN)

- Ref. (9): Bergmann 2015

This paper presents a growth model of the human heart. Determined that the number of cardiomyocytes is established at birth and remains stable throughout the human lifespan. Carbon dating and population modeling was used to determine that turnover was highest in infants and declined gradually throughout life span. The same hearts were examined by both Bergmann et al. and Mollova et al.
- Ref. (10): Mollova 2013

This paper presents a growth model of the human heart. Determined that the number of cardiomyocytes increases in infants and children. The percentages of cardiomyocytes in mitosis and cytokinesis were highest in infants and children; percentages of mitosis decreased to low levels and cytokinesis to zero by adulthood.
- Ref. (54): D'Uva 2015

NRG1 co-receptor ErbB2 enhances cardiomyocyte proliferation stimulated with recombinant neuregulin 1. Constitutively active ErbB2 is sufficient to reactivate proliferation in adult cardiomyocytes.
- Ref. (44): Polizzotti 2015

Application of recombinant NRG1 was sufficient to induce proliferation of cardiomyocyte in neonatal mice and infant myocardium.
- Ref. (90): Kang 2016

Identified a common regeneration enhancer mechanism for the regulation of genes in regenerating tissue. The mechanism has the potential to active clusters of genes, which will likely be necessary for meaningful proliferation.