Evolving approaches to heart regeneration by therapeutic stimulation of resident cardiomyocyte cell cycle

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Abstract

Heart has long been considered a terminally differentiated organ. Recent studies, however, have suggested that there is a modest degree of cardiomyocyte (CM) turnover in adult mammalian heart, albeit not sufficient for replacement of lost CMs following cardiac injuries. Cardiac regeneration studies in various model organisms including zebrafish, newt, and more recently in neonatal mouse, have demonstrated that CM dedifferentiation and concomitant proliferation play important roles in replacement of lost CMs and restoration of cardiac contractility. Further studies with neonatal cardiac regeneration mouse model suggested that major source of new CMs is existing CMs, with the possibility of involvement of cardiac stem cells. Numerous studies have now been conducted on induction of cardiac regeneration and have identified various cardiogenic factors, cardiogenic micro ribonucleic acid and cardiogenic small molecules. This report is a review of studies regarding generation of CM and prospects for application. *(Anatol J Cardiol 2016; 16: 881-6)*

Keywords: cardiac regeneration, cardiogenic factors, cardiogenic small molecules, cardiomyocyte proliferation, cardiomyocyte renewal, cell cycle activators

Introduction

Cardiovascular disorders are leading cause of death worldwide, with death rates up to 40% in some developing countries (1). Heart failure (HF) is a progressive and debilitating form of cardiovascular disorder with penetrance that rises exponentially with age. There are various causes of HF, but depressed ejection fraction following myocardial infarction (MI) is among the most common and can progressively lead to end-stage cardiomyopathy. Myocardium may be stunned and hibernate during ischemic MI or heart attack. If these cardiac muscle cells cannot recover their function, lack of viable myocardium leads to death in half of these patients within 5 years of diagnosis (2). Heart has been thought to be a terminally differentiated organ due lack of regeneration after HF, but there is some controversy, as recovery has also been observed following myocardial necrosis (3).

Heart transplantation is postulated as definitive treatment for HF. However, heart transplantation is limited by scarcity of human leukocyte antigen-compatible donors, as well as immunological complications that occur following transplantation (4). Thus, researchers seek alternative solutions for HF including induction of cardiac regeneration. In the last decade, promising studies related to cardiac regeneration have gained enormous momentum and have demonstrated cardiomyocyte (CM) renewal, either spontaneously or following cell-based therapies (5–7).

Different species have distinct regenerative capacities following various cardiac injury (8). Newts and zebrafish, for instance, regenerate their hearts following myocardial injury in as little as 60 days (9). Even though adult mammalian heart does not possess full regenerative capacity, it does has regenerative potential at a certain age and after specific stimulation (8) (Fig. 1). HF commonly originates from systolic HF due to lack of sufficient CM renewal following cardiac injury (10, 11). Recent landmark studies indicate that adult heart can produce new CMs (5, 6). The source of newly produced CMs is not yet evident, but pre-existing CMs or resident cardiac stem cells are thought to have a role in CM regeneration (5). During embryonic development and early postnatal period, CM proliferation (hyperplesia) is considered the main mechanism of cardiac growth. In the adult heart, cardiac hypertrophy becomes major growth mechanism subsequent to CM cell cycle arrest. Interestingly, cardiac development in Xenopus genus requires Hif-1α signaling, which acts upstream of Nkx2.5 (12). Concomitantly, it has also recently been demonstrated that adult mouse heart possesses hypoxic microenvironment where Hif-1α+ CMs are localized (labeled with TnnT) and glycolytic cardiac

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Accepted Date: 26.08.2016

DOI:10.14744/AnatolJCardiol.2016.7245

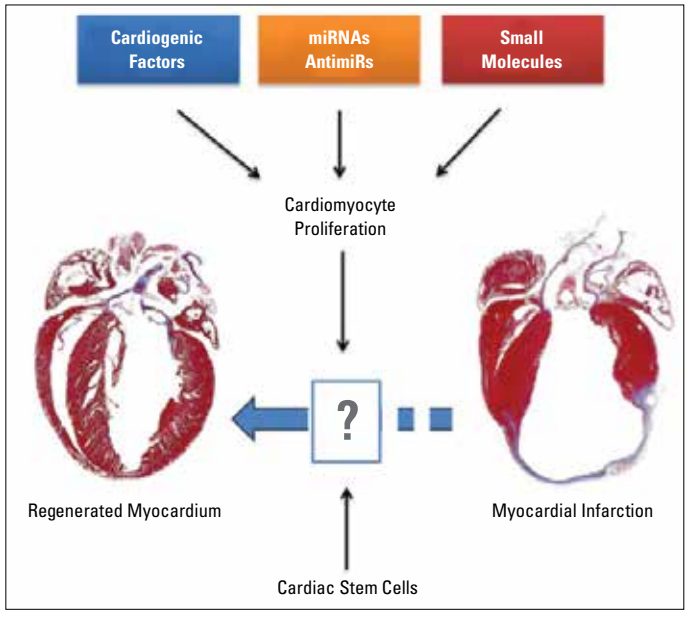

Figure 1. Therapeutic stimulation of resident cardiomyocyte cell cycle.

progenitors are housed in subepicardium (13). These hypoxic progenitors and CMs need to be further analyzed with lineage tracing studies to determine if they contribute to newly formed CMs. In addition, underlying mechanisms of CM turnover, role of CM proliferation, and contribution of extracardiac or resident stem cell population still remain to be determined (Fig. 1). Cardiogenic factors, micro ribonucleic acid molecules, antimiRs, and small molecules have been shown to induce cardiomyocyte proliferation and improvement of cardiac function following myocardial infarctions. In addition, studies showed that cellular therapies with stem cells could enhance renewal of cardiomyocytes.

Cardiomyocyte renewal

Regeneration capacity varies from organism to organism. Zebrafish and newt demonstrate remarkable heart regeneration after 20% amputation of the ventricular apex. Regeneration capacity of both organisms has been reported as capability for complete restoration over 2-month period (14). Restoration of cardiac function has been associated with proliferation of dedifferentiated CMs, characterized by dissolution of sarcomeric structures (15). In newts, however, regeneration of heart involves blastema formation with accumulation of dedifferentiated cells near edge of lesion (16). In mammals, on the other hand, regeneration capacity of adult mammalian heart is highly restricted; instead, it responds to cardiac injury with scarring (fibrosis). However, fate mapping techniques have provided evidence that low rates of CM turnover occur following cardiac injury (6). Several studies have suggested that myocardial recovery and CM renewal after heart injury might originate in stem cells. One study of patients with heart transplants indicated that vascular cells and small percentage (0.016–0.04%) of newly formed CMs were host-derived (7).

Rate of CM renewal has been estimated using a number of approaches such as autoradiographic measurements of deoxyribonucleic acid (DNA) synthesis (17). According to Bergmann et al. (5) CM turnover rates were estimated at 1% and 0.4% per year at the age of 20 and 75 years of age, respectively. On the other hand, studies by Anversa group demonstrated that CM turnover rates were specified respectively at 7%, 12%, and 32% per year at 20, 60, and 100 years of age in men, while higher rates were estimated in females (18). Although these studies provided further evidence of existence of CM turnover in human heart, whether the source of CMs is existing or newly produced CMs is not completely understood. To this end, 5-Bromo-2'-deoxyuridine (BrdU), which incorporates into newly synthesized DNA, has been used to assess DNA synthesis in CMs. BrdU pulse-chase analysis and lineage tracing studies in neonatal mouse heart regeneration model indicated that origin of newly formed CMs is likely to be pre-existing CMs (19).

Neonatal heart regeneration

CM cell cycle and activity change over course of cardiac growth. Growth phases of CMs can be categorized developmentally as fetal life, postnatal, and adult period. During fetal life, CMs proliferate rapidly. In early postnatal period of heart, murine CMs become binucleated around postnatal day 7 to 10. However, hypertrophy becomes major form of growth in adult heart. When regeneration capacity was analyzed, neonatal and adult hearts demonstrated unequal capacity following injury (20). Recent studies have demonstrated that neonatal heart could regenerate without noticeable fibrosis or cardiac dysfunction after removal of up to 15% of the ventricle apex or MI at postnatal day 1 (P1) (21). However, at P7, regeneration capacity of the heart appears to be lost. Rather, there is adult-like response to heart injury with scar formation and cardiac dysfunction. Renewal of CMs that occurs following myocardial injury is not sufficient in adult mammalian heart. This deficiency has been considered the primary limiting factor for adult heart regeneration. Therefore, a number of studies have sought means to reactivate intrinsic proliferative capacity of adult CMs.

Reactivating cardiomyocyte cell cycle with cardiogenic factors

MI causes loss of CMs through apoptosis and necrosis. Ideal cardiovascular therapies aim to both reduce CM death and induce proliferation by manipulation of CM cell cycle. Cell cycle is a highly regulated, complex process; prominent regulators include cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs), CDK-activating kinases (CAKs), and retinoblastoma (Rb) family. Cyclin/CDK complex is activated through periodic phosphorylation by CAKs. In addition, cell cycle is regulated by CDKIs including Cip/Kip family (p21Cip1, p27Kip1, p57Kip2) and Ink4 family members (p15Ink4b, p16Ink4a, p18Ink4c, p19Ink4d). Cell cycle comprises 4 phases: G1, S, G2, and M phases. There is also a Go phase when cells exit the cell cycle. Majority of adult CMs are ar-

Table 1. Factors involved in cardiomyocyte proliferation

Factors	Examples	References
Growth factors	Periostin, neuregulin FGF1, oncostatin-M	(25–27, 28)
Cell cycle activators	Cdk2, c-myc, cyclin D2, Cyclin A4, cyclin D1, E2F-1	(24, 29–32)
Gene deletion or inhibition	Meis1, antimiR-15, $p27^{Kip1}$ and $p21^{Cip1}$	(19, 23, 33–36)

Cdk2 - cyclin-dependent kinase 2; FGF1 - fibroblast growth factor 1; MEIS1 - meis homeobox 1

rested in Go/G1 cell cycle and marked by low proliferation index. Even though CDKIs are highly expressed and cell cycle activators are decreased in adult CMs, several reports have suggested that adult CMs can divide in injured heart (22). Studies have indicated that S phase progression in CMs can be induced with immunodepletion of p21Cip1 or deletion of CDKI p27Kip1 (23). In addition, overexpression or activation of E2F-1, cyclin D2, and cyclin D1 leads to DNA synthesis and CM mitosis, to some extent (Table 1) (19, 23–27, 28–36). Moreover, several studies have reported that growth factors and cytokines such as periostin, neuregulin, fibroblast growth factor 1, and oncostatin-M, as well as induction of Hippo pathway affect CM cell cycle progression (25–27, 37).

Cardiogenic miRNAs and anti-miRs

Micro ribonucleic acid (miRNA) molecules are small, single-strand, 22-nucleotide-long, non-coding RNAs. The most important role of miRNAs is target-specific inhibition of translation. This inhibition occurs through base pairing with specific binding sites located in the 3' untranslated region (UTR) of specific mRNA targets. MicroRNAs have significant role in many cellular and biological processes such as cell proliferation, differentiation, and apoptosis, as well as cardiac development. Moreover, miRNAs act as dynamic regulators in cardiomyopathy and HF (38). In study of mice, CM-specific deletion of miRNA processing-associated genes such as Dicer and Dgcr8 with α-myosin heavy chain promoter-driven Cre recombinase and muscle creatine kinase promoter-driven Cre-recombinase (MCK–Cre) led to lethality through P0 and P4, respectively (38). When miRNA expressions are analyzed in the mammalian heart, miR-1 was found to be highly expressed (Table 2) (19, 37, 38–41). miR133 and miR-1 deletion in CMs negatively affects CM proliferation and differentiation. This effect occurs through modulation of various myogenic transcription factors including serum-response factor, myocyte-enhancer factor 2, myogenic differentiation factor D, and Nkx2.5 (42). An important cluster of miRNAs in heart development is miR-17~92 cluster. Overexpression of miR-17 leads to growth retardation of various organs including the heart. MiR-17~92 cluster also affects myocardial differentiation of cardiac progenitors (43). In addition, miRNA-dependent therapeutic strategies, especially using miR-199a and miR-590, induce CM proliferation though stimulation of cell cycle re-entry without inducing CM apoptosis (44). On the other hand, miR-15

Table 2. miRNAs and antimiRs in cardiac regeneration

miRNA/ AntimiR	Effect on cardiomyocytes	Targets	References
miR-199a KO	Proliferation	Hopx, Homer1c	(40)
miR-590 KO	Proliferation	Hopx, Homer1c	(40)
mir-17-92 cluster KO	Proliferation	PTEN	(41)
miR-15 antimiR treatment	Proliferation	miR-15	(19
miR-133 KO	Inhibition of proliferation	Ccnd2, SRF, Hand2	(38)
miR-1 KO	Inhibition of proliferation	Hand2, PTEN	(38)
miR-15 KO	Inhibition of proliferation	Chek1, Arl2	(38)

Arl2 - adipose-ribosylation factor-like 2; Ccnd2 - cyclin D2; Chek1 - checkpoint kinase 1; Hand2 - heart and neural crest derivatives expressed 2; HOP homeobox; KO-knockout; miRNA/miR - micro ribonucleic acid; PTEN - phosphatase and tensin homolog; SRF – serum response factor

family downregulates cell cycle genes and induces cell cycle arrest postnatally (45). miR-15 family not only affects heart regeneration, but also regulates mitochondrial functions. For instance, overexpression of miR195 inhibits numerous mitochondrial and cell cycle genes. All of these effects suggest that repression of miR-15 family could lead to delay in CM mitotic arrest with administration of antimiRs (44).

Cardiogenic small molecules

Small molecules are chemically defined as low molecular weight organic compounds with upper limit of 900 Da. Given that many drugs are small molecules, they possess many advantages in terms of diffusion through the cell membrane, flexibility, ease of production and storage. In addition, one of the appealing characteristics of small molecules is lack of immune response against them. Thus, they are more conceivable than recombinant proteins or nucleic acid reagents. Small molecules rapidly influence a variety of cellular compartments in a reversible manner. They have been shown to modulate processes such as self-renewal, differentiation, and reprogramming mechanisms (46).

Small molecules demonstrate multiple effects by manipulating target proteins and modulating enzymatic activities and signaling pathways. The identification of small molecules has been accelerated with improved understanding of their cellular mechanisms. High throughput screening has been utilized to characterize small molecules for a specific phenotype or activity. For instance, Sadek et al. (47) characterized activators of cardiac phenotype in stem cells (Table 3) (13, 46–50). 5-Azacytidine (5-AzaC) has been widely used for CM differentiation of various cell types including glycolytic cardiac progenitors and Sca-1+ cardiac progenitors. In addition, 5-AzaC and oxytocin-treated Sca-1+ cardiac progenitors demonstrated an increase in expression of cardiac factors and spontaneous beating in vitro. 6-bromoindirubin-3'-oxime (BIO), defined as an inhibitor of glycogen synthase

Table 3. Cardiogenic small molecules

Small molecules	Effect on cardiomyocytes	Reference
BIO	GSK-3 inhibitor. Induces proliferation of mammalian cardiomyocytes	(48)
5-azacytidine	Induces cardiomyocyte differentiation of glycolytic cardiac progenitors	(13)
SAG, NBI-31772, SB-203580, and CHIR99021	Drive cardiomyocyte proliferation	Reviewed in (49)
Dorsomorphin	Inhibits the BMP signaling and induces cardiomyocyte differentiation in mouse ESCs	(50)
sulfonyl-hydrazone	Induces cardiac differentiation in M-PBMCs	(47)
RG108	Conversion of skeletal muscle stem cells into pluripotent state and use in cardiac regeneration	(46)

BIO - 6-bromoindirubin-3′-oxime; BMP - bone morphogenetic proteins; ESCs - embryonic stem cells, GSK-3 - glycogen synthase kinase 3; M-PBMCs - mouse peripheral blood mononuclear cells; SAG - smoothened agonist

Table 4. Approaches to cardiogenic small molecule discovery

Approaches	Method	Hurdles	Advantages
In silico	Molecular docking of small molecules to active site of target protein	Requires crystal structure of targeted protein	A large library of druggable small molecules may be computationally screened for in vitro verification
In vitro	Screening of small molecules against expressed target protein	Requires development of in vitro assay	A high throughput screening may be designed
Ex vivo	Screening of small molecules inducing neonatal rat CM proliferation	Neonatal proliferating CMs are used instead of adult CMs	Flow cytometric or fluorescent microscopy techniques may be used to determine proliferating CMs using markers such as Nkx2.5 and Phospho-H3
In vivo	Injection of small molecule into mouse	Costly. Requires use of a large number of animals	Provides in vivo stimulation effect of injected small molecules toward CM renewal

CM - cardiomyocyte

kinase-3 (GSK-3) pathway, is one of the most important small molecules that play a role in CM cell cycle. BIO induces maintenance of self-renewal in embryonic stem cells and proliferation of differentiated CMs (48). BIO treatment increases cardiac proliferation of rat neonatal CMs through stimulation of S phase entrance in dose-dependent manner, which increases expression levels of Ki67, cyclin D1, cyclin A, and also enhances activity of ß-Catenin. In search for cardiogenic small molecules, screening tests were applied to various heart models and 4 small molecules were identified: NBI-31772, an insulin-like growth factor; smoothened agonist (SAG), an activator of the smoothened protein that plays a role in Hedgehog signaling pathway; SB203580, an inhibitor of p83 mitogen-activated protein kinase; and CHIR99021, a GSK-3ß inhibitor. CHIR99021 was also used to generate primitive streak cells from human embryonic stem cells that would then undergo differentiation into CMs (49). Another small molecule, dorsomorphin, increases expression of cardiogenic markers such as Nkx2.5, troponin-T and Mhy6 (50). Various members of sulfonyl-hydrazone (Shz) family were applied on human mobilized peripheral blood mononuclear cells, and Shz-1 and Shz-3 treatments were found to stimulate expression of CM markers such as Nkx2.5 in a dose-dependent manner (47). Lastly, skeletal muscle stem cells (skeletal myoblasts) were reprogrammed into skeletal myoblast-derived induced pluripotent stem (SiPS) cells after treatment with RG108, a DNA methyltransferase inhibitor. Transplantation of simulated SiPS from skeletal myoblasts without any genetic modification induced repair in damaged myocardium (46).

Approaches to cardiogenic small molecule discovery

Development of therapeutics targeting CM renewal in the injured myocardium may be achieved with discovery of CM-specific cell cycle modulators (Table 4). Thus, recent studies have sought to determine cardiogenic small molecules that provide reactivation of cell cycle in adult heart. Integration of a variety of scientific approaches is required to identify cardiogenic small molecules. Candidate small molecules may first be identified using in silico methods. These methods rely on identification of active residues of target protein and utilization of molecular docking programs. However, crystal structure of target protein must be known for in silico screening approaches. Thus, target proteins that are druggable with small molecules may be computationally screened and selected hits may be further validated with in vitro protein or cell- based assays. Microarray and proteomics techniques may be utilized during validation and target identification. In addition, flow cytometric or fluorescent microscopy techniques may be used in ex-vivo neonatal CM cultures in order to

determine effects of small molecules on neonatal CM proliferation. However, target validations require preclinical studies in animals or humans. Ultimately, identified small molecules could be further validated in vivo using neonatal mouse cardiac regeneration model as well as adult myocardial injury models to assess effect on CM renewal and improvement of diastolic function.

Conclusion

CM renewal has been documented in adult mammalian heart, albeit inadequate for restoration of cardiac function following cardiac injury. Cardiac regeneration in zebrafish, newt, and neonatal mouse is associated with reactivation of CM cell cycle. Discovery of CM cell cycle modulators provided a new platform for development of cardiovascular therapeutics targeting CM cell cycle. Studies have demonstrated that CM cell cycle could be induced with small molecules.

Use of small molecules or miRNA to stimulate cardiac cell proliferation brings up questions regarding their involvement in the induction of tumor formation. It is noteworthy that small molecule treatments are designed to be short term; thus, their effect will be transient. During this period, if intended CM proliferation achieved, then small molecule treatment could be halted to avoid any side effects such as unwanted induction of cellular proliferation in other cell types or uncontrolled cell growth in other tissues. Small molecules must be mutagenic to cause cancer formation and tumuorogenicity in any tissue; however, it is possible that long-term exposure to such stimulating small molecules along with exposure to mutagens could lead to accumulated mutations in various cell types and eventually raise issues of tumor formation. More studies are needed to determine timing, dose, and route of administration of small molecules.

De novo CM proliferation and differentiation are thought to be a prospect for cardiac regeneration. Manipulations used for CM cell cycle modulation have yielded DNA synthesis, karyokinesis and cytokinesis in the heart to some extent. Inducible knockout systems used in adult mouse models further demonstrated that CM cell cycle re-entry may be achieved in adult mammalian heart. Discovery of small molecules that trigger and promote differentiation of stem cells into CMs and induce CM cell cycle re-entry brought further excitement for development of therapies targeting MI and HF. Overall, studies have proven feasibility of resident CMs and stem cell recruitment following therapeutic stimulation in heart regeneration.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – R.D.T., G.S.A., D.Y., R.D., F.K.; Design – F.K., R.D.; Supervision – F.K., R.D.; Data collection &/or processing – R.D.T., G.S.A., D.Y., R.D., F.K.; Literature search – R.D.T., G.S.A., D.Y., R.D., F.K.; Writing – R.D.T., G.S.A., D.Y., R.D., F.K.; Critical review – R.D.T., G.S.A., D.Y., R.D., F.K.

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