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MicroRNAs Inducing Proliferation of Quiescent Adult Cardiomyocytes

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Abstract

In the United States, each year over 700,000 people suffer from a heart attack and over 25% of deaths are related to heart disease, making it the leading cause of death. Following ischemic injury a part of the heart muscle is replaced by a scar tissue, reducing its functioning capacity. Recent advancements in surgical intervention and pharmacotherapy only provide symptomatic relief and do not address the root cause of the problem which is the massive loss of cardiomyocytes (CM). Therefore, the development of novel therapeutic intervention for the repair and regeneration of ischemic myocardium remains an area of intense research. While existing CM in zebra fish and neonatal mice are known to proliferate and replenish the infarcted heart, it has been shown that adult mammalian CM lose this ability, thus preventing regeneration of the scar tissue. There have been many attempts to facilitate regeneration of ischemic heart but have met with limited success. Micro-RNAs (miRNAs) are one of the promising candidates towards this goal as they are known to play important regulatory roles during differentiation and tissue regeneration, and regulate genetic information by post-transcriptional modification as well as regulation of other miRNAs. While previous work by Eulalio *et al*., showed miRNAs inducing proliferation in neonatal CM (NCM), we here identify miRNAs inducing proliferation of rat adult-CM (ACM). This commentary while analyses recent work by Eulalio *et al* [1] also shows some new data with microRNAs in rat adult-CMs. Further work into the mechanism of these miRNAs can determine their therapeutic potential towards regenerating cardiac tissue post ischemic injury.

Keywords

MicroRNA; Cardiomyocytes; Adult Cardiomyocytes; Myocardial Infarction; Cardiovascular Diseases

Cardiovascular Disease (CVD) and Ischemic Heart Failure

According to the NIH, cardiovascular disease is the biggest killer in the United States. Each year, a quarter of total lives lost are related to heart disease and over 700,000 people suffer from heart attacks which are distributed equally throughout ethnicities [2]. CVDs claim over

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17 million lives worldwide [3]. While the life expectancy in general has gone up over the last 50 years and was reported at a record high of 78.7 years in 2010, deaths related to “diseases of heart” have not decreased with the same rate [4]. In addition, in the United States alone cost associated with heart diseases exceed over $100 billion, annually [5] and there are about 700,000 new cases of heart attack each year; leaving those hearts with a large portion of dead cells causing a non-functional scar tissue. A major contributor leading to scar formation is ischemic injury, either acute or chronic like in coronary heart disease. An inflammation due to CVD narrows the arteries, allowing less blood and oxygen to reach the myocardium. This continued deprivation of blood flow causes ischemia and is often noticed with angina pectoris [6]. Substantial loss of CM leads to ventricular remodeling and wall thinning which are followed by decrease in pumping efficiency, leading to heart failure and even death [7, 8]. The biggest killer of humans in the 21st century needs to be dealt with highest and most intense research and by translating successful laboratory results from bench to bed-side.

Inadequate Therapeutic Options Post Ischemic Injury

While there has been intensive research in the field of regenerative medicine to regenerate the infarcted myocardium, it has met with limited success. Current first line of treatment for cardiovascular disease includes therapeutics like beta-blockers, diuretics, angiotensin-converting enzymes (ACE), and surgically placing a pacemaker or defibrillator. However, these options neither restore the lost CM, nor prevent ventricular wall thinning but rather results in overstretching of limited CM to meet the cardiac requirement, eventually leading to heart failure [7, 8]. All approaches from reprogramming the resident cardiac stem cells, mobilizing bone marrow cells to replenish the scarred region, to delivering cells directly to the heart have been extensively researched; however the improvements have been subpar [7, 9, 10]. Moreover when using stem cells, a need for an autologous match is also a concern. On the other hand using embryonic stem cells always raises ethical concerns and even when used their ability to differentiate into nonspecific cell-types and forming a tumor is a potential risk [8]. In addition, current embryonic stem cell therapy may also require immunosuppression, raising other concerns and side-effects associated with it [11]. Moreover, it has been shown that as much as 90% of transplanted cells do not survive long enough to regenerate the myocardium, due to hypoxic environment and inflammatory cytokines in the host tissue [8]. This led us to investigate the field of miR mediated proliferation of resident cardiomyocytes.

Limited Proliferation of Adult Cardiomyocytes

While CMs in zebra fish and neonatal mice are shown to proliferate and rejuvenate the infarcted heart, this capacity is diminished in adult mammals [12–14]. The proliferative capacity of NCM is maintained for a few days after birth and while there are reports of cardiogenesis in adult mammals, it is a rare phenomenon [15]. CMs maintain this regulation of cell cycle by altering the levels of cell-cycle regulatory genes, namely cyclin-CDK complex expressions, which were observed to be almost undetectable two weeks after birth [16]. However challenging this dogma recent studies using pulse-chase experiments have shown that the rate of CM turnover in young adults is 0.76% per year and it declines

with age but is shown to increase after myocardial injury. This study also proposes that myocardial homeostasis during health and injury is maintained by division of pre-existing cardiomyocytes and not through cardiac progenitors [17].

GenesInvolved in Cardiomyocytes Proliferation

Several groups have identified genes involved in cardiomyocyte cell cycle reactivation. Genes like neuregulin1 (through ErbB2/4 receptor) and FGF-1 (with inhibition of p38) [18, 19], and Meis1 deletion [20] have been shown to induce proliferation in otherwise quiescent CM. Cyclin-A2 (Ccna2) is silenced shortly after birth in mammalian CM and is one of the key players of cell cycle regulation. It is known to mediate G1-S and G2-M transitions and a number of studies have demonstrated that Ccna2 induces proliferation of cardiomyocytes following myocardial ischemia [21, 22] Additionally, Liu *et al*., reported that in the absence of miRNA-133a increased levels of Cyclin-D2 and SRF transcription factor initiates CM proliferation [23]. Interestingly, Eulalio *et al*., in their breakthrough finding of miRNAs that induce proliferation of neonatal CM, also identified several downstream genes including Homer1, Hopz, and Clic5. However none of these genes increased proliferation as robustly as observed with miR-590-3p and miR-199a-3p indicating CM proliferation is mediated through cumulative effect on multiple targets [1]. In this study we have identified miRNA that are specific for the induction of adult cardiomyocyte proliferation.

MicroRNA's Regulatory Roles

Since their discovery in *C. elegans* in 1990's, these small 21–25 nucleotide long, non-coding, single stranded ribonucleic acids have been shown to regulate gene expression in the most complex of life forms on earth [24, 25]. Majority of miR genes are located in the introns of protein-coding as well as non-coding genes and are matured through two steps of regulation. An RNAse III enzyme, DROSHA cleaves a long 'pri-miRNA' into a ~70 nucleotide long 'pre-miRNA', which can now be exported to cytoplasm by 'exportin-5'. This pre-miRNA gets cleaved by yet another RNAse III enzyme, DICER. This second cleavage leaves the small double stranded RNA only ~20 nucleotide long which is then processed and only one strand (guide strand) gets into a miRNA-induced silencing complex (miRISC) while the complementary passenger strand gets degraded (with exceptions of sometimes also binding the RISC complex) [26–28]. Mature miR can now bind without a perfect complementarity to the 3′ untranslated region (UTR) of its target mRNA, and repress gene expression [29]. Thus, it is clear that miRNAs can regulate gene expression and by repressing the repressor can also up-regulate their expression. There have been recent reports of miRNAs regulating other miRNAs through indirect regulation [30]. This complex mechanism of miR mediated regulation of biological processes therefore involves multiple steps of regulation and elucidating this mechanism could lead to improved therapeutics for a wide variety of biological disorders.

MicroRNAs inducing proliferation

MiRNAs have been shown to regulate key mechanisms involved in cell proliferation, apoptosis, autophagy, and other cell signaling pathways [1, 31–35]. Recent studies have identified of miRs involved in regulating cardiac regeneration [30], direct reprogramming of

cardiac fibroblast into CM [36], and metastatic cancers through their downstream mRNA targets [37]. Studies by Eulalio *et al* identified a panel of miRNAs associated with proliferation of NCM. From this panel we choose the top 25 NCM proliferation inducing miRs to identify their role in inducing ACM proliferation. We hypothesized that the rate and percentage of proliferation induction of these miRs will differ between NCM and ACM. Briefly, ACM from 8–10 week old rats were transfected with 50nM concentration of specific miRNA mimics (Life Technologies NY, USA), followed by replacing the medium 24 hour later with 5% FBS DMEM + 5μM EDU (5-ethynyl-2′-deoxyuridine; Life Technologies NY, USA) up to day 6. On day 7, cells were fixed in 4% PFA (paraformaldehyde) and immuno-staining was performed. Troponin-I (Santa Cruz Biotechnology, Texas, USA) was used as CM marker along with DAPI (4′,6-diamidino-2-phenylindole) for nuclear staining, as per standard protocol. As shown in table 1 these miRNAs induced proliferation of up to 22% in adult rat cardiomyocytes (as measure by EDU uptake). We saw a significant increase in proliferations with miR-1825, miR-199a-3p, miR-99a-5p, miR-548c-3p, miR-23b-3p, and many others in ACM. Additionally, we selected top five miRNAs from this list and measured phosphor-Histone-H3 (ser10), which is a marker of cells undergoing mitosis. As shown in table 1.2 these miRNAs showed a significant increase in p-Histone-H3 levels (compared to control miRNA). This confirmed that these miRNAs not only cause ACM to proliferate but also re-introduce them into the cell cycle, as a significant increase in mitotic marker was observed. Table 1 shows a full list of miRNAs tested and their corresponding proliferation percentage in ACM.

Mechanism of Action

Although we have shown these miRNAs to induce proliferation in adult CM, a thorough study to elucidate the mechanism of action is still required. However, an increased proliferation in CM was evident with a significant increase in EDU incorporation and p-Histone-H3 in actively proliferating and dividing ACM. Interestingly, one of the much known mechanisms of miRNA's action is through its binding to the untranslated region (UTR) at the 3′ end of the messenger RNA (mRNA), and perhaps it increase proliferation by altering the cell cycle genes. However, an exact mechanism of action for these miRNAs is still a big question and further work would determine critical genes involves.

Discussion and Future Directions

Previous attempts and approaches like homing bone marrow stem cells to the ischemic heart, injecting stem cells into the heart, and attempting to inject CM in the heart have limitations. While stem cells have to be autologous, can migrate to other tissues, and have adverse side effects like arrhythmia; 90% of cells injected in the heart are lost within few hours [7, 8, 11]. To address the issues associated with cell based therapy alternative approaches involving the proliferation of host cardiomyocytes surrounding the infarct zone and thereby regenerating the cardiac tissue looks promising. Towards this goal studies by Shapiro *et al* [38] have identified approaches for cardiac regeneration therapies by regulating the expression of a cell cycle protein Ccna2. As shown by Shapiro *et al* Ccna2 promotes cardiomyocyte mitoses, increases cardiomyocyte number and decreases fibrosis in a porcine myocardial ischemia model. Recent study by Puente *et al* [39] identified a mechanism for

prolonging the proliferative window in a postnatal cardiomyocyte through reduction of mitochondrial-dependent oxidative stress and oxidative DNA damage. Further studies need to be done to determine if quiescent cardiomyocytes can be brought back into cell cycle through this approach. In a study involving human patients with persistent postnatal cardiomyocyte replication Shenje *et al* [40] identified mutation in ALMS1 leading to deficiency of Alstrom protein and as a result impairing postnatal cardiomyocyte cell cycle arrest. The belief that postnatal cardiomyocytes are quiescent is challenged by a recent study by Naqvi *et al* [41] which identifies a one-time proliferative burst of cardiomyocyte at a small preadolescence window resulting in an increase in cardiomyocyte number by approximately 40%. Given all these ground breaking studies and the recent advances in identifying the role of miRNA in cell cycle regulation the approach of using miRNAs to promote cardiomyocyte proliferation can overcome the problems associated with cell transplantation. Since miRNAs can be readily available and can be used off the shelf along with a longer shelf life. Therefore, this study is important as it identifies miR that can cause ACM to proliferate and thereby have the potential to regenerate the lost functioning in a post-ischemia heart. In addition, more work to elucidate a mechanistic understanding of miRNA's mode of action is an ongoing an area of immense focus.

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Table 1.1

Proliferation inducing miRNAs; Table shows specific miRNAs with their respective percent proliferation of ACM

Micro-RNA	Proliferating ACM (%)
Cel-miR-67(control)	0.71±0.39
hsa-miR-1825	21.99± 1.19*
hsa-miR-199a-3p	16.71±2.38*
hsa-miR-99a-5p	14.64±3.15*
has-miR-548c-3p	11.99±1.81*
hsa-miR-23b-3p	12.71±0.87*
hsa-miR-495-3p	3.34±1.71*
hsa-miR-518f-3p	5.31±0.24*
hsa-miR-885-5p	5.29±0.28*
hsa-miR-302d-5p	5.52±1.14*
hsa-miR-498	6.82±1.75*
hsa-miR-1910	9.85±0.70*
hsa-miR-302b-5p	7.85±0.56*
hsa-miR-518c-3p	6.18±0.60*
hsa-miR-2053	10.15±0.41*
hsa-miR-1182	5.67±1.82
hsa-miR-513c-5p	2.35±1.44
hsa-miR-936	11.67±2.13*
hsa-miR-1224-3p	6.51±1.00*
hsa-miR-455-3p	5.76±1.15*
hsa-miR-1322	5.12±2.74
hsa-miR-1260a	3.75±0.93
hsa-miR-509-3p	7.16±0.65*
hsa-miR-361-3p	1.42±0.32
hsa-miR-590	11.59±0.89*

ACM transfected with specific miRNAs were measured for percent proliferation using EDU (5-ethynyl-2′-deoxyuridine).

Table 1.2

Proliferation inducing miRNAs; Table shows specific miRNAs with their respective percent proliferation of ACM

Micro-RNA	Proliferating ACM(% p-H3)
Cel-miR-67(control)	0.67±0.61
hsa-miR-1825	11.67±2.76*
hsa-miR-199a-3p	6.33±2.42*
hsa-miR-99a-5p	4.15±0.80*
hsa-miR-23b-3p	3.27±0.78*
hsa-miR-548c-3p	2.51±0.71*

Top five proliferation inducing miRNAs were used to measure percent of ACM positive for phospho-Histone H3 (Ser10) (p-H3) to measure active mitosis. N≥3;

* $p<0.05$.