The Next Regeneration: Identifying Cardiac Regeneration–Inducing MicroRNAs Using Functional Screening

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Study Hypothesis

As medical and interventional therapies improve, more patients are surviving myocardial infarction. As a consequence, the prevalence of patients surviving with compromised cardiac function is increasing, leading to significant morbidity and mortality. Unlike most organs in the body, it has long been accepted that the myocardium cannot regenerate in the adult because cardiomyocyte proliferation rapidly ceases after birth. Although this prevailing view has gradually given way to one that cardiomyocytes do have a modest capacity for regeneration, the factors that regulate these cells from entering the cell cycle have remained elusive. Eulalio et al hypothesized that microRNAs (miRNAs) serve as molecular regulators that maintain cardiomyocytes in quiescence and that manipulation of these regulators could improve cardiac regeneration and thus function.

How Was the Hypothesis Tested?

The investigators used an unbiased functional screening approach for miRNAs able to induce reentry of cardiomyocytes into the cell cycle. Cultures of neonatal rat cardiomyocytes were transfected with a whole-genome miRNA library of 875 mimics and stained for proliferative markers Ki67 and 5-ethynyl-2'-deoxyuridine (EdU) and cardiomyocyte marker α-actinin, followed by high-throughput microscopy. miRNAs that increased neonatal rat cardiomyocyte proliferation 2-fold were selected and then also tested in the mouse. The investigators next selected miRNAs to test in postnatal cardiomyocytes isolated from 7-day-old and 2-month-old rats. To confirm the capacity of the miRNAs to induce cell cycle reentry, the authors measured the expression of genes related to cardiac differentiation and proliferation. Next, to identify miRNA targets, they assessed global transcriptome changes by deep sequencing neonatal cardiomyocyte RNA after miRNA transfection. Among the downregulated genes, using short interference RNA (siRNA), they individually knocked down targets to assess specificity. To assess the in vivo use of 2 highly selected miRNAs, hsa-miR-590-3p and has-miR-199a-3p, miRNAs were injected directly into the heart of neonatal rats coadministered EdU and assessed for proliferation in the short term. In addition, to assess the effects of continuous miRNA expression on cardiomyocyte proliferation, the investigators generated adeno-associated virus 9 vectors expressing hsa-miR-590 and has-miR-199a and injected them directly into the left ventricle of neonatal hearts. Finally, to assess the therapeutic potential of these miRNAs, adeno-associated virus 9 was injected into the peri-infarct zone of mice that underwent left anterior descending coronary artery ligation, followed by examination of functional parameters by echocardiography and infarct size by histology.

Principal Findings

High-content, fluorescence-microscopy–based functional screening of 875 miRNAs transfected to neonatal rat cardiomyocytes identified 204 miRNAs that increased proliferation >2-fold without significantly affecting cell hypertrophy or viability.

Of these 204 miRNAs, 40 miRNAs also enhanced proliferative capacity at least 2-fold in mice. The 10 miRNAs that increased proliferation the most in both rat and mouse screens demonstrated increased staining for karyokinesis and cytokinesis markers histone H3 phosphorylated on serine 10 (H3S10ph), a marker of late G2/mitosis, and Aurora B kinase localization in midbodies, transient structures formed during cytokinesis, in addition to proliferative markers in neonatal cardiomyocytes without increases in fibroblast proliferation. Selected miRNAs, hsa-miR-590-3p, hsa-miR-199a-3p, hsa-miR-1825, and hsa-miR-33b, increased proliferation by up to 20% with corresponding increases in expression of β-myosin heavy chain, DAB2, destrin, and RUNX1 in 7-day-old postnatal cardiomyocytes, which showed almost undetectable proliferative capacity at baseline. Furthermore, when the miRNAs hsa-miR-590-3p and hsa-miR-199a-3p were transfected into 2-month-old fully differentiated rat cardiomyocytes, there was a time-dependent reentry of the cells into the cell cycle, leading to increased proliferation. Deep sequencing of neonatal cardiomyocytes transfected with hsa-miR-590-3p and hsa-miR-199a-3p identified 1056 upregulated transcripts rich in cell cycle, cellular growth, and cellular proliferation and DNA replication, recombination, and repair gene categories, as well as 697 downregulated.
transcripts rich in genes belonging to the skeletal and muscular system development and function and cellular assembly and organization categories. Selective inhibition of 601 of the downregulated genes by siRNA identified that 45 of these genes increased proliferation 2-fold, of which 5 siRNAs targeted transcripts downregulated by hsa-miR-590-3p, 43 by hsa-miR-199a-3p, and 3 by both. No siRNA induced increased proliferation to levels observed by miRNAs, suggesting that these miRNAs had cumulative effects on multiple targets. In vivo, injection of these miRNAs complexed to lipid transfection agents directly into the hearts of neonatal rats resulted in a marked increase in EdU-positive cells, confirmed to be cardiomyocytes, and increased left ventricular thickness after 4 days. Intraperitoneal injection of adeno-associated virus 9 vectors expressing hsa-miR-590-3p and hsa-miR-199a-3p resulted in overexpression of these miRNAs in both neonatal and adult animals and corresponding increases in numbers of proliferating cardiomyocytes, many of which showed H3S10ph positivity. Finally, when CD-1 mice that underwent left anterior descending artery ligation were injected with the 2 miRNA viral vectors in the peri-infarct zone, infarct size was significantly reduced, with evidence of EdU-positive cells in the peri-infarct zone 60 days after injection. Furthermore, mice injected with the active viral vectors showed improved left ventricular ejection fraction, improved fractional shortening, and increased wall thickness 12, 60, and 90 days after injection as assessed by echocardiography.

Implications
The authors demonstrate for the first time that miRNAs can induce reentry of postnatal cardiomyocytes into the cell cycle and that exogenous administration of these miRNAs can restore cardiac mass and promote a functional recovery after myocardial infarction. This study serves as a prime example of the advantages of unbiased genetic approaches. Using a library of nearly 900 synthetic miRNAs, the authors were able to probe an estimated 40% of the proteome and to identify >200 potential candidates, of which 40 showed cross-species reactivity. Deep sequencing of the changes that occurred with specific miRNAs demonstrated a skeletal and muscular system development and function, as well as cellular assembly and organization signature, among down-regulated genes. Specific transcripts were interrogated with siRNA that could only partially reproduce the effects of the miRNA, suggesting multiple downstream protein targets of the miRNAs. These findings highlight the complexity of cell quiescence and suggest the potential use of systems-based approaches to fully understand these complex pathways. This study represents an important step forward in identifying the self-renewal mechanisms in the adult heart and suggests that miRNAs are major regulators of this process.

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Disclosures
None.

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