Cellular Biology

MicroRNA-Mediated In Vitro and In Vivo Direct Reprogramming of Cardiac Fibroblasts to Cardiomyocytes

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Rationale: Repopulation of the injured heart with new, functional cardiomyocytes remains a daunting challenge for cardiac regenerative medicine. An ideal therapeutic approach would involve an effective method at achieving direct conversion of injured areas to functional tissue in situ.

Objective: The aim of this study was to develop a strategy that identified and evaluated the potential of specific micro (mi)RNAs capable of inducing reprogramming of cardiac fibroblasts directly to cardiomyocytes in vitro and in vivo.

Methods and Results: Using a combinatorial strategy, we identified a combination of miRNAs 1, 133, 208, and 499 capable of inducing direct cellular reprogramming of fibroblasts to cardiomyocyte-like cells in vitro. Detailed studies of the reprogrammed cells demonstrated that a single transient transfection of the miRNAs can direct a switch in cell fate as documented by expression of mature cardiomyocyte markers, sarcomeric organization, and exhibition of spontaneous calcium flux characteristic of a cardiomyocyte-like phenotype. Interestingly, we also found that miRNA-mediated reprogramming was enhanced 10-fold on JAK inhibitor I treatment. Importantly, administration of miRNAs into ischemic mouse myocardium resulted in evidence of direct conversion of cardiac fibroblasts to cardiomyocytes in situ. Genetic tracing analysis using Fsp1Cre-traced fibroblasts from both cardiac and noncardiac cell sources strongly suggests that induced cells are most likely of fibroblastic origin.

Conclusions: The findings from this study provide proof-of-concept that miRNAs have the capability of directly converting fibroblasts to a cardiomyocyte-like phenotype in vitro. Also of significance is that this is the first report of direct cardiac reprogramming in vivo. Our approach may have broad and important implications for therapeutic tissue regeneration in general. (Circ Res. 2012;110:1465-1473.)

Key Words: direct reprogramming ■ cardiac differentiation ■ microRNAs ■ tissue regeneration

Response to cardiac injury is characterized by inadequate cardiomyocyte regeneration and excessive fibrosis, resulting in significant impairment of tissue structure and function. Despite major developments in cardiac regenerative medicine, repopulation of the injured heart with new, functional cardiomyocytes remains a daunting challenge. Recently, landmark reports describing the reprogramming of somatic cells to induced pluripotent stem (iPS) cells have raised the possibility of using cellular reprogramming for cardiac tissue regeneration.1,2 These include directed cardiogenesis in vitro, of partially reprogrammed fibroblasts,3 as well as transplantation of the ischemic heart with undifferentiated iPS cells.4 However, concerns about the maturity and functional heterogeneity of stem cell–derived cardiomyocytes,5 the low survival and retention of delivered cells, as well as their potential tumorigenicity still exist.5,6 Some of these limitations could be overcome by direct reprogramming of somatic cells into cardiomyocytes, obviating an intermediate pluripotent stage. Indeed, a recent report from Ieda et al describes the use of 3 cardiac transcription factors (Gata4, Mef2c, and Tbx5) to reprogram mouse fibroblasts directly into cardiomyocytes.8 Importantly, there is no evidence reported as yet, for direct reprogramming in vivo.

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A promising alternative strategy for direct reprogramming is the use of microRNAs (miRNAs).9 The repressive actions

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of miRNAs on gene expression can be powerful as a single miRNA may target multiple pathways simultaneously. Chemically synthesized miRNA mimics are easily administered to cells via lipid-based transfection and exhibit low toxicity in animal models. Moreover, the small size of a single miRNA allows the packing of multiple transcripts in the same delivery vector to increase both reprogramming efficiency and functional homogeneity of reprogrammed cells.

In the present report, we present the first evidence of miRNA-mediated direct conversion of fibroblasts to the cardiomyocyte lineage, in vitro and in vivo. As such, our data show that mouse fibroblasts can be directly converted to a cardiomyocyte-like phenotype in vitro by a single transient transfection using a distinct combination of miRNAs (miRNAs 1; 126 to 3p; 133; 138, 206, 208a) enhanced further by JAK inhibitor I treatment. Reprogrammed cells express cardiomyocyte-specific genes and proteins and additionally exhibit sarcomeric organization, spontaneous calcium oscillations, and mechanical contractions characteristic of a cardiomyocyte-like phenotype. Importantly, studies using genetically traced fibroblasts also suggest that such conversion can be achieved directly in the injured myocardium after miRNA delivery. Together, our work provides insight into the role miRNAs may play in cardiac reprogramming biology as well as a novel and potentially more efficient method of achieving cardiomyocyte regeneration in situ.

Methods

An expanded Methods section is available in the Online Data Supplement.

MiRNA, Transfections, and Quantitative Real-Time PCR

Synthetic mimics of mature miRNAs (Ambion/Applied Biosystems) were introduced into cells via transient transfection with Dharmafect1 (ThermoScientific), using standard conditions and at a final concentration of 50 nmo/L. As a negative control, we used a random sequence nontargeting mimic (negmiR) miRNA (Ambion/Applied Biosystems). Total RNA was isolated using RNeasy-96 (Qiagen) and PicoPure RNA isolation kits (Applied Biosystems). JAK inhibitor I (EMD Biosciences) treatment (final concentration, 1 nmo/L) was initiated 2 days after transfection and continued daily for 5 to 8 days. Quantitative expression of each gene was assessed using Taqman Gene Expression Assays on the StepOnePlus Real-Time PCR System (Applied Biosystems).

Immunocytochemistry, Microscopy, and FACS

Cells were fixed in 4% paraformaldehyde and stained using the after primary antibodies α-actinin (Sigma), myosin heavy chain (MHC) (Developmental Studies Hybridoma Bank and Abcam), cardiac troponin I and T (Abcam), CD45 (BD Biosciences), Ckit (R&D), smooth muscle actin (Sigma), vimentin (Abcam), Ddr2 (R&D), and RFP (Rockland Immunochemicals). Confocal images were captured using an LSM 510 Meta DuoScan microscope (Zeiss) and processed using LSM 5 software, version 4.2. Images of cyan fluorescent protein (CFP) expression were captured using the AxioObserver wide-field fluorescence microscope (Zeiss). Three-dimensional images were reconstructed using Imaris software (Bitplane) of 20× confocal images, using the microscope mentioned above. FACS sorting was performed using a FACStar Plus cytometer (BD Biosciences). All FACS samples were gated against an untransfected cell control 7 to 10 days after transfection with miRNAs. The CFP+ cell population generated by FACS was first tested for the presence of Myh6 (because CFP is driven by the α-MHC promoter), using quantitative real-time PCR before evaluating for the expression of other genes.

Calcium Imaging and Contractility

Ca2+ signals in cardiac fibroblasts and myocytes were imaged using Fura-2, according to previously published protocols (see the Online Data Supplement for additional methods).

In Vivo Studies

Adult male fibroblast-specific protein 1 (Fsp1)Cre-ttdTOMATO transgenic mice were subjected to ligation of the left ascending coronary artery, using previously published procedures. Lentiviral miRNAs (2×10⁶ pfu in 40 μL) were injected once at the time of injury, at 2 sites 2 mm below site of ligation. Hearts were harvested after 4 weeks, fixed, paraffin-embedded, and stained with anti-RFP (Rockland Immunochemicals) and anti–cardiac troponin T (Abcam).

Results

Based on the potential of miRNAs for regulating cellular and developmental processes, we hypothesized that they may be capable of redirecting cardiac cell fate. In the present study, we developed a strategy to identify specific miRNAs capable of reprogramming cardiac fibroblasts directly to the cardiomyocyte lineage. We selected 6 candidate miRNAs, based on their roles in cardiac muscle development and differentiation. Synthetic, chemically modified mimics of miRNAs 1; 126 to 3p (126); 133a (133); 138, 206, 208a (208) were introduced into mouse cardiac fibroblasts via transient transfection, alone and in all possible double and triple combinations with each other, resulting in a total of 41 different compositions (Figure 1A). These were initially evaluated for their reprogramming potential by screening for induction of markers of cardiac differentiation in MEFs, neonatal and adult cardiac fibroblasts at 3 and 6 days after transfection, using quantitative real-time PCR (Figure 1A through 1C and Online Figure II). Markers included previously characterized cardiac reprogramming markers (Gata4, Mef2c, and Tbx5, as well as later stage mature cardiomyocytes markers such as Tnni3 (cardiac troponin I). To ensure that our starting population of cardiac fibroblasts was not...
Figure 1. Introduction of microRNA(s) into cardiac fibroblasts induces the expression of cardiac myocyte-specific markers. A, Cumulative gene expression data from miRNA-transfected adult cardiac fibroblasts are illustrated graphically in heat map form. These results depict a major shift in fibroblastic (Ddr2 and Vim) to early cardiomyocyte (Isl1, Nkx2.5, Gata4, Hand2, Mef2c) and late cardiac myocyte (Tnni3, Cx43) marker expression between 3 and 6 days after transfection. Fold change in normalized gene expression for each gene is shown on a green to black to red scale (minimum, green; maximum, red). Detailed color legends describing quantitative fold change data for each gene are represented in Online Figure XX. B, Representative bar graphs showing RNA expression profiles of...
contaminated with cardiomyocytes, we subjected our preps to Percoll gradient centrifugation to remove the cardiomyocyte fraction and further confirmed by FACS analysis that contamination by cardiomyocytes or c-Kit+/sca-1+ progenitor cells is insignificant (0.01% to 0.04%, data not shown).

Our results showed that as early as 3 days after transfection, miRNA-transfected cells exhibited a major shift in expression from fibroblastic genes such as Vimentin (Vim) and Ddr2 to cardiomyocyte-specific genes (Figure 1A). Additionally, controls (nontargeting miRNA (negmiR), mock, and untransfected cells, including the transfection of fibroblast-enriched miR-21, did not activate cardiomyocyte markers. Interestingly, gene expression data were clustered into defined groups where distinct miRNA compositions appeared to regulate the expression of stage-specific markers of cardiac differentiation. This approach identified miRNA combinations that consistently induced a fibroblast-cardiomyocyte phenotypic shift in both neonatal and adult cardiac fibroblasts (Figure 1A through 1C). Top candidates identified included miR-1 alone; miRs-1, 133, 206; miRs-1, 133, 208; and miRs-1, 133, 208, 206; miR-1, 133; and miRs-1, 138, 208.

To further validate these findings, we performed immunostaining on miRNA-transfected neonatal and adult cardiac fibroblasts for induced cardiomyocyte-specific markers, including MHC, Cardiac troponin I (TNNI3), and α-ACTININ. As shown in Figure 1D and 1E and Online Figures III and IV, immunostaining on adult and neonatal cardiac fibroblasts revealed that top miRNA candidates induced protein expression of MHC, TNNI3, and/or α-ACTININ as early as 6 days after transfection. Complementary experiments conducted using fibroblasts isolated from transgenic mice expressing α-myosin heavy chain–driven cyan fluorescent protein (αMHC-CFP) revealed that αMHC was activated as early as 4 days after transfection (Online Figure V). Moreover, transient transfection of neonatal cardiac fibroblasts isolated from double-transgenic mice carrying both the Fsp1-driven Cre recombinase gene25 and a floxed Ddr2–myosin heavy chain–driven cyan fluorescent protein (MHC-CFP) revealed that MHC was activated as early as 4 days after transfection (Online Figure VI). We chose to focus on miR-1 alone and the combination miRs-1, 133, 208, due to their overall potential at cell conversion and cardiac specificity. Interestingly, inclusion of myosin-regulated miR-49927 in the miRNA combination 1, 133, 208 did not change reprogramming efficiency as measured by CFP expression (Figure 2A). However, it did appear to increase the levels of cardiomyocyte marker expression (Figure 2B). Indeed, quantitative real-time PCR analysis of the CFP+ selected cells from fibroblasts transfected with miRs-1, 133, 208, 499 revealed a significant increase in expression of the cardiac developmental marker MeF2c and that of the late-stage marker, Tnni3. Overall, the above results provide strong evidence for the capacity of miRNAs to induce expression of cardiomyocyte markers in cardiac fibroblasts.

The data thus far have demonstrated that miRNAs are capable of inducing cardiomyocyte marker expression in fibroblasts albeit at low efficiency. Because it has been reported that small molecules might enhance cell conversion differentiation, we decided to investigate their effects on miRNA-induced cardiac reprogramming.17,28,29 Interestingly, Efe et al3 subjected partially reprogrammed mouse embryonic fibroblasts to sequential addition of JAK inhibitor I and BMP4 treatment to activate the cardiac progenitor program that yielded beating cardiomyocytes within 3 weeks. To evaluate the potential of this approach, neonatal cardiac fibroblasts from αMHC-CFP mice were treated with miRNAs and JAK inhibitor I. Cells were assayed by FACS for enhancement of a CFP+ cell population over transfection with miRNAs alone. Transfection with these miRNA compositions resulted in induction of CFP expression in approximately 1.5% to 7.7% of fibroblasts (Figure 2A). However, additional treatment with JAK inhibitor I dramatically shifted the profile of CFP+ cells (up to ~28% of CFP+ cells) and increased the efficiency of reprogramming by approximately 8 to 10 fold (Figure 2A and 2C). None of the other compounds tested seemed to enhance miRNA-induced reprogramming efficiency (data not shown). Remarkably, the combination of miRs-1, 133, 208, 499 plus JAK inhibitor I treatment also led to a significant elevation of ion channel expression in CFP+ cells resulting in an almost 10-fold increase in expression of the L-type calcium channel Cacna1c, when compared with non-JAK inhibitor I–treated miRNA-transfected cardiac fibroblasts (Figure 2D and Online Figure VIII).

Figure 1 (Continued). Gata4 and MeF2c in neonatal cardiac fibroblasts at 3 days after transfection. Highlighted are top miRNA combinations 1, 133, 206 and 1, 133, 208. All miRNA combinations represented by light gray bars. Dark gray bar represents averaged control values. Scale bar, 100 μm. A, TNNI3 (in green) immunostaining of DAPI-positive (blue) neonatal cardiac fibroblasts 1 week after transfection with miR-1; miRs-1, 133, 206; miRs-1, 133, 208; and miRs-1, 133, 206, 208. Scale bar, 100 μm. B, Representative scatterplot showing the geometric mean of normalized expression of Gata4, MeF2c, and Tbx5 at 3 days after transfection. Highlighted are miRs-1, 133, 206 (red); and miRs-1, 133, 206, (purple); mock, negmiR, and untransfected controls (green); remaining miRNA combinations (black). C, Representative scatterplot showing the geometric mean of normalized expression of Gata4, MeF2c, and Tbx5 at 3 days after transfection. Highlighted are miRs-1, 133, 206; miRs-1, 133, 208; and miRs-133, 206, 208. Scale bar, 100 μm. D, α-ACTININ (in green) immunostaining of DAPI-positive (blue) neonatal cardiac fibroblasts 1 week after transfection with miR-1; miRs-1, 133, 206; miRs-1, 133, 208; and miRs-133, 206, 208. Scale bar, 100 μm. E, TNNI3 (in green) immunostaining of DAPI-positive (blue) neonatal cardiac fibroblasts 1 week after transfection with miRs-1, 133, 208. Zoomed in area highlights the presence of prominent striations in TNNI3+ cells. F, TNNI3 (in green) immunostaining of DAPI-positive (blue) neonatal cardiac fibroblasts isolated from Fsp1Cre/dTomato (red) mice, 4 weeks after transfection with miRs-1, 138, 208. Scale bar, 100 μm.
To further validate the functional consequences of the above observations, we analyzed calcium signaling, excitation-contraction coupling, and spontaneous contraction. CFP+ cells were treated with miRNA combinations versus control, all in the presence of JAK inhibitor I, for up to 4 weeks after FACS (Figure 3). Spontaneous contraction was observed as early as 10 days in culture; however, this was sporadic (1% to 2% of total cell population). Rhythmic calcium oscillations were also often observed in miRNA-transfected cells by 14 days after FACS but were only rarely observed in control cells (negmiR and untransfected fibroblasts). Both the incidence and oscillation frequency of spontaneous calcium oscillations in miRNA-transfected fibroblasts increased with time in culture (not shown), suggesting an increase in the state of maturation. The average oscillation frequencies were significantly faster in CFP+ cells derived from fibroblasts transfected with miR-1 and miRs-1, 133, 208, 499 (1.31±0.31 oscillations/min; range: 0.15–4 oscillations/min and 1.54±0.14 oscillations/min; range: 0.1–3.15 oscillations/min, respectively) than in controls (0.45 and 0.6 oscillations/min) and reached about 50% of the average frequency observed in cultured neonatal cardiomyocytes (2.93±0.13 oscillations/min; range: 1–12 oscillations/min) (Figure 3A and 3B). There was also a substantial increase in the number of cells exhibiting oscillations in CFP+ reprogrammed cells (Figure 3C). Moreover, both the average oscillation frequency and the number of oscillating cells detected were greater in CFP+ cells derived from miR-1, 133, 208, 499–transfected fibroblasts than those transfected with miR-1 alone (Figure 3B and 3C), suggesting that the combination of miRNAs was more effective than miR-1 alone. To assess calcium entry through voltage-gated calcium channels, Cacna1c, Scn5a, and Kcnj2, in CFP+ cells sorted from JAK inhibitor I–treated neonatal cardiac fibroblasts, 1 week after transfection with miR-1 and miRs-1, 133, 208, 499. Both untransfected and negmiR controls were also treated with the JAK inhibitor I. For B and D, results are presented as mean±SEM, *P<0.05, 1-way ANOVA with Bonferroni correction. Stated probability value is versus negmiR control.
Calcium channels, we also measured responses to membrane depolarization (Figure 3D). Consistent with the increased expression of the L-type calcium channels in CFP+ cells, depolarization with high extracellular [K+] (60 mmol/L) produced a calcium transient or induced the onset of oscillations in CFP+ cells (Figures 2D and 3D and Online Figure VII), whereas control cells rarely exhibited a calcium response (Figure 3E). This suggests that miRNA-transfected fibroblasts develop the cellular machinery of excitation-contraction coupling, a hallmark of functional cardiomyocytes. Together, these findings provide functional support for reprogramming of miRNA-transfected fibroblasts toward a cardiomyocyte phenotype, albeit less fully matured, as evidenced by the presence of spontaneous calcium oscillations and excitation-contraction coupling, and further suggest that calcium entry through voltage-gated calcium channels plays a role in regulating calcium oscillations in these cells.

Additionally, to explore the route of cardiac reprogramming, we assessed for expression of the pluripotency markers Oct4 and Nanog immediately after miRNA introduction, at 24-hour intervals, until the induction of cardiomyocyte marker expression in both neonatal and adult cells, and did not observe any change in gene expression relative to experimental controls (data not shown). However, we did observe rapid induction of primitive cardiac mesodermal marker Mesp2 as early as 1 day after transfection in the case of miRs-1, 133, 208, 499 and by day 5 in the case of miR-1 (Online Figure IX). These data, along with an observed reduction in fibroblastic markers Ddr2 and Vim (Online Figure X) suggest that reprogramming events taking place in the fibroblasts are the result of a direct switch in cell fate circumventing passage through a pluripotent precursor cell type.

To evaluate the potential of our approach in the adult heart, we injected lentivirus encoding miR-1 or a combination of 4 lentiviruses encoding the miR combo (miRs-1, 133, 208, 499). A lentivirus encoding a nontargeting miRNA (negmiR) was used as a negative control. Viruses were injected intramyocardially, immediately after injury by permanent ligation of the left ascending coronary artery (2 injection sites 2 mm below ligation site). The recipient double-transgenic Fsp1-Cre/tdTomato mice allowed us to genetically trace the fate of endogenous cardiac fibroblasts in vivo and investigate whether they were reprogrammed to a cardiac phenotype. Four weeks after infarction and virus injection, hearts were harvested, fixed, and stained for cardiac troponin T (TNNT2) and tdTomato expression (Figure 4A and 4B and Online Figure XI). Analysis of the infarct areas in the left ventricular myocardium of mice injected with either miR-1 or the miR combo revealed the presence of large, striated, double-positive for CFP and tdTomato expression (Figure 4A and 4B and Online Figure XI). Coexpression of tdTomato and TNNT2 was further substantiated after 3D reconstruction of confocal images (Figure 4B). Moreover, cardiomyocytes were isolated 6 weeks after permanent ligation of aMHC-CFP/Fsp1-Cre/tdTomato mice that had been injected with either the miR combo or negmiR (at the time of injury). The number of CFP+/tdTomato+ cardiomyocytes was then quantified; approximately 1% of cardiomyocytes from an infarcted heart injected with the miR combo were double-positive for CFP and tdTomato, whereas only 1 double-positive cardiomyocyte was found from a negmiR-injected heart (Figure 4C). The background fluorescence of tdTOMATO in the CFP channel and vice versa was also.
Figure 4. MicroRNA-injected endogenous cardiac fibroblasts are reprogrammed in vivo after myocardial injury. A, Fsp1Cre/ttdTOMATO transgenic mice were injected with miRNAs intramyocardially at the time of permanent ligation of the left ascending coronary artery. Four weeks later, hearts were harvested, fixed, and immunostained with antibodies against ttdTOMATO (red) and cardiac troponin T (TNNT2) (green). Nuclear staining with DAPI (blue). For split channel images, please refer to Online Figure XI. Large, double-positive cells (red and green) with prominent striations (highlighted with arrows), often organized as part of clusters, were taken as evidence of reprogrammed cardiac fibroblasts. Shown are representative images from infarct and peri-infarct areas of miR-1 and miR-51, 133, 208, 499 (miR combo) injected hearts. Scale bar, 100 μm. Zoomed-in area highlights a representative example of the presence of prominent striations in ttdTOMATO+/TNNT2+ cells from miRNA-injected hearts. B, Zoom of selected confocal microscopy image from the heart of a miRNA-injected heart from a Fsp1Cre-ttdTOMATO animal; orthogonal slide of 3D reconstructed 20X confocal image using Imaris software. Maximum intensity projection of images to allow visualization of coexpression of fluorescence signals ttdTOMATO (red), and TNNT2 (green), and red and green coexpression (yellow) to ensure that the observed colocalization is not due to cell staggering (20X magnification of selected areas in the different planes X-Y, X-Z, and Y-Z). Panels show merged signal (yellow) in all the different planes. Scale bar, 20 μm. C, αMHC-CFP/Fsp1-Cre/ttdTOMATO transgenic mice were injected with either miR combo or negmiR after permanent ligation. Six weeks later, cardiomyocytes were isolated and CFP+/ttdTOMATO+ cells quantified. Shown is a representative example of a double-positive cardiomyocyte isolated from a miR combo-injected animal. Individual panels for CFP and ttdTOMATO are shown as well as a merged overlay of both channels. For background fluorescence of ttdTOMATO and CFP channels, please refer to Online Figures XII and XIII. D, Representative live cell images of αMHC-driven-CFP and Fsp1Cre-driven ttdTOMATO expression in adult tail-tip fibroblasts (ttf) 3 weeks after transfection with miR combo. Live cell images are merged displays that constitute CFP (in green), ttdTOMATO (in red), and Hoechst (in blue). Scale bar, 50 μm. For split channel images, please refer to Online Figure XVII. NegmiR-treated control is shown as a representative of all controls used including untransfected and mock controls.

examined and is provided in Online Figures XII and XIII. As shown, no background signal or overlap was detected in either the dtTomato or the CFP channels.

Although Fsp1-Cre is predominantly expressed in fibroblasts, activation in other cell types is a possibility. To address this, we did extensive studies using both immunocytochemistry and FACS analysis to determine coexpression of the tdTomato marker with markers for cardiomyocytes, cardiac stem cells, and inflammatory cells in the injured heart, but we did not observe coexpression with the above-mentioned cell types (Online Figures IV through VI). To further provide evidence and exclude the possibility of our observations being the result of cardiac differentiation from progenitor cells rather than the reprogramming of fibroblasts, we tested for the capacity of miRs-1, 133, 208, 499 to induce cardiac reprogramming in fibroblasts of extracardiac origin.

As shown in Figure 4D and Online Figures XVII through XIX, the combination of miRs-1, 133, 208, 499 was capable of mediating cardiomyocyte induction in adult tail-tip fibroblasts. As with neonatal cardiac fibroblasts, we also observed a significant elevation of %CFP induction with JAK inhibitor I treatment in reprogrammed adult tail-tip fibroblasts (Online Figures XVIII and XIX). We did find, however, that cells reprogrammed from adult tail-tip fibroblasts were at a less mature stage in their cardiac phenotype when compared with reprogrammed counterparts from neonatal cardiac fibroblasts (no spontaneous contraction was observed). Significantly, the use of adult fibroblasts isolated from the tails of αMHC-CFP/Fsp1Cre/ttdTOMATO mice (Figure 4D) further strengthens our observation that induced cells do not originate from contaminating cardiac progenitors. Additionally, the induction of cardiomyocyte-like cells in adult tail-tip fibroblasts strongly implies that reprogramming is not due to cell fusion between fibroblasts and cells of a cardiac lineage but is in fact consequential to cellular reprogramming.
Discussion
In summary, we provide evidence that distinct miRNAs are capable of inducing conversion of fibroblasts to cardiomyocyte-like cells. Moreover, cardiac direct reprogramming (in this case using miRNA) in vivo has not been demonstrated previously and this report serves as proof-of-concept that this distinctive, innovative approach can be used to switch cell fate and induce regeneration in the heart (obviating the use of cell transplantation methods used heretofore). Remarkably, our studies suggest that miR-1 alone is sufficient to induce cardiac reprogramming, but its effects are dramatically enhanced in combination with miRNAs 133, 208, 499 and even further with JAK inhibitor I treatment. Indeed, our analysis revealed a specific set of miRNAs (miR-1, mir 133, mir 208, and mir 499) that was efficient not only at inducing expression of cardiac markers in fibroblasts but also efficient at converting the fibroblasts into cells with functional properties characteristic of cardiomyocytes such as L-type channel expression, spontaneous calcium oscillations, and contractility. Interestingly, the full combination of miRNAs did not change the total number of reprogrammed cells (as measured by %CFP+ cells) but rather enhanced the maturation of converted cells. Moreover, the effectiveness of partnering miRNAs with JAK inhibitor I treatment led to a significant increase in efficiency via enhancement of both αMHC induction and expression of cardiac ion channels. Although the exact molecular pathways involved in these processes remain unresolved for now, our initial studies suggest that the miRNAs directly convert fibroblasts to cells of the cardiomyocyte lineage without intermediate conversion to a pluripotent state. In addition, the exact mechanism of JAK inhibitor I remains unclear. Recently, JAK inhibitor I has been reported to promote cardiomyocyte transdifferentiation of partially reprogrammed embryonic fibroblasts via suppression of pluripotency-promoting pathways.3 Whether its effects are similar in postnatal cardiac fibroblasts remains to be investigated. As such, this report describes evidence that the efficiency of miRNA-induced direct cardiac reprogramming is significantly increased by application of this particular small molecule and may represent a major advancement in this arena.

We also provide evidence of a nonviral method at accomplishing efficient miRNA-mediated direct cellular reprogramming of one somatic cell to another in vitro. This alone suggests that continual upregulation of miRNAs is not necessary for reprogramming to occur and that a single transient transfection of miRNAs is sufficient for cell fate switching, suggesting further that the latter may actually take place within the first days of miRNA introduction. Our work also demonstrates that distinct miRNAs are capable of inducing direct conversion of nonmyocytes to cardiomyocyte-like cells in vivo. Moreover, genetic tracing methods suggest that induced cardiomyocyte-like cells are probably of fibroblastic origin. We acknowledge that the use of Fsp1Cre might not serve as the ideal model for genetic tracing of cardiac fibroblasts. However, our preliminary data suggesting that Fsp1 is not expressed in nonfibroblasts (ie, stem cells, smooth muscle cells, and inflammatory cells) in the injured myocardium as well as evidence of reprogramming in adult tail-tip fibroblasts provides multiple lines of support for the occurrence of fibroblast reprogramming in the injured myocardium. Interestingly, dermal fibroblasts transfected with miRNAs, despite the presence of clear upregulation of cardiomyocyte markers, rarely exhibited induced calcium transients indicating that the reprogrammed phenotype is at a less mature stage when compared with cardiac fibroblasts as the originating cell. This is in line with the experience of others in the reprogramming field converting/driving tail-tip fibroblasts to a functionally responsive cardiomyocyte-like phenotype.8 Whether the propensity of those cells to become cardiomyocytes is correlated with their developmental origins remains to be determined. Importantly, our work also serves as proof-of-concept that it is possible to inject miRNAs directly in vivo and achieve fibroblastic conversion to cardiomyocytes in the scar and peri-infarct areas of the heart. Significantly, these events were a result of the actions of miRNAs alone, suggesting that the efficiency of reprogramming may be elevated in vivo by the dynamic cross-talk present within the microenvironment of a complex tissue versus the 2-dimensional cell culture dish. Further optimization of miRNA delivery methods combined with JAK inhibitor I treatment in vivo may amplify the therapeutic implications of our work. Collectively, this study presents the first evidence of miRNA-induced direct conversion to cardiomyocytes in vitro and in vivo and demonstrates the powerful effect of this group of small RNAs at mediating cellular reprogramming.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

- Cardiac injury is characterized by inadequate regeneration and excessive fibrosis.
- Cellular reprogramming could potentially be used for cardiac regenerative therapy.
- Cardiac fibroblasts represent therapeutic targets for in vivo reprogramming into cardiomyocytes.
- A promising strategy for direct reprogramming is the use of microRNAs (miRNAs).

What New Information Does This Article Contribute?

- Distinct miRNAs are capable of converting fibroblasts to cardiomyocyte-like cells in vitro.
- JAK inhibitor I treatment enhances miRNA-mediated reprogramming to cardiomyocytes.
- Cardiac fibroblasts could be reprogrammed to cardiomyocytes.
- In vivo reprogramming of fibroblasts in the heart using miRNAs may yield a more straightforward method for cardiac regeneration.

Response to cardiac injury is usually characterized by inadequate regeneration and excessive fibrosis, resulting in significant impairment of tissue structure and function. Although stem cell therapy holds promise, the data thus far suggest that authentic cardiac regeneration is modest at best. Recently, reports of reprogramming of somatic cells to inducible pluripotent stem cells or cells of other lineages have raised the possibility of using reprogrammed cells for cell therapy. A method to directly convert the injured areas to functional tissue in vivo would be the ideal. Using a model of cardiac injury, we provide evidence that fibroblasts can be directly reprogrammed to cardiomyocytes in the heart. Our studies show that (1) miRNAs are capable of converting fibroblasts to cardiomyocyte-like cells in vitro; (2) miRNAs can directly convert fibroblasts to cardiomyocytes in the adult mouse myocardium; and (3) a combination of miRNAs with JAK inhibitor I treatment significantly increases the efficiency of fibroblast reprogramming toward the cardiac fate. A therapeutic approach administering miRNAs would represent a novel and potentially simpler strategy to stimulate cardiac regeneration. This approach may have broad implications for therapeutic tissue regeneration in general.
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Supplementary Methods

**Calcium Imaging and Contractility**
Calcium signals in cultured cardiac fibroblasts and neonatal cardiomyocytes were imaged using Fura-2 according to previously published protocols (2, 3). Fura-2 AM loading (1 μM; Molecular Probes/Invitrogen) and calcium imaging were performed in Ringer solution. Standard Ringer contained (in mM): 140 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgCl₂, 10 glucose and 10 HEPES, pH 7.4. High [K⁺] Ringer contained (in mM): 80 NaCl, 62.8 KCl, 2 CaCl₂, 2 MgCl₂, 10 glucose and 10 HEPES, pH 7.4. Experiments were carried out at room temperature. To determine if cells exhibited spontaneous oscillations, at least 8 fields of cells in each dish were imaged for at least 3 minutes each. Once oscillations were observed, cells were imaged for at least 5 minutes and further fields were tested until no additional oscillating cells were found. For contractility, miRNA-induced CFP+ cells sorted by FACS were seeded at a density of 1.5x10⁴ cell/cm² on gelatin-coated plates and monitored every 1-2 days for evidence of beating cells/clusters.

**Transgenic Mouse Models and In vivo Studies**
Fsp1Cre transgenic mice (C57BL/6 background; inbred strain obtained from Harold Moses, Vanderbilt University) were bred to a homozygous Cre-reporter strain (Gt(ROSA)26Sor locus with loxP-flanked STOP cassette preventing transcription of CAG promoter-driven red fluorescent protein variant tdTOMATO) from The Jackson Laboratory (Stock number 007914). Cardiac and tail tip fibroblasts were isolated from neonatal mice positive for Fsp1Cre/tdTOMATO. Fsp1Cre heterozygous males were also bred to cMHC-CFP/βMHC-GFP (C57BL/6 background; inbred strain obtained from Kumar Pandya and Oliver Smithies, UNC) homozygous females and the resulting double positive offspring bred to tdTOMATO reporter mice for isolation of adult tail tip fibroblasts. Adult, male Fsp1Cre-tdTOMATO transgenic mice were subjected to LAD using previously published procedures (4). Lentiviral microRNAs (2x10⁶ pfu in 40µL) were injected once at the time of injury, at two sites 2mm below site of ligation (20µL at each site). Animals were harvested after 4 weeks, fixed, paraffin-embedded, and stained with anti-RFP (Rockland Immunochemicals) and anti-cardiac troponin T (Abcam).

**Gene Expression Analysis**
Expression of individual genes was normalized to Gapdh and the resulting numeric values range scaled, standardized, and graphically represented in heat map format using JMP statistical software (SAS, Cary, NC). Hierarchical clustering was performed using Ward’s method of linkage.
Online Figure I. Target gene knockdown in miRNA-transfected cardiac fibroblasts 48 hours post-transfection. Fold change of *Twf1* (target gene of miR-1) and *Col16a1* (target gene of miR-133a) relative to untransfected control was averaged from three independent experiments. NegmiR-transfected fibroblasts serve as an additional control. Results are presented as mean ± SEM.
Online Figure II. Relative gene expression of *Gata4* (upper); and *Vimentin* (lower) in mouse embryonic fibroblasts (MEFs) 4 days post-transfection with miRNAs. Reference control is comprised of the average of all controls used in this experiment including untransfected, mock, and negmiR-transfected samples.
Online Figure III. Representative images for immunostaining of Myosin heavy chain in DAPI-positive (blue) neonatal cardiac fibroblasts 7 days after transfection with miRNAs. Images of negmiR controls displayed are representative of all negative controls used including untransfected and mock-treated controls.
Online Figure IV. Representative images for TNNI3 or α-ACTININ (in green) immunostaining of DAPI-postive (blue) adult cardiac fibroblasts 6 days after transfection with miRNAs. Images of untransfected controls are representative of all negative controls used including negmiR- and mock-transfected controls.
Online Figure V. Representative images of induction of αMHC-driven-CFP in neonatal and adult cardiac fibroblasts (CF) after transfection with top miRNA combinations, 1-1.5 weeks post-transfection. Untransfected control cells are shown as representative of all other controls including negmiR- and mock-transfected controls. Scale bar, 100 μm.
Online Figure VI. α-ACTININ (green) immunostaining of DAPI-positive (blue) neonatal cardiac fibroblasts (red) isolated from FspCre/tdTOMATO mice, 2 weeks following transfection with miRs-1, 133, 206. Scale bar 100 μm. Representative negative control images for this experiment are provided in Online Figure VII.
Online Figure VII. Representative negative control images from DAPI-positive neonatal (blue) cardiac fibroblasts isolated from Fsp1Cre/tdTOMATO neonatal mice transfected with negmiR control. Images are representative of all other negative controls used including mock-transfected and untransfected controls. Note that as shown above, neither (a) α-ACTININ nor (b) cardiac troponin I (TNNI3) (b) are expressed in control fibroblasts. Scale bar, 100µm.
Online Figure VIII. RNA levels of cardiac marker expression from CFP+ neonatal cardiac fibroblasts 1 week following transfection with miRNAs with or without JAK inhibitor I treatment. Bar graphs represent fold change of RNA expression (relative to a negmiR control).
Online Figure IX. RNA levels of cardiac marker Mesp2 are increased as early as 1 day post-transfection with miRNA combination 1, 133, 208, 499. Higher conversion efficiencies associated with this combination appear to be associated with inclusion of miR-499. Similar experiments testing for Oct4 and Nanog showed no expression of these two early stem cell markers at the RNA level (data not shown). Results are presented as mean ± SEM.
**Online Figure X.** RNA levels of fibroblast marker *Ddr2* are decreased 7 days post-transfection in miRNA-induced CFP+ cells compared to untransfected and negmiR controls. All cells in this experiment were also treated with JAK Inhibitor I for 5 successive days prior to FACS. Results are presented as mean ± SEM.
Online Figure XI. Split channel images from Figure 4a indicating individual expression of Cardiac troponin (green), tdTOMATO (red), and DAPI (blue) in miR-1 and miR combo (miRs-1, 133, 208, 499)-injected hearts from Fsp1Cre/tdTOMATO animals (4 weeks post-ligation and injection). Large, double-positive cells (red and green) with prominent striations (highlighted with arrows), often organized as part of clusters, were taken as evidence of reprogrammed cardiac fibroblasts. Shown here are representative images from infarct and peri-infarct areas of miRNA-injected hearts. Scale bar, 100 μm.
**Online Figure XII.** Split channel, live cell confocal images showing endogenous expression of CFP and tdTOMATO from cardiac fibroblasts isolated from αMHC-CFP/Fsp1-Cre/tdTOMATO adult transgenic mice injected with negmiR (as a control) 6 weeks post-infarction. Do note that no expression of CFP is detected in cardiac fibroblasts at baseline. Scale bar, 100 μm.
Online Figure XIII. Split channel, live cell images showing endogenous expression of CFP and tdTOMATO from cardiomyocytes freshly isolated from αMHC-CFP/Fsp1-Cre/tdTOMATO adult transgenic mice injected with negmiR (as a control) 6 weeks post-infarction. Note that no expression of tdTOMATO can be detected at baseline. Scale bar, 200 μm.
Online Figure XIV. Representative images of injured, negmiR-injected control hearts (Fsp1Cre/tdTOMATO animals) stained with markers for myofibroblasts (Smooth muscle actin); blood cells (CD45); stem cells (ckit); and cardiomyocytes (Cardiac troponin T) to examine specificity/leakiness of the Fsp1Cre promoter. Note that co-expression of tdTOMATO was not observed for any of the markers tested. Nuclear staining with DAPI (blue). Scale bar, 100 μm.
Online Figure XV. Representative examples of isolated adult tail tip fibroblasts from Fsp1Cre-tdTOMATO mice stained for classic fibroblast markers VIMENTIN (green) and DDR2 (Green). Nuclear staining with DAPI (blue). Scale bar, 100 μm.
Online Figure XVI. Representative image from a tdTOMATO+ control (from a non-miRNA-injected, Fsp1Cre/tdTOMATO animal) heart illustrating an example of a cell stained positively for both tdTOMATO and cardiac troponin T (TNNT2). Such double-positive cells were rarely observed and if observed in miRNA-injected hearts were not taken as evidence of reprogramming unless organized clusters of larger, striated, double-positive cells were observed in the peri-infarct area or area away from the heart. Nuclear staining with DAPI (blue). Isolated double-positive cells of this nature were rarely observed, Scale bar, 100 μm.
Online Figure XVII. Upper panel, Split channel live cell images from Figure 4d, of miR-combo-transfected adult tail tip fibroblasts showing expression of individual fluorescent markers Hoechst (nuclear, blue), CFP (αMHC, green)) and tdTOMATO (red) (Fsp1Cre-driven). Scale bar, 50 μm. Lower panel, Additional examples of split channel live cell images of miR-combo-transfected adult tail tip fibroblasts showing expression of individual fluorescent markers Hoechst (nuclear), CFP (αMHC) and tdTOMATO (Fsp1Cre) as well as their merged overlay. Scale bar, 100 μm.
Online Figure XVIII. Summary from FACS analyses data of miRNA-transfected adult tail tip fibroblasts. Tail tip fibroblasts were isolated from αMHC-CFP adult transgenic mice, treated with JAK Inhibitor I for 5-8 days using standard culture conditions and/or gradual serum withdrawal. CFP+ cells were sorted 7-10 days post-miRNA-transfection and maintained in culture to observe for contraction. The average % of CFP induction for each experiment is provided above. Representative FACS overlay for the third experiment (8 day JAK Inhibitor I treatment) is provided in Online Figure XIX.
Online Figure XIX. Upper Panel, Representative FACS analyses demonstrating the induction of αMHC-driven-CFP+ cell population in miRNA-transfected adult tail tip fibroblasts 10 days post-transfection with miRNAs and treatment with JAK Inhibitor I. FACS traces are distinguished as follows: untransfected cells (green), non-targeting miRNA-transfected cells (red) and miRNA-transfected cells (dark blue). Lower panel, 2D FACS scatter plots displaying left: Forward Scatter (FSC) versus CFP; middle: Side scatter (SSC) versus CFP; and right: FSC versus SSC for miRNA-transfected adult tail tip fibroblasts 10 days post-transfection with miRNAs and treatment with JAK Inhibitor I (R1 in the right panel represents the gated CFP+ population and is equivalent to the cell populations marked in green on the left and middle panels). Cells were transfected with miRs-1, 133, 208, 499 and CFP induction is compared to untransfected and negmiR-transfected cell controls.
Online Figure XX. Color Legend for the heat map presented in Figure 1 indicating fold expression data for each gene examined in adult cardiac fibroblasts, 3 and 6 days post-transfection with miRNAs. Normalized (to GAPDH) expression data was obtained by qRT-PCR, scaled, standardized, and heat map was generated using JMP software.
References


