Tilanthi M. Jayawardena,* Elizabeth A. Finch,* Lunan Zhang, Hengtao Zhang, Conrad P. Hodgkinson, Richard E. Pratt, Paul B. Rosenberg, Maria Mirotou, Victor J. Dzau

Rationale: A major goal for the treatment of heart tissue damaged by cardiac injury is to develop strategies for restoring healthy heart muscle through the regeneration and repair of damaged myocardium. We recently demonstrated that administration of a specific combination of microRNAs (miR combo) into the infarcted myocardium leads to direct in vivo reprogramming of noncardiac myocytes to cardiac myocytes. However, the biological and functional consequences of such reprogramming are not yet known.

Objective: The aim of this study was to determine whether noncardiac myocytes directly reprogrammed using miRNAs in vivo develop into mature functional cardiac myocytes in situ, and whether reprogramming leads to improvement of cardiac function.

Methods and Results: We subjected fibroblast-specific protein 1-Cre mice/tandem dimer Tomato (tdTomato) mice to cardiac injury by permanent ligation of the left anterior descending coronary artery and injected lentiviruses encoding miR combo or a control nontargeting miRNA. miR combo significantly increased the number of reprogramming events in vivo. Five to 6 weeks after injury, morphological and physiological properties of tdTomato− and tdTomato+ cardiac myocyte–like cells were analyzed ex vivo. tdTomato+ cells expressed cardiac myocyte markers, sarcomeric organization, excitation–contraction coupling, and action potentials characteristic of mature ventricular cardiac myocytes (tdTomato− cells). Reprogramming was associated with improvement of cardiac function, as analyzed by serial echocardiography. There was a time delayed and progressive improvement in fractional shortening and other measures of ventricular function, indicating that miR combo promotes functional recovery of damaged myocardium.

Conclusions: The findings from this study further validate the potential use of miRNA-mediated reprogramming as a therapeutic approach to promote cardiac regeneration after myocardial injury. (Circ Res. 2015;116:418-424. DOI: 10.1161/CIRCRESAHA.116.304510.)

Key Words: cellular reprogramming ■ excitation–contraction coupling ■ guided tissue regeneration ■ microRNAs ■ myocardial infarction ■ myocytes, cardiac

The adult human heart has a limited capacity to regenerate lost or damaged cardiac myocytes after cardiac insult. Myocardial injury, characterized by excessive fibrosis and cardiac remodeling, eventually results in the deterioration of cardiac structure and function. The loss experienced by the cardiac myocyte cell fraction of the heart after myocardial infarction (MI) is permanent. This leads to a disproportionate thinning of the heart and severely reduced function. This impairment of the heart’s ability to pump blood has disastrous consequences on morbidity and mortality.¹

A major goal for the treatment of heart tissue damaged by cardiac injury is to develop strategies that restore healthy heart muscle through the regeneration and repair of damaged myocardium. Cellular reprogramming holds tremendous potential for promoting such cardiac repair. Recent advances have established that fibroblasts can be directly reprogrammed into cardiac myocytes by introducing distinct combinations of lineage-significant transcription factors²–⁵ or microRNAs.
(miRNAs). In the context of MI, it is, therefore, particularly alluring to use an analogous strategy in vivo by delivering a cocktail of reprogramming mediators directly into the border zone adjacent to the injured myocardium and thereby inducing fibroblasts to reprogram into cardiac muscle. This approach would be expected to lead to both a reduction in scar formation and an overall improvement in contractility resulting from a replenishment of cardiac myocytes within the heart. Recently, we and others demonstrated the feasibility of this approach by showing that introduction of either transcription factors or miRNAs into the infarcted myocardium leads to direct in vivo reprogramming of nonmyocytes to cardiac myocytes. With transcription factors, reprogramming was accompanied by a significant improvement in cardiac function. However, for miRNA-mediated reprogramming, it has not yet been established whether these reprogrammed cells develop into mature functional cardiac myocytes in situ, or whether reprogramming leads to an improvement of cardiac function in vivo.

We recently reported that a specific combination of miRNAs (miRNAs 1, 133, 208, and 499; miR combo) is capable of redirecting noncardiac myocytes to become cardiac myocyte–like cells in vitro and in vivo. In vitro, the induced cardiac myocyte–like cells possessed functional properties characteristic of cardiac myocytes, such as L-type channel expression, spontaneous calcium oscillations, and contractility, although their physiological properties seemed to be less mature than those of neonatal cardiac myocytes. We further showed that injection of lentiviruses encoding the miR combo into the peri-infarct area of the infarcted heart also induced the generation of new cardiac myocyte–like cells in this region from lineage-traced noncardiac myocytes by 4 weeks post infarct. This finding provided proof-of-concept that miRNA-mediated cardiac reprogramming can be achieved in vivo. Therefore, we sought to determine whether in vivo reprogramming with miRNAs improved cardiac structure and generated functionally mature ventricular cardiac myocytes. In this report, we characterize the morphological and physiological properties of reprogrammed cells ex vivo, and the consequences for left ventricular (LV) contractile function in vivo using serial echocardiography. We provide evidence that delivery of a specific combination of miRNAs to the injured myocardium yields reprogrammed cells that exhibit the characteristics of mature adult ventricular cardiac myocytes. This is associated with a progressive improvement of the cardiac function when compared with controls during a 3-month time period. Collectively, our findings further validate the potential use of miRNA-mediated reprogramming as a therapeutic approach to promote cardiac regeneration after myocardial injury.

### Methods

An expanded Methods is available in the Online Data Supplement.

### Animals and Surgery

Adult male fibroblast-specific protein 1 Cre-tandem dimer Tomato (tdTomato) transgenic mice (8–10 weeks) were subjected to permanent ligation of the left anterior descending coronary artery using previously published procedures. Lentivirus, consisting of either a combination of 4 individual lentiviruses expressing miRNAs 1, 133, 208, or 499 (miR combo), or a lentivirus expressing a random sequence nontargeting miRNA (negmiR), were injected once at the time of injury, at 2 sites 2 mm below the site of ligation, as previously described. The miR combo lentivirus significantly increased expression of miR-1, miR-133, miR-208, and miR-409 in cardiac fibroblasts (Online Figure 1A). Furthermore, in vivo, lentiviruses preferentially targeted cardiac fibroblasts, as determined by injection of a lentivirus-green fluorescent protein reporter (Online Figure 1B).

### Immunocytochemistry

Cells were fixed in paraformaldehyde and labeled using primary antibodies against sarcomeric β-actin, cardiac troponin T, N-cadherin, or Connexin-43 together with tdTomato. Confocal images were captured using an LSM 510 Meta DuoScan microscope (Zeiss) and processed using LSM 5 software, version 4.2.

### Ex Vivo Analysis

Animals were harvested 5 to 6 weeks after infarction and virus injection. Cardiac myocytes and other cells were isolated from the ventricles according to Louch et al with modifications and were analyzed within 8 hours of isolation. Wide field microscopy was used to image calcium dynamics and contraction; simultaneous high-speed fluorescence photometry and cell geometry measurements (IonOptix Calcium and Contractility System) were used to characterize excitation–contraction (EC) coupling. Whole-cell current clamp and voltage clamp recording were used to record action potentials and the current–voltage relationship, respectively.

### Serial Echocardiography

Adult male wild-type mice were subjected to left anterior descending coronary artery and lentivirus injection, as above. High-resolution 2-dimensional echocardiography was performed pre MI, and at 2 weeks and 1, 2, and 3 months post infarction. At each time point, the following information was acquired: fractional shortening, ejection fraction, LV mass, LV end-diastolic dimension, LV end-systolic dimension, heart rate, interventricular septum thickness, posterior wall thickness, and velocity of circumferential fiber shortening.

### Data and Statistical Analysis

Statistical analysis of cardiac, contractility, electrophysiological measurements, and echocardiography was performed using Student t test (2-sample equal variance, 2 tailed). Statistical analysis of serial echocardiography between groups was analyzed at each time point. ANOVA was used to compare multiple groups. P<0.05 was regarded as significant. Graphs are displayed as mean±SEM; asterisks indicate significance.

### Results

We recently reported that a specific combination of miRNAs (miRNAs 1, 133, 208, and 499; miR combo) is capable of redirecting noncardiac myocytes to become cardiac myocyte–like cells in vitro and in vivo. To investigate reprogramming efficiency and to determine whether cardiac myocyte–like cells generated by reprogramming in vivo possess morphological properties similar to wild-type mature cardiac myocytes, we subjected adult fibroblast-specific protein 1-Cre/tdTomato mice to cardiac injury by permanent left anterior descending coronary artery ligation and injected lentiviruses encoding the miR combo or a negmiR. This allowed us to analyze the lineage-traced in vivo reprogrammed noncardiac myocyte population.

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We first determined the number of reprogramming events in mice treated with miR combo or negmiR. tdTomato⁺ and tdTomato⁻ cardiac myocytes were counted in the peri-infarct regions of mice injected with miR combo and negmiR 7 weeks after infarction and viral injection. To avoid any potential bias in the counting, the entire infarct region was cut into a number of 0.5-mm sections (Online Figure IIA) and the number of cardiac myocytes counted in the peri-infarct region of each section, as has been described previously.° miR combo was associated with a ≈3-fold increase in the number of cells expressing both tdTomato and cardiac troponin-T (Figure 1A; 12% miR combo versus 4% negmiR, P = 0.0024; additional figures are supplied in Online Figure IIB and IIC) when compared with negmiR-injected mice. Intriguingly, in miR combo–injected mice, small tdTomato⁺ cardiac troponin-T⁺ cells were observed in the infarct region (Online Figure IID) suggestive of developing cardiac myocytes within the damaged area. Moreover, in miR combo–injected mice, tdTomato was also found to colocalize with α-sarcomeric actinin, another mature cardiac myocyte marker (Figure 1B). No such colocalization was observed in mice injected with negmiR (Figure 1B). tdTomato⁺ cardiac myocytes, in miR combo–injected mice, expressed the gap junction protein connexin-43. Importantly, gap junctions were observed between the pre-existing, tdTomato⁺, and new reprogrammed, tdTomato⁺, cardiac myocytes (Figure 1C). This suggests efficient integration with pre-existing cells. Several tdTomato⁺ cardiac myocytes in vivo were found to express DDR2 (discoidin domain–containing receptor 2), suggesting that a proportion of the cells reprogrammed by miR combo retained fibroblast markers/characteristics (Figure 1D). DDR2 positive cardiac myocytes were also observed in the negmiR-treated animals (Online Figure III), albeit at lower levels (≈25% of miR combo).

Five to 6 weeks after infarction and virus injection, ventricular cells were isolated from mice injected with either the miR combo or negmiR. In accordance with our previous study, tdTomato⁺ cardiac myocytes were rarely observed in preparations from negmiR-injected hearts. In cell preparations from miR combo–injected animals, tdTomato⁺ cells were rarely observed in preparations from negmiR-injected hearts. In cell preparations from miR combo–injected animals, tdTomato⁺ cells...
exhibited several morphologies. Mature cells with a clearly defined rod shape were observed (Online Figure IVA). In addition, we also noted tdTomato+ cells with an intermediate morphology (Online Figure IVB) reminiscent of developing or immature ventricular cardiac myocytes,11,12 which might represent differentiating, or intermediate, reprogrammed cells. These immature cells may be the small tdTomato+ cardiac troponin-T cells that were observed in vivo.

To determine whether the large rod-shaped tdTomato+ cardiac myocyte–like cells express proteins characteristic of mature cardiac myocytes, we performed immunocytochemical profiling for standard cardiac myocyte markers (Figure 2). Cardiac troponin T and sarcomeric α-actinin were localized in a striated pattern typical of mature cardiac myocytes. Connexin-43 and N-cadherin, proteins involved in gap junctional communication and cell adhesion between cardiac myocytes, were localized in a punctate pattern at the plasma membranes of tdTomato+ cardiac myocytes, as is characteristic of wild-type adult ventricular cardiac myocytes.

We next investigated whether induced cardiac myocytes exhibit the physiological characteristics of mature cardiac myocytes by analyzing EC coupling and the electrophysiological properties of rod-shaped tdTomato+ and tdTomato− cardiac myocyte–like cells isolated from miR combo–injected mice. To analyze calcium signaling and contractility, we first used a wide field imaging during pacing with electric field stimulation. This revealed
that the majority of rod-shaped tdTomato+ cells exhibited normal sarcomeres, rapid and large calcium transients, and concurrent contractions in response to depolarization (Online Figure V and Movie I). This indicates that tdTomato+ cardiac myocyte–like cells exhibit EC coupling. We quantified EC coupling parameters using simultaneous high-speed fluorescence photometry and sarcomeric length measurements. Figure 3A shows examples of simultaneous calcium transients and contraction generated by tdTomato− and tdTomato+ cardiac myocytes during pacing at 1 Hz. Our analysis revealed that there were no differences in basal cytosolic calcium levels or in the peak amplitudes and decay kinetics of depolarization-evoked calcium transients between tdTomato− and tdTomato+ cardiac myocytes (Figure 3B). There were also no differences in sarcomeric length at rest, fractional shortening, contraction decay kinetics, or EC coupling gain between tdTomato− and tdTomato+ cardiac myocytes (Figure 3C).

Simi-larly, these properties were not different during pacing at 0.5 and 2 Hz (not shown). These findings indicate that rod-shaped tdTomato+ cardiac myocytes exhibit EC coupling in response to depolarization and that the properties of EC coupling are similar to those of mature ventricular cardiac myocytes.

To compare the electrophysiological properties of tdTomato− and tdTomato+ cardiac myocytes, we next performed whole-cell patch-clamp recordings of cardiac myocytes dissociated from miR combo–injected hearts (Figure 4). Current injection elicited action potentials in both tdTomato− and tdTomato+ cardiac myocytes (Figure 4A). Although the action potentials evoked in tdTomato− cardiac myocytes were largely similar to those of control cells, the action potential amplitude and overshoot potential were reduced and resting membrane potential depolarized in tdTomato− when compared with tdTomato+ cardiac myocytes (Figure 4B). This would inactivate a fraction of the sodium channels, which might explain the smaller action potential amplitude and the trend toward smaller calcium transients and contractility in tdTomato− cardiac myocytes. We also compared the current–voltage relationship between tdTomato− and tdTomato+ cardiac myocytes (Figure 4C and 4D). This analysis showed that tdTomato− cardiac myocytes express voltage-gated calcium, inward rectifier potassium, and delayed rectifier potassium currents (Figure 4C) and that tdTomato− and tdTomato+ cardiac myocytes exhibit the same current–voltage relationship (Figure 4D). Thus, the electrophysiological properties of rod-shaped tdTomato+ cardiac myocytes further strengthen the conclusion that these cells are functionally similar to mature wild-type ventricular cardiac myocytes.

To determine whether miR combo can improve cardiac function after cardiac injury, we subjected adult male wild-type mice to cardiac injury (left anterior descending coronary artery) and miR combo delivery and examined cardiac function via echocardiographic analysis during the course of 3 months (Figure 5). Importantly, fractional shortening measurements (Figure 5A and 5B) indicate that miR combo significantly improves the heart’s pump function after MI relative to negmiR-injected animals. This improvement was time delayed and developed progressively, beginning between 1 and 2 months post surgery and was enhanced at 3 months (P=0.045 at 2 months; P=0.019 at 3 months). Velocity of circumferential fiber shortening, another measure of LV contractile function, was also significantly higher in miR combo–injected animals at the 3 months (Figure 5A; P=0.040). In addition, there was a trend toward a decrease in both LV end-systolic dimension (P=0.058) and LV mass (P=0.072) at 3 months post MI in miR combo–injected animals (Figure 5A). Moreover, 1 month post injury, fibrosis was significantly lower in miR combo–injected animals when compared with negmiR-injected mice (Figure 5C). Mice injected with PBS were not significantly different from negmiR-injected animals for any of the parameters described above (Online Figure VIA), indicating that viral delivery of miRs had no adverse effect on heart function.

To address the specificity of miR combo further, we measured cardiac myocyte apoptosis and vascular reprogramming. We found that in cultured neonatal cardiac myocytes, miR combo had no effect on apoptosis at baseline or in the presence of H2O2 (Online Figure VIB). Moreover, although miR combo had no effect on the expression of the endothelial progenitor marker Flk-1 (Online Figure VII), miR combo reduced the expression of key endothelial genes, VE (vascular endothelial)-cadherin and PECAM1 (platelet endothelial cell adhesion molecule) (Online Figure VII) in neonatal cardiac fibroblasts. Vascular density was increased in the peri-infarct region of miR combo reprogrammed hearts at 7 weeks; however, the effect did not reach significance (Online Figure VIII). Together, these findings suggest that miR combo promotes functional recovery of the damaged myocardium by specifically promoting cardiac reprogramming.

**Discussion**

We and others have shown that introduction of either transcription factors14 or miRNAs into the infarcted myocardium in vivo leads to direct reprogramming of noncardiac myocytes into cardiac myocytes. With transcription factors, reprogramming was accompanied by a significant improvement in cardiac function. Our results here demonstrate that miR combo can induce (1) the in vivo reprogramming of noncardiac myocytes in the heart to cardiac myocyte–like cells that possess the morphological and functional properties of mature adult ventricular cardiac myocytes; and (2) a significant improvement in cardiac function after myocardial infarct. These observations are compelling for the use of the identified miRNA combination for cardiac regeneration. The repressive actions of miRNAs on gene expression are powerful because a single miRNA may target multiple pathways simultaneously. As a therapeutic strategy, an miRNA-based approach has theoretical advantages over the use of transcription factors. 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. Moreover, nonviral delivery of chemically synthesized miRNA mimics, which can be more easily administered to cells and are suggested to also exhibit low toxicity in animal models,13,14 may be an attractive therapeutic strategy.

In our study, the majority of large rod-shaped tdTomato+ cells observed after miR combo treatment were striated and exhibited the morphological and functional characteristics of mature ventricular cardiac myocytes when analyzed 5 to 6 weeks post infarct. However, we also noted the presence of smaller tdTomato+ cells both in vitro and in vivo that had the appearance of immature or intermediate cardiac myocytes.3,11,15
Moreover, subtle changes in calcium transient and contractility properties may reflect delayed maturation of calcium-handling machinery. These findings suggest that at this time point, not all of the induced cardiac myocytes may have fully matured.

We found that miR combo was likely to be specific for cardiac reprogramming. Cardiac myocyte apoptosis was unaffected by miR combo. Similarly, miR combo did not induce an endothelial phenotype in neonatal cardiac fibroblasts. Vascular density was increased in the peri-infarct regions of miR combo–treated mice; however, the effect was not significant.

Intriguingly, the time frame we observed for the improvement of cardiac function with miRNAs is similar to that reported for reprogramming with exogenous transcription factors.3,4 This suggests that with current reprogramming methods, the time required for transdifferentiation, integration, and maturation of reprogrammed cells might explain the time delayed and progressive improvement of LV contractile function over a couple of months. Although the delivery of miRNAs has proven effective at improving cardiac function after MI, further optimization of reprogramming efficiency and efficacy is required for the advancement of this approach as a cardiac regenerative strategy. This may involve the delivery of higher and repeated doses of viral-expressing miRNAs, the use of different viral vectors for delivery, and the coadministration of additional cardiac reparative factors.16 Moreover, many questions

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relating to this therapy remain unanswered; these include the mechanisms that underlie reprogramming, long-term efficacy, temporal changes in cardiac performance after treatment, and how temporal changes in performance are correlated with the maturation of induced, or reprogrammed, cardiac myocytes. Although such questions must be addressed, initial studies using direct in vivo reprogramming with miRNAs as a therapeutic strategy for cardiac regeneration hold much promise.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- There is a critical need to develop strategies that can regenerate healthy heart muscle after injury.
- A specific combination of microRNAs (miR combo) can reprogram non-cardiac myocytes into cardiac myocytes in vivo.
- The functional consequences of such reprogramming remains unknown.

What New Information Does This Article Provide?

- Reprogramming was associated with improvement of cardiac function.

Direct reprogramming of cardiac fibroblasts into cardiac myocytes is emerging as important strategy to regenerate heart muscle after injury. We have found that a specific combination of microRNAs, miR combo, will reprogram cardiac fibroblasts into cardiac myocytes in vivo. The reprogrammed cardiac myocytes are fully mature and functionally normal. We also found that reprogramming was associated with an improvement in cardiac function after injury. These results further underscore the potential of miR combo as a therapeutic tool to treat cardiac injury.
MicroRNA Induced Cardiac Reprogramming In Vivo: Evidence for Mature Cardiac Myocytes and Improved Cardiac Function
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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Mouse Models
Fsp1Cre transgenic mice (B6.129S6-S100a4tm1Egn/YunkJ inbred strain obtained from Harold Moses, Vanderbilt University), backcrossed for 3 generations onto C57BL/6, 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). 40 mice were used for the ex vivo experiments in this study. Littermates were used for all experiments. Wild-type 57BL/6 mice (Charles River) were used for echocardiographic studies; a total of 153 mice were used.

Acute left anterior descending (LAD) coronary artery ligation myocardial infarction surgery and virus injection
Adult (8 – 10 weeks), male Fsp1CretdTOMATO transgenic mice were subjected to LAD using previously published procedures (4). Mice were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) by i.p. injection. Ophthalmic ointment was applied to both eyes to prevent corneal desiccation during the procedure. An initial injection of Buprenorphine (0.1 mg/kg) was given SQ and was repeated every 12 hours for a total of 5 days. The skin was prepared with an iodophore/alcohol three cycle wash. An endotracheal intubation was performed and the mouse connected to a ventilator as follows. In the supine position, endotracheal intubation was performed with a IV catheter (20 GA 1 IN) as the cannula under direct laryngoscopy. The cannula was connected to a volume-cycled rodent ventilator (model 683, Harvard Apparatus) on room air with a tidal volume of 0.7-1 mL and respiratory rate of 120 breaths per minute. Proper intubation was confirmed by observation of chest expansion and retraction during ventilated breaths. The chest cavity was opened, between the fourth and the fifth rib in the intercostals muscle, the heart externalized, and a 7-0 nylon suture placed through the myocardium into the anterolateral LV wall. This area corresponds to the course of the left anterior descending artery. The suture was positioned approximately midway between the apex and base, and a ligature was made. Before ligation, left coronary artery entrapment will be confirmed by upward traction. The suture was completely tied off (myocardial infarction), and the apex of the LV observed for evidence of myocardial blanching, indicating interruption in coronary flow. Lentiviral miRNAs (2×10^6 pfu in 40 μL) were injected once, 30 minutes after MI, at 2 sites 2 mm below site of ligation.

The chest cavity was closed with a 5-0 monofilament suture and the wound (chest muscles and skin) closed with 7-0 nylon. Negative pressure-established at closure, and the animal gradually weaned from the respirator. Once spontaneous respiration resumed, the endotracheal tube was removed and the animal placed on a deltaphase isothermal pad set at 37°C. The animal remained in a supervised setting until fully conscious, then returned to its cage and given standard chow and water. Post-operative analgesia was bupivicaine applied locally and buprenorphine SQ at BID for 5 days. All skin sutures were removed by 7 days post-operatively.

Ex vivo analysis
Animals were harvested 5 – 6 weeks after infarction and virus injection for ex vivo analysis of the physiological properties of reprogrammed cells. Cardiomyocytes were isolated according to Louch et al with modifications (1). Prior to harvest, the heart was flushed with Krebs-Henseleit buffer until clear of blood. The heart was then excised, carefully dissecting mediastinal adhesions, atria surgically removed, and the ventricles dissociated using standard
procedures. Following isolation, dissociated cells were suspended in a myocyte plating medium (0.9X MEM, 5% FBS, 1% Pen-Strep, and 2 mM L-Glutamine).

**Immunocytochemistry**

Cells were fixed in 4% v/v paraformaldehyde, permeabilized with triton, and incubated with primary antibodies against sarcomeric α-actinin (Sigma), cardiac troponin T (Abcam), N-cadherin (Abcam), or Connexin 43 (Sigma) together with RFP (Rockland Immunochemicals). Cells were then washed, incubated with secondary antibodies to cardiomyocyte markers (Alexa Fluor 488) and RFP (Alexa Fluor 594), and stained with DAPI. Confocal images were captured using an LSM 510 Meta DuoScan microscope (Zeiss) and processed using LSM 5 software, version 4.2.

**Calcium and contractility measurements**

Dissociated cells were plated on laminin coated dishes, and held in a tissue culture incubator until they were analyzed (within 12 hours of the isolation). Prior to analysis, dishes were screened to determine the number and location of rod-shaped tdTOMATO+ cardiomyocytes. Individual dishes were then loaded with the ratiometric calcium indicator Fura-2 (1 µM, 20 min), and perfused at room temperature with Tyrode solution containing 140 mM NaCl, 5.4 mM KCl, 1.05 mM MgCl2, 1.8 mM CaCl2, 0.33 mM NaH2PO4, 5 mM HEPES and 10 mM glucose (pH 7.4 with NaOH). Dishes were visually scanned, rod-shaped tdTOMATO+ or tdTOMATO- cardiomyocytes identified, and cells paced using electrical field stimulation with 1 ms voltage pulses at 150% of threshold (Warner Field Stimulation chamber). Cells were paced at paced at 0.5, 1, and 2 Hz. Wide field microscopy was used to image calcium dynamics and contraction. Simultaneous high-speed fluorescence photometry and cell geometry measurements (using an IonOptix Calcium and Contractility System) were used to characterize excitation-contraction coupling.

**Electrophysiological recordings**

For electrophysiological measurements, dissociated cardiomyocytes were plated in a recording chamber, perfused with Tyrode solution at room temperature, and scanned for tdTOMATO+ cardiomyocytes. Patch pipettes were pulled from borosilicate glass (Sutter) and had a resistance of 2–3 MΩ when filled with internal solution containing 130 mM K aspartate, 10 mM NaCl, 10 mM HEPES, 5 mM creatine phosphate, 0.04 mM CaCl2, 2 mM Mg·ATP, and 5 mM EGTA (pH 7.2 with KOH). Action potentials were recorded with an AXOPATCH 200B amplifier using the whole-cell current clamp technique. Action potentials of cardiomyocytes were triggered by injection of a brief depolarizing current (3 ms duration, 1–2 nA). The amplifier was then switched back to voltage clamp mode and the I-V relationship was recorded using 400 ms voltage steps from a holding potential of -40 mV, which inactivates Na+ currents, to between -120 and +60 mV in 10 mV increments. The I-V relationship was generated by measuring the steady-state current amplitudes at the end of each voltage step. All data were filtered at 5 kHz and analyzed using pCLAMP 10 software (Molecular Devices).

**Echocardiographic analysis**

Animals were anesthetized with isoflurane (via nose cone) and placed on a heating pad (Deltaphase pad) for the entire duration of procedure. To assess left ventricular mass, geometry, and function, echocardiographic analysis was performed with spontaneous respiration under light anesthesia using isoflurane to maintain a heart rate < 400 bpm. Short-axis two-dimensional images using an 8-12 MHz transducer placed at the midpapillary levels of the left ventricle were stored as digital loops. Echocardiographic evaluation was performed prior to injury and at 2 weeks and 1, 2, and 3 months post infarction. At each time point, fractional shortening, ejection fraction, left ventricular (LV) mass, LV end-diastolic dimension (LVEDD), LV
end-systolic dimension (LVESD), heart rate, interventricular septum thickness, posterior wall thickness, and velocity of circumferential fiber shortening (Vcfc) were acquired.

**Data and Statistical Analysis**

For analysis of serial echocardiography, variability of infarction severity was assessed on a total of 21 animals at 2 weeks post-MI by percent fractional shortening (%FS). Following interquartile range analysis several animals were identified that had either a severe (%FS 1-19%; n=4) or mild MI (%FS>31%; n=2). The majority of animals (n=15) had a %FS of 20-30% at 2 weeks post-MI. Further analysis at the later time-points was restricted to this group. Analysis could not be performed on the severe or mild groups as the n was too low for statistical analysis. The resulting values were plotted versus time for each group (negmiR versus miR combo). Statistical significance between groups at each time point was determined by conducting Student’s t-test; 2-sample equal variance, 2-tailed. ANOVA was used to compare multiple groups. Bonferroni correction was applied to determine significance between groups. Statistical analysis of calcium, contractility, and electrophysiological measurements was performed using Student’s t-test (2-sample equal variance, 2-tailed). Bar graphs are displayed as mean ± SEM. P < 0.05 was regarded as significant.
Online Figure I. Lentivirus delivery of miR combo preferentially targets cardiac fibroblasts in vivo.

(A) Neonatal cardiac fibroblasts were exposed to the lentiviruses expressing the miR combo at 100MOI. After 3 days expression of miR-1, -133, -208, and -409 was determined by qPCR. Expression of the miRs in cardiac fibroblasts transfected with the negmiR lentivirus was taken to be 1. A representative experiment is shown.

(B) FSP1-Cre/tdTomato mice were subjected to MI and then injected with a lentivirus containing a GFP expression cassette. Seven days following injury heart sections were immunostained for GFP and tdTomato. Representative images shown. Note that GFP solely co-expressed with tdTomato, GFP+ tdTomato- cells were not observed. Scale bar 100 microns.
Online Figure II. miRcombo promotes reprogramming in vivo.

(A) The infarcted area of the heart, seven weeks following injury, was cut into a set of 0.5 mm sections, starting 1 mm below the ligation site. Each individual section was used for immunostaining with the peri-infarct region being visualized. Sections were probed for tdTomato and cardiac troponin-T from both:

(B) miRcombo treated mice and,

(C) negmiR treated mice.

(D) Infarct region from a mouse injected with miRcombo, immunostained with tdTomato and cardiac troponin-T antibodies.

Representative images from n = 3 per group. Scale bar 200 μm for panels B and C, 100 μm for panel D.
Online Figure III. DDR2 immunostaining in FSP1-Cre/tdTomato mice injected with negmiR.

Seven weeks following injury the entire peri-infarct region was visualized by serial sectioning through the heart tissue. Sections were probed for tdTomato, cardiac troponin-T and DDR2. Representative images from n = 3 per group.
Online Figure IV. Morphology and expression of cardiomyocyte markers in tdTomato+ cells in vitro.

(A) Mature cardiomyocytes: Images of tdTomato+ and tdTomato− cardiomyocytes from dissociated cell preparations 5 – 6 weeks post-infarct. Upper, red fluorescence, showing tdTomato+ cardiomyocytes. Lower, corresponding brightfield images showing tdTomato+ and tdTomato− cardiomyocytes. Images 2 and 3 show tdTomato+ cardiomyocytes that appear to be coupled, which suggests cell-cell communication. Arrows in image 3 indicate tdTomato+ non-cardiomyocytes. Image 4 shows tdTomato+ cells that appear similar to fibroblasts. Image 5 shows well-developed sarcomeres in tdTomato+ cardiomyocytes. Scale bars: 50 µm, images 1-3; 25 µm, image 4; 10 µm, image 5.

(B) Immature cardiomyocytes: tdTomato+ cells with intermediate morphology. Images of tdTomato+ and tdTomato− cells from dissociated cell preparations 5 – 6 weeks post-infarct. Upper, red fluorescence, showing tdTomato+ cells. Lower, corresponding brightfield images showing tdTomato+ and tdTomato− cells. Arrow in image 2 indicates tdTomato+ cell that appears similar to a fibroblast. Scale bar, 50 µm.
Online Figure V. Imaging of excitation-contraction coupling in tdTomato+ cardiomyocytes.

(A) Image of tdTomato+ cardiomyocyte with striated sarcomeres, left panel: visible, right panel: tdTomato.

(B) Image of tdTomato+ cardiomyocyte that exhibited calcium transients and concurrent contraction during pacing at 0.5 Hz.

(C) Calcium transients obtained from Fura-2 loaded tdTomato+ cardiomyocyte in (B) during pacing at 0.5 Hz. Single wavelength emission was recorded to increase time resolution.

(D) Brightfield images illustrate contraction of cardiomyocyte in (B) during pacing at 0.5 Hz. Images correspond to frames in Online movie 1. Arrows are in same location in each of the 3 frames, highlighting shortening of cardiomyocyte during contraction (middle frame).
Online Figure VI. miR combo delivery improves cardiac function following myocardial infarction.

(A) Cardiac function was assessed in mice injected with negmiR (n = 9), miR combo (n = 6), or an equivalent volume of PBS (n = 3) by echocardiographic analysis before and at 2 weeks and 1, 2, and 3 months post-infarct. Fractional shortening, Vcfc, LV mass, and LVEDS of miR combo vs negmiR mice. * indicates p < 0.05 compared to negmiR group as determined by ANOVA.

(B) Cultured neonatal cardiomyocytes were transfected with negmiR or miR combo. After 72 hours cells were treated with 100 μM H_{2}O_{2} for 6 hours where appropriate. Apoptosis was determined by annexin-V staining and flow cytometry. Data is represented as the percentage of total cardiomyocytes (CM) that were annexin-V positive. No add - blank transfection with lipid only.
Online Figure VII. miR combo has no effect on endothelial reprogramming.

Neonatal cardiac fibroblasts were transfected with negmiR or miR combo. After 7 days, expression of the endothelial progenitor marker Flk-1 and the mature endothelial markers VE-cadherin and PECAM1 was determined by qPCR. Expression in untreated cells taken to be 1. N = 3 for Flk-1. Representative experiments are shown for VE-cadherin and PECAM1.
Online Figure VIII. Determination of vascular density in miR combo and negmiR treated mice following myocardial infarction. FSP1-Cre/tdTomato mice were subjected myocardial infarction and injected with lentivirus containing either miR combo or negmiR. Seven weeks following injury the peri-infarct region was visualized by serial sectioning through the heart tissue. Sections were probed with Isolectin-B4 and DAPI to stain vessels and nuclei respectively. N=3. Scale bar 100 microns.

SUPPLEMENT REFERENCES


VIDEO LEGEND

Movie of tdTomato+ cardiomyocyte in Online Figure II that illustrates contraction during pacing at 0.5 Hz.