Single-Dose Intracardiac Injection of Pro-Regenerative MicroRNAs Improves Cardiac Function After Myocardial Infarction

Pierluigi Lesizza, Giulia Prosdocimo, Valentina Martinelli, Gianfranco Sinagra, Serena Zacchigna, Mauro Giacca

Rationale: Recent evidence indicates that a few human microRNAs (miRNAs), in particular hsa-miR-199a-3p and hsa-miR-590-3p, stimulate proliferation of cardiomyocytes and, once expressed in the mouse heart using viral vectors, induce cardiac regeneration after myocardial infarction. Viral vectors, however, are not devoid of safety issues and, more notably, drive expression of the encoded miRNAs for indefinite periods of time, which might not be desirable in light of human therapeutic application.

Objective: As an alternative to the use of viral vectors, we wanted to assess the efficacy of synthetic miRNA mimics in inducing myocardial repair after single intracardiac injection using synthetic lipid formulations.

Methods and Results: We comparatively analyzed the efficacy of different lipid formulations in delivering hsa-miR-199a-3p and hsa-miR-590-3p both in primary neonatal mouse cardiomyocytes and in vivo. We established a transfection protocol allowing persistence of these 2 mimics for at least 12 days after a single intracardiac injection, with minimal dispersion to other organs and long-term preservation of miRNA functional activity, as assessed by monitoring the expression of 2 mRNA targets. Administration of this synthetic formulation immediately after myocardial infarction in mice resulted in marked reduction of infarct size and persistent recovery of cardiac function.

Conclusions: A single administration of synthetic miRNA–lipid formulations is sufficient to stimulate cardiac repair and restoration of cardiac function. (Circ Res. 2017;120:1298-1304. DOI: 10.1161/CIRCRESAHA.116.309589.)

Key Words: microRNA ▪ myocardial infarction ▪ regeneration ▪ therapy ▪ transfection

Mounting evidence indicates that RNA interference (RNAi) regulates virtually all aspects of cardiovascular function in both normal and pathological conditions. In particular, endogenous microRNAs (miRNAs) are crucial in regulating various aspects of cardiomyocyte biology, ranging from contractility to hypertrophy, both during development and in pathological conditions. Modulation of this miRNA network stands as an innovative therapeutic strategy against otherwise incurable pathological conditions.

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One of the paradigm-breaking concepts that has emerged recently is that the adult heart is still endowed with regenerative capacity, and that this capacity, albeit limited, increases after myocardial infarction (MI). In particular, 14C dating and imaging mass spectrometry studies concordantly show that, in normal conditions, ≈1% of cardiomyocytes are renewed annually, and that this percentage increases at least 3× after damage. Not surprisingly, cardiomyocyte replication is also under the control of the miRNA network; actually, various miRNAs have been shown to either increase or decrease replication of both fetal and postnatal cardiomyocytes. Our own work has revealed that at least 40 miRNAs, identified by a high throughput genomic screening performed with almost 1000 miRNAs, are capable of markedly boosting cardiomyocyte proliferation, promoting DNA synthesis passage through the G2/S phase of the cell cycle and karyokinesis.

miRNAs are short, 21 to 22 nucleotide-long, double-stranded RNAs that are physiologically generated by the cellular RNA-induced silencing complex by processing longer hairpin RNAs, which are, in turn, the processing products of upstream enzymes of the RNAi machinery. Intracellular overexpression of miRNAs in cardiomyocytes can effectively be achieved by transferring the respective coding genes using adeno-associated virus (AAVs) vectors, which efficiently
transduce cardiomyocytes, after either intracardiac or systemic delivery.10 Our previous work also showed that AAV9 vectors expressing miR-590 or miR-199a were capable of promoting cardiomyocyte proliferation and, thus, cardiac regeneration and functional recovery after MI in mice.9

Expressing miRNAs using AAV vectors, however, is not devoid of problems. In particular, these vectors persist epistemally and express their transgenes for prolonged periods of time, virtually coinciding with the life of the organism.10 These characteristics raise both safety and efficacy concerns. A more appealing delivery strategy for cardiac regenerative miRNAs would be the administration of these small nucleic acids as synthetic molecules, provided that their endogenous RNAi machinery for processing, either miRNA strand can be used according to the expressing cell type. These characteristics raise both safety and efficacy concerns.

A more appealing delivery strategy for cardiac regenerative miRNAs would be the administration of these small nucleic acids as synthetic molecules, provided that their endogenous RNAi machinery for processing, either miRNA strand can be used according to the expressing cell type. These characteristics raise both safety and efficacy concerns.

### Novelty and Significance

**What Is Known?**
- The heart has a poor regenerative capacity after damage.
- Previous studies have shown that the stimulation of cardiomyocyte replication using viral vectors expressing pro-proliferative microRNAs can lead to cardiac repair.
- Clinical use of viral vectors to administer microRNAs is still hampered by safety and technical issues.

**What New Information Does This Article Contribute?**
- A single injection of synthetic microRNA mimics for 2 specific microRNAs (miR-199a-3p and miR-590-3p) significantly improves cardiac function and stimulates cardiac repair after myocardial infarction in mice.
- Synthetic microRNA mimics might represent a novel class of biotherapeutics to stimulate cardiac regeneration after cardiac damage.

### Results

**Lipid Formulations for Small RNAs Cardiac Gene Transfer**
We compared transfection efficiency of small RNAs using 5 different commercial lipid formulations. These included Oligofectamine, Lipofectamine 2000, Lipofectamine RNAiMAX, Xtreme Gene, and INTERFERin. Efficiency of transfection of neonatal mouse ventricular cardiomyocytes was tested by assessing the effect of an siRNA targeting the Ubc gene in the ubiquitination cascade, which is essential for cell viability,11 including that of cardiomyocytes.9 We comparatively evaluated toxicity of transfection in the absence (lipid only) or presence of the anti-Ubc siRNA by measuring both the number of a-actinin-positive cardiomyocytes and ATP production as a surrogate for survival (the latter measurements also evaluates viability of other cell types eventually present in the primary cultures). We concluded that the most effective and less toxic formulation for neonatal cardiomyocyte transfection was Lipofectamine RNAiMAX (transfection efficiency >80% for both measurements; Figure 1A and 1B).

Three of the effective lipid formulations and an additional one reported to be active in vivo in other tissues (Invivofectamine) were then tested for their efficiency in delivering miR-199a-3p in 2-month-old female CD1 mice by intracardiac injection (n=6 per group). At day 3 after injection, we measured the levels of the injected miRNA mimic in the isolated left ventricular anterior wall and those of 2 miR-199a-3p targets that we had previously identified, the cellular genes Homer1 and Clic5,5 which were, thus, used here as surrogate markers to assess miRNA function. We detected a significant increase in the levels of miR-199a-3p using all 4 lipids, most notably with Lipofectamine RNAiMAX (P<0.01 in both cases; Figure 1C). Importantly, the levels of both analyzed cellular target genes were significantly downregulated, indicative of effective transfection (P<0.05; Figure 1D). Monitoring miRNA transfection using fluorescent miRNAs followed by fluorescence microscopy generated results that were largely inconsistent with the observed functional effects, suggesting...

### Methods

Expanded methods are presented in the Online Data Supplement. Isolation of cardiomyocytes and ex vivo siRNA transfection, intracardiac injections and MI, assays for cardiomyocyte proliferation and apoptosis, miRNA and miRNA quantification by reverse transcriptase polymerase chain reaction, echocardiography, and histological analysis were performed using standard procedures, as described.9

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Nonstandard Abbreviations and Acronyms

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<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>miRNA</td>
<td>microRNA</td>
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<td>RNAi</td>
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P <0.05; Figure 1D). Monitoring miRNA transfection using fluorescent miRNAs followed by fluorescence microscopy generated results that were largely inconsistent with the observed functional effects, suggesting...
that this is an ineffective method to evaluate in vivo transfection (data not shown).

Finally, in the group of mice injected in the left ventricle anterior wall with Lipofectamine RNAiMAX/miR199a-3p mimic, we measured the levels of the transfected miRNA in other organs to assess extracardiac dissemination. We found significantly increased levels of exogenous miR-199a-3p in liver (≈4.7-folds over endogenous expression) and kidney (≈8.3-folds; P<0.05). In the heart, miR-199a-3p exceeded over 200-folds the endogenous levels (P<0.01; Figure 1E).

Together, these results indicate that in vivo cardiac transfection of functional miRNAs can be achieved by intracardiac injection of cationic lipid formulations. Lipofectamine RNAiMAX was chosen for all the subsequent in vivo studies.

**In Vivo Persistence of Transfected miRNAs**

A crucial requisite for the development of an effective, miRNA-based, pro-regenerative therapy is to ensure proper persistence of the miRNA effect in vivo. We, therefore, injected the hearts of 2-month-old female CD1 mice (n=6 per group) with synthetic miR-199a-3p or miR-590-3p mimics in complex with the chosen lipid formulation, followed by quantification of the miRNAs in the isolated left ventricle free wall at 2, 4, 8, 12, and 20 days after injection; animals injected with a control, nontargeting mimic (Caenorhabditis elegans cel-miR-67, which has no homologue in vertebrates) served as a control.

The levels of transfected miR-199a-3p mimic progressively declined over time, but remained significantly higher than those of endogenous miR-199a-3p ≤12 days, as assessed by quantitative polymerase chain reaction amplification (Figure 2A). These levels were 269.0±21.9-fold over control at 2 days after injection, 37.0±4.0-fold at 4 days, 7.6±1.1-fold at 8 days, and 2.2±0.2-fold at 12 days (P<0.05 at all time points). A similar kinetics was also observed for miR-590-3p (Figure 2B; in this case, however, the endogenous miR-590-3p is not expressed at quantifiable levels in the heart).

Persistence of the injected miRNA mimics correlated with their functional effect, as concluded from the analysis of target
gene levels. In particular, we observed a significant downregulation of both Homer1 and Clic5 at both days 2 and 4 after transfection with either miR-199a-3p or miR-590-3p mimics. In the case of miR-590-3p, downregulation of Homer1 also continued at days 8 and 12 after myocardial injection (Figure 2C; P<0.05 in all cases).

Collectively, these results indicate that after direct intramyocardial injection, miRNA mimics exert a functional effect for several days.

### Functional Effect of Single-Dose, Intramyocardial Injection of miRNA Mimics After MI

Based on the above observations, we wanted to assess the efficacy of synthetic miRNA mimic injection after MI. Immediately after permanent ligation of the left anterior descending coronary artery, 2-month-old CD1 female mice were injected in the infarct border zone with 1.4 μg miR-199a-3p (n=20), miR-590-3p (n=13), or cel-miR-67 (n=13) mimics in a complex with Lipofectamine RNAiMAX; the last miRNA served as a control. Five and 3 animals died in the control and miR-199a-3p groups, respectively, within the first 20 days, while none died in the miR-590-3p group (Figure 3A).

Cardiac function was assessed by echocardiography over time. In contrast to control animals, in which there was a progressive deterioration, left ventricle ejection fraction was significantly preserved in the animals treated with miR-590-3p through the whole echocardiographic follow-up period, from week 1 to week 8 (left ventricle ejection fraction at week 8: 48.5±7.5% in miR-590-3p-injected animals versus 24±8.1% in control animals; P<0.01; Figure 3B). In the animals treated with miR-199a-3p, left ventricle ejection fraction was similar to that of controls during the first 2 weeks, but then remained constant up to week 8 (left ventricle ejection fraction at week 8 in the miR-199a-3 group: 37.5±4.5; P<0.05 versus control).

Analogous considerations on the effects of both miR-199a-3p and miR-590-3p also applies to the analysis of other parameters of cardiac function, including left ventricle fractional shortening (Figure 3C), left ventricle anterior wall and septum thickness (Online Figure I), and to the measurement of left ventricle systolic internal diameter, an indicator of cardiac dilation (Figure 3D). Both miRNAs reduced the hypertrophic response of cardiomyocytes observed after MI in control animals (Online Figure II).

At the end of the follow-up period (8 weeks), morphometric analysis indicated a highly significant reduction of the infarct size (28±4.8% of left ventricle in control treated animals versus 18±3.0% and 14±2.2% in the miR-199a-3p and miR-590-3p groups, respectively; P<0.05 in both cases; Figure 4A and 4B for representative images and quantification, respectively). A group of animals (n=6 per treatment) were injected with the thymidine analogue ethynyl-29-deoxyuridine from day 1 to day 12 after MI and miRNA treatment. The number of ethynyl-29-deoxyuridine–positive cardiomyocytes, a marker of cell passage through the S phase, was...
significantly increased at day 12 in the animals that had received miR-199a-3p or miR-590-3p (Figure 4C and 4D for representative images and quantification respectively; n=4 per group). Similarly, the number of cardiomyocytes positive for histone 3 phosphorylation at serine 10, a marker of late G2/mitosis, was increased at day 4 after MI and miRNA treatment (Figure 4E and 4F), as was the number of cardiomyocytes showing Aurora B positivity, including those showing localization of this protein in midbodies, which indicates passage through mitosis (Figure 4G and 4H). There was no evidence that either miRNA protected cardiomyocytes from apoptotic cell death immediately after MI, as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling staining (Online Figure III).

Taken together, these results are, thus, consistent with the conclusion that transfection of both pro-proliferative miR-199a-3p and miR-590-3p preserves cardiac function after MI and leads to cardiac repair.

Discussion
The results shown here indicate that miRNA mimics, which were previously selected for their property to stimulate proliferation of neonatal cardiomyocytes, can effectively be delivered to the adult heart in vivo by intracardiac transfection using cationic lipids. They also show that a single intracardiac injection of two of these miRNAs is sufficient to stimulate cardiac repair after MI.

Unlike small molecules (<500 Da) that passively diffuse across cellular membranes, even small nucleic acids, such as siRNAs or miRNAs, are too large (≈14 kDa) and too charged (≈40 charged phosphates) to spontaneously enter cells.14 In addition, because these molecules need to actively engage the cellular RNAi enzymatic machinery—in particular, to be loaded into the catalytic core of the RNAi effector complex RNA-induced silencing complex15—their phosphodiester backbone tolerates minimal chemical modifications; this renders these molecules prone to degradation by the nucleases present in serum and in the extracellular environment. These characteristics have hampered the rapid progression of miRNA mimic therapeutics toward the clinic. At the preclinical level, the use of viral vectors (in particular, AAVs for intracardiac delivery of the pri-miRNA genes) is commonly considered a more efficient tool to assess the miRNA effect in vivo.10

The use of AAV vectors for cardiac transduction, however, as mentioned previously, is not devoid of problems, especially because of the prolonged expression of the transgene they carry, which virtually coincides with the life of the transduced cells.16 Because the stimulation of cardiomyocyte proliferation is likely to require a partial dedifferentiation step (such as that occurring in zebrafish17,18), prolonged miRNA expression might be dangerous for myocardial function. Additionally, viral vectors accommodate and deliver both miRNA strands, which could be variably incorporated into RNA-induced silencing complex in different cell types, rendering the effect...
difficult to predict. To avoid all of these potential issues, transient administration of miRNA mimics seems highly desirable. Finally, one major difference between miRNA mimics and vector administration relates to cell specificity. Although miRNA lipofection is likely to affect all cell types in the heart (in particular, cardiac fibroblasts and endothelial cells in addition to cardiomyocytes), AAV9 vector transduction is selective for cardiomyocytes. Thus, we cannot formally exclude that the beneficial effects of the pro-regenerative miRNAs we describe here might depend, at least in part, also on the effects exerted in other cell types.

Our results not only challenge the unproven, but widespread, concept that miRNA mimic injection is volatile by showing that the injected mimics persist in the heart for at least 12 days, but also show that this time window is sufficient to drive a significant regenerative response. This result is consistent with the early proliferative response spontaneously occurring after damage in other experimental settings, including the mouse neonatal19 and zebrafish18,20 hearts.

From a technical point of view, our experiments indicate that a most effective manner to measure the results of an exogenously delivered miRNA is to evaluate the levels of its direct targets. Indeed, the analysis of efficiency of transfection using fluorescent miRNAs, in our experience, leads to highly erroneous conclusions, most likely because the signal detected by fluorescence microscopy corresponds to the amount of miRNAs accumulated and deposited into cell compartments other than the cytoplasm, where its effect should be exerted.
delivery; chemical stabilization and conjugation with functional molecules can be used to improve the stability and permeability of short RNA nucleic acids. Notwithstanding these possibilities for amelioration, our work shows that cardiac regeneration can be achieved by transient miRNA delivery to injured, adult hearts, pushing miRNA therapeutics a step forward toward clinical application.

Acknowledgments

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Disclosures

None.

References


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SUPPLEMENTAL METHODS

Isolation of ventricular cardiomyocytes from neonatal mice. Ventricular cardiomyocytes were isolated from neonatal CD1 mice (post-natal day 0). Ventricles were separated from the atria using scissors, cut into pieces and then dissociated in calcium and bicarbonate-free Hanks with HEPES (CBFHH) buffer containing 1.75 mg/ml trypsin (BD Difco) and 10 mg/ml DNase II (Sigma), under constant stirring. Digestion was performed at room temperature in eight to ten 10-min steps, collecting the supernatant into fetal bovine serum (FBS, Life Technologies) after each step. The collected supernatant was centrifuged to separate the cells, which were then suspended in Dulbecco’s modified Eagle medium 4.5 g/l glucose (DMEM, Life Technologies) supplemented with 5% FBS, 20 mg/ml vitamin B12 (Sigma), 100 U/ml of penicillin and 100 mg/ml of streptomycin (Sigma). The collected cells were passed through a cell strainer (40 μm, BD Falcon) and then seeded onto uncoated 100-mm plastic dishes for 2 h at 37°C in 5% CO2 and humidified atmosphere. The supernatant, composed mostly of cardiomyocytes, was then collected and pelleted. Cells were suspended in antibiotic-free media, counted and plated at the appropriate density.

siRNA transfection ex vivo. For anti-Ube siRNA (Dharmacon) transfection, cells were plated in 96-well plates at a density of 1.0x10^4 cells per well and transfected 24 hours later. The following lipids were comparatively tested: Lipofectamine® 2000, Lipofectamine® RNAiMAX Reagent and Oligofectamine Reagent® (Thermo Fisher Scientific), X-tremeGENE® siRNA Transfection Reagent (Roche) and INTERFERin®-HTS (Polyplus Transfection).

The amount of each lipid moiety used to transfect siRNA was calculated according to the manufacturers’ instructions (see table for final amount); the final concentration of siRNA was in all cases 50 nM. For each well transfection Opti-MEM (Thermo Fisher Scientific) was added to siRNA and lipids to reach the final volume of 50 μl.

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<thead>
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<th>FINAL siRNA CONCENTRATION: 50 nM</th>
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<td>96 multiwell</td>
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<td>Lipofectamine 2000 (µl)</td>
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<td>Oligofectamine (µl)</td>
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Cells treated with equal amount of lipid only served as controls. Cells were cultured in humidified atmosphere at 37°C in 5% CO₂ and 72 hours after transfection, were fixed with 4% PFA for 15 minutes, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, followed by 30 minutes blocking in 1% BSA (Roche). Cells were then stained overnight at 4°C with a mouse monoclonal antibody against sarcomeric α-actinin (Abcam) diluted in blocking solution, followed by incubation for 2 hours with a secondary antibody conjugated to Alexa Fluor-488 (Life Technologies). Cells were counterstained with Dapi (Life Technologies) to label nuclei. Cardiomyocyte area was then evaluated by automated high-content fluorescence microscopy using a Molecular Devices ImageXpress Micro screening microscope. Alternatively, for cell viability assay, cells were processed 72 hours after transfection using the ATPlite™ Luminescence Assay (PerkinElmer) according to the manufacturer’s protocol. Luminescence was measured by a Wallac EnVision™ 2104 MultiLabel Reader (PerkinElmer).

**Intra-cardiac miRNA injection and MI.** Animal care and treatment were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (European and Economic Council Directive 86/609, OJL 358, December 12, 1987). CD1 mice were purchased from Harlan Laboratories and maintained under controlled environmental conditions. All intracardiac injections were performed in 2 month-old female CD1 mice. Mice were anesthetized by intraperitoneal injection of ketamine-xylazine (40 mg/kg-100 mg/kg, Imalgene 1000 and Sigma respectively), at a dosage of 1.2-1.3 μl/g. When sleeping, mice were taken out from the cage and laid down in supine position on a dedicated pad at 37°C, fixed to the plate and intubated. To reach the anterior wall of the heart, a xifo-axillar incision was made, exposing the underlying muscles; the pectoralis major muscle was lifted up and fixed with a retractor, while the underlying pectoralis minor was cut to expose the ribs. The 5th intercostal space was pierced and enlarged with a retractor opening the thorax. The pericardium was stripped exposing heart anterior wall and 20 μl of a previously prepared mix of miRNA and lipids (ratio 1:1 in volume) was injected into the left ventricle anterior wall using a 0.3 ml insulin syringe with a 30-gauge needle. All anatomical structures were visualized with a stereomicroscope (Leica). After injection, intercostal space, muscles and skin were sutured and mice were extubated to re-establish normal breathing. Mice were then laid in prone position and kept on the warmed pad until awakening and later transferred to a new cage.

To evaluate efficiency of lipid-mediated, intracardiac miRNA delivery, animals (n=6 per group) received 200 pMoles hsa-miR-199a-3p, hsa-miR-590-3p or cel-miR-67 mimics (all from Dharmacon) mixed with 10 μl lipids, according to the manufacturers’ specific protocols, in a total volume of 20 μl.

To evaluate efficacy of miRNA transfection after MI, in a set of animals (n=13-20 per group) the anterior wall left anterior descending coronary artery was identified and ligated 1 mm below the left atrium auricula. Effective ligation of the coronary artery was confirmed by whitening of heart anterior wall. After ligation, intracardiac injection was performed as described above.

**Analysis of EdU incorporation and immunofluorescence.** To assess cardiomyocyte proliferation, infarcted animals (n=6 per group) received ethynyl-29-deoxyuridine (EdU, Life Technologies; 350 μg per animal intraperitoneally) every 2 days for 12 days. After euthanasia, mouse hearts were excised, briefly washed in PBS, fixed in 10% formalin at room temperature and embedded in paraffin. Samples were cut into 4 μm tissue sections, de-waxed in xylene for 30 min and rehydrated with alcohols at decreasing concentration (100, 90, 70, 50%) at room temperature. Antigen retrieval was performed on sections by boiling 20

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<th>X-tremeGENE (μl)</th>
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<td>INTERFERin (μl)</td>
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<td>Lipofectamine RNAiMax (μl)</td>
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minutes in 0.1 M sodium citrate buffer solution at pH 6.0 and letting cool down at room temperature for 3 hours. Sections were rinsed three times in water, permeabilized 30 min in 0.5% Triton X100 PBS, and then blocked for 1 h in 20% horse serum PBS. Tissue sections were stained overnight at 4°C in 1:100 anti-α-actinin monoclonal mouse antibody (Abcam) in 5% horse serum PBS, washed 2 times 5 min in 0.5% Triton X-100 at room temperature and incubated 1 h in 1:200 secondary antibody conjugated to Alexa Fluor-488 (Life Technologies). Tissue sections were further processed using the Click-IT EdU 555 Imaging kit to reveal EdU incorporation, according to the manufacturer’s instructions, and stained with Hoechst 33342 (Life Technologies). Histone H3 phosphorylation at serine 10 and Aurora B were recognized using primary antibodies purchased from Millipore and Abcam respectively and secondary antibody conjugated with Alexa Fluor-594 (Life Technologies). To measure cardiomyocyte cross sectional area, lectin Wheat Germ Agglutinin (WGA; Vector Labs) 1:100 was added together with the secondary antibody.

**RNA isolation and quantitative real-time PCR.** At the indicated time-points, animals were anaesthetized with 5% isoflurane and euthanized by cervical dislocation. Hearts were collected and briefly washed in PBS; the left ventricle anterior wall was separated from the rest of the heart and immediately frozen in liquid nitrogen. Total RNA, including the small RNA fraction was extracted using miRNeasy Mini Kit (Qiagen), according to manufacturer’s instructions. To quantify miRNAs, RNA was reverse transcribed using miRCURY LNA Universal cDNA synthesis kit (Exiqon) followed by qRT– PCR using pre-designed miRCURY LNA PCR primer sets (Exiqon) and miRCURY LNA SYBR Green master mix, according to manufacturer’s protocol. The levels of the analyzed miRNAs were normalized to those of the 5S rRNA. For the quantification of endogenous gene expression, 1 µg of RNA was first processed by adding 1 µL DNAse buffer and 0.2 µL DNAse (Roche) to a final volume of 10 µL to eliminate genomic DNA traces. Mixture was incubated for 15 minutes at room temperature and then heated for 10 minutes at 75°C to inactivate the reaction. Total RNA was reverse-transcribed using M-MLV (Invitrogen) random hexamers (10 µM) as a primer, 10 µM dATP, dTTo, dCTP and dGTP, and 20 U RNasin (Invitrogen). After 1 hour at 37°C the reaction was inactivated by heating 15 min at 70°C. For qRT–PCR, 1 µL presigned TaqMan assays (Applied Biosystems) were incubated with 10 ng cDNA, 10 µL iQ SuperMix (BioRad) in a 20 µL reaction mixture. PCR amplification protocol entails 1 step at 95°C for 3 minutes and then 44 cycles of 10 seconds at 95°C and 30 seconds at 60°C. The housekeeping gene GAPDH was used for normalization. Since the sequence of miR-199a-3p is 100% conserved in human and mice, the primer set for this miRNA recognizes both the endogenous and transfected miRNA species.

**TUNEL assay.** For analysis of TUNEL in vivo, hearts were collected 2 days after MI and miRNA mimic injection and fixed overnight in PFA 4%. Cryosections were obtained after 24 h incubation in sucrose 20%, rinsed in PBS, permeabilized 15 min in 0.5% Triton X100 PBS, and then blocked for 1 h in 2% BSA in PBS. Tissue sections were stained overnight at 4°C in 1:100 anti-α-actinin monoclonal mouse antibody (Abcam) in 2% BSA in PBS, washed 3 times in PBS at room temperature and incubated 1 h in 1:500 secondary antibody conjugated to Alexa Fluor-488 (Life Technologies). TUNEL assay (Roche) was performed, according to the manufacturer’s instructions. Nuclei were further stained with Hoechst 33342 (Life Technologies).

**Echocardiography analysis.** To evaluate heart function and size, transthoracic two-dimensional echocardiography was performed in mice anaesthetized with 5% isoflurane at 1, 2, 4 and 8 weeks after MI, using a Vevo 2100 Ultrasound (Visual Sonics) equipped with a MS550D 22–50 MHz linear array solid-state transducer. M-mode tracings in parasternal short and long axis views were used to measure left ventricular anterior and posterior wall thickness, septum thickness and left ventricular internal diameter at end-systole and end-diastole, which were used to calculate left ventricular fractional shortening and ejection fraction.
Heart collection and histological analysis. At the end of the studies, animals were anaesthetized with 5% isoflurane and then killed by cervical dislocation. The heart was excised, briefly washed in PBS, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology. Azan-Mallory’s trichrome staining (Bio Optica) was performed according to standard procedures, and analyzed for morphology and extension of fibrosis. Infarct size was calculated as the percentage of the total left ventricular area showing fibrosis.

SUPPLEMENTAL REFERENCES


Online Figure I. Cardiac wall thickness after MI and miRNA treatment. Left ventricular anterior wall (LVAW) thickness at end-systole and end-diastole and interventricular septum (IVS) thickness at end-diastole (A, B, C respectively) were assessed by echocardiography. Data are mean±s.e.m.; n=13-20 per group; *P<0.05 relative to Control (cel-miR-67).
Online Figure II. Visualization of cardiomyocytes cross-sectional area in non-infarcted animals and in infarcted animals after miRNA treatment at day 12 after MI and treatment. Representative images are in A (blue: Dapi, staining nuclei; red: α-actinin, staining cardiomyocytes; green: lectin WGA, marking cell membrane), quantification in B. *P<0.05 relative to Control (untreated animals).

Online Figure III. Visualization of TUNEL+ cardiomyocytes in infarcted animals at day 2 after miRNA treatment. Representative images are in B (blue: Dapi, staining nuclei; green: α-actinin, staining cardiomyocytes; red: TUNEL, marking cells that are undergoing apoptosis), quantification in A.