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

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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.)**

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[1] to hypertrophy,[2] both during development[3] and in pathological conditions.[4] 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[5,6] and imaging mass spectrometry[7] 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.[8] 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.[9]

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

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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.

There is a compelling need to develop innovative therapies promoting cardiac repair in patients with myocardial infarction; this can be achieved through the stimulation of the normally poor proliferative capacity of cardiomyocytes. Previous work has shown that a few pro-proliferative microRNAs, expressed from viral vectors, lead to cardiac regeneration after myocardial infarction in mice. Here we show that a single-dose, intramyocardial injection of synthetic microRNA mimics corresponding to 2 of the most effective of these microRNAs is sufficient to promote cardiac functional improvement and stimulate endogenous cardiomyocyte proliferation. These pro-proliferative microRNA mimics might constitute innovative nucleic acid drugs for patients with myocardial infarction.

Nonstandard Abbreviations and Acronyms	
AAV	adeno-associated virus
MI	myocardial infarction
miRNA	microRNA
RNAi	RNA interference

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 episomally and express their transgenes for prolonged periods of time, virtually coinciding with the life of the organism.[10] In addition, a portion of the injected vectors also transduces other organs, in particular, liver and skeletal muscle in the case of the AAV9 serotype.[11,12] Finally, because miRNA production after virus-mediated delivery of their coding genes depends on the 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 in vivo persistence is sufficient to induce the desired pro-proliferative effect. Here we show that a single intracardiac injection of miR-199a-3p and miR-590-3p in mice has prolonged effects on the downregulation of their respective target mRNAs and is sufficient to promote stable cardiac repair after MI in mice.

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, mRNA and miRNA quantification by reverse transcriptase polymerase chain reaction, echocardiography, and histological analysis were performed using standard procedures, as described.[9]

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,[13] 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 α-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,[9] 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 2000 and 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

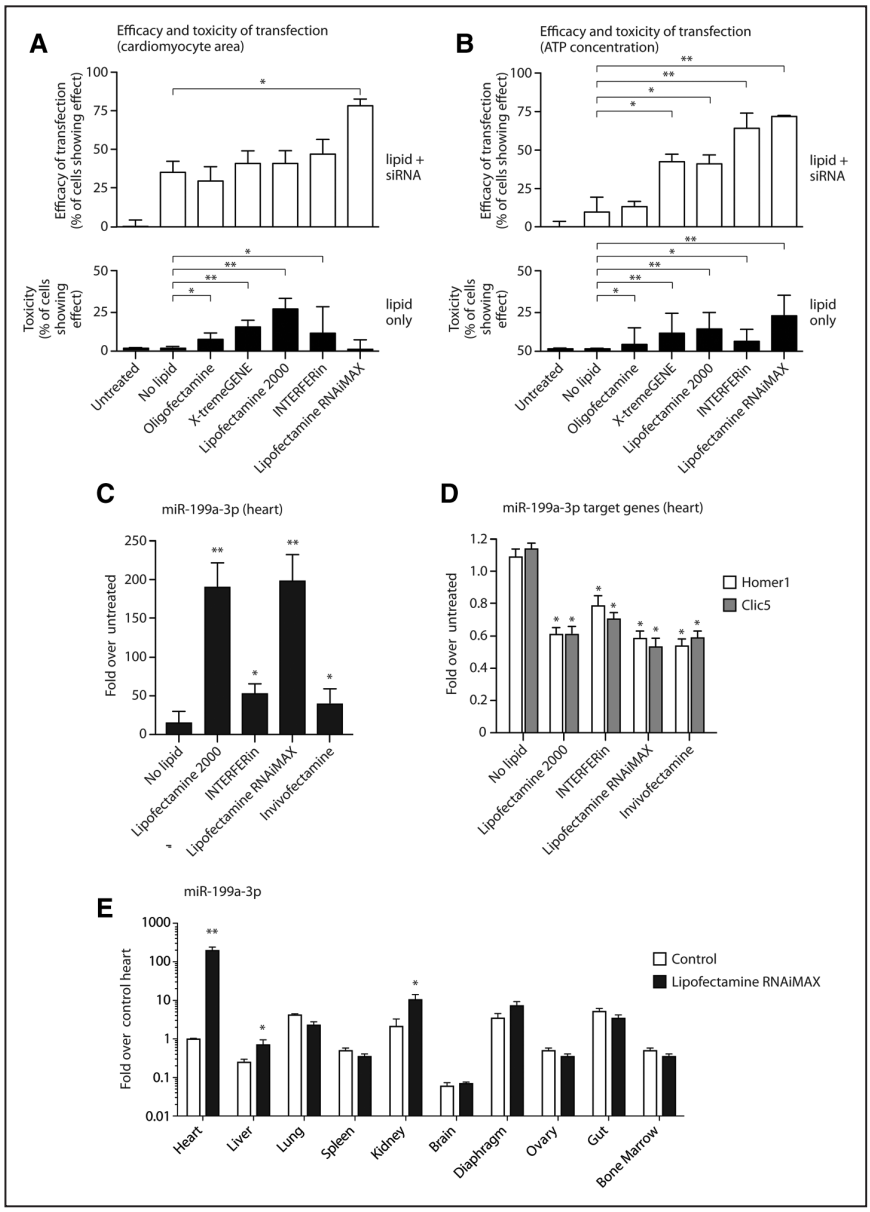

Figure 1. Efficacy of different lipid formulations on in vitro and in vivo delivery of small RNAs. **A** and **B,** Efficacy and toxicity of lipid-mediated microRNA (miRNA) transfection of cardiomyocytes. Neonatal mouse cardiomyocytes were transfected with an siRNA against the *Ubc* gene (which is essential for cell viability) using the indicated lipid formulations. The graphs report the percentage of cells, compared with untreated controls, showing the effect of treatment (cell death) using lipids or lipids plus siRNA (black and white bars, respectively), measured as cellular area (**A**) or levels of ATP production (**B**). Data are mean±SEM of 5 independent experiments; **P*<0.05, ***P*<0.01. **C,** Quantification of the levels of miR-199a-3p at 2 days after intracardiac injection using the indicated lipid formulations. n.t. indicates not tested. Data are mean±SEM; n=6 per group; **P*<0.05, ***P*<0.01. **D,** Quantification of the levels of 2 miR-199a-3p direct target mRNAs (Homer1 and Clic5) at 2 days after intracardiac miRNA injection using the indicated lipid formulations. Data are mean±SEM; n=6 per group; **P*<0.05. **E,** Levels of miR-199a-3p in different organs at 2 days after intracardiac miRNA injection using Lipofectamine RNAiMAX. The white bars (control, cel-miR-67) show the endogenous levels of miR-199a-3p. Data are mean±SEM; n=6 per group; **P*<0.05, ***P*<0.01.

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

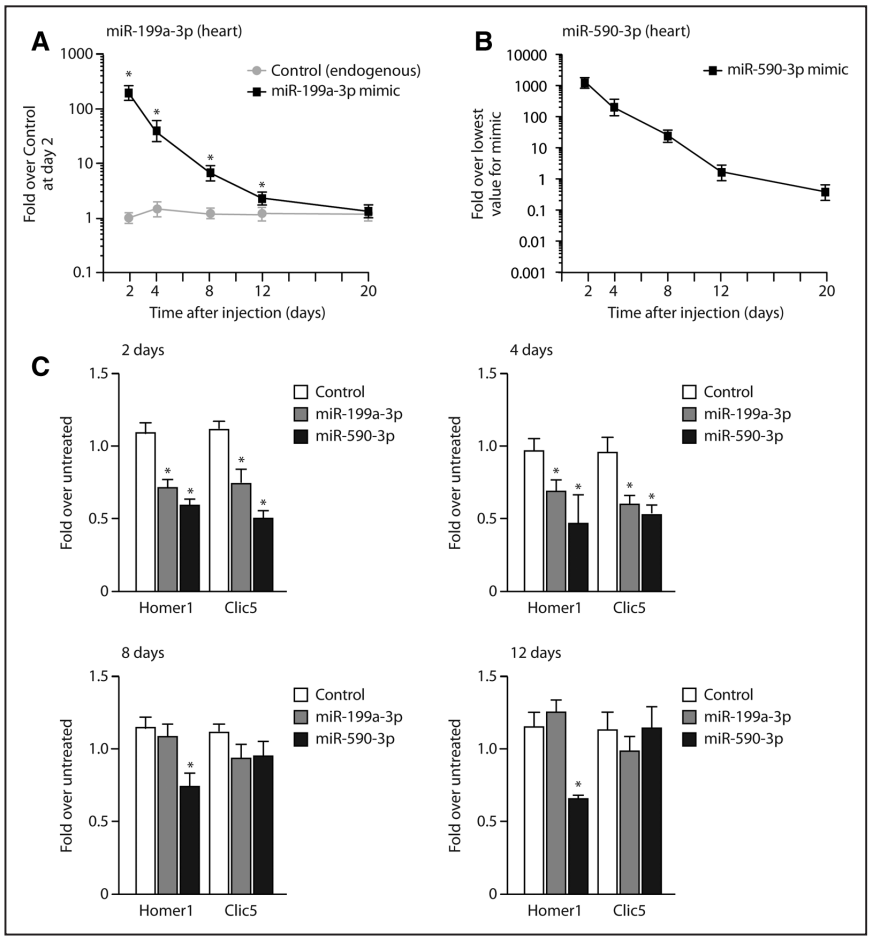

Figure 2. Prolonged effect of miRNA mimics after intracardiac transfection. **A** and **B,** Quantification of miR-199a3p (**A**) and miR-590-3p (**B**) mimics at different times after intracardiac injection. The gray line in (**A**) shows the levels of endogenous miR-199a-3p; polymerase chain reaction (PCR) quantification detects both endogenous and transfected mimics; **P*<0.05. Control refers to animals injected with cel-miR-67. **C,** Quantification of miR-199a-3p and miR-590-3p direct targets Homer1 and Clic5, as indicated, at 2, 4, 8, and 12 days after intracardiac injection. Data are mean±SEM; n=6 per group; **P*<0.05.

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

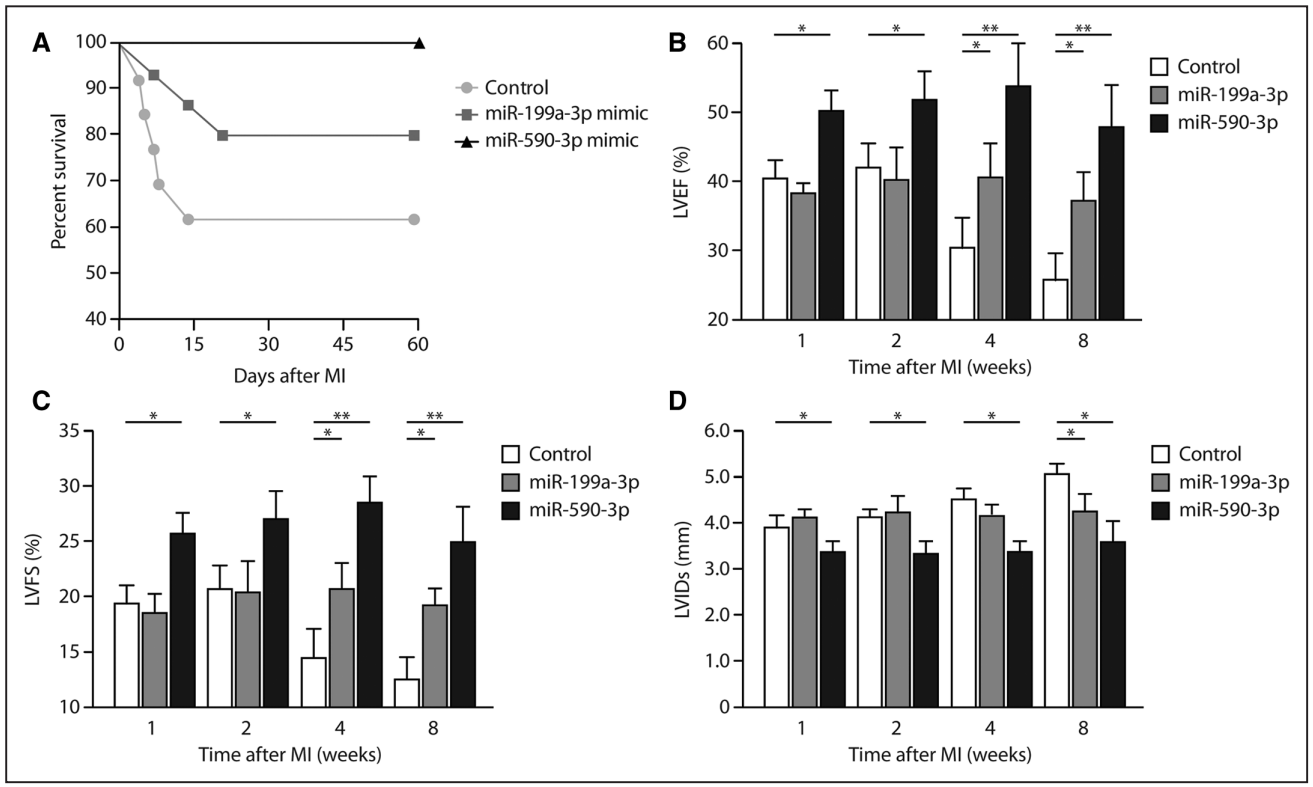

Figure 3. Delivery of miR-199a-3p and miR-590-3p mimics improves heart function and mice survival after myocardial infarction (MI). A, Mortality after MI and miRNA treatment. The Kaplan–Meier curves show mice survival after MI and single-dose miRNA injection (n=13 for miR-590a-3p and control (cel-miR-67), n=20 for miR-199a-3p). **B–D,** Cardiac function after MI and miRNA treatment. Left ventricular (LV) ejection fraction (LVEF), LV fractional shortening (LVFS), and LV internal diameter (LVID) at the end-systole (**B**, **C**, and **D,** respectively) were assessed by echocardiography. Data are mean±SEM; n=13 to 20 per group; **P*<0.05, ***P*<0.01 relative to control (cel-miR-67).

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 complex[15]—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 zebrafish[17,18]), 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

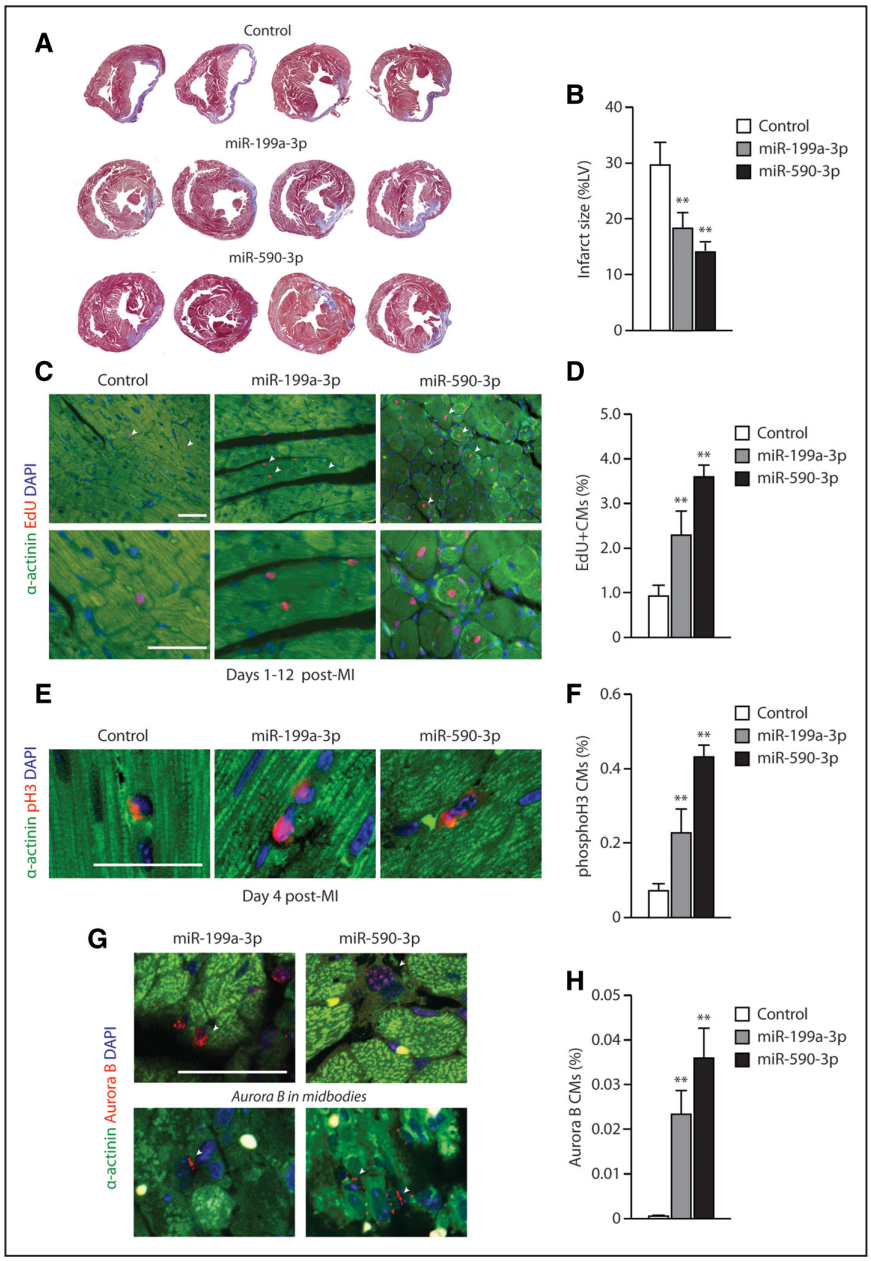

Figure 4. Administration of miR-199a-3p and miR-590-3p mimics after myocardial infarction (MI) reduces infarct size. **A** and **B,.** Histology of infarcted hearts at 8 weeks after MI and treatment. Azan–Mallory trichrome staining of representative cross-sections from 4 animals (**A**) and quantification of infarct size (**B**). Data are mean±SEM; n=13 to 20 per group; ***P*<0.01 relative to cel-miR-67 used as a control. **C** and **D**, Incorporation of ethynyl-29-deoxyuridine (EdU) in infarcted animals at day 12 after miRNA treatment. Representative images are in **C** (blue: Dapi, staining nuclei; green: α-actinin, staining cardiomyocytes; red: EdU, marking cells that have synthesized DNA; scale bar: 50 μmol/L), quantification in **D**. ***P*<0.01 relative to cel-miR-67, used as a control. Arrows point at EdU-positive nuclei. **E** and **F**, Visualization of phosho-histone H3 phoshorylation in infarcted animals at day 4 after miRNA treatment. Representative images are in **E** (blue: Dapi, staining nuclei; green: α-actinin, staining cardiomyocytes; red: phosho-H3, marking cells that are in late G2/M; scale bar: 50 μmol/L), quantification in **F**. ***P*<0.01 relative to cel-miR-67, used as a control. **G** and **H**, Visualization of Aurora B in infarcted hearts at day 4 after miRNA treatment. Representative images are in **G** (blue: Dapi, staining nuclei; green: α-actinin, staining cardiomyocytes; red: Aurora B, marking cells in late G2/M, including the end of cytokinesis when the protein marks midbodies; scale bar: 50 μmol/L), quantification in **H**. ***P*<0.01 relative to cel-miR-67, used as a control. Arrows point at Aurora B–positive nuclei (**upper** images) or midbodies (**lower** images).

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 neonatal[19] and zebrafish[18,20] 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.

We are well aware that there are many opportunities for improvement of transient miRNA delivery in terms of efficacy: limited modifications to the chemical miRNA scaffold can be tolerated and can extend the half-life of the nucleic acid[21]; different cationic lipids or cationic lipid–nanoparticle formulations can limit toxicity of lipofectamine and improve

delivery[22]; chemical stabilization and conjugation with functional molecules can be used to improve the stability and permeability of short RNA nucleic acids[14,23–25]. 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.

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Disclosures

None.

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