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

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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% CO_2 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-Ubc siRNA (Dharmacon) transfection, cells were plated in 96-well plates at a density of 1.0×10^4 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.

FINAL siRNA CONCENTRATION:	**50 nM**
	96 multiwell
Lipofectamine 2000 (µl)	0.2
Oligofectamine (µl)	0.5

X-tremeGENE (µl)	0.25
INTERFERin (µl)	0.875
Lipofectamine RNAiMax (µl)	0.4

Cells treated with equal amount of lipid only served as controls. Cells were cultured in humidified atmosphere at 37°C in 5% CO_2 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 intraperithoneal 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 then 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

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 predesigned 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.

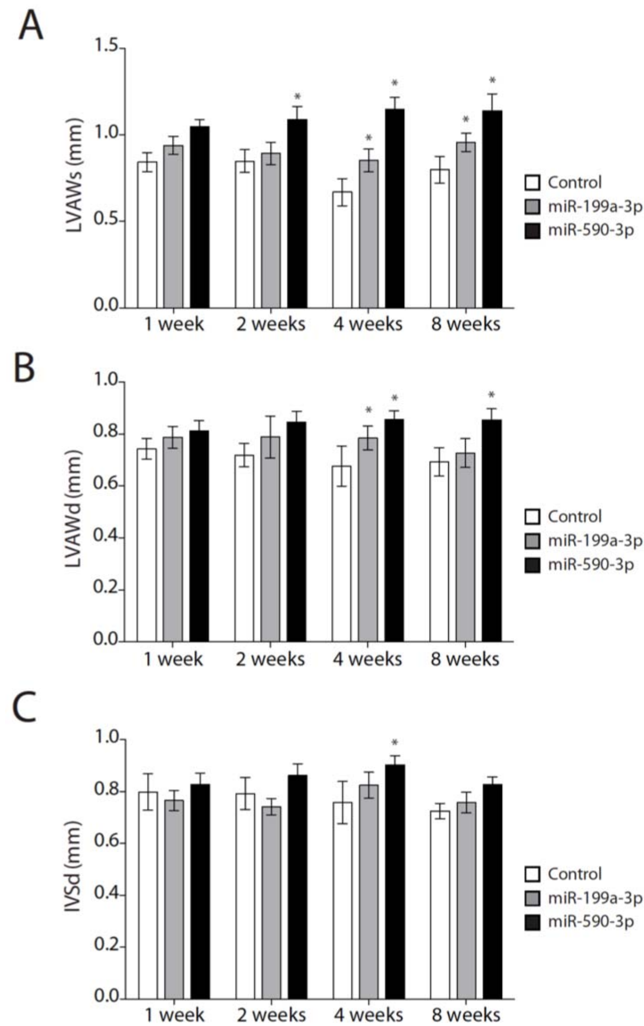
Heart collection and histological analysis. At the end of the studies, animals were anaesthetized with 5% isoflurane and then killed by cervical dislocation. The heart was excised, briefly washed in PBS, fixed in 10% formalin at room temperature, embedded in paraffin and further processed for histology. 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

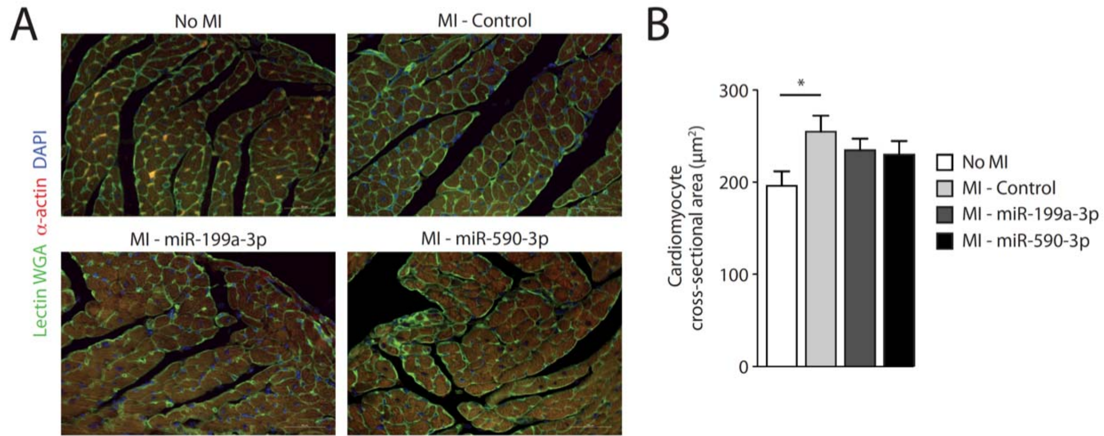
1. Collesi C, Zentilin L, Sinagra G, Giacca M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. *J Cell Biol.* 2008;183:117-128.

2. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. *Nature.* 2012;492:376-381.

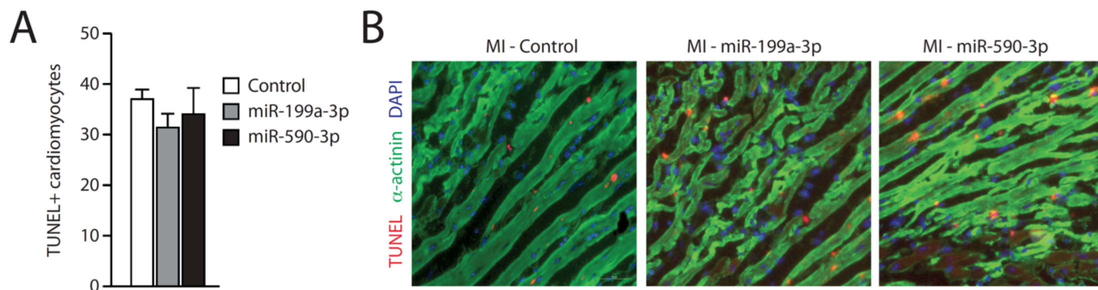
ONLINE FIGURES



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**.