MicroRNA-210 as a Novel Therapy for Treatment of Ischemic Heart Disease

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Background—MicroRNAs are involved in various critical functions, including the regulation of cellular differentiation, proliferation, angiogenesis, and apoptosis. We hypothesize that microRNA-210 can rescue cardiac function after myocardial infarction by upregulation of angiogenesis and inhibition of cellular apoptosis in the heart.

Methods and Results—Using microRNA microarrays, we first showed that microRNA-210 was highly expressed in live mouse HL-1 cardiomyocytes compared with apoptotic cells after 48 hours of hypoxia exposure. We confirmed by polymerase chain reaction that microRNA-210 was robustly induced in these cells. Gain-of-function and loss-of-function approaches were used to investigate microRNA-210 therapeutic potential in vitro. After transduction, microRNA-210 can upregulate several angiogenic factors, inhibit caspase activity, and prevent cell apoptosis compared with control. Afterward, adult FVB mice underwent intramyocardial injections with minicircle vector carrying microRNA-210 precursor, minicircle carrying microRNA-scramble, or sham surgery. At 8 weeks, echocardiography showed a significant improvement of left ventricular fractional shortening in the minicircle vector carrying microRNA-210 precursor group compared with the minicircle carrying microRNA-scramble control. Histological analysis confirmed decreased cellular apoptosis and increased neovascularization. Finally, 2 potential targets of microRNA-210, Efnα3 and Ptp1b, involved in angiogenesis and apoptosis were confirmed through additional experimental validation.

Conclusion—MicroRNA-210 can improve angiogenesis, inhibit apoptosis, and improve cardiac function in a murine model of myocardial infarction. It represents a potential novel therapeutic approach for treatment of ischemic heart disease. (Circulation. 2010;122[suppl 1]:S124–S131.)

Key Words: gene therapy • ischemic heart disease • microRNA • minicircle vector

Ischemic heart disease is the number 1 cause of morbidity and mortality in the United States owing to aging, obesity, diabetes, and other comorbid diseases. One potent therapeutic approach for ischemic heart disease is to reduce oxygen consumption, inhibit cardiomyocyte apoptosis, increase coronary flow, and induce revascularization. MicroRNAs (miRNAs), representing approximately 1% of the eukaryotic transcriptome, is an evolutionarily conserved family of non-coding RNAs of 20 to 22 nucleotides that negatively regulate the expression of protein-coding genes through translational inhibition and RNA decay. miRNAs are involved in diverse biological progresses, including cellular differentiation, proliferation, angiogenesis, and apoptosis. To date, 721 miRNAs have been discovered in human and 597 miRNAs in the mouse according to the miRBase Sequence Database Release 14 (www.mirbase.org/). miRNAs can regulate approximately 30% human protein-coding genes. Importantly, the successful suppression of murine liver cancer by systemic delivery of miR-26a suggests the potential of using miRNAs as a novel therapeutic tool.

In the cardiovascular field, miRNAs have also been implicated as a significant factor in various physiological and pathological diseases. For example, miR-21 expression is significantly downregulated in the infarcted heart but upregulated in border areas, hence serving a possible protective role in the early phase of acute myocardial infarction. Recently, several groups have reported miR-210 as 1 of several hypoxia-induced miRNAs critical for cell survival and angiogenesis. Huang et al demonstrated that miR-210 is hypoxia inducible factor-1α-dependent and provided further insight into its functional role during tumor initiation. They showed that increasing miR-210 expression gives the tumor cells an opportunity to prevail under initial stressful conditions.

In this study, we hypothesize that miRNA may play a significant role in regulating angiogenesis and apoptosis after myocardial infarction. We demonstrate for the first time that delivery of miR-210 through a nonviral minicircle vector in the ischemic heart can improve heart function by promoting angiogenesis and inhibiting apoptosis. Our results show miR-210 may lead to a novel therapy for ischemic heart disease.
Materials and Methods

Cell Culture, Cell Transduction, and Hypoxic Conditions

293FT cells (Invitrogen) were used to generate recombinant replication-deficient lentivirus used for in vitro assays as described.6 Mouse HL-1 cardiomyocytes were cultured in Clonetics media supplemented with 10% fetal bovine serum (Sigma), 0.1 μM l-norepinephrine (Sigma), 2 mM L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen) in a humidified 5% CO2 incubator at 37°C. Hypoxia was achieved by placing cells in a hypoxia chamber filled with 5% CO2, 1% O2, and 94% N2 at 37°C. At different time points during hypoxic treatment, cells were harvested for analysis of miR-210 levels. Angiogenesis factor antibody array (Panomics Inc, Fremont, Calif) was used to investigate the angiogenic potential of miR-210. Apoptosis assays were performed after 48 hours of transduction with lentivirus carrying miR-210 precursor (Pre-210), lentivirus carrying miR-scramble (Pre-Scr), lentivirus carrying antisense of miR-210 (Anti-210), and lentivirus carrying antisense of miR-scramble (Anti-Scr) using a Caspase-Glo 3/7 Assay (Promega) according to the manufacturer’s instructions. The caspase activities for all samples were normalized to that of an equal protein amount. The data obtained are from experiments performed in triplicate.

miRNA Microarray Data Analysis

Microarray assay was performed using a service provider (LC Sciences, Houston, Texas). The assay started with a 4-μg total RNA sample. Hybridization was performed overnight on a microfluidic chip consisting of chemically modified nucleotide coding segment complementary to target microRNA (miRBase 13.0) or other control RNA. Fluorescence images were collected using a laser scanner and digitized using Array-Pro image analysis software. Raw data matrix is then subtracted by the background matrix. Data adjustment includes data filtering, Log2 transformation, gene centering, and normalization. A 2-sample t-test was conducted for statistical analysis.

Preparation of Minicircle DNA

Minicircles are the product of site-specific intramolecular recombination between the attB and attP sites driven by bacteriophage ΦC31 integrase (Supplemental Figure 1; available at http://circ.ahajournals.org). The DNA fragment containing firefly luciferase and enhanced integrase (Supplemental Figure 1; available at http://circ.ahajournals.org). The assay started with a 4-μg total RNA sample. Hybridization was performed overnight on a microfluidic chip consisting of chemically modified nucleotide coding segment complementary to target microRNA (miRBase 13.0) or other control RNA. Fluorescence images were collected using a laser scanner and digitized using Array-Pro image analysis software. Raw data matrix is then subtracted by the background matrix. Data adjustment includes data filtering, Log2 transformation, gene centering, and normalization. A 2-sample t-test was conducted for statistical analysis.

Histological Examination

After imaging, mice were euthanized and left ventricular tissue was obtained at 8 weeks after myocardial infarction (MI). Tissue samples were embedded into optimal cutting temperature compound (Miles Scientific). Frozen sections (5 μm thick) were processed for immunostaining. Trichrome stain (Masson; Sigma) was used to determine collagen content of the infarct regions. For each heart, 8 to 10 sections from apex to base (1.2 mm apart) were analyzed. Images were taken for each section to calculate the fibrotic and nonfibrotic areas as well as ventricular and septal wall thickness. Infarct fraction was determined as [fibrotic area/(fibrotic areas as well as ventricular and septal wall thickness. Infarct fraction was determined as [fibrotic area/(fibrotic areas + nonfibrotic area)]×100% as previously described.9 To detect microvascular density in the peri-infarct area, a rat anti-CD31 (BD Pharmingen) was used. The number of capillary vessels was counted by a blinded investigator (Z.J.) in 10 randomly selected areas using the picture under a fluorescent microscope, as described previously.9 The formalin-fixed and paraffin-embedded explanted hearts were used for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Nuclear transduction with lentivirus carrying miR-210 precursor (Pre-210), miR-scramble (Pre-Scr), or miR-210 (Anti-Scr) for 48 hours were transfected via Lipofectamine 2000 according to manufacturer’s instructions. The caspase activities for all samples were normalized to that of an equal protein amount. The data obtained are from experiments performed in triplicate.

Surgical Model of Mouse Myocardial Infarction

Adult female FVB mice (10 weeks old) were purchased from Charles River Laboratories (Wilmington, Mass). Ligation of the mid-left anterior descending (LAD) artery was performed by a single experienced microsurgeon (X.W.). Myocardial infarction was confirmed by myocardial blanching and electrocardiographic changes. After 15 minutes, the animals were then injected intramyocardially with 25 μg of minicircles carrying miR-210 precursor (MC-210) or minicircles carrying Scramble (MC-Scr; n=15 per group). Injections were made near the peri-infarct region at 3 different sites with a total volume of 25 μL using a 29-gauge Hamilton syringe. The third group was performed sham, which underwent surgery but not LAD ligation (n=15). Study protocols were approved by the Stanford Animal Research Committee.

Echocardiographic Analysis of Left Ventricular Function

Echocardiography was performed before (Day −7) and after (Week 2, Week 4, and Week 8) the LAD ligation. A Siemens-Acuson Sequoia C512 system equipped with a multifrequency (8 to 14 MHZ) 15L8 transducer was used by an investigator (M.H.) blinded to group designation. Analysis of the M-mode images was performed using DicomWorks 1.3.5 (http://dicom.online.fr) analysis software. Left ventricular end-diastolic diameter and end-systolic diameter were measured and used to calculate left ventricular fractional shortening (FS) by the following formula: FS(%)=(LVEDD−ESD)/LVEDD×100%.

Pressure-Volume Loop Measurement

Invasive steady-state hemodynamic measurements were performed (n=5 mice per group for MC-210, MC-Scr, and sham) at Week 4 as described previously.10 Briefly, after a midline neck incision, a 1.4-Fr conductance catheter (Millar Instruments, Houston, Texas) was introduced into the left ventricle through the right carotid artery. The signals were continuously recorded using a PV conductance system coupled to a PowerLab/4SP analog to digital converter (AD Instruments, Colorado Springs, Colo). Data were analyzed by using a cardiac PV analysis program (PVAN 3.4; Millar Instruments) and Chart/Scope Software (AD Instruments).

In Vivo Optical Bioluminescence Imaging

Bioluminescence imaging was performed using the IVIS Spectrum system (Caliper Life Sciences). Recipient mice were anesthetized with isoflurane and injected intraperitoneally with β-luciferin (200 mg/kg body weight). Mice were imaged before surgery (baseline scan) and after surgery on Day 3, Week 1, Week 2, Week 4, Week 6, and Week 8. Peak signals from a fixed region of interest were evaluated and signals expressed as photons per second per centimeter square per steradian (p/s/cm²/sr) as described.11

Target Confirmation by Immunoprecipitation of c-myc-Ago2-Containing RNA-Induced Silencing Complex

Transient miR-210 overexpression was obtained by transfection of pSUPER-premiR-210 using Fugene6 (Roche) in HEK-239 cells
Induction of miR-210 by Hypoxia in Mouse HL-1 Cardiomyocytes

To identify potential miRNA targets in our study, we first set up miRNA expression profiling experiment. Murine HL-1 cardiomyocytes were subject to hypoxia for 48 hours. Using fluorescence-activated cell sorting, we obtained 2 main populations of cells, apoptotic cells and live cells. We performed miRNA microarray on these 2 populations of cells using the Sanger miRBase Version 13.0 miRNA expression microarrays (LC Sciences, Houston, Texas). We analyzed 679 unique mature miRNAs across biological duplicates of each cell type and found that 7 miRNAs were significantly upregulated and 13 miRNAs were significantly downregulated in live cells compared with apoptotic cells. In particular, miRNA-210 was upregulated approximately 11-fold in live cells (Figure 1A).

Figure 1. A, Schematic highlighting the miRNA microarray experimental design. HL-1 cells were subjected to hypoxia for 48 hours. After fluorescence-activated cell sorting, apoptotic cells and live cells were collected for miRNA microarray analysis. t test analysis demonstrates statistically significant differential miRNA expression across the 2 samples. miRNAs with \( P<0.05 \) were selected for cluster analysis. B, Quantitative reverse transcription–PCR showed miR-210 expression was 5.3±0.9-fold higher in live cells than in apoptotic cells. Welch t test was used. C, Quantitative reverse transcription–PCR showed miR-1187 expression was 8.4±0.9-fold higher in apoptotic cells compared with live cells. Welch t test was used. D, Time course regulation of miR-210 by hypoxia in HL-1 cells. Induction of miR-210 was discernible at 12 hours, becoming significant at 24 hours and increased progressively at 48 and 72 hour time points. One-way analysis of variance was used. *\( P<0.01 \) and **\( P<0.05 \).

Evaluation of miR-210 Proangiogenic and Antiapoptotic Functions in Cardiomyocytes

To assess angiogenic potential of miR-210, HL-1 cells were transduced by a lentivirus-carrying miR-210 precursor (Pre-210) or by a lentivirus-carrying miR-scramble (Pre-Scr). Under fluorescence microscopy, nearly all the HL-1 cells were green fluorescent protein-positive in both groups, indicating no significant difference in transduction efficiency (Supplemental Figure II). Using real-time PCR analysis, miR-210 expression was 124±15-fold higher in the Pre-210
group compared with the Pre-Scr group. Figure 2A shows that HL-1 transduced with miR-210 could release several angiogenic factors compared with control cells, including Leptin, interleukin-1-α, and tumor necrosis factor-α. In addition, Pre-210 reduced caspase 3/7 activity in HL-1 cells compared with Pre-Scr control under both normoxia (1505 ± 884 versus 649 ± 32 309; \( P < 0.01 \)) and hypoxia (2832 ± 509 versus 1886 ± 473; \( P < 0.01 \)) conditions. Conversely, inhibition of miR-210 (anti-210) increased caspase 3/7 activity compared with control anti-Scr in both normoxia and hypoxia conditions (Figure 2B). Moreover, fluorescence-activated cell sorting analysis confirmed that the miR-210-transduced group had more live cells (71.95 ± 1.69% versus 63.39 ± 0.95%; \( P < 0.05 \)) and less apoptotic cells (22.13 ± 0.48% versus 32.14 ± 1.52%; \( P < 0.05 \)). Student t test was used.

Figure 2. In vitro characterization of therapeutic potential for miR-210. A, Angiogenesis antibody array indicated miR-210 can release several angiogenic factors in HL-1 cells. Welch t test was used for statistical analysis. B, Caspase 3/7 activity assay demonstrated that miR-210 overexpression could inhibit caspase activity, whereas inhibition of miR-210 with anti-210 abrogated the favorable effect. Student t test was used. C, fluorescence-activated cell sorting analysis confirmed that the miR-210-transduced group had more live cells (71.95 ± 1.69% versus 63.39 ± 0.95%; \( P < 0.05 \)) and less apoptotic cells (22.13 ± 0.48% versus 32.14 ± 1.52%; \( P < 0.05 \)). Student t test was used. \( *P < 0.01 \) and \( **P < 0.05 \).

Figure 3. Transfection efficiency of minicircle in vitro and in vivo. A, HL-1 cells were transfected with MC-Fluc-eGFP (MC-LG) in a 6-well plate. B, A robust correlation exists between minicircle dosage and bioluminescence signals \( (r^2 = 0.96) \). Each data point is from an individual observation. Pearson correlation was used. (C), Bioluminescence imaging and (D) quantitative analysis indicate minicircle plasmid-mediated gene expression was stable for at least 8 weeks in the heart compared with <4 weeks using regular plasmid (data not shown).
Improvement of Cardiac Function After MI After Injection of miR-210

To examine whether miR-210 delivery can improve cardiac function after MI, nonviral minicircles were used to carry the miR-210 expression cassette (MC-210). As novel nonviral vectors, minicircles lack both an origin of replication and the antibiotic selection marker and carry only short bacterial sequences. Their smaller size confers greater transfection efficiency and the lack of bacterial backbone creates less immunogenicity and longer transgene expression.9 We first transfected HL-1 cells with different quantities of minicircles carrying Fluc-eGFP (MC-LG) in 6-well plate (Figure 3A). Data showed bioluminescence signals correlated robustly with in vitro Fluc enzyme activity ($r^2=0.96$; Figure 3B). Next, to monitor the duration of transgene expression mediated by MC vector in living animals, a subset of animals with LAD ligation (n=5) were injected with 25 μg of MC-LG into the heart. In vivo bioluminescence imaging indicated that minicircle vector-mediated gene expression was stable for at least 8 weeks in the animal heart (Figure 3C–D). To examine whether miC-210 can improve cardiac function, adult FVB mice underwent LAD ligation and were injected intramyocardially with (1) MC-210; (2) MC-Scr (control); and (3) sham operated animals (n=15 per group). Echocardiography was performed before and after (Week 2, Week 4, and Week 8) the LAD ligation. At baseline, left ventricular FS was comparable in all 3 groups (Figure 4A–B; Supplemental Table I). After LAD ligation, the MC-210 group had significantly higher left ventricular FS compared with the MC-Scr group at Week 4 (28.7±2.4% versus 25.1±1.9%; $P<0.05$) and Week 8 (27.8±1.9% versus 24.2±2.7%; $P<0.05$). This finding was further corroborated using invasive PV loops. The PV loop data showed that left ventricular end-diastolic volume and end-systolic volume in the MC-210 group were significantly lower than MC-Scr group, suggesting a more favorable left ventricular remodeling process after miR-210 treatment (Supplemental Table II).

Ex Vivo Histological Confirmation of Echocardiographic Data

After imaging, animals were euthanized and hearts explanted. Masson trichrome staining showed less infarction size for the MC-210 group compared with the MC-Scr group at Week 8 (Figure 4C), confirming the positive functional data seen in echocardiography. Calculated infarct fractions were significantly smaller in the MC-210 group compared with the MC-Scr group (26.5±2.4% versus 35.4±1.8%; $P=0.05$). TUNEL staining demonstrated significantly reduced apoptotic cells in the MC-210 group compared with the MC-Scr control group. E, Immunofluorescence staining of CD31 endothelial marker (green) demonstrated increased neovascularization in the myocardium after MC-210 delivery compared with the MC-Scr control. Cardiomyocyte staining is identified by α-sarcomeric actin (red) and nuclear staining is identified by 4',6-diamidino-2-phenylindole (blue).

Prediction and Confirmation of Target Genes of miR-210

To investigate the mechanism(s) of miR-210-based therapy, we predicted the target genes for miR-210 using both TargetScan and MicroCosm algorithms. Efn3, Dapk1, and Ctgf were predicted to be the putative target genes of miR-210 with high scores. The 3′-UTR segment of these 3 genes containing miR-210 putative binding site (“seed” sequence), which has a crucial role in miRNA:mRNA interaction, were very conserved in different species. Although it was reported that Ptp1b should be 1 of the miR-210 targets, the binding site
gets, biochemical assay based on the immunoprecipitation of the target genes of miR-210. These results demonstrate that Efna3, Ptp1b, Dapk1, and Ctgf are luciferase activity through seed-specific binding. Taken together, reporters with 4 nucleotides mismatching noncomplementary miR-scramble (Pre-Scr) control (Figure 5A). In contrast, mutant was not in the 3'-UTR segment. To confirm that they are direct miR-210 targets, the putative binding site of these target genes (Efna3, Ptp1b, Dapk1, and Ctgf) was amplified by PCR from mouse cDNA and inserted downstream of firefly luciferase reporter gene in the pGL3 control vector for dual-luciferase assay (see Supplemental Figure III). pRL-TK containing renilla luciferase was cotransfected for data normalization. Precursor miR-210 mimic (Pre-210) significantly reduced the luciferase activities of the wild-type Efna3, Ptp1b, Dapk1, and Ctgf reporters between 35% and 60% compared with the PremiR scramble control (Pre-Scr). However, mutant reporters (Pre-210+Mut) with noncomplementary seed binding site were not repressed by miR-210 precursor as expected. The blank vector (PGL3-control) has no seed binding site and therefore the luciferase activity was not affected by miR-210 precursor mimic. One-way analysis of variance was used. B, miR-210 targets were enriched in miR-210 containing RISC. Compared with cells transfected with a scramble sequence, immune precipitates of the miR-210 loaded RISC highly enriched its targets, including Efna3, Ptp1b, Dapk1, and Ctgf. Student t test was used. *P<0.05 and **P<0.01.

Figure 5. Confirmation of the target gene of miR-210. A, The binding segments of mouse Efna3, Ptp1b, Dapk1, and Ctgf interacting with miR-210 was amplified and inserted downstream of firefly luciferase reporter gene in the pGL3 control vector for dual-luciferase assay (see Supplemental Figure III). pRL-TK containing renilla luciferase was cotransfected for data normalization. Precursor miR-210 mimic (Pre-210) significantly reduced the luciferase activities of the wild-type Efna3, Ptp1b, Dapk1, and Ctgf reporters between 35% and 60% compared with the PremiR scramble control (Pre-Scr). However, mutant reporters (Pre-210+Mut) with noncomplementary seed binding site were not repressed by miR-210 precursor as expected. The blank vector (PGL3-control) has no seed binding site and therefore the luciferase activity was not affected by miR-210 precursor mimic. One-way analysis of variance was used. B, miR-210 targets were enriched in miR-210 containing RISC. Compared with cells transfected with a scramble sequence, immune precipitates of the miR-210 loaded RISC highly enriched its targets, including Efna3, Ptp1b, Dapk1, and Ctgf. Student t test was used. *P<0.05 and **P<0.01.

was not in the 3'-UTR segment. To confirm that they are indeed the target genes of miR-210, the putative binding site of these target genes (Efna3, Ptp1b, Dapk1, and Ctgf) was amplified by PCR from mouse cDNA and inserted downstream of the luciferase reporter gene in the pGL3 control vector for dual-luciferase assay (Supplemental Figure III). After NIH/3T3 cells were cotransfected with reconstructive vectors and normalizing vector pRL-TK containing Renilla luciferase, the precursor miR-210 mimic (Pre-210) significantly reduced the luciferase activities of the wild-type Efna3, Ptp1b, Dapk1, and Ctgf reporters by 35% to 60% (P<0.05) compared with the miR-scramble (Pre-Scr) control (Figure 5A). In contrast, mutant reporters with 4 nucleotides mismatching noncomplementary seed binding sites were not repressed by miR-210 precursor, confirming that the target site directly mediates repression of the luciferase activity through seed-specific binding. Taken together, these results demonstrate that Efna3, Ptp1b, Dapk1, and Ctgf are the target genes of miR-210.

To further demonstrate that they are direct miR-210 targets, biochemical assay based on the immunoprecipitation of RISC complexes enriched for miR-210 and its targets was used. To this end, a c-myc-tagged allele of Ago2, a core component of the RISC complex, was used. Easily transfectable HEK-293 cells were cotransfected with expression vectors for miR-210 and c-myc-Ago2, yielding cells enriched of miR-210/c-myc-Ago2-containing RISC complexes as well as miR-210 targets. We then immunoprecipitated c-myc-Ago2 and measured the levels of coimmunoprecipitated mRNAs by quantitative PCR. Background controls were represented by c-myc-immunoprecipitates derived from cells transfected with miR-210, but not c-myc-Ago2, and displayed low to undetectable signals for all the assayed genes (data not shown). Two known miR-210 targets (Efna3 and Ptp1) were used as positive controls. Figure 5B shows that Efna3, Ptp1b, Dapk1, and Ctgf were significantly enriched in immunoprecipitates of miR-210-overexpressing cells compared with cells transfected with a scramble sequence (Pre-Scr). We concluded that Efna3, Ptp1b, Dapk1, and Ctgf are all associated with miR-210 loaded RISC complexes and hence they are the real targets of miR-210.

Endogenous Regulation of Efna3 and Ptp1b by miR-210

Although these genes were identified as target genes for miR-210, it is still unknown whether miR-210 could regulate their expression endogenously. Because Efna3 and Ptp1b are involved in vascular remodeling and apoptosis in the heart, respectively, we selected these 2 genes for confirmation in HL-1 cells. HL-1 cells were transduced with Pre-210 to assess whether miR-210 could regulate endogenous Efna3 and Ptp1b. Compared with the control, the level of Efna3 mRNA was significantly downregulated by Pre-210 (Figure 6A) but not Ptp1b. However, we found the level of Ptp1b protein was downregulated on the Western blot (Figure 6B). These data suggest that endogenous Efna3 and Ptp1b are regulated by miR-210 at the level of mRNA and protein, respectively. Our data were also confirmed by immunofluorescence staining (Figure 6C). Furthermore, we also evaluated if miR-210 treatment in the heart would have an effect on Efna3 and Ptp1b. Western blot data from the peri-infarct regions of explanted hearts showed that Efna3 and Ptp1b in the MC-210 group are lower than in the MC-Scr group (Figure 6D). Taken together, these results suggest that overexpression of miR-210 led to downregulation of Efna3 on the mRNA level and downregulation of Ptp1b on the protein level. Efna3 is involved in inhibition of angiogenesis, whereas Ptp1b is involved in induction of apoptosis. Therefore, the suppression of these 2 targets by miR-210 delivery may contribute to the improvement of cardiac function after MI.

Discussion

Ischemic heart disease is the leading cause of human morbidity and mortality in the Western world, underscoring the need for innovative new therapies for heart disease. miRNAs are 21 to 23 nt noncoding small RNAs that act as negative regulators of the protein-coding gene by modulating the mRNA translation and stability. In this study, we report a novel therapeutic strategy for treatment of myocardial infarction based on miR-210. Using miRNA microarray analysis,
we found that miR-210 was upregulated in live HL-1 cells compared with apoptotic cells after 48 hours of hypoxia challenge, suggesting that miR-210 possesses antiapoptotic properties during cell stress conditions. These data are also confirmed by a recent report indicating that miR-210 is induced in ischemia-preconditioned bone marrow-derived mesenchymal stem cells and that its suppression abolished the protective effects of preconditioning due to the abnormal expression of its target FLASH/caspase-8-associated protein-2, which can activate caspase 8 and facilitate apoptosis.15

We also explored the time-course regulation of miR-210. Induction of miR-210 was discernible after 12 hours of hypoxia, and the upregulation was maintained for the next 72 hours. This predominant induction of miR-210 by hypoxia is consistent with reports involving other cell types such as embryo kidney cells, endothelial cells, breast carcinoma cells, colonic adenocarcinoma cells, and epithelial ovarian cells.7,12,21 The robust induction among various cell types is probably due to the highly conserved structure of hypoxia response element existing in miR-210 promoter.7 Under the diminished oxygen concentration of hypoxia, a variety of complex responses at both cellular and organism levels are activated, including endothelial cells proliferation, migration, and angiogenesis. The multiple lines of evidence prompted us to assess whether miR-210 delivery in vivo can improve heart function in a murine MI model.

Besides the therapeutic gene, the success of cardiovascular gene therapy also depends on effective delivery systems to target sites. Here we used a nonviral minicircle vector carrying miRNA because of its multiple advantages, including greater transfection efficiency (compared with regular plasmids) and less immunogenicity (compared with viral vectors).9 miR-210 delivery through the minicircle vector improved left ventricular function after MI, and ex vivo histological analysis indicated that miR-210 induced neovascularization and inhibited apoptosis in ischemic hearts. Interestingly, previous studies have shown that miR-210 can improve tubulogenesis12 and prevent mesenchymal stem cell apoptosis.15 A recent study also reported that miR-210 can modulate mitochondrial respiration, iron metabolism, and reactive oxygen species generation during hypoxia by repressing iron–sulfur cluster assembly proteins to influence cellular adaptation to hypoxia, accounting for its benefits in ischemia.22 Therefore, miR-210 delivery after MI may have several pleiotropic effects in addition to the proangiogenesis and antiapoptosis roles that were investigated here.

To study the potential molecular mechanism of miR-210 therapy in the heart, we performed an in silico search of potential targets using TargetScan and MicroCosm algorithms. We found several potential target genes for miR-210 after MI, including Efna3, Ptp1b, Dapk1, and Ctgf. Luciferase activities of these 4 putative wild-type target genes were downregulated by the miR-210 precursor (Pre-210) but not in mutant target sequences, suggesting that they are the real targets of miR-210 and that the inhibition was “seed” sequence-specific. Efna3 and Ptp1b are involved in inhibition of angiogenesis and induction of apoptosis, respectively. Efna3 suppression in human umbilical vein endothelial cells is vital for stimulation of tubulogenesis, indicating its crucial function in angiogenesis.12 Ptp1b is an ubiqui-
tously expressed 50-Kda enzyme that is the most widely studied prototype for the protein tyrosine phosphatase superfamily. Recently, it has been reported that Ptp1b inhibition by siRNA significantly decreased apoptosis in cardiomyocytes. Ptp1b has also been implicated as a negative regulator in vascular endothelial growth factor signaling in endothelial cells. Dapk1, which encodes a proapoptotic serine/threonine kinase, is critical for regulating the cell cycle, apoptosis, and metastasis, mainly functioning in the early stages of eukaryotic programmed cell death. Ctgf is a secreted cysteine-rich protein with major roles in angiogenesis, chondrogenesis, osteogenesis, tissue repair, cancer, and fibrosis. Ctgf expression is enhanced in cardiac myocytes and fibroblasts in the heart after myocardial infarction and is induced by transforming growth factor-β in heart fibrosis. Thus, the inhibition by miR-210 delivery after MI may favor the functional improvement of left ventricle through direct inhibition of these target genes, especially Efna3 and Ptp1b as investigated in this study.

In conclusion, we found that miR-210 can improve heart function by upregulating angiogenesis and inhibiting apoptosis. Because individual miRNAs can regulate the expression of multiple target genes, manipulating miRNA expression can influence an entire gene network and thereby modify complex disease pathology. Our approach shows that miR-210 delivery through nonviral minicircle may work as a novel therapeutic avenue for treatment of ischemic heart disease.

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Disclosures

None.

References

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Closed bacterial backbone

Minicircle
UB-miR-210

Used for *in vitro* and *in vivo* experimental studies

Supplemental Figure 1
Supplemental Figure 2

A

Pre-210  Pre-Scr

B

Relative expression of miR-210

Pre-210  Pre-Scr
Supplemental Figure 3

A

SV40 Pro → Fluc → ...3'UTR... → Poly A

PGL3-Control vector

SV40 Pro → Fluc → ...CAGGACCTATGCAACGCACAG... → Poly A

Wild-type recombinant vector

SV40 Pro → Fluc → ...CAGGACCTATGCAACCGTGAG... → Poly A

Mutant recombinant vector

B

Mutant

Wild type

Mature miR-210

CAGGACCTATGCAACCGTGAG
CAGGACCTATGCAACGCACAG

4 nt mutant Efn3 3' UTR
Position 761-767 of Efn3 3' UTR

Wild type

mature miR-210

mature miR-210

mature miR-210

CAGGACCTATGCAACCGTGAG
CAGGACCTATGCAACGCACAG

4 nt mutant Ptpn1
Position 1027-1033 of Ptpn1

mature miR-210

mature miR-210

mature miR-210

CAGGACCTATGCAACCGTGAG
CAGGACCTATGCAACGCACAG

4 nt mutant Dapk1 3' UTR
Position 834-840 of Dapk1 3' UTR

mature miR-210

mature miR-210

mature miR-210

CAGGACCTATGCAACCGTGAG
CAGGACCTATGCAACGCACAG

4 nt mutant Ctgf 3' UTR
Position 1549-1569 of Ctgf 3' UTR

mature miR-210

mature miR-210

mature miR-210

4 nt mutant Efn3 3' UTR
Position 761-767 of Efn3 3' UTR

mature miR-210

mature miR-210

mature miR-210

4 nt mutant Ptpn1
Position 1027-1033 of Ptpn1

mature miR-210

mature miR-210

mature miR-210

4 nt mutant Dapk1 3' UTR
Position 834-840 of Dapk1 3' UTR

mature miR-210

mature miR-210

mature miR-210

4 nt mutant Ctgf 3' UTR
Position 1549-1569 of Ctgf 3' UTR
<table>
<thead>
<tr>
<th></th>
<th>W4</th>
<th>W8</th>
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<tr>
<td></td>
<td>MC-Scr</td>
<td>MC-210</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.05±0.09</td>
<td>2.78±0.09*</td>
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<tr>
<td>LVEDD, mm</td>
<td>4.07±0.13</td>
<td>3.90±0.07</td>
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<tr>
<td>FS (%)</td>
<td>25.1±1.9</td>
<td>28.7±2.4*</td>
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Supplemental Table 1
<table>
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<tr>
<th></th>
<th>MC-Scr</th>
<th>MC-210</th>
<th>Sham</th>
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<tr>
<td>HR, beats/min</td>
<td>404 ± 32</td>
<td>393 ± 29</td>
<td>414 ± 47</td>
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<tr>
<td>ESV, μl</td>
<td>51.9 ± 2.8</td>
<td>45.3 ± 4.2*</td>
<td>33.2 ± 4.4†</td>
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<td>EDV, μl</td>
<td>64.3 ± 4.2</td>
<td>55.8 ± 6.5*</td>
<td>44.1 ± 3.2†</td>
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<tr>
<td>ESP, mmHg</td>
<td>54.3 ± 6.2</td>
<td>65.6 ± 3.1*</td>
<td>78.1 ± 7.3†</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>55.7 ± 2.8</td>
<td>45.3 ± 5.2</td>
<td>33.3 ± 4.4†</td>
</tr>
<tr>
<td>SV, ul</td>
<td>12.6 ± 2.4</td>
<td>13.7 ± 3.1</td>
<td>17.5 ± 2.6†</td>
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<tr>
<td>CO, ml/min</td>
<td>33813 ± 642</td>
<td>3962 ± 535</td>
<td>5496 ± 325.5†</td>
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<tr>
<td>dP/dtmax, mm Hg/μl</td>
<td>3073 ± 213</td>
<td>3674 ± 334*</td>
<td>4173 ± 634†</td>
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<tr>
<td>dP/dtmin, mm Hg/μl</td>
<td>-2834 ± 406</td>
<td>-3234 ± 395</td>
<td>-3925 ± 325†</td>
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<tr>
<td>SW, mm Hg/μl</td>
<td>379 ± 107</td>
<td>433 ± 163</td>
<td>638 ± 273†</td>
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<tr>
<td>Tau-Glantz, ms</td>
<td>13.4 ± 4.7</td>
<td>12.7 ± 3.7</td>
<td>11.3 ± 6.7</td>
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<td>PAMP, mW/ml²</td>
<td>9.3 ± 3.3</td>
<td>13.5 ± 2.9*</td>
<td>18.4 ± 5.2†</td>
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</table>

Supplemental Table 2
**Supplemental Figure 1:** Schema of the production process for minicircles carrying miR-210 precursor driven by ubiquitin promoter (UB-miR-210). Minicircles are the product of site-specific recombination between the attB and attP sites driven by bacteriophage ΦC31 integrase. By adding 1%-L-arabinose to the bacterial culture media, the att sites of p2øC31.UB-miR-210 undergo intramolecular recombination. The end result is two circular DNAs: one is the minicircle (MC), which contains the therapeutic gene cassette and the right hybrid sequence (attR), and the other is the closed bacterial backbone, which contains the origin of replication, the antibiotic marker, and the left hybrid sequence (attL). The larger size closed bacterial backbone plasmid is then removed by bacterial exonucleases at 37°C.

**Supplemental Figure 2:** Expression of miR-210 in HL-1 cell. (A) HL-1 cells were transduced by lentivirus carrying miR-210 precursor or scramble sequence. >90% of the cells were GFP positive, indicating high transgene expression efficiency. (B) Quantitative RT-PCR showed miR-210 expression was 124±15 folds higher in miR-210 transduced cells compared to miR-scramble transduced cells.

**Supplemental Figure 3:** Schematic diagram for constructing miR-210 binding site into pGL3-control vector (Promega). (A) Take Efna3 as an example. The blank vector for luciferase assay is PGL3-Control vector from Promega. For the wild type vector construction, the target binding sequence was amplified by PCR and cloned downstream of firefly luciferase (Fluc) stop codon as 3’ UTR of Fluc of PGL3-Control vector. This reconstructive vector plus normalizing vector pRL-TK (Promega) were transfected into NIH/3T3 cell for dual-luciferase assay. The mutant vector was reconstructed with 4 nucleotides mismatch using QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene). (B) Complementarity between miR-210 and binding sites
of the four target genes. Mutant sequences for mutant vector construction were marked in yellow.

**Supplemental Table 1:** Quantitative analysis of left ventricular function among the 3 groups through echocardiogram. LVESD, LV End-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening. Values are means ± SE. *P<0.05 vs MC-Scr.

**Supplemental Table 2:** Invasive pressure-volume measurements of cardiac function. HR, heart rate; ESV, end-systolic volume; EDV, end-diastolic volume; ESP, end-systolic pressure; EDP, end-diastolic pressure; SV, stroke volume; CO, cardiac output; dP/dtmax, maximum first derivative of change in pressure rise with respect to time; dP/dtmin, maximum first derivative of change in pressure fall with respect to time; SW, stroke work; Tau-Glantz, time constant of fall in ventricular pressure by Glantz method; PAMP, preload-adjusted maximal power. Values are means ± SE. *P<0.05 vs MC-Scr.