Novel MicroRNA Prosurvival Cocktail for Improving Engraftment and Function of Cardiac Progenitor Cell Transplantation

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Background—Although stem cell therapy has provided a promising treatment for myocardial infarction, the low survival of the transplanted cells in the infarcted myocardium is possibly a primary reason for failure of long-term improvement. Therefore, the development of novel prosurvival strategies to boost stem cell survival will be of significant benefit to this field.

Methods and Results—Cardiac progenitor cells (CPCs) were isolated from transgenic mice, which constitutively express firefly luciferase and green fluorescent protein. The CPCs were transduced with individual lentivirus carrying the precursor of miR-21, miR-24, and miR-221, a cocktail of these 3 microRNA precursors, or green fluorescent protein as a control. After challenge in serum free medium, CPCs treated with the 3 microRNA cocktail showed significantly higher viability compared with untreated CPCs. After intramuscular and intramyocardial injections, in vivo bioluminescence imaging showed that microRNA cocktail-treated CPCs survived significantly longer after transplantation. After left anterior descending artery ligation, microRNA cocktail-treated CPCs boost the therapeutic efficacy in terms of functional recovery. Histological analysis confirmed increased myocardial wall thickness and CPC engraftment in the myocardium with the microRNA cocktail. Finally, we used bioinformatics analysis and experimental validation assays to show that Bim, a critical apoptotic activator, is an important target gene of the microRNA cocktail, which collectively can bind to the 3’UTR region of Bim and suppress its expression.

Conclusions—We have demonstrated that a microRNA prosurvival cocktail (miR-21, miR-24, and miR-221) can improve the engraftment of transplanted cardiac progenitor cells and therapeutic efficacy for treatment of ischemic heart disease. (Circulation. 2011;124[suppl 1]:S27–S34.)

Key Words: cardiac progenitor cell ■ cell therapy ■ ischemic heart disease ■ microRNA

Coronary artery disease is a progressive disease with high morbidity and mortality rates in the United States. After myocardial infarction (MI), the limited ability of the surviving cardiac cells to proliferate renders the damaged heart susceptible to unfavorable remodeling processes and morbid sequelae such as heart failure. Current therapies for patients who have coronary artery disease or MI include lifestyle modification, drug treatments, percutaneous coronary intervention, bypass surgery, and orthotopic heart transplantation. In recent years, stem cell therapy has provided a promising means of replacing cardiomyocytes lost during MI through induction of endogenous regenerative processes and transferring of progenitor cells to new myocardium. Several types of stem cells have been shown to have a beneficial effect in small animal models, including bone marrow mononuclear cells, mesenchymal stem cells, endothelial progenitor cells, and human embryonic stem cell-derived cardiomyocytes, to name a few. However, large placebo-controlled clinical trials have not shown a consistent benefit in using stem cells to treat patients with ischemic heart disease.

In recent years, a number of studies have confirmed the presence of resident “progenitor cells” in the myocardium. These cells can be isolated and expanded ex vivo and can also differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells under the appropriate culturing conditions. However, the low survival of the transplanted stem cells, including cardiac progenitor cells (CPCs), in the infarcted myocardium is a major obstacle to achieving long-term...
improvements after stem cell therapy. Therefore, developing novel prosurvival strategies to boost stem cell survival will be highly beneficial to this field.

MicroRNAs (miRNAs) are short 20 to 22-nucleotide RNA molecules that are expressed in a tissue-specific and developmentally regulated manner. MiRNAs mainly function as negative regulators of gene expression in a variety of organisms, through binding with imperfect complementarity to the 3'UTR of their target mRNAs. In the heart, a large number of miRNAs are expressed and are considered to be important regulators in cardiac development and pathophysiology. In addition, recent studies indicate that miRNAs can be used as therapeutic targets for various cardiovascular diseases.

In this study, we hypothesize that miRNAs may play a significant role in regulating survival and apoptosis of CPCs and in improving engraftment posttransplantation. We demonstrate for the first time that CPCs treated with a miRNA prosurvival cocktail can significantly improve cell engraftment and functional recovery in a murine model of MI.

Methods

Isolation and Maintenance of Sca-1+ CPCs

Heart tissue explants are isolated from transgenic mice with ubiquitin promoter constitutively driving firefly luciferase (Fluc) and green fluorescent protein. The minced heart pieces are subjected to enzyme dissociation with 0.2% trypsin and 0.1% collagenase IV (Worthington Biochemical, Lakewood, NJ) in phosphate-buffered saline 3 times for 5 minutes at 37°C. Afterward, the remaining tissue fragments were cultured in the CPC medium (Iscove Modified Dulbecco IMDM with 15% fetal calf serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, and 0.1 mmol/L 2-mercaptoethanol) at 37°C. After approximately 2 weeks, we harvested small, phase-bright cells formed from adherent explants by washing with D-Hanks solution. We then enrich the Sca-1+ cells with anti-Sca-1 microbeads (Miltenyi Biotec) as previously described. The enriched Sca-1+ cells were seeded on gelatin coated dishes in CPC medium as described above.

In Vitro Differentiation of CPCs

For cardiac and smooth muscle differentiation, CPCs were cultured in poly-D-lysine-coated plates in differentiation medium containing 35% Iscove modified Dulbecco medium with 10% fetal bovine serum/65% Dulbecco modified Eagle medium-Ham F-12 mix containing 2% B27, 0.1 mmol/L 2-mercaptoethanol, 10 ng/mL epidermal growth factor (R&D Systems, Minneapolis, MN), 20 ng/mL basic fibroblast growth factor, 40 mmol/L cardiotrophin-1, 40 mmol/L thrombin (Sigma-Aldrich, St Louis, MO), 100 U/mL penicillin G, 100 g/mL streptomycin, and 2 mmol/L glutamine. For endothelial differentiation, CPCs were cultured on fibronectin-coated plates with 100 μg/mL streptomycin, and 2 mmol/L glutamine. After 7 days of differentiation into cardiomyocytes (Figure 1C–D). Next, we differentiated CPCs using induction medium. After 7 days of differentiation into cardiomyocytes and smooth muscle cells, the differentiated cells stained positive for connexin 43 (Figure 1E), cardiac troponin T (Figure 1F), myocyte-specific enhancer factor 2C (Figure 1G), and α-smooth muscle actin (Figure 1H). Furthermore, CPCs could be differentiated into endothelial cells (Figure 1I). The differentiated endothelial cells demonstrated Di-ac-LDL uptake (Figure 1J) and could form tube-like structures on Matrigel angiogenesis assay (Figure 1K). In contrast, the undifferentiated CPCs were not able to form similar tube-like structures (Figure 1L).

Intramuscular Injection of CPCs for Assessment of the MiRNA Cocktail

To compare the survival between CPCs treated with the miRNA prosurvival cocktail and CPCs alone, we injected the 2 cell types into the legs of the same animal. This method allowed us to avoid interindividual variation and perform an unbiased head-to-head comparison. We injected 5×10⁵ cells into the hind limb of adult female SCID/Beige mice (Charles River Laboratories, Wilmington, MA) with a total volume of 10 μL per injection leg (n=8 animals).

Surgical Model of Murine Myocardial Infarction and Cell Delivery

All animal research protocols were approved by the Stanford Animal Research Committee. Ligation of the midleft anterior descending artery was performed in adult female SCID Beige mice (Charles River Laboratories) by a single experienced microsurgeon (Y.G.). MI was confirmed by myocardial blanching and electrocardiographic changes. Mice (14 weeks old) were randomized into 3 groups (n=60 total): (1) phosphate-buffered saline (PBS); (2) 2×10⁵ CPCs alone; and (3) 2×10⁵ CPCs transduced with the 3 miRNA cocktail (n=20/group). After left anterior descending artery ligation, the animals were injected with CPCs or PBS in the peri-infarct zone at 2 different sites with total volume of 30 μL using a 31-gauge Hamilton syringe.

In Vivo Optical Bioluminescence Imaging

See Supplemental Methods (http://circ.ahajournals.org).

Echocardiographic Analysis of Left Ventricular Function

See Supplemental Methods.

Histological Examination

See Supplemental Methods.

Statistical Analysis

Statistics were calculated using SPSS 12.0 (SPSS Inc, Chicago, IL). Descriptive statistics included mean and SD. One-way analysis of variance and 2-way repeated-measures analysis of variance with post hoc testing were used. The initial F test was followed up with the Bonferroni multiple comparisons procedure. Differences were considered significant at probability values of <0.05.

Results

Characterization and Differentiation of CPCs

After purification by anti-Sca-1 microbeads, the typical CPCs showed high nucleus-to-cytoplasm ratios. The adherent cells sprouted new round bright cells at their cell body (Figure 1A). Fluorescence-activated cell sorting indicated that isolated CPCs were >95% Sca-1+ and CD45−; the latter is a marker for hematopoietic stem cells (Figure 1B). The isolated CPCs expressed robust levels of Fluc and green fluorescent protein (Figure 1C–D). Next, we differentiated CPCs using induction medium. After 7 days of differentiation into cardiomyocytes and smooth muscle cells, the differentiated cells stained positive for connexin 43 (Figure 1E), cardiac troponin T (Figure 1F), myocyte-specific enhancer factor 2C (Figure 1G), and α-smooth muscle actin (Figure 1H). Furthermore, CPCs could be differentiated into endothelial cells (Figure 1I). The differentiated endothelial cells demonstrated Di-ac-LDL uptake (Figure 1J) and could form tube-like structures on Matrigel angiogenesis assay (Figure 1K). In contrast, the undifferentiated CPCs were not able to form similar tube-like structures (Figure 1L).
Screening of MiRNA Candidates for Improving CPC Survival

To identify potential prosurvival miRNAs in CPCs, we tested the cell viability after serum-free challenge. We tested several miRNAs or miRNA clusters, including miR-21, miR24, miR-210, miR-221, miR-17-92, and miR106b-25, which have been shown to improve cell survival or inhibit cell apoptosis in previous studies. After transduction with individual miRNA, miRNA cluster, or control lentivirus, CPCs were challenged in serum-free condition for 24 hours. Cell viability was used as a criterion to evaluate miRNA capacity to improve cell survival. Overall, we found that CPCs treated individually with miR-21, miR-24, and miR-221 had significantly higher cell viability compared with control CPCs that were transduced with green fluorescent protein (Figure 2). CPCs treated with the 3 miRNA cocktail (miR-21, miR-24, and miR-221) showed the highest cell viability, suggesting synergistic benefits in action.

Comparison of CPC Survival After Intramuscular Transplantation

We first evaluated the correlation between Fluc expression and cell numbers (Figure 3A). Our data showed a robust linear correlation ($R^2=0.99$) between bioluminescence signals and cell numbers, which is crucial for accurate tracking of cell fate after in vivo imaging studies (Figure 3B). Next, we assessed cell viability in vivo of CPCs alone versus CPCs treated with the 3 miRNA cocktail after intramuscular injection by bioluminescence imaging. By injecting the 2 cell types into the same animal, we were able to avoid interindividual variation and perform a head-to-head comparison of cell survival attributed to the 3 miRNA cocktail. Bioluminescence imaging indicated Fluc activity from CPCs treated with miRNA cocktail was stable for at least 14 days after intramuscular injection. In contrast, CPCs alone lost their biolu-

Figure 2. Screening of microRNAs (miRNAs) for improving cell viability. After CPCs were transduced with several individual miRNAs or miRNAs cluster candidate, cells were exposed to serum-free medium. Cell viability was measured after 24 hours of serum-free challenge. Note the CPC viability is significantly higher in the presence of the miRNA cocktail (miR-21, miR24, and miR221). One-way analysis of variance was used. CPCs indicates cardiac progenitor cells.
baseline left ventricular fractional shortening was comparable in all 3 groups (Figure 5A–B). After left anterior descending artery ligation, CPCs treated with the miRNA cocktail had significantly higher fractional shortening compared with the PBS control group at Day 28 (28.8%±2.4% versus 24.6%±3.9%; P<0.05), whereas the CPC alone group showed a favorable trend toward fractional shortening improvement compared with the PBS control group (27.5%±2.5% versus 24.6%±3.9%; P=0.18).

Ex Vivo Histological Confirmation of Bioluminescence and Echocardiographic Data
After imaging, animals were euthanized and hearts explanted for histological analysis. Masson trichrome staining indicated a reduced infarction size for the CPC group treated with the miRNA cocktail (21.5%±3.4%) compared with the CPCs alone (26.5%±3.9%) and PBS control groups (32.5%±5.4%; P<0.05) at Day 28 (Figure 5C). Immunostaining of explanted hearts also confirmed that the population of green fluorescent protein™ cells in CPCs treated with the miRNA cocktail is significantly higher than CPC alone and PBS control groups (Figure 5D).

Prediction and Confirmation of MiRNA Targets
To elucidate the potential targets of these miRNAs, we predicted the target genes of miR-21, miR-24, and miR-221 using both TargetScan and MicroCosm, which are based on different prediction algorithms. Irrespective of site conservation, 1791 genes were predicted as potential target genes of mouse miR-21, 3048 genes as the potential target genes of mouse miR-24, and 2295 genes as the potential target genes of mouse miR-221. Bioinformatics analysis indicated many apoptotic genes are the targets of these 3 miRNAs, which are putatively suppressed by these miRNAs. These apoptotic genes include Casp3, Casp8ap2, Bax, Pdcd4, and Fasl. Interestingly, 106 genes are predicted as the common targets of all 3 miRNAs. Some of these 106 genes include apoptotic activators or effectors (including Bcl2l11, Foxo3, and Ak2), which are potential suppression targets by all of the 3 miRNAs (Supplemental Figure II). Bcl2l11, also named Bim, belongs to the Bcl-2 protein family. Interestingly, Bim has shown to be a critical apoptotic activator, which prompted us to perform further experimental analysis.

There are a total of 6 binding sites on the 3’UTR of Bim, including 1 binding site of miR-21, 2 binding sites of miR-24, and 3 binding sites of miR-221 (Figure 6A; Supplemental Figure III). To experimentally validate this bioinformatic prediction, we used the luciferase reporter assay. The 6 miRNAs binding segments of 3’UTR of Bim were amplified by polymerase chain reaction and inserted into downstream of the luciferase reporter gene separately in the pGL3 control vector for the dual-luciferase assay, including 21, 24_1, 24_2, 221_1, 221_2, and 221_3 recombinant reporter (Figure 6B). For reporters 21, 24_1, 24_2, 221_2, and 221_3, compared with the precursor miRNA control, their luciferase activity was significantly suppressed by their relative miRNAs. Furthermore, the luciferase activity could not be suppressed by using mismatched miRNAs. For example, luciferase activity of reporter 21 could be suppressed by miR-21, but not by...
Figure 4. Direct comparison of cell survival in vivo by bioluminescence imaging. A, Representative bioluminescence images of animals injected with CPCs alone (left) versus CPCs treated with the miRNA cocktail (right). Note this is in the setting of an uninjured hind limb injection model. B, Quantitative analysis of longitudinal bioluminescence imaging (BLI) signal after intramuscular hind limb injection. Two-way RMANOVA was used. C, After myocardial infarction, adult mice were injected intramyocardially with CPCs alone versus CPCs treated with the miRNA cocktail. D, Quantitative analysis of BLI signals demonstrated that CPCs treated with the miRNA cocktail showed significantly better survival at all time points. Two-way RMANOVA was used. CPCs indicates cardiac progenitor cells; miRNA, microRNA; RMANOVA, repeated-measures analysis of variance.
miR-24. However, for reporter 221_1, the luciferase activity was not suppressed by miR-221, indicating that this binding site is dispensable for Bim suppression by miR-221.

Next, we assessed whether miRNAs could regulate Bim expression endogenously. CPCs were transduced with these 3 miRNAs to evaluate whether miRNAs could regulate Bim expression by Western blotting (Figure 6C). Compared with control, the levels of Bim isoforms proteins were significantly downregulated by miRNA cocktails. Collectively, these data suggest that Bim is a real target of these miRNA cocktails, as confirmed by bioinformatic prediction and experimental validation. To further elucidate the downstream target of miRNA cocktail treatment, the transduced CPCs were exposed to a serum-free condition and the activated caspase 3 protein levels were evaluated by the Western blotting method. Compared with the control, the protein levels of active caspase 3 were downregulated by miRNA cocktails (Figure 6D).

### Discussion

MI is the leading cause of morbidity and mortality in the Western world. MI leads to thinning of the infarct wall, cardiac dilatation, and adverse cardiac remodeling. At the cellular level, MI causes cellular apoptosis, necrosis, and fibrosis. Stem cell therapy, which is designed to replenish the infarct area with new cells, is therefore a promising therapeutic strategy. Cardiac progenitor cells, which can be committed to cardiomyocytes, smooth muscle cells, and endothelial cell lineages, are 1 of many potential candidate cell types for cardiovascular regenerative medicine. Although several studies have demonstrated improvement in cardiac function after CPC transplantation, the low survival of the transplanted cells is believed to be one of the main hurdles for the failure to show long-term improvements after cell therapy.

MiRNAs are a class of small noncoding RNAs, mainly functioning as negative regulators of protein coding genes. In
this study, we report that a novel miRNA prosurvival cocktail can improve the engraftment of the transplanted CPCs. Consequently, the longer surviving CPCs could lead to better functional recovery. We first isolated CPCs from transgenic mice that constitutively express Fluc and green fluorescent protein. These isolated CPCs can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells under proper induction protocols.\(^7,28\) Afterward, we selected several miRNA candidates that have been shown to improve cell survival or inhibit cell apoptosis. Cell viability analysis demonstrated that miR-21, miR-24, and miR-211 were particularly potent compared with control. Furthermore, when transduced with these 3 miRNAs as a cocktail, we found that CPCs had significantly better cell viability both in vitro and in vivo, which led to improved function as demonstrated by echocardiography and histology.

MiR-21 displays strong evolutionary conservation across a wide range of species and is expressed in mammalian organ systems such as the heart, spleen, small intestine, and colon.\(^29\) MiR-21 is overexpressed in many tumor samples with apoptotic roles by inhibiting several apoptotic genes. Most recently, miR-21 has been found to play important roles in cardiovascular biology in the areas of vascular smooth muscle cell proliferation and apoptosis, cardiac cell growth and death, and cardiac fibroblast functions.\(^30\) Overexpression of miR-21 can significantly decrease infarct size in mouse acute MI models by targeting PDCD4 and its downstream molecule PTEN.\(^22\) MiR-24 also has been shown to be an antiapoptotic miRNA in the developing neural retina\(^22\) and tumorigenesis.\(^33\) miR-221 is involved as a miRNA in several cancer cells by functioning as an antiapoptotic regulator by suppressing PTEN.\(^34\) Antisense inhibition of miR-21 or miR-221 can arrest cell cycle and induce apoptosis in pancreatic adenocarcinoma.\(^21\)

To elucidate the molecular mechanism of CPC survival by the miRNA prosurvival cocktail, we performed target prediction of these 3 miRNAs. We found these miRNAs potentially suppress many classic apoptotic genes, including \(\text{Casp}3, \text{Casp8ap2}, \text{Bax}, \text{Pdcd4},\) and \(\text{Fasl}\) as well as \(\text{Bim}, \text{Foxo3},\) and \(\text{Ak2},\) which can be inhibited by all the 3 miRNAs. Bim is of particular interest because it belongs to the Bcl-2 family and can interact with other members of the family, including Bcl2, Bcl-xL, and Mcl-1.\(^25\) Bim mainly functions as a critical apoptotic activator. Bim gives rise to a variety of isoforms, including the most extensively studied cytotoxic splice variants, \(\text{BimL},\) and \(\text{BimEL}.\)\(^25\) Overexpression of Bim in cardiac myocytes can induce apoptosis.\(^35\) Our dual-luciferase assay indicated that all 3 miRNAs can bind to the 3'UTR of Bim transcript and suppress its expression. Bim has been shown to activate caspase cascades by inducing cytochrome \(c\) release from mitochondria and induce apoptosis of cells.\(^13,36,37\) Furthermore, Western blotting indicated that the miRNA cocktail inhibits Bim and subsequently suppresses activation of caspase 3, contributing to the survival of CPCs. Our data demonstrated that miR-21, miR-24, and miR-221 can inhibit the apoptotic Bim and can improve cell survival after challenge with serum-free stress in vitro and ischemic stress in vivo. Taken together, we believe the inhibition of potent proapoptotic Bim by the miRNA cocktail presents a novel and an important avenue for promoting cell survival.

Several cell types have been used for clinical trials.\(^37,38\) However, there is still little convincing evidence that stem cells can engraft significantly in the myocardium.\(^6\) It is thought that massive cell death after transplantation may be a major limiting factor that impedes long-term cardiac recovery. Therefore, developing prosurvival or antiapoptosis strategies to boost stem cell survival after transplantation may benefit this field. Cellular preconditioning by physical, chemical, genetic, and pharmacological manipulation has shown that the cells can better with-
stand the rigors of lethal ischemia in vitro as well as posttransplantation. We report that a novel miRNA prosurvival cocktail (miR-21, miR-24, and miR221) can also significantly improve CPC engraftment through suppression of proapoptotic Bim. Transplantation of CPCs treated with the miRNA cocktail can lead to significant improvement in cardiac function in a murine MI model. Hence, miRNAs may provide a valuable method to promote cell survival by regulating multiple target genes or even an entire gene network.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

Cell culture and lentiviral production of miRNA constructs. 293FT and NIH 3T3 were cultured in regular DMEM medium supplemented with 10% FBS and penicillin/streptomycin (Invitrogen) in a humidified 5% CO₂ incubator at 37°C. 293FT cells were used to generate recombinant replication-deficient lentivirus used for *in vitro* assays as previously described. Briefly, 293FT cells were seeded at about 80% confluence per 100-mm dish and transfected with 12 μg each lentiviral vectors (miR-21, miR-24, miR-210, miR-221, miR-17-92, miR-106b-25, and GFP) (System Biosciences, Mountain View, CA), as well as 4 μg pMD2.G and 7.5 μg psPAX2 vectors using Lipofectamine 2000 (Invitrogen) in Opti-MEM according to manufacturer’s instruction. The supernatant was collected at 36 h and 48 h after transfection. The supernatant was centrifuged at 3000 × g for 15 minutes to remove cells and cell debris. The supernatant was filtered through 0.45 μm PVDF filter to further eliminate cellular debris. The filtrate was mixed with PEG-it Virus Concentration Solution (System Biosciences) overnight at 4°C. In the next day, the filtrate/PEG-it mixture was centrifuged at 1500 × g for 30 minutes at 4°C. The lentiviral pellet was resuspended with Opti-MEM medium. The concentrated lentivirus was used directly or aliquoted and stored at -80°C for future usage.

Cell viability assay. Cardiac progenitor cells (CPCs) cells were seeded in 96-well plate and were transduced with lentivirus (miR-21, miR-24, miR210, miR-221, miR-17-92, miR-106b-25 and GFP) for 24 hours. Then the cells were exposed to serum free medium for 24 hours for stress. CellTiter-Fluor™ Cell Viability Assay (Promega) was used according to the manufacturer’s manual. Briefly, we added 100 μl CellTiter-Fluor™ Reagent (Promega) per well, mix by orbital
shaking, and then incubate for 1 hour at 37°C. The resulting fluorescence was measured using FlexStation II 384 fluorometer with excitation 380 nm and emission 505 nm.

**Western Blotting.** The CPCs alone and CPCs treated with miRNAs cocktail were collected in RIPA lysis buffer and briefly sonicated to shear DNA and reduce sample viscosity. The protein concentration was measured by Bio-Rad Protein Assay, which is based on the method of Bradford. The samples were run on a 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) and transferred onto Hybond™-ECL™ nitrocellulose membrane (Amersham™, GE Healthcare). After blocking with 5% non-fat dry milk (Bio-Rad) in PBS in 1 hour, the membranes were incubated with anti-mouse Bim antibody (Cell Signaling Technology) or Anti-mouse Caspase 3, Active (Sigma-Aldrich) 16 hours at 4 °C. After washing with PBS-T (0.1% Tween 20 in PBS) for 3 times, the membranes were incubated in donkey anti-rabbit antibody conjugated with house radish peroxidase (HRP) for 1 hour. After washing with PBS-T for 3 times, the membranes were developed with ECL™ Western Blotting Detection Reagents (Amersham™, GE Healthcare). Bands were visualized with Kodak X-OMAT 2000 processor.

**In vivo optical bioluminescence imaging (BLI).** BLI was performed using the Xenogen IVIS Spectrum system (Xenogen, CA). Recipient mice were anesthetized with isoflurane, and were intraperitoneally injected with D-Luciferin (200 mg/kg body weight). Mice were imaged after surgery on days 2, 5, 7, 10, and 14. Peak signals from a fixed region of interest (ROI) were obtained and signals quantified in photons/s/cm²/sr as previously described.²
Echocardiographic analysis of left ventricular function. Echocardiography was performed before (day -2) and after (days 7, 14, and 28) the LAD ligation. The Siemens-Acuson Sequioa C512 system equipped with a multi-frequency (8-14 MHZ) 15L8 transducer was used by an investigator (MH) 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 (EDD) and end-systolic diameter (ESD) were measured and used to calculate fractional shortening (FS) by the following formula: FS (%) = [(EDD - ESD)/EDD] x 100% as described.³

Histological examination. Mice were sacrificed and left ventricular (LV) tissue was obtained at 4 weeks after MI. Tissue samples were embedded into OCT compound (Miles Scientific). Frozen sections (5 μm thick) were processed for immunostaining. Anti-cardiac Troponin T antibody (Thermo Scientific) and anti-GFP antibody (Thermo Scientific) were used. Trichrome stain (Masson, Sigma) was used to determine collagen content of the infarct regions. For each heart, eight to ten sections from apex to base (1.2 mm apart) were analyzed. Images were taken for each section to calculate the fibrotic and non-fibrotic areas as well as ventricular and septal wall thickness. The NIH Image J software was used to quantify the infarct zones.
SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure 1. Ex vivo BLI of cell biodistribution. (A) Representative BLI in explanted tissues at 24 and 72 hours after CPCs were injected intramyocardially into mouse hearts following LAD ligation. (B) At day 1, quantitative analysis of BLI signal indicated that cell signal is mainly located in lung and heart. By day 3, significant cell signal is only detectable in the heart, which is consistent with our in vivo data. However, cells may still be in other organs but is below the detection threshold of BLI.

Supplemental Figure 2. Venn diagram of targets prediction of miR-21, miR-24, and miR-221. Based on TargetScan and MicroCosm prediction algorithms, there are 1791 genes predicted as miR-21 targets, 2048 genes predicted as miR-24 targets, and 2295 genes predicted as miR-221 targets. 106 genes can be predicted as the common targets of these 3 miRNAs, including apoptotic activators or effectors, Bcl2l11 (Bim), Foxo3, and Ak2.

Supplemental Figure 3. Complementarity between Bim and miRNAs. (A) 6 binding positions were distributed in the 3’UTR of Bim. (B) The details of individual binding sites are shown.
SUPPLEMENTAL REFERENCES


Supplementary Figure 1

A

Day 1

Day 3

B

Relative Luciferase Level

Day 1     Day 3

Heart     Lung     Liver     Spleen
Supplemental Figure 3