Regulation of Cardiac MicroRNAs by Bone Marrow Mononuclear Cell Therapy in Myocardial Infarction

Kazuma Iekushi, MD, PhD; Florian Seeger, MD; Birgit Assmus, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

Background—Cell therapy with bone marrow–derived mononuclear cells (BMCs) can improve recovery of cardiac function after ischemia; however, the molecular mechanisms are not yet fully understood. MicroRNAs (miRNAs) are key regulators of gene expression and modulate the pathophysiology of cardiovascular diseases.

Methods and Results—We demonstrated that intramyocardial delivery of BMCs in infarcted mice regulates the expression of cardiac miRNAs and significantly downregulates the proapoptotic miR-34a. In vitro studies confirmed that the supernatant of BMC inhibited the expression of H2O2-induced miR-34a and cardiomyocytes apoptosis. These effects were blocked by neutralizing antibodies directed against insulin-like growth factor-1 (IGF-1). Indeed, IGF-1 significantly inhibited H2O2-induced miR-34a expression, and miR-34a overexpression abolished the antiapoptotic effect of IGF-1. Likewise, inhibition of IGF-1 signaling in vivo abolished the BMC-mediated inhibition of miR-34 expression and the protective effect on cardiac function and increased apoptosis and cardiac fibrosis. IGF-1 specifically blocked the expression of the precursor and the mature miR-34a, but did not interfere with the transcription of the primary miR-34a demonstrating that IGF-1 blocks the processing of miR-34a.

Conclusions—Together, our data demonstrate that the paracrine regulation of cardiac miRNAs by transplanted BMCs contributes to the protective effects of cell therapy. BMCs release IGF-1, which inhibits the processing of miR-34a, thereby blocking cardiomyocyte apoptosis. (Circulation. 2012;125:1765-1773.)

Key Words: cell therapy ▪ microRNA ▪ growth factor ▪ myocardial infarction

Molecular Cardiology

Cell-based therapy is a promising option to treat cardiovascular diseases. Bone marrow–derived mononuclear cells (BMCs) improved the recovery after ischemia in various experimental studies and significantly, although modestly, improved cardiac function in patients after acute myocardial infarction (AMI).1–3 Multiple mechanisms have been proposed to mediate the therapeutic benefits of BMC therapy, including cell transdifferentiation, cell fusion, and the release of paracrine growth factors and cytokines.4 In fact, various cytokines and growth factors from transplanted progenitor cells have been shown to elicit positive effects by influencing cardiomyocyte survival, angiogenesis, and the recruitment of endogenous stem cells.5,6 Moreover, a recent study suggests that c-kit+ bone marrow–derived cells activate endogenous regeneration.7

Clinical Perspective on p 1773

miRNAs are small noncoding RNAs that negatively modulate gene expression by inhibiting protein translation or inducing degradation of the targeted mRNA.8 miRNAs play an important role in biological processes during development, tissue homeostasis, and disease.9–11 Several miRNAs are regulated after the induction of myocardial infarction (MI) and control neovascularization and fibrosis.12–14 For example, the increased expression of miR-21 and the downregulation of the miR-29 family members have been associated with cardiac fibrosis,15 whereas miR-92a and miR-126 regulate neovascularization after cardiac ischemia.12,15

Here, we show that administration of BMC in mice after AMI regulates cardiac miRNAs. BMC administration reduced the expression of the profibrotic miR-21 and the proapoptotic miR-34a. We further show that insulin-like growth factor (IGF-1), which is secreted from BMCs, inhibits miR-34a processing and blocks apoptosis of cardiomyocytes in vitro and in vivo.

Materials and Methods

Cell Culture and Conditioned Media Collection

BMCs from healthy volunteers were isolated as described previously.3 We used BMCs from a total of 18 healthy human volunteers...
and did not pool cells from individual donors. Cardiomyocytes were isolated from hearts of neonatal rats by the use of collagenase digestion. BMCs were cultured for 24 hours at 37°C in serum-free DMEM in 6-well plates (5×10⁶ cells in 2 mL per well) to obtain conditioned supernatants.

**RNA Isolation and Quantification**

Total RNA was isolated by using the miRNeasy kit (Qiagen, Hilden, Germany). Real-time polymerase chain reaction was performed for measurement of the expression levels of primary, precursor, and mature miRNAs. For detection of mature miRNAs and pri-miRNAs, TaqMan MicroRNA assay kits (Applied Biosystems, Carlsbad, CA) were used in accordance with the manufacturer’s protocol. For measurement of the pre-miRNAs, SYBR green was used. The primer sequences used were previously described. miRNA levels were normalized with RNU6 (human) and snoRNA202 (mouse) expression and mRNA levels with RPLP0.

**Transfection and Fluorescence-Activated Cell Sorter Analysis**

Transfection of cardiomyocytes with precursor miR (pre-miR) constructs (from Ambion, Carlsbad, CA) was performed with the use of HiPerfect reagent (Qiagen, Hilden, Germany). Apoptosis was quantified after incubation of cardiomyocytes with BMC supernatants, H₂O₂, with or without neutralizing antibody against IGF-1 (ab9572) or hepatocyte growth factor (ab10678) (both from Abcam, Cambridge, United Kingdom) by using Annexin V and 7-AAD staining and flow cytometry analysis by using a CantoII (BD Biosciences, San Jose, CA). Annexin V-positive/7-AAD–negative cells were considered apoptotic.

**Myocardial Infarction Model**

AMI was induced in male nu/nu mice (8–10 weeks; Charles River, Sulzfeld, Germany) by permanent ligation of the left anterior coronary artery under mechanical ventilation and anesthesia with isoflurane and analgesia with bupivacaine (1 mg/kg, 0.25% bupivacaine). An incision was made in the midscapular region under sterile conditions, and did not pool cells from individual donors. Cardiomyocytes were isolated from hearts of neonatal rats by the use of collagenase digestion. BMCs were cultured for 24 hours at 37°C in serum-free DMEM in 6-well plates (5×10⁶ cells in 2 mL per well) to obtain conditioned supernatants.

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**Histology**

Hearts were perfused with PBS by left ventricular cannulation, immersed in Roti-Histofix (Carl Roth, Karlsruhe, Germany) and then embedded in paraffin. Four-micrometer paraffin sections were cut with a cryotome, and infarct size was measured in 4 sections that were obtained from each heart (distance between sections ~200 μm). Infarct size was calculated as previously described. For apoptosis analysis, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling was performed according to the manufacturer’s protocol (In Situ Cell Death Detection Kit, Roche Diagnostics, Mannheim, Germany).

**MiR-34a Promoter-Luciferase Assay**

The miR-34a expression vector was kindly provided by Prof Joshua T. Mendell. For measuring luciferase activity, rat neonatal cardiomyocytes were grown in 12-well plates. Luciferase plasmid (0.2 μg) was cotransfected with 0.01 μg pGL4 Renilla plasmid (Promega, Madison, WI) as control for the transfection efficiency with the use of Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The activity of Luciferase and Renilla was assessed with the Dual-Luciferase Reporter Assay System (Promega).

**Statistical Analysis**

For 2 groups, we used 2-sample t test with equal/unequal variance version depending on variance ratio test. For 3 or more groups, Levene tests were performed to assess variance homogeneity. Standard 1-way ANOVA followed with Tukey-Kramer multiple comparison procedures were performed with homogeneous variances, and the Welch ANOVA followed by Dunnett T3 test was performed with heterogeneous variances. For comparisons of means between groups at different times (3, 5, and 7 days), linear regression with group, time and group*time interaction were performed. All results are presented as mean value±standard error. A final value of P<0.05 was considered significant. All statistical analyses were performed with SPSS for Windows 19.0 (SPSS, Inc., Chicago, IL).

**Results**

BMC Administration Regulates Cardiac miRNAs

To determine whether BMCs regulate miRNA expression in infarcted hearts, we injected human BMCs in infarcted hearts of nude mice and determined the expression of miRNAs. BMC administration improved cardiac function after 7 days induction of AMI in PBS-treated control mice increased the expression of miR-21 and miR-92a, whereas miR-29 levels were downregulated (Figure 1A). In accordance with previous studies, the induction of AMI in PBS-treated control mice increased the expression of miR-21 and miR-92a, whereas miR-29 levels were downregulated (Figure 1B). Interestingly, BMC administration induced a 1.7-fold (day 3), albeit non-
significant upregulation of miR-20a, which is known to augment cellular proliferation by targeting the E2F family of transcription factors and the cell cycle inhibitor p21\(^21\)–\(^23\) (Figure 1C, online-only Data Supplement Figure II B). Furthermore, BMC treatment abolished the AMI-induced upregulation of miR-34a (Figure 1D, online-only Data Supplement Figure II C), which is known to increase apoptosis of tumor cells\(^24\)–\(^25\) and inhibits angiogenesis in vitro.\(^26\) In contrast, the expression of miR-92a and miR-29b were not affected by BMC therapy (online-only Data Supplement Figure I A and I B). Together, BMC administration modulates the expression of several miRs, which regulate fibrosis, proliferation, and apoptosis. Because the expression of miR-34a was most efficiently suppressed by BMC treatment in vivo, we further elucidated the regulation and function of miR-34a.

**BMC Supernatants Inhibit Cardiac Myocyte miR-34a Expression and Apoptosis in Vitro**

To determine whether BMC may inhibit miR-34a expression in a paracrine manner, we induced apoptosis by hydrogen peroxide (H\(_2\)O\(_2\)) in neonatal rat cardiomyocytes and evaluated the effect of conditioned medium derived from cultured BMC. H\(_2\)O\(_2\) increased the expression of miR-34a in a time- and dose-dependent manner with maximal effects seen at 200 \(\mu\)mol/L after 12 hours (Figure 2A, data not shown). BMC supernatants significantly suppressed the H\(_2\)O\(_2\)-induced upregulation of miR-34a and reduced cardiomyocyte apoptosis (Figure 2B and 2C). To identify the factor(s) that mediate the protective effect of BMC supernatants, the antiapoptotic cytokines IGF-1 and hepatocyte growth factor, which are known to be released by BMC,\(^27\) were blocked by neutralizing antibodies. The antiapoptotic effect of BMC supernatants on miR-34a expression and apoptosis was significantly reversed by a neutralizing antibody directed against IGF-1, whereas anti-hepatocyte growth factor antibodies had no effect (Figure 2B and 2C). Indeed, recombinant IGF-1 protein inhibited H\(_2\)O\(_2\)-induced miR-34a expression (28.1 ± 5.7% inhibition, \(P<0.05\)). In addition, miR-34a overexpression significantly augmented baseline as well as H\(_2\)O\(_2\)-induced apoptosis in cardiomyocytes in comparison with control transfection, and miR-34a overexpression abrogated the antiapoptotic effect of IGF-1 (Figure 2D). Furthermore, the phosphatidylinositol 3-kinase inhibitor LY294002 signifi-
cantly reversed the inhibitory effect of IGF-1 on H$_2$O$_2$-induced miR-34a expression (46.7±13.0%, P<0.05), suggesting that IGF-1 mediates its effects predominantly via the Akt/phosphatidylinositol 3-kinase pathway. These data suggest that IGF-1, which is secreted by BMC, inhibits H$_2$O$_2$-induced miR-34 expression and blocks cardiomyocyte apoptosis in vitro.

**IGF-1 Mediates the Protective Effect of BMC in Vivo**

To investigate whether BMC-secreted IGF-1 is required for the cardioprotective responses of BMC treatment, we inhibited IGF-1 signaling in vivo by systemically applying the IGF-1 receptor antagonist JB1, which competes with IGF-1 to inhibit autophosphorylation of the receptor. The decrease in WMSI (−0.39±0.18) was reduced by JB1-treatment (−0.19±0.21) (Figure 3A and 3B). Furthermore, inhibition of IGF-1 signaling reversed the BMC-mediated inhibition of apoptosis (Figure 3C) and accelerated cardiac fibrosis in BMC-treated mice (Figure 3D). Consistently, in situ hybridization revealed that JB-1 treatment augmented miR-34a expression in the border zone of BMC-treated mice, and PBS-treated mice in comparison with BMC-treated mice receiving PBS, as well (online-only Data Supplement Figure III).

Together, these data demonstrate that inhibition of IGF-1 signaling reversed the effect of BMC therapy on cardiac apoptosis and miR-34a expression in mice after AMI.

**Figure 2.** IGF-1 secreted from BMC regulates H$_2$O$_2$-induced miR-34a expression in vitro. (A) The effect of H$_2$O$_2$ on miR-34a expression in neonatal rat cardiomyocytes. Neonatal rat cardiomyocytes were treated with H$_2$O$_2$ (50–500 μmol/L) for 12 hours. MiR-34a levels were determined by q-PCR. Analysis was performed with one-way ANOVA followed by Tukey-Kramer post hoc analysis. P<0.05 versus without H$_2$O$_2$. (B) The effect of BMC supernatants on H$_2$O$_2$-induced miR-34a expression (H$_2$O$_2$ 200 μmol/L, 12 hours). MiR-34a levels were determined by q-PCR. Analysis was performed with 1-way ANOVA followed by Dunnett T3 post hoc analysis. P<0.05 versus without H$_2$O$_2$ treatment; †P<0.05 versus BMC supernatants with H$_2$O$_2$ treatment; ‡P<0.05 versus BMC supernatants with IGF-1 antibody without H$_2$O$_2$ (n=9–15, each group). (C) The effect of BMC supernatants on H$_2$O$_2$-induced apoptosis (H$_2$O$_2$ 100 μmol/L, 24 hours). Apoptotic cells were identified as Annexin V-positive and 7AAD-negative cells. Analysis was performed with 1-way ANOVA followed by Tukey-Kramer post hoc analysis. *P<0.05 versus controls without H$_2$O$_2$ treatment; †P<0.05 versus BMC supernatants with H$_2$O$_2$ treatment; ‡P<0.05 versus BMC supernatants with IGF-1 antibody without H$_2$O$_2$ (n=9–13, each group). (D) The effect of IGF-1 on H$_2$O$_2$-induced apoptosis in neonatal cardiomyocytes with miR-34a overexpression (H$_2$O$_2$ 100 μmol/L, 24 hours). Apoptotic cells were identified as Annexin V-positive and 7AAD-negative cells. Analysis was performed with 1-way ANOVA followed by Tukey-Kramer post hoc analysis. *P<0.05 versus premiR-Co without H$_2$O$_2$ treatment; †P<0.05 versus premiR-Co with H$_2$O$_2$ treatment; ‡P<0.05 versus premiR-Co with H$_2$O$_2$ and IGF-1 treatment; §P<0.05 versus premiR-miR-34a without H$_2$O$_2$ treatment (n=5 per group). IGF-1 indicates insulin-like growth factor-1; BMC, bone marrow-derived mononuclear cell; q-PCR, quantitative polymerase chain reaction; premiR, precursor miR; HGF, hepatocyte growth factor.
Next, we investigated whether BMCs upregulate the expression of IGF-1 and thereby inhibit miR-34a expression in infarcted hearts. Consistent with a previous study, administration of BMCs significantly increased the expression of IGF-1 mRNA in the infarcted hearts in comparison with the PBS group (Figure 4A). To identify the source of increased IGF-1 expression in vivo, we measured the expression of BMC-derived human versus host-derived mouse IGF-1 mRNA by using human- and mouse-specific primers. The human IGF-1 originating from human BMC peaked at day 1 and subsequently declined within 3 days. In contrast, host-derived mouse IGF-1 mRNA levels were profoundly increased at day 3 and remained elevated up to 7 days (Figure 4B). To further investigate the possibility that BMC-derived IGF-1 might mediate the induction of endogenous IGF-1, we injected human recombinant IGF-1 protein in infarcted hearts of nude mice and determined the effect on host-derived mouse IGF-1 and miR-34a expression. Administration of recombinant IGF-1 protein significantly increased mouse IGF-1 expression and decreased miR-34a expression (Figure 4C and 4D). To further elucidate the causal role of BMC-derived IGF-1 in regulating miR-34a expression, we transfected human BMC with siRNA directed against IGF-1. siRNA directed against IGF-1 significantly suppressed IGF-1 expression in BMCs by \( \approx 65\% \) in comparison with control siRNA (Figure 4E). Inhibition of IGF-1 by siRNA in transplanted BMC significantly increased miR-34a expression in comparison with the control BMC treated hearts demonstrating that BMC-derived IGF-1 is required to reduce miR-34a in the infarcted heart (Figure 4F).

**BMC-Derived IGF-1 Mediates the Upregulation of Endogenous IGF-1 in Vivo**

The generation of mature miRs is regulated by the expression of the primary miRNA (pri-miR) and is additionally controlled at various steps of the miR biosynthesis pathways. Pri-miRs are cropped into a hairpin structure leading to the formation of the premiRs which are subsequently exported from the nucleus and processed into the \( \approx 22\)-nucleotide mature miR. \(^{30}\) MiR-34a is an intergenic miR that is controlled by its own promoter. \(^{20}\) To determine by which mechanism IGF-1 controls miR-34 synthesis, luciferase reporter constructs encoding the miR-34 promoter were used. However, IGF-1 did not significantly affect basal and H2O2-induced reporter gene activity (Figure 5A) suggesting that IGF-1 interferes with miR-34 processing. Consistent with this model, IGF-1 only slightly reduced the expression of the pri-miR-34 in H2O2-treated cardiomyocytes (Figure 5B), whereas the levels of the premiR-34 were significantly reduced by IGF-1 to 48.4±11.4% (Figure 5C). To demonstrate whether BMCs also affect miR-34 expression in vivo...
by a similar mechanism, we determined the expression of the pri-miR-34 and premiR-34 in vivo. Indeed, BMC administration did not affect the expression of the pri-miR-34, whereas the premiR-34 levels were significantly reduced in infarcted hearts (Figure 5D and 5E).

Discussion
The data of the present study demonstrate that BMC administration regulates the expression of miRs in the infarcted hearts in mice. In particular, the expression of the proapoptotic miR-34a was significantly reduced in the infarct border zone in mice after BMC therapy. The effects of BMC on cardiac miR expression are probably attributable to the release of paracrine factors, because BMC supernatants block H2O2-induced expression of miR-34a in cardiomyocytes in vitro. BMCs secrete various factors, which are known to provide protective effects on cardiomyocytes, including various antiapoptotic and anti-inflammatory cytokines.31,32 Among the factors tested, we showed that inhibition of the antiapoptotic cytokine IGF-1 by using neutralizing antibodies reduced the antiapoptotic effects of BMC supernatants on cardiac apoptosis and miR-34a expression in vitro. In addition, systemic inhibition of IGF-1 functions by antagonistic peptides reversed the beneficial effect of BMC on cardiac function and apoptosis in vivo. IGF-1 is well known for its cardioprotective effects under stress conditions.33–36 IGF-1 overexpression or local myocardial delivery of IGF-1 improved myocyte survival and protected against ventricular dilatation after infarction.37,38 IGF-1 additionally stimulates endogenous regeneration and increased the number and differentiation of cointegrated cardiac stem cells.39 BMCs secrete IGF-1 in vitro,27 and administration of BMCs increased the expression of IGF-1 mRNA in infarcted hearts (Figure 4A and 4B). Of note, consistent with previous studies,39 transplanted BMC directly provided IGF-1 as demonstrated by the increased expression of human IGF-1 mRNA, but also induced a longer-term expression of host tissue–derived murine IGF-1 mRNA, suggesting that BMC therapy directly and indirectly stimulated IGF-1 expression in the infarcted tissue. Together these data disclose a crucial function of IGF-1 in mediating the antiapoptotic and cardioprotective effects of BMC. However, given the reported pleiotropic effects of BMC, it is likely that additional mechanisms may contribute to the observed beneficial effects of BMC on angiogenesis and long-term remodeling. Indeed, blocking IGF-1 resulted in a higher mortality in the mice after AMI without BMC transplantation (38.4% survival) in comparison with JB-1-treated mice, which additionally receive BMC transplantation (70.5% survival) suggesting that BMCs even show an additional protective effect under conditions when IGF-1 signaling is blocked (online-only Data Supplement Figure IV).

IGF-1 improves cell survival by activating prosurvival kinases such as Akt.40 The present study now provides additional insights into the mechanism underlying the antiapoptotic activity of IGF-1. IGF-1 inhibits the stress-induced...
expression of miR-34 in a phosphatidylinositol 3-kinase–dependent manner. These findings are consistent with a recent study showing that phosphatidylinositol 3-kinase inhibition is associated with increased levels of miR-34.41 MiR-34 was shown to induce apoptosis in various cell types and targets protective proteins such as SIRT1.24 Interestingly, the overexpression of locally acting IGF-1 increased SIRT1 expression and activity, thereby preventing hypertrophy and oxidative stress in the heart.42 In addition, miR-34 regulates p53 activity and controls cell cycle progression and senescence.25 Therefore, one may speculate that inhibition of miR-34 processing may contribute to the well-established protective effects of IGF-1 under stress conditions.

Our data suggest that the inhibition of miR-34a expression is preferentially controlled by an inhibition of miR-34a processing. Although the expression of the pri-miR-34a is induced by H2O2 in a p53-dependent manner (online-only Data Supplement Figure V), IGF-1 did not reduce H2O2-induced induction of the pri-miR-34a and activation of the miR-34a promoter in luciferase assays in vitro. Likewise, BMC therapy did not reduce pri-miR-34a expression suggesting that both BMC and IGF-1 inhibit miR-34a processing. Several studies suggest a crucial role of miR metabolism by various mechanisms involving protein–protein and protein–RNA interactions.16,43,44 Therefore, further studies should elucidate the signaling pathways mediating the inhibitory effects of IGF-1 on miR-34 processing.

In summary, the present study demonstrates that administration of BMCs regulates cardiomyocyte miR-34a expression and apoptosis in a paracrine manner by secreting IGF-1. IGF-1–mediated inhibition of miR-34a may contribute to several of the known cardioprotective functions of IGF-1 (Figure 6).

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**CLINICAL PERSPECTIVE**

Cell-based therapy is a promising option to treat cardiovascular diseases. Multiple mechanisms have been proposed to mediate the therapeutic benefits of bone marrow–derived mononuclear cell therapy, including cell transdifferentiation, cell fusion, and the release of paracrine growth factors and cytokines. In fact, various cytokines and growth factors from transplanted progenitor cells have been shown to elicit positive effects by influencing cardiomyocyte survival, angiogenesis, and the recruitment of endogenous stem cells. MicroRNAs are small noncoding RNAs that negatively modulate gene expression by inhibiting protein translation or inducing degradation of the targeted mRNA. MicroRNAs play an important role in biological processes during development, tissue homeostasis, and disease. Several microRNAs are regulated after the induction of myocardial infarction and control neovascularization and fibrosis. Here, we demonstrate that the paracrine regulation of cardiac microRNAs by transplanted bone marrow–derived mononuclear cells contributes to the protective effects of cell therapy. In particular, bone marrow–derived mononuclear cell release insulin-like growth factor-1, which inhibits the processing of miR-34a, thereby blocking cardiomyocyte apoptosis. Insulin-like growth factor-1-mediated inhibition of miR-34a may contribute to several of the known cardioprotective functions of insulin-like growth factor-1.
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SUPPLEMENTARY MATERIAL

Paracrine regulation of cardiac miRNAs by bone marrow mononuclear cell therapy in myocardial infarction

Kazuma Iekushi, MD, PhD; Florian Seeger, MD; Birgit Assums, MD; Andreas M. Zeiher, MD; Stefanie Dimmeler, PhD

From the Institute for Cardiovascular Regeneration, Centre of Molecular Medicine, (K.I., F.S., S.D.), and Dept of Medicine III, Frankfurt University, Frankfurt, (F.S., B.A., A.M.Z.), Frankfurt University, Frankfurt,
Isolation of Bone Marrow Mononuclear Cells
Bone marrow aspirates were obtained from healthy volunteers. The Ethics Review Board of the Hospital of the Johann Wolfgang Goethe University of Frankfurt, Germany approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each volunteer. BMCs were isolated as described previously. Briefly, bone marrow aspirates were diluted with 0.9% NaCl (1:5), were filtrated (100 um) and mononuclear cells were isolated by density gradient centrifugation using Ficoll (Cambrex; 800g, 20 minutes, without brake). Mononuclear cells were washed 3 times with 50 mL PBS (800g), were counted, and used for the experiments1.

In situ hybridization
To detect miR-34a expression, tissue was processed and stained according to Obernosterer et al.2 10 µm sections were cut and thawed for 30 min at room temperature. After fixation with paraformaldehyde (4%) for 10 min, sections were washed 3 times for 5 min and incubated with protein kinase K (Sigma) for 5 min. After washing 3 times with PBS, sections were incubated with hybridization buffer for 4 h at room temperature. Meanwhile, probes (0.5 µl 3’-DIG labeled LNA probes, Exiqon) were mixed with 150 µl denaturation buffer, heated to 80 °C for 5 min, chilled on ice and added to the sections followed by incubation over night at 56 °C. After incubation for 1 h in 50 % formamide / 1x SSC at 56 °C, 1h in 0.2 SSC at 56 °C and 10 min in solution B1, sections were blocked for 1h at room temperature in blocking reagent. Then, anti-DIG AP (Roche) was added at a dilution of 1:500 for 1h at 37 °C. After washing with solution B1, sections were equilibrated in 1 M Tris, pH=8.3 for 10 min at room temperature and subsequently incubated for 15 min with Fast Red substrate (Dako) containing 1 drop levamisol. After washing in PBS/0.5% Tween-20, sections were mounted in DAPI mounting medium (Vector).

Fig. S1. Real time PCR analysis of cardiac miRs.

(A, B) Real time PCR analysis confirms the regulation of miR-92a (A), miR-29b (B) in response to treatment with BMC compared to treatment with PBS, (S, sham; BZ, border zone of infarcted area).
Fig. S2. Real time PCR analysis of cardiac miRs in the remote area. (A, B, C) MicroRNA expression was measured in the remote area by PCR analysis. miR-21 (A), miR-20a (B), miR-34a (C) in response to treatment with BMC compared to treatment with PBS or sham.
Figure S3. Localization of miR-34a expression in MI. Representative images showing miR-34a expression in ifarcted mice heart. Sections were counterstained with miR-34a (red), perfused vessels (lectin, green), and nucleus (blue). Scale bar: 20μm.
Survival (%)

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<tr>
<td>PBS+PBS</td>
<td>56.2%</td>
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<tr>
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<td>75.0%</td>
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<tr>
<td>BMC+JB-1</td>
<td>70.5%</td>
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Fig.S4. survival rate with JB-1 or PBS infusion in treatment with BMC or PBS after MI mice. Kaplan-Meier analysis with JB-1 or PBS in treatment with BMC or PBS after MI mice.
Fig. S5. Mir-34a promoter assay.
The luciferase assay of miR-34a promoter. The arrow above construct P1 indicates the position of the transcription start site. Filled circles show the position of the p53 binding site. Data represent mean ± SEM. Constructed plasmids were provided by Prof. Joshua T. Mendell. (Molecular Cell 2007).