Bone Marrow Stem Cells Prevent Left Ventricular Remodeling of Ischemic Heart Through Paracrine Signaling

Ryota Uemura,* Meifeng Xu,* Nauman Ahmad, Muhammad Ashraf

Abstract—In this study, we hypothesized that bone marrow stem cells (BMSCs) protect ischemic myocardium through paracrine effects that can be further augmented with preconditioning. In in vitro experiments, cell survival factors such as Akt and eNOS were significantly increased in BMSCs following anoxia. In the second series of experiments following coronary ligation in mice, left ventricles were randomly injected with the following: DMEM (G-1), BMSCs (G-2), and preconditioned BMSCs (G-3). Four days after myocardial infarction, BMSCs were observed within injured myocardium in G-2 and G-3. Apoptotic cardiomyocytes within peri-infarct area were significantly reduced in G-3. Four weeks after myocardial infarction, smaller left ventricular (LV) dimension and increased LV ejection fraction were observed in G-3. Infarct area was significantly reduced in G-3. However, GFP+ cardiomyocytes were observed in low numbers within peri-infarct area in G-2 and G-3. In conclusion, BMSCs secreted cell survival factors under ischemia, and they prevented apoptosis in cardiomyocytes adjacent to the infarcted area. Preconditioning of BMSCs enhanced their survival and ability to attenuate LV remodeling, which was attributable, in part, to paracrine effects. (Circ Res. 2006;98:0-0.)

Key Words: stem cells ■ paracrine effect ■ preconditioning ■ ischemia ■ remodeling

Myocardial infarction (MI) leads to cardiomyocyte loss and scar formation in the infarcted area. The large transmural infarction is associated with ventricular remodeling after MI. Ventricular remodeling is characterized by changes in left ventricular (LV) geometry, mass, volume, and function, which include hypertrophy and cellular apoptosis of cardiomyocytes, in response to myocardial injury or alteration in load. Ventricular remodeling is also a major factor in the progression of heart failure, and the prognosis for survival is poor.

One approach proposed to reverse myocardial remodeling is regeneration of new cardiomyocytes. Recent reports have shown that bone marrow stem cells (BMSCs) have multilineage differentiation potential and potentially cross the lineage restriction to form various nonhematopoietic tissues including heart. Most studies on BMSC therapy in experimental animal heart models have shown an improvement in cardiac function, signifying the safety and feasibility of this approach. However, the mechanism of BMSC therapy is still controversial. BMSCs repair the ischemic myocardium primarily by angioblast-mediated vasculogenesis, prevention of apoptosis of native cardiomyocytes, or direct regeneration of the lost cardiomyocytes.

We, therefore, hypothesized that BMSCs protected ischemic myocardium through paracrine effects that could be further augmented with preconditioning.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Coculture of BMSCs and Cardiomyocytes
Isolation and purification of BMSCs from C57B6 mice were performed as described previously. The details can be found in the online data supplement.

Detection of Cell Apoptosis: DNA Laddering and Annexin V+ Cell
For DNA laddering, after exposure to anoxia, 2×10⁶ cells were suspended in PBS and homogenized in buffer containing proteinase K and RNase. After 15 minutes of incubation at 37°C, NaI solution was added. Cell lysates were incubated at 50°C for 30 minutes, and isopropranol was added. DNA was precipitated and washed by 70% ethanol. DNA (8 μg) was then analyzed using 1.2% agarose gel electrophoresis. Annexin V staining was performed after cells were exposed to anoxia with a commercially available kit according to the protocols of the manufacturer (Roche).

Electroimmunoblotting
The details are provided in the online data supplement.

Induction of MI and BMSC Therapy
Myocardial infarction was created in wild-type C57B6 mice by permanent ligation of left anterior descending coronary artery (LAD). The animals were anesthetized with sodium pentobarbital (50 mg/kg IP) and mechanically ventilated. After a left-sided minithoracotomy, the heart was exposed and LAD was ligated by 7-0

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From the Department of Pathology and Laboratory Medicine, University of Cincinnati Medical Center, Ohio.
*Both authors contributed equally to this study.
Correspondence to Muhammad Ashraf, PhD, Department of Pathology and Laboratory Medicine, University of Cincinnati Medical Center, 231 Albert Sabin Way, Cincinnati, OH 45267-0529. E-mail muhammad.ashraf@uc.edu
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ethicon suture at just below the atrioventricular border. The mice were then randomly given an intraventricular injection with a 31-gauge needle of one of the following: 100 μL of DMEM (G-1), 1×10^6 BMSCs/100 μL (G-2), or 1×10^7 anoxic preconditioning (AP)-BMSCs/100 μL (G-3). Cultured BMSCs used for injection were taken from green fluorescent protein (GFP) transgenic mice. AP-BMSCs were exposed to 4 hours of anoxia followed by incubation in oxygenated medium for 2 hours. Exposure of BMSCs to anoxia for 4 hours did not cause irreversible damage. The chest was closed, and animals were weaned from the ventilator and allowed to recover.

**Echocardiography**

Mouse heart function was assessed by transthoracic echocardiography, which was performed at fifth day and 4 weeks after MI using HDI 5000 SonoCT (Phillips) with a 15-MHz probe. Details are given in the online data supplement.

**Infarct Size and Infarct Wall Thickness Measurement**

After echocardiographic measurements, the animals were euthanized and the hearts removed. The excised heart was cut into 3 transverse slices. Each slice was fixed in 4% paraformaldehyde and embedded in paraffin or frozen in optimal cutting temperature (OCT) compound. Sections (5-μm thick) were mounted on microscopic glass slides. Middle transverse section was stained with hematoxylin–eosin and Masson’s trichrome for both infarct size and wall thickness measurements. Infarct size was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total LV epicardial and endocardial circumferences using computer-based planimetry. The mean wall thickness of infarcted myocardium was measured from 3 equidistant points. Quantitative assessment of each parameter was performed with the use of image analysis software (version 1.6.0.65; NIH).

**Immunohistochemical Evaluation**

To evaluate blood vessel density and Ki-67–positive cardiomyocytes, the immunoperoxidase method was used. Serial paraffin heart sections were deparaffinized. Endogenous peroxidase activity was blocked by 3% H₂O₂ for 20 minutes. The sections were incubated with antibodies specific to Ki-67 (DAKO), α-smooth muscle actin (α-SMA) (Sigma), von Willebrand factor (vWF) (DAKO), and platelet endothelial cell adhesion molecule (PECAM)-1 (Santa Cruz Biotechnology) at room temperature for 1 hour. Then, the sections were treated with a biotinylated appropriate secondary antibody followed by incubation with avidin horseradish peroxidase complex (ABC method; Vector Labs). Finally, the sections were colored with diaminobenzidine. Blood vessel density in infarcted myocardium was calculated in at least 8 randomly high-power fields on each heart section.

For detecting GFP-positive cardiomyocytes and vessels, fluorescent immunostaining for vWF, α-SMA, α-sarcomeric actin, and desmin (Sigma) were performed. The heart section slides were incubated with goat serum for 20 minutes at room temperature. Then samples were incubated with primary antibodies and treated with respective secondary antibodies that conjugated with Alexa Fluor 488 or Alexa Fluor 633. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI) when necessary. Fluorescent images were obtained with an Olympus BX 41 microscope equipped with digital camera (Olympus) and Leitz DMRBE fluorescence microscopic equipped with a TCS 4D confocal scanning attachment (Leica Inc.).

**TUNEL Analysis**

Apoptotic myocytes after MI were evaluated by TUNEL assay in serial paraffin and cryosections with an ApopTag kit (Chemicon). More than 20 tissue sections in each group were examined microscopically. Four fields each were selected in noninfarction and border areas. The percentage of apoptotic cardiomyocyte was termed the apoptotic index.

**Statistical Analysis**

Data were expressed as mean±SEM. For the comparison of in vitro data, ultrasound parameters, and histological data, an ANOVA means table with Fisher's post hoc test was used for continuous data. For the comparison of GFP⁺ cell number, the unpaired Student's t test was used. Mann–Whitney U test was performed for nonparametric continuous data (the number of GFP⁺ cardiomyocytes). Differences with a value of P<0.05 were regarded as statistically significant.

**Results**

**Survival Gene and Transcription Factor Expression in BMSCs Subjected to Anoxia**

Typical BMSCs in primary culture are illustrated in Figure 1A. Immunostaining with antibodies against various markers
demonstrated that BMSCs expressed high levels of c-kit (>85%) and sca-1 (>95%). Expression of CD34, VE-cadherin, and Flk-1 ranged from 31% to 44% of the total population, which was in agreement with previously published data (Figure 1B). Therefore, isolated BMSCs comprised mostly mesenchymal stem cells.

Akt activity was determined in BMSCs before and after subjecting them to anoxia. Representative Western blots of Akt are illustrated in Figure 2A. Activation of Akt survival gene was strongly increased in BMSCs following anoxia, and phospho-Akt level of BMSCs was increased many folds after anoxia (arbitrary units, 1517 ± 149 in 2 hours of anoxia, *P* < 0.05; 3647 ± 816 in 4 hours of anoxia, *P* < 0.01; 1983 ± 329 in 6 hours of anoxia, *P* < 0.05; versus 670 ± 112 in 0 hour anoxia, respectively). Phospho–endothelial NO synthase (eNOS) level was also increased in BMSCs following anoxia (Figure 2B).

Our laboratory previously reported that the expression of cardiomyocyte transcription factors such as GATA-4 and MEF-2C was important in transdifferentiation of BMSCs into cardiomyocytes. Therefore, we next examined whether these factors were upregulated in BMSCs following anoxia. Western blot analysis showed that the expression of GATA-4 in BMSCs was slightly increased after anoxia, but the expression of MEF-2C was not increased following anoxia (Figure 2C).

**BMSCs Prevent Cardiomyocyte Apoptosis Induced by Anoxia**

It has been reported that Akt is involved in antiapoptotic signaling. Therefore, we examined the antiapoptotic effect of BMSCs. DNA fragmentation was assessed in cultured cells with or without being exposed to anoxia. Cardiomyocytes after 3 hours of anoxia displayed the typical nucleosome spacing ladder that is indicative of apoptosis (Figure 3A). The DNA laddering was not observed in normal cardiomyocytes and in BMSCs after 4 hours of anoxia (Figure 3B). When cardiomyocytes were cultured with BMSCs in a ratio of 10:1 and exposed to 3 hours of anoxia, DNA fragmentation was diminished (Figure 3C). Annexin V staining showed that the percentage of apoptotic cardiomyocytes was reduced (*P* < 0.01) in cocultured cells exposed to anoxia for 3 hours compared with cardiomyocytes alone (Figure 3D).

To explore the mechanism of protection, we next examined Akt activity in cardiomyocytes alone or in cardiomyocytes cocultured with BMSCs (Figure 3E and 3F). For coculture experiments, BMSCs and cardiomyocytes were cultured in 2
individual chambers separated by a semipermeable membrane (3-μm hole). This system allowed sharing the culture medium in 2 chambers but prevented cell contacts. Phospho-Akt level peaked in cardiomyocytes after 2 hours of anoxia but was decreased to very low levels after 4 hours of anoxia (arbitrary units, 2283±350 in 2 hours of anoxia, P<0.01; 962±243 in 4 hours of anoxia, 561±90 in 6 hours of anoxia, versus 940±230 in 0 hour of anoxia, respectively). However, the phosphorylation of Akt in cardiomyocytes cocultured with BMSC was significantly increased after 4 hours of anoxia (arbitrary units, 2685±350 in 2 hours, P<0.01; 2320±673 in 4 hours, P<0.01; 988±498 in 6 hours; versus 820±121 in 0 hour, respectively).

Secretion of Cytokines by BMSCs
Recently, it has been reported that BMSCs secrete a wide array of cytokines that exert beneficial effects on surrounding cells.14,15 We, therefore, examined the level of cytokines in either BMSC-conditioned medium (BM-M) or cardiomyocyte-conditioned medium (CM-M) with or without exposure to anoxia for 4 hours (Figure 4). Under normoxic conditions, vascular endothelial growth factor (VEGF) (8.14±0.70 pg/μg protein from BM-M versus 1.44±0.34 pg/μg protein from CM-M, P<0.05), basic fibroblast growth factor (bFGF) (32.4±4.0 versus 7.1±3.1, P<0.05), insulin-like growth factor (IGF) (81.0±15.7 versus 10.5±1.2, P<0.05), and stromal cell–derived factor (SDF) (0.13±0.01 versus 0.02±0.01, P<0.05) in BM-M were significantly higher compared with CM-M. After 4 hours of anoxia, these proteins were increased by 30% to 150% in BM-M. VEGF and SDF were increased, but IGF was decreased by 65% in CM-M under anoxia.

Recruited BMSCs to the Injured Myocardium Protect Cardiomyocytes in Early Phase of MI
Five mice in each group were examined for cardiac function by echocardiography at 5 days and were then euthanized. Immunofluorescent staining revealed that GFP-positive cells were immediately recruited after coronary ligation to the injured myocardium in both G-2 and G-3 animals (Figure 5A and 5F). The number of GFP-positive cells per field was higher in G-3 than in G-2 (54.1±9.9 versus 45.4±7.9,
P < 0.05). Our in vitro results suggested that the protective effects of BMSCs were attributable, in part, to reduction of cardiomyocyte apoptosis. To determine whether the recruited BMSCs mediated the antiapoptotic effects in the ischemic myocardium, TUNEL labeling was performed. The results showed that the number of TUNEL-positive cells in the perinfarct region was significantly different among the 3 groups (Figure 6A through 6D). Apoptotic cardiomyocyte index was significantly reduced in G-2 and G-3 (8.0 ± 0.74 in G-2, P < 0.05, and 4.1 ± 1.1 in G-3, P < 0.01, versus 11.1 ± 3.4 in G-1, respectively).

Heart function and hemodynamic parameters on day 5 were almost similar among the 3 groups. Initial infarct size on day 5 was also similar among the 3 groups.

**Improvement of Cardiac Function and Cardiac Morphology in Chronic Phase of MI**

LV internal dimension at both diastole and systole were significantly smaller in G-3 animals compared with both G-1 and G-2 animals 4 weeks after MI. Percent fractional shortening and left ventricular ejection fraction (LVEF) were also significantly higher in G-3 animals. G-2 animals had better LVEF compared with G-1 (supplemental Table 1). The percentage of infarct area was significantly reduced in G-3 (34.5 ± 4.8%) compared with G-1 (43.3 ± 4.5%; P < 0.01) and G-2 (40.4 ± 5.0%; P < 0.05) (Figure 7A through 7E). In G-3 animals, the cell structure was better preserved in the infarct area. The LV infarct wall was thicker in G-3 (0.38 ± 0.10 mm) compared with G-1 (0.29 ± 0.05 mm; P < 0.05).

**Regeneration of Cardiomyocytes and Angiogenesis by BMSCs**

GFP-positive cells were observed in the hearts of both G-2 and G-3 mice. Interestingly, they were mostly localized in the border area. However, the frequency of GFP-positive cardiomyocytes was very low in both G-2 and G-3 (3.8 ± 1.7 cells/4 × 10⁴ cardiomyocytes in G-2 and 4.3 ± 1.5 cells/4 × 10⁴ cardiomyocytes in G-3) (Figure 8A and 8B). Immunostaining for Ki-67 was used to determine cycling cells in the myocardium after MI. The percentage of Ki-67-positive cardiomyocytes was similar among the 3 groups (0.70 ± 0.34% in G-1, 0.76 ± 0.22% in G-2, and 0.95 ± 0.30% in G-3).

PECAM-1 was used as a marker for endothelial cells (Figure 8C through 8E). The mean number of microvessels per randomly chosen field did not differ between G-2 and G-3 mice (146.8 ± 20.4 in G-2 and 155.9 ± 23.6 in G-3), but it was increased 1.5-fold in both G-2 and G-3 compared with G-1 animals (101.0 ± 20.3, P < 0.01) (Figure 8F).
BMSCs are multipotent and can differentiate into several distinct cell types, including cardiac cell components, within the microenvironment of the heart. There appears to be general agreement that BMSCs therapy has potential to improve perfusion and contractile performance of the injured heart. However, accumulating evidence has questioned these previous reports. The mechanism underlying this therapeutic effect has not been clearly defined, with an intense debate over differentiation versus fusion, and it appears to be far more complex than previously anticipated. Recently, it has been reported that BMSCs provide protection by paracrine mechanisms involving release of a wide array of cytokines that exert their effects on surrounding cells.

We, therefore, hypothesized that BMSCs mediate their protection by paracrine mechanism under ischemic conditions and that preconditioning of BMSCs strongly enhances their survival and regenerative capacity. We have demonstrated here that (1) BMSCs showed strong upregulation of the cell survival gene Akt following brief anoxia. BMSCs prevented apoptosis in cardiomyocytes exposed to anoxia after coculture. Cardiac transcription factors such as GATA-4 and MEF-2C were not markedly upregulated after anoxia. (2) Recruited BMSCs following MI exerted a marked inhibitory effect on LV remodeling through paracrine mediators, which were released by BMSCs.

Our in vitro results showed that Akt activity in BMSCs was significantly increased following anoxia. Akt activity in cardiomyocytes peaked after 2 hours of anoxia and decreased after 4 hours of anoxia. However, under coculture conditions, Akt activity in cardiomyocytes was persistently increased.
Apoptosis in vivo after ischemia–reperfusion injury,24,25 and Akt signaling has been shown to minimize cardiomyocyte loss.24,26 More recently, Akt has been shown to modulate intracellular glucose metabolism, thereby enhancing energy production during hypoxia.22 More recently, an extended role for Akt has been established in a variety of cardiovascular events.23 Constitutive activation of Akt signaling has been shown to minimize cardiomyocyte apoptosis in vivo after ischemia–reperfusion injury.24,25 and Akt activation has been shown to preserve the function of hypoxic cardiomyocytes at levels comparable to those of normoxic controls.25

An earlier study reported that ex vivo hypoxia in BMSCs stimulated the synthesis of vascular endothelial cell growth factor mRNA, resulting in differentiation of BMSCs into endothelial cells in ischemic hindlimb.26 It suggests that BMSCs also comprise a population of endothelial progenitor cells (EPC). However, transcription factors such as GATA-4 and MEF-2C were insignificantly increased in BMSCs following ischemia. Although BMSCs were isolated by Dexter’s method, which excluded hematopoietic cells, they were positive for several phenotype markers. The precise nature of the bone marrow–derived cardiomyocyte precursor cells remains unknown, although the majority of isolated BMSCs might contain primarily nonmyogenic cells.

Our in vitro data further showed that anoxic preconditioning (AP) of BMSCs increased their effectiveness against ischemic injury by reducing cell apoptosis and LV remodeling. Ischemic preconditioning is known to promote synthesis of several proteins that enhance cell survival.27 Following AP, BMSCs expressed increased level of Akt and eNOS phosphorylation. Thus, these cells were able to survive better in ischemic environment and enhance their therapeutic potential. Several studies clearly indicate that NO plays an important role in cardioprotection against ischemic injury. Chronic hypoxia increases eNOS expression and confers resistance to ischemia in cardiomyocytes.28 Ischemic preconditioning protects heart against ischemia–reperfusion injury by increased synthesis of inducible NO synthase (iNOS).29,30 The protective role of EPC has been shown in a recent study in which ischemic preconditioning mobilized these cells in the ischemic myocardium where they acted as donors of eNOS, iNOS, and VEGF.31 In this study, AP-BMSC therapy significantly reduced infarct size and improved cardiac function. These benefits were mainly associated with reduction of apoptotic cardiomyocytes in perinfarct area. Recently, it has been reported that BMSCs release a wide array of cytokines that directly affect surrounding cells.15,16 Akt and possibly other cell survival pathways may be rapidly activated by AP-BMSCs in vivo model. Involvement of protective proteins may result in overall improvement of cardiac function caused by prevention of cardiomyocytes apoptosis and preservation of ischemic cardiomyocytes function. The cumulative effects resulted in attenuation of LV remodeling after MI.

LV remodeling is an important process affecting ventricular function and progression of cardiac failure1 and is characterized by an increase in myocardial mass associated with cardiomyocyte hypertrophy. There is evidence that pathological remodeling also involves the death of cardiomyocytes by apoptosis.2 The relative lack of oxygen to the hypertrophied cardiomyocytes might be an important etiological factor in their programmed death. Several studies have implicated cardiomyocyte apoptosis as the underlying mechanism responsible for LV remodeling after MI.32,33

Although BMSCs could differentiate into cardiomyocytes and vascular cells, thereby contributing to regeneration of myocardium4 and angiogenesis10,11 in ischemic hearts, recent evidence has questioned the role of BMSCs in cardiac regeneration.5,17 In our in vivo experiments, functioning
cardiac fibers developed from GFP \(^*\) cells could not be found on a large scale. The frequency of the BMSC-derived cardiomyocytes was very small. Our in vitro results also showed that cardiac transcription factors such as GATA-4 and MEF-2C were insignificantly increased in BMSCs following anoxia. These results support the concept that AP-BMSC therapy improves cardiac function, which is mediated by paracrine factors rather than myogenesis. Previous work has held the notion that the adult heart is terminally differentiated organ without self-renewal potential. However, recent studies have challenged these preexisting notions regarding cardiac regeneration and have identified resident stem/progenitor cell population capable of self-differentiation into cardiomyocytes in adult heart.\(^3\)–\(^7\) Furthermore, it has been reported that FGF-2 could regulate the fate and cardiogenic conversion of undifferentiated progenitors.\(^8\) In addition to the reduction of apoptotic cardiomyocytes, AP-BMSC therapy might act in a supportive paracrine manner that stimulates the mobilization and growth of resident cardiac stem cells, resulting in infarct size reduction.

Ischemic preconditioning is such a powerful stimulus for cardioprotection that no other therapeutic approach has matched its effect. Diverse signaling pathways have been involved in ischemic preconditioning. The latter phase of this protection is believed to be attributable to synthesis or secretion of proteins.\(^9\) Similarly, BMSCs also respond to ischemic preconditioning stimuli and become tolerant to lethal ischemia (unpublished data). Furthermore, recent reports have shown that cytokine-preconditioned adult stem cells can differentiate into large numbers of cardiomyogenic cells in vivo and in vitro.\(^10\) Preconditioning of BMSCs undergo upregulation of several cytokines, growth factors, and survival protein and secrete them in the immediate environment. These serve as antiapoptotic and myoangiogenic differentiation stimulants. Besides numerous benefits bestowed by preconditioning, the direct injection of preconditioned cells can be reduced because of their enhanced survival rate and perhaps higher potential for regeneration. Therefore, preconditioning appears to be an exciting and innovative approach in cell-based therapy.

Conclusion

Our results show that BMSCs exert their protective effects via upregulation of Akt and eNOS under ischemic conditions, thus preventing cardiomyocyte apoptosis. Preconditioning of BMSCs enhances their survival and ability to attenuate LV remodeling after MI by preventing cardiomyocyte apoptosis through paracrine mediators.

Acknowledgments

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**Co-culture of BMSCs and cardiomyocytes**

In brief, femoral and tibial bones were removed after euthanasia of animals with overdose of anesthesia. The bone-marrow plugs were flushed with phosphate-buffered saline solution (PBS). Cells were cultured with complete medium (Iscove’s Modified Dulbecco’s Medium containing 20% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml streptomycin) at 37°C in humid air with 5% CO₂. The cells adherent to the culture flask was maintained for propagation.

Cardiomyocytes were isolated from neonatal rat hearts (1 to 3 days old) using commercially available neonatal cardiomyocyte isolation kit (Worthington Biochemical Co) essentially as described by supplier’s protocol. Cardiomyocytes were co-cultured with BMSCs (10:1) and exposed to anoxia. Glucose-free Dulbecco’s Modified Essential Medium was deoxygenated by bubbling with purified nitrogen for 1 hr before the experiments. Cells were exposed to anaerobic medium and placed into the anoxic chamber for various periods (Forma 1025 anaerobic system).

**Electroimmunoblotting**

Cytoplasmic and nuclear proteins were extracted with different methods. To prepare cytoplasmic protein, cultured cells were homogenized in buffer (mM) 20 Tris [pH 7.4], 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 β-glycerolphosphate, 1 Na₃VO₄ containing proteinase cocktail. To prepare crude nuclear extracts, cells were homogenized in 1 ml of lysis buffer (mM): 10 HEPES [pH 7.9], 10 KCl, 0.1 EDTA, 1.5 MgCl, 0.2% Nonidet P-40, 1 dithiothreitol and incubated on ice for 5 min with intermittent vortexing. The nuclear pellets were obtained by centrifugation at 3,000 rpm for 5 min. The pellets were resuspended in 40 µl of extract buffer (mM): 20
HEPES [pH 7.9], 420 NaCl, 0.1 EDTA, 1.5 MgCl₂ 25 % glycerol, 1 dithiothreitol and incubated on ice for 10 min with intermittent gentle vortexing. After centrifugation, the supernatant was collected as crude nuclear extract.

The protein content of samples was determined according to the Bradford method. The denatured protein (25 µg for Akt, 30 µg for eNOS, 20 µg for GATA-4 and MEF-2C) were then analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were immunoblotted overnight at 4°C with antibodies including Akt (1:1000, Cell Signaling), Phospho-Akt [Ser473] (1:1000, Cell Signaling), eNOS (1:1000, BD phamingen), Phospho-eNOS [Ser1177] (1:1000, Cell Signaling), GATA-4 (1:1000; Santa Cruz) and MEF-2C (1:1000, Cell Signaling). Densitometric analysis for the blots was performed with NIH image software.

**Echocardiography**

Mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine. Mice were kept on a heating pad in a left lateral decubitus or supine position. LV parameters were obtained from two-dimensional image and M-mode interrogation in long-axis view. Interventricular septum thickness (IVST), LV posterior wall thickness (LVPWT), LV internal diastolic diameter (LVIDd), LV internal systolic diameter (LVIDs). LV percent fractional shortening (LV%FS) and LV ejection fraction (LVEF) were calculated as:

\[
LV\%FS= \frac{(LVIDd - LVIDs)}{LVIDd} \times 100; \quad LVEF= \frac{1}{3} \left( (LVIDd)^3 - (LVIDs)^3 \right) / (LVIDd)^3 \times 100.
\]

All echocardiographic measurements were averaged from at least 3 separate cardiac cycles.
### Online Table 1. Echocardiography parameters in various treatment groups

<table>
<thead>
<tr>
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<th>Day 5</th>
<th>4 week</th>
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<td></td>
<td>Med (n=5)</td>
<td>BM (n=5)</td>
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<td>IVSTd, mm</td>
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<tr>
<td>% EF</td>
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IVSTd, interventricular septum thickness; LVPWTd, left ventricular posterior wall thickness; LVIDd & LVIDs, left ventricular internal dimensions at end diastole and end systole; FS, fractional shortening; EF, ejection fraction. Data are expressed as mean±SD. *p < 0.05 vs Med(4week) mice, †p < 0.05 vs BM(4week) mice. ‡p < 0.05 vs Med(4week) mice.