(PBS). Bone marrow cells were cultured in a 10-cm dish with complete culture medium: α-minimal essential medium: αMEM (Invitrogen, Carsbad, CA), 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (MP Biomedicals, Solon, OH). Non-adherent hematopoietic cells were removed, and the medium was replaced every 3–4 days. The adherent, spindle-shaped MSC population expanded to >5 × 10^7 cells within 4–5 passages after the cells were first plated.

Preparation of MSC-derived conditioned medium. MSC (1 × 10^6 cells) were plated in 10-cm dishes and cultured in complete culture medium for 2 days. The attached cells were washed three times with PBS and the medium was replaced with basal culture medium: αMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, and after 48 h, conditioned medium was collected and centrifuged at 2000*g* for 10 min followed by filtering the supernatant through a 0.22-μm filtration unit (Millipore, Bedford, MA).

Isolation of CPC from neonatal rats. CPC were isolated from neonatal Lewis rats (Japan SLC), as reported previously with modification [11]. In brief, isolated myocardial tissue was cut into 1- to 2-mm^3 pieces and digested three times for 5 min at 37 °C with 0.2% trypsin (Invitrogen) and 0.1% collagenase II (Worthington Biomedical, Lakewood, NJ). These tissue pieces were washed with complete explant medium (Iscove's Modified Dulbecco's Medium, Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mmol/L 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan). Then, the remaining tissue fragments were cultured as explants in complete explant medium at 37 °C under 5% CO_2. After 1–2 weeks, a layer of fibroblast-like cells was generated from adherent explants and phase-bright cells migrated over a layer of fibroblast-like cells. These phase-bright cells were collected by washing with PBS. Isolation of the phase-bright cells was performed twice at 3- to 5-day intervals from the same dish.

Reverse transcription-polymerase chain reaction. RT-PCR assay was performed according to a previously described method [13]. In brief, total RNA was extracted from CSC using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was reverse-transcribed into cDNA using a QuantiTect reverse-transcription kit (Qiiagen) according to the manufacturer's instructions. PCR amplification was performed in 50 μl containing 1 μl cDNA and 2.5 U Taq DNA polymerase (Takara, Otsu, Japan). The oligonucleotides used in RT-PCR analysis are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA amplified from the same samples served as an internal control. PCR reaction mixtures were denatured at 95 °C for 5 min and cDNA templates amplified as follows: 35 cycles (21 cycles for GAPDH) of denaturation at 95 °C for 1 min, annealing at 55–66 °C for 45 s, and extension at 72 °C for 1 min. At the end of the cycling, the samples were incubated at 72 °C for 10 min.

Table 1
Primer pairs for RT-PCR

Primer	Sequence	Product size (bp)	Annealing temperature (°C)
ABCG2	5′-CAATGGGATCATGAAACCTG-3′	536	58
	5′-CAGGCTGATGAATGGAGAAA-3′		
c-Kit	5′-AGCAAGAGTTAACGATTCCGGAG-3′	300	53
	5′-CCAGAAAGGTGTAAGTGCCTCCT-3′		
c-Met	5′-CAGTGATGATCTCAATGGGCAAT-3′	725	60
	5′-AATGCCCTCTTGCTATGACTTC-3′		
CXCR4	5′-CAGAAGAAGCTGAGGAGCATGACA-3′	197	55
	5′-CTGATGAAGGCCAGGATGAGAACA-3′		
Flt-1	5′-CATGGTCAGCTGCTGGGACACCGCG-3′	400	62
	5′-GACTCCCTGCATCACTAACAATAT-3′		
IGF-1R	5′-ATTACGCACTGGTCATCTTC-3′	546	58
	5′-AAGCCATCTGAGTCACTGCT-3′		
MEF-2c	5′-GGCCATGGTACACCGAGTACAACGAGC-3′	401	62
	5′-GGGGATCCCTGTGTTACCTGCATTGG-3′		
GATA4	5′-CTGTCATCTCACTATGGGCA-3′	275	60
	5′-CCAAGTCCGAGCAGGAATTT-3′		
ANP	5′-CCGAGACAGCAAACATCAGATCG-3′	762	58
	5′-CCGTGGTGCTGAAGTTTATTCGG-3′		
β-MHC	5′-GCCAACACCAATGTCCAAGTTC-3′	205	66
	5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′		
GAPDH	5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′	983	51
	5′-CATGTAGGCCATGAGGTCCACCAC-3′		

Cell proliferation assay. Cell proliferation assay was performed using CellTiter96 AQueous One solution cell proliferation assay (Promega, Madison, WI). Briefly, isolated CPC were plated on 96-well plates (5 × 10^3 cells per well), and cultured in basal culture medium ($n = 6$) and MSC-derived conditioned medium ($n = 6$) for 48 h. The cellular level of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), indicative of the mitochondrial function of living cells and cell viability, was measured with a CellTiter96 AQueous One Solution Kit (Promega, Madison, WI) and a Microplate Reader (490 nm, Bio-Rad, Hercules, CA).

Terminal dUTP nick-end labeling (TUNEL) assay. TUNEL assay was performed to evaluate apoptosis of cells induced by serum starvation and hypoxia. After incubation of CPC in basal culture medium or MSC-derived conditioned medium under hypoxia (1% O_2) for 12 h, CPC were fixed in 1% paraformaldehyde. TUNEL assay was performed according to the manufacturer's instructions (ApopTag Fluorescein kit, Chemicon, Temecula, CA). The cells were then mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI).

Caspase-3 activity assay. Isolated CPC were plated on 6-well plates (2 × 10^5 cells per well), and cultured in complete culture medium under normoxia (Control), in basal culture medium under hypoxia (1% O_2, 5% CO_2) ($n = 6$), or in MSC-derived conditioned medium under hypoxia ($n = 6$) for 12 h. Caspase-3 activity was measured using a CaspACE Assay System Colorimetric (Promega, Madison, WI) according to the manufacturer's instructions.

Western blot analysis. To identify the protein expression of phosphorylated Akt, Western blotting was performed with rabbit antibodies against phosphorylated Akt (Ser473) and Akt (Cell Signaling Technology, Danvers, MA). After CSC had been cultured with basal culture medium for 24 h, cell lysates were extracted with sample buffer. Then, 2 μg of protein was transferred into sample buffer, loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore). After being blocked for 120 min, the membrane was incubated with primary antibody at a dilution of 1:500. The membrane was incubated with peroxidase labeled with secondary antibody at a dilution of 1:2000. Positive protein bands were visualized with an ECL kit (GE Healthcare, Buckinghamshire, UK) and measured by densitometry.

Cell migration assay. Migration assay was performed using Chemotaxicell96 (Kurabo, Osaka, Japan) composed of a membrane with 5-μm pores. CPC (5 × 10^3 cells per well) suspended with 50 μl basal culture medium were cultured in basal culture medium in the upper chamber and incubated in basal culture medium ($n = 6$) or MSC-derived conditioned medium ($n = 6$) in the lower chamber for 12 h at 37 °C. The filter was removed from the plate, and the number of the cells that migrated to the lower chamber was manually counted under a microscope.

Differentiation of CPC into caridomyocytes. Isolated CSC were plated on 6-well plates (1 × 10^5 cells per well) and differentiation of CPC into cardiomyocytes was induced (1) by incubation in complete culture medium, (2) by treatment with 3 μM 5-azacytidine (5-AZA, Sigma, Louis, MO) for 24 h [14], and (3) by incubation in MSC-derived conditioned medium for 2 weeks. After induction of differentiation, total RNA was extracted using an RNeasy Mini Kit (Qiagen).

Statistical analysis. All values were expressed as means ± standard error of the mean (SEM). Student's unpaired t test was used to compare differences between two groups. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $p < 0.05$.

Results

Isolation and features of CPC

One to two weeks after explantation, a layer of fibroblast-like cells from adherent explants was generated, over which small, round, phase-bright cells migrated (Fig. 1A and B). The number of these cells increased gradually, and these CPC could be harvested by washing with PBS from 10 to 14 days after explantation. The morphological features of these cells were similar to those of CPC reported previously [11]. RT-PCR analysis showed that these cells expressed stem cell markers such as c-kit and ATP-binding cassette (ABC) transporter subfamily G member 2 (ABCG2), which regulates the ability to exclude various molecules including Hoechst 33342 and defines side population stem cells [15]. In addition, they expressed various receptors of cytokines and chemokines including HGF receptor (c-Met), VEGF receptor (Flt-1), IGF-1 receptor, and stromal-derived factor (SDF-1) receptor (CXCR4 receptor) (Fig. 1C). Thus, we confirmed that our cultured cells were CPC based on their morphological features and gene expression.

Protective effect of MSC-derived conditioned medium on CPC

We examined the protective effects of MSC-derived conditioned medium on CPC. The number of viable CPC obviously increased when cultured in MSC-derived conditioned medium (Fig. 2A). Quantitative analysis using MTS assay demonstrated that the number of viable cells was significantly elevated when incubated in conditioned medium compared to basal culture medium (serum starvation) (Fig. 2B). Furthermore, MSC-derived conditioned medium significantly decreased the number of TUNEL/DAPI double-positive cells (Fig. 2C) and the caspase-3 activity of CPC compared to serum starvation (Fig. 2D). In order to investigate molecular mechanism of antiapoptoic effect of MSC-derived conditioned medium, we examined whether MSC-derived conditioned medium could phosphorylate Akt. Western blot analysis demonstrated that MSC-derived conditioned medium phosphorylated Akt (Fig. 2E).

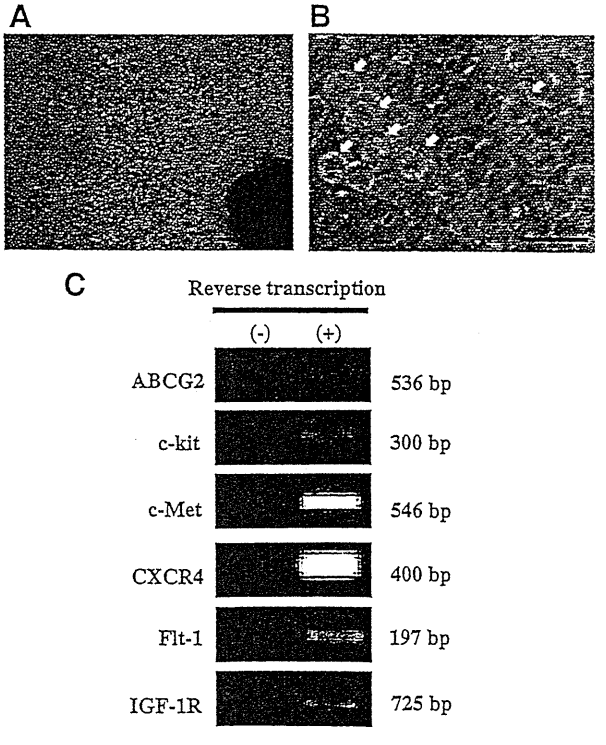

Fig. 1. Morphological features and gene expression of CPC derived from neonatal rat heart. (A,B) Representative photographs of CPC isolated by explant method. White arrows indicate CPC and black arrow indicates fibroblast-like cells. (A) Bar: 200 μm. (B) Bar: 20 μm. (C) Gene expression profile of CPC by RT-PCR.

Effect of MSC-derived conditioned medium on CPC migration

CPC migration was examined using chemotaxicell filters. The number of CPC that migrated through the filters significantly increased when incubated in the lower chamber in MSC-derived conditioned medium (124.8 ± 14.8 cells/well) compared with basal culture medium (Serum free) (4.7 ± 1.9 cells/well) (Fig. 3A and B).

Effect of MSC-derived conditioned medium on CPC differentiation

We examined whether CPC could differentiate into cardiomyocytes by treatment with MSC-derived conditioned medium. Two weeks after treatment with MSC-derived conditioned medium, CPC changed from having a spindle, multiangle appearance to a slim appearance. However, CPC did not beat spontaneously (Fig. 4A). The mRNA expression of GATA4, myocyte-enhancer factor-2c (MEF-2c), β-myosin heavy chain (β-MHC) and atrial natriuretic peptide (ANP) was determined by RT-PCR analysis. Before treatment, CPC expressed cardiomyocyte-related transcriptional factors: GATA4 and MEF-2c, but they did not express any late cardiomyogenic genes: β-MHC and ANP (Fig. 4B). Interestingly, the mRNA expression levels of β-MHC and ANP were upregulated after two weeks of treatment with MSC-derived conditioned medium or 5-AZA.

Discussion

In the present study, we demonstrated that (1) MSC-derived conditioned medium had protective effects on CPC under hypoxia and serum starvation, (2) MSC-derived conditioned medium enhanced CPC migration, and (3) CPC differentiation into cardiomyocytes phenotype was enhanced by MSC-derived conditioned medium.

Multipotent stem cells have been shown to reside in the heart in several species including rat, mouse, dog and human [9–12]. Beltrami et al. have reported that c-kit-positive cells isolated from adult rat heart are self-renewing and have the ability to differentiate into a variety of lineages, including cardiomyocytes, smooth muscle cells and vascular endothelial cells [9]. In the present study, we isolated CPC from neonatal rats according to a previously reported method with modification [11]. Messina et al. have reported that cells isolated using an explant method were a mixture of CPC, differentiating progenitor cells and even spontaneously differentiated cardiomyocytes. In this study, our isolated cells morphologically resembled CPC which Messina et al isolated from murine heart and expressed c-kit and ABCG2, which are stem cell markers [9,15]. Thus, we confirmed that our cultured cells were CPC based on their morphological features and gene expression.

MSC transplantation has been proved to be promising strategy to treat ischemic heart disease [1,16]. The effect of MSC transplantation is thought to be mediated mainly by the supply of cell protective, angiogenic, and mitogenic factors in a paracrine manner. In our previous reports, we demonstrated that MSC secreted a number of cytokines including HGF, VEGF, and IGF-1 [4–6]. In

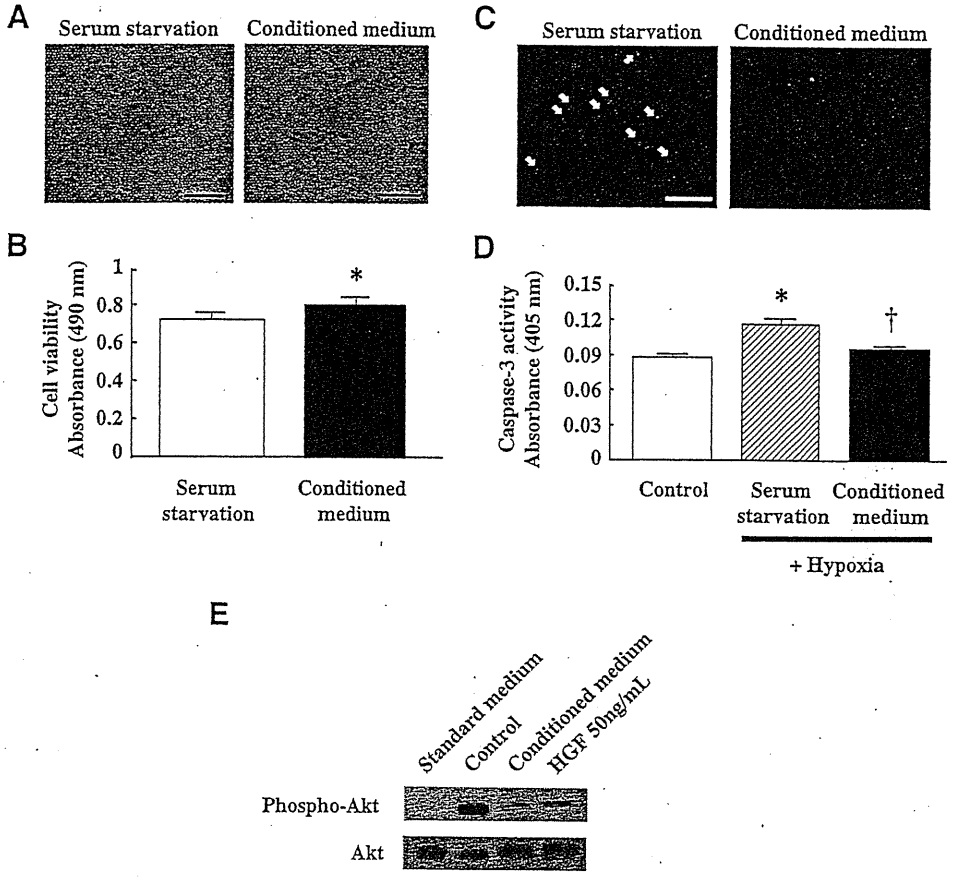

Fig. 2. Proliferative and antiapoptotic effect of MSC-derived conditioned medium on CPC. (A) Representative photographs of CPC incubated in basal culture medium (serum starvation) and MSC-derived conditioned medium for two days. Bar: 200 μm. (B) MTS assay of CPC. $^{*}p < 0.05$ vs serum starvation. (C) TUNEL staining of CPC. TUNEL-positive apoptotic CPC are stained green. Nuclei are stained with DAPI (blue). White arrows indicate TUNEL/DAPI double-positive cells. Bar: 50 μm. (D) Caspase-3 activity of cultured CPC. $^{*}p < 0.05$ vs control, $^{\dagger}p < 0.05$ vs serum starvation. (E) Western blot analysis. MSC-derived conditioned medium as well as 50 ng/mL HGF phosphorylated Akt compared to basal culture medium (standard medium).

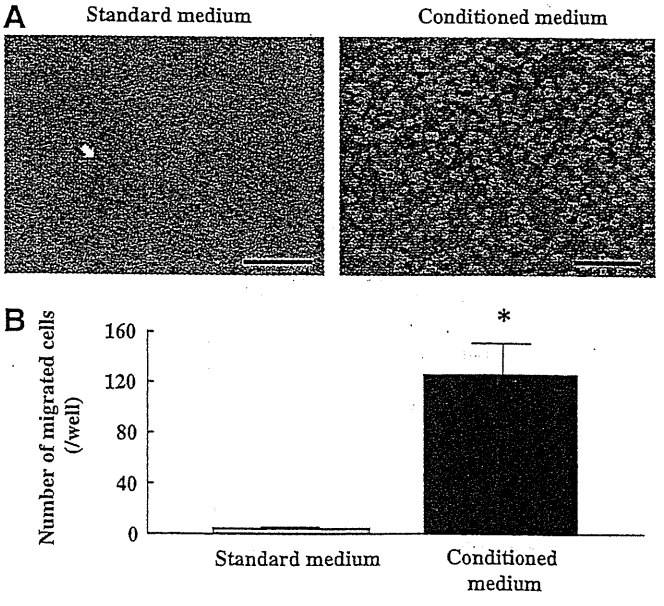

Fig. 3. Migration of CPC induced by MSC-derived conditioned medium. (A) Representative photographs of migrated CPC incubated in basal culture medium (standard medium) and MSC-derived conditioned medium. White arrows indicate migrated CPC (standard medium). Bars: 100 μm. (B) Quantitative analysis of migrated CSC. $^{*}p < 0.05$ vs standard medium.

the present study, isolated CPC expressed the mRNA of these receptors including c-Met, Flt-1, and IGF-1R. Therefore, we hypothesized that MSC-derived cytokines/chemokines may influence the survival and function of CPC. Quantitative analysis using MTS assay demonstrated that the number of viable cells was significantly elevated when incubated in conditioned medium compared to basal culture medium (serum starvation). In addition, we demonstrated that MSC-derived conditioned medium inhibited apoptosis induced by hypoxia and serum starvation, as indicated by decreases in the number of TUNEL-positive cells and caspase-3 activity. Western blot analysis demonstrated that MSC-derived conditioned medium phosphorylates Akt, a survival factor, which is activated by a variety of cytokines and growth factors including HGF and IGF-1 [17–19]. Considering that MSC secret a large amount of HGF and IGF-1 [6], MSC may have cytoprotective and antiapoptotic effects on CPC in a paracrine manner.

CSC are thought to be a population of quiescent stem cells, which reside in the niche of the heart [20]. In ischemic heart disease, CSC migrate to the region bordering the infarct possibly to regenerate and protect injured myocardium [9,21]. In the present study, MSC-derived conditioned medium markedly enhanced isolated CPC migration in comparison to basal culture medium. HGF has been shown to induce migration and invasion of CSC through activation of matrix metalloproteinase-2/9 by collagen-coated modified Boyden chamber assay [12,22]. SDF-1 has been shown to have protective effects on cardiomyocytes as well as chemoattract hematopoietic stem/progenitor cells [23,24]. Considering that

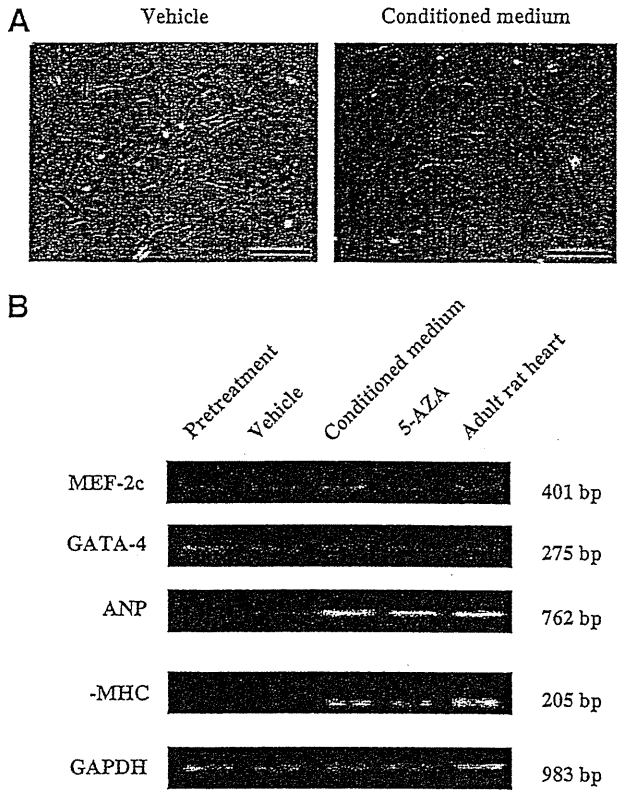

Fig. 4. Cardiomyogenesis of CPC induced by MSC-derived conditioned medium. (A) Morphological features of CPC after incubation in complete culture medium (Vehicle) and MSC-derived conditioned medium for two weeks. Cultured CPC did not beat spontaneously. (A) Bar: 50 μm. (B) RT-PCR analysis of CPC after induction of cardiomyogenesis. Adult rat heart extract was used as positive control.

MSC conditioned medium included a large amount of HGF and SDF-1, MSC might promote CSC migration possibly in a paracrine manner.

Messina et al. have reported that cultured murine CSC started beating, whereas human CSC beat only when co-cultured with rat cardiomyocytes [11,25]. In the present study, we examined whether MSC-derived conditioned medium could induce differentiation of isolated CPC into cardiomyocytes *in vitro*. At baseline, CPC did not express ANP and β-MHC, which are late markers of cardiac lineage. Before treatment with MSC-derived conditioned medium, however, CPC expressed several cardiac transcriptional factors including MEF-2c and GATA4, early phase markers of cardiomyogenic lineage. These results suggested that CPC isolated by explant method in this study were committed to cardiomyocyte differentiation to some degree. After two to four weeks of culture, the mRNA expression of ANP and β-MHC was actually upregulated in CPC by treatment with MSC-derived conditioned medium as well as 5-AZA. Unfortunately, we could not observe beating cells spontaneously even after four weeks of culture unlike the findings of a previous report [10,11]. Thus, MSC-derived conditioned medium could induce cardiomyogenesis of CPC, but might not have sufficient potential to induce CPC to differentiate into mature cardiomyocytes.

In conclusion, MSC-derived conditioned medium had protective effects on cultured CPC and enhanced their migration and differentiation into cardiomyocytes. Thus, MSC transplantation into the heart may have beneficial effects on endogenous cardiac stem/progenitor cells in a paracrine manner.

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