

Mesenchymal stem cells overexpressing MiR-126 enhance ischemic angiogenesis via the AKT/ERK-related pathway

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Abstract

Background: *This study was designed to examine whether transplantation of mesenchymal stem cells (MSCs) overexpressing miR-126 enhances angiogenesis in the infarcted myocardium of mice.*

Methods: *MSCs were harvested from mice using density gradient centrifugation and adherent culture. MSCs were transfected with lentiviral vectors carrying mature miR-126. Mice models of myocardial infarction were established by ligation of coronary artery. The ligated animals were randomly divided into three groups (15 in each) and after two weeks, were intramyocardially injected at the heart infarct zone with miR-126-transfected MSCs (the miR-126-MSCs group), MSCs (the MSCs group), or medium (the PBS group). Six weeks later, histological study and echocardiographic assessment were performed.*

Results: *Capillary density of the infarcted region was significantly improved in the miR-126-MSCs group compared to the MSC group and the PBS group (both $p < 0.01$). Western blot showed that ERK, pERK, AKT and pAKT gene were dramatically enhanced in the miR-126-MSC group compared to the MSC group and the PBS group (both $p < 0.05$). Echocardiography showed MiR-126 led to a sustained improvement in cardiac function for at least six weeks at the injected area, as assessed by left ventricular ejection fraction and fraction of shortening.*

Conclusions: *Transplantation of MSCs transfected with miR-126 can improve angiogenesis and cardiac function in the infarcted area of the hearts of mice, which may be due to stimulation of the AKT/ERK-related pathway. (Cardiol J 2011; 18, 6: 675–681)*

Key words: angiogenesis, cell transplantation, miRNA126, ischemic heart disease, MSCs, AKT, ERK

Introduction

Ischemic heart disease (IHD) which triggers dysfunction and the death of cardiomyocytes is the commonest cause of death throughout the world despite continued advances in the prevention and treatment of coronary artery disease [1]. Moreover, a large number of patients are not qualified for the conventional revascularization techniques of bal-

loon angioplasty and stenting, or coronary artery bypass grafting. Stem cells transplantation has been acclaimed as a promising means for the recovery of heart function [2–4]. Unfortunately, progress in stem cell therapy has been hampered by the poor survival of implanted cells.

MicroRNAs (miRNAs) are an abundant family of endogenous non-protein-coding small RNAs, which negatively regulate gene expression at the

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Received: 10.03.2011

Accepted: 09.08.2011

post-transcriptional level in various processes. Since certain miRNAs are expressed in specific stem cells and play an important role in regulating their survival and migratory functions [5], new therapeutic targets for IHD may be identified in these non-coding RNAs. MiR-126 is an endothelial cell-specific miRNA that plays an essential role in neoangiogenesis following myocardial infarction (MI) and in the maintenance of vascular integrity. We put forward the hypothesis that the combination of MSC transplantation and miR-126 transfer could be superior to MSC transplantation in the treatment of myocardial ischemia.

Methods

Cells and plasmid

MSCs from bone marrow aspirates were obtained from C57 mice and grown in MUBMX-90011 medium (Cyagen, Guangzhou, China) supplemented by 1X PBS (PBS-10001-500), 0.25% Trypsin-EDTA.

Lentiviral MicroRNAs expression vector was constructed using the Gateway system (Invitrogen). Mature miR-126, TRE promoter and eGFP sequences were inserted into plasmids to produce pUp-TRE, pDown-miR126 and pTail-IRES/eGFP; scramble sequence was set as negative control (NC). pLV.EX3d.P/puro-TRE > miR126 > IRES/eGFP was obtained with incubation of donors and acceptor vectors catalyzed by LR clonase (Gateway® LR Clonase™ Plus Enzyme Mix, Invitrogen). Plasmid was then sequenced and purified for lentivirus envelope.

Lentivirus envelope and bMSCs infection

Envelope helper plasmids: pLV/helper-SL3, pLV/helper-SL4, pLV/helper-SL5, with pLV.EX3d.P/puro-TRE-miR126-IRES/eGFP or pLVrtTA/neo which contains the imperative elements for virus packaging, were co-transfected into 293T cells with lipofectamine 2000, according to the manufacturer's instructions (Invitrogen) for the generation of Lenti-miR126-eGFP/puro or Lenti-rtTA/neo respectively. The culture supernatants containing lentivirus vectors were harvested and ultra-centrifuged. The virus titers of each viral preparation were determined.

To perform lentiviral infections, the mice MSCs cells were plated at 40–50% confluence and incubated overnight (16 h). On the day of infections, the culture medium was replaced by the appropriately titered viral supernatant (1.5 mL/well) and incubated at 37°C for 10 h; afterwards, the viral

supernatant was replaced by fresh media. Cells were firstly treated by Lenti-rtTA/neo; 48 h later, infected cell populations were selected in 0.5 mg/mL neomycin and refresh medium every two days. Selection was terminated when control cells were completely dead and antibiotic free medium were used for propagation. Neomycin resistant cells were then infected by Lenti-miR126-eGFP/puro and grown with 2 µg/mL Puromycin. Double resistance cells were ultimately obtained, and 2 µg/mL doxycycline was added to medium and intrigue expression of miR-126.

RNA extraction and quantitative real-time RT-PCR

Approximately 1.0×10^6 MSCs cells (doxycycline treated or not) were seeded into a six-well culture plate. Cells of each group were harvested after being cultured for 72 h. Two weeks after cell transplantation, hearts from the MSC group, the miR126-MSC group and the PBS group ($n = 4/\text{group}$) were excised for ERK₁ and pERK₁, AKT and pAKT assay. Hearts thus obtained were snap-frozen in liquid nitrogen. Specimens were homogenized in lysis buffer.

Total RNA was extracted from cells and hearts with All-in-One miRNA qRT-PCR Detection Kit (Genecopeia) according to the manufacturer's instructions. Expression of miR-126 was detected by quantitative real-time RT-PCR (qRT-PCR) using the All-in-One™ miRNA qPCR Primer (Genecopeia); the primer sequence is 5'-CATTAT-TACTTTTGGTACGCGAAA-3'. The relative levels of miR-126 transcripts were normalized to the control U6 mRNA; the primer sequence was 5'-TCGTGAAGCGTTCCATATTTTTAA-3'. Relative gene expression was quantified using the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA) and expressed as a percentage of the control.

Western blot analysis

Different cultures in 35 mm dishes were lysed in 0.2 mL lysis buffer (0.1% SDS, 1% NP-40, 50 mM HEPES, pH 7.4, 2 mM EDTA, 100 mM NaCl, 5 mM sodium orthovanadate, 40 µM p-nitrophenyl phosphate, and 1% protease inhibitor mixture set I; Calbiochem). Lysates were centrifuged at 12,000 rpm for 15 min. The supernatant was collected and denatured. Proteins were separated in 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membrane. The blot was blocked for 1.5 h at room temperature in 5% BSA, followed by overnight incubation at 4°C with AKT, pAKT, ERK₁ and

pERK₁ antibodies. Membranes were rinsed and incubated for 1 h with the corresponding peroxidase-conjugated secondary antibodies. Chemiluminescent detection was performed using the ECL kit (Pierce).

Myocardial infarction model

All animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources, National Research Council, and published as the 'Guide to the Care and Use of Experimental Animals' by the Chinese Council on Animal Care.

Myocardial infarction was induced in 60 experimental C57 mice by ligating the left anterior descending coronary artery (LAD) as previously described [6], with some modifications. Briefly, C57 mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and mechanically ventilated. Through a left thoracotomy in the fourth intercostal space, MI was induced by ligating the LAD 2 mm from the tip of the left auricle with 6–0 polypropylene (Ethicon, USA). Successful infarction was determined by observing a rapid occurrence of akinesia and/or pale discoloration of the left ventricular (LV) muscle [7].

Implantation of MSCs

Sixty C57 mice were used for construction of the ischemic model. 51 mice were selected from the 56 surviving ligated animals, six of which were randomly re-selected as the model-assessment group for baseline evaluation of the heart infarcted size.

Two weeks after the ligation, the 45 model AMI C57 mice were equally randomized to one of three groups: (1) the MSCs group wherein MSCs in suspension were injected intramuscularly at the left anterior free wall using an insulin syringe with a 30-gauge needle; (2) the miR-126-MSCs group, wherein the animals were injected intramuscularly with miR-126 transfected MSCs suspension; and (3) the PBS group, where the animals were injected with PBS. Cell implantation was performed within two weeks after induction of acute MI. PBS or cell solutions were injected at six injection sites into anterior and lateral aspects of the viable myocardium bordering the infarction (total 5.0×10^6 cells in 0.1 mL). After injection, the chest was closed and the animals were allowed to recover.

Capillary density

Capillary density was determined by immunohistochemical staining with anti-VIII factor antibody

42 days after MI as previously described. The tissue sections (5 μ m) were stained for factor VIII (Santa Cruz Biotech, Inc.) to identify capillaries. Immunohistochemical staining was performed using the two step immunohistochemical technique with DAB (Maixin bio, China) as described in the manufacturer's specifications. After being restained with hematine, the samples were coverslipped and photographed. The cytoplasm of the endothelial cells was stained red. The capillaries were counted with a $\times 200$ microscopic objective in ten randomly selected fields in two sections per animal and averaged. Criteria for being counted consisted of having diameters less than 50 μ m and including single or tiny vascular endothelial cells.

Cardiac function

Left ventricular function variables were assessed by transthoracic echocardiography, which was performed six weeks after cell transplantation using 7.5-MHz phased array transducer (Acuson Sequoia 256) at time endpoints of each subgroup [8]. Left ventricular internal dimensions (LVID) were measured as: shortening fraction — $FS (\%) = [(LVIDD - LVIDS)/LVIDD] \times 100$, ejection fraction — $EF (\%) = (EDV - ESV)/EDV \times 100\%$, where D stands for diastole, S for systole and EDV and ESV stand for end-diastolic and end-systolic LVID, respectively. All measurements were averaged for three consecutive cardiac cycles and were carried out by three experienced technicians who were unaware of the identity of the experimental groups.

The study was approved by the local bioethical committee and all patients gave their informed consent.

Statistical analysis

Data was expressed as mean \pm standard deviation. Statistical analysis was performed using SPSS software (Release 11.0, SPSS Inc.). The difference between two groups was analyzed by the Student's *t*-test. A value of $p < 0.05$ was considered as statistical significance.

Results

MSCs infection and selection

Transfecting MSCs using a traditional chemical method yielded few results, so we took advantage of lentivirus which declaimed tiny cell toxic and integrated introduction pattern. Furthermore, an off-on-off expression system was used in this study. Lenti-rtTA/neo infected cells were selected with 0.5 g/mL neomycin (2 mg/mL) addition. Non-treat-

ed MSCs were severely necrosed after 72 h, while most infected cells survived. Following treatment with Lenti-miR126-eGFP/puro introduced puromycin resistance into the cells, and controls were killed by 2 µg/L antibiotic. Next, expressions of miR-126 and green fluorescent protein (GFP) were intrigued, and doxycyclin was added to the medium. 24 h later, the test group showed strong GFP expression, which indicated that the off-on-off expression system functioned.

qPCR test of miR-126

To study the influence of miRNA on MSCs differentiation, a ten times higher expression level is necessary. The TRE promoter induced by doxycycline was proved to be a strong cis-element in miRNA expression. qPCR test verified that an approximately 16 times higher expression was obtained (Fig. 1). Primers for 5p of mature sequence was designed and all results were normalized by U6 expression in both doxycycline treated and control cells.

Western blot analysis

miRNAs post-transcriptionally regulate protein expression. Thus Western blot analysis is crucial validation before function analysis. We examined ERK₁, pERK₁, AKT and pAKT gene, which play key roles in neoangiogenesis pathways. Results showed that under higher expression of miR-126, ERK₁ and pERK₁, AKT and pAKT were dramatically increased (Fig. 2). The cells were harvested five days after infection, by when considerable miR-126 had accumulated.

Six weeks after cell transplantation, there were low protein levels of ERK₁ and pERK₁, AKT and pAKT in the PBS group's mice hearts. However, overall ERK₁ and pERK₁, AKT and pAKT protein level was significantly higher in the miR-126-MSCs group's hearts than in the PBS group's mice hearts ($p < 0.01$; Fig. 3). Moreover, there was also a higher level of expression of ERK₁ and pERK₁, AKT and pAKT in the miR-126-MSCs group's hearts compared to the MSCs group's hearts ($p < 0.01$; Fig. 3).

Capillary density

Semiquantitative analysis six weeks after transplantation *in vitro* demonstrated that the number of capillary density was significantly increased in the miR-126-MSCs group and the MSC group compared to the PBS group (Fig. 4). Moreover, the number of capillary density was significantly greater in the miR-126-MSCs group than in the MSC group ($p < 0.05$; Fig. 5).

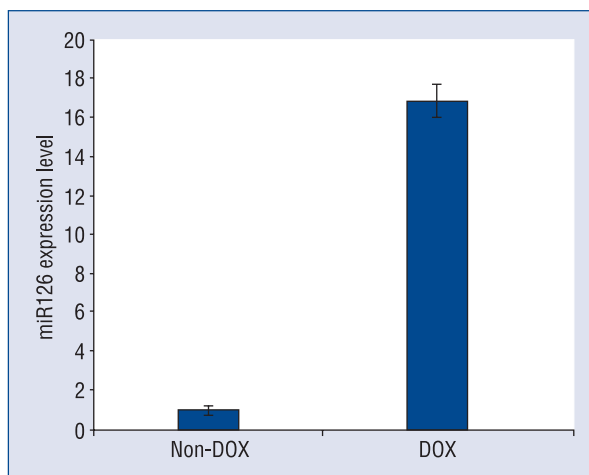


Figure 1. The miR-126 expression level of MSCs. Non-DOX indicates cells without doxycycline treatment; DOX indicates cells with doxycycline treatment. The expression level was normalized to the GAPDH RNA expression level and expressed as mean + SD ($p < 0.05$).

Cardiac function

Echocardiographic assessments of LV function are shown in Table 1. Results from infarcted C57 mice in the PBS group showed significant reduction of cardiac function in comparison with the miR-126-MSCs and MSCs group. In comparison with the PBS group, EF and FS in the MSCs group were improved significantly, by 25% and 24%, respectively ($p < 0.01$). EF and FS in the miR-126-MSCs group were 13% and 11% higher than those in the PBS group ($p < 0.05$). In comparison with the MSCs group, EF and FS in the miR-126-MSCs group were improved significantly, by 12% and 13%, respectively ($p < 0.05$).

Discussion

Previous studies have suggested a dramatic improvement in cardiac function after acute MI through regeneration of the myocardium or neovascularization by transfer of cells derived from bone marrow (BMC) generated clinical studies [9]. Moreover, growing evidence demonstrates that transplantation of stem cells into the ischemic myocardium with reperfusion is safe and effective.

However, in many studies, as has been shown in the case of MSC transplantation, the number of newly differentiated myocytes is too small to account for all the functional benefits observed with cell therapy. In addition to the quick loss of cells within 24 h of transplantation caused by cell leak-

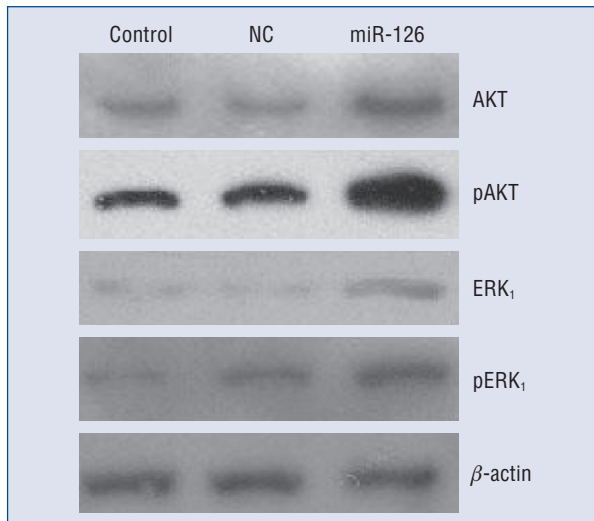


Figure 2. Western blot analysis of MSCs infected by lentivirus; NC — negative control, without doxycycline treatment; microRNA126 — double infected, with doxycycline treatment. Results revealed that under higher expression of miR-126, ERK and pERK, AKT and pAKT were dramatically increased ($p < 0.01$).

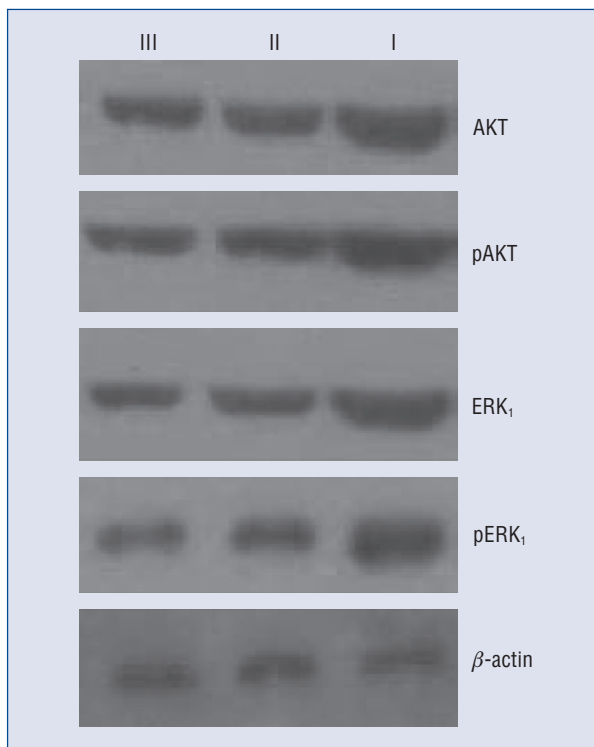


Figure 3. Western blot analysis of hearts; I — miR-126-MSCs group; II — MSCs group; III — PBS group. Overall ERK₁ and pERK₁, AKT and pAKT protein level was significantly higher in the miR-126-MSCs group hearts than in the PBS groups and the MSCs group mice hearts ($p < 0.01$).

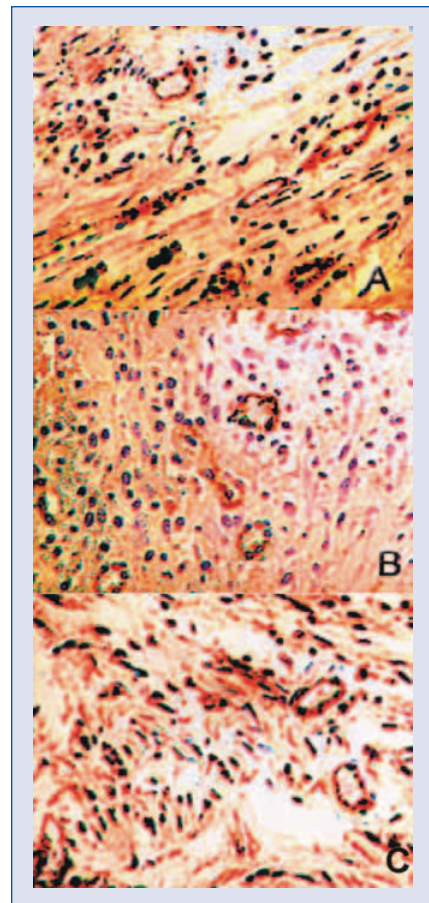


Figure 4. Factor VIII-related antigen immunohistochemical staining; the positive stain was identified by DAB which displayed endothelial cells as brown-red. A varying increase in the number of vessels was shown in the heart infarcted zone of the miR-126-MSCs (A) and MSCs (B) groups, while less growth was observed in the PBS (C) group (200 \times).

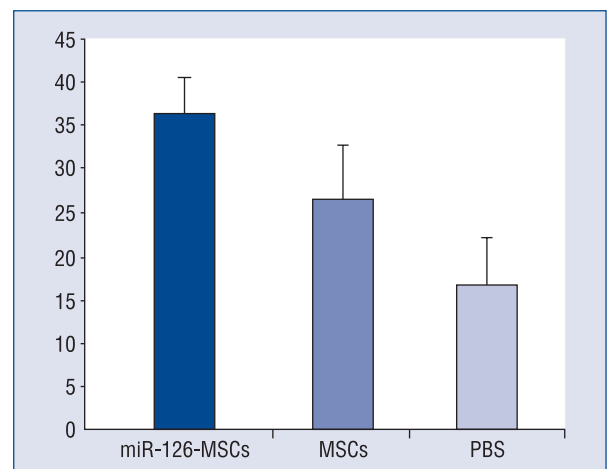


Figure 5. The miR-126-MSCs group had the greatest capillary density of all the groups, significantly greater than that of the MSCs and PBS groups ($p < 0.05$ for all).

Table 1. Cardiac function assessed by echocardiography ($X \pm S$, $n = 15$).

Cardiac function (42 days after cell transplantation)	miR-126-MSCs group	MSCs group	PBS group
Ejection fraction (%)	$87 \pm 8^{\#}$	$74 \pm 4^{\#}$	62 ± 12
Shortening fraction (%)	$56 \pm 7^{\#}$	$42 \pm 3^{\#}$	31 ± 8

* $p < 0.01$, and $^{\#}p < 0.05$ vs the PBS group; $^{\#}p < 0.05$ vs the MSCs group; PBS group, MSCs group and miR-126-MSCs group stand for the groups by injection of PBS, MSCs and miR-126-MSCs, respectively

age into the extra myocardial space, or being flushed out in the coronary vein, the major obstacle in the clinical application of stem cell-based therapy is the poor viability of the transplanted cells due to harsh microenvironments like ischemia in the infarcted myocardium.

The formation of new blood vessels through neo-angiogenesis is essential for cardiac repair following MI, when collateral vessels form at the site of the infarct and maintain blood flow to ischemic tissue [10]. MSCs may differentiate into vascular endothelium and incorporate into growing capillaries or promote angiogenesis, mitigating the adverse effects of ventricular remodeling through paracrine a series of angiogenic cytokines, including vascular endothelial growth factor (VEGF) [11]. Although MSCs are capable of producing a great variety of cytokines, including VEGF and HGF, their post-transplantation state may not support high metabolic activity.

MiR-126 is an endothelial cell-specific miRNA that plays an essential role in neoangiogenesis following MI and in maintenance of vascular integrity [12]. MiR-126 represses the expression of SPRED1 and PIK3R2, which negatively regulate VEGF signaling via the ERK and AKT pathways, respectively. VEGF, a highly specific mitogen for vascular endothelial cells, induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and plays a pivotal role in the regulation of vasculogenesis [13]. And the expression of the VEGF gene is restrictedly controlled by a hypoxia-inducible factor [14]. Thus, in the absence of miR-126, Spred-1/PI3k expression is elevated, resulting in repression of angiogenic signaling. Conversely, miR-126 overexpression relieves the repressive influence of Spred-1/PI3k on the signaling pathways activated by VEGF and FGF, favoring angiogenesis. A subset of miR-126-null mice dies during embryogenesis from vascular leakage, and those mutant mice that survive to adulthood are prone to cardiac rupture and lethality following MI, with defective neovascularization of the infarct [15]. Knockdown of miR-126 in zebrafish obtained a similar result.

These findings illustrate that miRNA can regulate vascular integrity and angiogenesis, improving blood flow to ischemic tissue for enhancing viability of the transplanted cells.

ERK and Akt are well known kinases that activate and promote cell proliferation by stimulating growth factors. In endothelial cells, VEGF promotes angiogenesis through the phosphorylation of ERK₁ and Akt in rabbit ischemic limb [16] and mouse ischemic hind limb [17]. Mangi et al. [18] demonstrated that transplanted Akt (pro-survival) modified MSCs could prevent remodeling and restore performance of infarcted hearts. We examined ERK₁, pERK₁, AKT and pAKT gene, which play key roles in neoangiogenesis pathways. Results revealed that under higher expression of miR-126, ERK₁ and pERK₁, AKT and pAKT were dramatically increased (Fig. 3). Moreover, overall ERK₁ and pERK₁, AKT and pAKT protein level was significantly higher in the miR-126-MSCs group's hearts than in the PBS group's or the MSCs group's mice hearts ($p < 0.01$; Fig 4). Therefore, ERK₁ and AKT were administered to improve the survival environment of MSCs and to achieve maximum functional benefits of MSCs.

In this study, we validated the efficacy and safety of MSC transplantation after MI in a mouse model. We characterized the mouse bone marrow-derived MSCs by immunophenotyping and confirmed that a population of MSCs can be obtained from a MSC fraction of mouse bone marrow by serial passage of adherent cells, as described by other investigators [19, 20]. We demonstrated that MSCs could be transfected with lentiviral vectors carrying miR-126 with high efficiency and without any adverse effect on cell viability. After transplantation of these cells, we also found that MSCs overexpressing miR-126 survived a long time and effectively expressed miR-126 for at least six weeks at the injected area.

We showed that the intra-myocardial injection of MSCs overexpressing miR-126 into ischemic myocardium significantly enhanced the vessel density of ischemic tissue after one month in the acute MI mouse model (Figs. 4A–C). In the *in vivo* experiment of the present study, the group receiving

MSCs that over-expressed miR-126 demonstrated a significantly greater angiogenic and arteriogenic capacity than the group transplanted with untreated MSCs.

The major conclusion of this study is that engraftment of MiR-126 overexpressing MSCs in the infarcted myocardium is effective for successful angiomyogenesis in the infarcted myocardium. In tandem with the continuous improvement of angiogenesis, MiR-126 overexpressing MSCs could sustain enhancement of cardiac function in the ischemic myocardium. In our study, at two weeks after cell transplantation, the cardiac function in the miR-126-MSCs group gradually recovered, whereas the PBS group demonstrated such effects (Table 1). In a parallel time frame with the observed enhancement of cardiac function in the miR-126-MSCs group, the amount of angiogenesis gradually increased. This suggests that angiogenesis played an important role in the enhancement of cardiac function. The increased vessel permeability is in favor of MSCs optimizing the arrangement of extracellular matrix associating with better support functions [21, 22], and thus better global heart performance. Neovascularization in the infarcted myocardium facilitates the survival and transdifferentiation of implanted stem cells. Therefore, it is reasonable to suggest that miRNA126 is a novel but practical regulator of stem cell implantation for IHD, and introduces a promising strategy for the treatment of MI.

Conclusions

In summary, our study suggests that MSCs overexpressing miR-126 transplantation stimulated vasculogenesis effectively via the AKT/ERK-related pathway. For these reasons, this combined strategy of cell transplantation with MiR-126 therapy should prove to be a useful approach in the treatment of IHD.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (No 30871053).

The authors do not report any conflict of interest regarding this work.

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