Endothelial growth medium suppresses apoptosis of mesenchymal stem cells in vitro via decrease of miR-29a

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Abstract. The administration of mesenchymal stem cells (MSCs) in cases of cardiac ischemia/reperfusion injury (IRI) has been associated with a significant reduction of myocardial cell death and an effective improvement in cardiac function. However, one major limiting factor in MSCs transplantation therapy is the low survival rate of the transplanted cells. The present study aimed to demonstrate that human amnion-derived mesenchymal stem cells (hAMSCs) cultured with endothelial growth medium (EGM-2) exhibited reduced apoptosis when exposed to serum-free and hypoxic conditions; and that the expression of microRNA (miR)-29a decreased significantly. Furthermore, miR-29a knockdown resulted in decreased apoptosis of hAMSCs and increased myeloid cell leukemia (MCL)-1 at the mRNA and protein levels. These results suggested that EGM-2 promoted survival of hAMSCs partly through the regulation of miR-29a and MCL-1 expression levels. These findings may provide a novel understanding of a potential effective therapeutic strategy for cardiac IRI.

Introduction

Mesenchymal stem cells (MSCs) represent a heterogeneous population of fibroblast-like multipotent cells which may differentiate into various mesodermal lineages and may be isolated from a number of tissues, including bone marrow, umbilical cord, amniotic membrane, or adipose tissue (1). Cardiac ischemia following acute myocardial infarction leads to impaired cardiac function and is associated with increased morbidity and mortality. MSCs administration in cases of cardiac ischemia/reperfusion injury (IRI) has been associated with a significant reduction of myocardial cell death and an effective improvement in cardiac function (2,3). However, a randomized control trial (4) and meta-analyses (5,6) have demonstrated that improvement of cardiac function resulting from MSCs transplantation is limited. One major limiting factor in MSCs transplantation therapy is the low survival rate of the transplanted cells (7,8). This is thought to be due to local inflammatory reactions, ischemia and hypoxia (9). Previous studies have focused on improving the survival of MSCs through various methods, including the use of genetically engineered MSCs, coupled with suitable tissue engineering materials, or preconditioning with optimal culture conditions (10-13).

miRNAs (miRs) are small (20-22 nucleotides long) noncoding RNAs that suppress protein translation by binding to target mRNAs, reducing their stability and/or inhibiting translation. Growing evidence indicates that miRs are involved in the regulation of cell survival, proliferation and migration, through mediating the expression of their target genes (14). Several miRs, including miR-29, miR-34a and miR-133, are involved in pathways modulating cellular apoptosis (15-17). However, the exact role of miRs in the survival of MSCs, in addition to the associated underlying mechanisms, remains to be elucidated.

Accordingly, the present study was designed to improve the survival of MSCs by cell culture. Specifically, it identified the expression of miR-29a in human amnion-derived (hA)MSCs cultured in different culture media. In addition, it also identified novel target genes of miR-29a and explored the underlying mechanisms associated with MSCs survival.

Materials and methods

Cell culture. Human amnion-derived (hA)MSCs were isolated from amniotic membranes of healthy donors using enzymatic digestion as previously described (18). All donors (between May 2015 and January 2016) gave their informed consent and the Ethics Committee of the First Affiliated Hospital of China Medical University (Shenyang, China) approved the study protocol. The hAMSCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) and 100 U/ml penicillin and streptomycin (Hyclone; GE Healthcare Life Sciences).
Healthcare Life Sciences) at 37°C in an incubator with 5% CO2. For the endothelial cell culture conditions, cells were cultured in endothelial growth medium (EGM-2) with 5% FBS and endothelial cell growth supplement (ECGS; ScienCell Research Laboratories, Inc., Carlsbad, CA, USA). All the subsequent assays were performed with 7-day cultured hAMSCs.

**HAMS C immunophenotyping.** The immunophenotype of hAMSCs cultured in either hAMSCs culture conditions or EC culture conditions was analyzed using flow cytometry. A total of 1x10^6 cells were detached from culture dishes using trypsin solution (Hyclone; GE Healthcare Life Sciences) and stained with 5 µl antibodies against cluster of differentiation (CD)31 (cat. no 303105), CD34 (cat. no 343505), CD73 (cat. no 344003), CD90 (cat. no 32810) and CD105 (cat. no 323205) (all undiluted; BioLegend, Inc., San Diego, CA, USA). Immunoglobulin G of the appropriate isotype was used as negative control. Data from 10,000 viable cells were acquired. List mode files were analyzed by FCS Express Software (version 3; BD Biosciences, Franklin Lakes, NJ, USA).

**RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from 1x10^6 hAMSCs using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration was determined by a NanoDrop ND-1000 (Nano-Drop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). For miR-29a detection, poly A tail was added to RNase-free DNase digested total RNA using the poly A tailing kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. SYBR RT-qPCR was used to assay miRNA expression with the specific forward primers and the universal reverse primer complementary to the anchor primer. U6 primer was used as an miRNA internal control. For the mRNA analysis of myeloid cell leukemia (MCL-1), cDNA was synthesized using PrimeScript™ RT reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Reactions were performed using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.) with an ABI 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The PCR reactions used for the amplification of miRNAs were conducted at 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 34 sec. As an internal control, β-actin level was quantified in parallel with the target genes. Normalization and fold alters were calculated using the 2^ΔΔCq method (19). All experiments were performed in triplicate and repeated three times. The primers used for RT-qPCR were as follows: Forward, 5'-AGG ATT CCT ATG TGG GCG AC-3' and reverse, 5'-CAC AAT CCT GCC CCA GTT TG-3' for U6; forward, 5'-GAG GAG GAC GAG TTG TAC ACG CTA CGT-3' for miR-29a; forward, 5'-CGC TTC GGC CTG AAA TCG GTT A-3' and reverse, 5'-GCT GTC AAC GAT TCC CAT ACC G-3' for MCL-1. The qPCR products were confirmed by DNA sequencing. HEK293T cells (American Type Culture Collection, Manassas, VA, USA; 80% confluence) were co-transfected with 100 ng reporter constructs and 50 nM miR-29a mimic, inhibitor, or control

miR-29a overexpression and suppression. miR-29a mimic, negative control (nc), miR-29a inhibitor and inhibitor nc were purchased from Suzhou GenPharma Co. Ltd. (Suzhou, China) and the sequences were as follows: 5'-UAGCACCACUGAAUCGGGUAA-3' for miR-29a mimic; 5'-UAACCGAUUCCAGAUGGGCUA-3' for miR-29a inhibitor; 5'-UCUCGAGUGUCUGACGT-3' for NC; and 5'-CAGUACUUUUGUAGUACAA-3' for inhibitor NC. Small interfering (si)RNA targeting human MCL-1 and control siRNA were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

hAMSCs at 80% confluence were transfected with miR-29a mimic, inhibitor, MCL-1 siRNA and their corresponding controls (50 nM for each) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Following 48 h, cells were incubated with serum-free DMEM with 300 µM cobalt (II) chloride for a further 48 h and examined with Hoechst 33258 staining or flow cytometry. Hoechst 33258 staining. Following cell incubation with serum-free DMEM with 300 µM cobalt (II) chloride for 48 h, a Hoechst 33258 staining kit (Beyotime Institute of Biotechnology, Haimen, China) was used to observe the apoptotic cells induced by mimic or inhibitor of miR-29a, according to the manufacturer's protocol. Each assay was performed at least three times.

**Caspases activities assay.** Caspase 3 and 7 activities were determined using Apo-ONE® Homogeneous Caspase-3/7 Assay according to the manufacturer's protocols (cat. no G7792; Promega Corporation, Madison, WI, USA). The plate was read by a microplate reader at a wavelength of 405 nm. Activities of caspases 3 and 7 were expressed as the ratio of treated cells to corresponding controls.

**Flow cytometry.** Following 48 h incubation in serum-free DMEM with 300 µM cobalt (II) chloride, a FITC Annexin V Apoptosis Detection kit (BD Biosciences) was used to double stain cells with FITC-Annexin V and propidium iodide according to the manufacturer's protocols. A total of 2.5x10^5 cells were seeded into 6-well plates for 48 h and removed using trypsin without EDTA, stained cells were analyzed using a flow cytometer (BD Biosciences). FCS Express Software (version 3; BD Biosciences) was used to observe cell apoptosis. In the graphs, cells were distinguished as dead, living, early apoptotic and late apoptotic cells. The aggregate of early and late apoptotic cells was regarded as an observation index to compare the experimental and negative groups. Each experiment was performed at least three times.

**Luciferase reporter assay.** The wild-type (WT) or mutant (MUT) 3'-untranslated region (UTR) segment of MCL-1 containing the putative miR-29a binding site was amplified and inserted into the pLUC Luciferase vector (Ambion; Thermo Fisher Scientific, Inc.). Site-directed mutagenesis of the miR-29a binding site in the MCL-1 3'-UTR was achieved with a commercially available kit (Beyotime Institute of Biotechnology). All the plasmids were confirmed by DNA sequencing. HEK293T cells (American Type Culture Collection, Manassas, VA, USA; 80% confluence) were transfected with 100 ng reporter constructs and 50 nM miR-29a mimic, inhibitor, or control
miRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 24 h posttransfection, cells were harvested and luciferase activity was assayed using the Dual Luciferase Reporter Assay System (Promega Corporation). A Renilla luciferase construct was used as the internal control. All experiments were repeated in triplicate.

Western blot analysis. Total proteins from hAMSC were harvested in lysis buffer (Beyotime Institute of Biotechnology) and quantified using the bicinchoninic acid method. Protein lysates were separated using 10% SDS-acrylamide gels and transferred onto Protran nitrocellulose membranes (Whatman; GE Healthcare Life Sciences, Little Chalfont, UK). For immunodetection, membranes were incubated with antibodies directed against MCL-1 (1:1,000; cat. no ab32087; Abcam), CD31 (1:1,000; cat. no ab28364; Abcam), CD105 (1:1,000; cat. no ab107595; Abcam), or β-actin (1:10,000; cat. no ab8227; Abcam). Signals from horseradish-peroxidase-conjugated secondary antibodies (1:5,000; cat. no KC-RB-035; KangChen Bio-tech, Shanghai, China) were generated by enhanced chemiluminescence solution (ECL; GE Healthcare Life Sciences) and recorded on film. Quantification was performed using ImageJ software (version 1.45S; National institutes of Health, Bethesda, MD, USA). Experiments were repeated in triplicate.

Statistical analysis and bioinformatics. TargetScan (www.targetscan.org), PicTar (pictar.mdc-berlin.de) and miRanda (www.microrna.org) were used to predict the target genes of miR-29a. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation and were analyzed by a Student's t-test or one-way analysis of variance followed by Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Phenotype characterization of hAMSCs is variable depending on growth medium. hAMSCs cultured in DMEM supplemented with 10% FBS revealed a spindle fibroblast-like morphology, whereas those cultured in EGM-2 exhibited a cobblestone-like morphology (Fig. 1A). Flow cytometry revealed the expression of surface markers. hAMSCs cultured in the two types of medium were positive for CD73, CD90 and CD105, but were negative for CD31 and CD34 (Fig. 1B). Western blotting revealed that hAMSCs cultured in the two types of medium were positive for CD105 expression, and negative for CD31 expression (Fig. 1C).

EGM-2 culture decreases apoptosis of hAMSCs and miR-29a expression. Flow cytometry results demonstrated that the apoptosis of hAMSCs was significantly decreased in hAMSCs cultured in EGM-2 compared with those cultured in DMEM (21.5±3.1%; Fig. 1D). Fig. 1E indicated that miR-29a expression was decreased in hAMSCs cultured in EGM-2 compared with hAMSCs cultured in DMEM (~2-fold change).

Suppression of miR-29a decreases hAMSCs apoptosis. To evaluate the ability of miR-29a to control hAMSCs apoptotic activity, these cells were transfected by miR-29a mimic, inhibitor or their corresponding controls and incubated with serum-free DMEM with 300 µM cobalt (II) chloride to undergo apoptosis. hAMSCs at 80% confluence were transfected with miR-29a inhibitor (50 and 100 nM) and 50 nM inhibitor successfully suppressed the expression of miR-29a, so 50 nM concentrations were selected for use in the present study (Fig. 2A). Following annexin-V/PI assays, miR-29a-transfected hAMSCs presented an apoptotic cell rate of 18.4±2.3% compared with hAMSCs transfected by the scramble oligos (12.7±1.3%). Meanwhile, knocking-out miR-29a by transfection with inhibitor suppressed the
apoptosis of hAMSCs (9.1±1.8%; Fig. 2B. The observations were confirmed by Hoechst 33258 staining, demonstrating a suppression of apoptosis in hAMSCs transfected with miR-29a inhibitor compared with the negative control (Fig. 2C). These observations were confirmed by caspase 3/7 activities measurements, demonstrating an apoptosis suppression of hAMSCs transfected with inhibitor (~2-fold) compared with the negative control (Fig. 2D).
MCL-1 is a target of miR-29a in hAMSCs. The expression of experimentally validated targets for miR-29a in hAMSCs were additionally examined. Potential targets of miR-29a were identified based on bioinformatics prediction. Through preliminary function screening, an anti-apoptotic protein, MCL-1, was selected to further study its interaction with miR-29a and its role in hAMSCs apoptosis. First, the basal level of MCL-1 mRNA in different culture mediums was observed by RT-qPCR. As presented in Fig. 3A, a significantly high level of MCL-1 mRNA was detected in the hAMSCs cultured in EGM-2 compared with DMEM (P<0.05). Similar results were obtained with western blotting analysis for MCL-1 protein expression (Fig. 3B). To ascertain if MCL-1 may be targeted by miR-29a, a gain-of-function experiment was performed with miR-29a in hAMSCs. MiR-29a or negative control was transiently transfected and endogenous MCL-1 expression was detected by western blotting. Densitometric expression was detected by western blotting. Densitometric analysis (13.2±1.4%) reversed the function of miR-29a inhibitor (9.7±1.6%) on cell apoptosis. The observations were confirmed by Hoechst 33258 staining (Fig. 4D) and Caspase 3/7 activity assay (Fig. 4E) demonstrating an apoptosis reduction with miR-29a inhibitor compared with controls. Taking these findings together, it is suggested that increased miR-29a results in increased levels of apoptosis via targeting of MCL-1 (decreasing its levels).

Discussion

The therapeutic potential of MSCs for cardiac repair is limited, partly due to their low survival rate within the ischemic myocardial microenvironment into which they are introduced (8,9). Hypoxia and serum deprivation, imitating the ischemic engraftment environment, induce the apoptosis of MSCs (20). To improve cardiovascular cell therapy, continuous efforts have been made to improve their function. The present study demonstrated that hAMSCs cultured in EGM-2 conferred a protective effect against serum-free and hypoxic conditions. In the specific culture condition, hAMSCs expressed a lower level of miR-29a compared with the normal conditions. This downregulation contributed in part to the overexpression of MCL-1, the primary anti-apoptotic B-cell lymphoma 2 (Bcl-2) family member and thus, to the hAMSCs survival.

It was demonstrated that hAMSCs cultured in EGM-2 conferred a protective effect against serum-free and hypoxic conditions, making them suitable for cell therapy. In endothelial culture conditions, hAMSCs exhibited a cobblestone-like morphology, yet expressed similar levels of surface makers...
compared with hAMSCs cultured in DMEM. The present study is consistent with previous studies demonstrating that EGM-2 mediated the morphological alterations of hAMSCs. However, hAMSCs did not express the mature endothelial cell markers, von Willebrand factor and vascular endothelial-cadherin, and hAMSCs resisted undergoing a complete differentiation into mature endothelial cells (21, 22). Therefore, hAMSCs cultured in EGM-2 medium still possess stem cell characteristics and are unable to differentiate into endothelial cells in the EGM-2 medium. Using RT-qPCR, a significantly lower expression of miR-29a in EGM-2 was observed compared with cells cultured in DMEM. The present study is the first, to the authors' knowledge, to demonstrate the difference in expression of miR-29a in hAMSCs cultured with EGM-2. Therefore miR-29a was selected for the current study. Notably, miR-29a-inhibitor mediated protective effects on the apoptosis of hAMSCs induced by serum starvation and hypoxia. Results from the current study demonstrated that endogenous MCL-1 protein expression was markedly decreased in hAMSCs transfected with miR-29a.

It was demonstrated that MCL-1 is regulated at the posttranscriptional level by miR-29a in hAMSCs, thus suggesting a survival advantage in the silencing of miR-29a. MCL-1, in absence of Bcl-2, promotes cell survival by inhibiting cell death (23, 24). It is known that miR-29 acts directly at the MCL-1 3'UTR. Mott et al. (25) were the first to demonstrate in a cholangiocarcinoma cell model that MCL-1 may additionally be regulated at the posttranscriptional level by miR-29b. The results of the present study are in accordance with those reported by Mott et al. (25), Garzon et al. (26) and Xiong et al. (27), they identified that miR-29a, another member of the miR-29 family, contributed to the downregulation of MCL-1 in hAMSCs. It was observed in the present study that
targeted knockdown of MCL-1 by specific siRNA evidently inhibited the protective effect of miR-29a-inhibitor on hypoxia- and serum deprivation-induced apoptosis of hAMSCs.

However, there are studies suggesting that loss of miR-29 results in cell death. In diabetic nephropathy, increasing miR-29a action may protect against diabetic podocytopathy (28). In addition, in vivo evidence demonstrated the neuronal cell death of brain-specific knockdown of miR-29 (29). It therefore remains to be elucidated whether the apoptotic action of miR-29a is dependent on cell types or different culture conditions.

In conclusion, the present study demonstrated that hAMSCs experienced decreased apoptosis when cultured in EGM-2, partly through low expression of miR-29a, and this may provide a novel tool to improve stem cell therapy in the future.

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