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Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury

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ABSTRACT

Background: Cardiac progenitors (CPC) mediate cardioprotection via paracrine effects. To date, most of studies focused on secreted paracrine proteins. Here we investigated the CPC-derived-exosomes on protecting myocardium from acute ischemia/reperfusion (MI/R) injury.
Methods and results: CPC were isolated from mouse heart using two-step protocol. Exosomes were purified from conditional medium, and confirmed by electron micrograph and Western blot using CD63 as a marker. qRT-PCR shows that CPC-exosomes have high level expression of GATA4-responsive-miR-451. Exosomes were *ex vivo* labeled with PKH26, We observed exosomes can be uptaken by H9C2 cardiomyoblasts with high efficiency after 12 h incubation. CPC-exosomes protect H9C2 from oxidative stress by inhibiting caspase 3/7 activation *in vitro*. *In vivo* delivery of CPC-exosomes in an acute mouse myocardial ischemia/reperfusion model inhibited cardiomyocyte apoptosis by about 53% in comparison with PBS control ($p < 0.05$).
Conclusion: Our results suggest, for the first time, the CPC-exosomes can be used as a therapeutic vehicle for cardioprotection, and highlights a new perspective for using non-cell exosomes for cardiac disease.

1. Introduction

Acute myocardial ischemia/reperfusion (MI/R) injury leads to cardiomyocyte apoptosis and death. Cardiac progenitor cells (CPC) derived from adult heart have emerged as one of the most promising stem cell types for cardioprotection and repair [1–5]. The therapeutic effect of CPC can be attributed to both cardiovascular lineage differentiation and paracrine effects [6–8].

Stem cells can secret exosomes, the 40–100 nm size membrane vesicles containing microRNA (miRNA) [9,10]. miRNA are evolutionarily conserved, 18–25 nucleotide, nonprotein coding transcripts that control gene expression via post-transcriptional repression [11,12]. In stem cells, miRNAs play significant roles in modulating differentiation [13], proliferation [14], and apoptosis [15]. Increasing evidence suggested that exosomes play an important role for miRNA communication between donor cells and recipient tissues [16,17], and exosomes mediated paracrine effect might be a novel mechanism for beneficial effect of CPC transplantation.

Exosomes from bone marrow derived mesenchymal stem cell (MSC) have been reported to reduce myocardial ischemia/reperfusion injury *ex vivo* [18], CPC express high level of early cardiac specific transcription factors, such as GATA4, Mef2c, Isl1, etc. [19–21], however, there is no report about whether exosomes secreted by CPC can protect myocardium from acute myocardial ischemia/reperfusion injury.

In this study, we show that the administration of purified CPC-exosomes can efficiently protects cardiomyocytes from oxidative induced apoptosis *in vitro* and acute myocardial apoptosis in mouse models of acute MI/R.

2. Methods

All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of University of Cincinnati.

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2.1. Isolation of cardiosphere derived cells

Cardiac progenitors were generated from the hearts of 2-month-old, male, C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) isolated via a 2-step procedure as described previously [5,22]. Briefly, in step 1, cardiac explants were minced and cultured for 2–3 weeks until the small, round, phase-bright cells migrated from the adherent explants and proliferated over a fibroblast layer. In step 2, Sca-1+ cells were isolated from the phase-bright cells through magnetic-activated cell sorting (MACS) with Sca-1 magnetic beads (Miltenyi Biotec Inc., Auburn, CA) as instructed by the manufacturers' protocols. The selected Sca-1 cells were cultured and maintained in complete media containing DMEM/F12, 10% exosomes-removed fetal calf serum (Exo-FBS, System Biosciences (SBI), Mountain View, CA), 200 mM L-glutamine, 55 nM ß-mercaptoethanol, and 1% MEM nonessential amino acids.

2.2. Exosome purification

The CPC-exosome isolation procedures were performed at 4 °C as described in the literatures with modification [23]. Briefly, 10 mL culture medium (CM) with 2% exosome-removed FBS was used for culturing 5×10^5 CPC in 10 cm dish. After 48 h, supernatant was centrifuged at 1000 rpm for 10 min to eliminate cells, followed by filtration through 0.22 μm filter to remove cell debris. Exosomes in medium were precipitated with 1/3 volume of polyethylene glycol (PEG) buffer (33.4% PEG 4000, 50 mM HEPES (pH 7.4), 1 M NaCl) overnight at 4 °C followed by centrifugation at 3000 rpm for 30 min and resuspension in 50 μL of PBS and stored at −80 °C.

2.3. Electron microscopy

For the transmission electron microscopy (TEM) morphology investigation, 3 μL of exosome pellet was placed on formvar carbon-coated 200-mesh copper electron microscopy grids, and incubated for 5 min at room temperature (RT), and then was subjected to standard uranyl acetate staining [24]. The grid was washed with three changes of PBS and allowed to semi-dry at room temperature before observation in transmission electron microscope (Hitachi H7500 TEM, Tokyo, Japan). Micrographs were used to quantify the diameter of exosomes.

2.4. Isolation and quantification of microRNAs

Total RNA from CPC or isolated CPC-exosomes were extracted by RNAzol RT (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer's instructions. Isolated RNAs were polyadenylated using the Ncode miRNA first-strand cDNA synthesis kit (Invitrogen). The cDNA synthesized was used to perform quantitative PCR on an Mx3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) using the SensiMix SYBR kit (Bioline, Tauton, MA). Amplification was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. For mature miRNA expression: the universal primer provided in the NCodeTM miRNA first-strand cDNA synthesis kit was used together with one of the following forward primer:

Mmu-miR-144: 5′ TAC AGT ATA GAT GAT GTA CT 3′

Mmu-miR-451: 5′ AAA CCG TTA CCA TTA CTG AGT T 3′

Mmu-miR-U6: 5′ ACA CGC AAA TTC GTG AAG C 3′

Fold-induction was calculated using the Ct method: $\Delta\Delta Ct = (Ct_{Target\ miRNA} - Ct_{U6})$ exosomes $- (Ct_{Target\ miRNA} - Ct_{U6})$ CPC, and the final data were derived from $2^{-\Delta\Delta Ct}$.

2.5. Western blotting

Exosome lysate supernatants were prepared, exosomes were assessed for their protein content using BCA Protein Assay Kit (Pierce, Rockford, IL), and then resolved on a 10% sodium dodecyl sulfate bis–tris gel, and transferred to an Immobilon® FL PVDF membrane (Millipore, Billerica, MA). For Odyssey technology, the membrane was blocked with Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE) and probed with rabbit anti-CD63 (1:250, SC-15363, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then, IRDye 680 goat anti-rabbit IgG at 1:5000 (LICOR Biosciences). Probed blots were scanned using an Odyssey infrared imager.

2.6. H9C2 treat with CPC exosomes and caspase assay

H9C2 cardiomyoblasts were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. H9C2 cells were pre-incubated with 2% Exo-FBS DMEM with or without CPC-exosomes for 24 h, then incubated with 200 μM H_2O_2 for 4 h. Following treatment, activity of CASPASE-3 and -7 in H9C2 was assayed with Caspase-Glo 3/7 Assay System (Promega, Madison) according to the instructions of the manufacturer.

2.7. Exosome Labeling with PKH26

Purified CPC-exosomes were labeled with PKH26 red fluorescent labeling kit (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions. The PKH26 concentration for exosome labeling is 2×10^{-6} M per exosomes from 5×10^5 cells. The labeled exosomes were stained with PKH26 dye in 400 μL Diluent C fluid supplied with the kit for 5 min at room temperature, an equal volume of serum was added to stop the labeling, the exosomes were re-purified via PEG precipitation. The labeled CPC-exosomes were incubated with H9C2 for 12 h at 37 °C, washed with PBS, the uptake of labeled exosomes by H9C2 was determined using an inverted fluorescence microscope.

2.8. Murine myocardial ischemia–reperfusion model

The mice were subjected to myocardial ischemia/reperfusion as described previously [25]. Briefly, male C57BL/6 mice weighing 25–30 g were anesthetized with intraperitoneal 100 mg/kg ketamine combined with 10 mg/kg xylazine. Mice were intubated transorally with a 24-gauge tube, and ventilated with oxygen-enriched room air using a Harvard rodent ventilator (Inspira Advenced Safety Ventilator Model 55-7058, Holliston, MA). The chest was opened via a lateral thoracotomy, and the heart was exposed through pericardiotomy. An 8-0 nylon suture (Ethicon, Somerville, NJ) was placed under the left anterior descending artery (LAD) and then threaded through a small plastic (PE10) tube to form a snare for reversible left coronary artery occlusion. The left coronary artery was occluded for 45 min, and then reperfusion was effected by gently removing the tube. The chest was closed and the mice allowed recovering. Animals were sacrificed at 24 h after reperfusion histology assay.

2.9. Intramyocardial delivery of CPC-exosomes into hearts

Myocardium exosome delivery was performed on mice immediately after induction of ischemia by LAD ligation. The mice were assigned to two groups based upon the injection ($n = 6$ per group):

1. *PBS group*: intramyocardial delivery of 25 μL PBS with MIR.
2. *CPC-exosomes group*: intramyocardial delivery of 25 μL exosomes from 5×10^5 CPC with MIR.

2.10. Histology

To quantify apoptotic cardiomyocytes, mouse hearts were removed 24 h after myocardial reperfusion, fixed with 30% sucrose, and routinely frozen embedded in OCT and processed for sectioning and staining with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and cardiac Troponin I (cTnI) (1:50; sc-15368, Santa Cruz Biotechnology, Santa Cruz, CA). TUNEL was performed using The DeadEnd™ Fluorometric TUNEL System (Promega) per the manufacturer's protocol. The slides were incubated with Alexa 555-conjugated anti-rabbit secondary antibodies (Invitrogen). Slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories). The staining was analyzed by a Zeiss 710 Laser Scanning Microscope (Carl Zeiss, Thornwood, NY). Apoptotic cardiomyocytes was quantified and classified as a TUNEL-positive cTnI-positive in a given field (×20).

2.11. Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). Student's *t*-test was used to compare two groups. A value of $p < 0.05$ was considered to indicate statistically significant differences.

3. Results

3.1. Characterization of CPC-exosomes isolated by PEG precipitation

CPC were obtained with a two-step procedure: cardiospheres were grown from enzymatically-digested minced mouse adult heart and expanded, then the CPC cells were isolated by using a hematopoietic lineage-depletion cocktail followed by enrichment for Sca-1+ cells via MACS sorting (Fig. 1A). RT-PCR shows that CPC over express cardiac transcription factor, GATA4 while bone marrow derived mesenchymal stem cell (BM-MSC) do not express GATA4 (Fig. 1B) (Supplemental Table 1). To character the CPC-exosomes, we first monitored the release of exosomes in conditioned media of mouse CPC. Exosomes were readily detectable in conditioned media from CPC after PEG precipitation (Fig. 1C). Morphological analysis of the CPC-exosomes using electron micrography revealed a heterogeneous population of vesicles comprising both round-shaped 40–100 nm diameter vesicles (Fig. 1D), Western blot analysis of CPC-exosomes revealed the presence of exosome marker CD63 (Fig. 1E).

3.2. Exosome labeling and uptake of exosomes by H9C2

To determine whether CPC-exosomes can be taken up by cardiomyocytes, we labeled CPC-exosomes with PKH26, a fluorescent cell linker compound that is incorporated into the cell membrane by selective partitioning. After labeling, the exosome pellet show strong red color. When we incubated exosomes labeled with PKH26 with H9C2, we observed a red fluorescence in the cytoplasm in almost every H9C2 (Fig. 2A), indicating that significant amounts of exosomes were taken up by the H9C2.

3.3. Effects of CPC-exosomes on H2O2-induced apoptosis in H9C2 cells

In order to determine the effects of exosomes on oxidative stress-induced myocyte apoptosis, the H9C2 cells were pretreated with exosomes or PBS in medium with 2% Exo-FBS for 24 h, and then coincubated with 200 μM of H2O2 for additional 4 h. Caspases-3/7 are executioner for the death program in many cells in response to oxidative stress, such as H2O2. Thus we examined the effect of CPC-exosomes on H2O2-stimulated caspase-3/7 activation, we compared Caspase 3/7 activity in H2O2-treated cells with or without CPC-exosomes. As shown in Fig. 2B, CPC exosomes pretreatment significantly decreased the levels of caspase-3/7

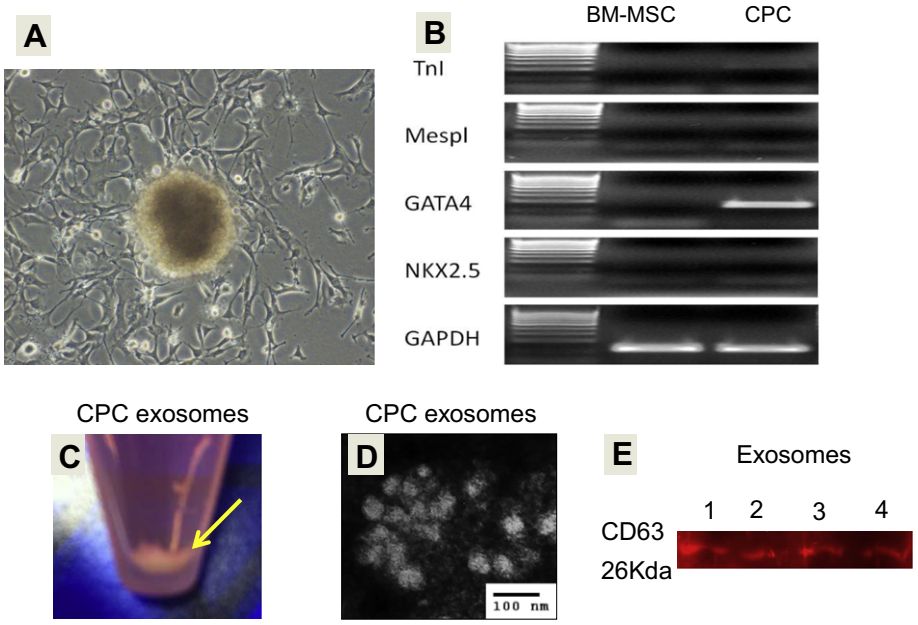

Fig. 1. Characterization of CPC and exosomes from CPC. (A) Mouse cardioprogenitor cells; (B) RT-PCR for mature cardiac marker (TnI) and early cardiac markers (Mesp1, GATA4 and Nkx2.5) in bone marrow derived mesenchymal stem cells (BM-MSC) and CPC. GATA4 mRNA was detected at high level by RT-PCR in CPC while absent in BM-MSC. (C) CPC-exosomes isolated by PEG precipitation; (D) electron micrograph image of CPC-derived exosomes. The image shows small vesicles of approximately 40–100 nm in diameter. Scale bar = 100 nm. (E) Western blot characterization of CPC-exosomes. CPC-exosome preparation was separated by SDS–PAGE, and electroblotted to the polyvinylidene fluoride (PVDF) membrane, and probed with exosome marker CD63.

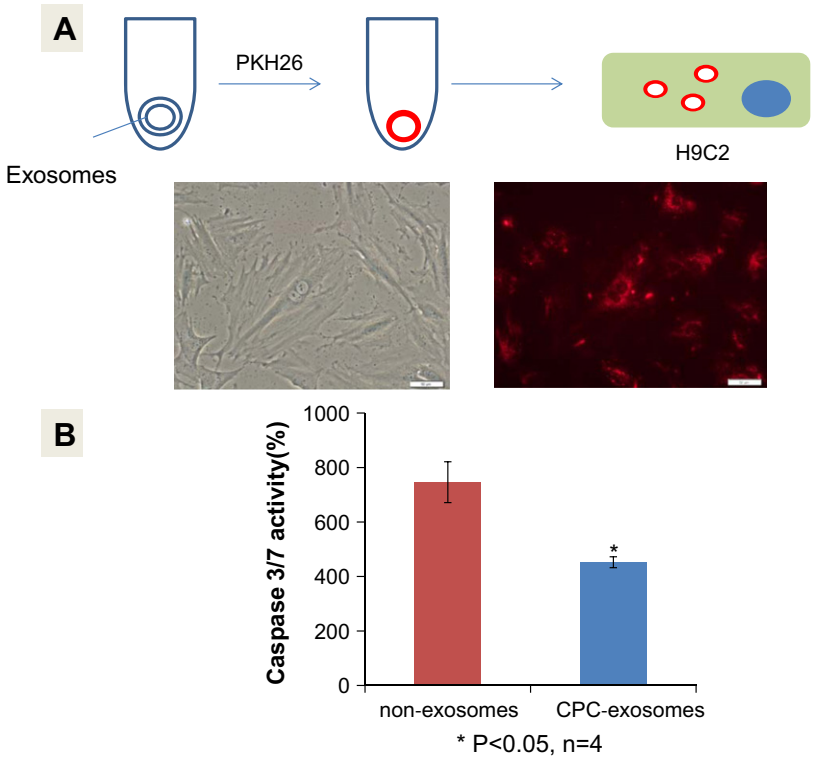

Fig. 2. Uptake of exosomes by H9C2 and CPC-exosomes protecting H9C2 from H2O2 induced apoptosis *in vitro*. (A) Uptake of PKH26-labeled-exosomes by H9C2 *in vitro*. H9C2 cells incorporated PKH26 labeled-exosomes from CPC after 12 h incubation. Red dots indicate CPC-exosomes incorporated in H9C2 cells. (B) CPC-exosomes protected H9C2 from H2O2 induced apoptosis *in vitro*. Caspase-3/7 activity assay was used to assess the effect of protection of CPC-exosomes on H9C2 cells treated for 4 h of H2O2 (200 μm). Data shown are mean ± SEM of 4 independent experiments.

activity in H2O2-treated H9C2, indicating that CPC-exosomes attenuated H2O2-induced apoptosis.

3.4. In vivo delivery of CPC-exosomes inhibits apoptosis in a mouse acute ischemia–reperfusion model

Apoptotic signals are induced in cardiomyocytes after acute ischemia/reperfusion. To determine whether CPC-exosomes treatment could inhibit ischemia/reperfusion-induced apoptosis of cardiomyocytes *in vivo*, we combined TUNEL labeling with the cardiomyocyte marker cTnI on reperfused mouse hearts treated with CPC-exosomes or control PBS. Delivery of CPC-exosomes to myocardium with ischemia–reperfusion produced a 53% reduction of the TUNEL apoptotic cells in reperfusion myocardium compared with the PBS control hearts (13 ± 1.5% in PBS group vs 6.1 ± 0.8% in CPC-exosome group, $p < 0.05$, respectively), indicating that exosomes from CPC could obviously inhibit apoptosis induced by ischemia–reperfusion injury in myocardium (Fig. 3 A–C).

3.5. Differential miR-451 content in CPC-exosomes vs CPC

Recent studies suggest that intercellular transfer of miRNAs by exosomes is able to modulate gene expression in recipient cells. CPC are progenitor cells with high level of GATA4 expression (Fig. 1B). The miR-144/451 cluster, an important GATA4-responsive miRNA cluster, has been reported to protect ischemic cardiomyocytes from mimic ischemia/reperfusion injury *in vitro*. To evaluate whether exosomes secreted by CPC can carry GATA4-responsive, cardioprotective miRNAs, we isolated exosomes from cell supernatants of the CPC. Exosomes were isolated by a series of microfiltration and PEG precipitation steps. We quantified relative levels of a miRNA-144/451 in CPC-derived exosomes and CPC. We found that miR-451 was significantly enriched in CPC-derived exosomes vs CPC, in contrast, miR-144 was more highly represented in CPC than in CPC-exosomes (Fig. 4), suggesting a distinct microRNA enrichment character of CPC-derived exosomes.

4. Discussion

This is the first observation that exosomes derived from CPC can protect cardiomyocytes from oxidative stress *in vitro* and ischemia–reperfusion injury *in vivo*. In our experiment, we found that PKH26, a fluorescent cell membrane linker compound, can be used to label exosomes, and cardiomyocytes can takeup exosomes at high efficiency. We also found that miR-451, one of the GATA4-responsive miRNA in miR-144/451 cluster, is enriched in CPC-exosomes while miR-144 is absent in CPC-exosomes. CPC-exosomes might provide a promising non-cellular approach for cardioprotection in patients with high risk of ischemic attack.

We have reported that CPC transplantation ameliorates the deleterious consequences of myocardial ischemia [5]. We found that transplantation of stem cells into ischemic myocardium decreased apoptosis [26–28]. Some of these beneficial effects can be better explained by a paracrine mechanism than by stem cell differentiation [28]. We have previously performed an unbiased antibody cytokine array analysis, and observed that CPC secreted higher levels of numerous factors, including chemokines (e.g., TCA-3, SDF-1, 6Ckine), vascular growth factors (e.g., VEGF, osteopontin, bFGF, erythropoietin, stem-cell factor), and factors involved in cardiac differentiation (e.g., Activin A, TGF-β, and Dickkopf homolog-1) that could potentially repair injured cardiac tissue mainly through endogenous stem cell homing and activation [5]. However, the paracrine effects of CPC may not be totally contributed to soluble cytokines. In addition to cytokines, exosomes is a lipid vesicle, which represents an ideal vehicle to deliver genetic materials, such as

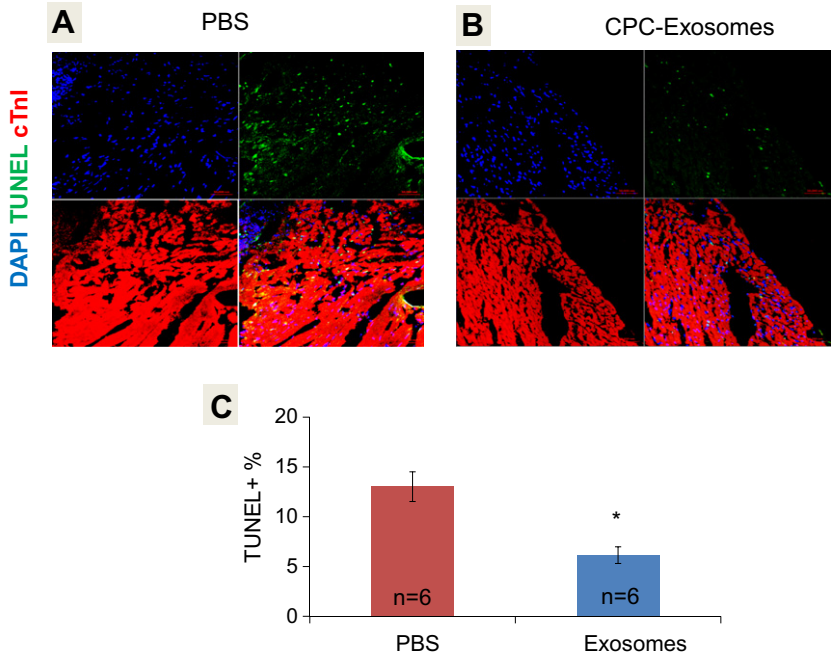

Fig. 3. Immunohistochemistry of control PBS or CPC-exosomes-treated heart sections marking TUNEL-positive cardiomyocytes costained with cardiac troponin I antibody within the border zone of infarcted hearts. (A and B) Representative illustration of TUNEL staining in heart section after 45 min ischemia and 24 h reperfusion. (C) Quantification of myocardial apoptosis. Green staining indicates TUNEL-positive cells, red staining indicating cTnI positive cardiomyocytes, Error bars indicate SEM (*$p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

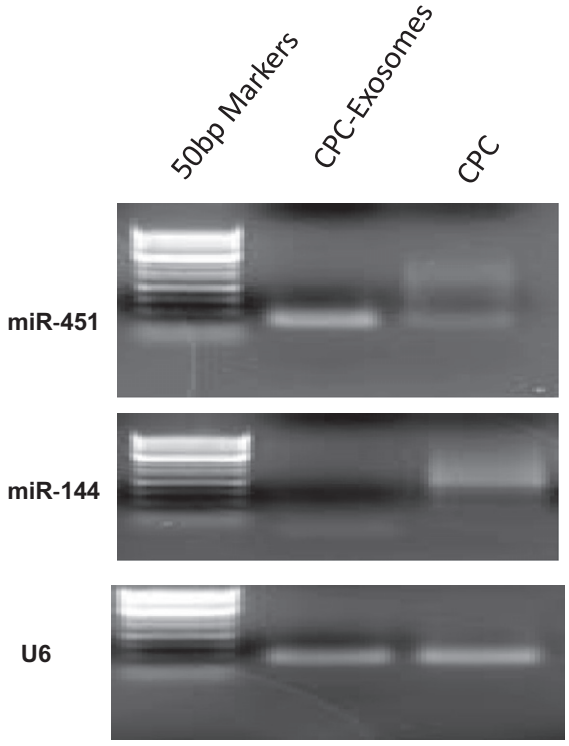

Fig. 4. Real-time RT-PCR validation of differential expression of miR-451 and miR-144 in CPC-exosomes versus CPC. U6 snRNA was used as a normalization reference in real-time PCR.

miRNAs, from one cell to another, and execute their functions [17]. Our observations demonstrated that exosomes with a diameter of 40–100 nm are present in the conditional medium of CPC and are therefore the major cardioprotective component in the conditional medium. We confirmed that the purified exosomes from conditioning medium of CPC, other than small soluble proteins, can protect H9C2 from apoptosis induced by H2O2 mediated oxidative stress, and enhance cardiomyocytes against apoptosis induced by acute myocardial ischemia/reperfusion.

Our findings demonstrated that the CPC-derived exosomes contained high level of miR-451 in comparison with CPC. Studies in rodents demonstrate that microRNA-144/451 (miR-144/451) cluster is regulated by GATA-4 [29], a critical transcription factor expressed in early heart development [30]. Recent study demonstrated that miR-451 confers protection against simulated ischemia/reperfusion (I/R)-induced cardiomyocyte death [29], however, miR-144, another GATA4-responsive miRNA within miR-144/451 cluster, is absent in CPC-exosomes. The mechanism why CPC-exosomes only enrich specific miRNA but not another from CPC is unknown, exploring this mechanism might help us optimizing the genetic components inside the exosomes for therapeutic purpose.

In summary, we have identified exosome as the cardioprotective component in CPC mediated paracrine effects. This finding broadens our current understanding of the beneficial paracrine effect of CPC transplantation on heart repair. It also highlights for the first time the important role of CPC-derived exosome as mediator of cardioprotection.

4.1. Perspectives

For myocardium with acute ischemia/reperfusion injury, myocardial injury is potential reversible if treated with cardioprotective exosomes early during the critical time window [31]. The CPC-derived exosomes therapy provides a non-cell therapy approach to deliver critical miRNAs to ischemic myocardium for cardioprotection. Since miRNAs are remarkably stable [32], both interventional cardiologists and cardiac surgeons could deliver them into the ischemic myocardium for protecting the heart from ischemia and reperfusion injury.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.015.

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