Original Paper

Loading MiR-210 in Endothelial Progenitor Cells Derived Exosomes Boosts Their Beneficial Effects on Hypoxia/Reoxygenation-Injured Human Endothelial Cells via Protecting Mitochondrial Function

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Key Words

Mir-210 • EPC-EXs • ECs • Mitochondrion

Abstract

Background/Aims: Stem cell-derived exosomes (EXs) offer protective effects on various cells via their carried microRNAs (miRs). Meanwhile, miR-210 has been shown to reduce mitochondrial reactive oxygen species (ROS) overproduction. In this study, we determined the potential effects of endothelial progenitor cell-derived EXs (EPC-EXs) on hypoxia/reoxygenation (H/R) injured endothelial cells (ECs) and investigated whether these effects could be boosted by miR-210 loading. **Methods:** Human EPCs were used to generate EPC-EXs, or transfected with scramble control or miR-210 mimics to generate EPC-EXs^{sc} and EPC-EXs^{miR-210}. H/R-injured human ECs were used as a model for functional analysis of EXs on apoptosis, viability, ROS production and angiogenic ability (migration and tube formation) by flow cytometry, MTT, dihydroethidium and angiogenesis assay kits, respectively. For mechanism analysis, the mitochondrion morphology, membrane potential (MMP), ATP level and the expression of fission/fusion proteins (dynamin-related protein 1: drp1 and mitofusin-2: mfn2) were assessed by using JC-1 staining, ELISA and western blot, respectively. **Results:** 1) Transfection of miR-210 mimics into EPCs induced increase of miR-210 in EPC-EXs^{miR-210} without change of average size; 2) EPC-EXs^{miR-210}, but not EPC-EXs or EPC-EXs^{sc}, significantly

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elevated miR-210 level in ECs; 3) EPC-EXs^{miR-210} were more effective than EPC-EXs and EPC-EXs^{sc} in reducing H/R-induced EC apoptosis, ROS overproduction and angiogenic dysfunction; 4) EPC-EXs decreased mitochondrial fragmentation, elevated MMP and ATP level, as well as improved mitochondrial mfn2 and drp1 dysregulation, which were more effective in EPC-EXs^{miR-210}. **Conclusion:** Our results suggest that EPC-EXs protect ECs against H/R injury via improving mitochondrial function and miR-210 enrichment could boost their effects.

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Introduction

Endothelial progenitor cells (EPCs) are known to play an important role in modulating the function of endothelial cells (ECs) [1]. The potential mechanisms have been attributed to their ability of differentiation into ECs to replace the injured or dysfunctional ECs [2, 3] and their secretion of growth factors (vascular endothelial growth factor et.) to promote angiogenesis [4]. Most recently, extracellular microvesicles have been recognized as a novel way of cell-cell communication [5, 6]. EPC-derived microvesicles can protect kidneys from ischemia/reperfusion injury [7] and improve neovascularization in a murine model of hindlimb ischemia through their shuttled microRNAs (miRs), miR-126 and miR-296 [8]. Our group has demonstrated that EPC-released extracellular vesicles could protect ECs against hypoxia/reoxygenation (H/R) injury, which is at least in part mediated by their carried miR-126 [9].

Exosomes (EXs) is one type of extracellular microvesicles which could modulate the functions of recipient cells via transferring their cargoes, such as miRs, proteins and mRNAs [5, 6]. Stem cells released EXs have been shown to execute the beneficial effects of their parent cells in stem cell-based therapy [10, 11]. Mesenchymal stem cell-derived EXs have been reported to promote neurological protection via conveying miR-133 to neural cells in a stroke rat model [12]. EXs derived from inducible pluripotent stem cells can prevent cardiomyocyte apoptosis through delivery miR-210 in the ischemic myocardium [13]. However, whether EPC-EXs have protective effects on H/R-injured ECs remains unknown.

Several studies have suggested that miR-210 plays a critical role in regulating the EC responses to hypoxia [14-18]. MiR-210 can decrease the apoptosis and angiogenic dysfunction of ECs induced by hypoxia [16]. Overexpression of miR-210 could enhance the microvessel density in the ischemic stroke mouse [17] and the angiogenesis in a myocardial infarction mice model [18]. A clinical study showed that the level of circulating miR-210 was positively related to the outcome of stroke patients [19]. Interestingly, EXs derived from breast cancer cells could enhance the angiogenic function of ECs by transferring miR-210 [20]. There is evidence showing that miR-210 promotes cell survival through reducing hypoxia-induced overproduction of mitochondrial reactive oxygen species (ROS) [21]. Therefore, upregulation of miR-210 in EPC-EXs might be a novel strategy to enhance the beneficial effects of EPC-EXs on H/R-injured ECs.

Mitochondria are essential organelles and play a significant role in a wide range of cells, including ECs and cardiomyocytes [22-24]. EC injury induced by ischemia-reperfusion and EC dysfunction in diabetes mellitus are associated with mitochondrial ROS overproduction [25] and mitochondrial fragmentation [24]. A recent study demonstrated that diffusion of mitochondria-derived ROS into the nucleus contributes to the regulation of hypoxia sensitive genes in rat pulmonary ECs [26]. Of note, miR-210 has been shown to be able to reduce mitochondrial ROS overproduction, suggesting the role of miR-210 in mitochondrial function [21]. Nevertheless, whether miR-210 could protect mitochondrial function to boost the beneficial effects of EPC-EXs on ECs remains unclear.

In this study, we determined whether EPC-EXs can protect ECs against H/R injury and investigate whether miR-210 enrichment could boost these effects through improving mitochondrial function.

Materials and Methods

Cell culture and transfection

Human EPCs were purchased from Celprogen (Torrance, CA) and cultured in complete growth medium (Celprogen; Torrance, CA) according to the manufacturer's protocol. Medium was changed every other day. Human brain ECs were purchased from Cell Systems (Kirkland, WA) and cultured with CSC complete medium (Cell Systems; Kirkland, WA) containing 10% serum, 2% human recombinant growth factors, and 0.2% antibiotic solution under standard cell culture conditions (5% CO₂, 37°C). Medium was changed twice a week.

To overexpress miR-210, human EPCs were cultured to 60-70% confluence, and transfected with miR-210 mimic or the scramble control (SC) (10 nM, Dharmacon, Lafayette, CO) using Dharmacon transfection reagents (Dharmacon, Lafayette, CO) for 48 hrs according to the manufacturer's instruction. EPCs cultured in complete culture medium served as control. The three types of EPCs were used for producing corresponding EXs.

Preparation and collection of EPC-EXs

The protocol for collecting EXs from serum-free culture medium has been reported in a previous study [27]. Briefly, the three types of EPCs were cultured in serum-free culture medium for 24 hrs. Then, the culture medium was centrifuged at 300 g, 15 mins to remove dead cells. The supernatants were then centrifuged at 2000 g, 30 mins to remove cell debris, followed by centrifugation at 20,000 g, 70 mins, and ultracentrifugation at 170,000 g, 90 mins to pellet EXs. The pelleted EXs were denoted as EPC-EXs, EPC-EX^{sc} or EPC-EX^{miR-210}. All EXs were resuspended with phosphate-buffered saline (PBS) and aliquoted for nanoparticle tracking analysis (NTA) and for co-culture experiments. PBS was filtered through 20 nm-filter (Whatman, Pittsburgh, PA).

Nanoparticle tracking analysis

The NanoSight NS300 (Malvern Instruments, Malvern, UK) was used to analyze the size and CD63 expression of EXs as previously described [27, 28]. To detect CD63 expression in the EXs, the collected EXs were incubated with CD63-conjugated microbeads (10 µl; Miltenyi Biotec) in a 100-µl reaction volume for 2 hrs. A magnet (DynaMag-2 magnet; Life technology) was then applied to separate the CD63⁺ EXs from the total EX suspension. After an overnight magnet separation, the CD63⁺ EXs were resuspended in 100 µl of filtered PBS and incubated with rabbit anti-goat IgG conjugated with Q-dot 655 (1:350; Life Technologies) for 90 mins at RT. All samples were then analyzed using the NTA fluorescence-scatter mode. Three 30-second videos of were taken with a frame rate of 30 frames per second. The Data were analyzed using NTA 3.0 software (Malvern Instruments, Malvern, UK) on a frame-by-frame basis.

Hypoxia/reoxygenation injury model in ECs

The EC injury model was induced as previously described [9]. In brief, ECs were cultured in hypoxia for 6 hrs in a hypoxic chamber (1% O₂, 5%CO₂, and 94%N₂; Biospherixhypoxia chamber, NY) and then reoxygenated by incubation in a standard 5% CO₂ incubator for 24 hrs. During the reoxygenation period, ECs were treated with the different types of EPC-EXs.

Co-incubation Design

H/R-injured ECs were divided into four co-culture groups: vehicle (co-culture medium only), EPC-EXs, EPC-EX^{sc}, or EPC-EX^{miR-210}. EPC-EXs were labeled with a red fluorescence dye PKH 26 and then were resuspended with CSC medium and added to the culture medium of ECs during the reoxygenation period. In brief, EXs were labeled with PKH 26 (2 × 10⁻⁶ M, Sigma-Aldrich) for 5 mins at 37 °C, followed by wash with 1 × PBS and ultracentrifuged at 170,000g for 90 mins. The concentration of EPC-EXs (50 µg/ml) was determined based on our previous study [29]. After 24 hr of co-culture, the incorporation of EPC-EXs into ECs was observed under a fluorescence microscope (EVOS; Thermo Fisher Scientific). The fluorescence intensity of ECs was analyzed using Image J as reported [28]. ECs were then used for various tests as described below.

Quantitative RT-PCR analysis

The RNAs from EPCs and their released EXs, as well as from ECs after co-incubation with various EPC-EXs were extracted using Trizol (Thermo Fisher Scientific). For determining miR-210 level, reverse transcription (RT) reactions were performed using PrimeScript™ RT reagent kit (TaKaRa, Japan) and PCR reactions were conducted using SYBR Premix EX Taq™ II kit (TaKaRa, Japan). The RT primer was: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTG GATACGACGACTGT-3'. The forward primer of miR-210 was: 5'-CACGCAGTCGTA TCCAGTGCAGG-3'. The reverse primer of miR-210 was: 5'-CCAGTGCAGGGTCCG AGGTA-3'. The expression of U6 was used as endogenous control for each sample. The forward primer of U6 was: 5'-CTCGCTTCGGCAGCAC-3', the reverse primer of U6 was: 5'-AACGCTTCACGAATTTGCGT-3'. Relative expression level of each gene was normalized to U6 and calculated using the $2^{-\Delta\Delta CT}$ method.

Apoptosis Assay

After 24 hr of co-culture, ECs were detached for apoptosis assay by using the FITC Annexin V apoptosis detection kit (BD Biosciences, CA) [29] and analyzed by flow cytometry. The percentage of apoptotic cells was calculated as: Annexin V+/PI- cells/ total cells x 100%.

Measurement of ROS production

Intracellular ROS production was determined by dihydroethidium (DHE) (Sigma) staining [9] and analyzed by flow cytometry. Data are expressed as fold of control in fluorescent intensity. ECs cultured with CSC medium under normoxic condition were used as controls.

Cell Viability Analysis

EC viability was measured using an MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyltetrazolium bromide, 5 mg/mL, Sigma) assay. Cells were seeded into 96-well plates at a concentration of 2×10^3 cells/well containing 100 μ L of CSC medium. After co-incubation, MTT solution (20 μ L) was added and incubated with cells for 4 hrs at 37 °C, then 150 μ L of DMSO was added to each well and incubated with the cells for 20 min at 37 °C. The optical density (OD) was read at 490 nm on a microplate reader (BioTek, USA). The percentage of cell proliferation was defined as the relative absorbance of treated cells versus untreated cells. Cells from 3 wells were counted at each time point, and the experiment was repeated 6 times.

The angiogenic functional assays of ECs

The tube formation assay was conducted using *in vitro* angiogenesis assay kit (Chemicon, Rosemont, IL) [9]. The migration ability of ECs was determined by scratch assay [30]. In brief, ECs were grown to confluence on 6-well cell culture plates. A scratch was made through the cell monolayer using a 1000 μ L pipette tip. After washing with PBS, cells were treated with the various types of EPC-EXs or culture medium only (vehicle). Photographs of the wounded area were taken immediately (0-hr time point) and 16 hr after making the scratch. The migration ability was determined by monitoring the invasion of cells into the scratched area.

Detection of ATP levels

Cellular ATP levels were measured using an ATP Assay Kit (Promega, WI, USA) according to the manufacturer's instruction. Briefly, the proteins of ECs treated with various types of EPC-EXs were harvested and determined by the BCA assay (Thermo Scientific). In 6-well plates, 200 μ L protein supernatant was mixed with 100 μ L ATP detection working solution. The luminance (RLU) was measured by a fluorescence microplate reader (Thermo Fisher Scientific, Varioskan Flash, USA). The total ATP levels were expressed as nmol/mg protein. The relative ATP level was calculated according to the following formula: relative ATP level = ATP value / protein concentration.

Analysis of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) of ECs treated with various types of EPC-EXs was measured using the lipophilic cationic dye JC-1 (1:1000, Invitrogen) [31]. In brief, after 24-hr of treatment, ECs were washed with PBS and incubated with 1 ml of medium containing JC-1 staining probe (2 μ M final concentration) for 20 mins at 37 °C. The cells were then washed with PBS and observed under a fluorescence microscope (EVOS; Thermo Fisher Scientific). The level of cellular fluorescence intensity was analyzed

by Image J (NIH, Bethesda, MD). The relative MMP was calculated as the ratio of J-aggregate to monomer (590/520 nm). Values are expressed as fold increase in the ratio of J-aggregate to monomer fluorescence relative to control cells. ECs cultured with CSC medium were used as controls. The original images were all taken with the same gain/intensity with the same threshold. The people who did the analysis was blinded to the grouping information.

Assessment of mitochondrial morphology

To assess mitochondrial morphology, ECs growing on coverslips were stained with MitoTracker as previous reports [32]. In brief, cells were pre-incubated with MitoTracker Green FM (100 nM; Invitrogen) for 15 mins, and then fixed in 4% paraformaldehyde (PFA) for 10 mins at RT. The nucleus was then counterstained with 4', 6-Diamidino-2-Phenylindole (DAPI). Mitochondrial morphology was observed under 60 x oil immersion objective of a confocal microscope (Olympus FV-1000, Japan). Digital images were subjected to a convolve filter through the IMAGEJ software (NIH) to isolate and equalize fluorescent pixels in the image [33]. After thresholding, individual particles (mitochondria) were analyzed for circularity ($4\pi \times \text{Area} / (\text{perimeter}^2)$) and lengths of major and minor axes. From these values, we calculated form factor (FF; the reciprocal of circularity value) and aspect ratio (AR; major/minor). Both FF and AR have a minimal value of 1 when a particle is a small perfect circle and the values increase as the shape becomes elongated. A minimum of 100 cells from 10 random microscopic fields (60x) were assessed for mitochondrial fragmentation per co-culture group in each independent experiment. Six independent experiments were conducted in each group.

Western blot analysis

After 24-hour of treatment, proteins of ECs in different groups were extracted with cell lysis buffer (Thermo Fisher scientific, Waltham, MA) supplemented with complete mini protease inhibitor tablet (Roche, Basel, Switzerland). Then, the protein lysates were electrophoresed through SDS-PAGE gel and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 hr at room temperature, and incubated with primary antibody against mitofusin-2 (mfn2: 1:1000; Thermo Fisher Scientific), dynamin-related protein 1 (drp1, 1:1000; Thermo Fisher Scientific) or β -actin (1:4000; Sigma, St. Louis, MO) at 4°C overnight. On the next day, membranes were washed and incubated with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:40000; Jackson Immuno Research Lab, West Grove, PA) for 1 hr at room temperature. Blots were developed with enhanced chemiluminescence developing solutions and images were quantified using ImageJ software.

Statistical analysis

All experiments were repeated for four to six times. Data are expressed as the mean $\pm$ SEM. Multiple comparisons were analyzed by one- or two-way ANOVA followed by Tukey post-hoc test. SPSS 23.0 statistical software was used for analyzing the data. For all measurements, $p < 0.05$ was considered statistic significant.

Results

Characterization of EPC-EXs

As shown in Fig. 1A, transfection of miR-210 mimics increased the level of miR-210 in EPCs as compared to the EPCs in vehicle or transfected with scramble control (SC). Meanwhile, we found the EXs released from EPCs overexpressed miR-210 (EPC-EXs^{miR-210}) were enriched with miR-210 as revealed by RT-PCR analysis.

According to the NTA analysis, the average diameter of the three types of EXs was less than 120 nm (Fig. 1B), and there was no difference of the size among EPC-

Fig. 1. Transfection of miR-210 mimics into EPCs increased miR-210 level in EPC-EXs. (A) The level of miR-210 in EPCs and their correspondences EXs after transfection; * $P < 0.05$ vs. vehicle or sc. (B-C) The size and CD63 expression of the various types of EPC-EXs; $n = 4/\text{group}$. Sc: scramble control.

EXs, EPC-EXs^{sc} and EPC-EXs^{miR-210}, reflecting that miR-210 transfection did not affect the size of the released EXs. In addition, our data showed that the majority (> 80%) of these EXs expressed EX specific marker CD63 as revealed by fluorescent NTA analysis (Fig. 1C).

EPC-EXs^{miR-210} co-incubation increased the miR-210 level in H/R-injured ECs

In order to track EPC-EXs in the co-culture system, we labeled the collected EXs with a red fluorescence dye PKH26. After 24-hr co-incubation, we observed red fluorescence in the cytoplasm of ECs (Fig. 2A). Meanwhile, we analyzed the fluorescence intensity of ECs in all groups and found that there was no significant difference of the fluorescence intensity (Fig. 2B).

As revealed by the qRT-PCR data, the level of miR-210 was slightly raised in ECs exposed to H/R than that in control ECs. After 24 hr co-incubation, there was no significant difference of miR-210 level in ECs between the vehicle and EPC-EXs or EPC-EXs^{sc}. However, co-cultured with EPC-EXs^{miR-210} significantly elevated both miR-210 level in H/R-injured ECs (Fig. 2C), indicating that co-incubation with EPC-EXs^{miR-210} could transfer their carried miR-210 to ECs.

EPC-EXs^{miR-210} were more effective than EPC-EXs and EPC-EXs^{sc} in attenuating H/R-induced EC apoptosis and ROS overproduction

As shown in Fig. 3A-3B, H/R induced a significant increase of EC apoptosis that was partially reduced by EPC-EX or EPC-EX^{sc} treatment. Moreover, we found that EPC-EXs^{miR-210} almost completely inhibited H/R-induced increase of EC apoptosis.

Similarly, ROS production was elevated in ECs exposed to H/R as compared to that in control. H/R-induced ROS overproduction was significantly reduced by EPC-EX or EPC-EX^{sc} treatment, which was further decreased by EPC-EXs^{miR-210} (Fig. 3C).

These data demonstrate that EPC-EXs^{miR-210} were more effective than EPC-EXs and EPC-EXs^{sc} in protecting ECs from H/R-induced apoptosis and oxidative stress.

EPC-EXs^{miR-210} were more effective than EPC-EXs and EPC-EXs^{sc} in promoting cell viability on both normal and H/R-injured ECs

According to the MTT assay, we found that H/R decreased EC viability (vs. control; $p < 0.05$; $n = 6$ /group; Fig. 4). EPC-EXs and EPC-EXs^{sc} increased the viability of ECs in both control (normoxia) and H/R treated cells (vs. vehicle; $p < 0.05$; $n = 6$ /group; Fig. 4). EPC-EXs^{miR-210} were more effective in increasing the viability of ECs (vs. EMVs; $p < 0.05$; $n = 6$ /group; Fig. 4).

Fig. 2. EPC-EXs^{miR-210} co-incubation increased miR-210 level in H/R-injured ECs. (A) Representative images showing the incorporation of PKH26 labeled EPC-EXs with H/R-injured ECs after 24 hrs. (B) Summarized data showing the fluorescence intensity of ECs co-incubated with the various EPC-EXs; Nucleus were counterstained with DAPI (blue). Scale bar: 100 µm. (C) Summarized data of the level of miR-210 in ECs. * $P < 0.05$ vs. control; * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. EPC-EXs or EPC-EXs^{sc}.

Fig. 3. EPC-EXs^{miR-210} were more effective on reducing H/R-induced EC apoptosis and ROS overproduction. (A-B) Representative images and summarized data of EC apoptosis. (C) ROS production in ECs. * $P < 0.05$ vs. control; * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. EPC-EXs or EPC-EXs^{sc}; $n = 6$ /group.

Angiogenic dysfunction of H/R-injured ECs was more effectively improved by EPC-EXs^{miR-210}

To assess the angiogenic ability of ECs, we measured their tube formation and migration abilities. Our data (Fig. 5) revealed that both tube formation and migration abilities of ECs were compromised by H/R as indicated by a decreased number of tubes per field and an increased gap distance as compared to that of the control. EPC-EX or EPC-EX^{sc} treatment could partially rescue the tube formation and migration abilities of ECs. What's more, there were no significant differences of the migration and tube formation abilities of ECs in the control and those treated by EPC-EXs^{miR-210}, indicating a prominent effect of EPC-EXs^{miR-210} on improving H/R-compromised EC angiogenic properties.

EPC-EXs^{miR-210} were more effective in increasing MMP and ATP level in H/R-injured ECs

To assess the MMP of H/R-injured ECs, we used JC-1 staining. As shown in Fig. 6A, red indicates aggregate while green indicates monomer. The MMP was calculated as the ratio of aggregate to monomer (red/green). Summarized data in Fig. 6B showed that H/R significantly decreased MMP ($\Delta\psi$ M, red/green) of ECs. After 24-hr treatment with EPC-EXs or EPC-EX^{sc}, there was an increase of $\Delta\psi$ M in ECs that were further increased by EPC-EX^{miR-210} treatment.

A comparison of the ATP synthetic ability of ECs was shown in Fig. 6C. ECs exposed to H/R displayed a decreased level of ATP as compared to that of ECs cultured in normoxic condition. EPC-EX or EPC-EX^{sc} treatment partially rescued H/R-decreased ATP level of ECs. Moreover, EPC-EX^{miR-210} further improved the ability of ECs to produce ATP. Taken together, EPC-EXs^{miR-210} were more effective than EPC-EXs in maintaining mitochondrial MMP balance and function.

EPC-EXs^{miR-210} prominently improved H/R-induced mitochondrial fragmentation

The representative pictures in Fig. 7A show the mitochondrial fragmentation (green) after different treatments. The summarized

Fig. 4. EPC-EXs^{miR-210} were more effective on promoting cell survival in normal and H/R-injured ECs. *P < 0.05 vs. vehicle; *P < 0.05 vs. EPC-EXs or EPC-EXs^{sc}; #P < 0.05 vs. control; n=6/group.

Fig. 5. EPC-EXs^{miR-210} were more effective in protecting ECs from H/R-induced angiogenic dysfunction. (A) Representative pictures of EC migration. Scale bar: 200 μ m. (B) Representative images of EC tube formation. Scale bar: 600 μ m. (C-D) Summary data. *P < 0.05 vs. control; *P < 0.05 vs. vehicle; *P < 0.05 vs. EPC-EXs or EPC-EXs^{sc}; n=6/group.

Fig. 6. EPC-EXs^{miR-210} were more effective in increasing $\Delta\psi$ M and ATP level compromised in H/R-induced ECs. (A) Representative JC-1 staining images; Bar: 50 μ m; (B) Summary data of $\Delta\psi$ M. (C) Summary data of ATP level. *P < 0.05 vs. control; *P < 0.05 vs. vehicle; *P < 0.05 vs. EPC-EXs or EPC-EXs^{sc}; n=6/group. $\Delta\psi$ M: change of mitochondrial membrane potential (red/green).

results (Fig. 7B) showed that ECs exposed to H/R exhibited a higher percentage of fragmented mitochondria in comparison to cells in the control. EPC-EX or EPC-EX^{sc} treatment decreased the percentage of cells with fragmented mitochondria. What's more, EPC-EX^{smiR-210} further decreased H/R-induced mitochondrial fragmentation. This data suggests that EPC-EX^{smiR-210} had better efficient in improving H/R-induced abnormality of mitochondrial morphology.

EPC-EX^{smiR-210} were prominent on modulating the expression of mitochondrial fusion and fission proteins in H/R-injured ECs

To further explore the mechanisms of fragments formation, we measured the levels of fission and fusion proteins in ECs. As shown in Fig. 8, H/R significantly upregulated the expression of mitochondrial fission protein Drp1 and downregulated the expression of mitochondrial fusion protein Mfn2 in ECs. EPC-EX or EPC-EX^{sc} treatment decreased Drp1 expression but increased Mfn2 expression. This effect was remarkably enhanced by EPC-EX^{smiR-210} treatment. The data indicate that EPC-EX^{smiR-210} had better efficacy in balancing the mitochondrial fusion and fission process in ECs.

Discussion

In the present study, we firstly found that EPC-EXs or EPC-EX^{sc} exhibited beneficial effects on protecting ECs from H/R-induced injury and dysfunction. More interesting, loading of miR-210 in EPC-EXs boosted these protective effects through improving mitochondrial function of ECs.

As previously reported [9], we reproduced the model of H/R-injured ECs which displayed increased apoptosis and ROS overproduction, accompanying with angiogenic dysfunction. It is known that MMP is a marker of mitochondrial health and mitochondrial fragmentation is a primary event in hypoxia [25]. Fusion and fission proteins, mfn2 and drp1, contribute to the maintenance of mitochondrial function [34]. In this study, we revealed that H/R-injured ECs had a dramatic loss of MMP, decreased ATP level and increased mitochondrial fragmentation. Meanwhile, mfn2 expression was significantly decreased, whereas drp1 expression was significantly increased, indicating the mitochondrion function of ECs was impaired. This is

Fig. 7. H/R-induced mitochondrial fragmentation in ECs was more effectively inhibited by EPC-EX^{smiR-210}. (A) Representative confocal images showing the morphology of mitochondrion. Enlarged images are magnifications of the mitochondria at the indicated areas; Bar: 10 μ m. (B) Summary data showing the percentage of cells with fragmented mitochondria. *P < 0.05 vs. control; *P < 0.05 vs. vehicle; #P < 0.05 vs. EPC-EXs or EPC-EXs^{sc}; n = 6/group.

Fig. 8. EPC-EX^{smiR-210} had a prominent effect on alleviating dysregulation of mitochondrial mfn2 and drp1 on H/R-induced ECs. (A) mfn2 expression. (B) drp1 expression. *P < 0.05 vs. control; *P < 0.05 vs. vehicle; #P < 0.05 vs. EPC-EXs or EPC-EXs^{sc}; n = 4/group.

supported by a previous study showing that decreased expression of *mfn2* occurred both *in vivo* and *in vitro* hypoxic models [35]. To test the protection effects of EPC-EXs, we co-cultured EPC-EXs with the H/R-injured ECs and found that EPC-EXs significantly decreased H/R-induced apoptosis and ROS overproduction. These findings indicate the anti-apoptotic and anti-oxidative effects of EPC-EXs on ECs. Besides, we found that H/R-compromised angiogenic function (tube formation and migration abilities) was significantly improved by EPC-EXs. This result is in agreement with previous reports showing the protective/therapeutic effects of stem cell-derived extracellular microvesicles [7-9, 12]. It is known that EXs can affect recipient cell function through conveying miRs [5, 6]. Interestingly, co-culture of EPC-EXs did not change the level of miR-210 in ECs, suggesting that the effects of EPC-EXs were independent on miR-210. Meanwhile, we did find that EPC-EXs improved H/R-compromised mitochondrial function as revealed by increased MMP and ATP level, reduced mitochondrial fragmentation, as well as increased *mfn2* expression and decreased *drp1* level. As we know, EXs carry massive RNAs and proteins. Previous reports by us and others showing that EPC-derived extracellular microvesicles shuttle miR-126 [7-9]. The protective effects of EPC-EXs on H/R-injured ECs is probably due to their carried miR-126 which has been shown to have beneficial effects on ECs/EPC survival and angiogenic function [30, 36] and have modulatory effects on mitochondrion [37]. However, whether these molecules are responsible for the observed effects of EPC-EXs need further investigation.

Using RT-PCR, we found that miR-210 was up-regulated in normal ECs compared to apoptotic ECs after H/R challenge, suggesting that miR-210 possesses anti-apoptotic properties during cell stress conditions. This data is also confirmed by a recent report indicating that miR-210 is induced in ischemia-preconditioned bone marrow derived mesenchymal stem cells [38] and hypoxia-challenged cardiomyocytes [18]. In the present study, we proposed to explore whether overexpressing miR-210 in EPC-EXs would enhance the protective of EPC-EXs. The rationale is based on followings: a) EPC transfusion has therapeutic effects on ischemic stroke [39]; b) The potential mechanisms of stem cell therapy for ischemic stroke have been partially attributed to their released microvesicles or EXs [10, 11]; c) EPC-released microvesicles have shown angiogenic function in ECs/EPCs by delivering their carried miR-126 [30]; d) miR-210 has shown therapeutic potential for ischemic disease [18]. To determine the role of miR-210 in EPC-EXs, we successfully produced miR-210 enriched EPC-EXs by transfecting EPCs with miR-210 mimics. As expected, EPC-EXs^{miR-210} were well incorporated with ECs after co-incubation. Moreover, EPC-EXs^{miR-210}, not EPC-EXs or EPC-EXs^{sc}, remarkably elevated miR-210 level in ECs, indicating EPC-EXs^{miR-210} can elevate miR-210 level in ECs. Functional studies revealed that EPC-EXs^{miR-210} had better effects than EPC-EXs on rescuing ECs from H/R-induced decrease of survival and angiogenic dysfunction. Our study is supported by a previous report showing that overexpression of miR-210 have therapeutic effects on the cerebral ischemic mice [17]. In addition, we found that the same amount of EPC-EXs, EPC-EXs^{sc} and EPC-EXs^{miR-210} was up-taken by H/R-injured ECs, which further supported our hypothesis that miR-210 played a boost effect on enhancing the beneficial effects of EPC-EXs. To further illustrate the mechanism of EPC-EXs^{miR-210}, we assessed the mitochondrial function of ECs. EPC-EXs^{miR-210} exhibited stronger effects than EPC-EXs or EPC-EXs^{sc} on increasing MMP and ATP level, as well as decreasing mitochondrion fragmentation, reflecting the boost effect of miR-210 on EPC-EXs. In addition, we found that EPC-EXs^{miR-210} prominently restored H/R-induced dysregulations of *mfn2* and *drp1* on ECs. These finding suggest that miR-210 enrichment can boost the beneficial effects of EPC-EXs through improving mitochondria function. However, although we demonstrated a specific effect of miR-210 in EPC-EXs, we could not rule out the downstream functional targets, such as *Efn3*, *Ptp1b*, *ISCU* (iron-sulfur cluster scaffold homolog) and *COX10* (cytochrome c oxidase assembly protein), which need further investigation. *Efn3* and *Ptp1b* are involved in inhibition of angiogenesis and induction of apoptosis, respectively. *Efn3* suppression in HUVEC cells is vital for stimulation of tubulogenesis, indicating its crucial function in angiogenesis [16]. Recently, it has been reported that *Ptp1b* is a negative regulator in VEGF signaling in ECs [40]. *ISCU* and *COX10* are two important factors of the mitochondria

electron transport chain and the tricarboxylic acid cycle, which have been identified as potential targets of miR-210 [41]. Thus, the enhanced protective effects of EPC-EXs^{miR-210} on H/R-injured ECs might through direct regulation of these target genes.

In conclusion, EPC-EXs have protective effects on ECs against H/R injury through decreasing apoptosis, ROS production and improving angiogenic function. MiR-210 could enhance the protection effects of EPC-EXs on H/R-injured ECs mainly through improving mitochondrial function.

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Disclosure Statement

No conflict of interests exists.

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