Bone marrow mesenchymal stem cell-derived exosomal miR-21 protects C-kit\(^+\) cardiac stem cells from oxidative injury through the PTEN/PI3K/Akt axis

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Abstract

Stem cell (SC) therapy for ischemic cardiomyopathy is hampered by poor survival of the implanted cells. Recently, SC-derived exosomes have been shown to facilitate cell proliferation and survival by transporting various proteins and non-coding RNAs (such as micro-RNAs and IncRNAs). In this study, miR-21 was highly enriched in exosomes derived from bone marrow mesenchymal stem cells (MSCs). Interestingly, exosomes collected from hydrogen peroxide (H\(_2\)O\(_2\))-treated MSCs (H-Exo) contained higher levels of miR-21 than exosomes released from MSCs under normal conditions (N-Exo). The pre-treatment of C-kit\(^+\) cardiac stem cells (CSCs) with H-Exos resulted in significantly increased levels of miR-21 and phosphor-Akt (pAkt) and decreased levels of PTEN, which is a known target of miR-21. AnnexinV-FITC/PI analysis further demonstrated that the degree of oxidative stress-induced apoptosis was markedly lower in H-Exo-treated C-kit\(^+\) CSCs than that in N-Exo-treated cells. These protective effects could be blocked by both a miR-21 inhibitor and the PI3K/Akt inhibitor LY294002. Therefore, exosomal miR-21 derived from H\(_2\)O\(_2\)-treated MSCs could be transported to C-kit\(^+\) cardiac stem cells to functionally inhibit PTEN expression, thereby activating PI3K/AKT signaling and leading to protection against oxidative stress-triggered cell death. Thus, exosomes derived from MSCs could be used as a new therapeutic vehicle to facilitate C-kit\(^+\) CSC therapies in the ischemic myocardium.

1. Introduction

Recently, cardiac stem cells (CSCs) residing in the adult mammalian heart have emerged as one of the most promising stem cell types for cardiac regeneration and repair\([1-7]\). However, the poor engraftment and viability of CSCs hamper functional improvements and optimal cardiac outcomes\([8-10]\). Preconditioning stem cells using various strategies could significantly enhance CSC survival after adoptive transfer in myocardial infarction patients\([11-14]\). Exosomes released from cells have been recently shown to mediate cell-cell communication to
ensure information transfer from donor cells to recipient cells and allow cells to react to environmental changes[15]. These exosomes constitute a delicate and complex system that can be used to control tissue regeneration and cell protection and survival[16–18].

Exosomes are membrane vesicles 30–100 nm in diameter that are released from many cell types under specific physiological or pathological states. Exosomes contain many protein factors, mRNAs, miRNAs, IncRNAs and other nutritional elements. These cargoes are selectively wrapped into the microbubble structure and finally secreted into the extracellular environment via exosomes[19, 20]. However, the contents of exosomes vary across different cell types and under different pathophysiological conditions, which may generate completely different outcomes in recipient cells[21, 22]. Hence, investigating the biological functions of exosomes under specific pathological conditions is imperative. MSC-released exosomes have been shown to improve cardiac function after myocardial infarction[18, 23]. Moreover, an injection of exosomes from exogenous MSCs could recruit endogenous CSCs to the ischemic and border zones of infarcted hearts and promote their expansion[24]. Additionally, exosomes released from MSCs could stimulate the proliferation, migration, and angiogenic potency of CSCs in vitro and in vivo[16]. Considering the potential therapeutic effects of MSC-exosomes (MSC-Exo) in cardioprotection and cell therapy, we sought to determine whether C-kit⁺ CSCs preconditioned with MSC-Exos could enhance survival and function under oxidative stress conditions.

miRNAs, which are among the many exosome cargo types, have been confirmed to play a pivotal role in improving the undesirable consequences associated with acute myocardial infarction[25]. miRNAs are endogenous single-stranded non-coding RNAs consisting of 20–22 nucleotides that play critical roles in mRNA inhibition and degradation[26]. miRNAs have been shown to be involved in the regulation of CSC apoptosis[8, 27]. miRNAs released by MSC-Exos may also regulate the proliferation, differentiation and survival of CSCs[16]. However, whether MSC-Exo-derived miRNAs protect against apoptosis induced by H₂O₂ in C-kit⁺ CSCs and specific miRNAs that play critical roles remain unknown. According to gain-of-function studies, miR-21 reduces cardiomyocyte apoptosis induced by oxidative stress [28, 29]. One study have confirmed that MiR-21 modulates the immunoregulatory function of MSCs by controlling the PTEN[30]. Furthermore, it is also reported that miR-21 via regulating the PTEN/HIF-1α/VEGF-A signaling cascade to enhances the therapeutic effects of human multipotent cardiovascular progenitors[31]. These studies have identified a potential exosomal miRNA target gene that likely regulates C-kit⁺ CSC apoptosis under oxidative stress conditions.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor gene that is involved in the regulation of cell proliferation, migration, differentiation and invasion in a variety of tumor cells[32, 33]. PTEN partially functions through the pro-survival pathway by inhibiting the phosphorylation of Akt to its active form (p-Akt)[33]. The inactivation of PTEN activates Akt signaling, which reduces apoptosis and increases survival[34–37]. PTEN is a well-documented target gene of miR-21[30, 38–40]. Moreover, miR-21 promotes cell proliferation via PTEN-dependent PI3K/Akt activation in cancer cells [41–45]. Additionally, in our previous study, miR-21 protected C-kit⁺ CSCs from H₂O₂-induced apoptosis and increases cell proliferation partially through the PTEN/PI3K/ Akt pathway[46–47]. The current study investigated the protective effects of MSC-Exos on C-kit⁺ CSCs under oxidative stress. These effects are mainly mediated through the transmission of exosomal miR-21, which inhibits PTEN and activates the PI3K/Akt pathway in C-kit⁺ CSCs. These findings provide a potential cellular therapeutic strategy for ischemic cardiomyopathy.
2. Materials and methods

2.1. Animals

Sprague-Dawley rats (males and females, approximately 3 weeks old, 45–60 g) were purchased from the Third Military Medical University (Chongqing, China) and housed at Zunyi Medical College. All experimental procedures were performed according to the “Guide for the Care and Use of Laboratory Animals” in China and approved by the local Experimental Animal Care and Use Committee.

2.2. Materials

Collagenase type II was obtained from Sigma (USA). Trypsin was obtained from Gibco (USA). Penicillin and streptomycin were obtained from Srlabio (China). Ham's/F-12 medium and fetal bovine serum were both purchased from HyClone (USA). Fibroblast growth factor was obtained from PeproTech (USA). Leukocyte inhibitory factor was obtained from Gibco (USA). The rabbit anti-rat C-kit+ primary antibody was supplied by Biorbyt (UK). The M-280 beads conjugated with sheep anti-rabbit secondary antibody were obtained from Dynal Biotech (Norway). The PE-conjugated anti-CD34 and anti-CD45, APC conjugated anti-CD29, and anti-CD90 primary antibodies were obtained from BioLegend (USA). The miR-21 mimics, miR-21 inhibitors and the negative control were synthesized by RIBOBIO (China). EXO quick TC was obtained from System Biosciences. SiRNA-PTEN and the scrambled siRNA were synthesized by GeneCopoeia (MD). The lentivirus and empty vector were synthesized by HANBIO (China). Lipofectamine 2000 was obtained from Invitrogen (USA). The primers and miRNA reverse transcript and qRT-PCR kits were obtained from Sangon Biotech (China). The anti-β-actin, anti-caspase-3, anti-cleaved-caspase-3, anti-PTEN, anti-P-Akt, and anti-Akt primary antibodies and additional secondary antibodies were obtained from Boster (China). The anti-CD63, anti-CD9, and anti-Hsp70 antibodies were purchased from Abcam (USA). DiI was obtained from Invitrogen (USA). The Annexin V-FITC apoptosis detection kit was obtained from Solarbio (China). The In-situ cell death detection kit was obtained from Sigma (USA). LY294002 (PI3K inhibitor) was obtained from Beyotime Technology (China). The unlisted reagents were of analytical grade.

2.3. In vitro culture of C-kit+ cells

CSCs were isolated[48] and purified[3] using previously published methods with some modifications. The rats were deeply anesthetized with sevoflurane, and the atrial appendage was sliced and digested with 0.1% collagenase type II (Sigma, USA). After a 40-min digestion at 37°C, the cells were collected by sedimentation at 1200 rpm for 5 min. Then, the cells from the atrial appendage were incubated in a humidified chamber in Ham’s F12 medium containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% L-glutamine, 20 ng/ml human recombinant fibroblast growth factor, 20 ng/ml leukocyte inhibitory factor, and 10 ng/ml epidermal growth factor (EGF). After reaching >90% confluence, the cells were resuspended by trypsinization. Subsequently, the CSCs were incubated with a rabbit anti-C-kit antibody (1:250 in F12 medium) for 1 h and sorted with anti-rabbit secondary antibody-conjugated 2.8 µm magnetic beads (Dynal Biotech, Norway) for 30 min as instructed by the manufacturer’s protocol. The purified C-kit+ CSCs were cultured in the previously mentioned F12 medium. Flow cytometry (FCM) was performed to confirm the surface markers on the C-kit+ CSCs. The cells were incubated with the fluorochrome-conjugated anti-CD34-PE, anti-CD45-PE, and anti-C-kit primary antibodies and the anti-C-kit IgG-allophycocyanin (APC) secondary antibody (all from BioLegend, USA).
2.4. Isolation and culture of MSCs

The culture medium was used to flush all bone marrow cells from the femurs and tibias of rats (2–4 months old) sacrificed with a sevoflurane overdose as previously described.[49] Low glucose-Dulbecco’s modified Eagle’s medium (L-DMEM) (GIBCO) complete medium containing 15% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin was used to resuspend the MSCs. Then, the cells were incubated in a humidified chamber. The first medium change was performed at 48 h to remove the non-adherent cells. Trypsin (0.25%, Sigma) was used to passage the cells at a ratio of 1:2 after reaching 90% confluence. FCM was used to analyze the MSC surface markers. The cells were incubated with the fluorochrome-conjugated anti-CD90-PE and anti-CD29 allophycocyanin (APC) or anti-CD45-PE primary antibodies (all from BioLegend, USA). MSCs between P3 and P5 were used for the subsequent experiments.

2.5. Purification and identification of MSC exosomes

The MSC-exosomes (MSC-Exos) extraction procedures were performed as previously described[23, 50]. The MSCs were cultured in L-DMEM supplemented with 10% FBS. Prior to use, all FBS was centrifuged at 100,000–110,000 g for 8 to 10 h to eliminate preexisting bovine-derived exosomes[7]. A 50-ml conditional culture medium containing 10% Exo-free fetal bovine serum (FBS) was used to culture the MSCs for 48 h. The supernatant was harvested and centrifuged at 500 g for 5 min and then 2000 g for 30 min at 4˚C to remove cell debris. Exo-Quick TC (System Biosciences) was applied to precipitate the exosomes according to the manufacturer’s instructions. Briefly, 50 ml supernatant were added to 10 ml ExoQuick-TC Exosomes Precipitation Solution. This cocktail was mixed well and refrigerated overnight. Subsequently, the cocktail was centrifuged at 1500 g for 30 min, and the supernatant was removed. The sediment was then centrifuged at 1500 g for 5 min and aspirated. Then, 50 μl phosphate-buffered saline (PBS) were used to resuspend the exosomes, and the resulting solution was stored at ~80˚C. The amount of MSC-Exo was detected by measuring the total protein content using a BCA protein assay kit (Pierce). Then, the exosomes were observed directly under a transmission electron microscope (Hitachi H7500 TEM, Tokyo, Japan). The MSC-Exos were also identified by Western blotting using the anti-CD63, anti-CD9, and anti-Hsp70 antibodies (all purchased from Abcam) previously described as specific exosome markers[51, 52].

2.6. Established H₂O₂-induced oxidative stress model in C-kit+CSCs and MSCs

The harvested CSCs and MSCs were treated with 100 μM H₂O₂ for 2 h as previously described [47]. FMC was used to determine early apoptosis and necrosis in C-kit+CSCs using an Annexin V-FITC/PI staining assay as reported elsewhere[5]. The phosphatidylserine levels on the surface of the C-kit+CSCs were estimated using the Annexin V- FITC and Propidium Iodide (PI) apoptosis detection kit (Solarbio, China) according to the manufacturer’s instructions. Apoptosis was analyzed in the C-kit+CSCs using a flow cytometer (BD Biosciences, USA). The results are expressed as the percentage of apoptotic cells among all cells. Flow cytometry was performed twice using C-kit+CSCs in three independent experiments. CCK-8 was used to determine MSC viability in three independent experiments.

2.7. Cell transfection

Fifty nanomoles of miR-21 mimics, inhibitors or negative control were added to 1.5 ml F12 medium in 6-well plates with 5 μl Lipofectamine 2000 transfection reagent (Invitrogen, USA)
and incubated with the C-kit⁺ CSCs or MSCs for 48 h according to the manufacturer’s instructions. The efficiency of the mimics or inhibitors was confirmed by RT-qPCR.

2.8. RNA interference
The synthesized siR-PTEN (siR-PTEN) and scramble (GeneCopoeia, MD) were transfected into C-kit⁺ CSCs using a lentiviral construct (HANBIO, China) according to the manufacturer’s instructions. Briefly, the lentiviral vector expressing PTEN(siR-PTEN) or PTEN negative control (siR-PTEN-NC) were constructed by inserting the siR-PTEN gene or siR-PTEN-NC into a Lv-EGFP vector using BamHI (FD0054) and EcoRI (N41890) restriction sites, all obtained from Invitrogen (Thermo Fisher Scientific). The lentiviral particles were prepared using a calcium phosphate method. The C-kit⁺ CSCs (1 × 10⁵ per well) were plated into 6-well plates and then treated with siR-PTEN and siR-PTEN-NC in the presence of 2 μg/ml polybrene (Sigma-Aldrich) at a multiplicity of infection of 50 MOI for 48 h. The siR-PTEN knockdown efficiency was confirmed by Western blotting and RT-qPCR.

2.9. Reverse transcription and Real-Time qPCR analysis of miR-21 and PTEN
The mRNA and miRNA levels were determined using quantitative RT-PCR as previously described[53, 54]. Briefly, the RNAs from the CSCs, MSCs and exosomes were isolated using the TRIzol (Invitrogen, USA) method. RT-PCR was performed on cDNA generated from 3 μg of the total RNA using a cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer’s protocol. RT-qPCR was performed using the CFX Connect Real-Time system (Bio-Rad, USA) and a SYBR green PrimScript RT kit (TaKaRa, Japan) according to the manufacturer’s instructions. U6 and β-actin were used as the internal controls for the miR-21 and PTEN mRNA quantification, respectively.

2.10. Internalization of Dil-labeled exosomes into C-kit⁺ CSCs
The C-kit⁺ CSCs were harvested and seeded in fibronectin-coated dishes and maintained at 37°C overnight. Briefly, the MSC-Exos were labeled with 1 g/ml Dil (Invitrogen, USA) as previously described[18]. Then, the exosomes were washed with PBS and centrifuged at 100,000 g for 2 h to remove the unbound Dil. Dil-labeled exosomes were added to the culture medium of C-kit⁺ CSCs at a concentration of 10 μg/ml for 24 h. Then, the C-kit⁺ CSCs were washed with PBS, fixed in 4% paraformaldehyde, and stained with 1 mg/ml 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA) for 10 min. Finally, the fluorescence was observed under a fluorescence microscope (Olympus).

2.11. Flow cytometry assay of apoptosis in C-kit⁺ CSCs
The C-kit⁺ CSCs were pre-incubated with different treatments (2 × 10⁹ particles per ml) and then incubated with 100 μM H₂O₂ for 2 h. Following treatment, the apoptosis rate was analyzed by flow cytometry using the Annexin V-FITC/PI kit (Solarbio, China) according to the manufacturer’s instructions. Flow cytometry was performed twice using C-kit⁺ CSCs in three independent experiments.

2.12. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for detecting the apoptosis of C-kit⁺ CSCs
The percentage of apoptotic cells was also detected by using In-situ cell death detection kit (Sigma, USA) following the manufacturer’s protocol. In brief, cells were planted on Petri dish.
After the cells were fixed with 4% paraformaldehyde and permeabilized by incubating with 1% Triton X-100, they were incubated in 50 μl/slide TUNEL reaction mixture (viaL1:viaL2 = 1:9) at 37°C with in darkness for 60 min under a humidified atmosphere. After incubation, the cells were stained with Hoechst33258 for 5 min. Apoptotic cells were counted in random fields by fluorescence microscopy; each experiment was performed in triplicate (×40 magnification, at least 6 fields per sample).

### 2.13. Western blotting

A Western blot analysis of the total protein from the C-kit⁺ cell lysates was performed as previously described[55]. The protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After blocking overnight in a nonfat milk solution, the membranes were probed with the anti-PTEN, -phospho-Akt, -Akt, -caspase-3, -cleaved caspase-3, -β-actin or -GAPDH primary antibodies. The PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and then exposed to an enhanced chemiluminescence substrate (Amersham Biosciences, USA). The immunoreactivity was visualized using a ChemiDoc MP system (Bio-Rad, USA). The protein levels were normalized to β-actin or GAPDH.

### 2.14. Statistical analysis

All data were analyzed by performing Student’s t-tests or one-way ANOVAs, followed by LSD or Dunnett’s T3 post-hoc test for multiple comparisons. A P-value less than 0.05 was considered statistically significant. The data analyses were performed using SPSS software (v.19.0, IBM, USA). The data are presented as the mean ± SD.

### 3. Results

#### 3.1. Isolated MSCs and C-kit⁺ CSCs

C-kit⁺ CSCs were purified using anti-rabbit secondary antibody-conjugated magnetic beads. The morphology of C-kit⁺ CSCs was triangular or polygonal (Fig 1(A)). According to flow cytometry analysis, 90.99% of the cells were positive for C-kit, 0.09% of the cells were positive for CD45, and 0.12% of the cells were positive for CD34 (Fig 1(B)). Primary MSCs isolated from the rats began adherent growth after 48 h of in vitro culture. Primary MSCs sub-cultured for 2–4 generations had a long spindle or polygonal appearance (Fig 1(C)). The following surface markers were identified on the MSCs by flow cytometry: (1) CD29 98.65%, (2) CD90 98.63%, and (3) CD45 0.09% (Fig 1(D)).

**3.2. Exosomes secreted by MSCs were isolated and identified**

MSC-Exos were obtained by precipitation. Then, the morphology of the exosomes was confirmed by performing transmission electron microscopy (TEM) and Western blotting as previously described[56]. The exosomes had a round or oval-shaped appearance and were approximately 30–100 nm in size as directly observed by TEM(Fig 1(E)-A), and the size of exosome was not changed when MSCs are exposed to H₂O₂ (Fig 1(E)-B). The exosome surface markers CD63, CD9 and HSP70 could be detected in MSC-Exos by Western blotting (Fig 1(F)).

**3.3. Oxidative stress induced apoptosis in the C-kit⁺ CSCs and altered the expression of miR-21 in MSCs, CSCs and exosomes**

We established an in vitro model of C-kit⁺ CSC apoptosis by treating the cells with 100 μM H₂O₂ for 2 h. Western blotting was performed to detect the expression of the mitochondria-
Fig 1. Characterization of C-kit+ CSCs, MSCs, and exosomes. (a) Phase morphology of C-kit+ CSCs (Olympus, Japan); scale bar = 100 μm. (b) Representative flow cytometric characterization of C-kit+ CSCs for the typical surface antigens and isotype control after magnetic bead sorting; surface expression of C-kit, and absence of surface expression of CD45, CD34. (c) MSC morphology was observed under a microscope (Olympus, Japan); scale bar = 100 μm. (d) MSCs were characterized by flow cytometric analysis for typical surface antigens or isotype control; surface expression of CD29, CD90, and absence of surface expression of CD45. (e) A transmission electron microscope was used to analyze MSC-derived exosomes. Images show a round-shaped vesicle with a diameter of approximately 100 nm. Scale bar = 100 nm/50 nm. (f) Western blotting characterization of the CD63, CD9, and Hsp70 MSC-Exos markers.

https://doi.org/10.1371/journal.pone.0191616.g001
Fig 2. $H_2O_2$ affects C-kit$^+$ CSC apoptosis and changes the expression of miR-21 in MSCs, C-kit$^+$ CSCs and exosomes. (a) and (b) Western blotting analysis of caspase-3 and cleaved caspase-3 in C-kit$^+$ CSCs cells after treatment with $H_2O_2$. (c) Rates of apoptosis in C-kit$^+$ CSCs exposed to 100 μM $H_2O_2$ for 2 h measured by performing an Annexin V-FITC/PI staining assay. The upper left quadrant (% Gated) shows the necrotic cells (Annexin V- /PI+), The upper right quadrant (% Gated) shows the late apoptotic cells (Annexin V+/PI+), The left lower quadrant (% Gated) shows the live cells (Annexin V- /PI-) and The right lower quadrant (% Gated) shows the early apoptotic cells (Annexin V+/PI-). PI = propidium iodide. (d) The percentage of apoptotic cells was representing as both early and late apoptotic cells compared with Control groups. (e) Effects of $H_2O_2$ on miR-21 expression in C-kit$^+$ CSCs. (f) Effects of $H_2O_2$ on miR-21 expression in MSCs (g) miR-21 expression levels in exosomes after exposure to $H_2O_2$ (n = 3, *P<0.05 versus control groups).

https://doi.org/10.1371/journal.pone.0191616.g002
Fig 3. MSC-Exos inhibit H$_2$O$_2$-induced apoptosis in C-kit$^+$ CSCs. (a) Cellular internalization of mesenchymal stem cell (MSC)-Exos into C-kit$^+$ CSCs. Dil-labeled MSC-Exos (red) were internalized into DAPI-labeled CSCs (blue). Bar = 20 μm. (b) Apoptosis rates of C-kit$^+$ CSCs were measured using the Annexin V-FITC/PI staining assay. The upper left quadrant (% gated) shows the necrotic cells (Annexin V-/PI-), The upper right quadrant (% gated) shows the late apoptotic cells (Annexin V+/PI+), The left lower quadrant (% gated) shows the live cells (Annexin V-/PI-), PI = propidium iodide. (c) The percentage of apoptotic cells was representing as both early and late apoptotic cells. (n = 3, *P<0.05 versus the H$_2$O$_2$ group). (d) Immunoblotting was performed to detect caspase-3 and cleaved caspase-3 in C-kit$^+$ CSCs (n = 3, *P<0.05 versus the control group). (e) And (f) Representative immunofluorescence staining of Hoechst33258 (blue), TUNEL (green) and merged images. Photos were taken randomly using fluorescence microscopy. Scale bar: 20 μm. (g) The panel shows the percentages of TUNEL positive cells. (n = 6, *P<0.05 versus the control group). (h) RT-qPCR analysis of miR-21 in C-kit$^+$ CSCs treated with 100 μM H$_2$O$_2$ after pre-protection with N-Exos or H-Exos (n = 9, *P<0.05 versus the H$_2$O$_2$ group).

related pro-apoptotic protein cleaved caspase-3, which is the active form of caspase-3. Treatment with 100 μM H$_2$O$_2$ up-regulated the levels of cleaved caspase-3 in C-kit$^+$ CSCs (Fig 2(A) and 2(B)). According to flow cytometry analysis, H$_2$O$_2$ challenge resulted in apoptosis increasing of C-kit$^+$ CSCs in comparison to the control (Fig 2(C) and 2(D)). miR-21 levels were also examined in H$_2$O$_2$-treated C-kit$^+$ CSC cells, and the result showed miR-21 levels were markedly reduced in C-kit$^+$ CSCs following H$_2$O$_2$ treatment (Fig 2(E)), suggesting that miR-21 is likely negatively correlated with apoptosis in C-kit$^+$ CSCs under oxidative stress conditions. In addition, compared with the controls, H$_2$O$_2$ treatment significantly reduced the expression of miR-21 in MSCs (Fig 2(F)). However, compared with the controls, the expression of miR-21 in MSC-Exos was up-regulated following H$_2$O$_2$-treatment (Fig 2(G)).

3.4. MSC-derived exosomes prevented H$_2$O$_2$-induced C-kit$^+$ CSC apoptosis

The unique biological function of exosomes is mainly to mediate cell-to-cell communication. The first step in the exchange of cargoes between cells is the internalization of exosomes by the target cells. To determine whether MSC-Exos can be internalized by C-kit$^+$ CSCs, MSC-Exos were labeled with Dil. After incubation, labeled MSC-Exos (400 μg/ml) were combined with C-kit$^+$ CSCs for 24 h and counterstained with DAPI to visualize the nuclei. Immunofluorescence staining showed strong red fluorescence in the cytoplasm and a blue nucleus in C-kit$^+$ CSCs (Fig 3(A)), indicating that many MSC-Exos were internalized by C-kit$^+$ CSCs. The anti-apoptotic effect of MSC-derived exosome was detected with Annexin V/PI assay. The Annexin V/PI assay showed that oxidative stress preconditioning MSC-derived exosome (H-Exo) decreased the percentage of the apoptotic cells compared with the normoxia preconditioning BMSC-derived exosome (N-exo) group and H2O2 group (Fig 3(B) and 3(C)). To examine whether BMSC-derived exosome protected against H$_2$O$_2$-Induced DNA fragmentation in C-kit$^+$ CSCs, As shown in (Fig 3(F) and 3(G)), the percentages of TUNEL$^+$ cells were significantly higher following treatment with H$_2$O$_2$ compared with control group, while H-Exo could significantly reduce the TUNEL$^+$ cells compared with H$_2$O$_2$ group or N-exo group. Expectedly, caspase-3 cleavage was suppressed (Fig 3(D) and 3(E)), and the decrease in miR-21 levels was significantly rescued (Fig 3(H)) in receptor cells under oxidative stress following pretreatment with exosomes derived from H$_2$O$_2$-treated MSCs. Therefore, H-Exo might exert a strong protective effect that helps C-kit$^+$ CSCs resist apoptosis caused by oxidative stress. During this process, miR-21 likely plays an important role.

3.5. miR-21 in MSC-Exos participated in the protection of C-kit$^+$ CSCs from apoptosis

We investigated whether the effects of MSC-Exos on H$_2$O$_2$-induced apoptosis in C-kit$^+$ CSCs were dependent on miR-21. MSCs were transfected with miR-21 mimics or inhibitors for 48 h. Then, MSC exosomes were harvested. The harvested exosomes were designated either
Fig 4. MSC-exosomal miR-21 inhibits apoptosis in C-kit$^+$ CSCs. (a) RT-qPCR was performed to analyze miR-21 levels in C-kit$^+$ CSCs (n = 9, $P<0.05$ versus the H-Exo group). (b) Apoptosis rates of C-kit$^+$ CSCs were measured using the Annexin V-FITC/PI staining assay. The upper left quadrant (% Gated) shows the necrotic cells (Annexin V- /PI-), the upper right quadrant (% Gated) shows the late apoptotic cells (Annexin V+/PI+), the left lower quadrant (% Gated) shows the live cells (Annexin V+/PI-), and the right lower quadrant (% Gated) shows the early apoptotic cells (Annexin V+/PI-). PI = propidium iodide. (c) The percentage of apoptotic cells was representing as both early and late apoptotic cells. (n = 3, $P<0.05$ versus the H-Exo group). (f) Representative immunofluorescence staining of Hoechst33258 (blue), TUNEL (green) and merged images. Photos were taken randomly using fluorescence microscopy. Scale bar: 20 μm. (g) The panel shows the percentages of TUNEL positive cells. (n = 6, $P<0.05$ versus the H$_2$O$_2$ group). (a) and (f) Western blot analysis of pro-apoptotic protein caspase-3 and cleaved caspase-3 in C-kit$^+$ CSCs (n = 3, $P<0.05$ versus the H$_2$O$_2$ group). 

Mimics-Exosome (M-Exo) or Inhibitor-Exosome (I-Exo). According to RT-qPCR analysis of miR-21, miR-21 significantly increased and decreased following the pretreatment of H$_2$O$_2$-treated CSCs with M-Exo or I-Exo, respectively (Fig 4(A)). We also blocked miR-21 in C-kit$^+$ CSCs using a miR-21 inhibitor for 48 h. Compared with that in the H-Exo or I-Exo groups, the miR-21 inhibitor treatment further decreased the expression of miR-21 after H$_2$O$_2$ insult (Fig 4(A)).

The anti-apoptotic effect of miR-21 in MSC-Exos was detected using the Annexin V-FITC/PI staining assay and the TUNEL measurement assays. M-Exos significantly decreased C-kit$^+$ CSCs after H$_2$O$_2$ insult, while I-Exos increased apoptosis in C-kit$^+$ CSCs (Fig 4(B)–4(E)). When we simultaneously inhibited miR-21 in CSCs, we found that C-kit$^+$ CSC apoptosis rates markedly increased under I-Exo+inhibitor conditions (Fig 4(B)–4(E)). Undoubtedly, the miR-21 inhibitor significantly increased the expression of pro-apoptotic protein—cleaved caspase-3, whereas H-Exos or M-Exo suppressed cleaved caspase-3 levels (Fig 4(F) and 4(G)) in C-kit$^+$ CSCs under oxidative stress conditions. Thus, the miR-21 inhibitor could partially block the anti-apoptosis properties of exosomal miR-21, further indicating that rescuing the decreased miR-21 levels in C-kit$^+$ CSCs by an H-Exo treatment might be a possible strategy to protect C-kit$^+$ CSCs against oxidative stress-induced apoptosis.

3.6. Contribution of PTEN to the anti-apoptotic effects of miR-21 in C-kit$^+$ CSCs

Because PTEN has been shown to be a target gene of miR-21[39, 57, 58], we performed gain- and loss-of-function assays to verify the effects of miR-21 inhibitors and mimics on PTEN expression in C-kit$^+$ CSCs. Compared with the control, the PTEN protein was significantly up-regulated in the inhibitor group and down-regulated in the mimic group, while the PTEN mRNA levels did not change (Fig 5(A)–5(C)) in the C-kit$^+$ CSCs. Furthermore, according to the RT-qPCR and Western blot analyses, the mRNA and protein levels of PTEN were significantly up-regulated in C-kit$^+$ CSCs after pretreatment with H$_2$O$_2$ (Fig 5(D)–5(F)). Therefore, miR-21 likely attenuates apoptosis by targeting PTEN. However, whether a relationship exists between PTEN and apoptosis in C-kit$^+$ CSCs remains unknown. Thus, EGFP-labeled siRNA PTEN lentiviruses (siR-PTEN) and EGFP-labeled siRNA PTEN Negative Control vector (siR-PTEN-NC) were transfected into C-kit$^+$ CSCs. The knockdown efficiency of siR-PTEN was detected by RT-qPCR and Western blotting, and the PTEN mRNA and protein levels were significantly down-regulated in siR-PTEN group (Fig 5(G)–5(I)). The percentage of apoptotic cells significantly decreased in the siR-PTEN group compared to the H$_2$O$_2$ group or the siR-PTEN-NC group (Fig 6(A)–6(D)) as demonstrated by the Annexin V/PI assay and TUNEL measurement assays. Moreover, according to the Western blot analysis, cleaved caspase-3 levels were down-regulated in the siR-PTEN group compared with those in the H$_2$O$_2$ groups (Fig 6(E) and 6(F)). Altogether, the anti-apoptotic effects of miR-21 in C-kit$^+$ CSCs were likely achieved via the inhibition of PTEN expression.
Fig 5. Effect of miR-21 on PTEN expression in CSCs. (a) PTEN mRNA did not significantly differ among groups by RT-qPCR. (b)and(c) the PTEN protein levels dramatically decreased after treatment with miR-21 mimics as demonstrated by Western blotting (n = 3, *P<0.05 versus control groups, #P<0.05 versus inhibitor groups). (d) Difference in PTEN mRNA expression between the Control group and the H2O2 group confirmed by RT-qPCR. (n = 3, *P<0.05 versus control groups) (e)and(f) Western blot analysis of PTEN in C-kit+ CSCs treated with 100 μM H2O2 (n = 3, *P<0.05 versus the control group). (g) RT-qPCR analyzed PTEN in CSC after different conditions treated (n = 9, *P<0.05 versus control group). (h)and(i) Western blot analysis of PTEN protein levels after transfection of C-kit+ CSCs with siR-PTEN (n = 3, *P<0.05 versus control group).

https://doi.org/10.1371/journal.pone.0191616.g005
3.7. MSC-derived exosomes protected CSCs from H$_2$O$_2$-induced apoptosis via the PTEN/PI3K/Akt pathway

To identify the mechanisms responsible for the MSC-derived exosomal miR-21-mediated anti-apoptotic effects in C-kit$^+$ CSCs, we blocked PI3K with the specific inhibitor LY294002. The Annexin V-FITC/PI staining assay and the TUNEL measurement assays were used to detect the apoptotic cells. LY294002 partially reversed the anti-apoptotic effects of H-Exos (Fig 7(A)–7(D)). Molecularly, LY294002 reversed the H-Exo-induced effects on cleaved caspase-3 expression (Fig 7(E)), which was demonstrated by an increase in cleaved caspase-3 levels (Fig 7(F) and 7(G)). Furthermore, the miR-21 inhibitor could also reverse the anti-apoptotic effects of H-Exo. In this experiment, RT-qPCR showed that compared with the $H_2O_2$ group, H-Exo, miR-21 inhibitors or PI3K inhibitor LY294002 did not influence PTEN expression levels (Fig 8(A) and 8(B)), PTEN protein was significantly down-regulated in the H-Exo group. Additionally, the exosomes incubation increased p-Akt levels (Fig 8(C)–8(F)), while the miR-21 inhibitor and PI3K inhibitor LY294002 dramatically decreased p-Akt levels (Fig 8(C)–8(F)).

4. Discussion

C-kit$^+$ cardiac stem cells (C-kit$^+$ CSCs) have emerged as some of the most promising CSCs for the prevention or treatment of myocardial remodeling and cardiac dysfunction after myocardial infarction[59]. However, after adoptive transfer, CSCs will encounter with various undesirable factors including oxidative stress, inflammation reactions and so on. all of which could decrease the cell viability and thereby compromise their therapeutic activities.[10]. Exosomes are intracellular messengers whose contents have been confirmed to be crucial signaling components for downstream reactions[20, 60]. Exosomes derived from MSCs could stimulate the proliferation, migration, and angiogenic potency of CSCs in vitro and in vivo and improve cardiac function[16]. However, very few studies have focused on the anti-apoptotic effects of MSC-Exos in C-kit$^+$ CSCs under oxidative stress conditions. Additionally, the underlying molecular mechanisms by which exosomes protect C-kit$^+$ CSCs must be elucidated. In this study, we obtained exosome vesicles (round, 30–100 nm) from conditioned MSC medium (Fig 1(E)) and confirmed the identity of these vesicles by detecting the expression of specific surface markers (Fig 1(F)).

Indeed, transplanted C-kit$^+$ CSCs and pretreatment conditions must remain in an oxidative stress environment. Thus, establishing a similar pathological state in which to study the effects of exosomes in transplanted C-kit$^+$ CSCs embedded in infarct zone or infarct border zones is imperative. miRNAs, which are shuttled by exosomes, are among the most important factors controlling gene expression. Additionally, exosomal miR-21 is up-regulated in many cell types under oxidative stress conditions[52, 61]. The exosome contents, however, greatly vary across different cell types and under different pathological conditions[22]. We also found that miR-21 reduces hydrogen peroxide-induced apoptosis and increases cell proliferation in c-kit cardiac stem cells in vitro through PTEN/PI3K/Akt signaling before[47, 62]. $H_2O_2$ has been widely used as an inducer of oxidative stress to mimic the pathophysiology of cardiovascular...
Exosome and cardiac stem cells
Fig 7. Contribution of the PTEN/P13K/Akt axis to H2O2-induced apoptosis in C-kit+ CSCs. (a) Flow cytometry was performed to detect apoptosis using Annexin V-FITC/PI staining in C-kit+ CSCs that underwent different treatments. The first quadrant (% Gated) shows the necrotic cells (Annexin V−/PI+). The second quadrant (% Gated) shows the late apoptotic cells (Annexin V+/PI+). The third quadrant (% Gated) shows the live cells (Annexin V−/PI−) and the fourth quadrant (% Gated) shows the early apoptotic cells (Annexin V+/PI−). PI = propidium iodide. (b) The percentage of apoptotic cells was representing as both early and late apoptotic cells. (n = 6, *P < 0.05 versus the H2O2 group. †P < 0.05 versus the H-Exo group). (c) Representative immunofluorescence staining of DAPI (blue), Cleaved–Caspase-3 (red) and merged images. Photos were taken randomly using fluorescence microscopy. Scale bar: 20 μm. (d) The panel shows the percentages of TUNEL positive cells. (n = 6, *P < 0.05 versus the H2O2 group. †P < 0.05 versus the H-Exo group). (e) Representative immunofluorescence staining of Hoechst33342 (blue), TUNEL (green) and merged images. Photos were taken randomly using fluorescence microscopy. Scale bar: 20 μm. (f-g) Apoptotic was further confirmed by immunoblotting for cleaved-caspase-3, (n = 3, *P < 0.05 versus the H2O2 group. †P < 0.05 versus the H-Exo group).

https://doi.org/10.1371/journal.pone.0191616.g007

disease and cause cell apoptosis[28]. Therefore, we evaluated miR-21 expression in MSC-derived exosomes treated with the same concentration of H2O2. The 2h H2O2 treatment significantly induced C-kit+ CSC apoptosis (up to 78.1%). Consistently, H2O2 treatment induced the up-regulation of the pro-apoptosis protein cleaved caspase-3, which was associated with significantly reduced miR-21 expression levels (Fig 2(A)–2(E)). The negative correlation between apoptosis and miR-21 expression suggests that miR-21 plays an important role in the regulation of C-kit+ CSC apoptosis under oxidative stress conditions. Moreover, compared with the control group, H2O2 significantly reduced miR-21 expression in MSCs (Fig 2(F)). Interestingly, compared with that in the untreated group, the expression of miR-21 in the MSC-exosomes was up-regulated after H2O2-treatment (Fig 2(G)).

The harsh ischemic microenvironment in acute MI, which kills most injected cells, is a primary barrier limiting the effectiveness of stem cell transplantation. Preconditioning C-kit+ CSCs with MSC-Exos may serve as a promising therapeutic approach because the useful cellular components encapsulated in MSC-Exos may greatly improve the survival rate of C-kit+ CSCs in ischemic environments. In this study, exosomes released from MSCs were internalized by C-kit+ CSCs (Fig 3(A)), and the levels of miR-21 were significantly increased in C-kit+ CSCs pre-treated with H-Exos and N-Exos (Fig 3(H)) prior to oxidative stress exposure. Moreover, the exosomes derived from H2O2-treated MSCs were more effective at increasing miR-21 levels in receptor cells and decreasing C-kit+ CSC apoptosis under oxidative stress conditions. Moreover, compared with the control group, H2O2 significantly reduced miR-21 expression in MSCs (Fig 2(F)).

To further confirm the anti-apoptotic effects of miR-21 from MSC-Exos after H2O2 treatment, we blocked miR-21 in C-kit+ CSCs and/or MSCs using a miR-21 inhibitor. Consistently, miR-21 inhibition significantly down-regulated miR-21 levels and partially reversed the anti-apoptotic effects of H-Exos (Fig 4(A)–4(G)). In conclusion, our data indicated an intricate exosome-mediated crosstalk interface between the MSCs and the CSCs that regulates the oxidative damage program, at least partly, via miR-21.

miRNA transfer between cells can activate the recipient cells to produce a series of biological effects by inhibiting miRNA target genes. PDCD4, PTEN, RECK and Bcl-2 can be regulated by miR-21 in many cell types. These genes are critical for promoting cell proliferation, differentiation and migration[63–65]. PTEN has been reported to be a target gene of miR-21 in many cell types[3, 39]. To further confirm that PTEN is a target of miR-21 in C-kit+ CSCs, gain- and loss-of-function studies were performed, and miR-21 inhibitors increased while miR-21 mimics decreased PTEN protein levels in C-kit+ CSCs; however, PTEN mRNA levels did not change (Fig 5(A)–5(G)). We further tested whether PTEN is involved in the regulation of H2O2-induced apoptosis in C-kit+ CSCs and showed that the PTEN expression is significantly up-regulated in C-kit+ CSCs following the H2O2 treatment (Fig 5(D)–5(F)). Furthermore, using siRNA-mediated gene silencing, the siR-PTEN vector efficiently infected the C-kit+ CSCs, and the PTEN mRNA/protein levels were efficiently inhibited(Fig 5(G)–5(I)). The inactivation of PTEN significantly decreased the rate of cell apoptosis (Fig 6(A)–6(F)). Altogether, miR-21 mediates cell protection by regulating PTEN.
Fig 8. Contributions of the PTEN/PI3K/Akt pathway to the anti-apoptotic effects of MSC-derived exosomal miR-21. (a) RT-qPCR was performed to detect PTEN expression at the mRNA level in cells that underwent different treatments (n = 3, * P < 0.05 versus the control group). (b) RT-qPCR was performed to detect
miR-21 affects the PI3K/Akt pathway by targeting the PTEN gene[39]. The activation of Akt can protect cells from apoptosis induced by H₂O₂[66, 67]. To study whether the PTEN/PI3K/Akt signaling is responsible for exosomal miR-21 mediated anti-apoptotic effect, we blocked PI3K with LY294002 and assessed Akt phosphorylation. LY294002 significantly reversed the anti-apoptotic effects of H-Exos (Fig 7(A)–7(D)), which were associated with increased levels of cleaved caspase-3 (Fig 7(E)–7(G)). Additionally, the miR-21 inhibitor could also block the anti-apoptotic effects of MSC-Exos. H-Exos decreased PTEN levels and increased p-Akt levels, while miR-21 inhibitors dramatically decreased p-Akt levels (Fig 8(A)–8(F)). This finding not only suggests that Akt is downstream of PI3K and PTEN but also indicates that the cellular protection provided by H-Exos likely occurs via miR-21 and its regulation of the PTEN/PI3K/Akt signaling pathway.

**Conclusion**

Exosomes carrying miR-21 can be effectively internalized into C-kit⁺ CSCs to protect these cells against apoptosis under stress conditions. This cargo successfully reduced PTEN expression, increased p-Akt levels, and exerted anti-apoptotic effects in C-kit⁺ CSCs. This effect can be compromised by miR-21 inhibitors and LY294002. Therefore, MSC-exosomes, particularly H-Exos, can rescue C-kit⁺ CSC apoptosis by regulating the miR-21/PTEN/PI3K/AKT axis under oxidative stress conditions. Although our data revealed that exosomal miR-21 derived from H₂O₂-induced MSCs plays a critical role in apoptosis regulation in recipient cells through the PTEN/PI3K/Akt pathway, we did not explore the function of other exosomal cargo. *In vivo* studies are warranted to further confirm that MSC-exosomes and changes in the PTEN/PI3K/Akt pathway have similar effects on the survival of C-kit⁺ CSCs.

**Supporting information**

S1 File. Apoptotic rates of CSCs detected by Annexin V-FITC PI dual staining assay. (ZIP)

S2 File. Identification of MSC, exosomes and CSCs. (ZIP)

S3 File. Internalization of DiI-labeled exosomes into CSCs. (ZIP)

S4 File. qRT-PCR analyzed miR-21 and PTEN mRNA. (ZIP)

S5 File. Apoptotic related genes detected by western blotting. (ZIP)

S6 File. TUNEL staining detected the apoptosis of CSCs. (ZIP)

S7 File. TUNEL staining detected the apoptosis of CSCs. (ZIP)
Acknowledgments

This study was supported by a grant from the National Natural Science Foundation of China (Grant No. 81360021).

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