

# hUMSC-sEVs protect the heart against ischemia/reperfusion injury by delivering circRNA to inhibit ferroptosis

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**ABSTRACT:** Human umbilical cord mesenchymal stem cell-derived small extracellular vesicles (hUMSC-sEVs) have been shown to protect against myocardial ischemia/reperfusion (I/R) injury; however, whether they can alleviate cellular ferroptosis in this process and the underlying molecular mechanisms remain unclear. Our results showed that hUMSC-sEVs reduced infarct size, improved cardiac function, and inhibited cardiomyocyte ferroptosis in mice. Our study screened for functionally active circular RNAs (circRNAs) in hUMSC-sEVs and microRNAs (miRNAs) with altered expression following myocardial I/R via sequencing and further validated the targeted binding interaction between has\_circ\_0049673\_STX10 (circSTX10) and miR-490-3p through integrated bioinformatics prediction. Overexpression of circSTX10 in HL-1 cardiomyocytes alleviated miR-490-3p-induced ferroptosis, characterized by reduced ferrous iron accumulation, suppressed lipid peroxidation, and reversed downregulation of GPX4 expression. Our results showed that miR-490-3p was upregulated in the I/R model and promoted cardiomyocyte ferroptosis by downregulating hephaestin-like 1 (Heph11), which induces intracellular ferrous iron accumulation and lipid peroxidation. Collectively, this study revealed a novel mechanism whereby hUMSC-sEVs inhibit myocardial I/R injury via circSTX10 binding to miR-490-3p, thereby exerting cardioprotection against cardiomyocyte ferroptosis, which offers new insights into the development of therapeutic strategies for this injury.

**KEYWORDS:** extracellular vesicles, cardiomyocyte, oxidative stress, cell death

## INTRODUCTION

Myocardial ischemia/reperfusion (I/R) injury remains a major clinical challenge that significantly impairs cardiac function and worsens the prognosis of patients with acute myocardial infarction (AMI) undergoing revascularization therapies, including percutaneous coronary intervention and coronary artery bypass grafting [1–3]. Cardiomyocyte death is a major component of myocardial I/R injury, and its mechanism was once thought to be restricted solely to apoptosis and necrosis [4, 5]. Recent studies have implicated ferroptosis in myocardial I/R injury; factors that promote lipid peroxidation or interfere with iron metabolism can exacerbate ferroptosis [6, 7].

Mesenchymal stem cell-derived small extracellular vesicles (MSC-sEVs) are being investigated in regenerative medicine for the treatment of various tissue injuries [8–10]. They contain high levels of proteins, lipids, and nucleic acid cargo, which can be delivered to recipient cells as messengers and signal mediators, thereby altering cellular functions under physiological and pathological conditions [8, 11, 12]. Studies

on various diseases have demonstrated that bioactive molecules carried by human umbilical cord mesenchymal stem cell-derived small extracellular vesicles (hUMSC-sEVs) can promote angiogenesis, restore blood supply to damaged tissues, and expedite tissue repair [13, 14]. However, the potential involvement of MSC-sEVs in regulating myocardial ferroptosis during I/R injury and the underlying molecular mechanisms remain largely unknown.

Recent studies have shown that circular RNAs (circRNAs) derived from stem cells or their EVs can mediate cell-to-cell communication and exert protective effects against cardiovascular diseases. CircRNAs, a class of non-coding RNAs with covalently closed loop structures, exhibit high stability and tissue-specific expression [15]. They primarily function as microRNA (miRNA) sponges to regulate target gene expression, thereby participating in various pathological processes including myocardial I/R injury [16]. However, whether hUMSC-sEVs can deliver functional circRNAs to myocardial cells to alleviate ferroptosis during I/R injury, as well as the specific molecular pathways involved, have not been fully elucidated.

In this study, by sequencing myocardial tissue three days after I/R injury, we found that miR-490-3p expression was significantly upregulated. We demonstrated that increased levels of miR-490-3p inhibited hephaestin-like 1 (Heph1l) expression, consequently inducing cardiomyocyte ferroptosis. Hephaestin (Heph), a member of the multicopper ferroxidase family, oxidizes  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  and facilitates cellular iron export through the membrane transporter ferroportin-1 (FPN1) [17, 18]. We further demonstrated that decreased levels of Heph1l resulted in ferrous ion accumulation, elevated malondialdehyde (MDA) levels, increased reactive oxygen species (ROS) production, reduced GPX4 expression, and compensatory upregulation of SLC7A11, ultimately leading to cardiomyocyte ferroptosis.

Herein, we screened for circRNAs in hUMSC-sEVs that could bind miR-490-3p and demonstrated their protective effects against cardiomyocyte ferroptosis. This study demonstrated that hsa\_circ\_0049673\_STX10 (circSTX10) in hUMSC-sEVs functioned as a molecular sponge for miR-490-3p. Additionally, we overexpressed circSTX10 in HL-1 cells using a lentiviral vector to assess whether circSTX10 in hUMSC-sEVs reversed miR-490-3p-induced ferroptosis. We found that miR-490-3p treatment increased MDA production and intracellular ferrous iron content, which were reversed by circSTX10 overexpression. Western blot (WB) analysis further revealed that circSTX10 elevation in HL-1 cells mitigated the miR-490-3p-induced reduction in GPX4 and the compensatory increase in SLC7A11. Therefore, hUMSC-sEVs could alleviate cardiomyocyte ferroptosis by transferring circSTX10.

## MATERIALS AND METHODS

### Animals

Eight-week-old male C57BL/6J mice were obtained from Shenzhen TOP Biotechnology Co., Ltd., China, and housed in a specific pathogen-free laboratory animal facility operated by the Company. The animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals, as outlined by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). A total of 60 mice were used, with 20 mice in each group. All animal operations and experimental protocols were officially permitted by the animal laboratory platform of Shenzhen TOP Biotechnology Co., Ltd. (Approval No.: TOP-IACUC-2023-0340) and were carried out in accordance with standard ethical guidelines for laboratory animal research.

### Myocardial I/R model

Adult male C57BL/6J mice were anesthetized with a 1.25% tribromoethanol solution (160 mg/kg, i.p.), followed by tracheal intubation and ventilation using

a small animal ventilator. Subsequently, we performed a left thoracotomy at the third intercostal space and reversibly ligated the left anterior descending (LAD) coronary artery using a slipknot. The ligation site was 2 mm below the inferior margin of the left atrial appendage and 5–6 mm away from the cardiac apex. After 2 h of ischemia, the chest was reopened under ventilation, and reperfusion was induced by severing the ligature. Three *in situ* injections were delivered into the myocardial infarct region in the hUMSC-sEVs group, including two sites at 4 mm and one site at 3 mm from the cardiac apex, with 10  $\mu\text{l}$  per site. hUMSC-sEVs at a concentration of  $3 \times 10^8$  particles/ $\mu\text{l}$  were used, and the vehicle control was given the same volume of PBS. A surgical incision was made in mice in the sham group, but the LAD was not ligated. All surgeries and analyses were performed by investigators who were blinded to treatment. The survival rate was 100% in the sham group, 77% in the hUMSC-sEVs group, and 71% in the PBS group.

### Analysis of infarct size

The mice were euthanized using  $\text{CO}_2$  three days after modeling. The hearts were subsequently collected and rinsed with phosphate buffered saline (PBS) before freezing at  $-80^\circ\text{C}$  for 3 min. After horizontal slicing of the heart, the slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, USA) and incubated at  $37^\circ\text{C}$  for 15 min to visualize the unstained infarcted region. Evans blue staining to determine the area at risk was not performed. The infarct area was quantified by planimetry using ImageJ software.

### Echocardiography

To check the heart function of each mouse three days after I/R injury, echocardiography was performed using the Vevo 2100 echocardiography machine (VisualSonics Inc., Toronto, Ontario, Canada). M-mode images were captured from the short-axis view at the papillary muscle levels in the parasternal position. The Vevo 2100 system and software were used for data acquisition and analysis. The operator and data analyzer were blinded to the sample grouping.

### Cell culture and treatment

HL-1, AC16, and 293T cells were acquired from Shanghai QuiCell Biotechnology (Shanghai, China). HL-1 and 293T cells were cultured in MEM and DMEM supplemented with 10% fetal bovine serum (BasalMedia, Shanghai, China) in a 5%  $\text{CO}_2$  atmosphere at  $37^\circ\text{C}$ . AC16 cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (BasalMedia). All cells underwent a maximum of 10 passages before the experiment. The CALNP™ RNAi reagent (D-Nano Therapeutics, Beijing, China) was used for miRNA transfection, and Heph1l mRNA expression, cell viability, ROS, and intracellular  $\text{Fe}^{2+}$  levels were detected at

48 h after transfection. Co-transfection of plasmid and miRNA was performed using Starvio DNA and siRNA transfection reagent (StarVio, Shanghai, China), and cell viability, MDA content, intracellular  $\text{Fe}^{2+}$  levels, and GPX4 protein expression were detected at 48 h after transfection.

#### Isolation of MSC-sEV

MSC-sEVs were isolated using ultracentrifugation. The supernatants were sequentially centrifuged at 300 and  $2,000 \times g$  for 10 min, followed by  $120,000 \times g$  for 1 h. Centrifugation was conducted at  $4^\circ\text{C}$ . Subsequently, the sEVs and the supernatant were separated, and the resulting hUMSC-sEVs pellets were collected and resuspended in PBS.

#### Particle measurement by nanoparticle tracking analysis (NTA)

Extracellular vesicles were resuspended in PBS. Nanoparticle tracking analysis was used to record the Brownian motion of particles in the solution over time. The hydrodynamic size and concentration of extracellular vesicles were calculated using the Stokes-Einstein equation. MSC-sEVs were characterized by OBiO Technology (Shanghai, China).

#### Transmission electron microscope (TEM)

Extracellular vesicles were resuspended in PBS and loaded onto carbon-coated copper grids for 1 min. The grids were then negatively stained with 10  $\mu\text{l}$  of 2% uranyl acetate. Finally, the grids were observed under a transmission electron microscope (Hitachi H-7500, Tokyo, Japan).

#### Extracellular vesicle uptake assay

Cells were seeded on coverslips overnight. DiO working solution was prepared and filter-sterilized the next day. sEVs were incubated with DiO (Servicebio, Wuhan, China), and excess dye was eliminated via 100 kDa ultrafiltration. The hUMSC-sEVs pellet was resuspended in PBS. After medium replacement, DiO-labeled sEVs were co-cultured with HL-1 cells or AC16 cells for 6 h. Cells were fixed with 4% paraformaldehyde, stained with phalloidin and DAPI (both from Servicebio), and finally examined microscopically.

#### Prussian blue staining

Paraffin-embedded tissues were cut into 3–7  $\mu\text{m}$  sections, followed by routine deparaffinization and rehydration. The Prussian Blue Iron Stain Kit (G1428, Solarbio, Beijing, China) was equilibrated at  $25\text{--}30^\circ\text{C}$  for 20 min, and a humidified box was prewarmed to  $37^\circ\text{C}$  for 20 min. Fresh Perls working solution (1:1, v/v) was applied to the sections and incubated for 20 min at  $37^\circ\text{C}$ . The sections were then rinsed with distilled water and treated with the secondary incubation solution for 10–20 min at  $37^\circ\text{C}$ . After washing with PBS, enhancement working solution (C1:C2:PBS

= 1:1:18, stable at  $2\text{--}8^\circ\text{C}$  for 7 days) was applied for 10–20 min. After PBS rinsing, sections were counterstained for 3–5 min, washed with water, dehydrated in graded ethanol, cleared in xylene, and mounted with neutral balsam.

#### Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA and miRNA were extracted using the RNA Simple Total RNA Kit (TIANGEN, Beijing, China) and the MiPure Cell/Tissue miRNA Kit (Vazyme, Nanjing, China). Subsequently, RNA samples were reverse-transcribed into first-strand complementary DNA (cDNA) using the 1st Strand cDNA Synthesis SuperMix (YEASEN, Shanghai, China) for total RNA and the miRNA 1st Strand cDNA Synthesis Kit (by stem-loop; Vazyme) for miRNA. Gene expression was quantified using a CFX Connect Real-Time PCR system (Bio-Rad, Hercules, USA) with the Hieff UNICON qPCR SYBR Green Master Mix (YEASEN). The target-gene expression levels were normalized to *Actb* (mouse cells and tissues) and quantified using the  $2^{-\Delta\Delta\text{Ct}}$  approach. The primer sequences are provided in Table S1).

#### Western blot analysis

Cells or tissues were washed three times with PBS and subsequently lysed on ice for 30 min using RIPA lysis buffer supplemented with a protease inhibitor (P0013, Beyotime Biotechnology, Shanghai, China). The protein concentration was measured using a BCA protein assay kit (Epizyme, Shanghai, China). Proteins were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequently transferred onto a 0.22  $\mu\text{m}$  nitrocellulose membrane (Beyotime Biotechnology). The membranes were blocked with protein-free rapid blocking solution (Epizyme) for 1 h. The membranes were washed three times with Tris-buffered saline Tween (TBST) and subsequently incubated overnight at  $4^\circ\text{C}$  with primary antibodies against GPX4 (A11243 1:1000, ABclonal, Wuhan, China), SLC7A11 (A2413, 1:1000, ABclonal), ALIX (ab275377, 1:1000, Abcam, Cambridge, UK), CD9 (CST13174S, 1:1000, CST, Danvers, USA), CD63 (ab134045, 1:1000, Abcam), calnexin (ab92573, 1:1000, Abcam), and  $\beta$ -tubulin (M20005S, 1:3000, Abmart, Shanghai, China). Subsequently, the membranes were incubated with secondary antibodies (ab205718, 1:3000, Abcam; LF101, 1:3000, Epizyme) for 1 h at room temperature. Protein bands were visualized using ChemiDoc MP (Bio-Rad).

#### Immunofluorescence staining

Cells on cover slips were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, and blocked with PBS containing bovine serum albumin to mask nonspecific binding sites. The samples were incubated overnight at  $4^\circ\text{C}$  with an anti-cTnI primary antibody (GB113806-50, 1:500, Servicebio), followed

by a 1 h incubation with a goat anti-rabbit secondary antibody (ab205718, 1:3000, Abcam). Nuclei were counterstained with DAPI, and the slides were examined under a fluorescence microscope.

#### Cell Counting Kit-8 assay

Cells were seeded at  $5 \times 10^3$  cells per well in 96-well plates; after the indicated transfection period, 10  $\mu$ l of CCK-8 reagent (C0038, Beyotime Biotechnology) was added to each well, followed by incubated at 37 °C for 1–2 h, and absorbance was measured at 450 nm.

#### Flow cytometry analysis

The cells were digested with Accutase and subsequently washed twice with PBS. Ferrous ions and ROS were detected by staining the cells with the Cellular Red Fluorescent Ferrous Ion Assay Kit with RhoNox-6 (S1070S) and the ROS Assay Kit (S0034S, 1:1000), both from Beyotime Biotechnology). All staining procedures were performed according to the manufacturer's protocols, and the labeled cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, USA).

#### MDA assay

Cell pellets were lysed using lysis buffer (P0013, Beyotime Biotechnology) and centrifuged at  $12,000 \times g$  for 10 min at 4 °C; the supernatant was then collected. The total protein concentration was measured using a BCA kit (Epizyme). The MDA content was measured using a commercial thiobarbituric acid (TBA) assay kit (S0131S, Beyotime Biotechnology) according to the manufacturer's instructions. Briefly, 100  $\mu$ l of the supernatant was mixed with 200  $\mu$ l of TBA working reagent, heated at 100 °C for 15 min in a heating block, cooled to room temperature in a water bath, and subsequently centrifuged at  $1,000 \times g$  for 10 min. The absorbance of the pink chromogen was measured at 532 nm using a Synergy H1 microplate reader (BioTek, Winooski, USA). MDA levels were calculated using a standard curve and normalized to the protein concentration (nmol/mg protein).

#### Package of lentivirus

A circ\_0049673-STX10 over-expression lentiviral vector (pSLenti-EF1-Puro-CMV-S-has\_circ\_0049673-WPRE) and corresponding empty control (pSLenti-EF1-Puro-CMV-MCS-WPRE) were constructed and packaged into lentiviruses. The sequence of has\_circ\_0049673-STX10 is provided below: has\_circ\_0049673-STX10: GTA TAG TGG AAG CCA ACC CAG GCA AGT TCA AGC TCC CAG CCG GGG ACC TGC AGG AGA GAA AGG TGT TCG TGG AGC GGA TGC GAG AGG CAG TCC AGG AAA TGA AGG ACC ATA TGG TCA GCC CAA CAG CCG TAG CAT TTT TGG AGA GGA ATA ACA GAG AGA TAC TCG CAG GCA AGC CAG CTG CCC AGA AGT CAC CCA GCG ACC TGC TGG ATG CCA GCG CAG TCT CGG CCA CAT CTC

GCT ACA TCG AGG AGC AGC AGG CCA CAC AGC AGC TGA TCA TGG ATG AAC AGG ATC AAC AGC TGG AGA TGG TGT CTG GGA GCA TCC AGG TTC TGA AGC ACA TGT CCG GCC GCG TTG GAG AAG AGC TGG ACG AGC AGG GCA TCA TGC TGG ATG CCT TCG CCC AAG AGA TGG ACC ACA CCC AGT CCC GCA TGG ACG GGG TCC TCA GGA AGT TGG CCA AAG TAT CCC ACA TGA CGA GTG ACC GCC GAC AGT GGT GTG CCA TCG CCG TGC TAG TGG GGG TGC TTC TCC TCG TTC TCA TCT TAC TAT TCT CTC TCT GA. The plasmids were transfected with pMD2.G and psPAX2. After 48 h, the supernatant was collected, and the lentivirus was isolated using a lentivirus concentration solution (YEASEN).

#### Dual-luciferase reporter assay

The wild-type and mutant miR-490-3p-binding sequences of circSTX10 were cloned into the pmirGLO dual-luciferase reporter vector (Obio Technology). Additionally, 293T cells were co-transfected with the resulting plasmids along with miR-490-3p mimic or miR-NC. Firefly and Renilla luciferase activities were measured 24 h post-transfection using the Dual-Luciferase Reporter Assay System (Shanghai Life-iLab Biotech Co., Ltd., China) according to the manufacturer's instructions.

#### Statistical analysis

GraphPad Prism software (version 9.0; GraphPad Software) was utilized for statistical analyses. Normally distributed data are presented as the mean  $\pm$  standard deviation. Independent-samples *t*-tests were utilized for comparisons between two groups, and one-way analysis of variance followed by Tukey's post hoc test was employed for comparisons among multiple groups. *p* < 0.05 was considered statistically significant.

## RESULTS

### hUMSC-sEV therapy protects the heart against I/R injury

A mouse model of myocardial I/R was established, with the sham group undergoing the same procedure but without coronary artery ligation. Three days after modeling, TTC staining, echocardiographic examination, and electrocardiography (ECG) confirmed successful establishment of the mouse myocardial I/R model used in this study.

To assess the efficacy of hUMSC-sEVs in treating myocardial I/R injury, we immediately performed an intramyocardial injection of hUMSC-sEVs after reperfusion, whereas the control group received vehicle PBS. Echocardiography conducted on day 7 post-treatment revealed that cardiac function in the hUMSC-sEV group was significantly better than that in the control group (Fig. 1A–D). On day 3 post-intervention, hearts were harvested for TTC staining

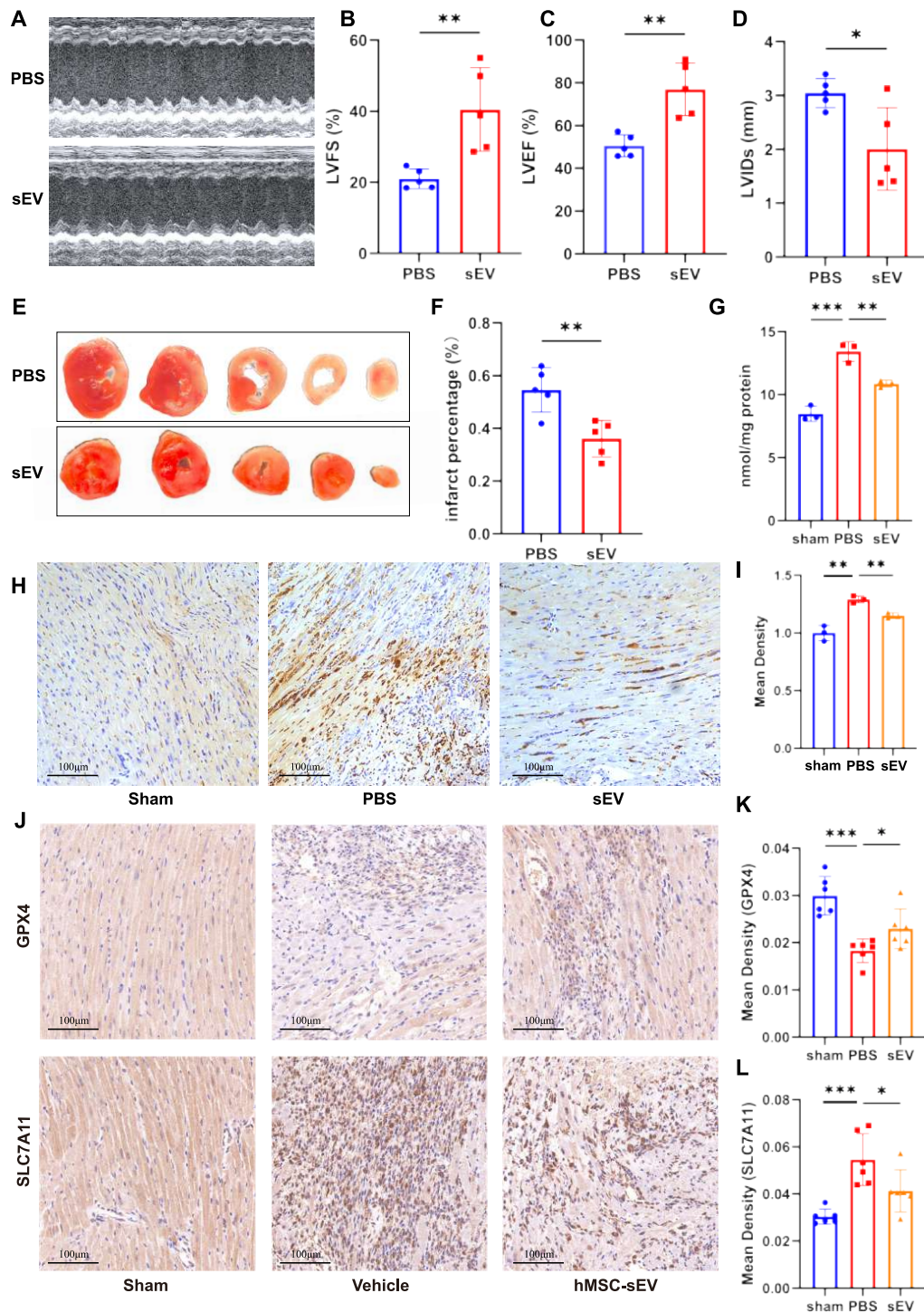


**Fig. 1** Intramyocardial injection of hUMSC-sEVs alleviating myocardial I/R injury in mice. (A) Representative M-mode echocardiographic changes in the hUMSC-sEV-treated group versus the control group. (B–D) Left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), and left ventricular internal diameter at end-systole (LVIDs) assessed using echocardiography in mice ( $n = 5$ ). (E) Representative images of TTC staining of the myocardium in the hUMSC-sEV-treated group versus the control group. (F) Statistical analysis of infarct area determined using TTC staining ( $n = 5$ ). (G) MDA content in myocardial tissue ( $n = 3$ ). (H) Representative images of Prussian blue staining for myocardial iron deposition. (I) Quantitative analysis of mean optical density from Prussian blue staining ( $n = 3$ ). (J–K) Statistical analysis of GPX4 and SLC7A11 expression levels from immunohistochemical staining ( $n = 5$ ). Scale bar = 100  $\mu\text{m}$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

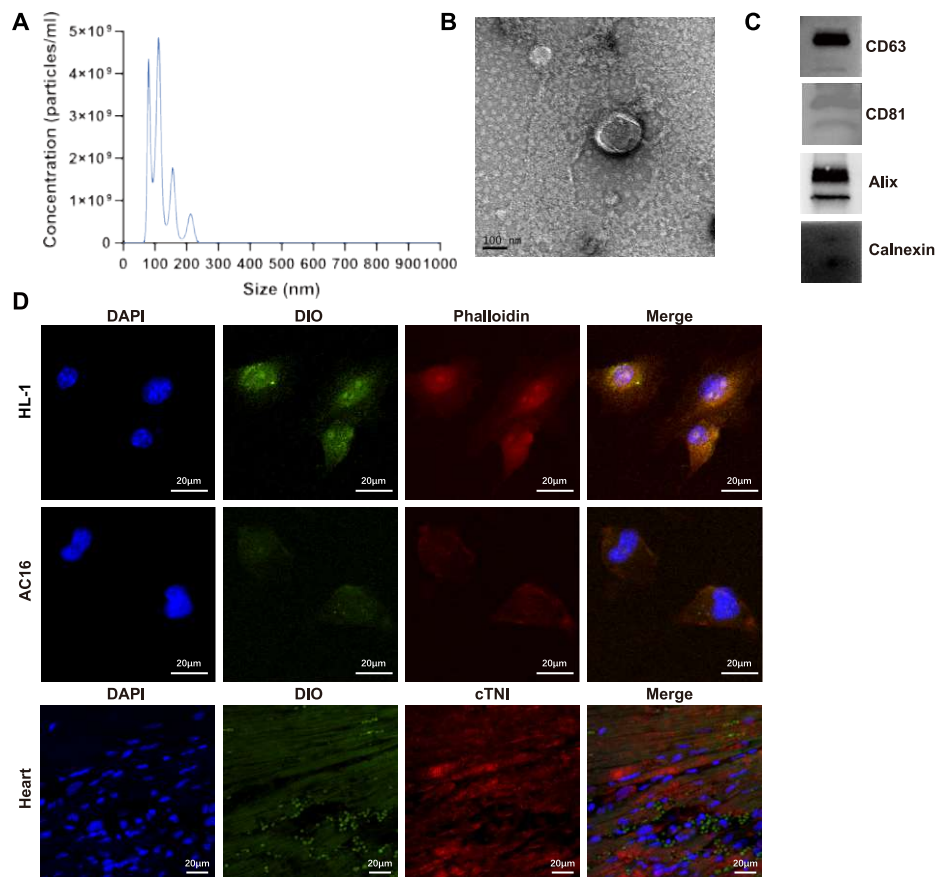


**Fig. 2** Isolation and identification of hUMSC-sEVs. (A) Results of a nanoparticle tracking analysis. (B) Results of TEM analysis. Scale bar = 100 nm. (C) WB analysis of the expression levels of CD63, CD81, Alix, and calnexin in hUMSC-sEVs. (D) EV uptake assay in HL-1 cells, AC16 cells, and cardiac tissue: DAPI (blue), cTNI (red), DiO-labeled hUMSC-sEVs (green); phalloidin and cTNI (red); scale bar = 20 μm.

to measure infarct size. The hUMSC-sEV-treated group exhibited a significantly smaller infarct area than the PBS control (Fig. 1E,F). Prussian blue staining was used to assess ferrous iron ( $\text{Fe}^{2+}$ ) deposition in myocardial tissues. The results revealed markedly reduced ferrous iron levels in the treatment group relative to the vehicle control group (Fig. 1H,I). Immunohistochemistry for ferroptosis-related proteins revealed that GPX4 expression decreased, whereas SLC7A11 expression increased in vehicle-treated hearts, and these alterations were notably reversed after hUMSC-sEV therapy (Fig. 1J-L).

sEVs were isolated and identified. NTA revealed a size distribution of 50–250 nm (Fig. 2A), and TEM demonstrated a typical cup-shaped morphology (Fig. 2B). WB analysis confirmed that hUMSC-sEVs expressed CD63, CD81, and Alix but not calnexin (Fig. 2C), indicating the successful isolation of hUMSC-sEVs which were then labeled with the lipophilic fluorescent dye DiO. DiO-tagged sEVs were co-cultured with HL-1 or AC16 cells *in vitro*, whereas *in vivo* hUMSC-sEVs administration was achieved via

*in situ* intramyocardial injection. These data demonstrated that hUMSC-sEVs can be internalized by cardiomyocytes both *in vitro* and *in vivo* (Fig. 2D).

### circSTX10 acts as a sponge for miR-490-3p

Recent studies have demonstrated the effectiveness of MSC-sEVs in reducing oxidative stress, decreasing lipid peroxidation, and inhibiting ferroptosis [19]. MSC-sEV-enriched miRNAs can target iron transporters in cardiomyocytes, thereby lowering intracellular  $\text{Fe}^{2+}$  levels, whereas the roles of circRNAs enriched in hUMSC-sEVs remain largely unknown. We then conducted transcriptome sequencing of the hUMSC-sEVs to identify potential therapeutic molecules. The data showed all circRNAs expressed in hUMSC-sEVs, ranked by normalized expression levels (Fig. 3A). Given their high stability and capacity to function as miRNA sponges, we hypothesized that sEV-enriched circRNAs might bind miRNAs. We performed miRNA sequencing of myocardial tissues from the sham and I/R groups to elucidate the mechanism of cardiac I/R injury. Sequence alignment demonstrated a significant upregu-

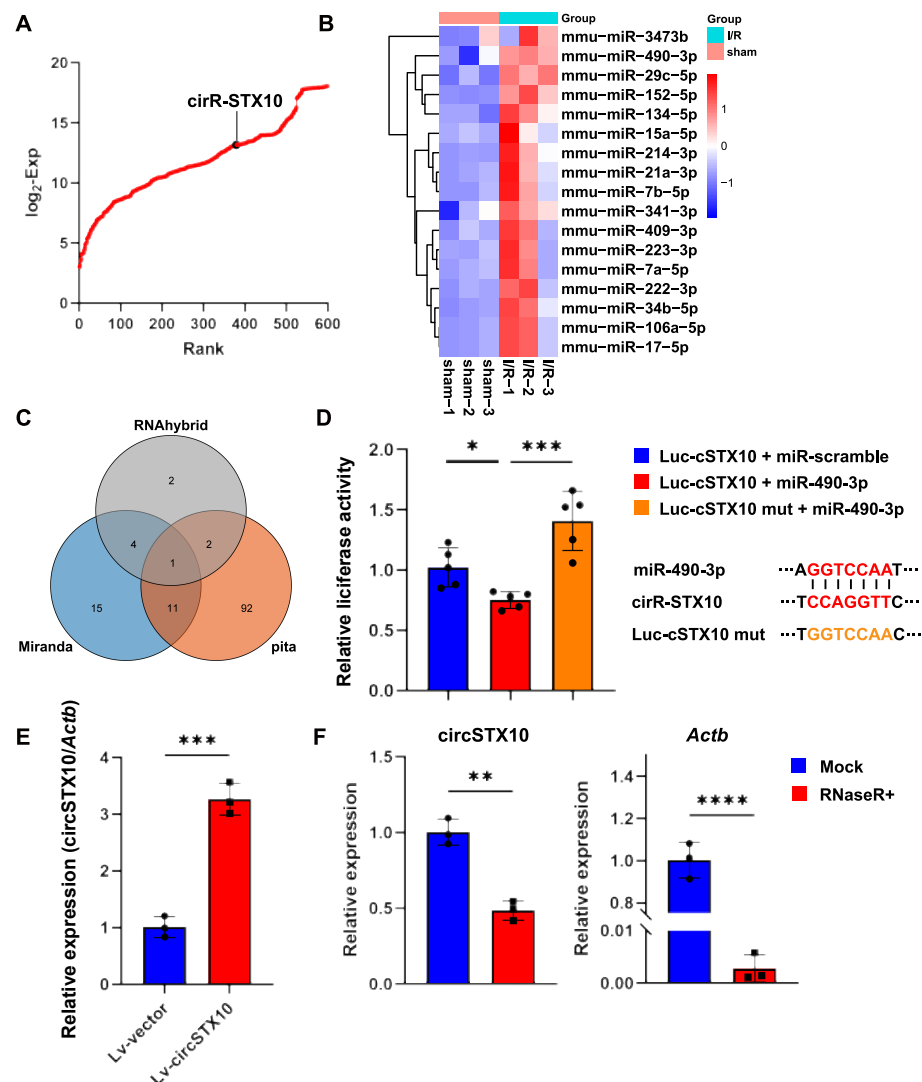


**Fig. 3** circSTX10 acting as a sponge for miR-490-3p. (A) All expressed circRNAs in hUMSC-sEVs, ranked by normalized expression levels. (B) miRNA sequencing of myocardium from the sham and I/R groups. (C) Screening of circRNAs in hUMSC-sEVs harboring potential binding sites for miR-490-3p using three independent prediction tools. (D) Dual-luciferase reporter assay of luc-cSTX10 or its mutant co-transfected with miR-490-3p mimics ( $n = 5$ ). (E) qPCR analysis of circSTX10 expression following lentivirus-mediated circSTX10 overexpression ( $n = 3$ ). (F) The abundance of circSTX10 and control Actb mRNA in RNase R-treated HL-1 cells normalized to the levels measured in mock-treated samples ( $n = 3$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

lation of miR-490-3p after I/R injury (Fig. 3B). Using three independent prediction tools, we identified putative binding sites for circSTX10 in sEVs and miR-490-3p (Fig. 3C). Sequence alignment analysis revealed that circSTX10 and miR-490-3p were evolutionarily conserved in humans and mice. To assess whether circSTX10 functions as a miRNA sponge, we mutated the predicted miR-490-3p-binding sites in the dual-luciferase reporter. Transfection with miR-490-3p resulted in a significant reduction in the luciferase signal in the wild-type circSTX10 reporter, whereas the mutant reporter remained unaffected (Fig. 3D), confirming that circSTX10 can directly sequester miR-

490-3p.

#### circSTX10 rescues HL-1 cells from miR-490-3p-induced ferroptosis

We overexpressed circSTX10 in HL-1 cells using a lentiviral vector to assess whether circSTX10 in hUMSC-sEVs reverses miR-490-3p-induced ferroptosis. Overexpression efficiency was confirmed using divergent primers that encompassed the back-splice junction, and lentiviral circSTX10 expression was approximately 3-fold higher than that in the empty-vector group (Fig. 3E). We performed an RNase R resistance assay to verify that the overexpressed circSTX10

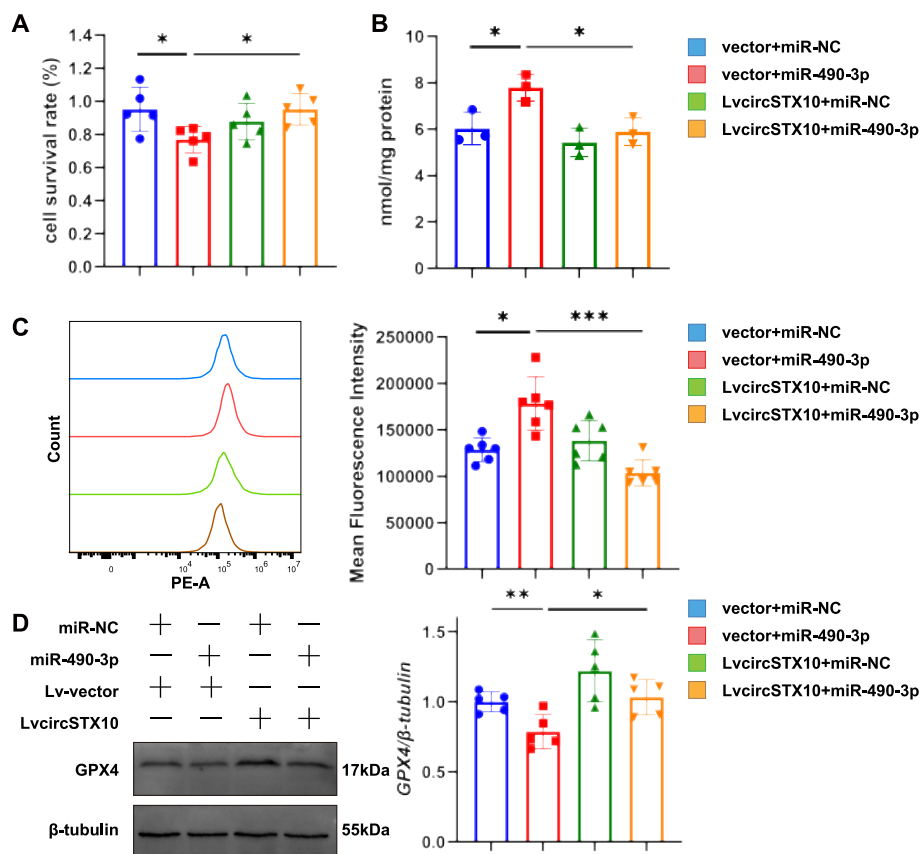


**Fig. 4** circSTX10 rescuing HL-1 cells from miR-490-3p-induced ferroptosis. (A) Cell viability ( $n = 5$ ). (B) MDA content ( $n = 3$ ). (C) Intracellular ferrous ion levels ( $n = 6$ ). (D) WB analysis of GPX4 protein levels ( $n = 5$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns: not significant.

formed a true circular transcript. Compared with the linear control *Actb* mRNA, the lentivirus-overexpressed circSTX10 remained intact after RNase R treatment, demonstrating its circular and highly stable structure (Fig. 3F). CCK-8 assays revealed that cell viability in HL-1 cells was comparable between the miR-NC mimic and circSTX10-overexpressing groups (Fig. 4A). However, circSTX10 overexpression restored cell viability, which was reduced by miR-490-3p (Fig. 4A). Furthermore, miR-490-3p treatment increased MDA production and intracellular ferrous iron content, both of which were reversed by circSTX10 overexpression (Fig. 4B,C). WB analysis further revealed that circSTX10 elevation in HL-1 cells mitigated the miR-490-3p-induced reduction in GPX4 (Fig. 4D). Collectively, our data demonstrated that circSTX10 overexpression mitigated miR-490-3p-induced ferrous iron accumulation, ROS elevation, lipid peroxidation, and ferroptosis.

#### Upregulated miR-490-3p induces ferroptosis by downregulating Heph11

We transfected HL-1 cells with the miR-490-3p mimic and found that miR-490-3p overexpression decreased

cell viability (Fig. 5A) and increased ROS production (Fig. 5B). Furthermore, transfection with miR-490-3p mimic resulted in elevated levels of intracellular ferrous iron in HL-1 cells (Fig. 5C). To investigate the specific mechanism by which miR-490-3p affects cell survival, cells treated with the miR-490-3p mimic were collected for RNA-seq. Sequence alignment revealed significant alterations in ferroptosis-associated gene sets; the downregulated genes included *Heph11* (Fig. 5D). Additionally, qPCR confirmed a significant reduction in *Heph11* mRNA levels (Fig. 5E).

Individual siRNA knockdown of *Heph11* in HL-1 cells decreased cell viability (Fig. 6A). Furthermore, silencing of *Heph11* resulted in increased intracellular ferrous iron (Fig. 6B), ROS production (Fig. 6C), and elevated levels of the lipid-peroxidation product MDA (Fig. 6D). WB analysis revealed that *Heph11* knockdown decreased the ferroptosis-related protein GPX4 expression (Fig. 6E). We explicitly examined *Heph11* expression in normal versus ischemic myocardium, and ischemia/hypoxia led to a significant downregulation of *Heph11* (Fig. S1). SLC7A11 is upregulated as a compensatory response. As a member of the multicopper ferroxidase family, Heph11 governs intracellular iron

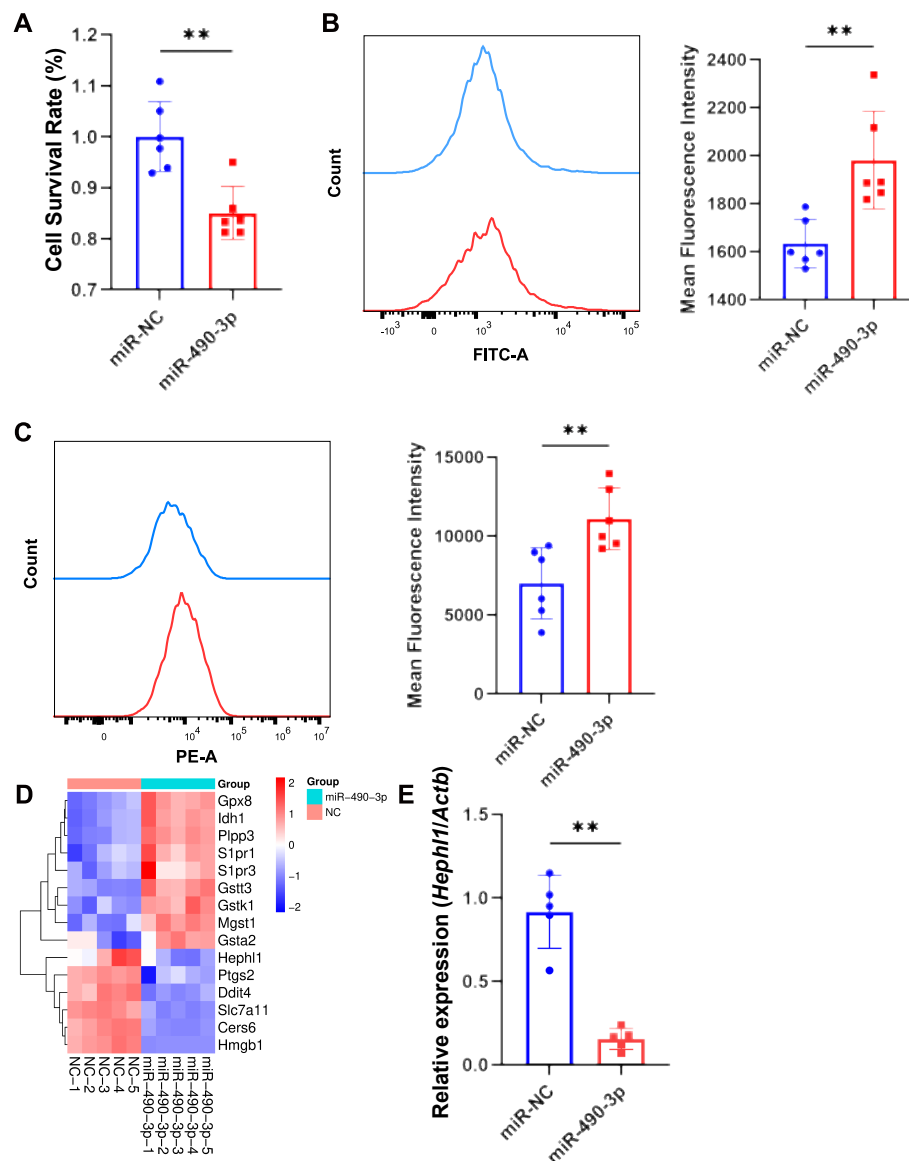


**Fig. 5** Upregulation of miR-490-3p after myocardial I/R leading to decreased Heph1 expression. (A) Effect of miR-490-3p on HL-1 cell proliferation ( $n = 6$ ). (B) Flow cytometry analysis of ROS in cells transfected with miR-490-3p mimics or miR-NC mimics and statistical analysis ( $n = 6$ ). (C) Flow cytometry analysis of ferrous iron in cells transfected with miR-490-3p mimics or miR-NC mimics and statistical analysis ( $n = 6$ ). (D) Heatmap of differentially expressed genes in cells following treatment with miR-490-3p mimics or miR-NC mimics, based on published RNA-seq data. (E) RT-qPCR analysis of Heph1 after treatment of cells with miR-490-3p mimics or miR-NC mimics ( $n = 5$ ). \*\*  $p < 0.01$ .

homeostasis. Mechanistically, as a transmembrane ferroxidase, Heph1 oxidizes labile  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , reduces iron-dependent ROS production and lipid peroxidation, and may mitigate oxidative stress-mediated degradation of SLC7A11.

## DISCUSSION

Ferroptosis-targeted therapies are beneficial for mitigating myocardial I/R injury [20, 21], while new anti-ferroptosis drugs are still under development. Remark-

able progress has been made in the translational application of MSC-sEVs for various disorders, and MSC-sEVs can be delivered post-intervention via intranasal, oral, or biomaterial-based approaches to treat cardiac I/R damage. In this study, we systematically investigated the role and underlying mechanisms of MSC-sEVs in mitigating myocardial I/R-induced ferroptosis.

This study highlights the innovative finding that MSC-sEVs alleviate I/R-triggered myocardial ferroptosis. Mechanistically, circSTX10 acts as a competing en-

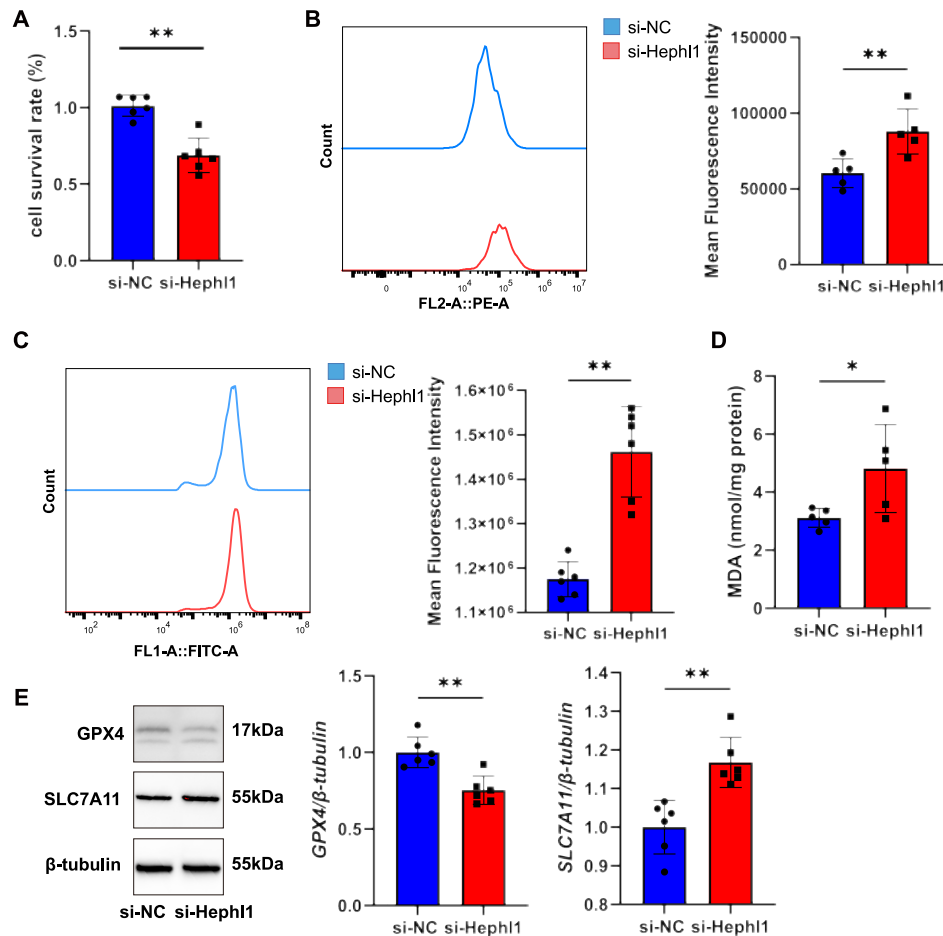


**Fig. 6** Knockdown of *Heph1* inducing ferroptosis in HL-1 cells. (A) Individual knockdown of *Heph1* followed by CCK-8 assay for cell viability ( $n = 6$ ). (B) Analysis of intracellular ferrous ion levels using flow cytometry in cells treated with si-Heph1 or si-NC, with statistical comparison ( $n = 5$ ). (C) Flow-cytometric quantification of ROS in cells after si-Heph1 or si-NC treatment ( $n = 6$ ). (D) MDA content in cells after si-Heph1 or si-NC treatment ( $n = 5$ ). (E) WB analysis and statistical analysis of GPX4 protein levels ( $n = 5$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

dogenous RNA (ceRNA) to sponge miR-490-3p. miR-490-3p was upregulated in the myocardium following I/R injury, and its overexpression led to the downregulation of *Heph1*. When *Heph1* expression was suppressed, intracellular ferrous ion levels increased, ROS release increased, cardiomyocytes underwent ferroptosis, GPX4 protein expression decreased, and SLC7A11 expression increased. Our *in vitro* experiments further confirmed that overexpression of circSTX10 in HL-1 cells reversed miR-490-3p-induced lipid peroxidation and GPX4 reduction. The decrease in GPX4 level is consistent with ferroptosis [22], and previous studies have revealed that SLC7A11 is protectively upregulated during ferroptosis [19].

This study has several limitations. First, this study was conducted *in vitro* using cardiomyocyte cell lines and *in vivo* mouse models of myocardial I/R injury. Further validation in large animal models with more

clinically relevant I/R models is required to confirm their translational potential. Second, although we identified miR-490-3p as a downstream target of circSTX10, other potential signaling pathways involved in circSTX10-mediated ferroptosis inhibition cannot be excluded. Third, we did not confirm the direct targeting of *Heph1* by miR-490-3p, and their regulatory interaction remains putative. Prediction and alignment using mainstream bioinformatics databases revealed no canonical conserved binding sites between miR-490-3p and the 3' UTR of *Heph1*. Given the lack of a theoretical basis for their direct targeting interaction, a dual-luciferase reporter assay was not performed in this study. Fourth, we acknowledge that whether miR-490-3p directly regulates the cGAS-STING pathway in myocardial I/R injury remains unexplored in the current study, and related functional verification will be implemented in our future investigations.

As a member of the multicopper ferroxidase (MCF) family, Heph11 was originally recognized for its regulatory role in placental iron transport and is widely expressed in vertebrate tissues, with low basal expression in the heart [23]. Notably, Heph11 acts as an irreplaceable modulator of intracellular iron homeostasis, and its physiological function cannot be rescued by the compensatory upregulation of other ferroxidase homologs [24]. Cellular iron homeostasis is precisely orchestrated by iron absorption, transport, storage, and efflux processes. FPN1 is responsible for intracellular  $\text{Fe}^{2+}$  export, and MCF family members including Heph11 catalyze the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ . Mechanistically, Heph11 reduces intracellular iron overload and relieves oxidative stress. Myocardial ischemia markedly downregulates Heph11 expression, leading to iron accumulation and aggravated cardiomyocyte ferroptosis. Furthermore, Heph11 upregulates SLC7A11 expression and maintains the activity of system  $\text{Xc}^-$ , thereby preserving GPX4 activity and suppressing ferroptosis progression. Indeed, preservation of GPX4-mediated antioxidant defense to suppress ferroptosis has been widely recognized as a pivotal protective mechanism across diverse pathological contexts, including cancer [25].

Considering the issue of cross-species therapy, circSTX10, miR-490-3p, and Heph11 are evolutionarily conserved between humans and mice. hsa\_circ\_0049673 has only been identified in humans so far, whereas the full-length sequence of miR-490-3p shows 100% sequence identity between humans and mice. A previous study revealed that miR-490-3p was downregulated in the heart 45 min after I/R, and that inhibition of miR-490-3p attenuated myocardial I/R injury by upregulating ATG4B and increasing autophagy [26]. Here, we observed that miR-490-3p was upregulated three days after I/R in mice. Given that miR-490-3p possesses numerous potential downstream targets and participates in the regulation of multiple cell death pathways, we selected 72 h after reperfusion as the experimental endpoint to exclude the interference from other cell death modes and specifically investigate the biological function of miR-490-3p in cardiomyocyte ferroptosis.

hUMSC-sEVs have been proven to ameliorate myocardial I/R injury [27–30]. However, whether hUMSC-sEVs exert cardioprotective effects by regulating cardiomyocyte ferroptosis and the underlying molecular mechanisms remain largely unclear. Theoretically, this study further complements and refines the pathological regulatory network of myocardial I/R injury and enriches our understanding of the molecular mechanism by which small extracellular vesicles mediate myocardial protection. Clinically, this study identifies promising therapeutic targets for myocardial I/R injury, providing a potential therapeutic strategy to improve long-term cardiac function and reduce the

incidence of heart failure in patients with myocardial infarction and those undergoing cardiac surgery.

## CONCLUSION

In conclusion, our study demonstrated that hUMSC-sEVs alleviate myocardial I/R injury by delivering circSTX10, which acts as a ceRNA to regulate miR-490-3p. These findings demonstrate that hUMSC-sEVs exert cardioprotective effects by inhibiting cardiomyocyte ferroptosis, enriching our understanding of the pathological regulatory network of myocardial I/R injury. This work also offers a potential therapeutic option to improve long-term cardiac function and reduce heart failure incidence in patients with myocardial infarction or in patients after cardiac surgery.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found at <https://dx.doi.org/10.2306/scienceasia1513-1874.2026.083>.

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Appendix A. Supplementary data

**Table S1** Primer sequences for RT-qPCR.

| Gene                               | Type    | Sequence (5' to 3')     |
|------------------------------------|---------|-------------------------|
| <i>Heph11</i>                      | Forward | TGCAGTCACCAGAACATACTACA |
|                                    | Reverse | CCCGGTAATAACATCCTTCCCT  |
| <i>Lcn2</i>                        | Forward | TGGCCCTGAGTGTCATGTG     |
|                                    | Reverse | CTCTTGTAGCTCATAGATGGTGC |
| <i>Birc5</i>                       | Forward | GAGGCTGGCTTCATCCACTG    |
|                                    | Reverse | ATGCTCCTCTATCGGGTTGTC   |
| <i>Pi16</i>                        | Forward | GGGGCCACAACAAAGAACG     |
|                                    | Reverse | CACATCTGGTTCGGATCGCA    |
| <i>circSTX10</i> divergent primers | Forward | GTGCTTCTCCTCGTTCTCATCT  |
|                                    | Reverse | CACCTTTCTCTCCTGCAGGT    |
| <i>Actb</i>                        | Forward | GGCTGTATTCCCCTCCATCG    |
|                                    | Reverse | CCAGTTGGTAACAATGCCATGT  |
| <i>BCL2L1</i>                      | Forward | GAGCTGGTGGTTGACTTTCTC   |
|                                    | Reverse | TCCATCTCCGATTCAGTCCCT   |

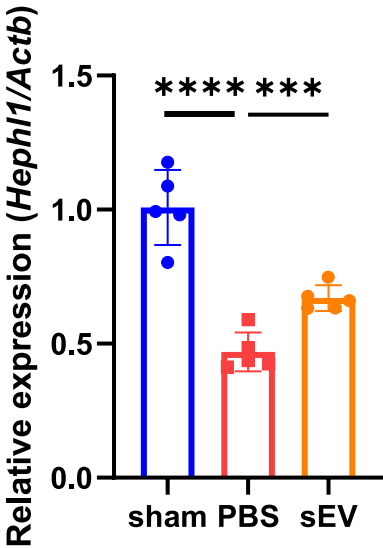


**Fig. S1** RT-qPCR analysis of *Heph11* in normal versus ischemic myocardium.