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Hypoxic cardiomyocyte-derived exosomes regulate cardiac fibroblast activation, apoptosis, migration and ferroptosis through miR-208a/b

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Abstract. Studies have found that cardiomyocytes and cardiac fibroblasts (CFs) can communicate through exosomes, thereby affecting each other's biological functions, but there are few studies on the mechanism. miR-208a/b are specifically expressed in the heart and highly expressed in exosomes derived from various myocardial diseases. Hypoxia induced cardiomyocytes to secrete exosomes (H-Exo) with high expression of miR-208a/b. When H-Exo were added to CFs for co-culture, it was found that CFs took up exosomes, thereby upregulating the expression of miR-208a/b. H-Exo significantly promoted the viability and migration of CFs, enhanced the expression of α-SMA, collagen I and III, and promoted the secretion of collagen I and III. miR-208a or/and miR-208b inhibitors significantly attenuated the effects of H-Exo on CF biological functions. miR-208a/b inhibitors significantly enhanced the levels of apoptosis and caspase-3 activity in CFs, while H-Exo significantly attenuated the pro-apoptotic effects of miR-208a/b inhibitors. Further treatment of CFs with ferroptosis inducer Erastin found that H-Exo further enhanced the accumulation of ROS, MDA and Fe^{2+}, the main indicators of ferroptosis, and inhibited the expression of GPX4, a key regulator of ferroptosis. miR-208a or/and miR-208b inhibitors significantly attenuated the effects of Erastin and H-Exo on ferroptosis. In conclusion, hypoxic cardiomyocyte-derived exosomes can regulate the biological functions of CFs through highly expressed miR-208a/b.

Key words: Cardiac fibroblasts — Exosome — miR-208a/b — Hypoxia — Ferroptosis

Introduction

Myocardial fibrosis is not only the result of various heart diseases, but also the inducement of many heart diseases (Gyöngyösi et al. 2017). Myocardial fibrosis can cause serious complications such as heart failure, arrhythmia and sudden cardiac death (González et al. 2018). Prevention and reversal of myocardial fibrosis is one of the key points in clinical treatment.

At present, a large number of studies have confirmed that extracellular vesicles dominated by exosomes play an important role in the communication process of cells and tissues because of the cell-specific proteins, liposomes and genetic materials carried by them (Kalluri and LeBleu 2020). Exosomes are involved in the regulation of important cellular physiological activities, and their roles in immune responses, apoptosis and angiogenesis have been reported (Kalluri and LeBleu 2020). They have become early diagnostic markers for various diseases and used as carriers for targeted drugs in the treatment of diseases, and transmission of genetic information through exosomes has become a new field of epigenetic regulation (Lin et al. 2015; Familtseva et al. 2019). Experiments have shown that after myocardial infarction, cardiomyocytes release a large number of exosomes into the intercellular space and serum, and these exosomes participate in the patho-

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physiological process of cardiovascular diseases, which has become a hot spot in the diagnosis and treatment of clinical heart diseases (Nguyen et al. 2021). Several studies have shown that there are abundant miRNAs in circulating exosomes (Henning 2021). For example, Xiao et al. (2016) found that exosomes derived from cardiac precursor cells played the role of anti-apoptosis by downregulating programmed cell death 4 (PDCD4) expression through highly expressed miR-21, thereby protecting cardiomyocytes from oxidative stress damage. Therefore, exosomal miRNAs have important significance in heart physiological and pathological process.

miR-208a/b are heart-specific miRNAs, which are involved in the occurrence of myocardial fibrosis, myocardial hypertrophy, heart failure and myocardial ischemia (Huang and Li 2015). Studies have shown that miR-208 level is an independent risk factor for sudden cardiac death and heart failure after acute myocardial infarction (AMI) (Zhao et al. 2020). Deddens et al. (2016) found that myocardial tissues secreted extracellular vesicles during ischemia-reperfusion injury (IRI), and the expression of miR-208b in extracellular vesicles was much higher than that in blood. Cheng et al. (2019) pointed out that the expression of miR-208a was significantly increased in plasma-derived exosomes from AMI mice. Therefore, in the pathological process, myocardial tissues can secrete exosomes containing high expression of miR-208, thereby affecting the physiological processes of other cells. However, there is little research on whether exosome-derived miR-208a/b can affect the biological function of cardiac fibroblasts (CFs).

Recent studies have shown that the activation of fibroblasts is closely related to ferroptosis (Gong et al. 2019). Ferroptosis is a newly discovered cell death mode regulated by various systems, and its occurrence mainly depends on the action of related iron substances and the continuous generation of reactive oxygen species (ROS) (Qiu et al. 2020). A study reported that in a rat model of pulmonary fibrosis, the degree of pulmonary fibrosis was improved after the intervention of ferroptosis inhibitor Fer-1, suggesting that ferroptosis plays an important role in the process of pulmonary fibrosis (Li et al. 2019). However, whether miR-208a/b are also involved in the regulation of ferroptosis has not yet been reported. In this study, hypoxia-induced cardiomyocytes secreted exosomes and these exosome-derived miR-208a/b promoted CF activation, migration and ferroptosis, and inhibited apoptosis, indicating that miR-208a/b carried by hypoxic cardiomyocyte-derived exosomes has important biological significance. This finding will provide new ideas and new targets for the prevention and treatment of myocardial infarction, and provide better guidance for the clinical application of exosomes and their derived miRNAs.

Materials and Methods

Cell culture and treatment

Human cardiac myocytes and human CFs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), and cultured in the cardiac myocyte medium (ScienCell Research Laboratories) and fibroblast medium-2 (ScienCell Research Laboratories) supplemented with 5% fetal bovine serum (FBS; ScienCell Research Laboratories). Human cardiac myocytes were transfected with 50 nmol/l miR-208a or/and miR-208b inhibitors (GenePharma, Shanghai, China) or added with 10 μmol/l GW4869 (an inhibitor of neutral sphingomyelinase that impairs exosome biogenesis/release; Sigma-Aldrich, St. Louis, MO, USA), and then subjected to hypoxic treatment (1% oxygen concentration) for 24 h.

Exosome isolation and identification

CM or CF supernatant was collected and centrifuged at 3,000 × *g* for 15 min, followed by filtration through a 0.22 μm membrane. After that, exosomes were isolated with ExoQuick-TC (System Biosciences, Palo Alto, CA, USA) as instructed by the manufacturer. Exosome morphology was characterized using transmission electron microscopy *via* a JEM-2100 microscope (JEOL, Tokyo, Japan), and exosome size distribution and quantification were analyzed by nanoparticle tracking analysis (NTA) using a Nanosight NS300 nanoparticle tracking analyzer (Malvern Panalytical, Malvern, UK). Meanwhile, exosomes were lysed and Western blot assays were carried out to detect the expression of exosome markers CD63 (1/1000; Abcam, Cambridge, UK) and tumor susceptibility 101 (TSG101, 1/1000; Abcam).

Exosome uptake assay

Hypoxic cardiomyocyte-derived exosomes (H-Exo) were labelled with 2 μl CellTracker CM-DiI (Invitrogen, Carlabad CA, USA) at 37°C for 1 h, and these DiI-labelled exosomes were precipitated with ExoQuick-TC and then added to CFs for co-culture for 12 h. After that, CFs were fixed and washed, and 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) was added to stain the nuclei for 10 min. Finally, CFs were visualized using fluorescence microscopy.

Quantitative real-time PCR (qRT-PCR)

Total RNA extraction from cardiac myocytes, CFs and exosomes was carried out using the miRNeasy Mini Kit (QIAGEN, Germany) in accordance to manufacturer's instructions. RNA was reverse transcribed into cDNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA),

oligo(dT) and random primer, or miR-208a/b stem-loop RT primer. RNA expression levels were determined by qRT-PCR using SYBR Green qPCR SuperMix (Invitrogen). U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls for miRNA and mRNA. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ value.

Western blotting analysis

Total proteins were extracted from CFs and exosomes using RIPA buffer (Beyotime, Shanghai, China) with protease inhibitors (Pierce, Rockford, IL, USA). 8% SDS-PAGE was used to separate equal amount of each protein sample and separated proteins were transferred to a PVDF membrane (Pierce). Western blotting analysis was performed using CD63, TSG101, glutathione peroxidase 4 (GPX4, 1:500; CST, Beverly, MA, USA) or β-actin (1:1000, CST) primary antibody and horseradish peroxidase-conjugated secondary antibody (1:2000, CST). The blot signals were visualized using the BeyoECL Plus (Beyotime).

Transfection and cell viability assay

50 nmol/l miR-208a inhibitors, miR-208b inhibitors, miR-208a/b inhibitors or negative controls (NC) were transfected into CFs using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. After that, CFs were co-cultured with normoxic cardiomyocyte-derived

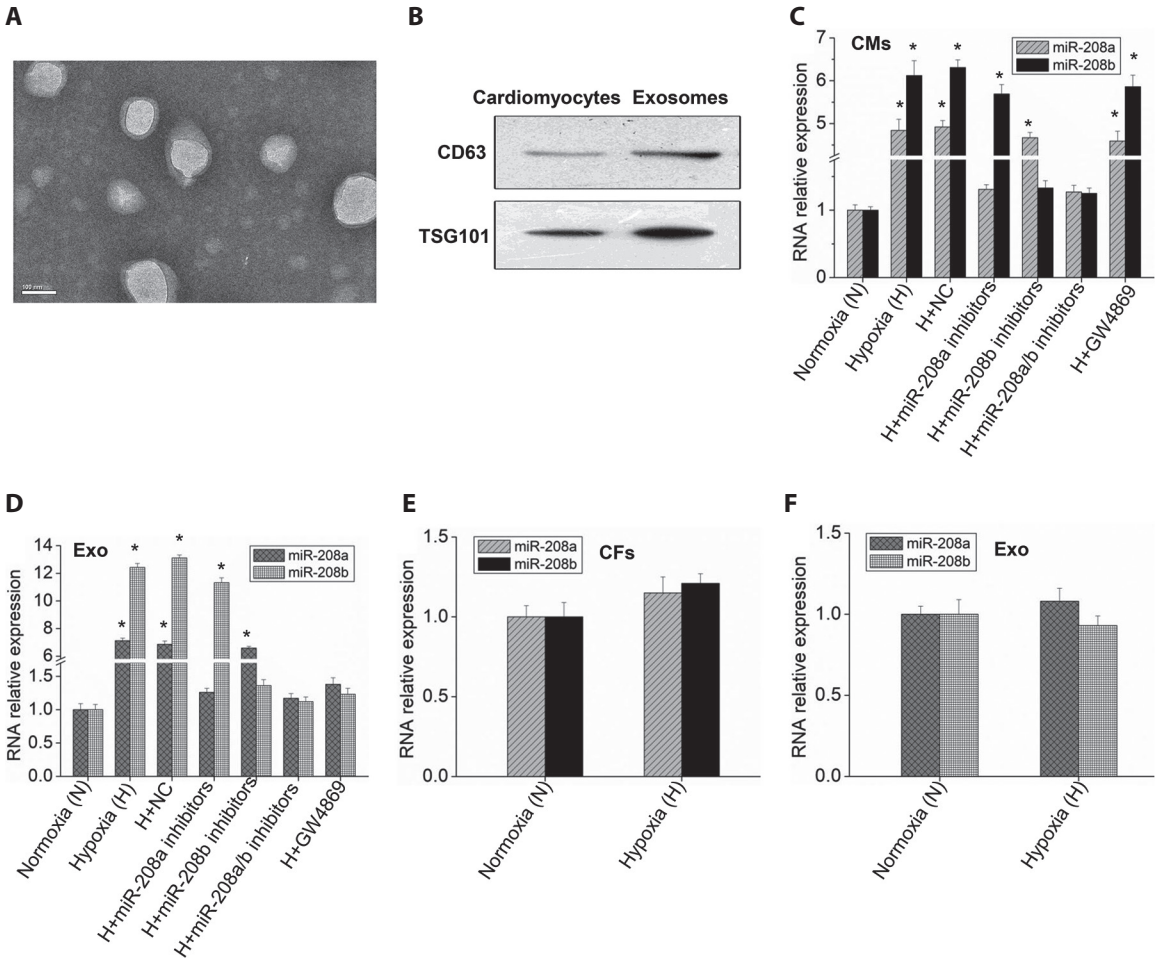
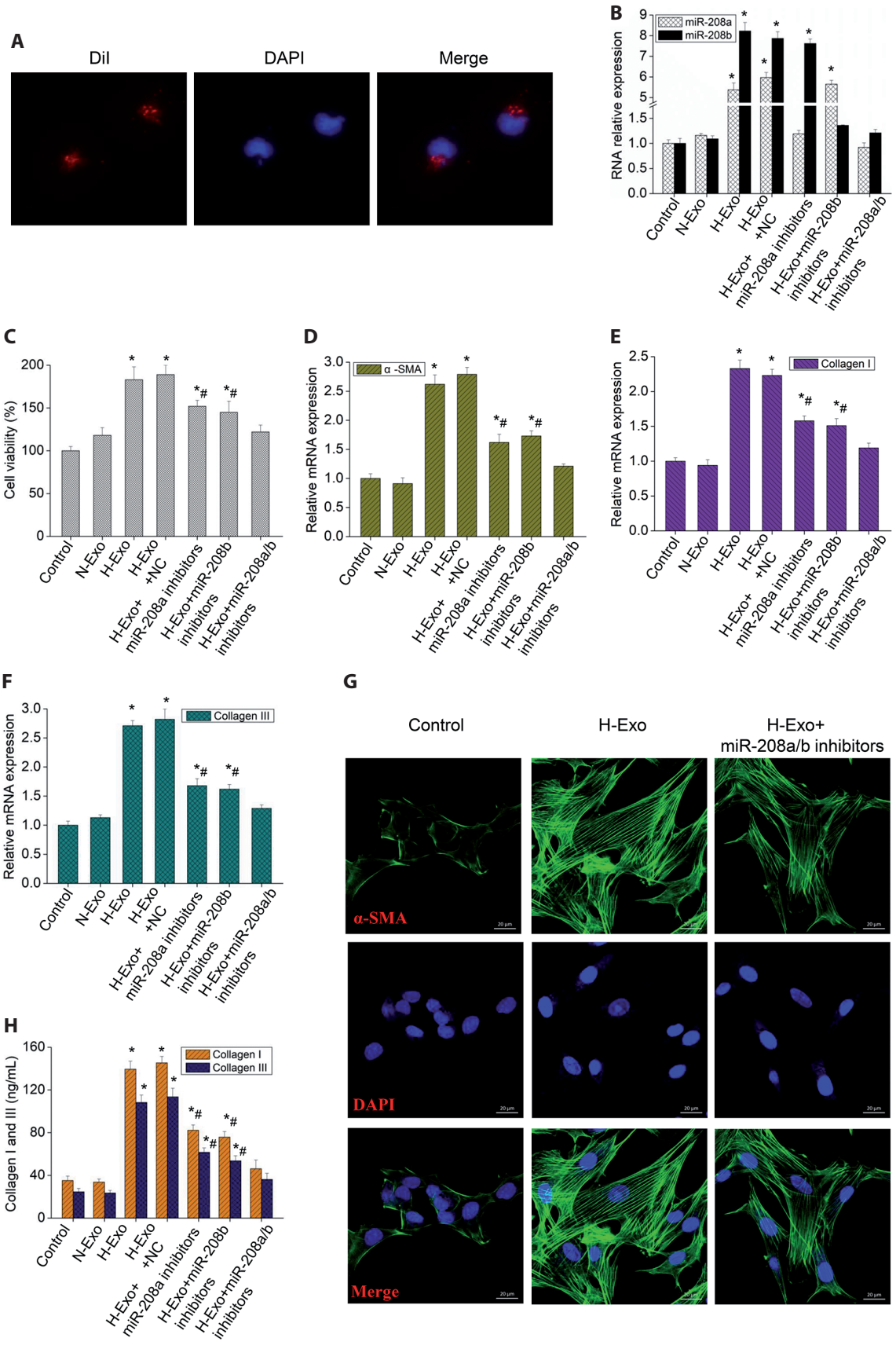


Figure 1. Hypoxia induces cardiomyocytes to secrete exosomes with high expression of miR-208a/b. **A.** Electron microscope images of exosomes secreted by cardiomyocytes. Scale = 100 nm. **B.** Western blot detection of exosome marker CD63 expression levels. **C, D.** Cardiomyocytes (CMs) were transfected with 50 nmol/l miR-208a inhibitors, miR-208b inhibitors, miR-208a/b inhibitors and negative control (NC) miRNA, respectively, or added with 10 μmol/l GW4869, and then subjected to hypoxic treatment (1% oxygen concentration) for 24 h. The expression of miR-208a and miR-208b in CMs (C) and exosomes (Exo, D) was detected using qRT-PCR. **E, F.** Cardiac fibroblasts (CFs) were subjected to hypoxic treatment for 24 h, and the expression of miR-208a and miR-208b in CFs (E) and exosomes (Exo, F) was detected using qRT-PCR. * $p < 0.05$ compared to Normoxia (N).


A
DiI
DAPI
Merge
B
miR-208a
miR-208b
RNA relative expression
C
Cell viability (%)
D
α -SMA
Relative mRNA expression
E
Collagen I
Relative mRNA expression
F
Collagen III
Relative mRNA expression
G
Control
H-Exo
H-Exo+ miR-208a/b inhibitors
α-SMA
DAPI
Merge
H
Collagen I
Collagen III
Collagen I and III (ng/mL)
Control
N-Exo
H-Exo
H-Exo +NC
H-Exo+ miR-208a inhibitors
H-Exo+miR-208b inhibitors
H-Exo+miR-208a/b inhibitors

exosomes (N-Exo), H-Exo, Erastin (10 μmol/l; Sigma-Aldrich), or Erastin plus H-Exo. After 24 h of incubation, cell viability was detected using a cell counting kit-8 (CCK-8, Beyotime). After adding 10 μl CCK-8 solution to each well, the absorbance at 450 nm was measured with a microplate reader.

Immunofluorescence and enzyme linked immunosorbent assay (ELISA)

The slides were added with 4% paraformaldehyde and fixed for 15 min, and then permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Normal goat serum (Beyotime) was added to the slides and blocked at room temperature for 30 min. Then the diluted alpha smooth muscle actin (α-SMA) primary antibody (1:100, Abcam) was added and incubated at 4°C overnight. After slide washing, diluted fluorescent secondary antibody (1:500, Abcam) was added and incubated at 37°C for 1 h. DAPI was added and incubated for 5 min in the dark for nuclei staining, the slides were mounted with a mounting solution containing an anti-fluorescence quencher, and photographed and analyzed under a fluorescence microscope (Olympus IX71, Tokyo, Japan). Collagen I and III in the supernatant of CFs were measured using the human pro-collagen I alpha 1 DuoSet ELISA (R&D Systems, Minneapolis, MN, USA) and human collagen III ELISA kit (Novus Biologicals, Centennial, CO, USA) according to the manufacturer's protocol.

TUNEL and caspase-3 activity assays

CF apoptosis was detected using an *in situ* cell death detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the principle of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). First, the cultured CFs were washed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 1 h at 25°C. After incubating with 0.1% Triton X-100 for 2 min on ice, CFs were further incubated with 50 μl TUNEL reaction mixture containing the nucleotide mixture and TdT at 37°C for 60 min. Then the cells were treated with 0.3% H_2O_2 and streptavidin working solution at room temperature for 10 and 30 min, respectively. Finally, 0.5 μg/ml DAPI was added and incubated for 5 min, and the samples were observed and photographed under a fluorescence microscope. Caspase-3 activity was quantified in CF lysates using a caspase-3 colorimetric activity assay kit (Roche Diagnostics GmbH) as instructed by the manufacturer.

Transwell migration assay

200 μl serum-free medium containing 2×10^5 CFs were seeded into the upper chamber (8 μm pore size; Corning, NY, USA), and the lower chamber was added with 2.5% FBS medium. After incubated for 8 h, the migrated cells were fixed with 4% paraformaldehyde and stained with crystal violet for 10 min. The number of cells in 5 random fields was counted to evaluate cell migration.

Analysis of lipid peroxidation and Fe^{2+}

The intracellular ROS levels were analyzed using a ROS assay kit (Beyotime). CFs were incubated with 10 μmol/l ROS fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) in the dark for 20 min at 37°C. After that, CFs were washed with PBS and observed under a fluorescence microscope, or the fluorescence intensity was detected using a fluorescence spectrometer (F-7000, Hitachi, Japan) at excitation and emission wavelength of 488 and 525 nm, respectively. The malonaldehyde (MDA) content in CF lysates was examined using a lipid peroxidation MDA assay kit (Beyotime) as instructed by the manufacturer. The levels of iron (Fe^{2+}) in CFs were measured using an iron assay kit (Sigma-Aldrich) as instructed by the manufacturer.

Statistical analysis

All results were obtained through at least three independent experimental replications and presented as mean ± SD. All statistical analyses were performed using the GraphPad Prism software (La Jolla, CA, USA), and significance was tested using an unpaired t-test between two groups and one-way analysis of variance (ANOVA) between multiple groups. Significant differences were established a value of $p < 0.05$.

◀ **Figure 2.** Hypoxic cardiomyocyte-derived exosomes promote cardiac fibroblast activation *via* miR-208a/b. **A.** Cardiac fibroblasts were co-cultured with DiI-labeled hypoxic cardiomyocyte-derived exosomes (H-Exo). DAPI was used for nuclear staining. **B–H.** Cardiac fibroblasts were transfected with miR-208a inhibitors, miR-208b inhibitors, miR-208a/b inhibitors and NC miRNA, respectively, and then treated with H-Exo, or only treated with normoxic cardiomyocyte-derived exosomes (N-Exo) or H-Exo. The expression of miR-208a and miR-208b in cardiac fibroblasts was detected using qRT-PCR (B), cell viability was measured using CCK-8 assay (C), the expression of α-SMA (D), collagen I (E) and III (F) was detected using qRT-PCR, α-SMA expression was also detected by immunofluorescence (scale = 20 μm; G), and the expression of collagen I and III (H) was also detected by ELISA. * $p < 0.05$ compared to Control; # $p < 0.05$ compared to H-Exo.

Results

Hypoxia promotes miR-208a/b expression in cardiomyocytes and their secreted exosomes

Cardiomyocyte-secreted exosomes displayed round-like morphology with a diameter distribution ranging from 30–150 nm (the average diameter was about 100 nm) and a content of 1×10^8 particles (Fig. 1A), and these exosomes significantly expressed relevant markers (Fig. 1B). Hypoxia promoted the expression of miR-208a/b in cardiomyocytes and also enhanced miR-208a/b expression in exosomes secreted by cardiomyocytes (Fig. 1C,D). When cardiomyocytes were transfected with miR-208a/b inhibitors and then treated with hypoxia, the expression of miR-208a/b in cells and exosomes was not changed significantly (Fig. 1C,D). In addition, when cardiomyocytes were treated with hypoxia after adding the exosome inhibitor GW4869, although the expression of miR-208a/b in cells was still significantly increased, miR-208a/b expression in exosomes was not changed significantly (Fig. 1C,D). These results showed that hypoxia promoted the expression of miR-208a/b in cardiomyocytes and their secreted exosomes. When CFs were treated with hypoxia, the expression of miR-208a/b in cells and secreted exosomes was not changed significantly, indicating that hypoxia did not affect the expression of miR-208a/b in CFs and their secreted exosomes (Fig. 1E,F).

H-Exo promote the activation of CFs through miR-208a/b

When H-Exo were added to CFs for culture, it was clearly observed that CFs took up exosomes (Fig. 2A). Meanwhile, H-Exo significantly increased the expression of miR-208a/b in CFs, while N-Exo had no such effect (Fig. 2B). H-Exo significantly promoted the viability of CFs (Fig. 2C), enhanced the expression of α-SMA, collagen I and III (Fig. 2D–G), and promoted the secretion of collagen I and III (Fig. 2H); after transfection of miR-208a and miR-208b inhibitors respectively, the regulation of H-Exo on cell viability and fibrosis marker expression was significantly attenuated (Fig. 2C–H); when miR-208a and miR-208b inhibitors were co-transfected, the above-mentioned effects of H-Exo were further inhibited (Fig. 2C–H). However, N-Exo did not affect the viability and activation of CFs (Fig. 2C–H). Therefore, hypoxic cardiomyocytes can secrete exosomes with high expression of miR-208a/b, thereby promoting the activation of CFs.

H-Exo regulate CF apoptosis and migration through miR-208a/b

Apoptosis was significantly increased when co-transfected with miR-208a and miR-208b inhibitors, but H-Exo significantly attenuated the pro-apoptotic effects of miR-208a/b inhibitors (Fig. 3A–B). In addition, when CFs were transfected with miR-208a or miR-208b inhibitors, caspase-3 activity was significantly increased; when co-transfected with miR-208a and miR-208b inhibitors, caspase-3 activity was further enhanced; similarly, H-Exo significantly attenuated the above effects of miR-208a/b (Fig. 3C). At the same time, the detection of cell migration showed that H-Exo significantly promoted cell migration, and miR-208a/b inhibitors attenuated the pro-migration effect of these exosomes, while N-Exo had no effect on CF migration (Fig. 3D–E). Therefore, H-Exo can affect the apoptosis and migration of CFs through miR-208a/b.

H-Exo enhance CF ferroptosis through miR-208a/b

The latest studies found that ferroptosis, a new way of cell death, is involved in the activation of fibroblasts (Gong et al. 2019). Therefore, this study continued to explore whether H-Exo miR-208a/b was associated with ferroptosis. Treating CFs with ferroptosis inducer Erastin or H-Exo found that the contents of ROS, MDA and Fe^{2+}, the main indicators of ferroptosis, were significantly increased (Fig. 4A–D), while the expression of GPX4, a key factor regulating the process of ferroptosis, was significantly down-regulated (Fig. 4E,F). When Erastin and H-Exo were added simultaneously, the accumulation of ROS, MDA and Fe^{2+} was further enhanced, while the expression of GPX4 was further downregulated (Fig. 4). When CFs were transfected with miR-208a or miR-208b inhibitors and then added with Erastin and H-Exo, the changes of the above-mentioned ferroptosis-related indicators were still higher than those in Erastin-treated group, but lower than those in Erastin and H-Exo-treated group (Fig. 4). When miR-208a and miR-208b inhibitors were co-transfected and then Erastin and H-Exo were added, the changes of the above-mentioned ferroptosis-related indicators were not significantly different from those in Erastin-treated group (Fig. 4). Therefore, hypoxic cardiomyocyte exosome-derived miR-208a/b can enhance the ferroptosis process of CFs.

Discussion

Myocardial fibrosis is a key factor determining the prognosis of cardiovascular diseases, and it is one of the key topics in the field of cardiovascular research (Gyöngyösi et al. 2017). A detailed understanding of myocardial fibrosis pathogenesis will help to find intervention targets for the prevention and treatment of myocardial fibrosis, which is beneficial to the comprehensive prevention of heart diseases.

Studies have found that in the process of myocardial diseases, different cells can communicate information

through exosomes, etc., thus affecting disease progression (Nguyen et al. 2021). For example, Luo et al. (2019) found that ischemic postconditioning upregulated the expression of miR-423-3p in exosomes/microvesicles secreted by CFs, and these exosomes/microvesicles played a cardioprotective role in the acute phase of IRI by targeting the downstream effector Ras-related protein Rap-2c (RAP2C) through miR-423-3p. Angiotensin II (Ang II) stimulated

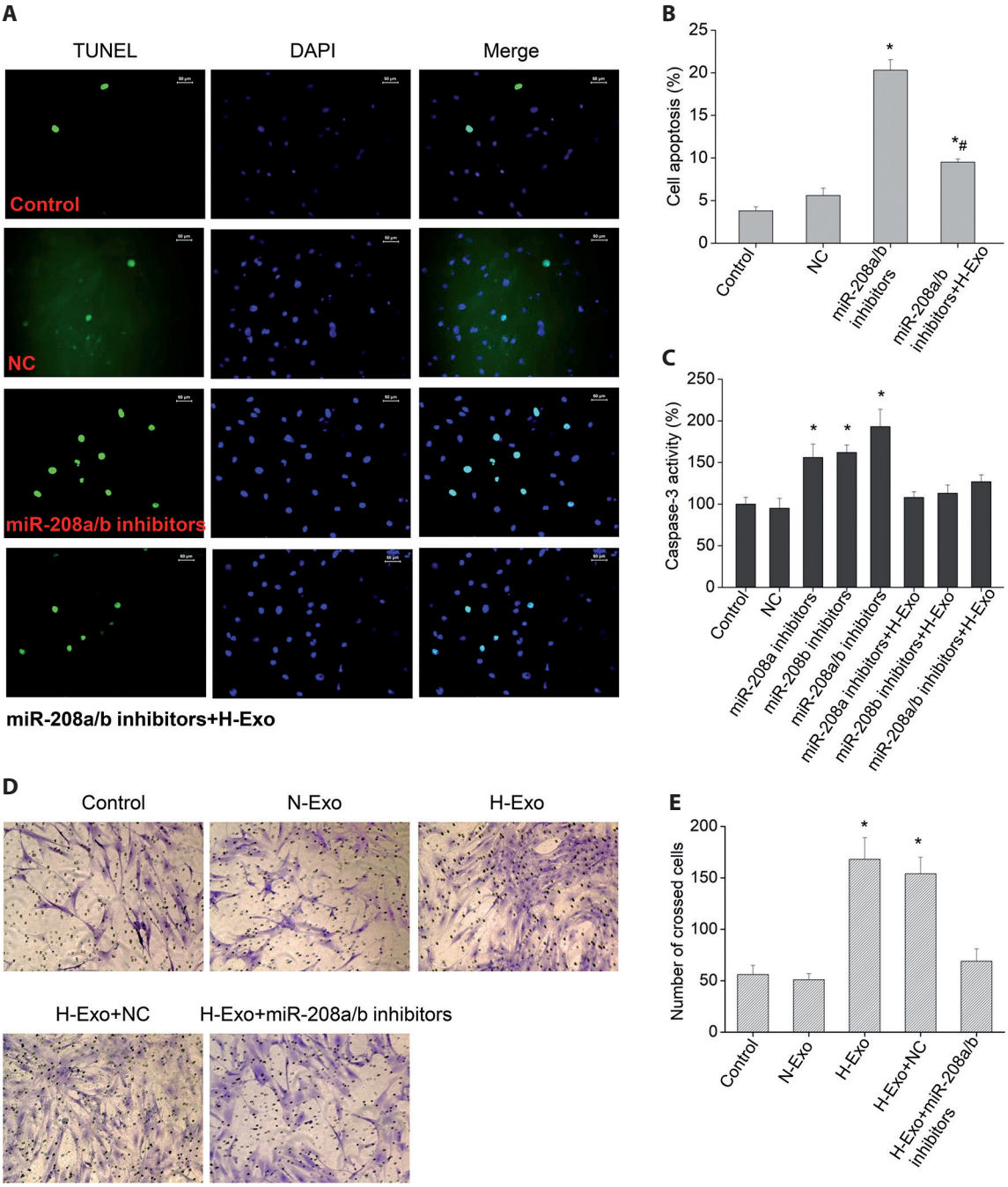


Figure 3. Hypoxic cardiomyocyte-derived exosomes inhibit apoptosis and promote migration of cardiac fibroblasts. Cardiac fibroblasts were transfected with miR-208a inhibitors, miR-208b inhibitors, miR-208a/b inhibitors and NC miRNA, respectively, and then treated with cardiomyocyte-derived exosomes (H-Exo), or only treated with normoxic cardiomyocyte-derived exosomes (N-Exo) or H-Exo. **A, B.** Cardiac fibroblast apoptosis was detected by TUNEL assay (scale = 50 μm). **C.** Caspase-3 activity was quantified using a caspase-3 colorimetric activity assay kit. **D, E.** Cell migration was evaluated by Transwell assay. * $p < 0.05$ compared to Control; # $p < 0.05$ compared to miR-208a/b inhibitors.

CFs to release exosomes, which in turn increased the production of Ang II and the expression of its receptors in cardiomyocytes by activating Akt and mitogen-activated protein kinases (MAPKs), thereby exacerbating Ang II-induced cardiomyocyte hypertrophy (Lyu et al. 2015). This study found that hypoxia induced cardiomyocytes to secrete exosomes with high expression of miR-208a/b. The expression of miR-208a in serum and serum-derived exosomes of acute coronary syndrome (ACS) patients was significantly higher than that of healthy controls (Bi et al. 2015). This indicates that myocardial tissues can secrete exosomes with high expression of miR-208a/b under various pathological conditions. However, Tony et al. (2015) found that miR-208a affected the expression of apoptotic genes, and increased expression of miR-208a promoted the apoptosis of ischemic cardiomyocytes. This shows that miR-208a/b play different roles in cardiomyocytes and CFs, and the differences are worthy of further study.

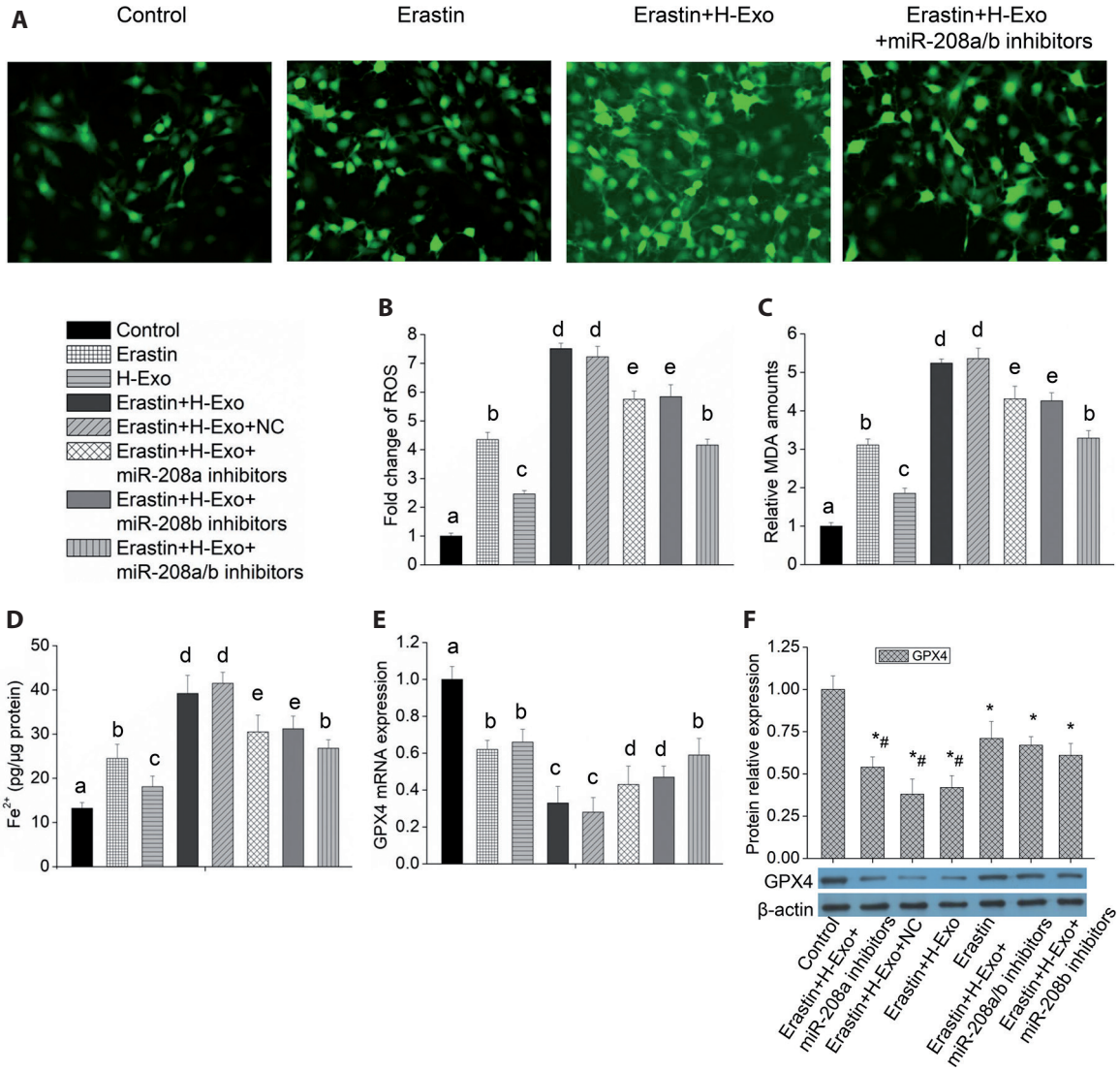


Figure 4. Hypoxic cardiomyocyte-derived exosomes enhance cardiac fibroblast ferroptosis. Cardiac fibroblasts were transfected with miR-208a inhibitors, miR-208b inhibitors, miR-208a/b inhibitors and NC miRNA, respectively, and then treated with Erastin plus cardiomyocyte-derived exosomes (H-Exo), or only treated with Erastin, H-Exo or Erastin plus H-Exo. The intracellular ROS levels were analyzed using a ROS probe DCFH-DA under a fluorescence microscope (**A**) and the fluorescence intensity was detected using a fluorescence spectrometer (**B**), the MDA content was examined using a lipid peroxidation MDA assay kit (**C**), the levels of Fe^{2+} were measured using an iron assay kit (**D**), and the expression of GPX4 was detected using qRT-PCR (**E**) and Western blot (**F**). ^{a,b,c,d,e} statistically significant differences between two groups; * $p < 0.05$ compared to Control; $^{\#}$ $p < 0.05$ compared to Erastin.

Wang et al. (2017) also found that hypoxia caused H9c2 cells to secrete extracellular vesicles with high expression of miR-208a/b, and these extracellular vesicles targeted to inhibit the expression of RNA-binding protein Quaking (QKI) through miR-208a/b, thereby aggravating hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis. Yang et al. (2018) showed that during the progression of myocardial fibrosis, cardiomyocytes secreted exosomes containing miR-208a, and miR-208a could be transferred to CFs through exosomes, thereby promoting cell proliferation and differentiation into myofibroblasts; *in vivo* experiments also showed that exosomes containing miR-208a led to deterioration of cardiac function in normal rats, while inhibition of miR-208a expression improved cardiac function and reduced cardiac fibrosis in rats after myocardial infarction. This study further found that hypoxic cardiomyocyte-derived exosomes significantly promoted the viability and migration of CFs, enhanced the expression of α-SMA, collagen I and III, while miR-208a or/and miR-208b inhibitors significantly attenuated the effects of these exosomes on the biological functions of CFs. miR-208a or/and miR-208b inhibitors significantly enhanced CF apoptosis, whereas hypoxic cardiomyocyte-derived exosomes significantly attenuated the pro-apoptotic effect of miR-208a or/and miR-208b inhibitors. Therefore, cardiomyocyte exosome-derived miR-208a/b is of great significance in myocardial diseases.

This study also found that when CFs were treated with ferroptosis inducer Erastin, hypoxic cardiomyocyte-derived exosomes could further enhance the accumulation of ROS, MDA, and Fe^{2+}, the main indicators of ferroptosis, and inhibit the expression of GPX4, a key regulator of ferroptosis; miR-208a or/and miR-208b inhibitors significantly attenuate the effects of Erastin and hypoxic cardiomyocyte-derived exosomes on ferroptosis. This study found for the first time that hypoxic cardiomyocyte-derived exosomes could affect CF ferroptosis through miR-208a/b. And studies have shown that ferroptosis can affect the activation of fibroblasts (Yang et al. 2020; Zhuang et al. 2022). For example, miR-375-3p accelerated cardiomyocyte ferroptosis by inhibiting GPX4 expression in the ischemia-reperfusion rat model and Ang II-induced cardiac fibrosis cell model, thus promoting fibrosis; miR-375-3p inhibitors and Fer-1 promoted CF antioxidant capacity, reduced GPX4-mediated ferroptosis and attenuated ischemia-reperfusion-induced cardiac fibrosis (Zhuang et al. 2022). Long non-coding RNA (lncRNA)-ZFAS1 was upregulated in transforming growth factor-β1 (TGF-β1)-treated HFL1 cells and bleomycin (BLM)-induced pulmonary fibrosis rat lung tissues, and induced ferroptosis through the miR-150-5p/SLC38A1 axis, thereby promoting TGF-β1-induced fibroblast activation, lipid peroxidation and inflammation, and enhancing BLM-induced lipid peroxidation and pulmonary fibrosis progression (Yang et al. 2020). In addition, mixed lineage kinase 3 (MLK3) mainly regulated ferroptosis-induced myocardial fibrosis in the advanced stages of chronic heart failure and oxidative stress mediated by JNK/p53 signaling pathway, thereby promoting adverse myocardial fibrosis induced by pressure overload (Wang et al. 2020). All these studies indicate that ferroptosis can promote the activation of fibroblasts, suggesting that ferroptosis is an important inducement of fibrosis.

Conclusion

In summary, H-Exo can regulate the biological functions of CFs such as viability, apoptosis, migration and ferroptosis through highly expressed miR-208a/b, indicating that exosome-derived miR-208a/b have important functions and may be key regulators closely related to myocardial diseases. However, this study only isolated exosomes secreted by cardiomyocytes *in vitro*, then added these exosomes to CFs for co-culture, and investigated the effects of cardiomyocyte-derived exosomes on the biological functions of CFs. As for whether cardiomyocyte-derived exosomes can be taken up by CFs *in vivo* and affect their physiological functions during the progression of myocardial diseases, further research is needed.

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Conflict of interest. The authors declare that they have no competing interests.

References

Bi S, Wang C, Jin Y, Lv Z, Xing X, Lu Q (2015): Correlation between serum exosome derived mir-208a and acute coronary syndrome. Int. J. Clin. Exp. Med. **8,** 4275-4280

Cheng M, Yang J, Zhao X, Zhang E, Zeng Q, Yu Y, Yang L, Wu B, Yi G, Mao X, et al. (2019): Circulating myocardial micrornas from infarcted hearts are carried in exosomes and mobilise bone marrow progenitor cells. Nat. Commun. **10,** 959 https://doi.org/10.1038/s41467-019-08895-7

Deddens JC, Vrijsen KR, Colijn JM, Oerlemans MI, Metz CH, van der Vlist EJ, Nolte-'t Hoen EN, den Ouden K, Jansen Of Lorkeers SJ, van der Spoel TI, et al. (2016): Circulating extracellular vesicles contain mirnas and are released as early biomarkers for cardiac injury. J. Cardiovasc. Transl. Res. **9,** 291-301 https://doi.org/10.1007/s12265-016-9705-1

Familtseva A, Jeremic N, Tyagi SC (2019): Exosomes: Cell-created drug delivery systems. Mol. Cell Biochem. **459,** 1-6 https://doi.org/10.1007/s11010-019-03545-4

Gong Y, Wang N, Liu N, Dong H (2019): Lipid peroxidation and gpx4 inhibition are common causes for myofibroblast differentiation and ferroptosis. DNA Cell Biol. **38,** 725-733

https://doi.org/10.1089/dna.2018.4541
González A, Schelbert EB, Díez J, Butler J (2018): Myocardial interstitial fibrosis in heart failure: Biological and translational perspectives. J. Am. Coll. Cardiol. **71,** 1696-1706 https://doi.org/10.1016/j.jacc.2018.02.021
Gyöngyösi M, Winkler J, Ramos I, Do QT, Firat H, McDonald K, González A, Thum T, Díez J, Jaisser F, et al. (2017): Myocardial fibrosis: Biomedical research from bench to bedside. Eur. J. Heart Fail. **19,** 177-191 https://doi.org/10.1002/ejhf.696
Henning RJ (2021): Cardiovascular exosomes and micrornas in cardiovascular physiology and pathophysiology. J. Cardiovasc. Transl. Res. **14,** 195-212 https://doi.org/10.1007/s12265-020-10040-5
Huang Y, Li J (2015): Microrna208 family in cardiovascular diseases: Therapeutic implication and potential biomarker. J. Physiol. Biochem. **71,** 479-486 https://doi.org/10.1007/s13105-015-0409-9
Kalluri R, LeBleu VS (2020): The biology, function, and biomedical applications of exosomes. Science **367,** eaau6977 https://doi.org/10.1126/science.aau6977
Li X, Duan L, Yuan S, Zhuang X, Qiao T, He J (2019): Ferroptosis inhibitor alleviates radiation-induced lung fibrosis (rilf) via down-regulation of tgf-β1. J. Inflamm. (Lond) **16,** 11 https://doi.org/10.1186/s12950-019-0216-0
Lin J, Li J, Huang B, Liu J, Chen X, Chen XM, Xu YM, Huang LF, Wang XZ (2015): Exosomes: Novel biomarkers for clinical diagnosis. The Scientific World Journal **2015,** 657086 https://doi.org/10.1155/2015/657086
Luo H, Li X, Li T, Zhao L, He J, Zha L, Qi Q, Yu Z (2019): Microrna-423-3p exosomes derived from cardiac fibroblasts mediates the cardioprotective effects of ischaemic post-conditioning. Cardiovasc. Res. **115,** 1189-1204 https://doi.org/10.1093/cvr/cvy231
Lyu L, Wang H, Li B, Qin Q, Qi L, Nagarkatti M, Nagarkatti P, Janicki JS, Wang XL, Cui T (2015): A critical role of cardiac fibroblast-derived exosomes in activating renin angiotensin system in cardiomyocytes. J. Mol. Cell Cardiol. **89,** 268-279 https://doi.org/10.1016/j.yjmcc.2015.10.022
Nguyen BY, Azam T, Wang X (2021): Cellular signaling cross-talk between different cardiac cell populations: An insight into the role of exosomes in the heart diseases and therapy. Am. J. Physiol. Heart Circ. Physiol. **320,** H1213-H1234 https://doi.org/10.1152/ajpheart.00718.2020
Qiu Y, Cao Y, Cao W, Jia Y, Lu N (2020): The application of ferroptosis in diseases. Pharmacol. Res. **159,** 104919 https://doi.org/10.1016/j.phrs.2020.104919
Tony H, Meng K, Wu B, Yu A, Zeng Q, Yu K, Zhong Y (2015): MicroRNA-208a dysregulates apoptosis genes expression and promotes cardiomyocyte apoptosis during ischemia and its silencing improves cardiac function after myocardial infarction. Mediators Inflamm. **2015,** 479123 https://doi.org/10.1155/2015/479123
Wang F, Yuan Y, Yang P, Li X (2017): Extracellular vesicles-mediated transfer of mir-208a/b exaggerate hypoxia/reoxygenation injury in cardiomyocytes by reducing qki expression. Mol. Cell Biochem. **431,** 187-195 https://doi.org/10.1007/s11010-017-2990-4
Wang J, Deng B, Liu Q, Huang Y, Chen W, Li J, Zhou Z, Zhang L, Liang B, He J, et al. (2020): Pyroptosis and ferroptosis induced by mixed lineage kinase 3 (mlk3) signaling in cardiomyocytes are essential for myocardial fibrosis in response to pressure overload. Cell Death Dis. **11,** 574 https://doi.org/10.1038/s41419-020-02777-3
Xiao J, Pan Y, Li XH, Yang XY, Feng YL, Tan HH, Jiang L, Feng J, Yu XY (2016): Cardiac progenitor cell-derived exosomes prevent cardiomyocytes apoptosis through exosomal mir-21 by targeting pdcd4. Cell Death Dis. **7,** e2277 https://doi.org/10.1038/cddis.2016.181
Yang J, Yu X, Xue F, Li Y, Liu W, Zhang S (2018): Exosomes derived from cardiomyocytes promote cardiac fibrosis via myocyte-fibroblast cross-talk. Am. J. Transl. Res. **10,** 4350-4366
Yang Y, Tai W, Lu N, Li T, Liu Y, Wu W, Li Z, Pu L, Zhao X, Zhang T, et al. (2020): Lncrna zfas1 promotes lung fibroblast-to-myofibroblast transition and ferroptosis via functioning as a cerna through mir-150-5p/slc38a1 axis. Aging (Albany NY) **12,** 9085-9102 https://doi.org/10.18632/aging.103176
Zhao X, Wang Y, Sun X (2020): The functions of microrna-208 in the heart. Diabetes Res. Clin. Pract. **160,** 108004 https://doi.org/10.1016/j.diabres.2020.108004
Zhuang Y, Yang D, Shi S, Wang L, Yu M, Meng X, Fan Y, Zhou R, Wang F (2022): Mir-375-3p promotes cardiac fibrosis by regulating the ferroptosis mediated by gpx4. Comput. Intell. Neurosci. **2022,** 9629158 https://doi.org/10.1155/2022/9629158

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