CircRNA1615 Inhibits Ferroptosis via Modulation of Autophagy by the miRNA152-3p/LRP6 Axis in Cardiomyocytes of Myocardial Infarction

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Research

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Abstract

Background Searching for new molecular targets of ferroptosis is gradually becoming the focus in the field of cardiovascular disease research. This study was aimed to explore the biological function and molecular mechanism of ferroptosis of circRNA modulation in cardiomyocytes of myocardial infarction (MI).

Method We explored the regulatory effect and molecular mechanism of LPR6 on myocardial cell ferroptosis by establishing a model of MI in vivo and in vitro, constructed the regulatory network of circRNA-miRNA-LRP6 by the bioinformatics analysis, and focused on the biological function and molecular mechanism of circRNA1615 regulating ferroptosis in MI by the overexpression or knockdown of circRNA1615, the RIP experiments, and double luciferase reporter gene assay.

Results Ferrostatin-1 (ferroptosis inhibitor) can improve the pathological process of MI; LRP6 was involved in the process of ferroptosis in cardiomyocytes; LRP6 deletion regulates ferroptosis in cardiomyocytes through autophagy; Screening and identification of circRNA1615 targets LRP6; circRNA1615 inhibits ferroptosis in cardiomyocytes; circRNA1615 regulates the expression of LRP6 through sponge adsorption of miR-152-3p, and then prevent LRP6-mediated autophagy-related ferroptosis in cardiomyocytes, finally regulate the pathological process of MI.

Conclusions CircRNA1615 inhibits ferroptosis via modulation of autophagy by the miRNA152-3p/LRP6 molecular axis in cardiomyocytes of myocardial infarction.

Introduction

Myocardial infarction (MI) is the main cause of high incidence and sudden cardiac death, and ischemia induces a variety of complications such as cardiomyocytes injury and heart failure, which further aggravates the problem of the treatment of MI and a poor prognosis.

Ferroptosis is caused by iron-dependent lipid peroxidation. As a way of programmed cell death, ferroptosis is involved in lipid metabolism, amino acid metabolism and iron metabolism, which is different from other forms of cell death in biochemistry and morphology. The inhibitors of apoptosis, necrosis and autophagy can not prevent ferroptosis, but the iron chelating agents and antioxidants can inhibit it.

Iron is also an independent predictor of poor left ventricular remodeling after MI. In addition, ferroptosis is an major form of cardiomyocyte death in the infarcted area, which may play an important role in the pathological process of heart disease. However, it is not clear about the effect of ferroptosis and its molecular mechanism in the process of MI.

Low density lipoprotein receptor related protein 6 (LRP6) is involved in the process of cardiomyopathy. The down-regulation of LRP6 can activate Drp1 (Dynamin-related protein1), and then inhibit
cardiomyocyte survival under the condition of glucose deprivation,\(^5\) suggesting that LRP6 may be associated with the regulation of cardiomyocyte death. Moreover, LRP6 deletion promotes autophagy,\(^6\) autophagy is a necessary factor in ferroptosis,\(^7\) and the autophagy agonist rapamycin was loaded into mouse cardiomyocytes to increase the sensitivity of ferroptosis, indicating that LRP6 may be closely related to autophagy and ferroptosis of cardiomyocytes.\(^8\)

Autophagy-related Cyclic RNAs (ACR circRNAs) attenuates cardiomyocyte autophagy and cell death by regulating Pink1 (Phosphatase and tensin homolog-induced putative kinase1) / FAM65B (Family with sequence similarity 65 member B) pathway, protects the heart from ischemia/reperfusion injury, and reduces MI size.\(^9\) These findings highlight the physiological role of circRNAs in cardiac repair, and the regulation of circRNA expression may represent a potential strategy for promoting cardiac function and ventricular remodeling after MI. However, it has not been reported whether circRNA is involved in the pathological process of MI by regulating ferroptosis in cardiomyocytes.

The study aimed to explore the effect and mechanism of LRP6 on cardiac myocyte ferroptosis in MI, to screen and identify circRNA targeting LRP6, and to investigate the biological function and molecular mechanism of ferroptosis of circRNA modulation through LRP6 and autophagy.

**Methods**

**Animal models**

The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals (US and National Institutes of Health). C57BL/6J male mice of SPF grade, 8–10 weeks old. The day before operation, the mice in MI + ferrostatin-1 (Fer-1) group were injected a dose of 1mg/kg ferroptosis inhibitor Fer-1 (SML0583-5MG, Sigma, USA). Fer-1 was dissolved in Dimethyl sulfoxide (DMSO), then diluted in sterile saline, Sham group and MI + NS group were injected with the same dose of saline. The mice were anesthetized by 3% pentobarbital sodium of intraperitoneal injection, and the MI model was established by ligating the anterior descending branch of left coronary artery (LAD). The mice in the sham group were not ligated with LAD.

**Echocardiography measurement**

On the 7th day of MI, the left ventricular end-diastolic and end-systolic diameters (LVIDd and LVIDs) of mice were measured by small animal ultrasound Shenzhen Mairui biomedical DP-50ev (Shenzhen Mindray Bio-Medical Electronics Co., China), and the ejection fraction (EF%) and left ventricular short axis shortening (FS%) were calculated using edge-detection software and standard techniques.

**Histology**

Midventricular short-axis heart sections (5mm) from MI model hearts were fixed in 2% paraformaldehyde (PFA) overnight at 4°C, dehydrated in 70% ethyl alcohol, and embedded in paraffin. The Sections were stained with H&E and examined under a light microscope.
Cells culture and intervention

The mouse cardiomyocytes (HL-1 cells, ZQ0920, Shanghai Zhong Qiao Xin Zhou Biotechnology, China) were cultured with DMEM (10-013-CVRC, Corning, USA), 10% FBS (GIBCO, ThermoFisher Scientific, USA) and 1% PBS (E607011, Shanghai Sangon Biotech, China) at 95% air, 5% CO₂, and 37°C.

The HL-1 cells was adjusted and made into single cell suspension. After counting, each group of cells was made into 2×10⁴ cells/ml single cell suspension. Cardiomyocytes were treated with 0, 2.5, 5, 10, 20 and 40 µM erastin (HY-15763, MCE, USA), 10 µM ZVAD-FMK (219007-250UG, MCE, USA), and 0.5µM necrosulfonamide (1360614-48, MCE, USA). After the cells were made into 1 × 10⁴ cell/ml, each well was covered with 100ul cells, namely 1000 cell/well. There were 6 multiple wells in each sample, and the edge wells were added with 100ul sterile water or PBS, which were cultured at 37 °C in 5% CO₂ incubator.

HL-1 cells were transfected with mimics/inhibitor of NC (Negative Control), LRP6, and ATG5 (Autophagy Related 5) using Lipofectamine™ 2000 Transfection Reagent Kit (Thermo Fisher Scientific, Waltham, MA, USA) via the reverse transfection method according to the manufacturer's protocol.

Cell viability assay

After experimental treatment conditions were finished, the cells were made into 1×10⁴ cell/ml, each well was covered with 100ul cells. There were 6 multiple well in each sample, and the edge wells were added with 100ul PBS. The cells were cultured in hypoxia bag at 37 °C in 5% CO₂ incubator. The cells were treated with CCK-8, and detected by enzyme labeling instrument: 10ul CCK-8(Beyotime Biotechnology, China) was added into the well 48 hours later, and there was no need to change the liquid. After 1 hour, the OD value was detected by 450nm Enzyme labeling instrument (Infinite M1000, TECAN, Switzerland).

Lipid peroxidation assay

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) using a Lipid Peroxidation assay kit (s0131, Beyotime Biotechnology, China) according to the manufacturer's instructions. Briefly, HL-1 cells were homogenized with lysis buffer, and the supernatant was prepared with a thiobarbituric acid (TBA)-glacial acetic acid reagent. After incubation at 95°C for 1 h, the MDA TBA adduct was quantified colourimetrically at 532 nm using an Enzyme labeling instrument (Infinite M1000, TECAN, Switzerland). After calculating the MDA content in the sample solution, the MDA content in the initial sample was expressed by the tissue weight per unit weight, µ mol/mg.

Detection of iron by colorimetry

The pretreatment of iron-containing DMEM cultured HL-1 cells were described as the foregoing method. Iron was detected by the Iron Colorimetric Assay Kit (E1042, Beijing applygen, China) according to the manufacturer's instructions. The absorbance was determined by 550nm, which drew the standard curve and calculated the concentration of iron.

Western blot analysis
Briefly, after collection of the supernatants of the tissue or cell lysates, protein samples (20–25 mg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed with primary antibodies overnight. Diluted secondary antibodies were used to detect the matching primary antibodies. Further analysis was carried out using chemiluminescence imaging analysis system (Shanghai Clinx Science Instruments Co, China) to quantify the protein bands.

The following blotting reagents and antibodies were used: Prepare cell lysate (RIPA cracking solution, Thermo, USA), the concentrated glue and separation glue (Thermo, USA), anti-GAPDH (60004-1-Lg, Proteintech, USA), anti-LRP6 (sc-25317, Santa Cruz Biotechnology, USA), Goat anti-Mouse IgG HRP (ab205719 or ab6721, abcam, Britain), anti-LC3-A/B (12741T, CST, USA), ATG5 (ab109490, abcam, Britain), P62 (5114T, CST, USA), High sensitive ECL luminescence kit (Thermo, USA).

RNA extraction, RT–PCR and sequencing of samples

Total RNA was isolated using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA using the reverse transcription kit (Thermo#K1622, Thermo, USA) according to the manufacturer's protocol. Amplified cDNA was analysed via real-time PCR (ABI Q6, Applied Biosystems Inc.,USA) with the following primers: GAPDH (forward),

CAAAATGGTGAAGGTCGGTGT; GAPDH(reverse), GAGGTCAATGAAGGGGTCGTT; circ_0001615(forward), ATGTTTCTGGAGCAGCAAGTG; circ_0001615(reverse), CGAGAGCTGTGTAATGGAG; NC (forward),

UUCUCCGAACUGUGACUGGT; NC (reverse), ACGUGACACGGUCGGAGAATT; miR-152-3p(forward),

GCAGTCAGTGCATGACAGA; miR-152-3p(reverse), AGTGCGTGTCGTGGAGTCG; LRP6 (forward),

AATAATATGCGGCTGCTGCTT; LRP6 (reverse), GAAGACATCAATGGGAGAAT; ATG5(forward),

TGGCATGAGCCAACTGGT; ATG5(reverse), GGTGATGGCCAAACTGGT

Double luciferase reporter experiments

3’UTR sequences of circRNA1615 containing wildtype (WT) or mutant (MUT) binding sites of miR-152-3p were amplified and cloned into PUC57 vector (target DNA fragment vector) to generate circRNA1615-WT or circRNA1615-MUT Luciferase reporter vector, respectively. The transfection plasmids were PsiCHECK-2 Vector (Promega, Madison, WI, USA). The HL-1 cells were co-transfected with circRNA1615-WT, or circRNA1615-MUT, and miR-152-3p mimics (miR-152-3p) or miR-152-3p negative control (miR-NC) according to the manufacturer’s instruction of Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA). So was the transfection of LRP6-WT, or LRP6-MUT in the HL-1 cells. After 48 h transfection, the relative Luciferase activity was detected with Dual-Luciferase reporter assay Kit (Promega, Madison, WI, USA). The firefly Luciferase activity was normalized to the Luciferase activity.

RNA Binding Protein Immunoprecipitation (RIP) Assay

RIP assay was performed using the Magna RI RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions. The magnetic beads (Bio-Rad, Hercules, CA, USA) were incubated with Argonaute-2 antibody (Anti-Ago2) or Immunoglobulin G antibody (Anti-IgG). Then, HL-1 cells extract was incubated with RIP buffer containing magnetic beads conjugated to human
anti-AGO2 antibody (Millipore, Billerica, MA, USA) and IgG control (Millipore, Billerica, MA, USA). The co-precipitated RNA was purified and quantified by qRT-PCR. RNA integrity was assessed with an Agilent 2100 Bioanalyzer.

**Statistical analysis**
Continuous data were expressed as the means ± standard error (SEM). Data were analyzed using GraphPad Prism (version 8.0) and the plots were generated by GraphPad Prism. The comparisons between two groups were calculated using the two tailed t-test. For comparison across multiple experimental groups, one-way ANOVA was used, and followed by Bonferroni's post hoc test. Statistical significance was set at p < 0.05.

**Results**

**Ferroptosis inhibitor improved the pathological process of MI**

The LVEF% and LVFS% in the MI group were significantly lower than those in the control group (Fig. 1A and 1B). The cardiomyocytes were closely arranged, and its morphology was natural in the sham group, but in the MI group, the injured myocardial cells presented the coagulative necrosis (black arrow), the disappearing cardiomyocytes with new granulation tissue and gradual fibrosis (blue arrow), the inflammatory cells infiltration (orange arrow), and bleeding (green arrow) (Fig. 1C). Further the MI group had higher levels of MDA and Fe$^{2+}$ in infarcted myocardial tissue (Fig. 1D). The ferroptosis inhibitor reversed the low LVEF% and LVFS% in infarcted mice (Fig. 1A and 1B), and showed the mild cells injury, and a few coagulative necrosis of cardiomyocytes (black arrow) (Fig. 1C), and decreased the levels of MDA and Fe$^{2+}$ in infarcted myocardial tissue (Fig. 1D). Moreover, Fer-1 significantly improved the viability of cardiomyocytes treated with erastin (ferroptosis inducer), but ZVAD-FMK (apoptosis inhibitor) and necrosulfonamide (necrosis inhibitor) did not have similar effect (Fig. 1E).

Thus, in the established mouse MI model, we found that ferroptosis occurred in mouse MI. Further ZVAD-FMK (apoptosis inhibitor) and necrosulfonamide (necrosis inhibitor) did not improve the viability of cardiomyocytes treated with erastin (ferroptosis inducer), but Fer-1 (ferroptosis inhibitor) could improve left ventricular function and pathological changes of MI.

**LRP6 was involved in the process of ferroptosis in cardiomyocytes through autophagy**

The expression of LRP6 in the infarcted myocardial tissue was significantly lower in comparison to the control group (Fig. 2A). LRP6 interference fragment siRNA was transfected into cardiomyocytes, and the expression of LRP6 in cardiomyocytes was significantly down-regulated after transfection with LRP6-siR-mus-4649 compared with LRP6-siR-mus-2488 or LRP6-siR-mus-513 (Fig. 2B). After induction of hypoxia and ferroptosis, compared with the control group, the activity of cardiomyocytes interfered by LRP6 was lower (Fig. 2C), and the levels of MDA and Fe$^{2+}$ in cardiomyocytes were higher (Fig. 2D and 2E).
In the cardiomyocytes treated with hypoxia and erastin, the expression of autophagy-related proteins LC3-
A/B (microtubule associated protein 1 light chain 3-A/B) and ATG5 after LRP6 interference was higher in
comparing with the control group, but the expression of p62 (sequestosome-1) was lower (Fig. 3A). ATG5
siRNA significantly decreased the expression of ATG5 in cardiomyocytes (Fig. 3B). In cardiomyocytes	
treated with hypoxia and erastin, the cardiomyocytes with LRP6 siRNA and ATG5 siRNA could repair the
decreasing survival rate induced by LRP6 deletion (Fig. 3C), and reverse the increase of Fe^{2+} and MDA
induced by LRP6 deletion (Fig. 3D and 3E).

Thus, to explore whether LRP6 regulates ferroptosis through autophagy, we detected the expression of
LRP6 in myocardial tissue, and found the low expression of LRP6 in MI group, interference with LRP6
could promote ferroptosis of cardiomyocytes, increase the expression of autophagy-related proteins LC3-
A/B and ATG5, and decrease the expression of p62. In addition, the cell was interfered with LRP6 and
inhibited autophagy simultaneously, its survival rate increased, and the level of ferroptosis decreased.

**Screening and identification of circRNA1615 plays a role as a miRNA sponge by targeting LRP6**

We screened the miRNA targeting LRP6 and the circRNAs, that bind to these miRNA through CLIP
database to construct circRNA-miRNA-LRP6 regulatory network (Fig. 4A). Based on the analysis of LRP6
regulatory network, it was found that mmu-miR-466c-5p, mmu-miR-466o-5p, and mmu-miR-679-5p
miRNA had strong binding ability, while GEO database data (GSE81636) showed that the expression of
miR-152-3p in myocardial tissue of MI mice was significantly higher than that of sham-operated group at
2 days after MI, and it significantly rose at 7 day of MI. In addition, the expression of miR-152-3p was
significantly increased in MI group and hypoxia-treated cardiomyocytes (Fig. 4B and 4C). The expression
of five circRNAs targeting miR-152-3p was detected in mouse myocardial tissue, and the expression of
circRNA1615 was significantly down-regulated in the MI group (Fig. 4D). Further the expression of
circRNA1615 decreased significantly after hypoxia (Fig. 4E). Using polymeric primers and divergent
primers to amplify gDNA and cDNA of mouse myocardial tissue, only divergent primers amplified
circRNA1615 in cDNA. Sequencing of PCR products revealed that circRNA1615 was a circular RNA
derived from Copb1 gene (Fig. 4F and 4G).

We constructed the overexpressed circRNA1615 plasmids, then transfected it to cardiomyocytes. It was
found that the expression of circRNA1615 in mouse cardiomyocytes transfected with overexpressed
plasmids was higher than that of control plasmids (Fig. 5A). The survival rate of overexpressed circRNA
cells increased in cardiomyocytes treated with hypoxia and erastin (Fig. 5B), suggesting that
circRNA1615 inhibits ferroptosis in cardiomyocytes.

Thus, to study whether the circRNAs are involved in the regulation of ferroptosis of cardiomyocytes by
LRP6, we constructed a circRNA-miRNA-LRP6 regulatory network. Bioinformatics analysis combined with
experimental tests showed that the expression of miR-152-3p in myocardial tissue of infarcted mice and
hypoxia-treated cardiomyocytes was increased, while the matching circRNA1615 (circBase:
mmu_circ_0001615) expression was down-regulated. Noticeably, the overexpression of circRNA1615 could inhibit cardiomyocyte death (Figs. 4 and 5).

In addition, we studied whether circRNA1615 has the ability to bind to miRNA. The binding sites of circRNA1615 and miR-152-3p were analyzed in database, and psichек-2 reporter gene wild-type vector (including miR-152-3p target sequence) and mutant vector (miR-152-3p target sequence point mutation) of LRP6 were constructed and cotransfected with miR-152-3p mimics. Compared with the negative control RNA (NC), miR-152-3p decreased the luciferase activity of the wild-type vector (Fig. 6A); RIP experiment showed that circRNA1615 could be precipitated by miR-152-3p and AGO2 antibodies, indicating the sponge adsorption ability of circRNA1615 to miR-152-3p (Fig. 6B). The expression of miR-152-3p in cardiomyocytes was decreased after overexpression of circRNA1615, while the expression of miR-152-3p was up-regulated by knocking down circRNA1615 (Fig. 6C). To test whether miR-152-3p targets the binding sites of miR-152-3p and LRP6 in the database, the wild-type psichек-2 reporter gene vector and mutant vector of LRP6 were constructed and cotransfected with miR-152-3p mimics and miR-NC. Compared with the negative control RNA (NC), miR-152-3p decreased the luciferase activity of the wild-type vector (Fig. 6D). Overexpression of miR-152-3p in cardiomyocytes inhibited the expression of LRP6, while the knocking down miR-152-3p upregulated the expression of LRP6 (Fig. 6E).

Therefore, further the overexpression or knockdown of circRNA1615 in cardiomyocytes negatively regulated the expression of miR-152-3p, the luciferase and RIP experiments verified the spongy adsorption of circRNA1615 to miR-152-3p. In addition, the overexpression or knockdown of miR-152-3p in cardiomyocytes could negatively regulate the expression of LRP6, and luciferase reporter gene assay confirmed the targeting effect of miR-152-3p on LRP6 (Fig. 6). These results suggest that circRNA1615 regulates the expression of LRP6 through sponge adsorption of miR-152-3p, and then regulates ferroptosis in cardiomyocytes.

**Discussion**

Searching for new molecular targets of ferroptosis is gradually becoming the focus in the field of cardiovascular disease research. However, the effect and molecular mechanism of ferroptosis in the pathological development of MI are not clear. We explored the regulatory effect and molecular mechanism of LPR6 on myocardial cell ferroptosis by establishing a model of MI in vivo and in vitro, constructed the regulatory network of circRNA-miRNA-LRP6 by the bioinformatics analysis, and focused on the biological function and molecular mechanism of circRNA1615 regulating ferroptosis in MI by the overexpression or knockdown of circRNA1615, the RIP experiments, and double luciferase reporter gene assay. Based on our findings, circRNA1615 regulates the expression of LRP6 through sponge adsorption of miR-152-3p, and then regulates LRP6-mediated autophagy-related ferroptosis in cardiomyocytes of MI. (Fig. 7)

In the reperfusion injury caused by heart transplantation or coronary occlusion, cardiomyocytes will incur ferroptosis and release inflammatory mediators to aggravate the myocardial injury. Ferroptosis inhibitor
Fer-1 reduced cardiomyocyte death and prevented the recruitment of neutrophils, reduced the infarct size, improved left ventricular systolic function and reduced left ventricular remodeling. Quantitative proteomic analysis shows that the down-regulation of glutathione peroxidase 4 (Gpx4) in MI contributes to the ferroptosis of cardiomyocytes, and clinical studies have shown that myocardial iron is a risk factor for left ventricular remodeling after MI. In our study, we also found that ferroptosis occurred in the MI mouse, and ferroptosis inhibitor Fer-1 could improve left ventricular function and pathological changes of MI (Fig. 1), suggested that ferroptosis might be a novel therapeutic target for MI.

LRP6 is a member of the low-density lipoprotein receptor, which focuses on metabolic regulation, especially in lipid homeostasis and glucose metabolism associated with rapamycin target protein (mTOR) pathway, regulating a variety of cellular mechanisms, and LDLR cycle in hepatocytes. Targeting LRP6-mediated signaling pathways can improve aortic pressure and lipid metabolism, and reduce neointimal formation and myocardial ischemia/reperfusion injury. These suggest that LRP6 is an important factor in the development of heart disease.

Further cardiac LRP6 deletion inhibited autophagy degradation and fatty acid utilization, togethether with the activation of Drp1 and down-regulation of nuclear TFEB (transcription factor EB), subsequently brought fatal dilated cardiomyopathy and cardiac dysfunction. Importantly, the mitochondrial DNA stress triggers autophagy-dependent ferroptotic death. ROS-mediated autophagy increases intracellular iron levels and ferroptosis by ferritin and transferrin receptor regulation.

In our study, the interference with LRP6 could promote ferroptosis of cardiomyocytes, increase the expression of autophagy-related proteins LC3-A/B and ATG5, and decrease the expression of p62. In addition, the cell was interfered with LRP6 and inhibited autophagy simultaneously, its survival rate increased, and the level of ferroptosis decreased (Figs. 2 and 3). These results confirmed that LRP6 deletion could promote autophagy, then trigger ferroptosis of cardiomyocytes, and participate in the regulation of MI pathological process.

circRNAs is a kind of non-coding RNA molecules with closed ring structure produced by special selective splicing, which modulate the transcriptional expression of genes like miRNA sponge and the splicing of pre-RNA, or protein production by binding to ribosomal related proteins. CircNfix (circRNA Nfix) deletion can induce myocardial regeneration after MI in adult mice. Overexpression of circFndc3b (derived from exons2 and3 of the Fndc3b gene) in infarcted heart can reduce cardiomyocyte apoptosis, enhance neovascularization and improve left ventricular function. The expression of circNFIB (circBase: mmu_circ_0011794) in mouse heart after MI is decreased, and the up-regulation of circNFIB can reduce myocardial fibrosis by inhibiting miR-433.

In our study, we constructed a circRNA-miRNA-LRP6 regulatory network. Bioinformatics analysis combined with experimental tests showed that the expression of miR-152-3p in myocardial tissue of the infarcted mice and the hypoxia-treated cardiomyocytes was increased, while the matching circRNA1615 expression was down-regulated. Noticeably, the overexpression of circRNA1615 could inhibit
cardiomyocyte death (Figs. 4 and 5). Further the overexpression or knockdown of circRNA1615 in cardiomyocytes negatively regulated the expression of miR-152-3p, and the spongy adsorption of circRNA1615 to miR-152-3p. In addition, the overexpression or knockdown of miR-152-3p in cardiomyocytes could negatively regulate the expression of LRP6, and the targeting effect of miR-152-3p on LRP6 (Fig. 6), thereby our findings suggested that circRNA1615 would regulate the expression of LRP6 through sponge adsorption of miR-152-3p.

**Conclusion**

CircRNA1615 inhibits ferroptosis via modulation of autophagy by the miRNA152-3p/LRP6 molecular axis in cardiomyocytes of myocardial infarction.

**List Of Abbreviations**
| Abbreviation | Non Abbreviation |
|--------------|------------------|
| ACR circRNAs: | autophagy-related Cyclic RNAs |
| ANOVA: | analysis of variance |
| Anti-Ago2: | argonaute-2 antibody |
| ATG5: | autophagy Related 5 |
| circFndc3b: | derived from exons2 and3 of the Fndc3b gene |
| CircNfix: | circRNA Nfix |
| DMSO: | dimethyl sulfoxide |
| Drp1: | dynamin-related protein1 |
| EF%: | ejection fraction |
| FAM65B: | family with sequence similarity 65 member B |
| FBS: | fetal bovine serum |
| Fer-1: | ferrostatin-1 |
| FS%: | left ventricular short axis shortening |
| Gpx4: | glutathione peroxidase 4 |
| LAD: | left coronary artery |
| LC3-A/B: | microtubule associated protein 1 light chain 3-A/B |
| LRP6: | low density lipoprotein receptor related protein 6 |
| LVIDd: | left ventricular end-diastolic diameters |
| LVIDs: | left ventricular end-systolic diameters |
| MDA: | malondialdehyde |
| MI: | myocardial infarction |
| mTOR: | rapamycin target protein |
| MUT: | mutant |
| NC: | negative Control |
| PBS: | phosphate buffered solution |
| PFA: | paraformaldehyde |
| Pink1: | phosphatase and tensinhomolog-induced putative kinase1 |
| PVDF: | polyvinylidene difluoride |
Abbreviation | Non Abbreviation
--- | ---
p62: | sequestosome-1
RIP: | RNA Binding Protein Immunoprecipitation
SDS-PAGE: | sodiumdodecyl sulfate polyacrylamide gel electrophoresis
SEM: | standard error
TBA: | thiobarbituric acid
TFEB: | transcription factor EB
WT: | wildtype

**Declarations**

**Ethics approval and consent to participate**

The study was approved by the ethics board of Laboratory Animal Research Center, Tongji University (No. TJTJ00621401).

**Consent for publication**

All of authors have read and approved the manuscript.

**Data Availability Statement**

The data used to support the findings of this study are available from the corresponding author upon request.

**Competing interests**

No authors have any potential conflicts of interest to be disclosed.

**Authors' Contributions**

S.K. conceived and designed the study. R.L.L., C.H.F. and S.Y. G. participated in the experiment and data collection. S.K was responsible for quality control. S.K. analyzed data and drafted the manuscript. S.K. and R.L.L. participated in the fund support of the study.

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**Figures**
Figure 1

Ferroptosis inhibitor improves the pathological process of MI Fig.1(A and B) The LVEF% and LVFS% in both the MI group and the control group. Time stamp, 200ms; Scale bar, 1 mm. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m.**P<0.01 compared with ctrl by one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test. Fig.1(C) Using immunohistochemistry staining cardiomyocytes death in
both the sham group and the MI group. Scale bar, 50 um. The presented images were representative of three separate experiments. Fig.1(D) Evaluation of MDA and Fe2+ levels in infarced myocardial tissue. Upper, MDA level; bottom, Fe2+ level. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Fig.1(E) Effect of Fer-1 mediated the viability of cardiomyocytes treated with erastin, ZVAD-FMK, or necrosulfonamide. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl by one-way ANOVA with Bonferroni’s post hoc test.

Figure 2
LRP6 deletion promotes ferroptosis in cardiomyocytes Fig.2(A) The protein expression of LRP6 in the infarcted myocardial tissue was detected using western blot analysis with SDS-PAGE. Top, typical blots; bottom, pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. Fig.2(B) Analysis of expression of LRP6 transfected with LRP6-siR-mus-4649, LRP6-siR-mus-2488 or LRP6-siR-mus-513. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl by one-way ANOVA with Bonferroni's post hoc test. Fig.2(C) Evaluation of the activity of cardiomyocytes interfered with LRP6 following induction of hypoxia and ferroptosis. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl by two-tailed unpaired Student's t-test. Fig.2(D and E) Representative the levels of MDA and Fe2+ in cardiomyocytes following induction of hypoxia and ferroptosis. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl by one-way ANOVA with Bonferroni's post hoc test.
LRP6 deletion regulates ferroptosis in cardiomyocytes through autophagy Fig.3(A) The presented blots were obtained from the expression of autophagy-related proteins LC3-A/B, ATG and p62 after LRP6 interference in the cardiomyocytes treated with hypoxia and erastin. Top, typical blots; Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Fig.3(B) Effect of ATG5 siRNA mediated the expression of ATG5 in
cardiomyocytes. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl by one-way ANOVA with Bonferroni’s post hoc test. Fig.3(C) Analysis of the cardiomyocytes with LRP6 siRNA and ATG5 siRNA influence on survival rate in cardiomyocytes treated with hypoxia and erastin. Statistical analysis were obtained from five mice in each group and three separate experiments. Fig.3(D and E) In this case, the presented Fe2+ and MDA level induced with LRP6 deletion. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. *P<0.05 and **P<0.01 compared with ctrl or Si-LRP6 by one-way ANOVA with Bonferroni’s post hoc test.
Figure 4

Screening and identification of circRNA1615 targeting LRP6 in the process of MI Fig.4(A) To construct circRNA-miRNA-LRP6 regulatory network through CLIP database. Fig.4(B and C) Based on the analysis of LRP6 regulatory network, Evaluation of the binding ability of mmu-miR-466c-5p, mmu-miR-466o-5p, mmu-miR-679-5p miRNA, and the representative of expression of miR-152-3p in GEO database data (GSE81636). Fig.4(D) The expression of five circRNAs targeting miR-152-3p in mouse myocardial tissue.
Fig.4(E) Effect of expression of circRNA1615 after hypoxia. Pooled data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times in Fig.4(B,C,D, and E). Data were means±s.e.m. ***P<0.001 compared with ctrl by two-tailed unpaired Student's t-test. Fig.4(F and G) Representative of circRNA1615 derived from Copb1 gene.

**Figure 5**

CircRNA1615 inhibits ferroptosis in cardiomyocytes Fig.5(A) The presented expression of circRNA1615 in mouse cardiomyocytes transfected with overexpressed plasmids. Fig.5(B) Analysis of the survival rate of overexpressed circRNA cells treated with hypoxia and erastin. Pool data, biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. **P<0.01 compared with ctrl by two-tailed unpaired Student's t-test.
Fig.6(A) Luciferase assay for the ability of circRNA1615 binding to miR-152-3p. Fig.6(B) circRNA1615 precipitated with miR-152-3p and AGO2 antibodies in RIP experiment. Fig.6(C) The expression of miR-152-3p in cardiomyocytes after overexpression or knocking down circRNA1615. Fig.6(D) Analysis of the binding sites of miR-152-3p and LRP6. Fig.6(E) The presented expression of LRP6 after overexpression or knocking down miR-152-3p. Left, pool data; Right, the presented typical blots were representative of three separate experiments. Pool data Fig.6(A,B,C,D and E), biological replicate indicated five mice in each group, the number of technical repeated experiments presented three times. Data were means±s.e.m. ***P<0.001 compared with ctrl by one-way ANOVA with Bonferroni's post hoc test.
The diagram of mechanism hypothesis circRNA1615 may inhibit autophagy via the spongy adsorption of miR-152-3p targeting LRP6, and then prevent LRP6-mediated autophagy-related ferroptosis in cardiomyocytes, finally regulate the pathological process of MI.