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ORIGINAL ARTICLE

LncRNA RNA ROR Aggravates Hypoxia/Reoxygenation-Induced Cardiomyocyte Ferroptosis by Targeting miR-769-5p/CBX7 Axis

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Abstract

Ferroptosis is a new way of cell death which is reported to participate in the pathology of myocardial ischemia–reperfusion (MI/R) injury, but it's mechanism remains unclear. The present investigation is to study the emerging role of long non-coding RNA (lncRNA) regulator of reprogramming (ROR) in cardiomyocyte ferroptosis after hypoxia/reoxygenation (H/R) administration. RT-qPCR and/or Western blot methods were performed to examine the gene/or protein levels, and CCK-8, ELISA, and DCFH-DA staining determined the cellular viability and ferroptosis. Dual-luciferase and RNA immunoprecipitation were applied to verify molecular interaction. LncRNA ROR and miR-769-5p were overexpressed and reduced in blood samples from MI patients and H/R-treated AC16 cells, respectively. Mechanistically, lncROR sponged to miR-769-5p, thus upregulating CBX7 expression. Functional experiments presented that lncRNA ROR silence mitigated H/R-stimulated inflammatory damage, oxidative stress, and ferroptosis in AC16 cells, whereas these roles could be reversed by co-downregulation of miR-769-5p or co-overexpression of CBX7. These data uncovered that lncRNA ROR prevented against H/R-induced cardiomyocyte ferroptosis by modulating miR-769-5p/CBX7 signaling, emphasizing the therapeutic value of lncRNA ROR in MI/R injury.

Keywords Ferroptosis · Myocardial ischemia–reperfusion injury · Cardiomyocytes · lncRNA ROR

Abbreviations

MI/R	Myocardial ischemia–reperfusion
lncRNA	Injury; long non-coding RNA
ROR	Regulator of reprogramming

Guorong Lai and Jie Shen are the co-first authors.

Extended author information available on the last page of the article

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MI	Myocardial infarction
H/R	Hypoxia-reoxygenation
GPX4	Glutathione peroxidase 4
ROS	Reactive oxygen species
ACSL4	Acyl-CoA synthetase long-chain fatty acyl-CoA synthetase 4
ceRNA	Competitive endogenous RNA
CBX7	Chromobox protein homolog 7
FBS	Fetal bovine serum
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
siRNA	Small interfering RNA
CCK-8	Cell counting kit 8
ELISA	Enzyme-linked immunosorbent assay
RIP	RNA immunoprecipitation
PRC1	Polycomb repressive complex 1

Introduction

Cardiovascular diseases are considered to be a most common and serious disease threatening human health worldwide (Flora and Nayak 2019). Myocardial infarction (MI) caused by insufficient blood supply to the heart is a major event of cardiovascular disease (Orlandi et al. 2020). Coronary aortocoronary bypass, thrombolytic therapy, percutaneous transluminal coronary angioplasty, etc. can effectively restore blood flow to the heart and decrease MI size (Neri et al. 2017). However, these therapies inevitably lead to a lots of additional damages, which known as myocardial ischemia/reperfusion (MI/R) injury (Li et al. 2021). Although hypoxia-reoxygenation (H/R)-triggered excessive reactive oxygen species (ROS), cardiomyocyte apoptosis, inflammatory responses, intracellular calcium stress, and ferroptosis were proved as main pathology of MI/R injury (Li et al. 2020; Peng et al. 2020; Lu et al. 2010), but the detailed regulatory network and effectively strategies remain limited. It is important to study novel molecular target and pathway to improve the prognosis and survival of MI/R injury patients.

Ferroptosis is a new type of iron-dependent cell death characterized by the accumulation of ROS and large amounts of lipid peroxides (Wu et al. 2021). It has been shown that iron content was increased in cardiac tissue in rat models of cardiac arrest and deferoxamine and UAMC-3203, specific ferroptosis inhibitors, could suppress cardiomyocyte ferroptosis to restore cardiac function (Jin et al. 2022). Glutathione peroxidase 4 (GPX4) is mainly responsible for the removal of lipid peroxide products and is a vital repressor of ferroptosis (Seibt et al. 2019). Loss of GPX4 was proved to promote cardiomyocyte ferroptosis during MI/R injury (Sun et al. 2021a; Yu et al. 2022). In addition, acyl-CoA synthetase long-chain fatty acyl-CoA synthetase 4 (ACSL4) is an enzyme that regulates lipid biosynthesis and known as an essential component for ferroptosis execution (Wang et al. 2022). Activation of ASCL4-mediated ferroptosis worsened the myocardial damage caused by MI/R

(Zhang et al. 2022a; Fan et al. 2021). These observations suggested that targeting ferroptosis is a critical pathway to improve MI/R.

Long non-coding RNA (lncRNAs), a class of RNA molecules with molecular weight of more than 200 nucleotides, are key regulatory factors in the progression of MI/R injury. For example, lncRNA Gm4419 was elevated in myocardial tissues of MI/R rat model and H/R-induced cardiomyocytes and its depletion could improve myocardial infarction and apoptosis via regulating miR-682/TRAF3 cascade (Zhao et al. 2020). Enforced expression of lncRNA HULC could directly sponge miR-337-5p to repress NLRP3/Caspase1/IL-1βsignaling, thus attenuating MI/R- or H/R-triggered cardiomyocyte apoptosis (Liang et al. 2021). Regulator of reprogramming (ROR, gene ID: 100885779, NR_048536.2) was a lincRNA capable of regulating various physiological processes. There were several reports described that lncRNA ROR was greatly overexpressed in myocardial tissues and could exacerbate myocardial tissues inflammation, apoptosis, and oxidative stress (Zhang et al. 2018; Sun et al. 2022; Liang et al. 2019). Whereas, whether lncRNA ROR is related to cardiomyocyte ferroptosis after MI/R has not been reported so far.

Here, we sought to probe the underlying roles and mechanism of lncRNA ROR in cardiomyocyte ferroptosis after H/R treatment. Our work demonstrated lncRNA ROR positively triggered H/R- challenged ferroptosis through acting as a competitive endogenous RNA (ceRNA) to target miR-769-5p/Chromebox protein homolog 7 (CBX7). These results uncovered a novel regulatory axis about lncRNA ROR/miR-769-5p/CBX7 in pathogenesis of MI/R injury, which provided some convictive target for treating MI/R injury.

Methods

Specimen Collection

A total of 48 patients diagnosed with myocardial infarction (MI) and 48 cases of healthy volunteers were included in the study. The samples collection criteria of the subjects were referred to the previously described (Tong et al. 2021). Blood samples (about 5 mL) were obtained from each participator and centrifuged (2000 g, 10 min) for isolating serum and then stored at -80℃ until use. The experimental protocol was acquired the approvement of Second Affiliated Hospital of Nanchang University hospital. All participator was signed the informed consent.

Cell Culture and Administration

AC16 cells, a human cardiomyocyte cell line, was acquired from BeNa Culture Collection (Beijing, China). Cells were growth in conventional DMEM medium (Gibco, Thermo, MA, USA) maintaining 10% fetal bovine serum (FBS, Gibco), penicillin (100U/ml), and streptomycin (100 μg/ml) at a 5% CO_2, 37℃ incubator. For H/R treatment, AC16 cells were maintained in DMEM medium with free of glucose and serum for 5 h under hypoxic atmosphere (95% N_2 and 5% CO_2). Afterward, AC16

cells were transferred into normal complete DMEM medium under reoxygenation incubator (95% O_2 and 5% CO_2) for another 2 h.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol® reagent (Invitrogen; Thermo) was applied to extract total RNA samples and a PrimeScript RT Master Mix Kit (TaKaRa, Japan) or PrimeScript miRNA cDNA Synthesis Kit (TaKaRa) was utilized to generate cDNA synthesis in accordance with protocol instruction. Then, the RT-qPCR assay was conducted using SYBR Green reagent (TaKaRa). The relative gene expression was calculated by e $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH or U6, respectively. The primer sequences were presented as follows (5′-3′): lncRNA ROR (F): CAAACA CATCGCCACTCTGC, (R): AGGAGTCAGGAGAAGGTGCT; miR-769-5p (F): GGCTGAGACCTCTGGGITC, (R) CAGTGCGTGTCGTGGAGT; CBX7 (F) CATGGAGCTGTCAGCCATC, (R) CTGTACTTTGGGGGCCATC; GAPDH (F) CCTGTTCGACAGTCAGCCG, (R) GAGAACAGTGAGCGCCTAGT; and U6 (F) CTCGCTTCGGCAGCACA, (R) AACGCTTCACGAATTTGCGT.

Cell Transfection

The miR-769-5p mimics or inhibitor, small interfering RNA (siRNA) against lncRNA ROR (si-lncRNA ROR#1, sense: AAGACUACAACUUCCAGCUUC CUUU, antisense: AAAGGAAGCUGGAAGUUGUAGUCUU; si-lncRNA ROR#2, sense: CACUCUUAUGGAAGGAGGAAAUCUA, antisense: UAGAUU UCCUCCUUCCAUAAGAGUG; si-lncRNA ROR#3, sense: UAGCUUCCAGAA GACAGAAUCCUUU, antisense: AAAGGAUUCUGUCUUCUGGAAGCUA), overexpressing vectors of CBX7 (pc-CBX7), and their related negative controls (mimics/inhibitor NC; siNC, sense: CACAUUGGUGAAGAGAAGUAUCCUA, antisense: UAGGAUACUUCUCUUCACCAAUGUG; pc-NC) were all acquired from GenePharma (Shanghai, China). For cell transfection, AC16 cells were plated into 6-well plates at the density of 10^6 cells/well, and then the above molecules (50 nM) were transfected into cells using Lipofectamine 3000 (Invitrogen). The transfection efficiency was measured utilizing RT-qPCR assay, and the cells were followed by exposing to H/R treatment.

Cell Counting Kit 8 (CCK-8) Assay

AC16 cell viability was quantified by CCK-8 assay. In brief, AC16 cells with different treatments were plated into 96-well plates at 5000 cells/well and cultured for overnight. Then, 10-μL CCK-8 (Sigma) was added to each well and incubated for another 4 h. Finally, the absorbance of each well was recorded using a microplate reader (Bio-Tek).

Enzyme-Linked Immunosorbent Assay (ELISA)

After different treatments, AC16 cells were harvested and lysed with the lysis buffer. Then, the cell supernatant was collected for the assessments of IL-1β (BMS224-2, Invitrogen), IL-18 (A35613, Invitrogen), IL-6 (ab178013, Abcam, Cambridge, MA, USA), CK-MB (ab193696, Abcam), and LDH (A020-2-2, Jiancheng Bioengineering Institute, Nanjing, China) according to responding protocols. A microplate reader (Bio-Tek, WA, USA) was used to determine the OD value.

Lipid Peroxidation and Iron Content Assay

Commercial kits for determining MDA (A003-1-2, Jiancheng Bioengineering Institute), GSH (A006-1-1, Jiancheng Bioengineering Institute), and SOD (A001-3-2, Jiancheng Bioengineering Institute) were purchased to assess the cellular oxidative stress levels. Moreover, the iron content in AC16 cells was determined by an Iron assay kit (ab83366, Abcam) described in manufacturer's instructions.

ROS Assessment

2,7-dichlorofluorescein diacetate (DCFH-DA) Cellular ROS Assay kit (ab113851, Abcam) was acquired to measure the ROS level in AC16 cells. In brief, AC16 cells was incubated with 10-μM DCFH-DA for 30 min and then washed by PBS solution for twice. Subsequently, cells were followed by stimulated LPS/ATP. Finally, the cells were imaged by a fluorescent microscope.

Western Blot Assay

The total proteins of AC16 cells were extracted and quantified the concentration by a BCA kit (Bey time, Shanghai, China). The equal loading number of proteins in each group was separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% non-fat milk for 1 h and then incubated with the primary antibodies against GPX4 (ab219592, Abcam), ASCL4 (SAB2701949, Sigma, St. Louis, MO, USA), CBX7 (ab178411, Abcam), and GAPDH (ab8245, Abcam) at 4 ℃. On the next day, the membrane was further incubated with the secondary antibody for anti-Rat IgG (HRP; ab288151, Abcam) for another 60 min.

RNA Immunoprecipitation (RIP) Assay

Magna RIP kit was obtained from Merck (Darmstadt, Germany) to examine the binding relationship between lncRNA ROR and miR-769-5p. In brief, AC16 cells were lysed in RIP lysis buffer and centrifuged for collecting cell lysates. The rabbit

anti-Ago2 (5 μg, ab186733, Abcam) and normal rabbit IgG (5 μg, ab37415, Abcam) were mixed with protein A/G magnetic beads for preparing the magnetic bead-antibody complexes. The RNA–protein complexes were then immunoprecipitated by magnetic bead-antibody complexes, and the RNA was extracted as above described.

Dual-Luciferase Reporter Assay

The wild and mutant binding sites for miR769-5p in lncRNA ROR (ROR-wt/mut) and CBX7 (CBX7-wt/mut) were inserted into the pmirGLO vector (Promega, Madison, WI, USA). Cells were co-transfected with miR-769-5p mimics and the recombinant reporter by Lipofectamine 3000 (Invitrogen). At 48-h post-transfection, the luciferase activity was analyzed with Dual-Glo Luciferase Assay System (Promega).

Data Analysis

All data were repeated at least for three independent times, the assumption of normality was analyzed by Shapiro–Wilk test, and the homogeneity of variance was performed by Bartlett test. Then, data that meet the requirements were calculated by GraphPad Prism 8.0 software and expressed as "Mean ± SD." If the data do not meet the normal distribution and variance homogeneity test, the number of repetitions of the experiment would be increased until the requirements were met. Data comparison between two groups were performed using Student's t test, and data variance among multiple groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P < 0.05$ was statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Table 1 Characteristics of enrolled participants in this work

Clinical characteristics	Normal ($n = 48$)	MI ($n = 48$)	P value
Age, years	61.7 ± 2.6	50.7 ± 2.7	< 0.001
Sex, male/female	30/18	25 ± 23	0.302
Heart rate, beats per minute	74.9 ± 6.7	77.1 ± 7.2	0.123
BMI (kg/m^2)	21.6 ± 2.7	22.1 ± 2.3	0.298
Systolic blood pressure, mmHg	133 ± 13	131 ± 12	0.335
Diastolic blood pressure, mmHg	74 ± 3	75 ± 4	0.055
TC, mmol/L	1.8 ± 0.2	4.3 ± 0.5	< 0.001
TG, mmol/L	1.7 ± 0.8	2.2 ± 1.0	0.010
HDL, mmol/L	0. 7 ± 0.1	1.1 ± 0.2	< 0.001
LDL, mmol/L	3.1 ± 0.3	3.7 ± 0.6	< 0.001

MI myocardial infarction, *BMI* body mass index, *TC* total cholesterol, *TG* triglycerides, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

Results

Expressions of lncRNA ROR and miR-769-5p After MI/R Injury

We firstly compared the expressions of lncRNA ROR and miR-769-5p in serum samples between MI and healthy control group by RT-qPCR assay. As presented in Table 1, the total cholesterol (TC), triglycerides (TG), low-/high-density lipoprotein (LDL, HDL) were exhibited higher levels in MI patients compared to those in healthy control. Then, the transcript of lncRNA ROR was significantly overexpressed and miR-769-5p was markedly decreased in serum samples of MI patients than those in healthy control (Fig. 1A, B). Moreover, a negative correlation between lncRNA ROR and miR-768-5p was observed after Pearson analysis (Fig. 1C). In vitro, it also observed that lncRNA ROR was upregulated but miR-769-5p was reduced in a time-dependent manner after H/R treatment (Fig. 1D, E). These results indicated the potential regulatory relationship between lncRNA ROR and miR-769-5p in MI/R damage.

LncRNA ROR Directly Targeted miR-769-5p to Repress Its Expression

The sequence of lncRNA ROR (NR_048536.2) was acquired from NCBI database. After comparison, the binding bases between lncRNA ROR and miR-769-5p were shown in Fig. 2A. Results of RIP assay revealed that both lncRNA ROR and

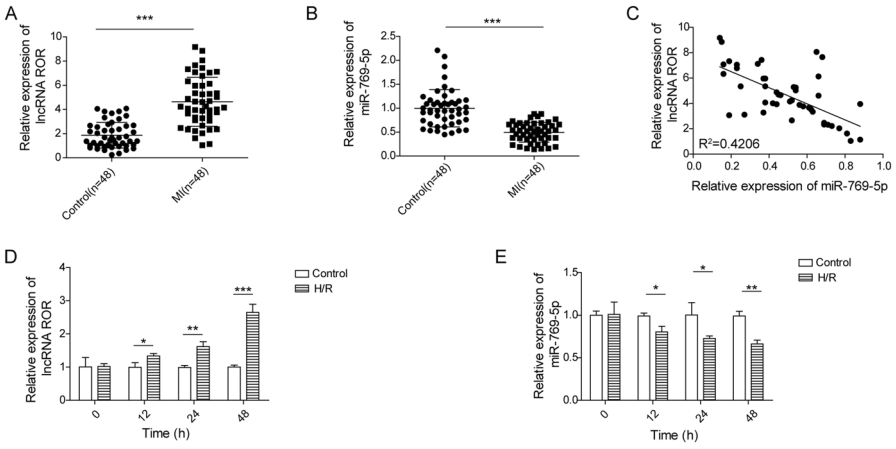

Fig. 1 Expressions of lncRNA ROR and miR-769-5p after MI/R injury. **A**–**C** The serum samples were harvested from MI ($n=48$) and healthy control ($n=48$). **A**, **B** Serum expression of lncRNA ROR and miR-769-5p were quantified employing RT-qPCR. **C** The clinical correlation between lncRNA ROR and miR-769-5p was analyzed by Pearson analysis. **D**–**E** After H/R treatment for 12, 24, and 48 h, the levels of lncRNA ROR and miR-769-5p in AC16 cells were determined by RT-qPCR. Data were showed as Mean ± SD. $*P<0.05$, $**P<0.01$, and $***P<0.001$. *MI* myocardial infarction, *ROR* regulator of reprogramming, *RT-qPCR* Reverse transcription-quantitative polymerase chain reaction, *H/R* hypoxia-reoxygenation

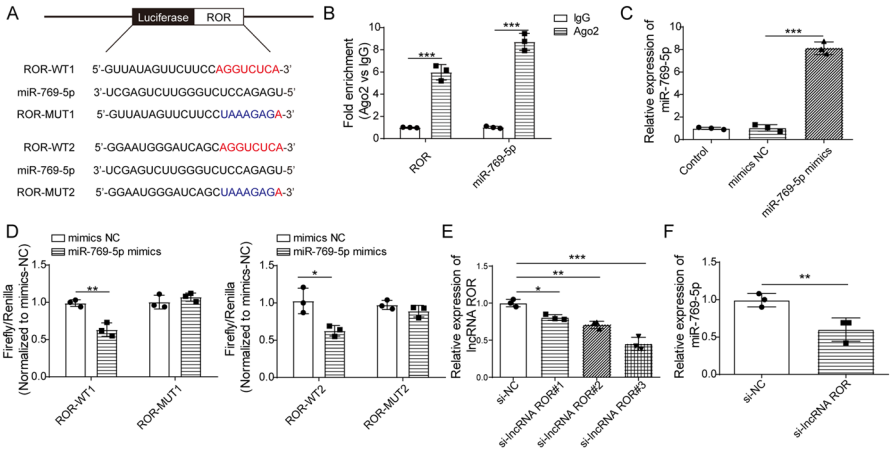

Fig. 2 LncRNA ROR directly targeted miR-769-5p to repress its expression. **A** The putative binding site between lncRNA ROR and miR-769-5p were presented. **B** RIP experiment was performed for validating the interaction between miR-769-5p and lncRNA ROR, and the targeted lncRNA ROR and miR-769-5p immunoprecipitated by anti-Ago2/anti-IgG were quantified by RT-qPCR. **C** Mimics NC and miR-769-5p mimics were transfected into AC16 cells, respectively, and the expression of miR-769-5p was detected by RT-qPCR. **D** Dual-luciferase assay verified the binding relationship between lncRNA ROR and miR-769-5p. **E**, **F** SiRNAs against lncRNA ROR was transfected into AC16 cells and the molecules of lncRNA ROR and miR-769-5p were measured by RT-qPCR. Data were showed as Mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *RIP* RNA immunoprecipitation, *ROR* regulator of reprogramming, *RT-qPCR* Reverse transcription-quantitative polymerase chain reaction, *NC* negative control

miR-769-5p were enriched in precipitated RNA complex of anti-Ago2 (Fig. 2B). Next, we established miR-769-5p overexpressing cells by transfection with miR-769-5p mimics, and the transfection efficiency was examined by RT-qPCR. It had confirmed that miR-769-5p was dramatically elevated in presence of miR-769-5p mimics (Fig. 2C). Moreover, miR-769-5p overexpression both repressed the luciferase activities of AC16 cells transfected with ROR-wt1/2, whereas it had little impact on the luciferase activities on AC16 cells expressing ROR-mut1/2 (Fig. 2D). Subsequently, siRNAs against lncRNA ROR were designed and transfected into AC16 cells and obviously inhibited lncRNA ROR expression, especially in si-lncRNA ROR#3 group (Fig. 2E), which was chosen for next experiments (named si-lncRNA ROR). Importantly, we had found that the loss of lncRNA ROR could increase miR-769-5p expression in AC16 cells (Fig. 2F), further supporting that miR-769-5p was a negative target of lncRNA ROR.

Effects of lncRNA ROR/miR-769-5p Axis in H/R-Induced Cell Damage and Inflammation in AC16 Cells

This part was aimed to probe the regulatory roles of lncRNA ROR/miR-769-5p in AC16 cells under H/R treatment. Data from RT-qPCR assay demonstrated that miR-769-5p was markedly decreased after miR-769-5p inhibitor transfection (Fig. 3A). Moreover, compared to control group, H/R treatment significantly repressed cell

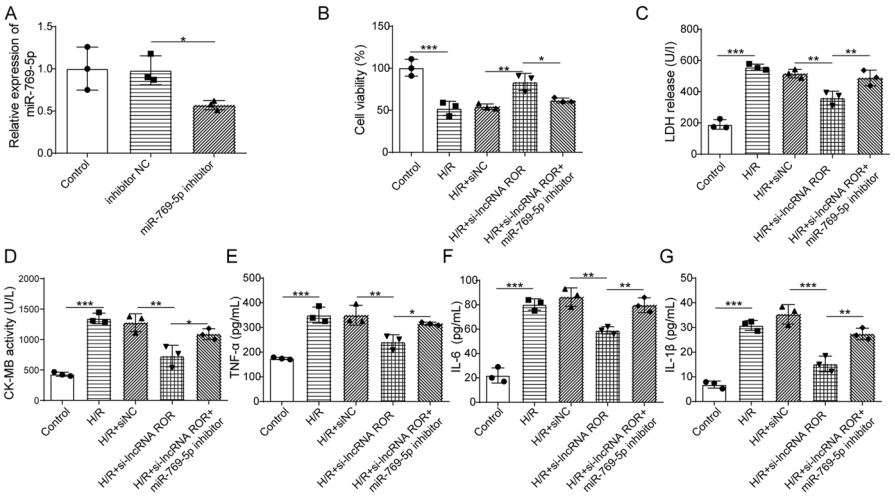

Fig. 3 Effects of lncRNA ROR/ miR-769-5p axis in H/R-induced cell damage and inflammation in AC16 cells. **A** MiR-769-5p inhibitor and inhibitor NC were transfected into AC16 cells, respectively. The expression of miR-769-5p was detected by RT-qPCR. **B**–**F** AC16 cells were transfected with siNC or si-lncRNA ROR or co-transfected with si-ROR + miR-769-5p inhibitor, followed by H/R treatment. **B** Cell viability was detected by CCK-8 analysis. **C**, **D** The cardiomyocyte damage was assessed by examining LDH and CK-MB contents. **E**–**G** The level of pro-cytokines factors (TNF-α, IL-1β, and IL-6) was evaluated by ELISA detection. Data were showed as Mean ± SD. *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. ROR, regulator of reprogramming; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; NC, negative control; H/R, hypoxia-reoxygenation; CCK-8, Cell counting Kit 8

viability, and lncRNA ROR depletion could greatly reverse this effect; however, the protective roles of lncRNA ROR blockage on cell viability of AC16 cells were abolished by miR-769-5p co-downregulation (Fig. 3B). In addition, the contents of myocardial damage markers (LDH and CK-MB) and inflammatory factors (TNF-α, IL-1β, IL-6) were greatly increased upon H/R treatment, and these changes could be abolished after lncRNA ROR blockage (Fig. 3C–G). Notably, the suppressive roles of lncRNA ROR knockdown on these damage and cytokines factors were impeded by miR-769-5p inhibitor (Fig. 3C–G). These results confirmed that lncRNA ROR could alleviate H/R-induced cell damage and inflammation via repressing miR-769-5p.

Effects of lncRNA ROR/miR-769-5p Axis in H/R-Induced Oxidative Stress and Ferroptosis in AC16 Cells

As shown in Fig. 4A, B and C, H/R treatment led to the significant increase of MDA and reduction of SOD and GSH levels, and these phenomena could be rescued upon lncRNA ROR downregulation. Interestingly, the above regulatory roles mediated by lncRNA ROR downregulation were diminished by miR-769-5p downregulation (Fig. 4A–C). Similarly, ROS level and iron content were enhanced in

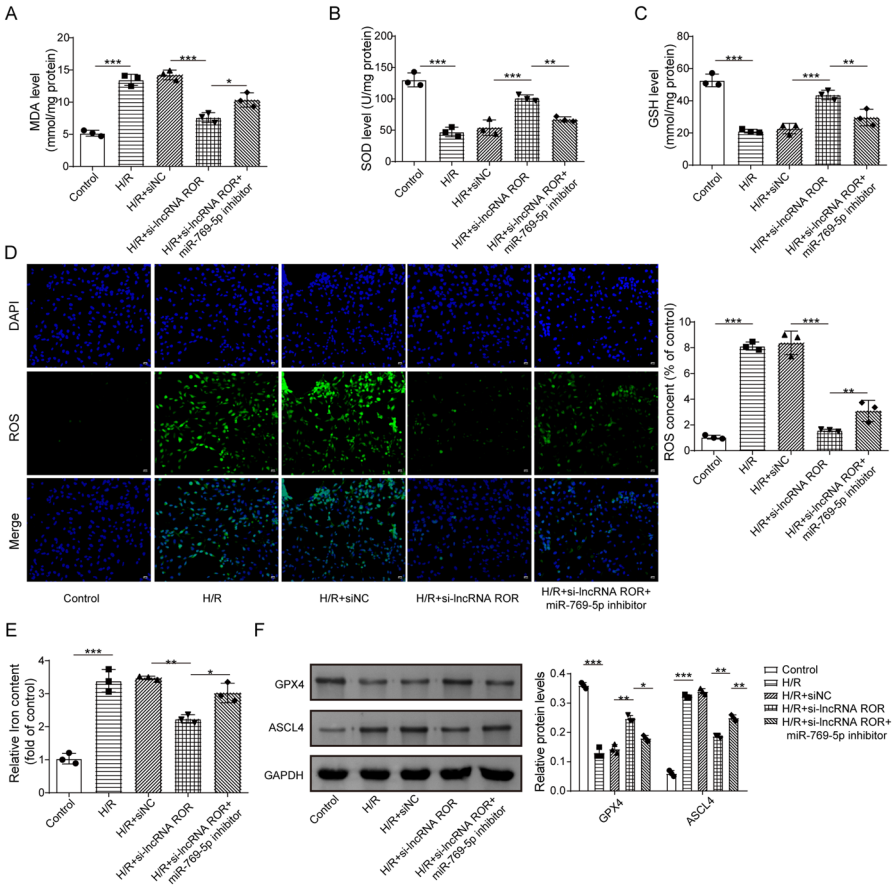

Fig. 4 Effects of lncRNA ROR/ miR-769-5p axis in H/R-induced oxidative stress and ferroptosis in AC16 cells. AC16 cells were transfected with siNC or si-lncRNA ROR or co-transfected with si-ROR+miR-769-5p inhibitor, followed by H/R treatment. **A**–**C** ELISA determined the contents of MDA, SOD, and GSH. **D** ROS accumulation was detected by DCFH-DA staining. **E** Iron content was examined by Iron assay kit. **F** ASCL4 and GPX4 protein levels were assessed by western blot. Data were showed as Mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *ROR* regulator of reprogramming, *H/R* hypoxia-reoxygenation, *ELISA* Enzyme-linked immunosorbent assay, *ROS* reactive oxygen species, *GPX4* Glutathione peroxidase 4, *ASCL4* acyl-CoA synthetase long-chain fatty acyl-CoA synthetase 4

H/R-challenged AC16 cells, and it was decreased significantly after ROR silencing; however, they were further elevated upon miR-769-5p was co-downregulated (Fig. 4D–E). Western blot analysis also demonstrated that GPX4 was reduced and ASCL4 was increased in H/R group than control group, and knockdown of lncRNA ROR remarkably reversed the protein changes caused by H/R treatment (Fig. 4F). Whereas, the upregulated GPX4 and downregulated ASCL4 caused by lncRNA ROR silence were further diminished by miR-769-5p knockdown (Fig. 4F). These finding demonstrated that lncRNA ROR silence relieved H/R-stimulated cardiomyocyte ferroptosis though upregulating miR-769-5p.

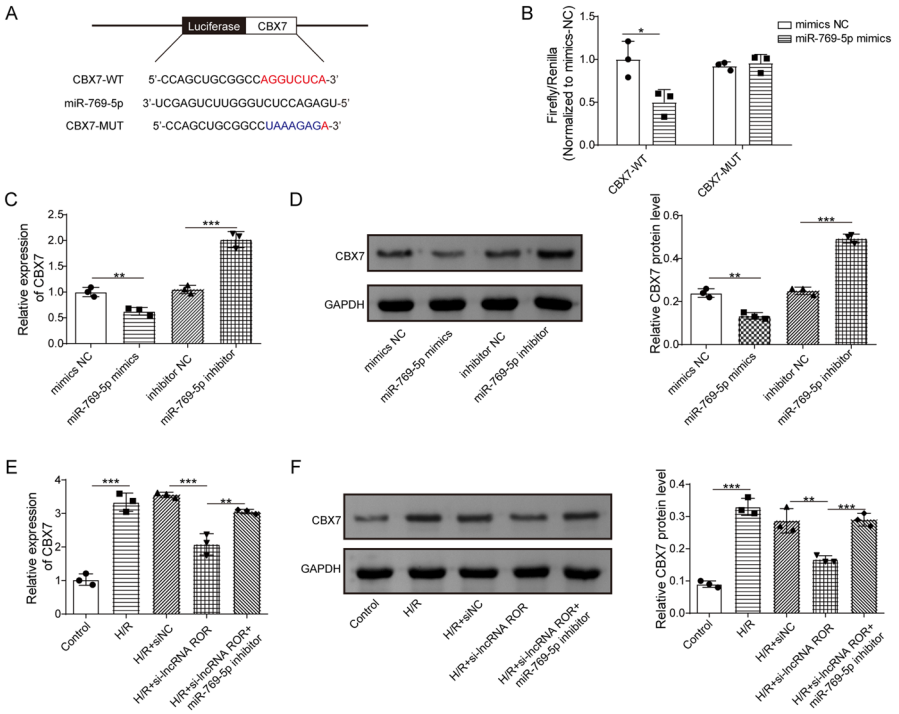

Fig. 5 CBX7 was a downstream target of miR-769-5p. **A** Schematic diagram of the binding site between miR-769-5p and CBX7 3'-UTR. **B** Dual-luciferase assay verified the binding relationship between miR-769-5p and CBX7. **C**–**D** MiR-769-5p mimics and miR-769-5p inhibitor as well as their negative controls were transfected into AC16 cells, respectively. Then, the mRNA and protein level of CBX7 were quantified by RT-qPCR and western blot assays. **E**–**F** AC16 cells were transfected with siNC or si-lncRNA ROR or co-transfected with si-ROR+miR-769-5p inhibitor, followed by H/R treatment. The mRNA and protein level of CBX7 were quantified by RT-qPCR and western blot assays. Data were showed as Mean ± SD. *$P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. *ROR* regulator of reprogramming, *RT-qPCR* Reverse transcription-quantitative polymerase chain reaction, *NC* negative control, *H/R* hypoxia-reoxygenation, *CBX7* chromobox protein homolog 7

CBX7 was a Downstream Target of miR-769-5p

After prediction by Starbase database, the putative site between miR-769-5p and CBX7 3'-UTR is displayed in Fig. 5A. Dual-luciferase assay validated that overexpression of miR-769-5p signally repressed the luciferase activity in AC16 cells transfected with CBX7-wt plasmids, whereas no change was observed in CBX7-mut plasmid-transfected AC16 cells (Fig. 5B). Subsequently, overexpression of miR-769-5p markedly repressed CBX7 mRNA and protein levels, while miR-769-5p inhibition showed opposite effects (Fig. 5C, D). Moreover, both CBX7 mRNA and protein levels were elevated in H/R-challenged AC16 cells (Fig. 5E, F). On this basis, depletion of lncRNA ROR repressed CBX7 expression in H/R-treated AC16 cells, while this effect was reversed by miR-769-5p inhibition (Fig. 5E, F).

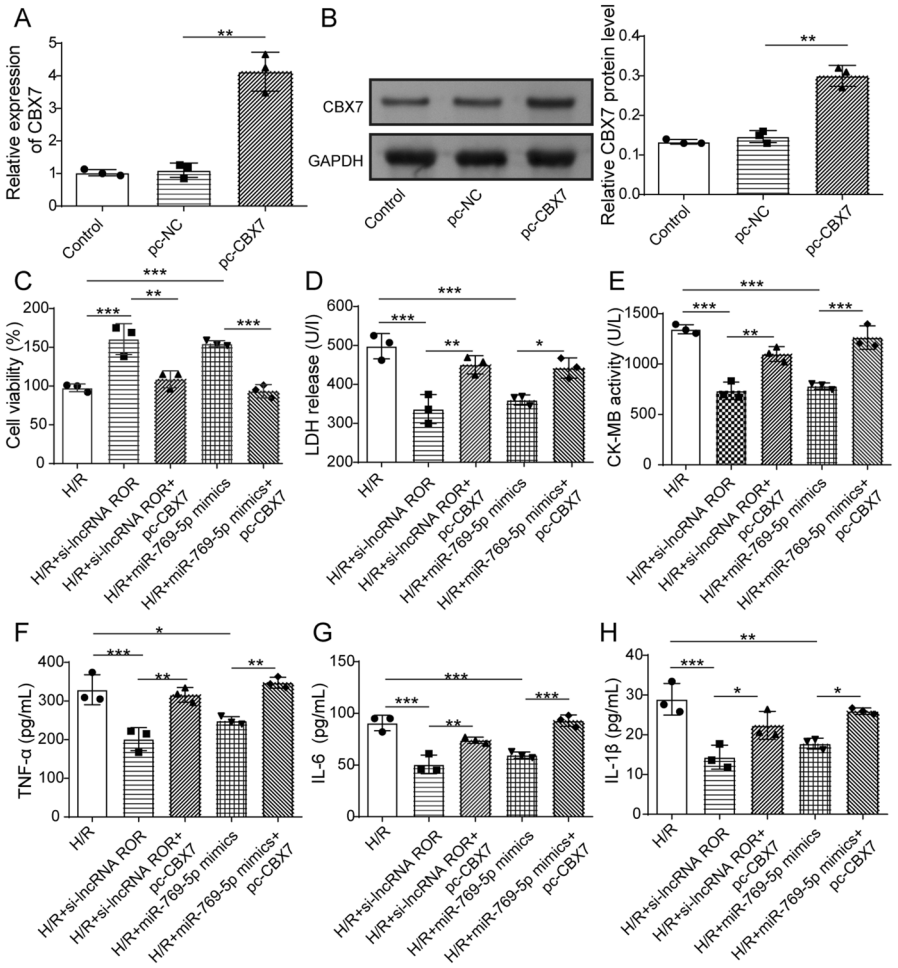

Fig. 6 Effects of lncRNA ROR/ miR-769-5p axis in H/R-induced cell damage and inflammation in AC16 cells. **A**, **B** The overexpressing vectors of CBX7 and empty vector were transfected into AC16 cells. The mRNA and protein level were examined by RT-qPCR and western blot assay. **C**, **H** AC16 cells were transfected with si-lncRNA ROR or miR-769-5p mimics or co-transfected with si-lncRNA ROR+pc-CBX7 or co-transfected with miR-769-5p mimics+pc-CBX7, respectively. **C** Cell viability was detected by CCK-8 analysis. **D**, **E** The cardiomyocyte damage was assessed by examining LDH and CK-MB contents. **F**–**H** The level of pro-cytokines factors (TNF-α, IL-1β, and IL-6) was evaluated by ELISA detection. Data were showed as Mean ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. *CBX7* chromobox protein homolog 7, *ROR* regulator of reprogramming, *RT-qPCR* Reverse transcription-quantitative polymerase chain reaction, *CCK-8* Cell counting Kit 8, *ELISA* Enzyme-linked immunosorbent assay

CBX7 Overexpression Reversed the Regulatory Effects of lncRNA ROR/miR-769-5p Axis in H/R-Induced Inflammatory Damage

The overexpressing vectors of CBX7 was transfected into AC16 cells and strikingly elevated CBX7 mRNA and protein levels (Fig. 6A, B). Functional assay showed

that both lncRNA ROR silence and miR-769-5p overexpression could enhance cell viability of H/R-treated AC16 cells, while this effect was lightened upon CBX7 was enforced (Fig. 6C). Compared to H/R group, lncRNA ROR loss and miR-769-5p overexpression resulted in the decrease of cardiomyocyte damage-related markers (LDH and CK-MB) and pro-cytokines factors (TNF-α, IL-1β, and IL-6), whereas these changes were all restrained by CBX7 overexpression (Fig. 6D–H). These findings suggested that CBX7 was downstream target of lncRNA ROR/miR-769-5p in regulating H/R-mediated cardiomyocyte inflammation.

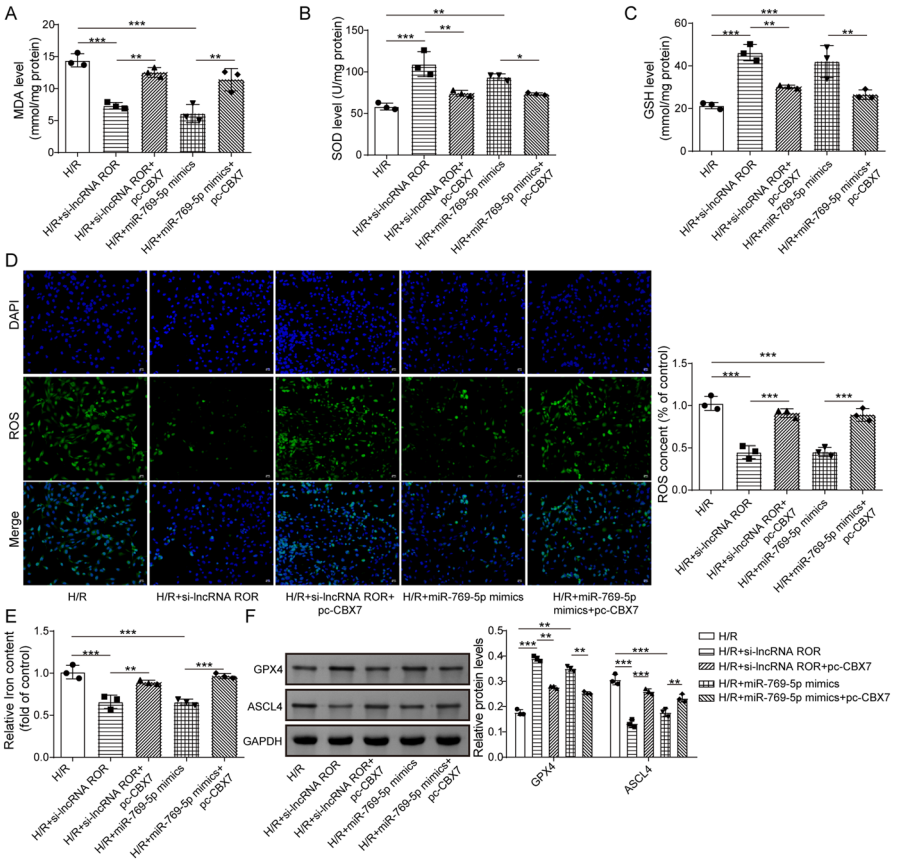

Fig. 7 CBX7 was involved in the regulation of lncRNA ROR/miR-765-5p axis on H/R-induced cardiomyocyte ferroptosis. AC16 cells were transfected with si-lncRNA ROR or miR-769-5p mimics or co-transfected with si-lncRNA ROR+pc-CBX7 or co-transfected with miR-769-5p mimics+pc-CBX7, respectively. **A–C** ELISA determined the contents of MDA, SOD, and GSH. **D** ROS accumulation was detected by DCFH-DA staining. **E** Iron content was examined by Iron assay kit. **F** ASCL4 and GPX4 protein levels were assessed by western blot. Data were showed as Mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. *CBX7* chromobox protein homolog 7, *ROR* regulator of reprogramming, *H/R* hypoxia-reoxygenation, *ELISA* Enzyme-linked immunosorbent assay, *ROS* reactive oxygen species, *GPX4* Glutathione peroxidase 4, *ASCL4* acyl-CoA synthetase long-chain fatty acyl-CoA synthetase 4

CBX7 was Involved in the Regulation of lncRNA ROR/miR-765-5p Axis on H/R-Induced Cardiomyocyte Ferroptosis

In H/R-challenged AC16 cells, either lncRNA ROR silence or miR-769-5p overexpression could repress MDA content and enhance GSH and SOD levels, and these changes could be reversed after CBX7 co-overexpression (Fig. 7A–C). Likewise, the reduced ROS and iron levels in H/R-induced AC16 cells caused by lncRNA ROR silence or miR-769-5p mimics were greatly mitigated by CBX7 upregulation (Fig. 7D, E). In addition, compared to H/R group, lncRNA ROR silence and miR-769-5p mimics led to the increase of GPX4 and reduction ASCL4 protein levels, and these changes could be abolished by CBX7 overexpression (Fig. 7F). It could be demonstrated that CBX7 could reverse the regulation of lncRNA ROR/miR-765-5p axis on cardiomyocyte ferroptosis.

Discussion

LncRNA is one of most typical non-coding RNA which have been variously reported to participate in the progression of MI/R injury. Generally, many lncRNAs were proved to play essential roles in multiple biological processes (proliferation, apoptosis, autophagy, oxidative stress, ferroptosis, etc.) during MI/R injury via modulating gene transcription or post-transcriptional modification (Zhao et al. 2021; Xiong et al. 2019). Due to the complex downstream action pathways and targets of lncRNA, the functions of lncRNA in MI/ R-induced ground damage are often multifaceted. For instance, Yu et al. proved that enforced expression of lncRNA MALAT1 could facilitate the NLRP3 inflammasome activation in MI/R injury by sponging miR-133 (Mavraev 1986); Wang et al.'s work confirmed that lncRNA MALAT1 also enhanced cardiomyocytes autophagy to aggravate MI/R injury through regulating miR-206/ATG3 cascade (Jing et al. 2021). Moreover, lncRNA HOTAIR was also reported to promote cardiomyocyte survival via functioning as a ceRNA to regulate miR-126/SRSF1 cascade or acting as RNA scaffold of FUS (Sun and Hu 2020; Liu et al. 2023a). These references fully demonstrated that lncRNAs play an essential role during MI/R injury progression, suggesting effective targeting of key pathologic lncRNAs may be an important way to treat MI/R injury.

LncRNA ROR was initially identified as a regulator which is capable of reprogramming differentiated cells to induced pluripotent stem cells (Zhang et al. 2013) and was confirmed to involve in various tumor developments (Chen et al. 2020). Recently, lncRNA ROR was also proved to be a key regulator in I/R-induced myocardial injury (Zhang et al. 2018) and cerebral injury (Chen and Li 2019). Several investigations showed that lncRNA ROR was upregulated in MI/R-induced myocardial tissues and H/R-induced cardiomyocytes and could aggravate cardiomyocyte pyroptosis, inflammation, oxidative stress, and apoptosis (Zhang et al. 2018; Sun et al. 2022; Liang et al. 2019), suggesting lncRNA ROR might be a key contributor during MI/R injury. In our work, we had observed that lncRNA ROR was highly expressed in serum samples of MI patients and H/R-challenged AC16 cells, and loss of function of lncRNA ROR repressed H/R-triggered cardiomyocytes damage,

inflammation, and oxidative stress, which further supported previous reports (Sun et al. 2022; Liang et al. 2019). Biochemically, the accumulation of ROS and oxidative expression levels were the key driving factors of ferroptosis (Liu et al. 2023b). However, there were a lack of direct evidence linking ROR to ferroptosis. Based on the previous data, our work also presented for the first time that lncRNA ROR silencing decreased the level of iron content and ASCL4 expression but enhanced GPX4 level in H/R-induced cardiomyocytes, suggesting that lncRNA ROR knockdown repressed H/R-induced cardiomyocytes ferroptosis. Overall, these findings proposed a new pathogenic pathway of MI/R injury aggravated by lncRNA ROR and implying inhibition of lncRNA ROR might be a promising therapeutic strategy for improving MI/R injury.

MiRNAs are small non-coding RNAs with length about 18 ~ 22 nt. There were increasing evidence that revealed the vital roles of miRNAs in pathogenesis mechanism of various cardiovascular diseases. As Wang et al.'s reported, miR-135a was a heart-specific miRNA and could decrease MI/R-induced infarct size and suppress H/R-triggered cardiomyocytes apoptosis (Wang et al. 2019). In addition, miR-1 was upregulated in MI/R-induced rat model and aggravated cardiomyocytes apoptosis by negatively targeting Hsp90 and Cx43 (Bian et al. 2017; Zhu et al. 2016). In the present work, we had observed that miR-769-5p was greatly decreased in MI patients and H/R-treated cardiomyocytes and showed a negative correlation with lncRNA ROR. Considering that lncRNAs usually acted as molecular sponges for miRNA and regulated their function and expression, we following the data also validated the direct binding relationship between miR-769-5p and lncRNA ROR. Recently, miR-769-5p was more commonly reported to be involved in the occurrence and development of tumors as tumor suppressor or oncogene (Luan et al. 2020; Sun et al. 2021b). The detailed roles and mechanism of miR-769-5p in I/R injury remains rare. Here, our data for the first time uncovered that miR-769-5p improved H/R-induced cardiomyocytes inflammation, oxidative stress, and ferroptosis and reversed the protective roles of lncRNA ROR silence in H/R-triggered inflammatory damage and ferroptosis in cardiomyocytes. Additionally, a recent work highlighted that miR-769-5p play an anti-inflammatory and anti-apoptotic impact in lipopolysaccharide-induced periodontal ligament cells (Sun et al. 2023), which partly supported the protective roles of miR-769-5p on cell survival. These findings revealed for the first time the roles of miR-769-5p in MI/R injury and the functional correlation with lncRNA ROR, suggesting miR-769-5p might be a potential diagnostic and therapeutic target in the development of MI/R injury.

CBX7, a member of the CBX family, is a typical component of Polycomb repressive Complex 1(PRC1) and can regulate heterochromatin, gene expression, and developmental progress (Zhang et al. 2020). Existing references confirmed that CBX7 was taken part in a series of cellular processes, such as endoplasmic reticulum stress, differentiation, angiogenesis, and apoptosis (Zhang et al. 2020; Forzati et al. 2014). Over the past few years, CBX7 was identified to participate in I/R-induced organ injury. Zhang et al. showed that CBX7 depletion could decrease cerebral infarct size and neurological injury through activating Nrf2/HO-1 signaling-mediated oxidative stress (Zhang et al. 2022b). Moreover, CBX7 knockdown also prevented ferroptosis and endoplasmic reticulum stress

and improved cognitive dysfunction in I/R-induced cerebral injury (Zhang et al. 2020, 2022). In addition, it has been found that CBX7 could repress cardiomyocyte proliferation via regulating TARDBP/RBM38 signaling (Cho et al. 2023). Whereas, whether CBX7 was involved in MI/R injury remain investigation. In our work, our findings revealed that CBX7 was a directly target of miR-769-5p and could be positively regulated by lncRNA ROR. Importantly, CBX7 ectopic expression reversed the protective roles of lncRNA ROR silence and miR-769-5p overexpression in H/R-triggered inflammatory injury, oxidative stress, and ferroptosis in cardiomyocytes. To sum up, these data provided key evidences for revealing the important role of lncRNA ROR/miR-769-5p/CBX7 signaling axis after MI/R injury. Moreover, it further supported an important theoretical reference for elucidating the pathogenesis after MI/R injury and developing new therapeutic strategies.

Conclusion

Collectively, we elucidated that lncRNA ROR accentuated H/R-driven cardiomyocyte inflammation and ferroptosis through regulating miR-769-5p/CBX7 signaling cascade. Together, our study firstly illustrated a novel pathogenic pathway of lncRNA ROR miR-769-5p/CBX7 axis in MI/R injury, which provided in-depth insights into uncover pathogenesis of MI/R injury, and implied inhibiting lncRNA ROR might be a potential therapeutic pathway for MI/R injury. However, there are still some shortcomings in the current study: In future work, we will further explore whether lncRNA ROR could affect MI/R progression by regulating the miR-769-5p/CBX7 signaling axis at the animal level. In addition, there were multiple targets of miRNAs or RNA-binding proteins on the lncRNAs sequence. Therefore, are there other mechanisms of action of lncRNA ROR after MI/R injury? We would endeavor to make that clear as well.

Author Contributions GL and JS: conceived and designed the experiments. YL and DL: wrote the manuscript. YH, FY, QL, and CZ: performed all experiments, collected data and performed the statistical analysis. All authors read and approved the final version of the manuscript and agree to take responsibility for the published article.

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Data Availability All data collected and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

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

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication All the authors approved the publication.

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