Suppression of microRNA-27a protects against liver ischemia/reperfusion injury by targeting PPARγ and inhibiting endoplasmic reticulum stress

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Abstract. Liver ischemia-reperfusion (I/R) injury is an important clinical issue related to liver transplantation. Recent studies suggest that microRNAs are implicated in various biological and pathological processes, including liver I/R injury. This study aimed to investigate the role and potential mechanism of miR-27a during liver I/R injury. A liver I/R model was induced via 60 min of ischemia and reperfusion for 6 h in rats. Cells were transfected with miR-27a mimics or the miR-27a inhibitor to examine the effect of miR-27a on liver I/R. Apoptotic cells were detected by flow cytometry and TUNEL staining. The expression of miR-27a was measured by real-time PCR. The expression of peroxisome proliferator-activated receptor γ (PPARγ); gastrin-releasing peptide 78 (GRP78) and C/EBP homologous protein (CHOP) were detected by western blot analysis. The results showed that miR-27a was significantly upregulated during I/R injury *in vivo* and *in vitro*. In addition, miR-27a inhibitors attenuated hypoxia/reoxygenation (H/R)-induced oxidative stress, endoplasmic reticulum stress (ERS) and apoptosis in AML12 cells. By contrast, miR-27a mimics promoted hypoxia/reoxygenation-induced ERS, and apoptosis. Furthermore, PPARγ was identified as a target gene of miR-27a using bioinformatic analysis and a dual-luciferase reporter assay. Knockdown of PPARγ significantly abrogated the inhibitory effect of miR-27a inhibitors on the ERS pathway. Moreover, the miR-27a antagomir attenuated liver I/R injury in rats, a finding manifested by reduced ALT/AST, hepatocyte apoptosis, oxidative stress and inhibition of the ERS pathway. Taken together, these findings demonstrate that suppression of miR-27a protects against liver I/R injury by targeting PPARγ and by inhibiting the ERS pathway.

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Introduction

Liver ischemia-reperfusion (I/R) injury is a common clinical issue that occurs as a consequence of liver transplantation, hepatic resection and trauma (1,2). Compared with pure ischemia, liver I/R injury can result in more serious liver damage and even liver failure after reperfusion. A large number of studies have shown that the mechanism of liver I/R injury involves complex and multiple pathways, including oxidative stress, inflammation and cell apoptosis (3-5). Recent studies have found that gastrin-releasing peptide (GRP) and the GRP receptor, bone marrow mesenchymal stem cells, cobalt-protoporphyrin and glycogen synthase kinase 3β are also involved in liver I/R injury (6-8). In addition, various approaches have been reported towards the prevention or attenuation of liver I/R injury, including ischemic preconditioning (IPC), antioxidant preconditioning, pharmaceutical preconditioning and a gene targeting approach (9). However, effective strategies and drugs for liver I/R injury are still lacking. Therefore, elucidating the possible molecular mechanism underlying liver I/R injury is necessary for the sake of developing effective drugs and approaches.

In recent years, several studies have suggested that endoplasmic reticulum stress (ERS) plays a critical role in the progression of liver IR injury (10,11). ERS, also known as the unfolded protein response, contributes to the protection of cells against toxic stimuli or cellular stress-induced accumulation of misfolded proteins (12,13). Under ERS, the BiP (GRP78) chaperone binds to misfolded proteins to generate an adaptive mechanism by activating a series of signaling pathways such as PERK, ATF6 and IRE1α (14,15). However, excessive ERS can lead to cell damage or death by activating pro-apoptotic factors, including the C/EBP homologous protein (CHOP) and caspase-12 (16,17). Thus, inhibiting ERS may provide novel insight into the treatment of liver I/R injury (18).

MicroRNAs are a class of small non-coding RNAs that bind to the 3'UTR (untranslated region) of target mRNAs, thereby regulating target gene expression (19-21). Several reports have suggested that microRNAs (miRs) can

contribute to liver I/R injury by the regulation of several key signaling pathways (22-25). miR-27a is a member of the miR-23a~27a~24-2 cluster, and plays a crucial role in cell survival and death (26-28). In addition, it has been demonstrated that peroxisome proliferator-activated receptor γ (PPARγ) is a target of miR-27a, and PPARγ is a therapeutic target for liver I/R injury (29-32). Recently, miR-27a-5p was found to have protective effects against liver I/R-induced apoptosis by targeting Bach1 (33). However, many aspects of the molecular mechanisms underlying the effect of miR-27a in liver I/R injury remain largely unknown, and whether this effect is associated with PPARγ and ERS needs to be further clarified. Therefore, in this study, we aimed to investigate the effect and relevant molecular mechanism of miR-27a in response to liver I/R injury *in vitro* and *in vivo*. Our study may provide new insight into the development of novel therapeutic strategies for clinical interventions to reduce liver I/R injury.

Materials and methods

Materials and reagents. miR-27a mimics, inhibitors, PPARγ siRNA and matched negative control (NC) were synthesized by GenePharma, Shanghai, China: miR-27a mimics (5'-AGG GCUUAGCUGCUUGUGAGCA-3' and 3'-CUCACAAGC AGCUAAGCCCUUU-5'); miR-27a mimic NC (5'-UUUGUA CUACACAAAAGUACUG-3' and 3'-AAACAUGAUGUG UUUUCAUGAC-5'); miR-27a inhibitor (5'-UGCUCACAA GCAGCUAAGCCCU-3'); miR-27a inhibitor NC (5'-CAGUAC UUUUGUGUAGUACAAA-3'); PPARγ siRNA (5'-AATATG ACCTGAAGCTCCAAGAATAAG-3'); siRNA NC (5'-GAG GCGGACTAATATCTAACAACAAAT-3'). Malondialdehyde (MDA) and superoxide dismutase (SOD) commercial kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Annexin V-FITC/propidium iodide (PI) apoptosis kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). PPARγ (cat. no. sc-271392; dilution 1:200), GRP78 (cat. no. sc-13539; dilution 1:200), CHOP (cat. no. sc-7351; dilution 1:200) and GAPDH (cat. no. sc-66163; dilution 1:200) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies used in the western blot analysis included Alexa Fluor Plus 800 anti-rabbit IgG (H+L) (cat. no. A32735; dilution 1:1,000) and Alexa Fluor Plus 800 anti-mouse IgG (H+L) (cat. no. A32730; dilution 1:1,000). A dual luciferase reporter assay kit was purchased from Promega (Madison, WI, USA).

Cell culture and transfection. AML12 cells, which were obtained from the Cell Bank of the Chinese Academy of Sciences, were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) that was supplemented with 10% fetal bovine serum (FBS, Hyclone, South Logan, UT, USA) plus 1% penicillin and streptomycin (Thermo Fisher Scientific, Inc.). The cells were cultured in a humidified atmosphere containing 5% CO_2. at 37˚C. AML12 cells were seeded into 6-well plates and transfected with miR-27a mimics, miR-27a mimic NC, miR-27a inhibitor, or miR-27a inhibitor NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For gene silencing, 50 nm PPARγ siRNA or siRNA NC was transfected into AML12 cells using Lipofectamine 2000 according to the manufacturer's instructions. Subsequent experiments including H/R, RNA/protein extraction and apoptosis analysis were performed 24 h after miRNA transfection.

In vitro hypoxia/reoxygenation (H/R) model. Briefly, AML12 cells were firstly perfused in normal Hank's solution with a gas mixture of 95% O_2-5% CO_2. at 37˚C, at pH 7.4. To simulate ischemia, the Hank's solution was switched to pH 7.4 at 37˚C without glucose or calcium and then the cells were aerated with a gas mixture of 95% N_2-5% CO_2. for 4 h. To simulate reperfusion, the cells were again treated with normal Hank's solution with a gas mixture of 95% O_2-5% CO_2. at 37˚C at pH 7.4. Cells under normoxia throughout the experiments were included as a control.

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was determined by the CCK-8 assay kit (Dojindo, Kumamoto, Japan). Briefly, 10 µl CCK-8 reagent was added to transfected AML2 cells at 0, 2, 6, 12 and 24 h, respectively and incubated for 2 h. The absorbance was detected at 450 nm. Each group was analyzed in triplicate.

Flow cytometry. The Annexin V-FITC/PI apoptosis detection kit was used to determine the cell apoptosis, according to the manufacturer's instructions. After transfection, cells were harvested and resuspended in 200 µl binding buffer. Then, the cells were incubated with 10 µl Annexin V-FITC and 5 µl PI in the dark for 15 min. The stained cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA).

Luciferase reporter assay. The predicted 3'UTR sequences of PPARγ that interacts with miR-27a and its mutated sequences within the predicted target sites were synthesized and inserted into the pRL-TK control vector. AML12 cells were transfected with 120 ng of miR-27a mimics, miR-27a inhibitors or the negative control (NC), followed by co-transfection with 30 ng of the wild-type or mutant 3'UTR of PPARγ using 0.45 µl of Fugene (Promega). The luciferase assay was carried out on cell extracts 24 h post-transfection, and the signals were measured using the Dual-Luciferase Assay System. The pRL-TK expressing *Renilla* luciferase was co-transfected as an internal control (29). The relative luciferase activity (RLA) was calculated as the ratio of firefly luciferase activity (Ff) to *Renilla* luciferase activity (Rn).

Animals and experimental design. Male Sprague-Dawley (SD) rats (weight, 150-200 g; age, 8 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Rats were housed under appropriate conditions (25±2˚C and 12-h light/dark cycle) with free access to water and food before the experiment. All of the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Fuzhou General Hospital for Accreditation of Laboratory Animal Care.

The rats were randomly divided into four groups (n=8 per group) as follows: i) Sham group; ii) I/R group; iii) I/R + miR-NC group and iv) I/R + miR-27a antagomir

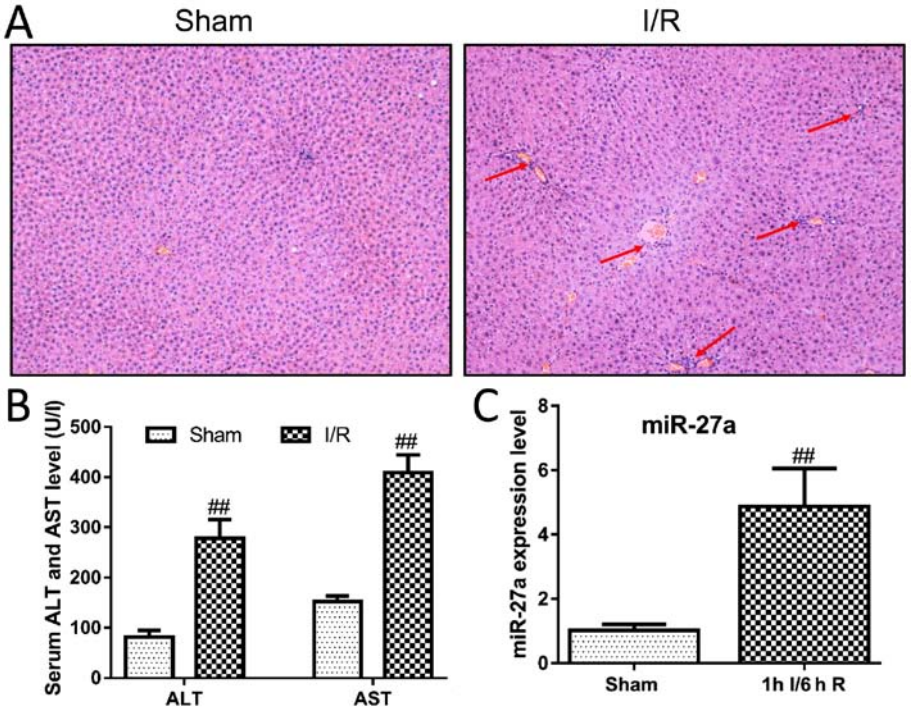

Figure 1. Expression of miR-27a in liver I/R injury. (A) H&E staining of liver sections (red arrows, necrotic area; original magnification x100). (B) Serum ALT and AST levels. (C) Relative miR-27a levels in the liver. Data are expressed as the mean ± SD (n=6). $^{\#\#}$P<0.01 vs. the sham group. I/R, ischemia-reperfusion; H&E, hematoxylin and eosin; ALT, alanine transaminase; AST, aspartate transaminase (AST).

group. The I/R group was anesthetized by an intraperitoneal injection of 3 ml/kg chloral hydrate. The liver hilum was subsequently exposed, and the portal structures of the left and median lobes were occluded. After 60 min of ischemia, the clamp was removed, and the liver was reperfused for 6 h. The sham group underwent abdominal surgery without liver ischemia/reperfusion. In the miR-27a antagomir group, the rats were given the miR-27a antagomir (20 μl of 500 pmol miR-27a antagomir/day) by intraperitoneal injection for 7 days before ischemia. Rats in the I/R + miR-NC group were given an equal amount of miR-NC.

After the surgery, the animals were immediately sacrificed. The serum was prepared and stored at -80˚C until the biochemical assays, and the left liver tissues were used for biochemical analyses, real-time PCR and western blot analysis.

ALT/AST assessment. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were determined using a commercial reagent kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

H&E staining and TUNEL assay. Liver tissues were fixed in 10% formalin for 24 h, dehydrated and embedded in paraffin. The liver sections were subsequently cut from each paraffin-embedded tissue and were stained with hematoxylin and eosin (H&E) to evaluate the degree of liver damage. The sections were imaged under a microscope (Olympus, Tokyo, Japan). TUNEL staining was performed using a commercial reagent kit (Beyotime Biotechnology) according to the protocols in the manual. The nuclei were stained with DAPI to assess nuclear morphology. All of the slices were imaged with a microscope (Olympus, Tokyo, Japan; magnification, x100).

Real-time PCR (qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed into cDNA using the PrimeScriptRT reagent kit (Takara, Tokyo, Japan). The expression of miRNA was quantified using the SYBR Green PCR Master Mix (Takara). The qPCR protocol was 95˚C for 5 min followed by 40 cycles of 95˚C for 30 sec and 60˚C for 1 min conducted in the Step One Real-Time PCR System (Applied Biosystems, Warrington, UK). The following primers were synthesized by Sangon Biotech (Shanghai, China) and were used in the PCR: miR-27a-F, 5'-TTCACAGTGGCTAAG-3' and miR-27a-R, 5'-CCAGTGCAGGGTCCGAGGT-3'; U6-F, 5'-GCTTCGGCA GCACATATACTAAAAT-3' and U6-R, 5'-CGCTTCACG AATTTGCGTGTCAT-3'. The relative expression of miR-27a was determined in relation to U6 by the $2^{-\Delta\Delta Cq}$ method (34).

Western blot analysis. Total proteins were extracted using lysis buffer (Beyotime), and the protein concentration was quantified with the BCA protein kit (Beyotime). Equal amounts of protein (10 μg total protein/well) were mixed with the SDS loading buffer and boiled for 5 min at 100˚C. The proteins were resolved on a 10% SDS-PAGE gel for 30 min at 80 V and then at 120 V for 1 h. The proteins were transferred onto an NC membrane for 60 min at 250 ma. The NC membranes were subsequently blocked with 5% skim milk at room temperature for 2 h and incubated with primary antibodies at 4˚C overnight. The membranes were washed with TBST thrice for 5 min and incubated with the corresponding secondary antibodies for 1 h. Protein expression was imaged using the Odyssey Infrared Imaging System (Lincoln, NE, USA). Protein levels were measured using the Quantity One software (version 4.62; Bio-ra Bio-Rad Laboratories, Inc.).

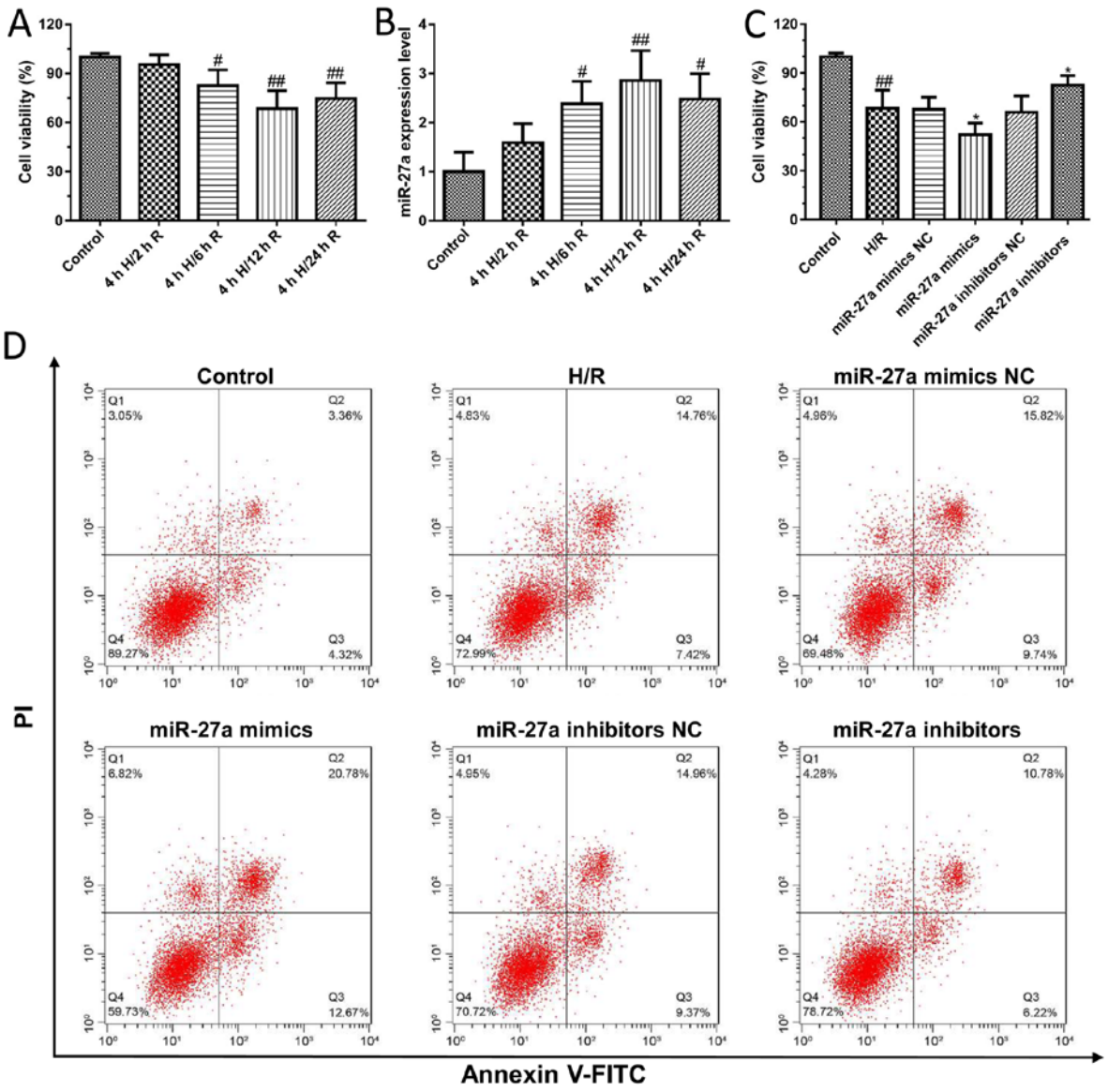

Figure 2. Effects of miR-27a on H/R-induced hepatocyte injury. (A) Cell viability of the AML12 cells following H/R at different times. (B) Relative miR-27a levels in the AML12 cells following H/R at different times. (C) Effects of miR-27a mimics and inhibitors on cell viability after H/R. (D) Effects of miR-27a mimics and inhibitors on cell apoptosis after H/R. Data are expressed as the mean ± SD (n=6) $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the control group; *P<0.05 vs. the H/R group. H/R, hypoxia/reoxygenation.

Statistical analysis. Experiments were performed in triplicate, and the data are expressed as the mean ± standard deviation (SD). The differences between two groups was analyzed using the Student's t-test. The differences among multiple groups were determined by ANOVA followed by Student-Newman-Keuls post hoc test in GraphPad Prism 5 (GraphPad Software, Inc.) P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-27a upon liver I/R injury. To investigate the potential effect of miR-27a on liver I/R injury, the I/R-induced liver injury model was constructed in rats. As shown in Fig. 1A, H&E staining of livers revealed large areas of hepatocyte necrosis and inflammatory infiltration in the I/R group. Moreover, the serum levels of ALT and AST were significantly increased after liver I/R surgery compared with the sham group (Fig. 1B). These results suggest that we successfully constructed a rat model of I/R in this study. We then examined the expression levels of miR-27a in the I/R model. As shown in Fig. 1C, miR-27a in the I/R group was significantly upregulated at least 5-fold when compared with the sham group. These findings indicate that miR-27a may play an important role in I/R-induced liver injury.

Effects of miR-27a on H/R-induced hepatocyte injury. To further confirm the effect of miR-27a on liver I/R injury, an H/R-induced cell model in AML12 cells was established. As shown in Fig. 2A, the viability of AML12 cells was significantly decreased after H/R treatment compared to the control group. In addition, we found that the expression of miR-27a was significantly increased in a time-dependent manner after H/R treatment compared with control cells

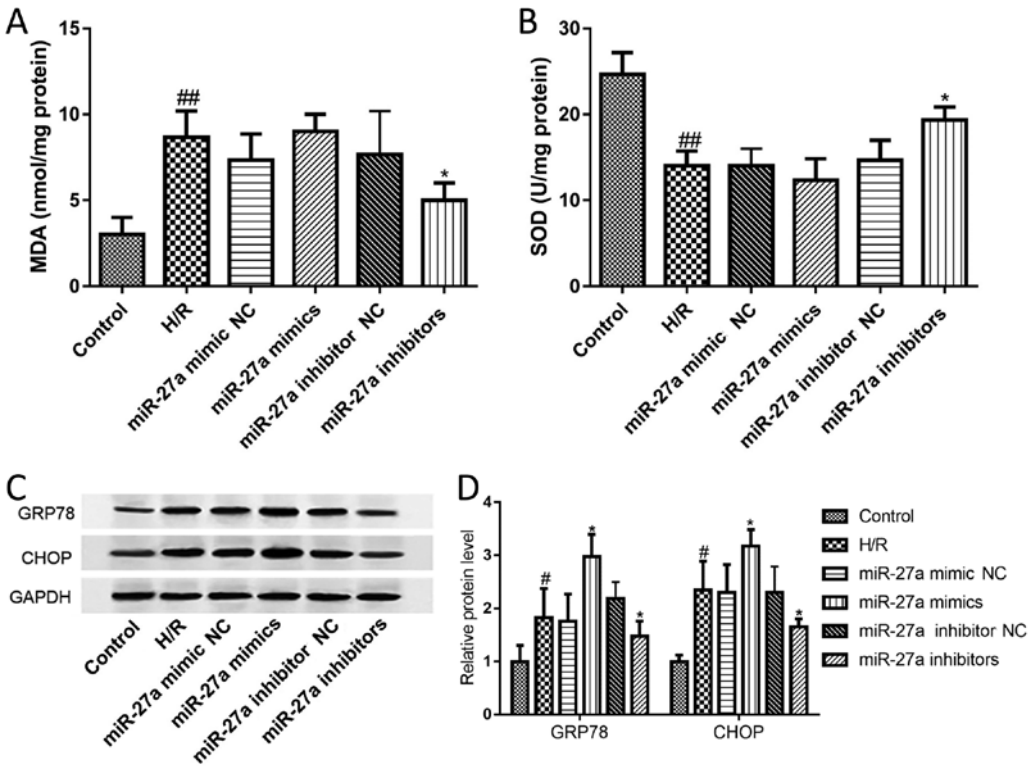

Figure 3. Effects of miR-27a on H/R-induced oxidative stress and ERS. (A) MDA levels, (B) SOD activity levels and (C) western blot analysis and (D) quantification of GRP78 and CHOP protein levels in the presence of miR-27a mimics and inhibitors. Data are expressed as the mean ± SD (n=3) $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the control group; *P<0.05 vs. the H/R group. ERS, endoplasmic reticulum stress; MDA, malondialdehyde; SOD, superoxide dismutase; H/R, hypoxia/reoxygenation; GRP78, gastrin-releasing peptide 78; CHOP, C/EBP homologous protein.

(Fig. 2B). Furthermore, AML12 cells were transfected with miR-27a mimics and inhibitors, and the cell viability was detected using the CCK-8 kit. We first detected the effects of miR-27a mimics and inhibitors on the expression of miR-27a by qPCR. The results found that miR-27a mimics significantly increased the expression of miR-27a, and the miR-27a inhibitor decreased the expression of miR-27a, which showed that transfection of the miR-27a mimics and inhibitors was successfully carried out (Fig. S1). The results showed that miR-27a inhibitors significantly increased cell viability compared with what was observed in the control group. In contrast, the cell viability was significantly decreased following treatment with miR-27a mimics (Fig. 2C). In addition, we detected their effect on cell apoptosis by flow cytometry. Compared with the control group, the apoptotic cells were markedly increased in the H/R group, which was effectively attenuated with the miR-27a inhibitor or significantly aggravated by miR-27a mimics (Fig. 2D). These results suggest that suppression of miR-27a may exert a protective effect against liver I/R injury.

Effects of miR-27a on H/R-induced oxidative stress and ERS. A large number of studies have suggested that oxidative stress and ERS play an important role in the development of liver I/R injury. First, the MDA and SOD levels were examined to investigate the effect of miR-27a on oxidative stress. The results showed that compared with the control group, MDA levels were significantly elevated, while SOD was significantly decreased. However, these changes were restored with the miR-27a inhibitor (Fig. 3A and B). Furthermore, the expression of GRP78 and CHOP was assessed to study the effect of miR-27a on ERS. As shown in Fig. 3C and D, the expression levels of GRP78 and CHOP were both significantly increased after H/R treatment. As expected, the expression levels of GRP78 and CHOP were also significantly decreased with miR-27a inhibitor treatment or further increased by treatment with miR-27a mimics. Our data suggest that miR-27a regulates oxidative stress and ERS signaling in H/R-induced hepatocyte injury.

PPARγ is a potential target gene of miR-27a. Bioinformatic analysis revealed that the 3'UTR of PPARγ mRNA contains one binding site for miR-27a; thus, PPARγ is one of the candidate target genes of miR-27a (Fig. 4A). To validate this prediction, the PPARγ luciferase reporter gene assay was used to analyze the relationship between miR-27a and PPARγ. The results showed that miR-27a mimics significantly suppressed the luciferase activity of the 3'UTR of wild-type (WT) PPARγ, which had no effect on the mutated (MUT) 3'UTR of the PPAR-γ transfected group. By contrast, the miR-27a inhibitor markedly increased the luciferase activity in WT-transfected cells (Fig. 4B). Furthermore, the expression of the PPARγ protein was detected by western blot analysis. As shown in Fig. 4C and D, the expression of PPARγ was significantly downregulated after transfection with the miR-27a mimic, indicating a negative association between miR-27a and PPARγ expression. Taken together, these results indicate that PPARγ is a target gene of miR-27a.

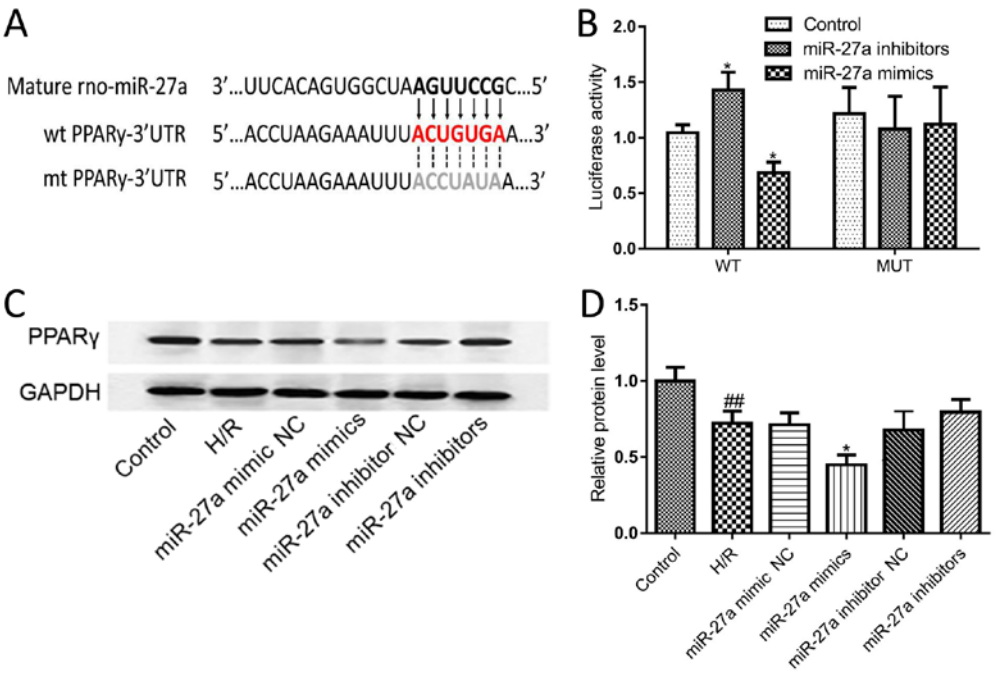

Figure 4. PPARγ is a potential target gene of miR-27a. (A) Prediction of miR-27a binding sites in the PPARγ sequence. (B) Relative luciferase activity in AML12 cells with co-transfection of miR-27a mimics or inhibitors. (C and D) Western blot analysis of the PPARγ protein levels in AML12 cells transfected with miR-27a mimics and inhibitors. Data are expressed as the mean ± SD (n=3). $^{\#\#}$P<0.01 vs. the control group *P<0.05 vs. the H/R group. PPARγ, peroxisome proliferator-activated receptor γ; H/R, hypoxia/reoxygenation; MUT, mutant; WT, wild-type.

miR-27a regulates ERS by PPARγ. To verify that miR-27a regulates ERS via PPARγ, PPARγ siRNA was used to determine the effect of miR-27a on the ERS pathway. We first tested the effect of PPARγ siRNA on PPARγ expression, and the results showed that PPARγ siRNA could significantly silence the expression of PPARγ (Fig. 5A and B). Furthermore, we found that silencing of PPARγ significantly blocked the inhibitory effect of the miR-27a inhibitor on the ERS pathway, as indicated by the increased expression of CHOP and GRP78 (Fig. 5C and D). The results suggest that miR-27a regulates the ER stress pathway by targeting PPARγ.

Suppression of miR-27a protects against liver I/R injury in rats. To study the therapeutic effect of miR-27a on liver I/R injury *in vivo*, the I/R rats were treated with the miR-27a antagomir and miR-NC by intraperitoneal injections. As shown in Fig. 6A and B, serum ALT and AST levels in the miR-27a antagomir group were significantly decreased, compared with the miR-NC group. Moreover, the liver histology of H&E staining showed an obvious reduction in I/R-induced hepatocellular necrosis and an improvement in cell integrity in the miR-27a antagomir group (Fig. 6C). In addition, the TUNEL results demonstrated that miR-27a antagomir markedly decreased I/R-induced apoptosis (Fig. 6D). These results suggest that the miR-27a antagomir effectively alleviated liver I/R injury in rats.

Suppression of miR-27a inhibits I/R-induced oxidative stress and ERS in rats. We further explored the molecular mechanism underlying the effect of miR-27a in I/R rats. Compared with the sham group, I/R significantly elevated the level of MDA, but miR-27a antagomir treatment significantly inhibited I/R-induced elevation of MDA in the liver (Fig. 7A). Meanwhile, the activity of SOD was decreased in I/R-exposed livers, and this decrease was significantly increased by miR-27a antagomir treatment (Fig. 7B). Furthermore, the expression of CHOP, GRP78 and PPARγ was examined in rat liver. As shown in Fig. 7C and D, the PPARγ level was decreased in the I/R rats, and the expression of CHOP and GRP78 was significantly increased compared with the sham group. After miR-27a antagomir treatment, the PPARγ level in the rat liver was significantly upregulated, and CHOP and GRP78 was significantly reduced compared to the miR-NC group. This finding indicated that the inhibition of miR-27a protected the rat liver against I/R injury by regulating oxidative stress and the ERS signaling pathway.

Discussion

Liver ischemia-reperfusion (I/R) injury is one of the major issues during liver surgery and greatly affects surgical outcomes. Recent studies suggest that miRNAs play an important role in liver I/R injury, and miRNAs have become potential molecular targets for therapeutic intervention (35-38). In the present study, it was demonstrated that inhibition of miR-27a protects the liver against I/R injury by targeting PPARγ and inhibiting the endoplasmic reticulum stress (ERS) pathway *in vitro* and *in vivo*.

In the present study, a model of liver I/R injury was successfully constructed in rats according to the findings from a previous study (7), as evidenced by the elevated serum activities of ALT/AST and extensive hepatocyte necrosis. Importantly, it was found that miR-27a was markedly increased at least 5-fold in the liver I/R group when compared with the

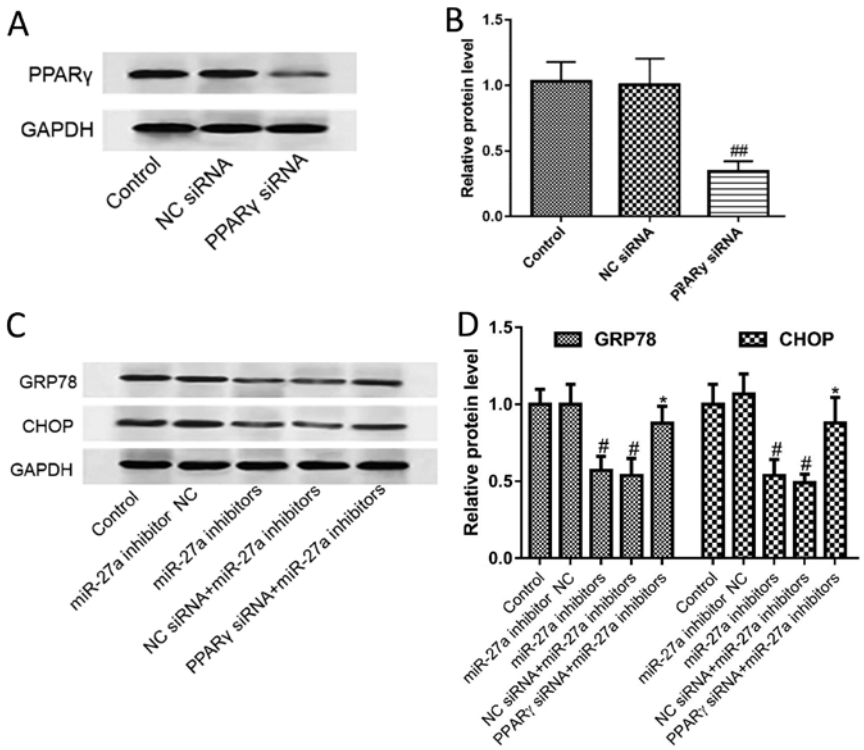

Figure 5. miR-27a regulates ERS by PPARγ. (A) Western blot analysis of the protein levels of PPARγ in AML12 cells transfected with PPARγ siRNA or siRNA NC. (B) Quantification of A. (C) Western blot analysis and (D) quantification of the protein levels of CHOP and GRP78 in AML12 cells transfected with PPARγ siRNA + miR-27a to verify that miR-27a regulates ERS via PPARγ. Data are expressed as the mean ± SD (n=3). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the control group; *P<0.05 vs. the miR-27a inhibitor group. ERS, endoplasmic reticulum stress; PPARγ, peroxisome proliferator-activated receptor γ; GRP78, gastrin-releasing peptide 78; CHOP, C/EBP homologous protein.

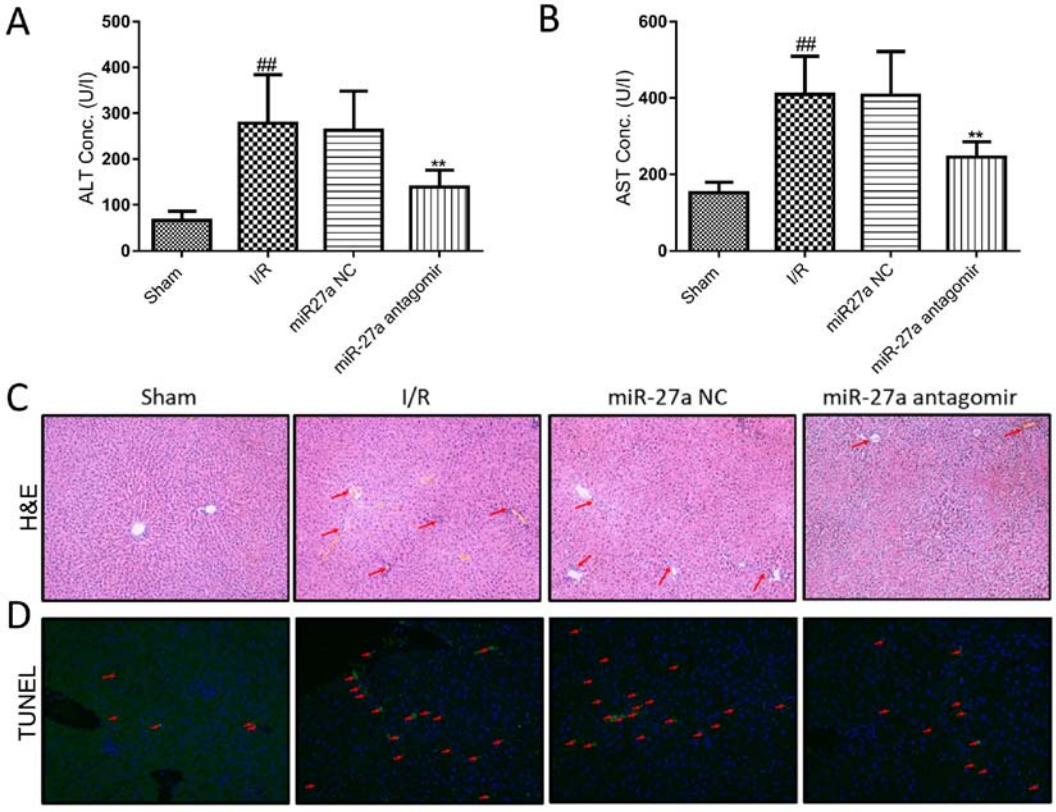

Figure 6. Suppression of miR-27a protects against liver I/R injury in rats. (A) Serum levels of ALT. (B) Serum levels of AST. (C) H&E staining of liver sections (red arrows, necrotic area; original magnification x100). (D) TUNEL staining of hepatocyte apoptosis (red arrows, apoptotic cells; original magnification x100). Data are expressed as the mean ± SD (n=3). $^{\#\#}$P<0.01 vs. the sham group; **P<0.05 vs. the I/R group. I/R, ischemia-reperfusion; ALT, serum alanine transaminase; AST, aspartate transaminase; H&E, hematoxylin and eosin.

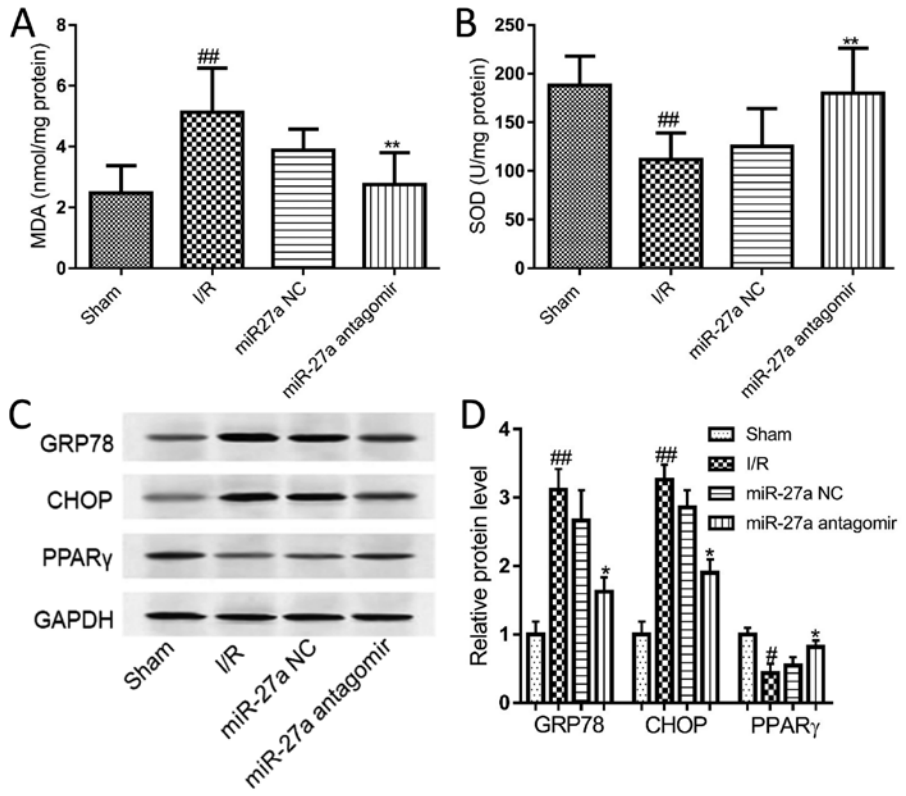

Figure 7. Suppression of miR-27a inhibits I/R-induced oxidative stress and ERS in rats. (A) MDA levels. (B) SOD activity levels. (C) Western blot analysis and (D) quantification of the GRP78, CHOP and PPARγ protein levels. Data are expressed as the mean ± SD (n=3). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the sham group; *P<0.05 and **P<0.01 vs. the I/R group. I/R, ischemia-reperfusion; ERS, endoplasmic reticulum stress; MDA, malondialdehyde; SOD, superoxide dismutase; GRP78, gastrin-releasing peptide 78; CHOP, C/EBP homologous protein; PPARγ, peroxisome proliferator-activated receptor γ.

sham group. To investigate the potential role of miR-27a in liver I/R injury, a hypoxia/reoxygenation (H/R)-induced cell model in hepatic AML12 cells was used to simulate liver I/R injury *in vitro*. The results showed that the expression of miR-27a was time-dependent and was increased in hepatic AML12 cells during H/R, a finding that was consistent with the *in vivo* results. Furthermore, it was also found that miR-27a inhibitors significantly improved proliferation and decreased apoptosis in the H/R-exposed AML12 cells when compared with the NC group. These results indicate that the suppression of miR-27a exerted a protective role against H/R-induced hepatocyte injury in AML12 cells.

Oxidative stress is an important factor that leads to liver I/R injury (39,40). In this study, miR-27a inhibitors reduced MDA content and enhanced SOD activity. This trend was reversed by miR-27a mimics. Since SOD is an antioxidant enzyme that acts against superoxide, increased SOD activity resulted in decreased oxidative stress. Previous research has also shown that miR-27a-induced cell apoptosis was associated with the ERS signaling pathway in 293T cells. The ERS pathway is an important signaling pathway in the regulation of cell survival and apoptosis (5). Studies have shown that inhibition of the ERS pathway can significantly prevent I/R-induced cell apoptosis (41-43). To validate the possible role of miR-27a in ERS in liver I/R injury, the expression of GRP78 and CHOP (the main signaling pathways of ERS) was detected. In line with the findings in previous studies (15), the protein levels of GRP78 and CHOP were increased during liver I/R. Our study showed that the expression levels of GRP78 and CHOP were significantly decreased by the miR-27a inhibitor or were further increased by treatment with miR-27a mimics. These results suggest that the suppression of miR-27a exerted a protective role against H/R-induced hepatocyte injury, which may inhibit oxidative stress and the ERS pathway.

Next, the target gene of miR-27a was investigated. The results of our bioinformatic analysis revealed that PPARγ is one of the target genes of miR-27a. Furthermore, a dual-luciferase reporter assay also confirmed that miR-27a specifically targeted the 3'UTR of the PPARγ gene, findings that are consistent with those in previous studies (43,44). In addition, *in vitro* experiments showed that the protein expression of PPARγ was markedly decreased after treatment with the miR-27a mimics and was increased after treatment with the miR-27a inhibitor *in vitro*, further confirming PPARγ as a downstream target of miR-27a. Several reports have suggested that PPARγ plays an important role in the regulation of ERS and cell apoptosis (45,46). To confirm that miR-27a regulates the ERS pathway through PPARγ, PPARγ was knocked down using PPARγ siRNA. The results showed that knockdown of PPARγ significantly abrogated the inhibitory effect of the miR-27a inhibitor on the ERS pathway. Taken together, these findings indicate that miR-27a increased ERS by negatively regulating PPARγ, and PPARγ is a target gene of miR-27a.

To study the therapeutic effect of miR-27a on liver I/R injury *in vivo*, we treated I/R rats with antagomir-miR-27a by intraperitoneal injection. It was found that the miR-27a antagomir alleviated liver I/R injury as evidenced by lower serum ALT/AST levels and improved liver morphology

and histology. In addition, I/R increased the number of TUNEL-positive cells compared with what was observed in normal rats, while miR-27a antagomir pretreatment decreased this trend. Furthermore, oxidative stress and the ERS pathway were evaluated to explore the molecular mechanism of miR-27a. The results demonstrated that miR-27a antagomir treatment significantly decreased MDA content and increased SOD activity. Moreover, our study showed that the miR-27a antagomir reduced CHOP and GRP78 and increased PPARγ expression. These results suggest that the suppression of miR-27a effectively alleviated liver I/R injury by regulating oxidative stress and ERS *in vivo*.

In conclusion, the persent study demonstrated that miR-27a mediates liver I/R injury by oxidative stress and ERS, and the suppression of miR-27a protects against liver I/R injury. Therefore, miR-27a inhibitors have therapeutic potential for liver I/R injury and warrant further research interest. Macrophage infiltration plays a critical role in the pathogenesis of liver I/R injury and inflammation response (47), thus the effect of miR-27a on the inflammatory response and macrophage infiltration will be investigated in future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC and YJ conceived and designed the study. XC, YC, FY, QC and FP performed the experiments. XZ and LL analyzed the data. XC and LL wrote the paper. XC, XZ and YJ reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All of the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Fuzhou General Hospital for Accreditation of Laboratory Animal Care.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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