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Overexpression of miR-638 attenuated the effects of hypoxia/reoxygenation treatment on cell viability, cell apoptosis and autophagy by targeting ATG5 in the human cardiomyocytes

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Abstract. – OBJECTIVE: Myocardial ischemia/reperfusion (I/R) injury largely contributed to the damage of myocardial tissues in patients with coronary disease, which may subsequently lead to heart failure. MicroRNAs (miRNAs) are considered to be involved in the process of myocardial I/R injury. The present study aimed to investigate the *in vitro* functional role of miR-638 in the myocardial I/R injury in the human cardiomyocytes (HCMs).

PATIENTS AND METHODS: MTT assay and flow cytometry assay were performed to determine cell viability and apoptosis of HCMs. Real Time-quantitative Polymerase Chain Reaction was used to determine miRNA and mRNA expression levels. The protein levels were determined by Western blot assay.

RESULTS: Hypoxia/reoxygenation (H/R) treatment suppressed cell viability, increased cell apoptotic rate and suppressed miR-638 expression in the HCMs. The downregulation of miR-638 suppressed cell viability and induced cell apoptosis in the HCMs. The overexpression of miR-638 attenuated the effects of H/R treatment on the cell viability and cell apoptosis in the HCMs. In addition, miR-638 suppressed the expression of autophagy-related 5 (ATG5) by targeting the 3'untranslated region of ATG5. Enforced expression of ATG5 reversed the effects of miR-638 overexpression on cell viability and cell apoptosis in H/R-treated HCMs. More importantly, H/R treatment promoted autophagy in the HCMs, and this effect was significantly reversed by miR-638 mimic transfection.

CONCLUSIONS: Our results suggested that the overexpression of miR-638 attenuated the effects of H/R treatment on cell viability, cell apoptosis and autophagy, at least partly by regulating the ATG5 expression in the HCMs.

Key Words:
Heart failure, MiR-638, Hypoxia/reoxygenation, Apoptosis, ATG5, Autophagy.

Introduction

Coronary heart disease is one of the leading causes of human morbidity and mortality[1]. Myocardial ischemia/reperfusion (I/R) is a major process of myocardial infarction, and largely contributed to the damage of myocardial tissues in patients with coronary disease[2]. Patients who underwent percutaneous coronary intervention, coronary artery bypass surgery, or extracorporeal circulation surgery often suffered from myocardial I/R injury[3,4]. The underlying mechanisms of myocardial I/R injury are believed to be caused by impaired function of mitochondria, intracellular overload of calcium, excessive production of free radicals, which subsequently induce cardiomyocyte apoptosis and may ultimately cause heart failure in patients with coronary disease[5-7]. Therefore, to fully understand the complicated molecular signaling pathways of myocardial I/R injury may help us have better opportunities to prevent heart failure. The role of microRNAs (miRNAs) in the regulation of gene expression

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has been extensively investigated in a great number of studies[8-11]. MiRNAs are generally containing about 22 nucleotides and repressed the gene expression by targeting the 3'untranslated region (3'UTR) of the targeted genes, which enables miRNAs to regulate many cellular processes such as cell proliferation, cell apoptosis, cell differentiation and so on[12-14]. The functional roles of miRNAs in the process of myocardial I/R injury have also been examined. In the clinical aspect, some circulating miRNAs were proposed to be associated with heart failure. In a investigation[15] involving 96 acute heart failure patients, higher serum miR-30d levels predicted lower mortality in patients with acute heart failure. In the genome-wide prospective study, Scrutinio et al[16] demonstrated circulating miR-150-5p as a novel biomarker for advanced heart failure. In the *in vitro* functional studies, Wu et al[17] showed that miR-365 could accelerate cardiac hypertrophy by inhibiting autophagy *via* the modulation of Skp2 expression. MiR-218 was found to be involved in the cardiomyocytes hypertrophy by regulating the expression of RE1-silencing transcription factor gene[18]. MiR-638 was found to regulate cell proliferation in different types of cancers, and various mechanisms of miR-638-involved in cancer cell proliferation have been proposed[19-21]. Nevertheless, no researches have been conducted to investigate the role of miR-638 in heart failure. In this work, we aimed to investigate the role of miR-638 related to heart failure by using the hypoxia/reoxygenation (H/R) human cardiomyocytes (HCMs) *in vitro* model. We found that H/R treatment reduced cell viability, induced cell apoptosis and suppressed the expression of miR-638 in the HCMs. The inhibition of miR-638 reversed the effect of H/R treatment on cell viability and cell apoptosis in the HCMs. In addition, miR-638 was found to regulate cell viability, cell apoptosis and autophagy by targeting the autophagy-related 5 (ATG5) gene.

Patients and Methods

Cell Culture

HCMs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA), and the cells were cultured in cardiac myocyte medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% of fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 1% of cardiac myocyte growth supplement (Sigma-Aldrich, St. Louis, MO, USA) and 1% of penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

Hypoxia/Reoxygenation (H/R) Treatment

For the H/R treatment, cells were incubated with serum-free cardiac myocyte medium in 5% CO_2 at 37°C for 12 h, and then cells were subjected to the culturing condition in 1% O_2/94% N_2/5% CO_2 at 37°C for 4 h. After that, the cells were again cultured in the cardiac myocyte medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 4 h.

MiRNAs, Plasmids and Transfection

The miR-638 mimic, miR-638 inhibitor and the negative controls (named as "mimic NC" and "inhibitor NC", respectively) and the empty vector, pcDNA3.1 as well as the ATG5-overexpressing vector, pcDNA3.1-ATG5 were purchased from a commercial manufacturer (GenePharma, Shanghai, China). MiRNAs transfection or MiRNAs and plasmids co-transfection were performed by using the Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instruction. At 24 h after transfection, cells were processed for further experiments.

3-(4,5-Dimethyl-Thiazol-2-yl) 2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

Cell viability was determined by using the MTT (Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay. Briefly, cells were seeded in 96-well plates and incubated for 24 h, and cells were subjected to different treatment regimens. After the treatments, cells were washed with phosphate-buffered saline (PBS) and then incubated in MTT solution (Sigma-Aldrich, St. Louis, MO, USA) for 3 h. After that, dimethyl sulfoxide was added into each well. Finally, the absorbance was measured at 450 nm to determine the cell viability by using a microplate reader (BioTek, Winooski, VT, USA).

Cell Apoptosis Determination (Flow Cytometry)

The cell apoptosis was determined by flow cytometry by using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, after different treatments, cells were harvested and washed twice with cold

PBS. Then, cells were re-suspended in 200 µl binding buffer with 10 µl Annexin V-FITC and 5 µl Propidium Iodide (PI), and further incubated for 30 min in the dark. After that, 300 µl binding buffer was added and cell apoptosis was determined by using the flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Real Time-Quantitative PCR (RT-qPCR)

Total RNA was extracted and isolated from HCMs by using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). For the analysis of miR-638 expression, an ABI miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to transcribe RNA into cDNA. For the analysis of ATG5 expression, the TaqMan Reverse Transcript Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to transcribe RNA into cDNA. PCR was performed by using a Power SYBR green kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Bio-Rad MiniOption thermocycler (Bio-Rad, Hercules, CA, USA). U6 was used as an internal control for miR-638 expression, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for ATG5 expression. For the miR-638 and ATG5 expression, the relative expression was analyzed by using the $2^{-\Delta\Delta Ct}$ method. The primers for miR-638 were: F, 5'-ATCCAGTGCGTG TCGTG-3', R, 5'-TGCTAGGGATCGCGGGCGGGTG-3'; the primers for U6 were: F, 5'-CTCGCTTCGGCAGCACA-3', R, 5'-AACGCTTCACGAATTTGCGT-3'; the primers for ATG5 were: F, 5'-GCTTCGAGATGTGTGGTTTGG-3', R, 5'-ACTTTGTCAGTTACCAACGTCA-3'; the primers for GAPDH were: F, 5'-ACCCACTCCTCCACCTTTGAC-3', R, 5'-TGTTGCTGTAGCCAAATTCGTT-3'.

Western Blot Assay

Proteins from the HCMs were extracted with lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) and separated by gel electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Subsequently, the separated proteins on the SDS-PAGE gel were transferred onto polyvinylidene difluoride membranes. After blocking, membranes were probed with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies conjugated with horseradish peroxidase and detected by enhanced chemiluminescence (ECL). All the antibodies were purchased from the Abcam Company (Cambridge, MA, USA).

Dual-Luciferase Reporter Assay

The mutant 3'UTR of ATG5 was generated by using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The 3'UTR of ATG5 harboring either the miR-638 binding site or the mutant 3'UTR of ATG5 was cloned into the psiCHECK-2 vectors (Promega, Madison, WI, USA) to generate the psiCHECK-ATG5 3'UTR Luciferase reporter plasmid. Luciferase reporter plasmids and miR-638 mimic or miR-638 inhibitor were co-transfected into HCMs by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in according with the manufacturer's instruction. At 48 h after transfection, a Dual-Luciferase reporter gene assay kit (Promega, Madison, WI, USA) was used to determine the Luciferase activities, and Renilla Luciferase activity was normalized to firefly Luciferase activity.

Statistical Analysis

For the results, all the data were expressed as mean ± standard deviation. Statistical significance between groups was determined by unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. All the experiments were repeated at least three times. The data analysis was performed by using the GraphPad Version 6.0 software (La Jolla, CA, USA). $p<0.05$ was considered statistically significant.

Results

Effects of H/R on Cell Viability, Cell Apoptosis and MiR-638 Expression in HCMs

The cell viability of HCMs following H/R treatment was determined by MTT assay. The cell viability was significantly suppressed following H/R treatment in the HCM (Figure 1A). Cell apoptosis of HCMs following H/R treatment was determined by flow cytometry. Cell apoptotic rate was significantly increased following H/R treatment in the HCMs (Figure 1B). MiR-638 expression following H/R treatment was also explored by the RT-qPCR assay, and H/R treatment significantly suppressed the expression level of miR-638 in the HCMs (Figure 1C).

Effects of MiR-638 on Cell Viability, Cell Apoptosis in HCMs

To determine the role of miR-638 in the cell viability and apoptosis, miR-638 was ove-

rexpressed or down-regulated by transfecting with miR-638 mimic or miR-638 inhibitor. As shown in Figure 2A, miR-638 mimic transfection significantly increased the expression level of miR-638 in the HCMs, while miR-638 inhibitor transfection significantly suppressed the expression level of miR-638 in the HCMs (Figure 2A). Firstly, the effects of miR-638 down-regulation on cell viability and cell apoptosis in the HCMs were examined. The down-regulation of miR-638 by miR-638 inhibitor transfection significantly suppressed the cell viability and increased the cell apoptosis in the HCMs (Figure 2B, 2C). In addition, the effects of miR-638 overexpression on cell viability and cell apoptosis in H/R-treated HCMs were determined by MTT assay and flow cytometry, respectively. Transfection with scrambled miRNA for miR-638 mimic (Mimic NC) had no effect on the cell viability and cell apoptosis in the H/R-treated HCMs, and the overexpression of miR-638 by miR-638 mimic transfection significantly attenuated the effects of H/R treatment on the cell viability and cell apoptosis in the HCMs, in which miR-638 mimic transfection increased the cell viability and decreased the cell apoptosis in the H/R-treated HCMs (Figure 2D, 2E).

ATG5 Was a Target Gene of MiR-638

By using TargetScan, ATG5 was predicted to be a target of miR-638. To further confirm that ATG5 is a direct target of miR-638, we examined if miR-638 could regulate ATG5 expression by binding to the 3'UTR of ATG5 (Figure 3A). We first generated Luciferase reporters containing the wild-type 3'UTR of ATG5. The transfection with miR-638 resulted in a significant reduction in Luciferase activity compared with mimic NC group in HCMs, while the transfection with miR-638 inhibitor increased the Luciferase activity compared with inhibitor NC in the HCMs (Figure 3B). In addition, when the Luciferase reporters containing a mutated segment of ATG5, miR-638 mimic or miR-638 inhibitor transfection had no effect on the Luciferase activity compared with their respective negative controls (Figure 3C).

MiR-638 Suppressed the Expression of ATG5 and ATG5 Reversed the Effects of MiR-638 Overexpression on Cell Viability and Apoptosis in H/R-Treated HCMs

The effects of miR-638 on the mRNA and protein expression of ATG5 were determined by RT-qPCR and Western blot, respectively. The transfection with miR-638 mimic significantly suppressed the mRNA and protein expression levels of ATG5 compared with mimic NC group in the HCMs, while the downregulation of miR-638 increased the mRNA and protein expression levels of ATG5 compared with inhibitor NC group in the HCMs (Figure 4A, 4B). To further determine the *in vitro* functional role of ATG5, we tested if the enforced expression of ATG5 could reverse the effect of miR-638 mimic transfection on cell viability and apopto-

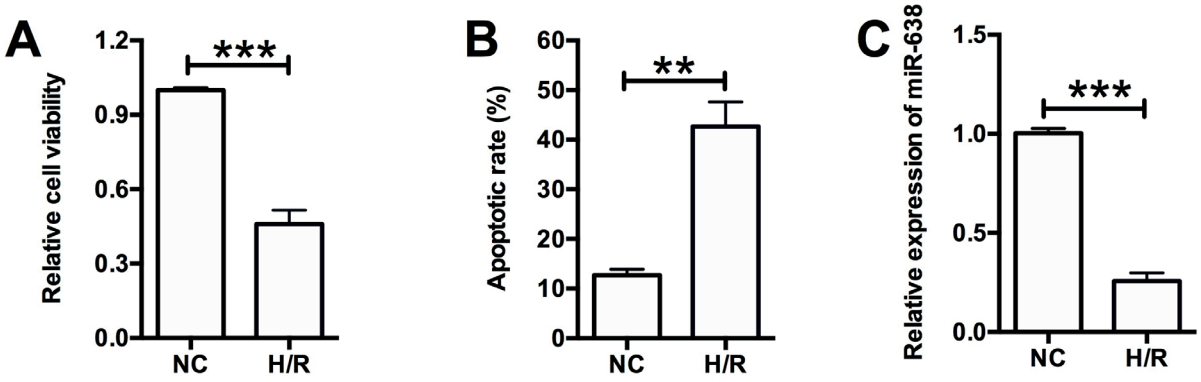

Figure 1. H/R reduces cell viability, induces apoptosis and down-regulates miR-638 expression in HCMs. ***A,*** MTT analysis was performed to determine cell viability in H/R-treated HCMs. ***B,*** Flow cytometry was used to determine the apoptotic rate in H/R-treated HCMs. ***C,*** RT-qPCR was performed to determine the expression levels of miR-638 in H/R-treated HCMs. NC=negative control, H/R=hypoxia/reoxygenation. N=3. Significant differences between treatment groups were indicated as *p<0.05, **p<0.01, ***p<0.001.

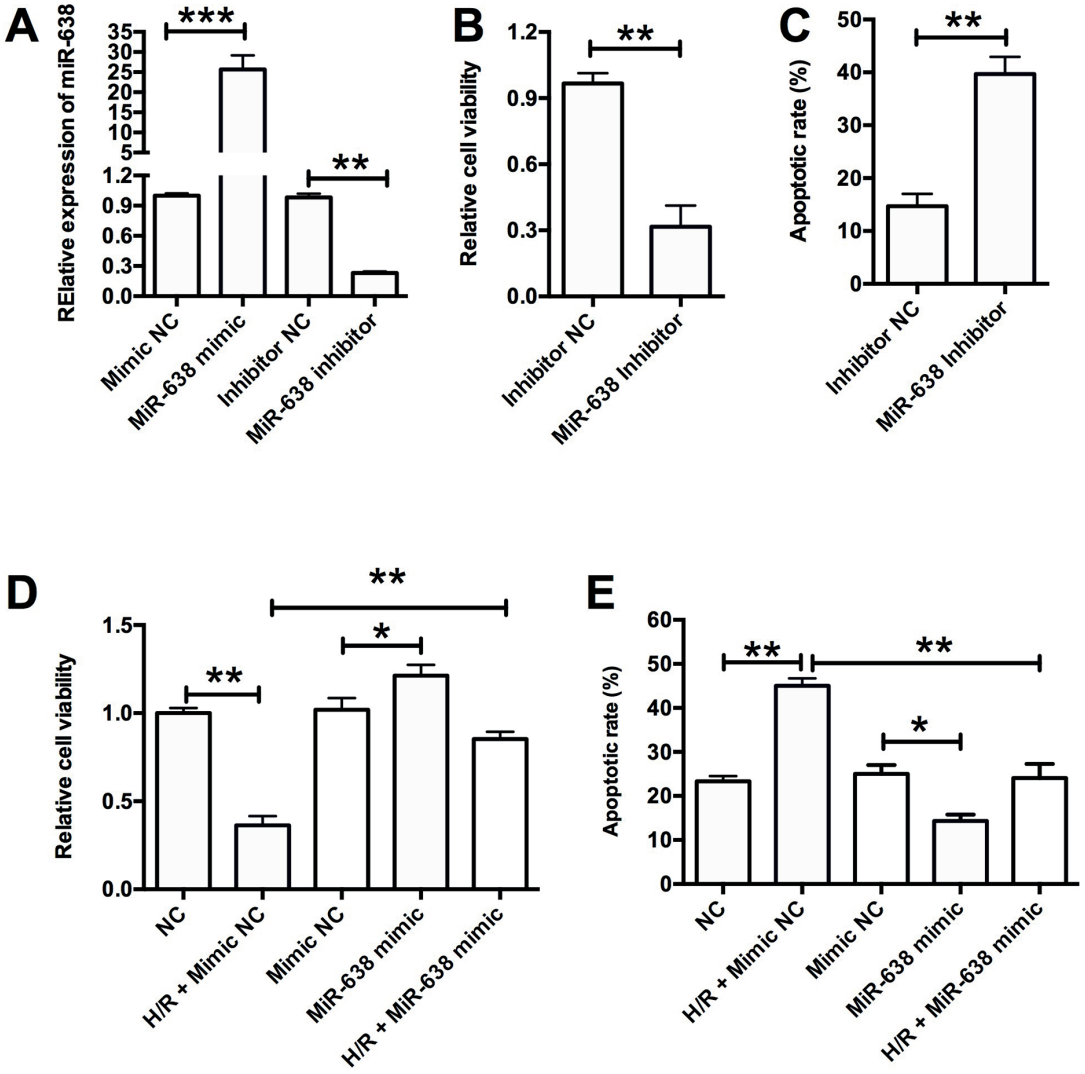

Figure 2. Effects of miR-638 on cell viability and cell apoptosis in HCMs. ***A,*** RT-qPCR was performed to determine the expression levels of miR-638 in HCMs transfected with miR-638 mimic or miR-638 inhibitor. ***B,*** MTT analysis was used to determine the cell viability in HCMs transfected with miR-638 inhibitor. ***C,*** Flow cytometry was used to determine the cell apoptosis in HCMs transfected with miR-638 inhibitor. ***D,*** MTT analysis was performed to determine the cell viability in H/R-treated HCMs transfected with miR-638 mimic. ***E,*** Flow cytometry analysis was performed to determine the cell apoptosis in H/R-treated HCMs transfected with miR-638 mimic. N=3. Significant differences between treatment groups were indicated as *p<0.05, **p<0.01, ***p<0.001.

sis in the H/R-treated HCMs. Consistently, the co-transfection with miR-638 mimic and the empty vector (pcDNA3.1) significantly reduced cell viability and increased cell apoptosis in the H/R-treated HCMs compared with mimic NC + empty vector group; co-transfection with miR-638 mimic and pcDNA3.1-ATG5 attenuated the effects of miR-638 overexpression on the cell viability and cell apoptosis in the H/R-treated HCMs (Figure 4C, 4D).

Effects of MiR-638 on the Protein Levels of Autophagy-Related Factors in H/R-Treated HCMs

Since ATG5 is an important factor in the process of autophagy, we tested if modulation of miR-638 expression could affect the autophagy in the H/R-treated HCMs. H/R-treatment significantly increased the ratio of LC3-II to LC3-I and suppressed the protein levels of p62; miR-638 mimic transfection decreased the ratio of LC3-II

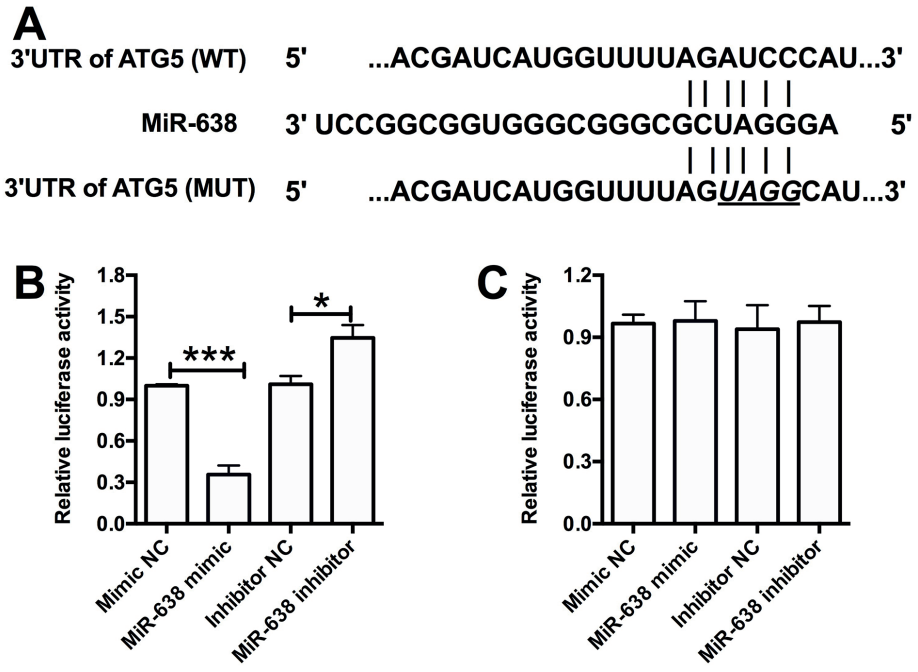

Figure 3. ATG5 was target gene of miR-638. ***A,*** Sequence alignment of miR-638 and 3'UTR of ATG5 using TargetScan algorithm. ***B,*** HCMs were co-transfected with miR-638 mimic or miR-638 inhibitor + Luciferase reporter containing a fragment of the ATG5 3'UTR harboring the miR-638 binding site (3'UTR of ATG5-WT), and Luciferase activity was determined by Luciferase assay. ***C,*** HCMs were co-transfected with miR-638 mimic or miR-638 inhibitor + Luciferase reporter containing a fragment of the ATG5 3'UTR harboring the miR-638 binding site (3'UTR of ATG5-MUT), and Luciferase activity was determined by Luciferase assay. WT=wild-type, MUT=mutant. N=3. Significant differences between treatment groups were indicated as *p<0.05, ***p<0.001.

to LC3-I and increased the expression of p62 (Figure 5). In addition, miR-638 mimic transfection also attenuated the effects of H/R treatment on the autophagy-related proteins expression in the HCMs (Figure 5).

Discussion

The abnormal expression of miRNAs was found to contribute to myocardial I/R injury and subsequent heart failure[22,23]. Herein, we demonstrated that H/R treatment suppressed cell viability, induced cell apoptosis and decreased the expression level of miR-638 in the HCMs. MiR-638 down-regulation suppressed cell viability and induced cell apoptosis in the HCMs. The overexpression of miR-638 reversed the effects of H/R treatment on the cell viability and apoptosis in the HCMs. By using bioinformatics prediction, ATG5 was found to be one of the potential targets of miR-638. The Luciferase reporter assay results showed that miR-638 suppressed the expression of ATG5 by targeting the 3'UTR of ATG5. MiR-638 was found to suppress the mRNA and protein levels of ATG5 in the HCMs. Enforced expression of ATG5 reversed the effects of miR-638 overexpression on cell viability and cell apoptosis in the H/R treated HCMs. More importantly, by examining the autophagy-related factors, we found that H/R treatment promoted autophagy in the HCMs, which can be prevented by miR-638 overexpression. Our work confirmed that miR-638 is a key regulator in the myocardial I/R injury and thus may be a potential therapeutic target for the treatment of heart failure. Accumulating evidence has indicated that miRNAs play important roles in both physiological and pathological processes of heart disease[24,25]. The functional role of miR-638 was first identified in the cancer studies. Based on previous researches, miR-638 can function to be oncogenic to promote cell proliferation in melanoma and esophageal squamous cell carcinoma[20,26]; while in other types of cancers, such as gastric cancer, osteosarcoma, and colorectal cancer miR-638

plays a tumor-suppressive role in these cancer cells[27-29], suggesting the complexity of miR-638 in the regulation of cancer cell proliferation. Up to date, the role of miR-638 was not examined in the HCMs subjected to the H/R treatment. We demonstrated that H/R treatment reduced cell viability and induced apoptosis accompanied by the suppression of miR-638 expression in the HCMs. The restoration of miR-638 in the H/R-treated HCMs reversed the effects of H/R treatment on the cell viability and apoptosis, suggesting that miR-638 promotes cell viability and inhibits cell apoptosis in the H/R treated HCMs. Autophagy is a conserved catabolic process and it is essential for maintaining normal cardiac function[30]. Basal autophagy is important for cell survival, and de-regulation of autophagy can trigger autophagic cell death, which has been shown be to be associated with heart disease[31]. Recently, accumulating evidence has suggested the role of autophagy in the myocardial I/R injury. Scholars[32] revealed that autophagy exerted both beneficial and detrimental effects in the pathology of myocardial I/R injury. Fan et al[33] demonstrated that H/R treatment increased autophagy with an increase in the cell apoptotic rate in the neonatal rat cardiomyocytes. Our results also showed that H/R treatment in the HCMs promoted autophagy and increased apoptosis. Furthermore, by using bioinformatics analysis, we found that ATG5 was a downstream target of miR-638, which was confirmed by Luciferase reporter assay, *in vitro*

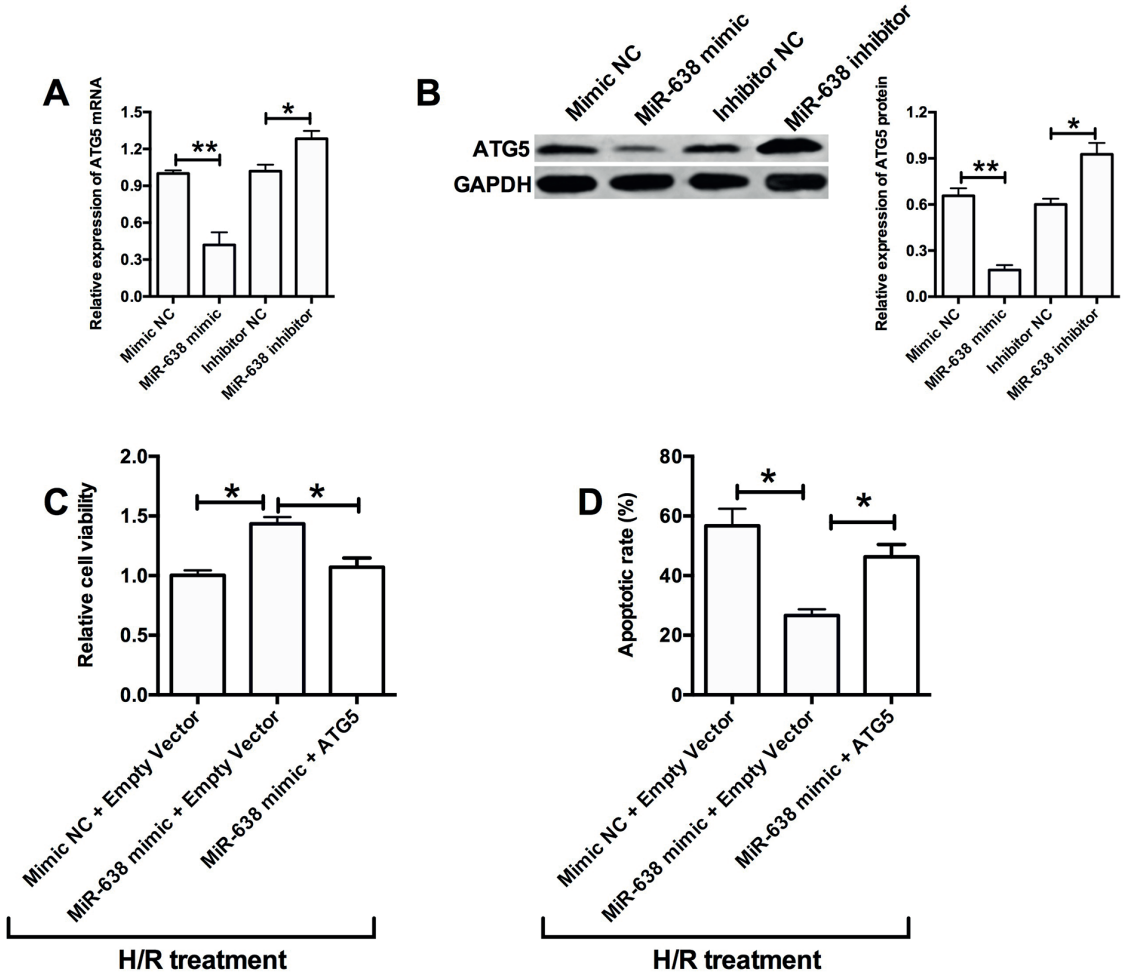

Figure 4. MiR-638 suppressed the expression of ATG5 and ATG5 reversed the effects of miR-638 overexpression on cell viability and apoptosis in H/R-treated HCMs. ***A,*** RT-qPCR was used to determine the mRNA expression of ATG5 in HCMs transfected with miR-638 mimic or miR-638 inhibitor. ***B,*** Western blot was used to measure the relative protein levels of ATG5 in HCMs transfected with miR-638 mimic or miR-638 inhibitor. N=3. Significant differences between treatment groups were indicated as *p<0.05, **p<0.01.

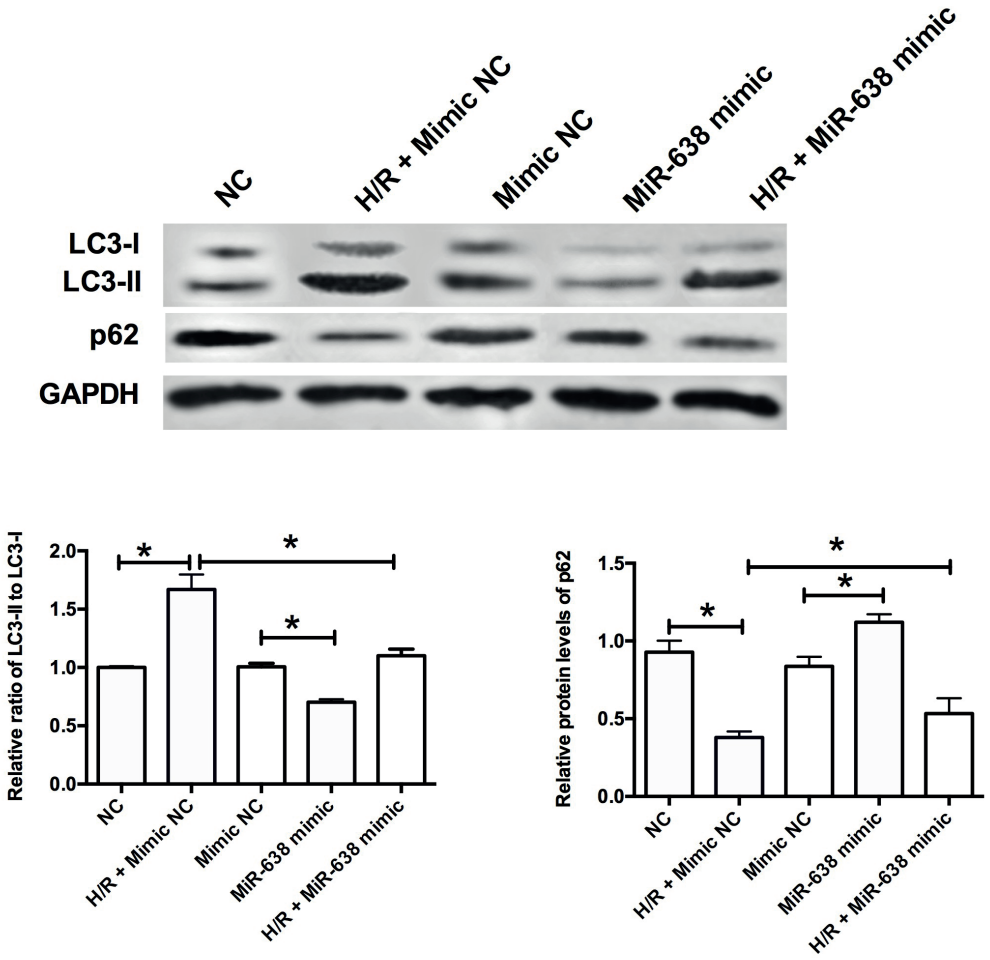

Figure 5. Effects of miR-638 on the protein levels of autophagy-related factors in H/R-treated HCMs. Western blot assay was performed to analyze the protein levels of autophagy-related factors in H/R-treated HCMs transfected with miR-638 mimic. N=3. Significant differences between treatment groups were indicated as *p<0.05.

RT-qPCR and Western blot studies. More importantly, enforced expression of ATG5 restored effects of miR-638 overexpression on cell viability and cell apoptosis in H/R-treated HCMs. The molecular mechanism of autophagy involves several conserved autophagy-related genes (ATG), and among these genes, ATG5 protein in a conjugated form with ATG12 and LC3 involved in the early stages of autophagosome formation[34] ATG5 was also found to be up-regulated in failing hearts, which was believed to be an adaptive response for protecting cardiomyocytes from hemodynamic stress[35]. In this investigation, we further found that the overexpression of miR-638 inhibited autophagy in the HCMs and also reversed the effects of H/R treatment on the autophagy in the HCMs, suggesting that miR-638 had an inhibitory effect on the autophagy in the HCMs. Consistent with the previous study, depletion of miR-638 induced autophagy in the melanoma cells[20]. Conversely, the overexpression of miR-638 promoted starvation- and rapamycin-induced autophagy in the esophageal squamous cell carcinoma and breast cancer cells[26]. These results suggest that the effects of miR-638 on autophagy may depend on different cell types or different stimuli. Further studies may be conducted to look into the details on miR-638-regulated autophagy in the HCMs.

Conclusions

We suggested that the overexpression of miR-638 attenuated the effects of H/R treatment on cell viability, cell apoptosis and autophagy, at le-

ast partly by regulating the ATG5 expression in the HCMs, and miR-638 may represent a novel therapeutic target for the prevention or treatment of heart failure.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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Author Contributions

PZ and ZL conceived and designed the experiments. PZ and KL performed the experiments and analysed the data. BQ analysed the data and proofread the manuscript. ZL wrote the manuscript.

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