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# Effect on hypoxia/reoxygenation-induced cardiomyocyte injury and Pink1/Parkin pathway

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**Abstract.** Our study aimed to detect the effects of proprotein convertase subtilisin/kexin type 9 (PCSK9) on exacerbating cardiomyocyte hypoxia/reoxygenation (H/R) injury and the possible mechanism. A cell model of H/R was constructed. PCSK9 mRNA and protein levels were significantly upregulated during AC16 cardiomyocyte H/R. Flowmetry detection of apoptosis, as well as JC-1, confirmed that PCSK9 upregulation of autophagy levels was accompanied by apoptosis. Furthermore, in the H/R+si-PCSK9 group, the expression of autophagy-related protein LC3 decreased and P62 increased. At the same time, the presentation of the autophagic pathway Pink1/Parkin was also downregulated. In conclusion, in AC16 cardiomyocytes treated with H/R, PCSK9 expression and autophagy levels were increased; a possible molecular mechanism was the activation of the Pink1/Parkin pathway.

**Key words:** PCSK9 — Pink1/Parkin — Small interfering RNA — Autophagy — Cardiomyocyte cytology

**Abbreviations:** H/R, hypoxia/reoxygenation; I/R, ischemia-reperfusion; OMM, outer mitochondrial membrane; PCSK9, proprotein convertase subtilisin/kexin type 9.

## Introduction

Under myocardial ischemia-reperfusion (I/R) injury, cardiomyocytes may undergo autophagy, apoptosis, and necrosis (Du et al. 2020). Autophagy refers to the process by which autophagic vesicles produced in cells encapsulate damaged, degenerated, senescent long-lived proteins and organelles and then transport them to lysosomes for enzymatic digestion and reduction of the substrate to its constituent parts for cellular reuse (Jiang 2017; Ji and Bai 2018). The role of autophagy varies in different cells and different pathophysiological states. For example, in heart failure, abnormal enhancement of autophagy will further lead to cardiomyocyte damage, while in cardiac hypertrophy, weakened autophagy will exacerbate cardiac failure (Azad et al. 2008; Du et al. 2020).

The proprotein convertase subtilisin/kexin type 9 (PCSK9) has received widespread attention because of its important impact on LDLC metabolism, and current guidelines suggest that in patients with the very high-risk acute coronary syndrome (ACS) or very high-risk familial hypercholesterolemia (FH), LDLC remains high after maximum tolerated doses of statin combined with ezetimibe (Cohen et al. 2006). The current guidelines recommend the combination of PCSK9 inhibitors in patients with very high-risk ACS or the very high-risk FH in cases that LDLC remains

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high after the maximum tolerated dose of statin combined with ezetimibe (Pradhan et al. 2018). It has been shown that PCSK9 is an independent risk factor for prognostic adverse events in patients with cardiovascular disease, and the increasing search for effective drugs to reduce I/R injury is of clinical importance in the treatment of patients with heart attack (Ding et al. 2018).

Mitochondria-specific autophagy (mitophagy) is a fundamental process critical for maintaining mitochondrial fitness in a myriad of cell types. Particularly, mitophagy contributes to mitochondrial quality control by selectively eliminating dysfunctional mitochondria. In mammalian cells, the Ser/Thr kinase Pink1 and the E3 ubiquitin ligase Parkin act cooperatively in sensing mitochondrial functional state and marking damaged mitochondria for disposal *via* the autophagy pathway. Notably, ubiquitin and deubiquitinases play vital roles in modulating Parkin activity and mitophagy efficiency.

The PTEN-induced kinase 1 (Pink1)/Parkin-mediated mitophagy pathway has been reviewed extensively (Pickrell and Youle 2015; Yamano et al. 2016; Harper et al. 2018), because these proteins were the first identified to specifically target damaged mitochondria for engulfment by the autophagosome for lysosomal degradation (Narendra et al. 2008, 2010; Jin et al. 2010; Pickrell and Youle 2015; Yamano et al. 2016). Pink1 is continuously imported through the translocase of the outer membrane (TOM) complex, processed, and released into the cytosol through the N-end rule pathway to undergo proteasomal degradation when mitochondria are healthy (Narendra et al. 2010; Vives-Bauza et al. 2010; Pickrell and Youle 2015). However, upon mitochondrial depolarization or damage, Pink1 is stabilized on the outer mitochondrial membrane (OMM) (Lazarou et al. 2012; Okatsu et al. 2013) and phosphorylates ubiquitin (Ub) chains on OMM proteins and the E3 ubiquitin ligase Parkin at serine 65 (Kane et al. 2014; Kazlauskaite et al. 2014a, 2014b). Interactions between phosphorylated Ub and Parkin act as a primer for recruiting cytosolic Parkin onto depolarized mitochondria. These events activate Parkin, which attaches poly Ub chains to its substate proteins on the OMM (Sarraf et al. 2013). This starts a positive feedback loop for further recruitment of cytosolic Parkin to rapidly coat the mitochondria for autophagy receptor attachment for autophagosomal degradation.

These studies reveal that there is much to learn regarding the relationship among Pink1, Parkin, and mitophagy under physiological conditions.

Both *in vivo* and *in vitro* experiments have shown that cellular autophagy plays an important role after myocardial I/R injury. In this study, we used *in vitro* experiments to simulate cardiomyocyte I/R injury by H/R and regulate cardiomyocyte PCSK9 expression to observe the changes in cellular autophagy and related experimental parameters, and to verify whether PCSK9 is involved in cardiomyocyte injury after H/R.

## Materials and Methods

### *Cell culture and treatment protocol*

The human cardiomyocytes AC16 were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco-Laboratories, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories, USA) and 100 U/ml penicillin/100 mg/ml streptomycin in an atmosphere of 90% air and 10% $CO_2$ at 37°C as described previously. The medium was replaced every 2 days, and the cells were digested with 0.05% trypsin when the density of the cells reached 80–90%. AC16 cells were seeded in six-well plates or 96-well plates and treated as used for the following experiments.

### *CCK-8 assay to determine the H/R time of AC16 cells*

Cell viability was determined by using a CCK-8 assay kit (Jiancheng, Nanjing, China) in 96-well plates according to the manufacturer's instructions. Cardiomyocytes in the logarithmic growth phase were inoculated with $3\times10^3$/100 µl in well plates, and when the cells reached 80% or more, the original culture medium was aspirated and discarded, and the cells were washed once with PBS and divided into normal control group and H/R group (after 8, 10, and 12 h of hypoxia and 8 h of reoxygenation, respectively). The control group was incubated in a normal incubator, while the H/R group was incubated with serum-free DEME low sugar medium and placed in a three-gas incubator containing 94% $N_2$, 1% $O_2$ and 5% $CO_2$ for 8, 10 and 12 h of hypoxia respectively, then the cell culture medium was aspirated and discarded, PBS was washed once, serum-containing DEME high sugar medium was added, and placed in the normal incubator for 8 h of reoxygenation, and after the end of H/R, the cell cultures were removed and 110 µl (containing 10 µl of CCK-8) of new serum-containing DEME high sugar medium was added and incubated in a normal incubator, and CCK-8 was added to the normal control group at the same time point as the H/R group. After incubation in the incubator at 37°C with 5% carbon dioxide for 3 h, the absorbance was determined at 450 nm using a PerkinElmer microplate reader (PerkinElmer VIC-TOR 1420, USA).

Based on the cell viability, the effect of different hypoxic times on cell viability was judged to determine whether the H/R model was successful, i.e., the greater the cell viability, the less the damage on cells, and *vice versa*, the smaller the cell viability, the greater the damage of H/R on cells. Synthesis and selection of siRNA for PCSK9: (PCSK9_1): GAGGTGTACTCCTAGACA; (PCSK9_2): CCCATGTCGACTACATCGA; (PCSK9_3): GGTCACCGACTTCGAGAAT.

PCSK9_1, PCSK9_2, PCSK9_3 were synthesized by RiboBio (Guangzhou, China), AC16 cells were chosen to

test the transfection and inhibition efficiency of the three sequences. RT-qPCR was used to validate the highest inhibition efficiency of PCSK9 and a 50 nM dose was selected. AC16 cardiomyocytes were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The cells were used for experiments 48 h after siRNA transfection.

*Experimental groups*

1) Control group (blank).
2) H/R group (hypoxia/reoxygenation group).
3) H/R+si-NC group (small interfering RNA negative control group).
4) H/R+si-PCSK9 group (small interfering RNA H/R group).

*Cell viability*

Cells from different experimental subgroups were collected and according to the manufacturer's instructions, a CCK-8 assay kit (Jiancheng, Nanjing, China) was used to determine the cell viability in 96-well plates (the experimental procedure is as above).

*Assessment of apoptotic cell death in cultured AC16 cells*

Apoptotic AC16 cardiomyocytes were measured using both flow cytometry. For flow cytometry, cells were harvested and stained with Annexin V-FITC and propidium iodide (PI) (Thermo Fisher Scientific, Shanghai, China) for 20 min at room temperature. The cells were then washed twice with PBS, and the fluorescence was analyzed with CellQuest (BD Biosciences, Franklin Lakes, NJ) software on data obtained from the cell population.

*Early and late apoptosis detection via flow cytometry*

AC16 cells were harvested with 0.25% trypsin and washed with PBS. Following staining with 5 μl 7-Amino-Actinomycin D and 5 μl PE Annexin V (cat. no. BD 559763; BD Biosciences) for 18 min in the dark at 37°C, early and late apoptosis was examined using a FACSMelody flow cytometer (BD Biosciences) with FlowJo software (version 7.6.1; FlowJollc).

*Real-time fluorescent quantitative PCR (RT-qPCR)*

After collecting AC16 cells in each group, TRIzol reagent was used to extract the total RNA in each group of cells, and the RNA was then reverse transcribed into cDNA by following the PrimeScript RT Master Mix Kit instructions. β-actin was then used as an internal reference to measure the expression levels of PCSK9, P62m, Parkin, LC3B and Pink1 in cells by following the SYBR Green kit instructions. The primer sequences used in this study are shown in Table 1. The reaction system contained 2 μl reverse transcription product, 10 μl SYBR Green Mix reagent, 0.5 μl (10 μmol/l) of forward and reverse primers, and 7 μl of $dH_2O$. The reaction conditions for a total of 45 cycles were set as follows: 95°C predenaturation for 5 min, denaturation at 94°C for 30 s, and annealing at 60°C for 30 s. The relative expression levels of miR-16-3p and miR-16-5p were calculated by the $2^{-\Delta\Delta Ct}$ method.

*Western blot assay for protein expression*

After the modelling of AC16 cells, the cells were washed 2~3 times with PBS, digested with trypsin, and the digested solution was discarded, then the appropriate volume of RIPA was added, and the cells and reagent solution were collected. The protein content was measured by a protein content meter. The protein samples were diluted by adding the loading buffer and electrophoresis solution, denatured by boiling water for 15 min, and then stored in the refrigerator at −80°C. Then add primary antibody PCSK9 (1:1000), P62 (1:1000), Pink1 (1:1000), Parkin (1:1000), LC3 (1:1000) or β-actin (1:2000), and incubate overnight at 4°C. After washing with PBS containing 0.2% Tween, and incubating with a secondary antibody (1:10,000) at room temperature for 1 h, the final color was developed with ECL luminescent solution and photographed on a Bio-Rad gel imager.

*Measurement of mitochondrial membrane potential (ΔΨ) by flow cytometry analysis*

JC-1 is a carbon cyanide fluorescent dye, which exists in two different physical forms, polymer and monomer, at different fluorescence emission peaks in the cell. When the concentration of JC-1 (i.e. the level of membrane potential) is low, it

**Table 1.** Primer sequences

| Gene | Sequence |
|---|---|
| PCSK9 | F: 5'-ACCCACCTCTCGCAGCAGA-3'<br>R: 5'-CGCCACTCATCTTCACCAGGC-3' |
| P62 | F: 5'-GACTACGACTTGTGTAGCGTC-3'<br>R: 5'-AGTGTCCGTGTTTCACCTTCC-3' |
| Parkin | F: 5'-CCCACCTCTGACAAGGAAACA-3'<br>R: 5'-TCGTGAACAAACTGCCGATCA-3' |
| Pink1 | F: 5'-GGAGGAGTATCTGATGGGCAG-3'<br>R: 5'-AACCCGGTGCTCTTTGTCAC-3' |
| LC3 | F: 5'-ACCAGCACCCCAGCAAA-3'<br>R: 5'-TCACCAGCAGGAAGAAGGC-3' |
| β-actin | F: 5'-CTCGCTTCGGCAGCACA-3'<br>R: 5'-AACGCTTCACGAAATTGCGT-3' |

F, forward; R, reverse.

exists mainly as a monomer with an excitation wavelength of 527 nm and a green fluorescence; when the concentration of JC-1 (i.e. the level of mitochondrial membrane potential) is high, it forms a polymer and emits a red fluorescence with an excitation wavelength of 590 nm. After the modelling of AC16 cells is completed, the cells are digested by trypsin, the supernatant is discarded by centrifugation, rinsed once with PBS, and resuspended with 500 µl of medium. The cells were resuspended with 500 µl of culture medium; 500 µl of staining solution was added, mixed and incubated for 20 min at 4°C and centrifuged for 5 min at 300 × *g*. The supernatant was discarded and rinsed twice with precooled 1× buffer; 500 µl of 1× buffer was resuspended, and the percentage of red fluorescent cells in AC16 cells was detected by flow cytometry to analyze the changes in mitochondrial membrane potential.

*Statistical analysis*

All experiments were repeated, and data were obtained by repeating them with similar quality. Data were displayed as mean ± SD. Statistical analysis was performed using GraphPad Prism 7.0 software (CA, USA). The unpaired Student's *t* test was used to perform a statistical comparison between two groups. The ANOVA test was used when performing multiple comparisons. The level of significance was set at $p < 0.05$.

## Results

*The effect of H/R on cell viability in AC16 cells*

Compared with the normal control group, the cell viability of AC16 cells was lower after 10 h of hypoxia and 8 h of reoxygenation, and the cell viability decreased to the lowest after extending the hypoxia time (Fig. 1A). In order to facilitate the development of the next experiment, 10 h of hypoxia and 8 h of reoxygenation were chosen.

To investigate the effect of PCSK9 on AC16 cells viability, AC16 cells were directly exposed to different methods. CCK-8 measurement was performed to assess the effect of PCSK9 on cell viability. In comparison to the control group, the H/R group dramatically decreased the cell viability (Fig. 1C). Meanwhile, after transfection treatment of PCSK9, H/R+si-PCSK9 group cell viability was significantly upregulated. These results indicated that PCSK9 downregulated cell viability under H/R conditions. Increased levels of PCSK9-induced autophagy may be accompanied by apoptosis.

Low-level autophagy is important for maintaining cellular homeostasis, but in inflammation and various inflammatory and oxidative stress conditions, excessive autophagy can induce apoptosis. As shown in the Figure 2A, the apoptosis rate was significantly increased under H/R conditions. Compared with the control group (Fig. 2B), AC16 cardiomyocytes in H/R+si-PCSK9 group significantly declined (Fig. 2B). We speculate that autophagy triggered by PCSK9 under H/R conditions may also be accompanied by apoptosis of AC16 cardiomyocyte cells.

Manipulation of PCSK9 expression was achieved through transfection of cells with siRNA. The RT-qPCR results indicated that PCSK9 overexpression during H/R significantly up-regulated the levels of Pink1, Parkin mRNA and LC3 mRNA, and down-regulated the level of P62 mRNA. Conversely, the levels of Pink1, Parkin mRNA and LC3 mRNA decreased, while P62 mRNA levels increased upon the siRNA-mediated knockdown of PCSK9 (Fig. 3). Collectively,

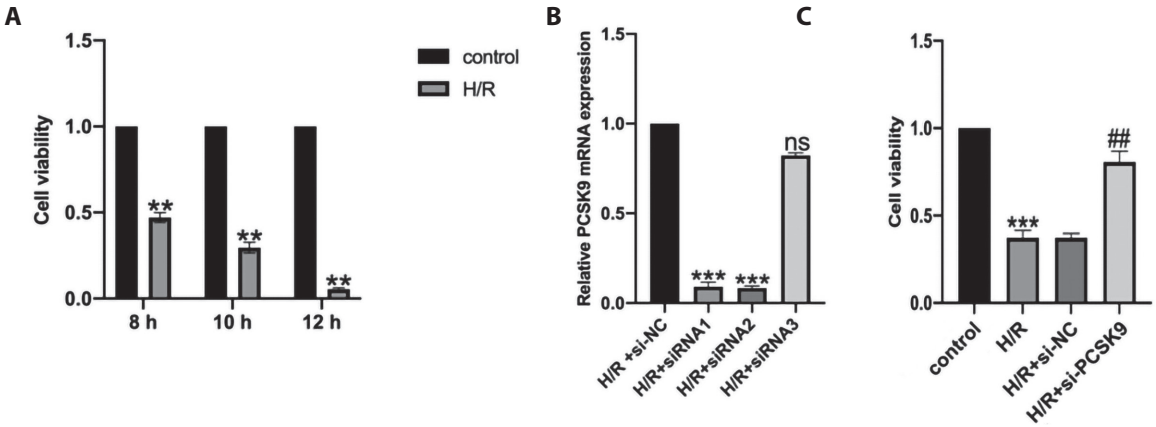


**Figure 1.** PCSK9 aggravated H/R-induced AC16 cells injury. **A.** Effect of H/R on cell viability in AC16 cells. ** $p < 0.01$ *vs*. control. **B.** The most efficient transfection siRNA for PCSK9 selected by RT-qPCR method. *** $p < 0.001$ *vs*. H/R+si-NC, $^{ns}$ $p > 0.05$ *vs*. H/R+si-NC. **C.** The viability of cardiomyocytes measured by CCK-8 method in the different groups. $^{***}$ $p < 0.001$ *vs*. control, $^{\#\#}$ $p < 0.01$ *vs*. H/R. H/R, hypoxia/reoxygenation group; H/R+si-NC, small interfering RNA negative control group; H/R+si-PCSK9; small interfering RNA H/R group.

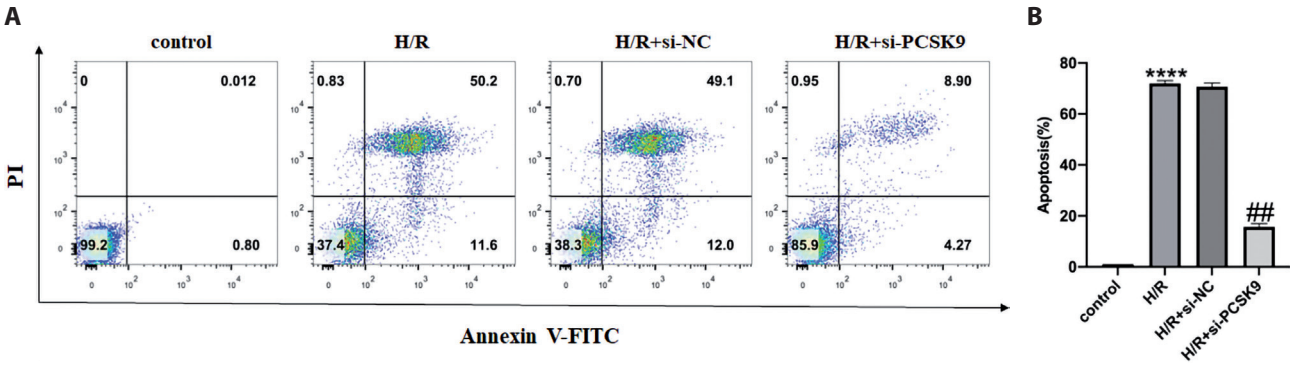


**Figure 2.** Increased levels of PCSK9-induced autophagy may be accompanied by apoptosis. **A.** Cell apoptosis in AC16 cells in different groups determined by flow cytometry assay **B.** Quantitative analysis. Data are expressed as mean ± SD. **** $p < 0.0001$ *vs.* control, ## $p < 0.01$ *vs.* H/R. For abbreviations, see Figure 1.

these results revealed that PCSK9 is involved in regulating the transcriptional expression of Pink1, Parkin, LC3 and P62 following H/R in AC16 cardiomyocytes.

To investigate the role of PCSK9 aggravated AC16 cell autophagy under H/R stimulation, we next measured the production of cellular PCSK9, P62, LC3, Pink1 and Parkin protein expression in AC16 cells. The results showed that the production of cellular PCSK9 was increased under H/R conditions compared with the control group (Fig. 4A). As shown in Figure 4E, H/R stimulation could significantly increase the expression of LC3. H/R stimulation could significantly decrease the protein expression of P62 – a marker protein of autophagy pathway (Fig. 4F). AC16 cells in H/R+si-PCSK9 group significantly decreased PCSK9 and LC3 protein levels. Subsequently, compared with the H/R group, P62 protein levels significantly increased (Fig. 4F). Many studies have reported that mitophagy is activated through the Pink1/Parkin signaling pathway in cancer cells. Under stress, Pink1 is translocated and stabilized on the OMM to recruit Parkin. Subsequently, Pink1 phosphorylates the poly-Ub chains, which are formed through ubiquitination of several components on the OMM by Parkin, thereby initiating mitophagy. Thus, PCR (Fig. 3A–E) and Western blotting were used to determine if PCSK9 induced mitophagy *via* the Pink1/Parkin signaling pathway. We have found that in different experimental groups, Pink1 and Parkin protein expression level also existed in different variations. Compared with control group, Pink1, Parkin protein levels have significantly increased (Fig. 4C, D). After interference with H/R+si-PCSK9, stimulation could signifi-

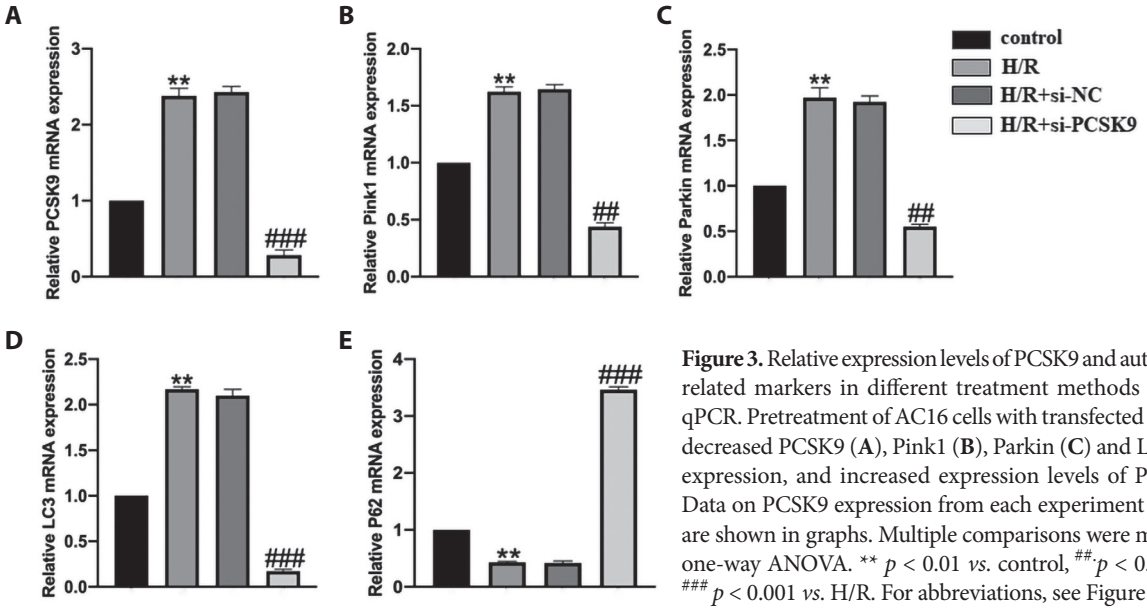


**Figure 3.** Relative expression levels of PCSK9 and autophagy related markers in different treatment methods by RT-qPCR. Pretreatment of AC16 cells with transfected PCSK9 decreased PCSK9 (**A**), Pink1 (**B**), Parkin (**C**) and LC3 (**D**) expression, and increased expression levels of P62 (**E**). Data on PCSK9 expression from each experiment ($n = 7$) are shown in graphs. Multiple comparisons were made by one-way ANOVA. ** $p < 0.01$ *vs.* control, ## $p < 0.01$ and ### $p < 0.001$ *vs.* H/R. For abbreviations, see Figure 1.

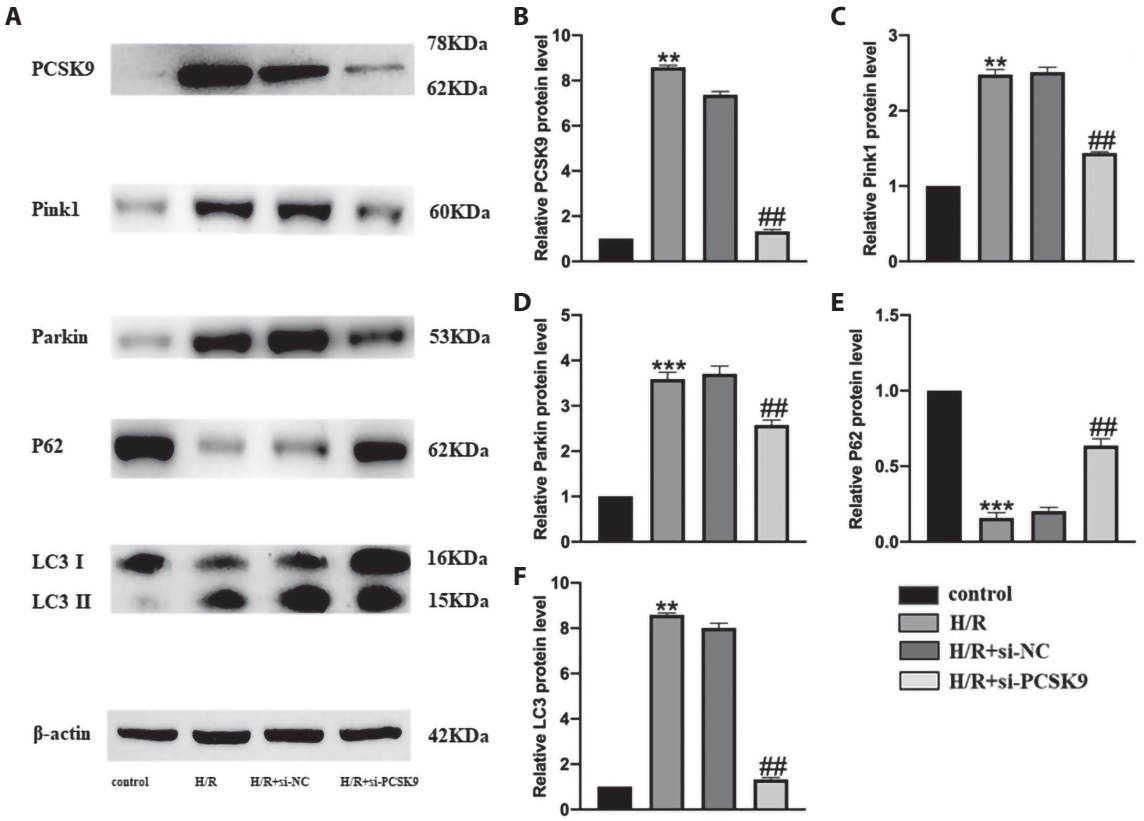


**Figure 4.** Expression and relationship of PCSK9, autophagy markers assessed by Western blotting. Protein expression was measured by Western blot (**A**) and quantified for each group (**B–F**). Data are expressed as the mean ± SEM, β-actin was used as the control. ** $p <$ 0.01 *vs.* control, ## $p < 0.01$ *vs.* H/R. For abbreviations, see Figure 1.

cantly downgrade the expression of Pink1 and Parkin (Fig. 4C, D). These results indicated that PCSK9 further inductions autophagy production, then PCSK9 siRNA inhibits PCSK9-triggered autophagy in the H/R-induced cell injury. As a part from this, PCSK9-induced autophagy and Pink1/Parkin pathway are closely related.

PCSK9 promotes autophagy levels and leads to a decrease in mitochondrial membrane potential. We know that the occurrence of autophagy is closely related to mitochondria, and in order to explore the relationship between PCSK9 and autophagy under the conditions of H/R (Fig. 5A); when autophagy occurs in mitochondria, the number of red cells shifting to green fluorescence gradually increases, the percentage of green fluorescent cells increases, and the mitochondrial membrane potential decrease. Compared with the control group, in AC16 cells with H/R conditions, the percentage of red fluorescence/green fluorescence was significantly downgrading, AC16 cells in H/R+si-PCSK9 group, the percentage of red fluorescence/green fluorescence was significantly increased. These results indicated that on the basis of cardiomyocyte H/R, PCSK9 further induces mitochondrial autophagy level upregulated.

## Discussion

Ischemia-reperfusion stimulates myocardial cells to produce large amounts of oxygen radicals, which can induce oxidative damage and activate the mitochondrial autophagy pathway to induce cellular autophagy (Khuanjing et al. 2021). By reducing the oxygen supply to myocardial cells and restoring the oxygen supply within a certain time frame, it is a common experimental method to simulate the reperfusion therapy of clinical ischemic heart disease (Khuanjing et al. 2021; Ou et al. 2021). Currently, there are two main methods to establish H/R: physical and chemical, especially the physical gas mixture model is common. In this experiment, we chose different times of purely physical damage to building a cellular H/R, and combined with CCK8 assay results, we finally chose 10 h hypoxia and 8 h reoxygenation. After the experiment, the cell survival rate decreased significantly, indicating that the cellular H/R model was successfully built.

Intracellular autophagy and apoptosis are not independent and unrelated regulatory processes (Li et al. 2016; Ding et al. 2020). More and more studies have begun to focus on the

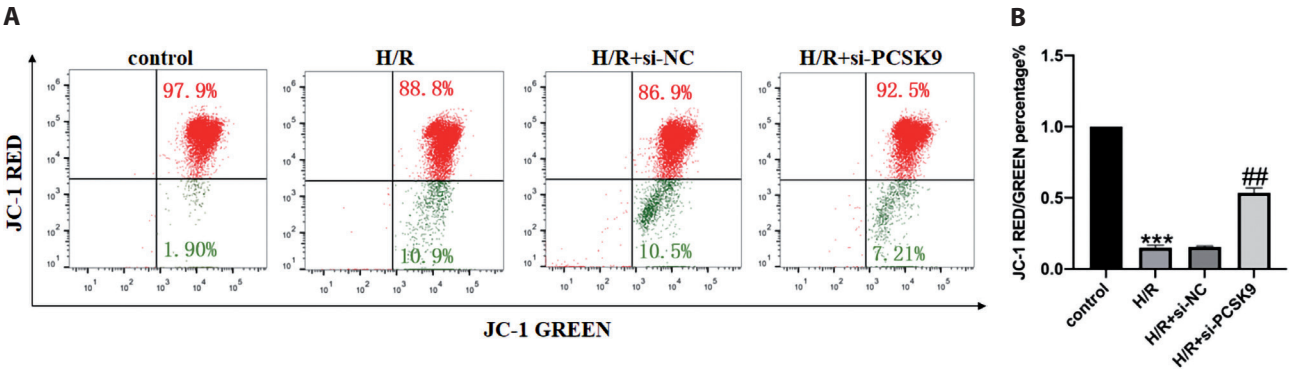


**Figure 5.** PCSK9 upregulates the level of autophagy and reduces the mitochondrial membrane potential under H/R conditions. Detection of changes in mitochondrial membrane potential in different experimental groups (**A**) and quantified for each group (**B**). Data are expressed as mean ± SD. *** $p < 0.001$ *vs.* control, ## $p < 0.01$ *vs.* H/R. For abbreviations, see Figure 1.

'crosstalk' between autophagy and apoptosis and the complex influence of the interaction on the occurrence and development of cardiovascular diseases (Dong et al. 2019). However, evidence about the two responses and their interplay is not fully defined and understood. In the process of myocardial remodelling, due to hypertrophy of myocardial cells, and extensive intracellular metabolic remodelling, mitochondria can be damaged due to toxic effects such as imbalance of energy metabolism, oxidative stress and calcium overload. Mitochondrial autophagy, as a kind of selective autophagy reaction against mitochondria, can clear the damaged mitochondria in the cell and participate in maintaining energy homeostasis and cell vitality, but excessive activation of autophagy or autophagy flux defects accelerates cell death. The results of the CCK8 and flow cytometric analysis of our study also showed an increase in the level of autophagy in H/R AC16 cardiomyocytes along with AC16 cardiomyocyte apoptosis.

In our study, it was found that after PCSK9 overexpression, the mitochondrial membrane potential of cardiomyocytes decreased, suggesting that mitochondrial membrane permeability is abnormal.

The decrease in mitochondrial membrane potential ΔΨm by JC-1 detection not only indicates the early apoptosis of cardiomyocytes, but also reflects the changes in mitochondrial membrane permeability and the mitochondrial dysfunction in cardiomyocytes of the PCSK9 overexpression group. The Western blot analysis on supported the results that the overexpression of PCSK9 induced autophagy upward in AC16 cardiomyocytes.

Sequestosome 1 (p62/SQSTM 1) is a multidomain protein that interacts with the autophagy machinery as a key adaptor of target cargo. It interacts with phagophores through the LC3-interacting (LIR) domain and with the ubiquitinated protein aggregates through the ubiquitin-associated (UBA) domain (Aparicio et al. 2019).

In our study, it was found that P62 protein levels have significantly downregulated after H/R treatments. We also found that after pretreatment of AC16 cells with H/R+si-PCSK9, its protein expression levels are upregulated. During mitochondrial autophagy, in mitochondrial autophagy, Pink1 degradation on the outer mitochondrial membrane is inhibited and the cytoplasmic Parkin is recruited to the mitochondria. Parkin translocation to mitochondria promotes mitochondrial matrix protein ubiquitination and recruitment of P62 to mitochondria. P62 acts as a signal junction protein to further mediate mitochondrial autophagy and lysosomal binding and promote mitochondrial degradation (Huang et al. 2011). LC3 is a marker protein on the autophagosomal membrane, its high or low protein expression is also parallel to the protein expression of PCSK9 in the experimental subgroups of different treatments.

The Pink1/Parkin pathway is a well-studied mitochondrial autophagy pathway, and its expression reflects the level of mitochondrial autophagy to a certain extent. In the normal physiological state of mitochondria, Pink1 is constantly transferred to the inner mitochondrial membrane and cleaved by the mitochondrial membrane protease. Therefore, under normal conditions, Pink1 remains at low levels in cells. Parkin is an E3 ubiquitin ligase with 465 amino acids and has ligase activity (Quinn et al. 2020; Paul and Pickrell 2021) .When the body is stimulated by stressors, Pink1 is blocked from entering mitochondria, accumulates in the outer mitochondrial membrane, and recruits Parkin to the mitochondria. Therefore, when mitochondrial homeostasis is disrupted, Pink1 is heavily concentrated in the cell. This is consistent with the increased expression of Pink1 protein in the H/R group as detected by Western blot. When mitochondrial autophagy is initiated, Pink1 protein accumulates Parkin protein on the outer mitochondrial module (Shao et al. 2021), resulting in increased Parkin expression in the

cells. In conclusion, under the condition of H/R pretreatment, Pink1 and Parkin protein expression in PCSK9 and cells were closely related to each other.

There is intense interest in the role of PCSK9 in hypercholesterolemia and atherosclerotic cardiovascular disease. Here, we used H/R pretreatment of AC16 cells to induce PCSK9 overexpression and specific siRNAs to inhibit PCSK9 expression. As we expected, its expression of PCSK9 is associated with the development of autophagy, and the degree of autophagy parallels PCSK9 expression, suggesting a link between PCSK9 expression/release and the development of autophagy. A strong evidence for the link between PCSK9 and autophagy came from studies in AC16 cells treated with its inhibition by a specific siRNA significantly reduced the expression of both PCSK9 and autophagy.

In conclusion, our results suggest that overexpression of PCSK9 in the presence of H/R pretreatment can upregulate the level of autophagy, induce cardiomyocyte injury and reduce cardiomyocyte survival. This phenomenon was also accompanied by apoptosis of cardiomyocytes, and antagonizing PCSK9 could ameliorate cardiomyocyte injury and increase cardiomyocyte survival. The mechanism may be related to the Pink1/Parkin signaling pathway. These results provide new insights into PCSK9 as a potential target for myocardial ischemia-reperfusion and heart failure.

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**Authors' contributions.** WL and XL provided the hypothesis, and handled funding and supervision. GH and XL collected and analyzed the data. XL drafted and revised the manuscript. All authors approved the final version to be published.

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

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