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Abstract	This study investigates the effect and mechanism of proprotein convertase subtilisin/Kexin type 9 (PCSK9) on myocardial ischemia–reperfusion injury (MIRI) and provides a reference for clinical prevention and treatment of acute myocardial infarction (AMI). We established a rat model of myocardial ischemia/reperfusion (I/R) and AC16 hypoxia/reoxygenation (H/R) model. A total of 48 adult 7-week-old male Sprague–Dawley rats were randomly assigned to three groups ($n = 16$): control, I/R, and I/R + SiRNA. In I/R and I/R + siRNA groups, myocardial ischemia was induced via occlusion of the left anterior descending branch (LAD) of the coronary artery in rats in I/R group for 30 min and reperfused for 3 days. To assess the myocardial injury, the rats were subjected to an electrocardiogram (ECG), cardiac function tests, cardiac enzymes analysis, and 2,3,5-triphenyl tetrazolium chloride (TTC)/Evan Blue (EB) staining. Meanwhile, differences in the expression of autophagy-level proteins and Bcl-2/adenovirus E1B 19-kDa interacting protein (Bnip3) signaling-related proteins were determined by protein blotting. In vitro and in vivo experimental studies revealed that siRNA knockdown of PCSK9 reduced the expression of autophagic protein Beclin-1, light chain 3 (LC3) compared to normal control-treated cells and control-operated groups. Simultaneously, the expression of Bnip3 pathway protein was downregulated. Furthermore, the PCSK9-mediated small interfering RNA (siRNA) group injected into the left ventricular wall significantly improved cardiac function and myocardial infarct size. In ischemic/hypoxic circumstances, PCSK9 expression was dramatically increased. PCSK9 knockdown alleviated MIRI via Bnip3-mediated autophagic pathway, inhibited inflammatory response, reduced myocardial infarct size, and protected cardiac function.

Keywords (separated by '-')	Autophagy - Bnip3 - Knockdown - Myocardial ischemia–reperfusion injury - PCSK9 - SiRNA
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PCSK9 Knockdown Can Improve Myocardial Ischemia/Reperfusion Injury by Inhibiting Autophagy

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Abstract

This study investigates the effect and mechanism of proprotein convertase subtilisin/Kexin type 9 (PCSK9) on myocardial ischemia–reperfusion injury (MIRI) and provides a reference for clinical prevention and treatment of acute myocardial infarction (AMI). We established a rat model of myocardial ischemia/reperfusion (I/R) and AC16 hypoxia/reoxygenation (H/R) model. A total of 48 adult 7-week-old male Sprague–Dawley rats were randomly assigned to three groups ($n = 16$): control, I/R, and I/R + SiRNA. In I/R and I/R + siRNA groups, myocardial ischemia was induced via occlusion of the left anterior descending branch (LAD) of the coronary artery in rats in I/R group for 30 min and reperfused for 3 days. To assess the myocardial injury, the rats were subjected to an electrocardiogram (ECG), cardiac function tests, cardiac enzymes analysis, and 2,3,5-triphenyl tetrazolium chloride (TTC)/Evan Blue (EB) staining. Meanwhile, differences in the expression of autophagy-level proteins and Bcl-2/adenovirus E1B 19 kDa interacting protein (Bnip3) signaling-related proteins were determined by protein blotting. In vitro and in vivo experimental studies revealed that siRNA knockdown of PCSK9 reduced the expression of autophagic protein Beclin-1, light chain 3 (LC3) compared to normal control-treated cells and control-operated groups. Simultaneously, the expression of Bnip3 pathway protein was downregulated. Furthermore, the PCSK9-mediated small interfering RNA (siRNA) group injected into the left ventricular wall significantly improved cardiac function and myocardial infarct size. In ischemic/hypoxic circumstances, PCSK9 expression was dramatically increased. PCSK9 knockdown alleviated MIRI via Bnip3-mediated autophagic pathway, inhibited inflammatory response, reduced myocardial infarct size, and protected cardiac function.

Keywords Autophagy · Bnip3 · Knockdown · Myocardial ischemia–reperfusion injury · PCSK9 · SiRNA

Introduction

Annually, more than 17 million people die due to cardiovascular disease, making it the leading cause of death worldwide [1]. Myocardial infarction (MI) significantly impacts cardiovascular disease-related mortality. Timely and effective myocardial reperfusion is the key to rescuing ischemic cardiomyocytes and limiting infarct size. However, the abrupt restoration of blood flow often aggravates the structural and functional damage of ischemic myocardium, leading to cardiomyocyte apoptosis and necrosis, potentially leading to reduced mitochondrial membrane heart failure, arrhythmias, and eventually cardiac systolic dysfunction [2, 3]. Although MIRI mechanism has not been fully elucidated, studies have demonstrated that it is closely associated with Ca^{2+} overload, reactive oxygen species (ROS) accumulation, reduced adenosine triphosphate (ATP) production, and reduced mitochondrial membrane potential [4, 5]. Under normal conditions, autophagy in the myocardium occurs at a basal level and participates in cellular homeostasis by removing excess or long-lived proteins as well as aging organelles [6]. Autophagy is activated by regulating Sirt3

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concentration in muscle cells. Cardiomyocytes have almost no degenerative changes, autophagosomes exist at different maturation stages, reduced infarct size, and enhanced cardiac function [6]. However, the mechanism underlying the link between autophagy and myocardial ischemia–reperfusion injury is unknown.

PCSK9 is an amino acid serine protease encoded by PCSK9 gene on human chromosome 1 p32.3, mainly expressed in hepatocytes [7]. PCSK9 competes with low-density lipoprotein cholesterol (LDL) to bind to the low-density lipoprotein receptor (LDLR) on the surface of hepatocytes and guides LDLR internalization to lysosomal degradation, reducing LDLR number on the cell membrane, thereby upregulating cholesterol in the body s level [8]. As research deepens, increasing evidence implies that PCSK9 expression may also be associated with inflammation, independent of low-density lipoprotein cholesterol regulation.

A class of PCSK9 inhibitors is used in clinical trials to lower cholesterol levels by inhibiting the hepatic LDL receptors, raising serum LDL-c levels in the process [9]. Salvage kinase, a key enzyme in insulin signaling, may negatively affect susceptibility to myocardial reperfusion injury. Activating salvage kinase or lower pH in the first phase of reperfusion after ischemia maintenance can reduce myocardial infarct volume caused by a high-fat diet [10, 11]. In contrast, increased myocardial infarct volume in hypercholesterolemia is associated with increased ROS formation during reperfusion [12]. PCSK9 is thought to protect the myocardium by preventing autophagy from occurring, which is why it is upregulated in MIRI hearts [13]. Consequently, how PCSK9 affects ischemia–reperfusion injury, thereby improving the mechanism of myocardial infarction size, has not been fully understood.

In past studies, atherosclerotic cardiovascular disease and myocardial infarction were reduced when LDL-C levels were lower. The benefits of PCSK9 inhibitors in lowering LDL-C and cardiovascular risk are undeniable. Therefore, in August 2019, the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) issued a joint recommendation to develop stricter LDL-C level targets for patients with recent MI [14]. During ischemia–reperfusion injury, PCSK9 inhibitors can reduce the incidence of myocardial infarction and arrhythmias [15]. Notably, evolocumab may effectively reduce myocardial infarct size and severity. Evolocumab is a human monoclonal immunoglobulin G2 (IgG2). The mechanism of action of evolocumab is to increase LDLR number that can clear LDL from the blood by inhibiting PCSK9 binding to LDLR, thereby significantly reducing LDL-C levels and further reducing the risk of myocardial infarction and stroke. Evolocumab has become the only PCSK9 inhibitor approved in China for treating homozygous familial hypercholesterolemia in adults or adolescents over 12 years of age. It is well known that myocardial infarct size is linked to reperfusion opening time, and mortality may be further lowered if myocardial infarct size can be significantly reduced [16, 17].

Using RNA interference (RNAi) pathways to silence disease-causing genes holds great promise for developing therapeutics for targets that current drugs cannot address [18]. siRNAs are widely used to silence target genes. This process involves introducing the double-stranded RNA corresponding to the target gene into the organism, resulting in the corresponding mRNA degradation, thereby silencing the target gene. Here, we revealed that siRNAs, when delivered systemically in a liposomal formulation, can silence the disease target PCSK9 in rodent primates with MIRI to prove whether PCSK9 could be expressed in the myocardial cells of rats.

This study seeks to answer the following questions: (1) how does PCSK9 relate to MIRI and autophagy; (2) how does PCSK9 affect the size and cardiac function of myocardial infarction; and (3) can PCSK9 inhibitors inhibit the inflammatory response, thereby alleviating MIRI, thereby decreasing the size of a MI; and thereby decreasing mortality from myocardial ischemia–reperfusion injury?

Materials and Methods

Animals

In total, 48 adult male Sprague–Dawley rats, weighing 250–300 g, were purchased from Suzhou Xishan Biotechnology Co., LTD. [License No: scxk (Xiang) 2019-0014]. All rats were housed in a temperature-controlled environment with a 12:12 h light–dark cycle, with free access to food and water. After the experiments, the rats were sacrificed with an intravenous injection of 10% chloral hydrate at 8 mL/kg. The animal experiments were approved by the Animal Experimentation Committee of the Institutional Review Board of Guizhou Medical University and complied with the guidelines of Guizhou Medical University for the care and use of animals.

Myocardial Ischemia–Reperfusion Protocol

The rats were anesthetized with chloral hydrate (3 mL/kg), and heparin was used to avoid blood clotting during the surgery. When the rats were mechanically ventilated after endotracheal intubation, the tidal volume was 1.0 mL per min, and the breathing rate was 100 breaths/min. A left-sided chest opening was performed at the fourth intercostal space, and the pericardium was opened. An 8-0 filament gently crossed the LAD 2/3 of the way around LAD, located between the starting points near the pulmonary cones. LAD occlusion caused epicardial cyanosis

with local hypokinesis and typical ECG changes of acute myocardial infarction (marked ST-segment elevation with T-wave changes). LAD was ligated for 30 min, following which the ligation wires were released, and reperfusion was performed for 3 days [19]. A total of 48 adult male Sprague–Dawley rats were randomly divided into three experimental groups using the methodology of random number table, as follows: (I) the control group in which the rats were subjected to the same manipulation but without LAD ligation ($n = 16$); (II) I/R group ($n = 10$); and (III) I/R + siRNA groups, where the left ventricular wall was injected with siRNA (1 μg/10 g) at multiple points, using an insulin needle, end of ischemia at 30 min, within 3 min after the start of reperfusion. For 3 days, the rats were cared after the chest was closed, and they began to recover. The sequence of rat-derived PCSK9 siRNA was as follows: sense 5′-GGAGGUGUAUCUCUUAGAUTT-3′ and antisense 5′-AUCUAAGAGAUACACCUCCTT-3′. The sequence of rat-derived scrambled siRNA was as follows: sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

Assessment of the Size of Myocardial Infarct

Infarct size was estimated using Evans blue (Beijing Solarbio Science & Technology Co., Ltd., China)/2,3,5-triphenyltetrazolium staining (Beijing Solarbio Science & Technology Co., Ltd., China). Following reperfusion, the rats were re-anesthetized and LAD re-ligated; they were then injected with 2 mL 2% Evans blue via the tail vein. After the skin of lips and distal limbs was stained blue, the hearts were removed, rinsed with 4 °C phosphate-buffered saline (PBS), and frozen at − 80 °C for 30 min before being cut into 5–7 slices. The sections were immersed in 1% TTC buffer (pH 7.5) for 30 min at 37 °C. The area at risk (AAR) was defined as the area not stained by Evans blue, and the infarct area (IA) was defined as the area not stained by TTC. Images of the stained slices were captured with a digital camera, quantified using Image-Pro Plus, and presented as a percentage [20].

Echocardiographic Assessment of LV Function

A VINNO6 high-resolution ultrasound system was employed to perform an echocardiographic analysis on day 3 after IRI to assess cardiac function in anesthetized rats. Ejection fraction (EF) and shortening fraction (FS) measurements were performed to assess the left ventricular (LV) systolic function of the heart. The average of three consecutive cardiac cycles was used for each measurement.

Cell Culture, Simulated Ischemia and Transfection of siRNA

AC16 cells (American Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and streptomycin. The cells were maintained in a humidified 95% air/5% CO_2 incubator at 37 °C. Briefly, cardiomyocytes were exposed to a glucose-free, serum-free medium and transferred to a hypoxic modular incubator for 10 h at 37 °C with 5% CO_2 and 95% N_2. After hypoxia, the medium was replaced with a fresh oxygenated normal or high glucose medium, and the dishes were transferred to a normoxic incubator (95% air/5% CO_2) for 8-h reoxygenation. All cells were starved for 12 h with serum-free media before being subjected to normoxia or hypoxia.

siRNA duplexes corresponding to human-derived PCSK9 were purchased from RiboBio Biotechnology (Guangzhou, CHN). The sequence of overexpressed human-derived PCSK9 siRNA1(Ps1) was CCCATGTCGACTACATCGA, and for silenced siRNA2(Ps2), it was GGTCACCGACTT CGAGAAT. Cardiomyocytes were transfected with 50 nM of each siRNA for 48 h using siRNA transfection reagent to overexpress and inhibit PCSK9 gene expression. The medium was replaced with a normal medium, and the cells were subsequently exposed to hypoxia for specified times. As a control, the cells were transfected with sequence-disordered siRNA control. To confirm the efficiency of protein knockdown using siRNA, cell lysates were used for Q-PCR or Western blot analysis.

Western Blot Analysis

Human-derived cardiomyocytes and infarcted tissue in the left ventricular of the rat were extracted with RIPA lysis buffer (Beyotime, China) and PMSF (Roche, USA) to extract total protein. Protein content was measured using a BCA protein assay, and protein samples were separated by electrophoresis on SDS–PAGE and transferred to a polyvinylidene difluoride membrane.

After 2-h blocking with 5% skim milk, the membranes were incubated overnight at 4 °C with the primary antibody at a dilution of 1:1000. After washing with PBS containing 0.2% Tween, the membrane was incubated with a secondary antibody for 1 h at room temperature. Then, signals were detected with Pierce ECL Western Blotting Substrate. Intensity quantitation of the bands was captured using Image J software and normalized to β-actin.

Antibodies directed at PCSK9 (ab31762, ab84041), BNip3 (ab109362), and Beclin-1 (ab210498) were purchased from Abcam (San Francisco, CA), LC3 (CST#4599) was purchased from Cell Signaling (Danvers, MA).

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Real-Time Q-PCR

The peri-infarct area and border zone area of the left ventricle of the rats was removed and homogenized. Total RNA was extracted with TRizol reagent (ThermoFisher). cDNA was synthesized with Hiscript III-RT Supermix for the qPCR kit (Vazyme) following the manufacturer's instructions. Relative β-actin levels were quantified for each sample based on Ct (amplification cycle threshold), normalized to a value of 1 as an endogenous mRNA standard, and the relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method. Real-time quantitative PCR using gene-specific primers was employed, as demonstrated in Table 1.

Histopathological Change

Hematoxylin–eosin (H&E) staining and Masson trichrome staining were used to examine myocardial tissue's pathological and morphological changes. The hearts obtained from each group were left overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin blocks. Subsequently, all myocardial tissues were cut into 5% thick slices, mounted on glass slides, dried and stained. A light microscope was used to examine paraffin-embedded myocardial tissue slices stained with hematoxylin for 5 min, eosin for 2 min, or Masson trichrome staining kit (Beijing Sola Biotechnology Co., Ltd., China). Finally, the stained slides were immersed in xylene, gradient concentrations of ethanol, according to the instructions, and then sealed with resin.

Immunohistochemistry (IHC)

The left ventricular infarct tissue in the rats was prepared into 5-μm thick paraffin sections, dewaxed, and antigenically repaired in 1 mM EDTA (pH 9.0) for 15 min. The slides were then incubated with 10% goat serum for 1 h at room temperature, with primary antibody overnight at 4 °C and secondary antibody for 30 min. Hematoxylin was used to re-stain the nuclei for 2 min; the cells were differentiated with a differentiation medium for 5 s and then returned to blue with ammonia. Finally, using alcohol, the eluate was dehydrated and clear, and the neutral resin was used to seal the slides for fixation, microscopic observation (Lycra), and analysis using Image Pro6.0.

Measurement of cTnT, CK-MB

Myocardial injury was evaluated by measuring the plasma concentrations of cardiac troponin T (cTnT) creatine kinase-MB (CK-MB). At the end of the reperfusion period, blood was collected and centrifuged at 1500 rpm for 10 min to obtain plasma. CKMB and cTnT levels and activity were measured using specific ELISA kits (Quanzhou Ruixin Biological Technology Co., LTD, Quanzhou,China) according to manufacturer'sprotocols.

Statistical Analysis

All experiments were conducted three times at least ($n = 3$). All data were analyzed using GraphPad Prism 7.0 software (CA, USA). Comparisons between groups were performed via the *t*-test or one-way ANOVA test for continuous numerical variables. Values were expressed as mean ± standard deviation, and $p < 0.05$ was considered statistically significant.

Results

Upregulation of PCSK9 and Autophagy Levels Under Hypoxia

AC16 cells were subjected to 10-h hypoxia and 8-h reoxygenation, while total cellular proteins, lysate, and protein

Table 1 List of primers used in this study

Gene	Species	Forward	Reverse
PCSK9	Rat	TGGAACCTGGAGCGGATTAC	TTCCCGGTGGTCACTCTGTA
BNIP3	Rat	GGGCTCCTGGGTAGAACT	AGACGGAAGCTGGAACG
Beclin-1	Rat	GCGTCAGCTCTCGTCAA	GCCCGGTCTTCAGCTAC
LC3	Rat	GGAGTCCTGTGTCTACGG	AAAAGCTGGGGTGTTCCT
β-Actin	Rat	CACCCGCGAGTACAACCTC	CCCATACCCACCATCACACC
PCSK9	Human	GCTGTGCCTTGGTTTCCT	TGTGAAGTAGGGGTGCGA
BNIP3	Human	CCAGCCTCGGTTTCTATTT	TATCTTGGTGTCTGCGA
Beclin-1	Human	GGATGGTGTCTCTCGCA	CAGTCTTCGGCTGAGGTT
LC3	Human	GGCACCAACCCACCTACTC	ATCCCACCAGCCAGCAC
β-Actin	Human	CTCGCTTCGGCAGCACA	AACGCTTCACGAAATTGCGT
IL-1β	Rat	CCCTTGACTTGGGCTGT	CGAGATGCTGCTGTGAGA
NLRP3	Rat	TGTTGTCAGGATCTCGCA	AGTGAAGTAAGGCCGGAAT

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buffer were extracted and prepared into upper samples, as revealed in Fig. 1. PCSK9, Beclin-1, and LC3II/I expressions were upregulated in H/R group compared with the normal control (NC) group ($p<0.001$, $n=4$), thus suggesting a potential relationship between PCSK9 and autophagy. Bnip3 is known to be induced by hypoxia, and autophagy is protective against Bnip3-induced autophagy and cell death [21]. Additionally, PCSK9 may affect hypoxia-reoxygenated cardiomyocytes by mediating autophagy through Bnip3 pathway.

PCSK9 Synchronization Regulate BNIP3 and Autophagy Levels in AC16 Cells

A higher or lower expression level of PCSK9 was observed in HR group than in the control group (Fig. 2A, B, $p<0.05$). Furthermore, we used PCSK9 overexpression to enhance Bnip3 expression. On the contrary, Bnip3 expression was significantly reduced after using PCSK9 knockdown (Fig. 2A, B, $p<0.05$). Furthermore, the expression level of Bnip3 increased significantly along with PCSK9 upregulation during HR. Additionally, the expression level of Bnip3 was lower along with PCSK9 downregulation. In contrast, PCSK9 knockdown in cardiomyocytes drastically reduced the expression of LC3-II, Beclin-1, and autophagic flux, while it increased P62 expression, as well as the expression of Bnip3 pathway proteins, which was confirmed at RNA levels (Fig. 2D).

In vitro experiments verified the correlation between PCSK9 and the autophagic pathway, which could affect autophagy via Bnip3 pathway, thereby influencing cell death induced by hypoxic reoxygenation. We then performed in vivo experiments in SD rats to further validate this.

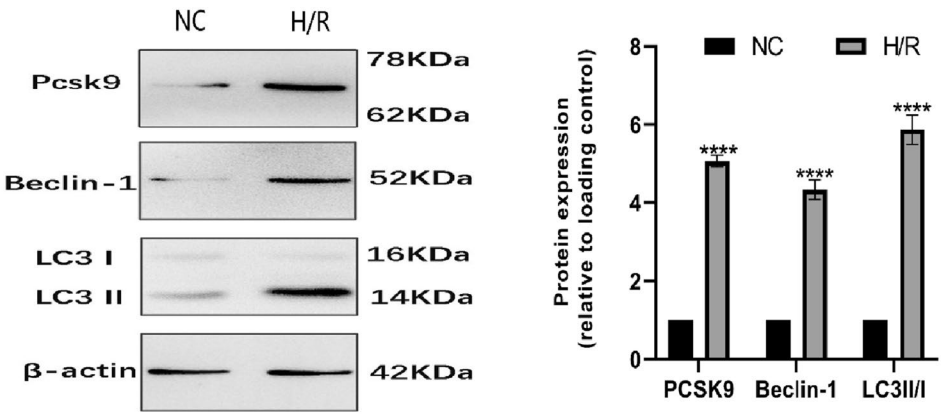

Fig. 1 Upregulation of PCSK9 and autophagy levels under hypoxia. Western Blot analysis of cellular proteins shows significant elevation of PCSK9, Beclin-1, and LC3 with 10 h of hypoxia ($n=4$ in each group). Data are expressed as the mean ± SEM, ****$p<0.0001$ vs. NC

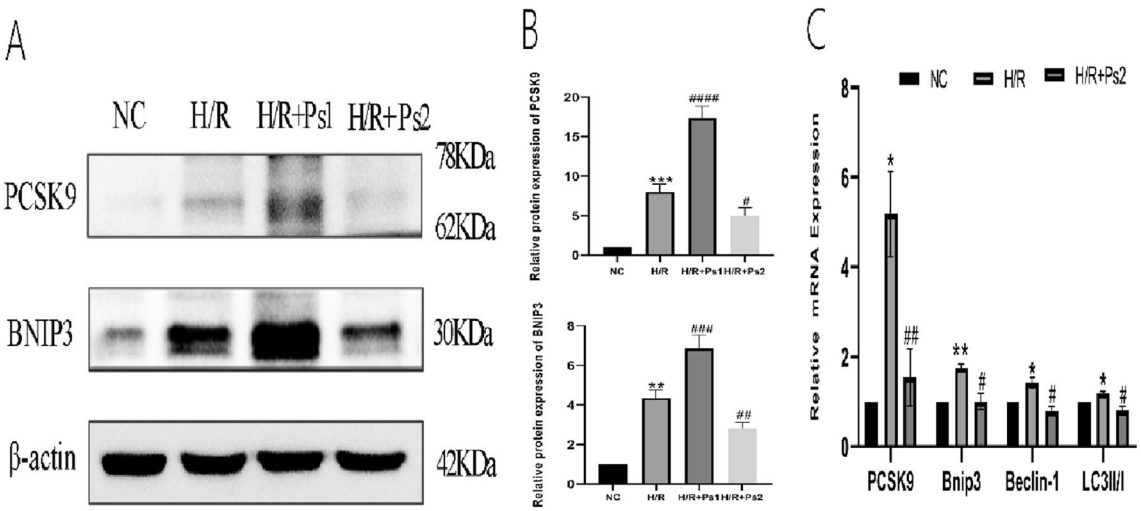

Fig. 2 PCSK9 synchronization regulate BNIP3 and autophagy levels in AC16 cells. **A**, **B** The level of PCSK9 protein, BNIP3 protein in the normal control, H/R, H/R + Ps1 and H/R + Ps2 groups. **C** RNA expression and analysis of pcsk9, Bnip3, Beclin-1, lc3 and P62. Data are expressed as the mean ± SEM, $N=4$. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. NC, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ and $^{\#\#\#\#}P<0.0001$ vs. H/R

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PCSK9 is Knocked Down to Restrict MI Region

As depicted in the schematic diagram in Fig. 3A, MIRI was induced in rats and confirmed via electrocardiography (Fig. 3B). Myocardial infarct size, myocardial damage marker expression, cardiac function, and histomorphological heart alterations were measured. TTC/EB staining revealed that PCSK9 knockdown significantly reduced the myocardial infarct size compared with I/R group ($p < 0.001$; Fig. 3C, D). The findings of cardiac enzyme tests revealed that knocking down PCSK9 lowered the severity of myocardial infarction ($p < 0.0001$; Fig. 3E, F).

PCSK9 Knockdown Ameliorated MIRI in Rats

Echocardiography was used to investigate whether PCSK9 knockdown influenced cardiac function. After 3 days of ischemia, the reperfused simultaneous left ventricular wall multipoint injection of PCSK9 siRNA resulted in significantly less unfavorable remodeling as well as better ejection fraction (EF) and shortening fraction (FS) compared to I/R group ($p < 0.001$; Fig. 4A–C). Compared with the control group, the values of left ventricular end-diastolic diameter (LVDd) and left ventricular end-systolic diameter (LVDs) in I/R group were significantly increased, and LVDd and LVDs were significantly decreased after PCSK9 knockdown, compared with I/R group ($p < 0.05$; Fig. 4D, E). Meanwhile, cardiac enzymes such as CK-MB and CTNT showed that knockdown of PCSK9 resulted in a reduced degree of myocardial infarction compared to I/R group ($p < 0.0001$; Fig. 3E, F).

PCSK9 Knockdown Reduces Autophagy Expression and Improves Myocardial Fibrosis

We performed HE, Masson staining, and immunohistochemistry tests to further evaluate the effect of PCSK9 knockdown on myocardial histological structure and fibrosis. Additionally, HE staining revealed that the cardiac tissue exhibited a clear and well-organized structure with little inflammatory infiltration or cardiac necrosis in control. However, myocardial structural abnormalities and histological changes, including perinuclear vacuolization, necrosis, cardiac intercellular spaces, myofibrillar thinning and wavy pattern consistent with infiltration and transmigration of inflammatory cells, were detected in IR group (the arrow in Fig. 5A). In contrast, PCSK9 knockdown reduced myocardial inflammatory infiltration with a more transparent structure and less tissue necrosis (Fig. 5A). IR group's Masson stain revealed a significantly increased fibrosis ratio, which was also alleviated by PCSK9 knockdown (Fig. 5B, F). In IHC staining (Fig. 5C–E, G–I), PCSK9, Bnip3, and Beclin-1 meaningfully rose in

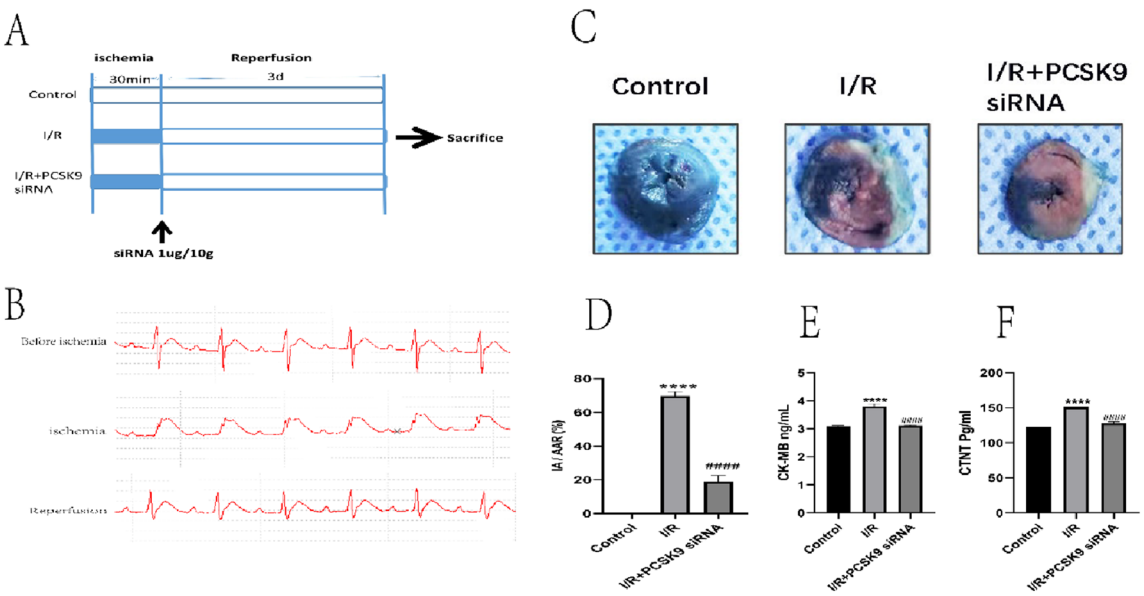

Fig. 3 PCSK9 is knocked down to restrict the MI region. **A** Schematic diagram of the myocardial ischemia–reperfusion process. **B** Representative electrocardiograms before ischemia, during myocardial ischemia, and after reperfusion. **C**, **D** The infarct area was determined by TTC/EB staining. The ischemic area showed pale, and the viable myocardium showed red. The infarct area was quantified as a percentage of the total slice area. The infarct area and area at risk were quantified via Evans blue and TTC double staining. Graphic representation of the infarct size expressed as a percentage of infarct area over the area at risk ($n = 3$). Blue, non-blue and white areas represent non-ischemic, AAR and IA areas. **E** Serum CK-MB levels. **F** Serum CTNT levels

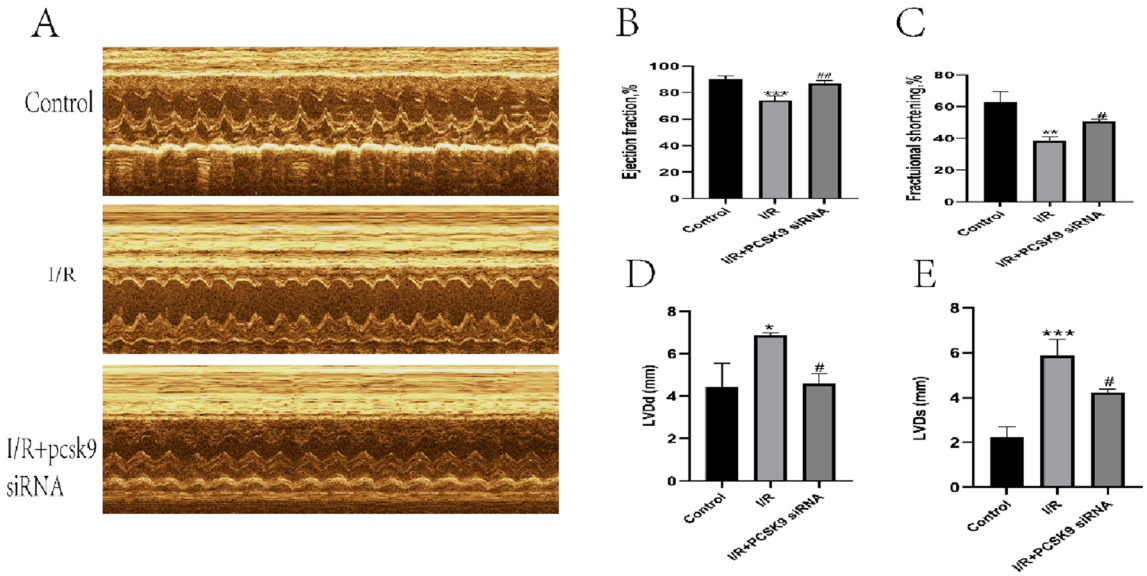

Fig. 4 Knockdown of PCSK9 ameliorated MIRI. **A** Representative echocardiograms between the groups. **B** The percentage of ejection fraction. **C** The percentage of fractional shorting. **D** Left ventricle end-diastolic diameter (LVDd). **E** Left ventricle end-systolic diameter (LVDs). Data are expressed as mean ± standard deviation. $N=4$. $^{**}P<0.01$ and $^{***}P<0.001$ vs. Control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. I/R

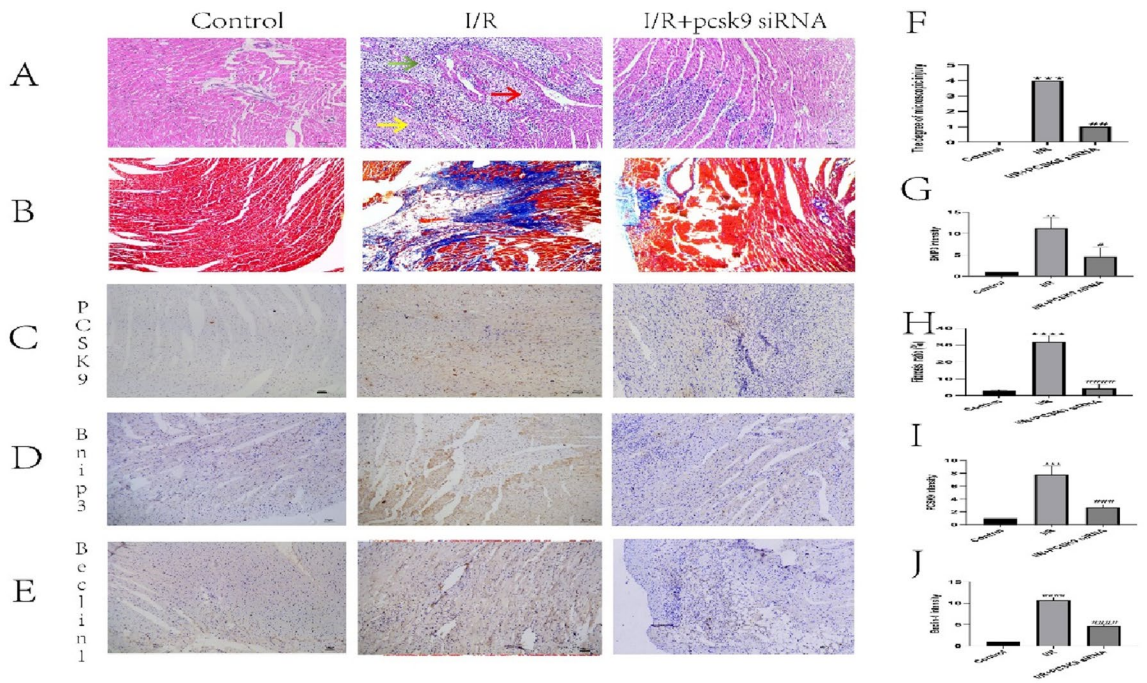

Fig. 5 Knockdown of PCSK9 reduces autophagy expression and improves myocardial fibrosis. Three days after the operation, the heart was isolated, and the thin paraffin section (4 μm) were made. **A** Results of HE staining (×100). **B**, **G** Fibrosis ratio was compared among the three groups (×100). **C**–**E**, **H**–**J** PCSK9, Bnip3, and Beclin-1 were stained by immunohistochemistry, compared the three groups and quantitative analysis (×100). **F** The degree of microscopic injury of the heart evaluated and graded on a scale of 0–4 with 0 = no injury; 1 = injury to 25% of the field; 2 = injury to 50% of the field; 3 = injury to 75% of the field; and 4 = severe injury. Data are expressed as the mean ± standard deviation ($n=5$). $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. Control, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ and $^{\#\#\#\#}P<0.0001$ vs. I/R. *HE* hematoxylin–eosin

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IR group ($p < 0.05$), although both autophagy levels and Bnip3 pathway protein expression were downregulated via the knocking down of PCSK9 ($p < 0.05$).

PCSK9 Knockdown Inhibits Autophagy via Bnip3 Signaling Pathway

To further study the mechanism of PCSK9 in vivo, rat models were constructed in this work. The knockdown efficiency was validated via Western Blot and Q-PCR findings. After transfection with PCSK-siRNA, the protein and mRNA expression of Bnip3, Beclin-1 and LC3 were significantly downregulated ($p < 0.05$; $N = 4$; Fig. 6A–C). We discovered that inhibiting the Bnip3 pathway significantly decreased Beclin-1 expression ($p < 0.05$; $N = 4$; Fig. 6A–C). According to the findings, PCSK9 may suppress autophagy levels by activating the Bnip3 pathway, thereby reducing the damage caused by I/R to cardiomyocytes.

PCSK9 Knockdown Attenuates Myocardial Inflammatory Response

The inflammatory response during myocardial ischemia–reperfusion injury is critical for cardiac healing, whereas excessive inflammation prolongs infarction and promotes poor cardiac remodeling. Understanding the mechanisms underlying these uncontrolled inflammatory processes has significant implications during MIRI treatment [22]. It has been revealed that interleukin-1β (IL-1β) and Nod-1-like receptor protein 3 (NLRP3) are significantly elevated in ischemia, and we have also performed experiments with inflammatory mediators; the results are displayed in Fig. 7. Compared with the control, IL-1 and NLRP3 expressions were significantly upregulated in IR group ($p < 0.05$, $n = 5$; Fig. 7A); however, compared with IR + PCSK9 siRNA group, expression was significantly downregulated ($p < 0.05$; $n = 5$; Fig. 7). indirectly suggesting that PCSK9 knockdown could improve MIRI by suppressing autophagy levels and attenuating the inflammatory response.

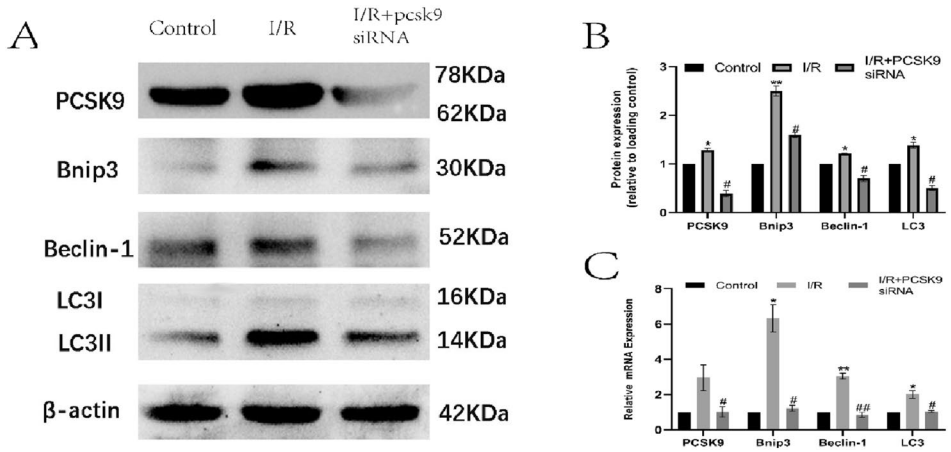

Fig. 6 Knockdown of PCSK9 inhibiting autophagy via Bnip3 signaling pathway. **A**, **B** In each group, expression and analysis of PCSK9, Bnip3, Beclin-1 and LC3 proteins were expressed. **C** Expression analysis of mRNA levels of PCSK9, Bnip3, Beclin-1, and LC3. Data are expressed as the mean ± standard deviation. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. I/R

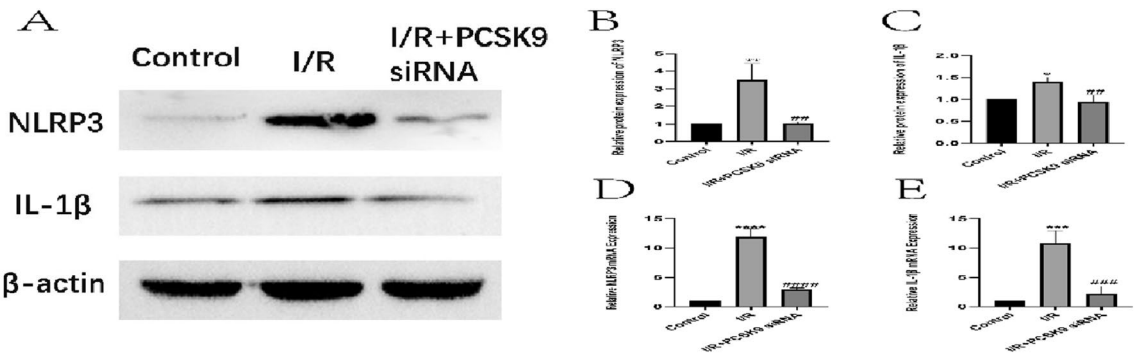

Fig. 7 Knockdown of PCSK9 attenuates myocardial inflammatory response. **A**–**C** Protein expression and analysis of NLRP3 and IL-1β between the three groups. **D**–**E** Expression and analysis of mRNA of NLRP3 and IL-1β between the three groups. Data are expressed as the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{***}P < 0.0001$ vs. Control, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ vs. I/R

Discussion

In vitro and in vivo experimental studies revealed that siRNA knockdown of PCSK9 resulted in reduced expression of the autophagic protein Beclin-1, light chain 3 (LC3) compared to normal control-treated cells and control-operated groups. Simultaneously, Bnip3 pathway protein expression was downregulated. Furthermore, PCSK9-mediated small interfering RNA (siRNA) group injected into the left ventricular wall significantly improved cardiac function and myocardial infarct size.

Cardiovascular disease accounts for one-third of all death, and ischemic heart disease is the primary cause of death. To the best of our understanding, I/R damage frequently occurs in clinical circumstances with complex mechanisms [23]. PPCSK9 inhibitor therapy may be useful in reducing biochemical and physiological problems associated with cardiac MIRI [15]. Recently, studies have focused on PCSK9 effects on cholesterol and low-density lipoprotein. This work demonstrated that PCSK9 knockdown could ameliorate MIRI by inhibiting Bnip3 pathway-mediated autophagy. When one suffers external damage, autophagy is a self-repair mechanism; nevertheless, excessive autophagy can lead to increased cell death, reversing the process. Mitophagy is a type of selective autophagy involved in ischemia–reperfusion injury [24]. PCSK9 was considerably upregulated in ex vivo tests after ischemia/hypoxia, whereas autophagy levels were recruited to further damage mitochondrial structure and metabolic function. Consistently, the results of TTC/EB staining, H&E staining, Masson's trichrome staining, and cardiac enzymes measured in this study demonstrated that the myocardial infarction area was enlarged, while structural abnormalities and cardiac enzyme (cTnT, CK-MB) levels increased significantly in I/R group. However, PCSK9 knockdown regulated autophagy levels through Bnip3 pathway, and, in contrast with IR group, MIRI severity was reduced, as was myocardial infarct size, thus resulting in improved cardiac function and reduced mortality. These results suggest that cardiac structures are protected, and PCSK9 knockdown increases myocardial viability.

Extensive evidence indicates that autophagy is critical in cardiomyocyte apoptosis during MIRI [25, 26]. Yu et al. discovered that a lack of Mammalian STE20-like kinase1 provides a pro-survival signal to the reperfused heart by reversing FUN14 domain containing one related mitophagy, thereby decreasing cardiomyocyte mitochondrial apoptosis [27]. Furthermore, I/R and oxygen–glucose deprivation/recovery damage increased hypoxia-inducible factor-1 (HIF-1) expression, activated downstream Bnip3, and induced mitochondria-dependent autophagy. HIF-1α upregulation and Bnip3 expression may contribute to I/R-injured SD rat cardiomyocytes and OGD/R injury-induced autophagy in H9C2 cells. We hypothesize that myocardial viability is enhanced through knockdown of PCSK9 regulation associated with autophagy and Bnip3 pathway. To verify this idea, we investigated the relationship between PCSK9 and autophagy and Bnip3 pathway. WB analysis revealed increased Bnip3, Beclin-1, and LC3II/I ratios, thus indicating that PCSK9 activated the Bnip3 signaling pathway and increased autophagy levels. PCSK9 siRNA was injected into the left ventricular wall at numerous stages during reperfusion to confirm the role of Bnip3 pathway in PCSK9 cardioprotection, while PCSK9 siRNA was transfected into cells before intrinsic hypoxia-reoxygenation. As expected, in the presence of Bnip3-siRNA, Bnip3 and Beclin-1 protein expressions were repressed, P62 was boosted, and the upregulated effect of PCSK9 on autophagy was significantly reduced. These findings show that the key protective mechanism of PCSK9 against MIRI is autophagy inhibition via the Bnip3 pathway.

Inflammasome activation plays a vital role in host defense. Simultaneously, autophagy is naturally linked to the adaptive immune system and strongly linked to inflammation. Zhang et al. discovered that knocking down lincRNA-Cox2 activated caspase-1, resulting in decreased IL-1 production and increased autophagy [28]. Furthermore, Wang et al. discovered that neuregulin-1 could reduce reactive oxygen species formation by inhibiting NADPH oxidase 4 and inhibiting NLRP3/caspase-1 pathway in MIRI to minimize oxidative damage and inflammation [29]. A recent study disclosed that normocholesterolemic subjects with lower plasma PCSK9 and higher white adipose tissue surface expression of LDLR and CD36 had higher NLRP3 inflammasome activation [30]. To test whether PCSK9 can control inflammatory factors and hence worsen MIRI, PCSK9 knockdown was followed by NLRP3 and IL-1 downregulation, as confirmed by WB and Q-PCR. The above data display that PCSK9 knockdown inhibits autophagy and attenuates the inflammatory response, thereby ameliorating MIRI.

PCSK9 is mainly secreted by the liver and can be released into the blood. Circulating PCSK9 levels are associated with LDL-c levels. LDL-c is readily oxidized by ROS, and LOX-1 can be activated by autophagy, thereby regulating infarct size [31]. Based on this information, we hypothesize that MIRI can be controlled indirectly by modulating autophagy and LDL-c levels. PCSK9 inhibitors are commonly used in lipid-lowering therapy, although their use in individuals with myocardial infarction is debatable. In the Atherosclerosis Risk in Communities trial, deletion of one copy of PCSK9 saved 88% of human cardiovascular events [32]. This study provided experimental evidence for reducing reperfusion injury in patients with acute myocardial infarction. A significant number of clinical trials remain

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required to validate the clinical use of PCSK9 inhibitors in patients with myocardial infarction.

Conclusion

In ischemic/hypoxic circumstances, PCSK9 expression was dramatically increased. PCSK9 knockdown alleviated MIRI via Bnip3-mediated autophagic pathway and improved inflammatory response, myocardial infarct size, and cardiac function.

Author Contributions GWH, XYL, and WL designed and performed experiments, analyzed and interpreted data, and prepared the manuscript. KZ, ZGD, XLX, MZL, CL, YQL and LX participated in the design of the study and performed the statistical analysis. HYZ and ZHL conceived of the study, and participated in its design and coordination and review of this manuscript. All authors read and approved the final manuscript.

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Data Availability All data, models, or code generated or used during the study are available from the corresponding author by request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval and Consent to Participate The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Animal Experimentation Committee of the Institutional Review Board of Guizhou Medical University (License No. 2019(105)) and complied with the guidelines of Guizhou Medical University for the care and use of animals. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Consent for Publication Not applicable.

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