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Original Paper

Suppression of Long Non-Coding RNA LINC00652 Restores Sevoflurane-Induced Cardioprotection Against Myocardial Ischemia-Reperfusion Injury by Targeting GLP-1R Through the cAMP/PKA Pathway in Mice

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Key Words
Long-non-coding RNA LINC00652 • Sevoflurane • Glucagon-like peptide 1 receptor (GLP-1R) • CAMP/PKA pathway • Myocardial ischemia-reperfusion

Abstract
Background/Aims: Long non-coding RNA (lncRNA) and glucagon-like peptide 1 receptor (GLP-1R) are crucial for heart development and for adult heart structural maintenance and function. Herein, we performed a study to explore the effect of lncRNA LINC00652 (LINC00652) on myocardial ischemia-reperfusion (I/R) injury by targeting GLP-1R through the cyclic adenosine monophosphate-protein kinase A (cAMP/PKA) pathway. ***Methods:*** Bioinformatics software was used to screen the long-chain non-coding RNAs associated with myocardial ischemia-reperfusion and to predict target genes. The mRNA and protein levels of LINC00652, GLP-1R and CREB were detected by RT-qPCR and western blotting. In order to identify the interaction between LINC00652 and myocardial I/R injury, the cardiac function, the hemodynamic changes, the pathological changes of the myocardial tissues, the myocardial infarct size, and the apoptosis of myocardial cells of mice were measured. Meanwhile, the levels of serum IL-1β and TNF-α were detected. ***Results:*** LINC00652 was overexpressed in the myocardial cells of mice with myocardial I/R injury. GLP-1R is the target gene of LINC00652. We also determined higher levels of LINC00652 and GLP-1R in the I/R modeled mice. Additionally, si-LINC00652 decreased cardiac pathology, infarct size, apoptosis rates of myocardial cells, and levels of IL-1β and TNF-α, and increased GLP-1R expression cardiac function, normal hemodynamic index, and the expression and phosphorylation of GLP-1R and CREB proteins. ***Conclusion:*** Taken together, our key findings of the present highlight LINC00652 inhibits the

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activation of the cAMP/PKA pathway by targeting GLP-1R to reduce the protective effect of sevoflurane on myocardial I/R injury in mice.

Introduction

It is widely accepted that an ischemic response to stress testing identifies an increased risk of death or myocardial ischemia among patients with coronary artery disease, which is associated with worse prognosis and plays a role in predicting myocardial revascularization [1, 2]. Blood incapable of flow to the heart can cause an imbalance in oxygen demand and supply, which is called ischemia, and results in dysfunction or damage of the cardiac tissue; therefore, prompting blood flow restoration has been established as an effective treatment to prevent further tissue injury [3]. In addition, reperfusion involves restoring blood flow to the ischemic myocardium, which can also induce injury, causing additional cardiomyocyte death and increasing infarct size in a process known as myocardial ischemia reperfusion (I/R) injury, among which potential mediators of reperfusion injury include inflammation and oxidative stress [4-6]. Some studies have suggested that sevoflurane pretreatment has a protective effect on animal hearts after myocardial I/R injury [7, 8]. Another study suggests that sevoflurane could protect chronically infarcted mouse hearts against I/R injury [9]. Recently, studies have indicated that long non-coding RNAs (lncRNA) have an influence in myocardial I/R injury [10, 11].

LncRNA in the mammalian genome is characterized by a tissue-specific expression pattern, functionality and/or ectopic expression that play roles in various pathological processes, for example, oxidative stress responses, inflammation and ischemic injury [12]. A previous study indicated that dysregulated lncRNAs in the plasma have become potential novel biomarkers to evaluate liver I/R injury [13]. Glucagon-like peptide-1 (GLP-1) is a gut incretin hormone that has an anti-oxidative protective effect on various tissues [14]. The GLP-1 receptor (GLP-1R) is a key physiological regulator of insulin secretion and a major therapeutic target for the treatment of diseases such as diabetes and ischemic stroke [15, 16]. A previous study demonstrated that preconditioned activation of the GLP-1R with exendin-4 in the kidney could significantly protect against I/R injury in mice [17]. Additionally, a study has suggested that the cAMP/PKA signaling pathway plays an important role in dealing with the myocardial I/R injury [18]. Furthermore, renal cAMP and PKA activity were reduced in GLP-1R knockout C57B/6-Akita mice [14]. In this paper, the long non-coding RNAs were screened by GEO analysis, and the target genes of lncRNA were predicted by Multi Experiment Matrix software (MEM, http://biit.cs.ut.ee/mem/). The gene was analyzed by KEGG enrichment, and the genes related to myocardial infarction were analyzed by WebGestalt database. The lncRNA Targets Website was used to verify the binding sites between the lncRNA and target genes. We studied the effect of lncRNA LINC00652 on myocardial I/R injury in mice treated with sevoflurane by targeting GLP-1R through the cAMP/PKA pathway, which will be promising in finding more effective treatments for patients with myocardial I/R injury.

Materials and Methods

Ethics Statement

The experiment was approved by the Ethics Committee of North China University of Science and Technology Affiliated Hospital, and the protocol was conducted in strict accordance with the recommendations in the Institutional Animal Care and Use Committee.

Bioinformatics prediction

Initially, The GEO database (http://www.ncbi.nlm.nih.gov/geo) was accessed for downloading the myocardial I/R-related chip data related to the chip expression profile data (GSE6381), and probe files that were detected by Affymetrix Human Genome U133A Array chip were annotated. The Affy installation package from R software was used to perform background correction and normalization of each chip data [19]. Then, the non-specific filtering of the expression data was carried out using the linear model of the Limma installation package and the empirical Bayesian statistical method combined with a traditional *t*-test to screen out the differentially expressed lncRNA [20]. The differentially expressed lncRNA was measured by using the Multi Experiment Matrix website (http://biit.cs.ut.ee/mem/). As a network-based instrument

that used for co-expression inquiries about a large number of researches on gene expression, MEM offers hundreds of publicly available gene expression datasets of varying tissues, diseases and conditions, according to types and microarray platform types [21]. KEGG enrichment analysis of the target genes was performed using the WebGestalt database (http://www.webgestalt.org) to determine the most significant biochemical metabolic pathways and signaling pathways involved in the target genes [22]. The LncRNA Targets website (http://www.herbbol.org:8001/lrt/index.php) was employed to predict the targets of lncRNA on the basis of nucleic acid nearest-neighbor thermodynamics, indicating excellent ability in predicting RNA' targets in large scale with no size restrictions. In the process of calculating, lncRNA was applied as a primer in the algorithm to scan the target sequence from the first nucleotide, which connects two trains heading toward mutually. Every combinatorial structure was assigned a ΔG value using a Nearest-Neighbor method on the basis of nucleic acid thermodynamics [23]. To better evaluate the stability of every combinatorial structure, a new parameter was introduced, ΔG/n, as average free energy, where n represents the amount of base pairs of each combinatorial structure including matches and mismatches.

Experimental animals

A total of 108 male C57BL/6 mice aged 10 ~ 12 weeks old weighing 20 ~ 30 g were purchased from the Third Military Medical University Medical Laboratory Animal Center. The mice were kept for one week adaptively at a room temperature of 22 - 25°C with normal circadian rhythm and free access to food and water in the clean room. Then, 36 mice were selected to establish the myocardial I/R model. The remaining 72 mice were followed by successful modeling for the subsequent sevoflurane treatment.

Establishment of a mouse model with myocardial I/R

To verify the feasibility of constructing a myocardial ischemia-reperfusion model using left coronary artery ligation, we conducted preliminary experiments. Cardiac ultrasonography and hemodynamics monitoring were used to identify if the model was constructed successfully. Thirty-six mice were grouped into the normal, sham and myocardial I/R groups, and each group contained 12 mice. The mice were fasted for 8 hours, weighed and labeled before modeling. At a dose of 50 ml/kg, 1% pentobarbital sodium solution (Sigma-Aldrich Chemical Company, St Louis MO, USA) was injected into the mouse intraperitoneally with 1-ml sterile syringes. The mice were fixed in the supine position, and the skin of the neck and thorax of the mice were disinfected. Then, the skin of the anterior cervical area was cut by an ophthalmic scissor. The tissues and muscles were separated, and the trachea was exposed. Tracheal intubation and a respirator were connected from the mouth into the tracheal intubation to observe whether the chest breathing of mouse and the frequency of breathing machine were consistent or not. The mice were placed and fixed in the right lateral position. The left side of the chest skin was cut along the direction of the ribs, free from the ectopectoralis and entopectoralis. The fourth intercostal space was exposed, and the intercostal muscle was separated. A hook was used to fully expose the heart, the left coronary artery was ligated, the knot was placed in the lower left heart or pulmonary artery cones at 0.5 cm, and a PE-10 hose was placed between the knot and the heart. The left ventricular surface turned white, which was a symbol of successful ligation. The chest was closed temporarily by a metal clip for 45 min. After opening the knot, the incision was not sutured until the heart surface showed restored blood flow. The endotracheal intubation was pulled out after the complete recovery of the mice, and an intraoperative electric blanket was used continuously for insulating the mice to keep the temperature no less than 37°C. The same thoracotomy procedure was also used in the sham group, but the left coronary artery was not ligated.

Construction and transfection of overexpression vectors and interference vectors of LINC00652

According to the known transcript sequence of LINC00652 in GenBank, the full-length sequence, siRNA sequence and nonsense negative control sequence (si-NC) of lncRNA LINC00652 were designed online by the American Ambion website. The synthesis was performed by Shanghai Gemma Biology Co., Ltd, (Shanghai, China). The correct sequence was cloned into the plasmid vector pcDNA3.1 (VPI0001, Invitrogen Inc., Carlsbad, CA, USA) with Hind III and XHo I restriction sites and ligated at 16°C for 1 h. The ligation product was added to competent E. coli DH5α (D9052, Takara) cells and transformed, and the resistant colonies were screened and identified by colony PCR. The positive clones were selected and sequenced, and the correct recombinant plasmids were named LINC00652 vector and si-LINC00652. The plasmid was quickly and quantitatively extracted and stored at -20°C. The H9C2 cells (purchased from the US ATCC cell bank) were inoculated in a 6-well plate with 1×10^5 cells in each well, and the cell transfection was conducted when cell density reached 60% - 70%. A total of 10 μl liposomes was mixed in 250 μl OPTI-MEMI and incubated at room temperature for 5 min. Then, 100 pmol si-LINC00652 and si-NC were diluted in 250 μl OPTI-MEMI. The mixture of liposomes and si-LINC00652 and si-NC solutions was maintained at room temperature for 20 min, and then added to wells (containing 1.5 ml OPTI-MEMI) drop by drop, followed

by gentle shaking. The cells were cultured at 37°C with 5% CO_2 for 6 - 8 h. Later, the medium in each group was replaced by complete medium. After being cultured for 48 h, the transfection efficiency was detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Dual-luciferase reporter gene assay

The online prediction site RNA22 (https://cm.jefferson.edu/rna22/Interactive/RNA22Controller) was used to analyze the predicted target gene for LINC00652. HEK-293T (AT-1592, ATCC, Manassas, VA, USA) cells were cultured in 24-well plates for 24 h. The dual-luciferase reporter gene vector (pmiRRB-GLP-1R-3'UTR) of GLP-1R was constructed and co-transfected into HEK-293T cells with LINC00652 vector, siRNA-LINC00652 or negative control respectively. After transfection for 48 h, the medium was aspirated and washed twice with PBS. The cells were lysed and collected. The luciferase activity was measured by using a dual luciferase reporter assay system (Dual-Luciferase® Reporter Assay System, E1910, Promega). Firefly luciferase working solution (50 μl) was added to each 10 μl cell sample to detect firefly luciferase activity, and then bacillus luciferase solution (50 μl) was added to detect Renilla luciferase activity. The ratio of firefly luciferase activity to Renilla luciferase activity was used as relative luciferase activity. The experiment was repeated three times, and the average data were presented.

Experimental animals grouping and processing

To investigate the protective effect of sevoflurane preconditioning on myocardial I/R injury and to investigate the effect of lncRNA LINC00652 on myocardial I/R injury in mouse models treated with sevoflurane by targeting GLP-1R through the cAMP/PKA pathway, 72 mice were divided into 6 groups as follows (12 mice in each group): (1) simple model group (I/R): the left coronary artery was ligated, then the ligation line was released after 45 min, followed by reperfusion for 24 h; (2) model control group (NC): intraperitoneal injection of saline performed in the mice 24 h before modeling, the left coronary artery was ligated, the ligation line was released after 45 min, followed by reperfusion for 24 h; (3) sevoflurane treatment (Sevo + I/R): the left coronary artery was ligated, the ligation line was released after 45 min, while inhalation of 2.4% sevoflurane (Maruishi Pharmaceutical Co., Ltd, Japan) was performed for 5 min, reperfusion for 24 h; (4) LINC00652 treatment group (si-LINC00652 + Servo + I/R): 20 μg si-LINC00652 was injected intraperitoneally in the mice 24 h before modeling; the left coronary artery was ligated, and the ligature was released after 45 min, while inhalation of 2.4% sevoflurane was performed for 5 min, reperfusion for 24 h; (5) signal pathway inhibition group (H89 + Servo + I/R): 20 nmol PKA (protein kinase A) inhibitor H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl hydrate) (Biosource, Camarillo, CA, USA) was injected into the abdomen of the mice 24 h before modeling, the left coronary artery was ligated, then the ligation line was released after 45 min, while inhalation of 2.4% sevoflurane was performed for 5 min, reperfusion for 24 h; (6) LINC00652 treatment and signal pathway inhibition group (si-LINC00652 + H89 + Sevo + I/R): 20 μg si-LINC00652 and 20 nmol PKA inhibitor H89 were injected into the mice 24 h before modeling, the left coronary artery was ligated, then the ligation line was released after 45 min, while inhalation of 2.4% sevoflurane was performed for 5 min, reperfusion for 24 h. After the end of the experiment, the following indicators were detected.

Echocardiographic detection

Before the myocardial I/R model was established, mild depilatory was used to strip the chest area hair of mice; anesthesia was performed by isoflurane inhalation, the heart rate of the mice was used as the criterion of anesthesia depth, and the heart rate was controlled in the range of 350 ~ 400 times/min. The mouse was fixed on the ultrasound platform in the supine position, and the heart slices were positioned in the lower edge of the papillary muscle using the B-mode ultrasound. The probe was kept stable and converted to the M-mode for section scan to take the pictures. The left ventricular end-diastolic volume and left ventricular end-systolic volume were measured. The left ventricular ejection fractions (LVEF) and left ventricular fractional shortening (LVFS) were calculated. LVEF (%) = (left ventricular end-diastolic volume - left ventricular end-systolic volume) / (left ventricular end-diastolic volume) × 100% [24].

Hemodynamics monitoring

At a dose of 50 ml/kg, 1% pentobarbital sodium solution was injected into the mice intraperitoneally with 1-ml sterile syringes. The neck tissue was separated bluntly to expose the the mouse carotid artery, the distal end of the heart was ligated and the proximal end with the wire was pulled, and then a 1.4 F Millar catheter was inserted into the mouse carotid artery. The left ventricular end diastolic pressure (LVEDP) and left ventricular pressure rise ($+dp/dt_{max}$) and descending ($-dp/dt_{max}$) maximal rate were recorded after the Millar catheter entered the left ventricle. Whether the catheter was in the left ventricle was determined according to the blood pressure waveform.

Hematoxylin-eosin (HE) staining

After 24 hours of I/R, the hearts of the mice were cut from the root of the aorta and quickly rinsed in phosphate buffer saline (PBS) at 4°C to wash away blood. Then the hearts were fixed by paraformaldehyde (Xi'an Chemical Reagent Factory, Shannxi, China) for 24 h, followed by paraffin embedding and slicing into pieces at a thickness of 5 μm by using a paraffin microtome. HE staining and histopathological examinations were performed to observe the coloration of myocardial tissues in the mice, and its distribution range and staining intensity.

2, 3,5-Triphenyltetrazolium chloride (TTC) staining

The freshly 1% sodium pentobarbital solution prepared by PBS was intraperitoneally injected into the mice with 1-ml sterile syringes at a dose of 50 ml/kg; the mouse thoracic cavity was opened again, the distal end of the aorta was ligated by a hemostatic forceps, the insulin injector was inserted into the autonomic artery root reversely with the Evans blue dye (Sigma-Aldrich Chemical Company, St Louis, MO, USA), and the heart was cut from the root of main autonomic artery and frozen in the refrigerator at -80°C for 5 min. The frozen hearts were quickly sliced in the mouse heart microtome, and the entire heart was cut into 5 slices with the thickness of 1 mm; the sliced myocardium were placed in 2% freshly prepared TTC dye (Sigma-Aldrich Chemical Company, St Louis MO, USA) that pre-heated at 37°C and then incubated in the water bath at 37°C for 10 ~ 15 min until the ischemic tissue showed bright red. Lastly, pictures were taken and analyzed by ImageJ software.

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) Staining

The dewaxed tissue sections were rinsed with PBS three times (5 min for each time). After the water was sucked to dry, sections were added with the protease K working solution (Sigma-Aldrich Chemical Company, St Louis MO, USA) at 21 - 37°C for 15 ~ 30 min, the paraffin sections were immersed in PBS 3 times × 5 min. After the surface water was dried, the sections were immersed in 3% H_2O_2 methanol blocking solution and incubated at room temperature for 10 min. The sections were rinsed in PBS 3 times for 5 min for each time, added with 5% bovine serum albumin (BSA) blocking solution at room temperature for 1 h. The sections were rinsed again in PBS 3 times × 5 min. After the α-actin primary antibody working solution (Sigma-Aldrich Chemical Company, St Louis MO, USA) was added, the sections were incubated in the wet box at 37°C for 1 h and then rinsed in PBS 3 times for 5 min for each time. The fluorescent secondary antibody was added, and the sections were then incubated in the wet box at 37°C for 1 h avoiding exposure to light followed by PBS washing 3 times (5 min for each time). The TUNEL dyeing mixture (Sigma-Aldrich Chemical Company, St Louis MO, USA) was added, then sections were incubated at room temperature for 1 h avoiding exposure to light, followed by PBS washing (3 times × 5 min); 4',6-diamidino-2-phenylindole (DAPI) dye (F. Hoffmann-La Roche Ltd., Basel, Switzerland) was added, sections were incubated for 5 min avoiding exposure to light, followed by PBS washing (3 times × 5 min). Next, the water was dried, the sections were mounted with the neutral balsam and photographed. ImageJ software was used for image analysis, and the apoptosis rate (%) was calculated as the ratio of TUNEL positive cells to the total number of cells.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the mouse myocardium by the one-step method of Trizo (Invitrogen Inc., Carlsbad, CA, USA) and the high quality RNA was confirmed by ultraviolet analysis and formaldehyde denaturing electrophoresis. RNA (1 μg) was taken, and cDNA was obtained by AMV reverse transcriptase. PCR primers were designed and synthesized by Invitrogen Inc., Carlsbad, CA, USA with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference (Table 1). The PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The product was electrophoresed on an agarose gel, and the RT-qPCR results were analyzed using OpticonMonitor3 software (Bio-Rad, Inc., Hercules, CA, USA). The threshold cycle (Ct) value of each reaction tube was selected and obtained manually at the lowest point where the logarithmic amplification curve was raised in a parallel manner. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method, and $2^{-\Delta\Delta Ct}$ was the ratio of the expression of the gene in the experiment group and the control group. The formula was as follows: ΔΔCt = [Ct (target gene) - Ct (reference gene)] experimental group - [Ct (target gene) -Ct (reference gene)] control group. The experiment was repeated 3 times, and the average data were collected.

Western blotting

Myocardial tissue protein was extracted, and the protein concentration was determined by using a BCA kit (Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China). The extracted protein was added

with loading buffer and boiled at 95°C for 10 min, and 30 μg of protein was added in each well. Proteins were separated by 10% polyacrylamide gel (Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China) electrophoresis, and the electrophoresis was conducted at a voltage from 80 V to 120 V wetly, and the membrane was transferred at the voltage of 100 mv for 45 - 70 min. Polyvinylidene fluoride (PVDF) membrane was transferred, incubated in 5% BSA for 1 h at room temperature, added with primary anti-GLP-1R, cAMP response element binding protein (CREB) and phosphorylated (p)-CREB (1: 1000 dilution; Cell Signaling Technologies (CST), Beverly, MA, USA) and anti-β-actin (1: 3000 dilution; Becton, Dickinson and Company, NJ, USA) at 4°C overnight. Tris-buffered saline and tween-20 (TBST) were used for membrane rinsing (3 times/5 min). Membrane was added with the corresponding secondary antibody (Miao Tong Biotechnology Company, Shanghai, China) and incubated at room temperature for 1 h. After the membrane was washed (3 times/5 min), the chemiluminescent reagent was used to develop the membrane. β-actin was used as the internal reference. A Bio-Rad Gel EZ Dol imager (GEL DOC EZ IMAGER, Bio-Rad, California, USA) was used to develop the image. ImageJ software was applied to analyze the gray value of the targeted band. The experiment was repeated three times, and the average data were presented.

Table 1. Primer sequence for RT-qPCR. Note: F, forward; R, reverse; RT-qPCR, everse transcription quantitative polymerase chain reaction; GLP-1R, glucagon-like peptide 1 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Gene	Sequence
LINC00652	F: 5'-GAGAGACGGAAGCATTAAGAAT-3'
	R: 5'-ATTCTTAATGCTTCCGTCTCTC-3'
GLP-1R	F: 5'-AATGGCGGCACTCCAGATG-3'
	R: 5'-GACCTGCCCTTGGAACCTCA-3'
GAPDH	F: 5'-CAAGTTCAACGGCACAGTCA-3'
	R: 5'-CCCCATTTGATGTTAGCGGG-3'

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1β and TNF-α in serum were measured by ELISA. The concentration of standard samples of serum samples was determined, and the ordinate curve was drawn. The concentrations of IL-1β and TNF-α in the serum of the mice were determine according to the instructions of the ELISA kits (all purchased from R & D Systems, Inc., Minneapolis, MN, USA) and the blank wells were used for calibration. A microplate reader (Bio-TEK Co., Ltd, Vermont, USA) was used to measure the absorbance (Optical Density [OD]) value of each well at 450 nm. The experiment was repeated three times, and the average data were presented.

Statistical analysis

The data were analyzed by statistical software SPSS 21.0 (IBM Corp Armonk, NY, USA). The data were expressed as the mean ± standard deviation, and comparisons between two groups with normal distribution were detected using the Student *t* test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), and the least significant difference (LSD) method was used among groups. *p* represents a bilateral test, and $p < 0.05$ was statistically significant for the difference.

Results

GLP-1R is a target gene of LINC00652

Initially, the analysis of the myocardial I/R chip (GSE6381) revealed that lncRNA LINC00652 was highly expressed in myocardial I/R (Fig. 1A). The MEM website predicted that GLP-1R was the target gene of LINC00652 (Fig. 1B). KEGG enrichment analysis by using the WebGestalt database showed that GLP-1R was involved in the cAMP/PKA signaling pathway (Fig. 2). The analysis of online prediction site (https://cm.jefferson.edu/rna22/Interactive/RNA22Controller) indicated the existence of binding sites between LINC00652 and GLP-1R 3'UTR (Fig. 1C), and the free energy between them was ΔG = -119.04, ΔG/n = -0.018. All the findings suggested that GLP-1R was the target of LINC00652. In addition, the results of co-transfecting the dual-luciferase reporter gene vector (pmiRRB-GLP-1R-3'UTR) with the LINC00652 vector, siRNA-LINC00652 or NC into HEK-293T cells showed that the relative luciferase activity of the LINC00652 vector group was significantly lower while of the siRNA-LINC00652 group was significantly higher than that of the NC group ($p < 0.05$)

(Fig. 1D), indicating that LINC00652 targeted to inhibit GLP-1R activity and GLP-1R was a direct target of LINC00652.

The myocardial I/R injury mice decreased cardiac function and hemodynamics and increased myocardial cells apoptosis increased

To identify whether the model of myocardial I/R was successfully established, we examined the cardiac function, hemodynamics and myocardial cells apoptosis of mice before and after modeling. The results showed that compared with the normal group, the mice in I/R groups had decreased cardiac function, lower LVEF (57.24 ± 3.98% vs. 32.68 ± 2.15%, $p < 0.05$), lower LVFS (38.61 ± 2.47% vs. 21.54 ± 1.89%, $p < 0.05$), lower maximum rising rate in left ventricular pressure ($+dp/dt_{max}$) (7502.46 ± 297.15 mmHg/s vs. 5261.35 ± 237.92 mmHg/s, $p < 0.05$) and lower maximum descent rate of left ventricular pressure ($-dp/dt_{max}$) (4982.61 ± 218.24 mmHg/s VS 3017.26 ± 124.72 mmHg/s, $p < 0.05$), while had higher LVEDP (4.07 ± 0.32 mmHg vs. 16.19 ± 1.25 mmHg, $p < 0.05$) and more apoptotic myocardial cells. There was no significant difference in the cardiac function, hemodynamics and myocardial cells apoptosis of the mice between the sham group and the normal group ($p < 0.05$) (Fig. 3). All the results above indicated that the method of ligating left coronary artery of mouse with ligature being released after 45 min and then conducting reperfusion for 24 h could be used to successfully establish the model of myocardial I/R in mice.

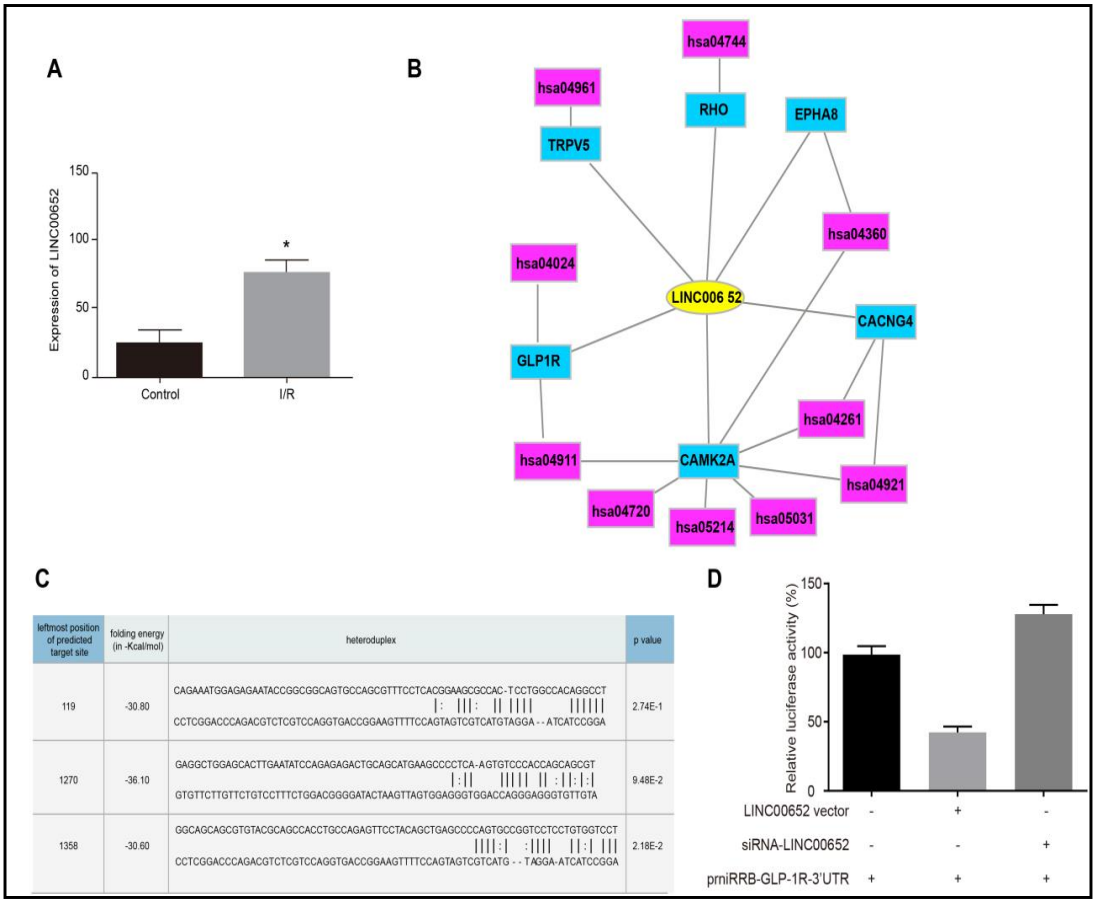

Fig. 1. GLP-1R is a target gene of LINC00652. Note: Panel A, differences of LINC00652 expression in the myocardial I/R group and the NC groups; Panel B, prediction of target gene of LINC00652; Panel C, three binding sites between LINC00652 and GLP-1R-3'UTR; Panel D, activity of HEK293T cell co-transfecting with pmiRRB-GLP-1R-3'UTR measured by a dual luciferase reporter gene assay; **, p<0.01 compared with the NC group. GLP-1R, glucagon-like peptide 1 receptor; cAMP/PKA, cyclic adenosine monophosphate-protein kinase A; NC, negative control.

Expression of LINC00652 and mRNA and protein levels of GLP-1R in the myocardium of mice after myocardial I/R were increased

RT-qPCR was used to detect the lncRNA LINC00652 expression in the myocardium of mice before and after myocardial I/R. The results showed that compared with the normal group, the lncRNA LINC00652 expression in the myocardium of mice in the I/R group was significantly increased ($p < 0.05$). Meanwhile, to observe the GLP-1R expression in the myocardium of mice before and after myocardial I/R, the mRNA and protein levels of GLP-1R in the myocardium of mice were measured by RT-qPCR and western blotting, respectively. The results showed that the mRNA and protein levels of GLP-1R in the myocardium of mice after myocardial I/R were significantly higher than those in the normal group ($p < 0.05$). The levels of lncRNA LINC00652 and GLP-1R in the myocardium were not significantly different between the sham group and the normal group ($p > 0.05$) (Fig. 4). Therefore, the LINC00652 expression and the mRNA and protein levels of GLP-1R in the myocardium of mice after myocardial I/R were all increased.

Si-LINC00652 had lower transfection efficiency and sevoflurane reduced expression of LINC00652 and up-regulated GLP-1R and CREB expression

To detect the interference efficiency of si-LINC00652 sequence, the RNA interference fragments of LINC00652 was exogenously transfected in the H9C2 cells and the cells were collected after 48 h. The RT-qPCR results showed that the interference efficiency of si-LINC00652 was 68% ± 3% compared with the negative control (si-NC). The cells could be used for the following experiments. RT-qPCR and western blotting were applied the LINC00652 expression and mRNA expression of GLP-1R and the levels and phosphorylation of the GLP-1R

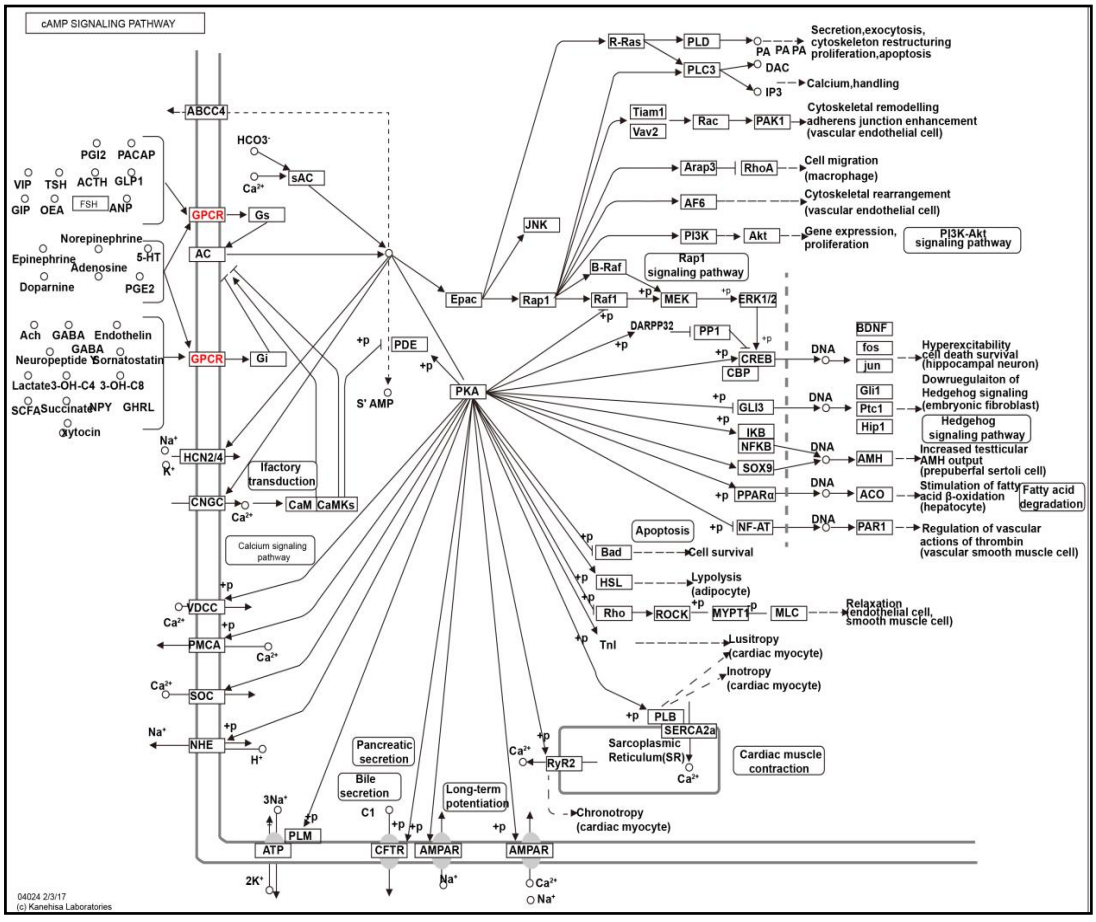

Fig. 2. The mechanism of GLP-1R in the cAMP/PKA signaling pathway by KEGG analysis in the WebGestalt database.

and CREB proteins respectively, and the results are shown in Fig. 5. There were no significant differences in the LINC00652 expression, mRNA and protein expression of GLP-1R, or the expression and phosphorylation of CREB protein in the myocardium of mice between the I/R and NC groups (all $p > 0.05$). Compared with the I/R group, the expression of LINC00652 in the Sevo + I/R group was significantly decreased, but the mRNA and protein expression of GLP-1R and the phosphorylation of CREB protein were significantly increased (all $p < 0.05$), indicating that sevoflurane treatment could decrease LINC00652 expression in the myocardium of mice with myocardial I/R and upregulate GLP-1R and CREB expression. Compared with the Sevo + I/R group, the expression of LINC00652 in the si-LINC00652 + Sevo + I/R group was decreased, but the mRNA and protein expression of GLP-1R, and the phosphorylation of CREB protein were increased (all $p < 0.05$), indicating that there was a certain relationship between LINC00652 and GLP-1R in mice with myocardium I/R, and LINC00652 could inhibit the activation of the cAMP/PKA pathway by targeting the GLP-1R gene. There was no significant difference of the expression of LINC00652 and mRNA and protein expression of GLP-1R, and the phosphorylation of CREB protein between the Sevo + I/R group and the H89 + Sevo + I/R group, nor did that between the si-LINC00652 + H89 + Sevo + I/R group and the si-LINC00652 + Sevo + I/R group (all $p > 0.05$), which indicated that inhibition of the cAMP/PKA pathway did not affect the upstream expression of

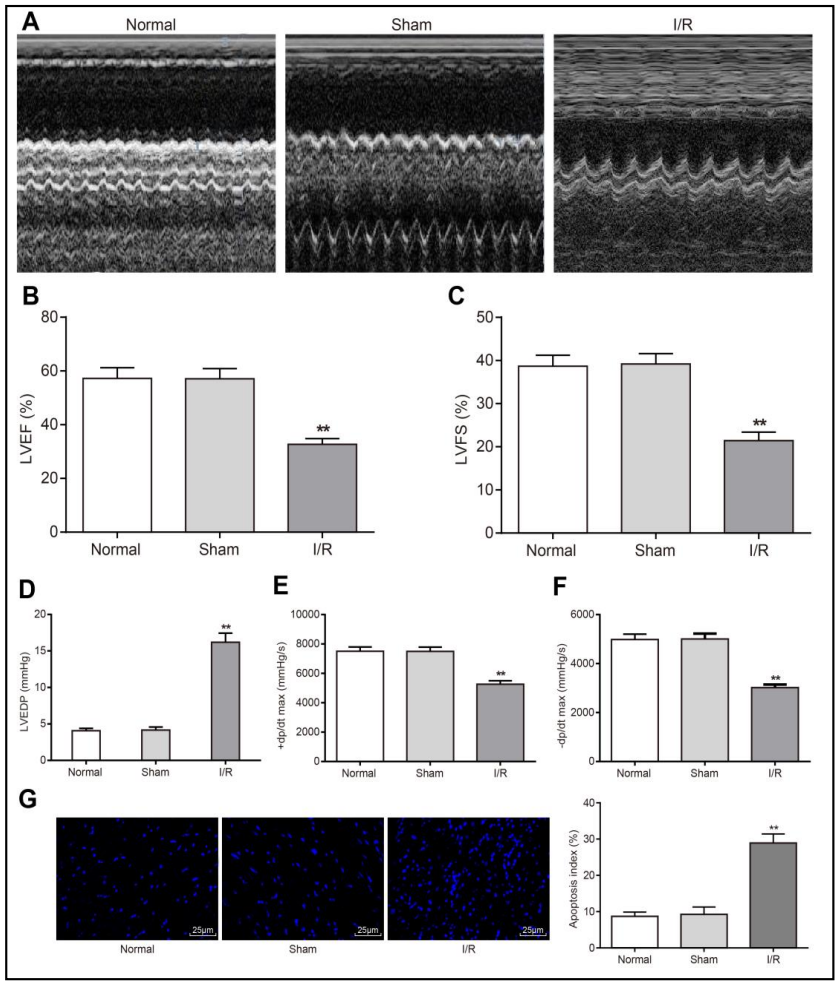

Fig. 3. The myocardial I/R injury mice decreased cardiac function and hemodynamics and increased myocardial cells apoptosis increased. Note: Panel A, cardiac ultrasonography of mice after myocardial ischemia and reperfusion; Panel B, LVEF of mice after myocardial ischemia and reperfusion; Panel C, LVFS of mice after myocardial ischemia and reperfusion; Panel D, changes in LVEDP in mice after myocardial ischemia and reperfusion; Panel E, changes in +dp/dt_{max} in mice after myocardial ischemia and reperfusion; Panel F, changes in -dp/dt_{max} in mice after myocardial ischemia and reperfusion; Panel G, myocardial cells apoptosis measured by TUNEL; **, P<0.01 compared with the NC group. I/R, ischemia-reperfusion; LVEF, left ventricular ejection fractions; LVFS, left ventricular fractional shortening; LVEDP, left ventricular end diastolic pressure; TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling; NC, negative control.

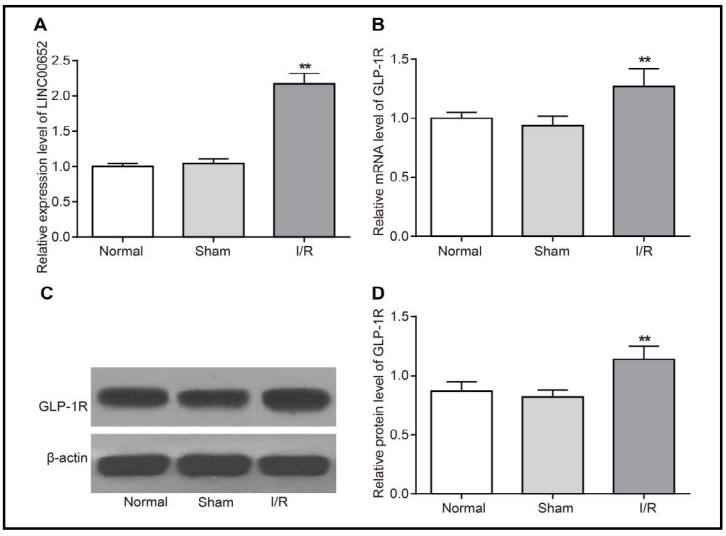

Fig. 4. The expression of LINC00652 and the mRNA and protein levels of GLP-1R in the myocardium of mice after myocardial I/R were increased. Note: Panel A, the LINC00652 expression in the myocardium of mice before and after modeling; Panel B, mRNA expression of GLP-1R in the myocardium of mice before and after modeling; Panel C, GLP-1R protein in the myocardium of mice measured by western blotting before and after modeling; Panel D, the expression of GLP-1R protein in the myocardium of mice before and after modeling; **, P<0.01 compared with the NC group; I/R, ischemia-reperfusion; GLP-1R, glucagon-like peptide 1 receptor; NC, negative control.

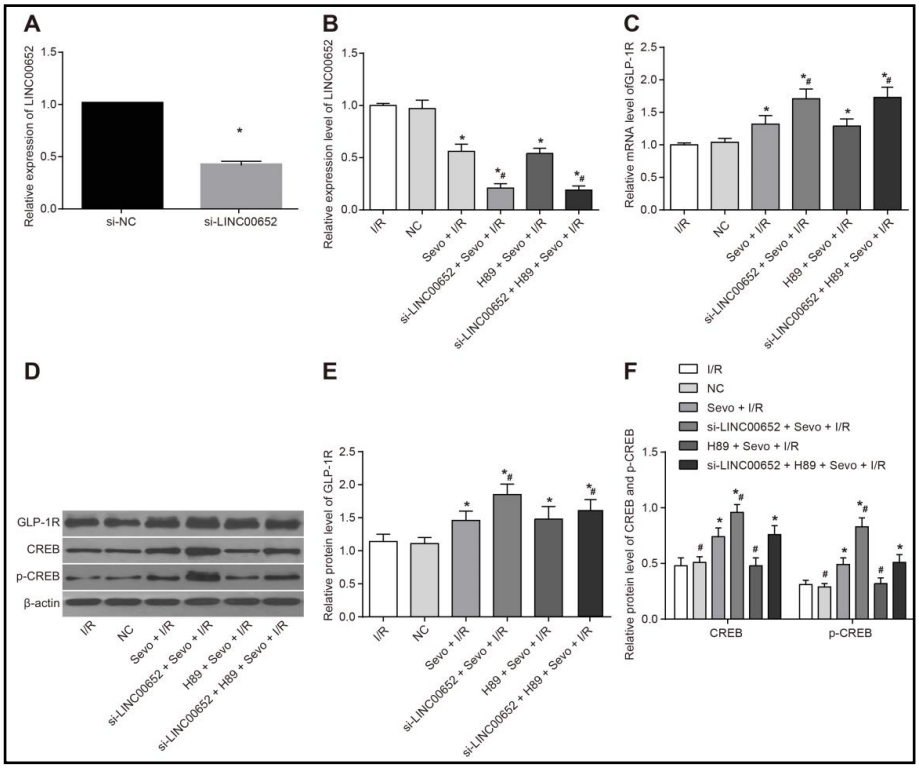

Fig. 5. Si-LINC00652 decreased transfection efficiency and sevoflurane reduced expression of LINC00652 and up-regulated GLP-1R and CREB expression. Note: Panel A, the results of transfection efficiency; Panel B, LINC00652 expression in the myocardium of mice in each group; Panel C, mRNA expression of GLP-1R in the myocardium of mice in each group; Panel D, GLP-1R and CREB proteins in the myocardium of mice in each group examined by western blotting; Panel E, GLP-1R protein expression in the myocardium of mice in each group; Panel F, expression and phosphorylation of CREB protein in the myocardium of mice in each group; *, p<0.05 compared with the I/R group; #, p<0.05 compared with the Sevo + I/R group; I/R, ischemia-reperfusion; Sevo, sevoflurane; GLP-1R, glucagon-like peptide 1 receptor; CREB, cAMP responsive element-binding protein; NC, negative control.

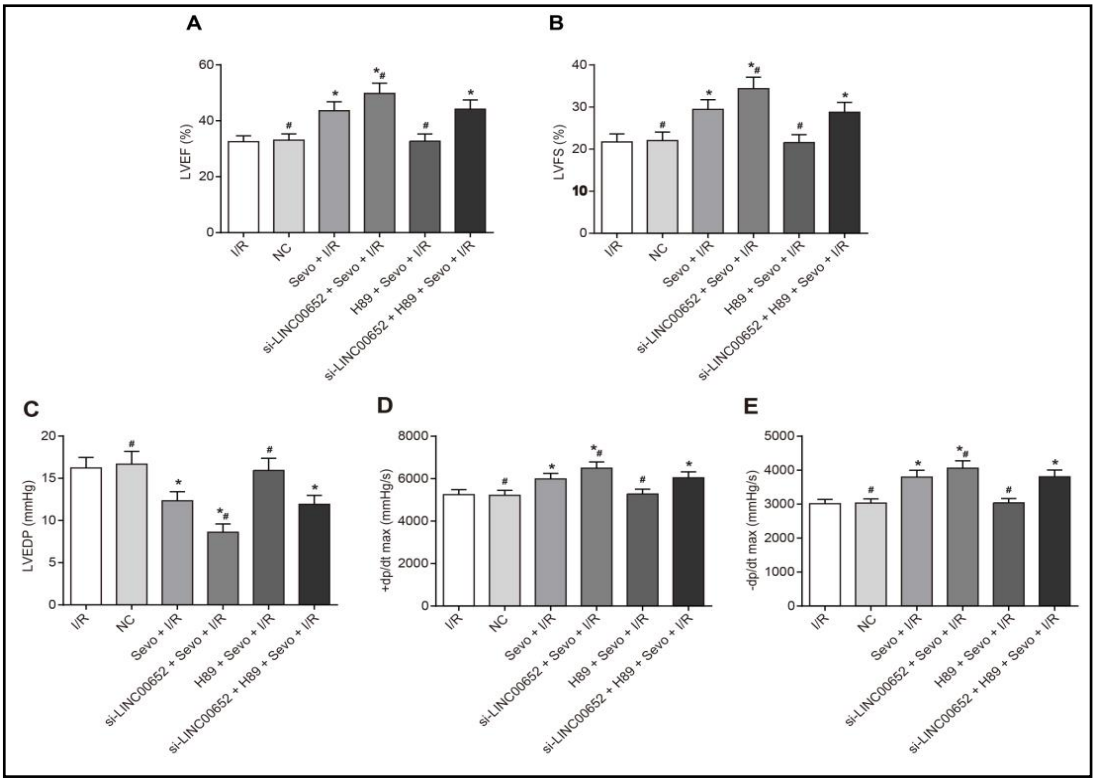

Fig. 6. LINC00652 reduced myocardial I/R injury and promoted cardiac function and hemodynamics of mice. Note: Panel A, LVEF of mice in each group; Panel B, LVFS of mice in each group; Panel C, changes of LVEDP of mice in each group; Panel D, changes of +dp/dt_{max} of mice in each group; Panel E, changes of -dp/dt_{max} of mice in each group; *, p<0.05 compared with the I/R group; #, p<0.05 compared with the Sevo + I/R group; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; I/R, ischemia-reperfusion; Sevo, sevoflurane.

LINC00652 and GLP-1R. Here, it can be concluded that si-LINC00652 increased transfection efficiency, and sevoflurane decreased LINC00652 expression and up-regulated GLP-1R and CREB expression.

LncRNA LINC00652 reduced myocardial I/R injury and increased increased cardiac function and hemodynamics of mice

In order to investigate whether lncRNA LINC00652 can affect the myocardial I/R injury in mice treated with sevoflurane by targeting GLP1R through the cAMP/PKA pathway, changes in the cardiac function of mice in each experimental group were examined. The test results showed that compared with the I/R group, the LVEF and LVFS of mice in the NC group had no significant change ($p > 0.05$), while the LVEF and LVFS of the modeled mice treated with sevoflurane were significantly increased ($p < 0.05$), indicating that sevoflurane treatment can protect the myocardial function in myocardial I/R mice. Besides, compared with the Sevo + I/R group, the LVEF and LVFS of mice in the si-LINC00652 + Sevo + I/R group showed an increasing trend, while of the H89 + Sevo + I/R group were significantly decreased and returned to the level of the I/R group, indicating that interference of LINC00652 could reduce the myocardial I/R injury in mice, while inhibition of the cAMP/PKA pathway increased myocardial I/R injury. There was no significant difference between the si-LINC00652 + H89 + Servo + I/R group and the Servo + I/R group, indicating that inhibition of the cAMP/PKA pathway could counteract the protective effect of si-LINC00652 on myocardial I/R injury in mice. Furthermore, the results of hemodynamics monitoring revealed that Compared with the I/R group, the LVEDP of mice in the Sevo + I/R and si-LINC00652 + H89 + Sevo + I/R groups was significantly decreased, and the +dp/dt_{max} and -dp/dt_{max} were significantly increased (all $p < 0.05$). The LVEDP of mice in the si-LINC00652 + Sevo + I/R

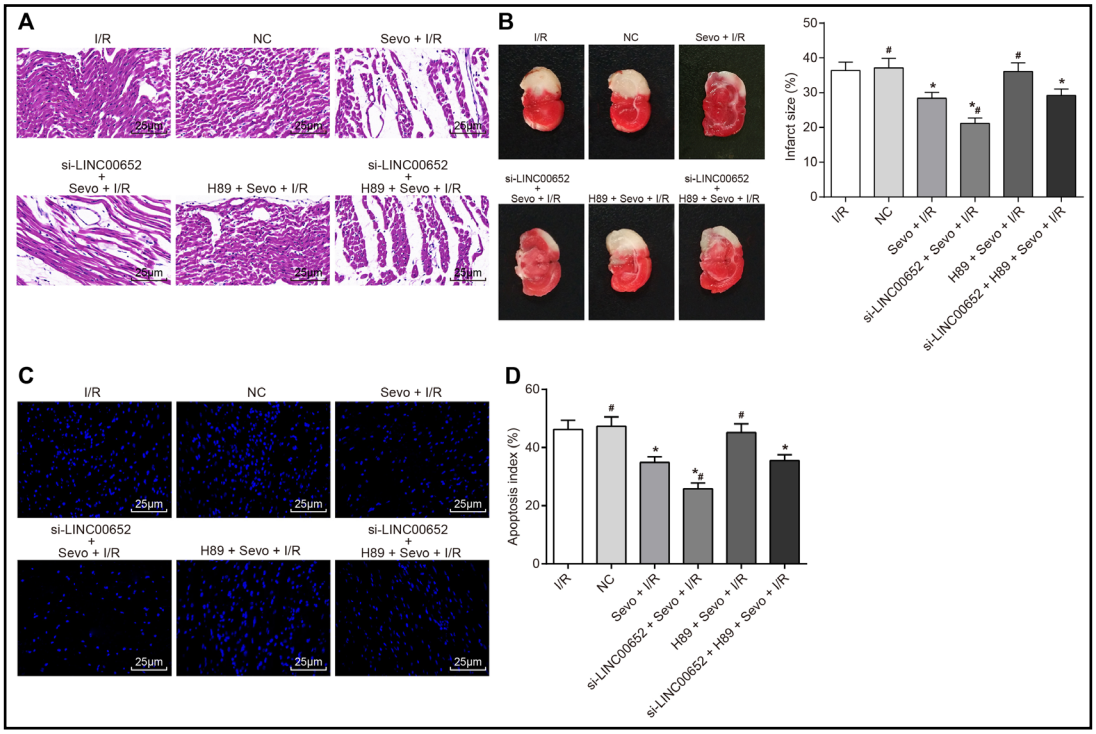

Fig. 7. Inhibition of LINC00652 activated the cAMP/PKA pathway and promoted the inhibitory role of sevoflurane on reducing myocardial injury, infarct size and rate of cell apoptosis in myocardial I/R mice. Note: Panel A, cardiac pathological changes of mice in each group measured by HE staining; Panel B, infarct size in mice of each group measured by TTC; Panel C, myocardial cell apoptosis of mice in each group; Panel D, statistical analysis of myocardial cell apoptosis in mice; *, p<0.05 compared with the I/R group; #, p<0.05 compared with the Sevo + I/R group; I/R, ischemia-reperfusion; Sevo, sevoflurane; cAMP/PKA, cyclic adenosine monophosphate-protein kinase A; HE, hematoxylin-eosin; TTC, 2,3,5-Triphenyltetrazolium chloride.

group continued to decrease compared with that in the Sevo + I/R group, and both the $+dp/dt_{max}$ and $-dp/dt_{max}$ continued to increase ($p < 0.05$). Compared with the I/R group, the LVEF, $+dp/dt_{max}$ and $-dp/dt_{max}$ of mice in the H89 + Servo + I/R group and the NC group were not significantly different (all $p > 0.05$) (Fig. 6). From above, these suggested that interfering the expression of LINC00652 could alleviate the myocardial I/R injury of mice and increased cardiac function and hemodynamics

Suppressed LINC00652 activated the cAMP/PKA pathway and promoted the inhibitory role of sevoflurane on reducing myocardial injury, infarct size and rate of cell apoptosis in myocardial I/R mice

The cardiac pathological changes, the infarct size and the myocardial cell apoptosis of mice were measured and analyzed by HE, TTC and TUNEL staining assays, respectively. HE results showed that the myocardial fibers of mice in the I/R group, the NC group and the H89 + Sevo + I/R group were irregularly

Table 2. The level of inflammatory factors in the serum of each group (ng/ml). Note: *, p < 0.05 compared with the I/R group; #, p < 0.05 compared with the Sevo + I/R group; I/R, ischemia-reperfusion; Sevo, sevoflurane; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; NC, negative control

Groups	IL-1β	TNF-α
I/R	0.81 ± 0.07	1.92 ± 0.16
NC	0.79 ± 0.08#	1.94 ± 0.14#
Sevo + I/R	0.62 ± 0.05*	1.69 ± 0.12*
si-LINC00652 + Sevo + I/R	0.41 ± 0.04*#	1.43 ± 0.11*#
H89 + Sevo + I/R	0.82 ± 0.06#	1.89 ± 0.15#
si-LINC00652 + H89 + Sevo + I/R	0.60 ± 0.05*	1.67 ± 0.13*

arranged and in structural disorder, the wave-like degeneration was observed, the local stripes disappeared, extensive swelling, the fusion of rupture and necrosis, and the obvious hemorrhagic foci were observed, while no significant differences in the infarct size and cell apoptosis were observed. Compared with the I/R group, the mice in the Sevo + I/R, si-LINC00652 + Sevo + I/R and si-LINC00652 + H89 + Sevo + I/R groups showed a reduced degree of myocardial injury, infarct size and rate of cell apoptosis (all $p < 0.05$). Compared with the Sevo + I/R group, mice in the si-LINC00652 + Sevo + I/R group had a lower degree of myocardial injury, infarct size and rate of cell apoptosis (all $p < 0.05$), indicating that inhibition of LINC00652 expression could activate the cAMP/PKA pathway, thereby promoting the inhibition role of sevoflurane on a reduced degree of myocardial injury, infarct size and rate of cell apoptosis in myocardial I/R mice (Fig. 7).

Si-LINC00652 alleviated inflammatory damage in mice by targeting GLP-1R through the cAMP/PKA pathway

ELISA was used to detect the levels of the inflammatory factors IL-1β and TNF-α in the serum of the mice in each group. Results showed that there was no obvious difference in the levels of serum IL-1β and TNF-α among the I/R group, the NC group and the H89 + Sevo + I/R group (all $p > 0.05$). The levels of IL-1β and TNF-α in the serum of the Sevo + I/R group were decreased significantly (all $p < 0.05$), indicating that sevoflurane treatment could alleviate the inflammatory damage in mice. Also, The levels of IL-1β and TNF-α in the serum of the si-LINC00652 + Sevo + I/R group were lower than the levels in the Sevo + I/R group (all $p < 0.05$), which demonstrated that the si-LINC00652 treatment could also alleviate the inflammatory damage in mice. However, there was no significant difference of the levels of IL-1β and TNF-α in the Sevo + I/R group and the si-LINC00652 + H89 + Sevo + I/R group (all $p > 0.05$), indicating that si-LINC00652 treatment reduced inflammatory damage in mice by targeting GLP-1R through the cAMP/PKA pathway (Table 2).

Discussion

Myocardial ischemia can cause arrhythmias, cardiac dysfunction, myocardial infarction, and even sudden death; its various clinical manifestations are usually caused by thrombosis, obstruction of coronary blood flow by coronary plaques, or hyper-constriction/vasospasm of epicardial and microvascular coronary arteries [3, 25]. I/R can cause additional cell death and increased infarct size, which is called I/R injury [26]. Studies have proven that lncRNA and GLP-1R are essential in myocardial I/R injury [27, 28]. Therefore, our study aimed to determine the effect of lncRNA LINC00652 on myocardial I/R injury in mice with sevoflurane treatment by targeting GLP-1R through the cAMP/PKA signaling pathway.

In the present study, we first observed that the cardiac function, LVEF and LVFS of mice decreased after myocardial I/R, but the LVEF and LVFS of mouse models treated by sevoflurane increased, which could indicate that sevoflurane treatment has a protective effect on the cardiac function of mice after myocardial I/R. Previous studies showed that sevoflurane could completely prevent the neuronal eosinophilic damage of the brain in incomplete cerebral ischemia and improve neurological outcomes after severe cerebral ischemia in rats compared with animals anesthetized with Zoletil [29, 30]. A previous study conducted by Cao et al. demonstrated that sevoflurane post-conditioning could significantly attenuate myocardial I/R injury and inhibit cardiac cell apoptosis and excessive autophagy [31]. However, compared with the Sevo + I/R group, the LVEF and LVFS of the si-LINC00652 + Sevo + I/R group increased, whereas the LVEF and LVFS of the mice in the H89 + Sevo + I/R group decreased and returned to the level of the I/R group compared with the Sevo + I/R group, suggesting that interfering with the expression of LINC00652 could alleviate the degree of myocardial I/R injury, while inhibiting the cAMP/PKA signaling pathway could strengthen the degree of myocardial I/R injury in mice. LncRNA constitutes a novel class of noncoding RNAs that regulate gene expression and might be associated with cardiac disease [32]. It was also believed that the expression levels of selected lncRNA in peripheral blood cells are correlated with myocardial ischemia and the prediction of prognosis [32]. RNA interference is an endogenous mechanism of cellular RNA control through the degradation of specific mRNA sequences, among which gene silencing might be exploited by siRNA to mediate the precise control of targeted cellular functions [33]. Some previous studies have

shown that cAMP/PKA could protect livers from I/R injury [18, 34]. The cAMP/PKA pathway was also proven to play a role against myocardial I/R injury in mice [35].

In addition, compared with the I/R group, the LINC00652 expression in the myocardium of the Sevo + I/R group decreased, and the GLP-1R expression increased, the cardiac function increased, the hemodynamic index trended towards normal, while infarct size was reduced and the myocardial cell apoptosis decreased significantly, the L-1β and TNF-α level decreased in the serum, and the percentage of myocardial cells decreased. The LINC00652 expression in the myocardium of the H89 + Servo + I/R group decreased, and expression and phosphorylation of GLP-1R and CREB proteins increased. Compared with the Sevo + I/R group, the LINC00652 expression in the myocardium of the si-LINC00652 + Sevo + I/R group further decreased, the expression of the GLP-1R and CREB proteins further increased, the infarct size further reduced, the number of apoptotic myocardial cells and inflammatory factors IL-1β and TNF-α in serum significantly decreased. A previous study showed that GLP-1 and its receptor GLP-1R exhibited cardio-protective effects after myocardial I/R in animal studies and clinical trials [28]. Several lncRNAs are dysregulated during acute myocardial infarction or heart failure, whereas others could control hypertrophy, mitochondrial function and cardiomyocyte apoptosis, and identified cardiovascular lncRNAs might play significant roles in cardiovascular diseases [36]. Previous studies have indicated that sevoflurane post-conditioning could protect isolated mouse hearts against I/R injury [9, 37]. A previous study showed that cAMP/PKA activation as a regulator could halt pathological cell recruitment, prevent destructive immune reactions, and promote hepatocyte survival, which might raise defensive thresholds to inflammatory responses [34].

In conclusion, the present study provided evidence that lncRNA LINC00652 might decrease the protective effect of sevoflurane in mouse models of myocardial I/R injury by targeting GLP-1R through the cAMP/PKA pathway and that lncRNA LINC00652 might be a promising target for patients with myocardial I/R injury. However, future studies are required to understand the specific regulation mechanism due to the complex pathogenesis of mice with myocardial I/R injury.

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Disclosure Statement

No conflict of interests exists.

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