Microarray Analysis Reveals a Potential Role of lncRNA Expression in remote ischemic preconditioning in Myocardial ischemia-reperfusion injury

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Research

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Abstract

Background

Avoiding or reducing cardiopulmonary bypass-related injuries of important organs is the eternal topic in the field of cardiovascular surgery. Remote ischemic preconditioning (RIPC) is a promising strategy whose clinical application seems more realistic and extensive compared with other conservative or surgical strategies. However, considering its complex process which involves the expression of a variety of molecules and even genes and little is known about its underlying mechanism after years of researches, new train of thought and method must be developed to explore its potential in clinical application. Long noncoding RNA (LncRNA) is a kind of RNA that has been proven be exert efficacy in the occurrence and development of cardiovascular disease. The differential expression of LncRNAs and their biological effects during RIPC have not been reported.

Methods

We first verified the protective effect of RIPC on myocardial ischemia-reperfusionby western blot, ELISA and hematoxylin-eosin(HE) staining. Mouse and human LncRNA arrays were used to investigate the expression signatures of LncRNAs and mRNAs in myocardial tissue after RIPC. Homology comparison was used to screen homologous genes in total differentially expressed LncRNAs. Competing endogenous RNA(ceRNA) mechanism analysis helped us find the matching relationship among homologous LncRNA, mRNA and microRNA.

Results

554 differentially expresssed mouse LncRNAs(281 up-regulated / 273 down-regulated) and 1392 differentially expresssed human LncRNAs(635 up-regulated / 757 down-regulated) were selected from LncRNA microarrays. Quantitative real-time polymerase chain reaction(qRT-PCR) was used to verify these selected LncRNAs, after homology comparison and ceRNA mechanism analysis, we got a pair of homologous LncRNAs(ENST00000574727 & ENSMUST00000123752), predicted corresponding matching relationship and potential competitive binding sites.

Conclusions

In this study, we identify a number of differentially expressed LncRNAs, which may be closely related to the regulation of inflammation and cell proliferation. In addition, we take several pairs of homologous LncRNAs as the next research object. By continuously exploring the function and mechanism of these LncRNAs, we hope to unveil the mystery of RIPC and discover a new protective mechanism of cardiovascular ischemia-reperfusion disease.

Background

How to avoid or reduce the injury of important organs caused by cardiopulmonary bypass has always been the focus of clinical and basic research in the field of cardiovascular surgery, and ischemic preconditioning is considered to be the best strategy of endogenous protection so far. As the implementation of direct ischemic preconditioning is greatly limited in clinical application, researchers turn their attention to the more realistic one, remote ischemic preconditioning (RIPC).

RIPC, is a low-risk, non-invasive, economical treatment[1], *it can protect heart, brain, liver, kidney and other organs after ischemia-reperfusion(I/R)*[2] *and its protective effect has been proved in many animal experiments and clinical practice*[3]. *However, the molecular mechanism of the function of RIPC remains unclear, and its process is complex, involving many factors, including the expression of a variety of molecules and even genes. With the deepening of the understanding of RIPC, the research in this area is gradually increasing in recent years.*

RIPC mainly achieves its protective effect through three steps: signal generation, signal transmission to the target organ, and target organ response to the signal[4]. *In the past five years, RIPC has been used in the conservative treatment of heart disease, however, the results are disappointing, mainly due to a thorough understanding of its pathogenesis and, which resulted in the lack of strong intervention.*

LncRNA is a kind of RNA molecules with a length of more than 200 bp. Like circRNA and tiRNA, its importance has been ignored for a long time, though it was early discovered. Existing studies have shown that LncRNA plays an important role in the occurrence and development of cardiovascular disease by regulating the proliferation, apoptosis and autophagy of various kinds of cells[5]. *The role of LncRNA in organ ischemia-reperfusion injury(IRI) is gradually confirmed by some experiments. A few articles have reported that LncRNA can alleviate MIRI by regulating apoptosis*[6]. *LncRNA can also be used as a molecular sponge for adsorbing microRNA and an epigenetic regulator to regulate MIRI*[7]. *LncRNA microarray analysis has showed that there are significant changes in the expression profile of LncRNA in myocardium at the early stage of reperfusion and these genes are related to immune response, spermine metabolism, chemotactic activity and chemokine receptor binding which are closely related to several MIRI-related signal pathways. LncRNA may play a role in myocardial ischemia-reperfusion through gene coexpression network*[8]. *The regulatory effect of LncRNA on MIRI is gradually being recognized, but its mechanism is largely unknown. In addition, what role does LncRNA play in RIPC? What is the specific mechanism? Can it be used as a therapeutic target for MIRI? At present, there is no relevant research and conclusion. In view of the above, in this study, LncRNA microarray has been used to analyze the changes of myocardial LncRNA expression in early stage after RIPC in mice and humans. By bioinformatics analysis and homology analysis, obvious differentially expressed LncRNAs have been screened out. These highly maladjusted LncRNAs are further verified by qRT-PCR to confirm candidate LncRNAs and lay an indispensable foundation for further functional research, mechanism research and clinical research in the future.*

LncRNA contains more information, and involve much more diversified molecular mechanism than microRNA, but little is known about the regulatory network of gene expression mediated by LncRNA and its mechanism. Exploring the mechanism of RIPC from the perspective of gene regulation may push the study of RIPC to a whole new level. Thus, the study of underlying mechanism of RIPC related LncRNA has strong clinical implications and practical significance for discovering new intervention strategy as well as exploring new therapeutic targets for MIRI.

Methods

Surgical management and Grouping of patients

24 children patients with non-cyanotic congenital heart disease were from the Department of Cardiothoracic surgery, the second affiliated Hospital of Wenzhou Medical University and Yuying Children's Hospital. Exclusion criteria: 1) patients with severe complications such as severe liver disease, kidney disease or lung disease affect the peripheral vascular disease of the upper limb; 2) patients or their families refuse to participate in the clinical trial. Withdrawal criteria: patients with anesthesia, surgical accidents and other adverse events during the experimental observation had to stop the trial. All the expenses for the treatment of the patients are paid by the Children's Heart Center of the second affiliated Hospital of Wenzhou Medical University, which does not increase the additional financial burden of the subjects. This study has been approved by the Ethics Committee of the Department of Cardiothoracic surgery of the second affiliated Hospital of Wenzhou Medical University and the Ethics Committee of Yuying Children's Hospital.

Self-control study on pre and post RIPC is performed in these patients, the cardiac tissues obtained before and after RIPC were randomized into control group and RIPC group respectively. After anesthesia and thoracotomy, a small amount of right atrial tissue is taken from above the purse-string suture area of the right atrium intubation. Placing the cuff on patients' upper arm, inflate to 200mmHg to block the blood flow for 5 minutes then relax the cuff to restore perfusion for 5 minutes, repeated 4 times, and took a small amount of right atrial tissue again. After the specimen is collected, the operation begins. Echocardiography and electrocardiogram will be used to detect heart rhythm, cardiac correction and recovery of cardiac function during operation or at least 3 days after operation.

Grouping and Treatment of experimental animals

C57BL/6 mice, 6-8 weeks old, weighing 22-26g (provided by Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), Mice are kept in a quiet room with 12/12h cycle light/dark at a constant temperature of 20~22 °C and provided with adequate water and food. All surgical procedures and postoperative care were carried out in accordance with the guidelines for the Care and use of Experimental Animals issued by the Chinese Academy of Health. All the operations involving mice were approved by the Animal Research Committee of Wenzhou Medical University(wydw2016-0334). Forty C57BL/6 mice are randomly grouped as follows: 1) Sham group(n=10): only undergo dissociation of the left femoral artery and thoracotomy.; 2) MIRI group(n=10): ligation and reperfusion of the left anterior

descending branch are given on the basis of the separation of the left femoral artery and thoracotomy.; 3) RIPC+MIRI group(n=10): separating left femoral artery for RIPC, then ligate left anterior descending branch and reperfuse by thoracotomy; 4) RIPC group(n=10): separating left femoral artery for RIPC, then obtain the heart by thoracotomy.

RIPC in C57BL/6 mice

After endotracheal intubation and anesthetized with isoflurane, the limbs of mice are fixed with tape, a small incision is made along the left groin, the femoral artery is freed, and the left femoral artery is gently clamped with a microvascular clamp for 5 minutes, then relax the vascular clamp and restore perfusion for 5 minutes. Repeat 4 cycles.

MIRI model of mice

After isoflurane induced anesthesia, the mice are endotracheal intubated and adjusted to right lateral position. Thoracotomy is performed on the third or fourth intercostal on the left side to expose the pericardium. Open the pericardium with microscopic forceps and ligate the left anterior descending branch of coronary artery with 6-0 silk thread to induce myocardial ischemia, After 30 minutes of myocardial ischemia, loose ligation so that the heart can be reperfused. Meanwhile, indwell a small hose in chest to extract residual air in the chest, suture layer by layer rapidly to chest cavity. Then mice are resuscitated by 100% oxygen ventilation and transferred to a warm pad. 180 minutes later, the re-anesthetized mice are killed and the samples needed for the experiment were taken. The occlusion and reperfusion of the left anterior descending branch can be judged by the significant changes in myocardial color.

Total RNA Purification

Total RNA was extracted from collected myocardial tissue with the Trizol reagent (15596026; Ambion, Cambridge, UK). RNA content is quantified with 260/280 UV spectrophotometry. After being qualified, isolated high-purity total RNA is subjected to LncRNA microarray analysis or qRT-PCR testing.

LncRNA Microarray Analysis

Arraystarhuman and mouse LncRNA microarrays V3.0 provided by KangChen Bio-tech Co. Ltd. (Shanghai, China) are used to detect our RNA samples. RNA labeling and array hybridization

are conducted as previously described[9, 10]. Differential expression levels of LncRNAs and mRNAs between the RIPC group and the Sham group are compared. Among detected up-regulated RNAs, adjusted P value □0.05 and either whose FC value□fold change□□2 & Chip intensity □500 or FC value □3 & Chip intensity □50 are considered as differentially expressed. As for down-regulated RNAs, adjusted P value □ 0.05 and either whose FC value□2 & Chip intensity□1000 or FC value□3 & Chip intensity□50 are defined differentially expressed.

GO and KEGG Pathway Analysis

KangChen Bio-tech Co. Ltd. (Shanghai, China) is commissioned to conduct GO and KEGG pathway analysis to systematically analyze differentially expressed genes and enrich important GO terms and KEGG pathways□P□0.05□[11]. The significance of the P value is evaluated by FDR (false discovery rate) and recommended FDR value is less than 0.05.

Western Blot Analysis in vivo

Extract total protein from mice's myocardial tissue (5 mg) and determinate the protein concentration with Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). The following primary antibodies were used: 1:1000 diluted Rabbit anti-Bax (50599-2-Ig; Proteintech Group, Inc), Bcl2(26593-1-AP; Proteintech Group, Inc), Caspase3(66470-2-Ig; Proteintech Group, Inc), cleaved-Caspase3(WL01992; Wanlei Biotechnology Co., Ltd.), TNF-α (17590-1-AP; Proteintech Group, Inc), IL-6(WL02841; Wanlei Biotechnology Co., Ltd.)primary antibody. The next day, added 1:3000 diluted Goat Anti-Rabbit antibody (SA00001-2; Proteintech Group, Inc), use ECL for coloration, scan and analyze the results with the Gel imaging system. After quantification, ratio of each protein to GAPDH is to be used to compare and analyze.

Myocardial infarct size measurement

Assess myocardial infarct size through Evans Blue (E2129; Sigma, St. Louis, MO) / TTC (2,3,5-triphenyltetrazolium chloride, T8877; Sigma) double staining method. The viable tissue is stained red and white by TTC was defined as area at risk (AAR). Non-ischemic myocardial tissue is stained deep blue by Evans Blue. Infarct area (INF) looks pale after staining. The percent of infarcted area over area at risk (INF/AAR ratio, IAR%) is calculated[12].

Hematoxylin-eosin staining

Mice's hearts are fixed in 4% paraformaldehyde. After 24h, embed fixed hearts with paraffin and slice the embedded hearts (5 μm thick). Sections are stained with HE according standard protocol and analyzed by light microscopy.

Plasma cTnI, TNF-α concentration

The serum levels of TNF-α, cTnI were measured using a mouse Enzyme-Linked ImmunoSorbent Assay kit (Boyun Biotech Co., Ltd, Shanghai) in accordance with the manufacturer's instructions.

qRT-PCR analysis

Extract total RNA from myocardial tissue using the Trizol reagent (15596026; Ambion, Cambridge, UK), quantatify RNA content by 260/280 UV spectrophotometry. The same volume of RNA solution was used to reverse transcriptase by the RT-PCR kit (FSQ-101; TOYOBO, Kita-ku, Osaka, Japan). The expression of these LncRNAs are quantified by SYBR Green (170-8882AP; Bio-Rad, Hercules, CA) two-step, real-time RT-

PCR using CFX96 Touch Real-Time PCR Detection System. The expression of LncRNA is normalized to GAPDH mRNA content and calculated using comparative methods.

Genes Homology Analysis & CeRNA Mechanism Analysis

Genes Homology Analysis and CeRNA Mechanism Analysis are performed by KangChen Bio-tech Co. Ltd. (Shanghai, China). Detected up-regulated homologous LncRNAs, whose chip intensity≥500, FC value ≥1.0, P≤0.05 and E-value ≤0.05 are selected. Detected down-regulated homologous LncRNAs, whose chip intensity≤50, FC value ≥1.0, P≤0.05 and E-value ≤0.05 are selected. Finally, the ceRNA mechanism analysis of stable differentially expressed homologous LncRNAs is performed.

Statistical analysis

All data in this article are expressed as mean±SEM. Comparisons between groups are assessed by T-test. All statistical analyses were performed using Graph Pad Prism Software (Version 8.0, La Jolla, CA). P < 0.05 was considered statistically significant.

Results

RIPC attenuate myocardial apoptosis and inflammation after MIRI in vivo

The levels of serum TNF-α and cTnI were measured by enzyme linked immunosorbent assay (Elisa) kit. Compared with the sham group, RIPC group did not have significantly elevated serum TNF-α and cTnI levels but in MIRI group and RIPC+MIRI group, serum TNF-α and cTnI levels were significantly increased while those in RIPC+MIRI group were significantly lower than MIRI group(Figure1.A). The results showed that MIRI was accompanied by the increase of troponin and inflammatory factors which indicates myocardial injury and inflammatory state and effective RIPC operation could inhibit this process and protect myocardium. Western blot showed that cleaved Caspase-3, Bax, TNF-α and IL-6 were up-regulated, while Bcl-2 was down-regulated(Figure1.C,D), local myocardium was in the state of inflammatory microenvironment and myocardial apoptosis, while RIPC significantly inhibit the expression of these pro-apoptotic and pro-inflammatory factors and alleviate myocardial damage.

RIPC attenuates myocardial structural changes after MIRI in mice

HE staining showed that MIRI caused local swelling, myocardial necrosis, myocardial fiber disorder and rupture, accompanied by inflammatory cell infiltration(Figure1.B). In contrast, these pathological changes were greatly weakened in the RIPC group, the structure and morphology of cardiomyocytes were relatively normal, with mild swelling, partial rupture of myocardial fibers, a small amount of inflammatory cell infiltration in interstitium. The myocardial fibers in sham group and RIPC group were normal and the stripes were clear and no myocardial remodeling, necrosis or neutrophil infiltration were observed.

RIPC reduces the infarct size after MIRI

After TTC-Evans blue staining, as shown in the figure, compared with sham operation group and RIPC group, IAR% increased remarkably in MIRI group and RIPC+MIRI group while IAR% in RIPC+MIRI group was smaller than MIRI group to a certain extent（Figure1.C）. The results showed that the infarct size increased during MIRI, and RIPC could protect the myocardium and significantly reduce the infarct size.

Microarray hybridization data of mouse & human

Arraystar mouse LncRNA microarray v3.0 and arraystar human LncRNA microarray v3.0 are specially designed for the global profiling of mouse & human LncRNAs and protein-coding transcripts. LncRNAs are carefully selected from authoritative public transcriptome databases (including UCSC knowngenes, Gencode, Refseq, etc.) and papers with high impact factors. Heat maps of hierarchical clustering results were performed to show remarkably different-regulated lncRNA and mRNA between the two groups. We could learn from results of scatterplot that expression of log-2 ratio and distribution of lncRNA and mRNA in RIPC group and nc group were almost the same in both mouse and human.

Differentially regulated lncRNAs and mRNAs of human and mouse

Presupposed（2-fold differential expression as screening condition. 1392 differentially regulated LncRNAs were selected from human LncRNA microarray, including 635 up-regulated and 757 down-regulated. 554 differentially regulated LncRNAs from mouse lncRNA microarray, including 281 up-regulated and 273 down-regulated(Table1). Meanwhile, we analyzed the differential expression profile of mRNA, top10 differentially expressed LncRNAs and mRNA after RIPC in both Human and Mouse were listed(Table2).

GO and KEGG Analyses of differentially regulated mRNAs of human and mouse

GO analysis (Biological processes, Cellular components, Molecular function) was performed to analyze the main potentially relevant functions of the closest coding genes according to the GO database provided the essential functional classification for the National Center for Biotechnology Information (NCBI). In our study, GO analysis initially reflected the function of differentially expressed (up-regulated and down-regulated) mRNA in myocardium which experienced RIPC in both human and mouse. In human and mouse groups, the most enriched GO terms (top10) of 3 structured networks were shown in Figure 3. And the top10 enriched pathways were shown in Figure 4. Pathway analysis showed that most of up-regulated mRNAs were involved in MAPK signal pathway and TNF signal pathway, while most down-regulated mRNAs were involved in NF-κB signal pathway and apoptosis in humans. Pathway analysis of mice revealed that most down-regulated mRNAs participated in cAMP signaling pathway, inflammation-mediated TRP pathway and so forth, up-regulated mRNAs related signaling pathways did not seem to be closely related to myocardial ischemia-reperfusion injury.

Homology analysis between human LncRNAs & mouse LncRNAs

Among preliminary screened mouse and human LncRNAs, homology analysis was performed to select homologous LncRNAs. We noticed that there were 5 pairs of up-regulated homologous LncRNAs, meanwhile, there were 6 pairs of down-regulated homologous LncRNAs. Homologous genes are highly

conserved in the process of biological evolution, may have important biological functions, and may become targets for disease treatment. Therefore, we believed that the selected 11 pairs of homologous LncRNAs might have potential significance for further study. 11 pairs of homologous LncRNAs are listed in Table 3.

Verification of differentially expressed LncRNAs in microarray by qRT-PCR

Rmoveing mitochondrial LncRNAs and those which cannot be designed with suitable primers, taking in detected differentially expressed 11 pairs of homologous LncRNAs, another 17 LncRNAs with more stable expression but lower FC. ENSMUST00000163913, ENST00000565841, ENSMUST00000126460 cannot be designed with suitable primers. Finally, only 36 LncRNAs left for qRT-PCR verification(sequence name and primers were shown in Table4). According to the Genebank sequence, each primer sequence is designed and synthesized by Shanghai Zhili Biology Co., Ltd. Relative expression of top 9 differentially expressed LncRNAs after qRT-PCR were listed(Figure5A), microarray consistency was validated reliable.

CeRNA mechanism analysis of Homologous LncRNA ENSMUST00000123752

In order to facilitate the follow-up exploration and verification, we only performed the ceRNA mechanism analysis and binding site prediction of mouse LncRNA (LncRNA ENSMUST00000123752), we named LncRNA ENSMUST00000123752 ischemic preconditioning-related LncRNA1(IPCRL1), corresponding homologous LncRNA ENST00000574727 were named ischemic preconditioning-related LncRNA2(IPCRL2). There is a complementary binding site between IPCRL1 and miR-128-3p. Predicted target protein JNK-interacting protein3(JIP3, Mapk8ip3), its mRNA could bind to the same binding site(Figure5C). CeRNA mechanism was shown in endogenouse competing relation conception map(Figure5D).

Discussion

MIRI is a common problem in clinical practice and it is the basic pathophysiological process that occurs in the treatment of cardiopulmonary resuscitation, thrombolysis of myocardial infarction, cardiopulmonary bypass and so on. Therefore, finding safe and effective treatments and drugs to alleviate MIRI has always been the focus of research in the field of cardiovascular medicine. When myocardial tissue is subjected to non-fatal ischemia-reperfusion, it will show adaptive compensatory response with defensive and protective significance, which is called ischemic preconditioning. In recent years, myocardial ischemic preconditioning has become one of the effective strategies for the prevention and treatment of MIRI. However RIPC has a more realistic and extensive application space as a result of the implementation of direct ischemic preconditioning is greatly limited in clinical application. The protective effect of RIPC has been confirmed in many animal experiments and clinical practice. Such as, protecting heart, brain, liver, kidney and other organs after ischemia-reperfusion. We found that after MIRI, myocardial tissue swelling, myocardial necrosis, myocardial fiber disorder and rupture.

Besides protein-coding genes, non-coding RNA increasingly attracted people's attention recently. According to the Human Genome Project, the number of total protein-coding genes in humans accounts for less than 2% of the entire human genome sequence, and the remaining 90% of the non-coding sequences are transcribed, resulting in a large number of non-coding RNAs. Due to the rapid development of high-throughput RNA sequencing technology, a large number of new non-coding RNAs have been discovered. The most famous non-coding RNAs is microRNA (miRNA), which has been shown to play a key role in a variety of biological and pathological processes[13, 14]. *Yu et al have shown that silencing LncRNA AK139328 can significantly up-regulate the expression of miR-204-3p and then inhibit cardiomyocyte autophagy, thus attenuating MIRI in diabetic mice*[15]. *Zhu et al have shown that LncRNA AK139128 mediates autophagy and apoptosis of cardiomyocytes after MIRI by targeting miR-499/FOXO4 axis*[16]. *Liang et al have revealed that the LncRNA ROR /miR-124-3p/TRAF6 axis regulates apoptosis and inflammation of human cardiomyocytes induced by hypoxia/reoxygenation*[17]. *We hypothesized that differentially expressed LncRNAs after RIPC may also play an important role in MIRI, so we used high-throughput LncRNA microarray to screen and found the differentially expressed LncRNAs in mouse and human myocardium in the early stage after RIPC.*

Without existing databases and limited reference information being used to reveal their functional annotations, we try to construct a correlation between LncRNA and mRNA and systematically analyzed the function of differentially expressed mRNA through GO annotation and pathway analysis.

In human's pathway analysis, MAPK signaling pathway, cAMP signaling pathway, Toll-like receptor signaling pathway and TNF signaling pathway are mostly enriched in up-regulated genes, while NF-κB signaling pathway and Apoptosis pathway are mostly enriched in down-regulated genes. For example, NF-κB signaling pathway, we notice that mRNAs of activating factors of this pathway such as TRADD, TNF R1, RANKL, LTB, LTA and crucial promoting factor of it IKKβ are markedly down-regulated. As for Apoptosis pathway we find p53 mRNA is down-regulated, in addition, down-regulated TRADD, TNF R1 can also be coupled with apoptosis pathway. Meanwhile, the four pathways enriched in up-regulated genes seem to be activated, however, this result cannot be explained by the activation of pathways. In fact, among this up-regulated genes in these pathways, several product proteins of them belong to anti-injury factors, and their up-regulation means the antagonism of damage-related signal pathways to some extent such as HSP72 in MAPK pathway, Hip1 at the end of cAMP signaling pathway. Hip1 then plays as a role in Hedgehog signaling pathway by which improve cardiac angiogenesis, promote cardiac repairment and improve cardiac function after myocardial infarction. The activation of Epac2-Rap1 signal in cAMP pathway is generally considered to have adverse effects but existing studies have shown that the existence of Epac2-Rap1 signal can reduce the production of mitochondrial ROS and reduce the susceptibility to myocardial arrhythmia[18]. *Numerous studies have shown that inhibition of cAMP-PKA pathway can significantly inhibit apoptosis. Wu et al found that the inhibition of miR-200a can inhibit the cAMP/PKA signal pathway by up-regulating the expression of DRD2 in PD rats, thus inhibiting the apoptosis of striatum*[19]. *Xu et al showed that MIRI increased DRG cAMP, and intrathecal injection of selective cAMP-PKA inhibitors could reduce myocardial injury*[20].

Everything has two sides, activation of these pathways reflects that the human's heart in the early stage of RIPC is in a state of co-expression of pro-injury factors and anti-injury factors. Because of the particularity of sample collection, we cannot completely rule out the influence of patients' inherent cardiac pathological state on production of pro-injury factors.

In mouse's pathway analysis, Hedgehog signaling pathway is mostly enriched in up-regulated genes, while cAMP signaling pathway, Dilated cardiomyopathy related pathway, Adrenergic signaling in cardiomyocytes and Inflammatory mediator regulation of TRP channels are mostly enriched in down-regulated genes.

Most of significant signaling pathways above are well known for their close relations with apoptosis, inflammation and oxidative stress which are the most common pathological processes in MIRI. The remaining signaling pathways are directly or indirectly involved in cardiac structure, myocardial angiogenesis, myocardial regeneration and repairment. Although the regulatory relationship and specific mechanism between these differentially expressed LncRNAs and these signaling pathways cannot be determined, what is certain is that these enriched signaling pathways are inextricably linked with MIRI, so we preliminarily believe that the differentially expressed LncRNA after RIPC has potential functions, and our work has its own unique significance. We further deepen the association through the homologous genes in these differentially expressed LncRNAs and find the most clinically meaningful targets and pathways for these LncRNAs, so as to establish a complete evidence chain of RIPC-LncRNAs-biological targets-signaling pathways.

In fact, after homology analysis and qRT-PCR verification, we identified a pair of human and mouse homologous LncRNAs (ENST00000574727 & ENSMUST00000123752) as our further research object. ENST00000574727, human LncRNA, also known as ALOX12P2-003 or ALOX12E, is located at Chromosome 17 forward strand, with a length of 4326 bp. ENSMUST00000123752, mouse LncRNA, also known as Alox12e-002, Alox12-ps1; Alox12-ps2, is a 793 bp LncRNA, located at Chromosome 11 reverse strand. The functions of them have not been discovered and confirmed. We observe that they have the same down-regulated trend as JNK mRNA. Then we try to analyze their functions through a ceRNA mechanism analysis and match complementary binding sites. Finally, we found a possible target for LncRNA ENSMUST00000123752 through ceRNA mechanism analysis. In recent years, miR-128-3p has been proved to be associated with myocardial infarction and myocardial ischemia-reperfusion injury. Liu et al. found that Selenomethionine attenuates LPS-induced myocardial inflammation and oxidative stress through miR-128-3p/p38MAPK/NF-κB pathway, also clarified the negative regulation relationship between miR-128-3p and p38MAPK[21]. *Our ceRNA mechanism analysis shows that there are complementary paired sequences between miR-128-3p and JIP3 mRNA, and the JNK pathway is an important part of the MAPK pathway. In the differential expression profile of mRNA, we observe that JIP3 may be down-regulated through negative regulation, thus affecting the phosphorylation activation of JNK, but further regulation of LncRNA and miR-128-3p is needed to confirm our hypothesis.*

Our analysis of ceRNA mechanism shows that there are complementary paired sequences between miR-128-3p and JIP3 (Mapk8ip3) mRNA. JNK pathway is an important part of MAPK pathway, and in the differential expression profile of mRNA, we observe the down-regulation of JNK3. JIP3, as the direct substrate of JNK[22], *plays an important role in the activation of JNK pathway. Ma et al found that silent JIP3 can reduce myocardial hypertrophy by inactivating JNK*[23]. *Yin et al used JIP3 siRNA to reduce the expression of JIP3 and found that the level of MA2K7 / p-JNK / cleaved caspase-3 subsequently decreased*[24] *and the activation of JNK pathway was suppressed. Interestingly, the predicted binding site of LncRNA ENSMUST00000123752 and miR-128-3p was the same as that of JIP3 mRNA and miR-128-3p, in other words, there may be competitive adsorption of miR-128-3p between LncRNA ENSMUST00000123752 and JIP3 mRNA. Therefore, we can establish a new hypothesis of RIPC and then through the LncRNA/miR-128-3p/JIP3 mRNA ceRNA mechanism to inhibit the activation of JNK pathway to reduce MIRI, but we need further experimental demonstration in gene knockout animals, which is what we are now working on.*

The result of the study of microRNA, which is also one kind of non-coding RNA, is that anti-microRNA therapy has been successfully and safely used in phase II clinical trials of hepatitis[25]. *We have reason to believe that through in-depth study of the role of LncRNA in RIPC and understanding the its mechanism of LncRNA, the development of targeted therapy for cardiovascular diseases undergoing MIRI will be greatly promoted.*

Conclusion

In summary, we identifed and validated a number of differentially expressed LncRNAs, which may be closely related to MIRI. After qRT-PCR, we selected several LncRNAs with stable expression and significant FC, includeing homologous LncRNAs such IPCRL1 and IPCRL2. It suggests that IPCRL1 may mediate MIRI by targeting JIP3 through a ceRNA regulation mechanism. As the next research object, this hypothesis still needs to be proved by further experimental research. By continuously exploring the function and mechanism of these LncRNAs, we hope to unveil the mystery of RIPC and discover a new protective mechanism of cardiovascular ischemia-reperfusion disease.

Abbreviations

RIPC: Remote ischemic preconditioning; LncRNA:Long noncoding RNA; MIRI:Myocardial ischemia-reperfusion injury; I/R:ischemia-reperfusion; IRI:ischemia-reperfusion injury; ELISA:enzyme linked immunosorbent assay; qRT-PCR:Quantitative real-time polymerase chain reaction; CeRNA:Competing endogenous RNA; FC:fold change; AAR:Area at risk; INF:Infarct area; HE:hematoxylin-eosin; GO:Gene ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; NCBI:National Center for Biotechnology Information; FDR:false discovery rate; IPCRL1:Ischemic preconditioning-related LncRNA1; IPCRL2:Ischemic preconditioning-related LncRNA2; JIP3:JNK-interacting protein3;

Declarations

Availability of data and materials

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

Ethics approval and consent to participate

The human procedures are approved by Ethics Committee of The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University. Animal procedureswere approved byWenzhouMedical University Animal Care and Use Committee,which are certified by the Chinese Association of Accreditation of Laboratory Animal Care and were consistent with the Guide for the Care and Use of Laboratory Animals(updated [2011] version of the NIH guidelines).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

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Authors’ contributions

Qifeng Zhao conceived of the study, and participated in its design and coordination and helped to modificated the manuscript. Qifeng Zhao, Weijia Wu, Hanwei Ge, Zhiyong Lin and Hanlei Wang complete doctor-patient communication, sign the informed consent form, perform the operation and collect samples of children patients, provide postoperative recovery treatment and free postoperative imaging examination. Zhiling Lou, Jiyang Xue performed the animal model experiments and collected the samples. Jiyang Xue performed RNA extraction and purification, Zhiling Lou performed qRT-PCR and statistical analysis then drafted the manuscript. Hanlei Wang, Ruiheng Chen and Haochun Shi participated in the statistical analysis. Zhiling Lou and Jie Xia prepared all figures. All authors read and approved the final manuscript.

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Tables

Table1: Number of differentially expressed LncRNAs

LncRNAs	Spacies	FC2-5	FC5-10	FC≥10	Total
Up	Mouse	247	24	10	281
Down	Mouse	239	29	5	273
Up	Human	617	13	5	635
Down	Human	731	21	5	757

Table2: Top10 differentially expressed LncRNAs and mRNA after RIPC in Human and Mouse

Sepuence name	Gene symbol	FC
Top10 Up-regulated human LncRNAs		
ENST00000507926	CTD-2306M5.1	61.2128103
NR_028308	BRE-AS1	30.9319247
ENST00000564531	RP11-1100L3.8	14.7943499
uc001jpa.1	BC022313	14.3805754
NR_026860	LINC00473	12.6209272
TCONS_00019322	XLOC_009167	9.7478118
ENST00000457058	RP11-109I13.2	9.2358625
ENST00000417650	RP11-439L18.1	8.5209731
ENST00000508443	CTB-47B11.3	7.243086
TCONS_00017313	XLOC_008149	7.0343197
Top10 Up-regulated human mRNAs		
ENST00000338488	NR4A3	350.7761867
NM_005252	FOS	264.2330283
NM_173200	NR4A3	92.7188392
NM_001114171	FOSB	62.3703608
NM_006732	FOSB	58.6511585
NM_002089	CXCL2	42.6043755
ENST00000307407	IL8	29.8900665
ENST00000294829	FAM71A	22.987126
NM_000450	SELE	21.876733
NM_001174090	SLC4A11	20.5018814
Top10 Down-regulated human LncRNAs		
uc001ukd.1	AX747752	19.4261082
uc001tdk.2	KRT19P2	15.7030871
ENST00000519609	RP11-32D16.1	13.8072969
ENST00000552167	RP11-359M6.1	13.1343691
ENST00000431401	RP1-290I10.5	10.7059655

uc003cmy.1	DQ592230	9.8065073
NR_024089	LINC00162	9.4575664
ENST00000464537	RP11-420J11.2	8.9155883
ENST00000581210	KRT16P1	8.2424453
chrX:136212300-136224525+	chrX:136212300-136224525	7.8726809
Top10 Down-regulated human mRNAs		
NM_001083537	FAM86B1	57.6879463
NM_005823	MSLN	17.0867096
NM_016352	CPA4	15.2902808
NM_002276	KRT19	13.1581887
NM_001126102	HP	13.1148645
NM_005143	HP	12.4122693
ENST00000322165	HSD17B6	12.0160265
NM_005556	KRT7	11.9192372
NM_013404	MSLN	11.5713044
NM_017625	ITLN1	10.5037021
Top10 Up-regulated mouse LncRNAs		
ENSMUST00000136102	Plekhm2	54.2768466
NR_045285	Gm5083	39.6142257
BG068257	mouselincRNA1118	19.6325586
ENSMUST00000153836	Asxl3	16.6523791
BF180946	mouselincRNA0005	16.1865307
TCONS_00032441	XLOC_024323	14.4913109
ENSMUST00000175707	Vmn1r-ps142	12.7990311
TCONS_00004283	XLOC_003982	11.4696996
mouselincRNA0499-	mouselincRNA0499	11.1981243
uc007tsn.1	Tcrd	10.8516461
Top10 Up-regulated mouse mRNAs		
NM_009341	Tcp10b	16.1522272

NM_011325	Scnn1b	13.3807131
NM_153524	Mrgpra4	9.0141778
NM_146334	Olfr1330	8.9121771
NM_001081449	Vmn2r54	7.8662994
NM_011456	Serpinb9e	7.587836
NM_001099301	Obp2b	7.2571405
NM_001159422	Ccdc58	6.7617881
NM_146789	Olfr1230	5.6599084
NM_001005748	Phactr1	5.4333252
Top10 Down-regulated mouse LncRNAs		
ENSMUST00000173563	Gm2302	43.4009886
AK017761	AK017761	16.2941162
mouselincRNA0933-	mouselincRNA0933	13.2961955
ENSMUST00000152439	Gm14009	11.5085008
TCONS_00004249	XLOC_003927	11.1955942
uc009qiv.2	DQ690356	9.9517056
ENSMUST00000152128	Gm16080	9.6625515
NR_045965	1700084F23Rik	9.6499683
ENSMUST00000157528	Gm26348	9.6431601
ENSMUST00000123663	Gm13707	9.5857546
Top10 Down-regulated mouse mRNAs		
NM_177855	Med12l	28.4145113
NM_001163028	Bcmo1	27.8440333
NM_147038	Olfr1416	19.690464
NM_010199	Fgf12	16.3330758
NM_008081	B4galnt2	12.6905967
NM_010990	Olfr48	12.6787376
NM_001166375	March1	11.8556742
NM_053015	Mlph	11.3830897

NM_001081153	Unc13c	8.8654521
NM_009411	Tpbpa	7.2739333

Table3: Detected Homologous LncRNAs

No.	Sequence name	Regulation	FC	Spacies
1	NR_026778	Up	2.7203589	human
	ENSMUST00000154437	Up	1.0567851	mouse
2	Uc010rog.2	Up	2.1087689	human
	NR_003513	Up	1.0640434	mouse
3	ENST00000446406	Up	2.2950602	human
	NR_001592	Up	1.3577539	mouse
4	ENST00000527314	Up	1.5297628	human
	ENSMUST00000163913	Up	9.7726336	mouse
5	NR_037719	Up	3.1086571	human
	NR_033133	Up	5.9252023	mouse
6	ENST00000565841	Down	3.9193896	human
	AK050947	Down	8.2915752	mouse
7	NR_073081	Down	2.5207779	human
	AK158081	Down	1.2406486	mouse
8	ENST00000509649	Down	2.3379888	human
	NR_002928	Down	1.1492311	mouse
9	uc001tdk.2	Down	15.7030871	human
	ENSMUST00000126460	Down	1.0307194	mouse
10	ENST00000574727	Down	7.2125214	human
	ENSMUST00000123752	Down	1.1493588	mouse
11	NR_037845	Down	1.0515052	human
	AK133331	Down	4.1421506	mouse

Table4: Primers of selected LncRNAs

Sequence name	Forward （5'-3'）	Reverse （5'-3'）
uc010rog.2	TGACTGACTATGACTGCTGAG	TGCTATCTTCCTGTTGCCATT
ENST00000566457	TCTGCTCGGCTCTGTCAT	GGCTCTTGGTTATGGTTCTTG
ENST00000509649	GCACATTTGACTTGGAAGCA	CGCCCAGCCAACATTCTT
ENST00000527314	TAGGAGATCGGGACCAGCTT	GTGCGGACAACCTCAGCTAC
NR_037845	GTGGCGTGATGTCTGCTTA	GAGGCTGAAGTAGGCGAATC
ENST00000574727	CCTGTTCTGTGTATTGCTTCC	CTGACTCTCCTTGCTACCATT
ENSMUST00000123752	AAGACTGCTCTGCCTGGT	GCTCTGAAGACATCCGTTGA
NR_040512	GCAGGAATATGGAGCACAGT	ACTTCAGGAGACAGTAACAGAC
ENSMUST00000173563	TCAGAGTGATTCAGACAGTGTT	AAGGTCCAGCCAGCAGAT
ENSMUST00000154036	CTGGCTGAACTGGAACTTAGA	CCAAGGAACCTGACATCTTCT
AK133331	TGGTGCTTATCTAACTGGATGT	TGCTGTGGTCTCGTCTTAC
ENSMUST00000148722	GTGTCTCCTGTACTGACTTGT	CTTCGCCATCCATCCTGAA
TCONS_00003974	TCATGGTTCTTCAGGCTGTATA	ATGCTGGCTCCTCTTCTCT
NR_001592	GAGACTCAAAGCACCCGTGA	AGATGGACGACAGGTGGGTA
ENST00000446406	GGAGCACCTTGGACATCT	CCTACTCCACACTCCTCAC
NR_033133	CCAACAGCCATCTCCGTAG	AGACAGACACAGAAACAAAGAC
NR_037719	GCTCACTGACACCGTTTG	TCATTGGCTTCCAGGACTG
ENSMUST00000130038	CCTTACTCCCTGCCCTTCT	ATCCACTTGTCTCTGCTTCTC
ENSMUST00000134981	TGCTGCTTCCTGTTGTGT	TGTTCTTCCTTCGTCTATATGG
NR_046186	ACTTACCCTTGAAGAGGAAATG	CTCATCAGTGCTATCATCATCA
TCONS_00023846	GATGTAATTGTATGCGACCTG	AACCTCTCAATCCCAGATTT
TCONS_00019322	AGCAGAAGCAACAGGTCAA	ACGGAGTAAGCAAGTCACAA
ENSMUST00000136102	AGCAGGTGCAGTTGAGTG	GGAACTTGTTATGTAGCAGAGG
ENSMUST00000128411	AGAAGTGTTATTGCCACCAGAT	TGCTGACCAGGATAGACCAA
AK050947	TTGGTATGAAGGTGTGAGAGG	TGGAGGTCTGTAGCACTGTA
NR 073081	AGGACTGGCACAAGTTCTG	TTCTCAGCGAAGTACACCTT
AK158081	TGAGAGGCAGGCAAGGAA	TAGCGGTGGAGTGGTCAA
uc001tdk.2	AGCCACTACTACAGGACCAT	GCCAGTTGAGCATTGTTGAT

ENSMUST00000145313	AATGCTGTCTCACCTACCAT	CAGAGTAGATGCTTGAAGGAG
ENSMUST00000146263	CTGTGGCTGAAGAGTCTCC	CAATTCTGGTGATGATGTCTGA
ENST00000420279	AACCACATGAAGCAACTTAGC	TCGGTAGACTCCAAGCAGAA
NR_026778	GGCGGCAAGACAGACATT	CCTTCTAGGTATCACCTCATCC
ENSMUST00000154437	AAAGCCCTTCAAGCTCCTAG	TCCAAGTCCAGTCACAATAGAT
NR_003513	GGCAGAGCAGCAGTTGAT	GACCTCCACTACGCACCTA
NR_002928	GCTGTCACCATTGCTTGTT	TCCTAGAACTGCCAACCATC
AK133331	TGGTGCTTATCTAACTGGATGT	TGCTGTGGTCTCGTCTTAC

Figures

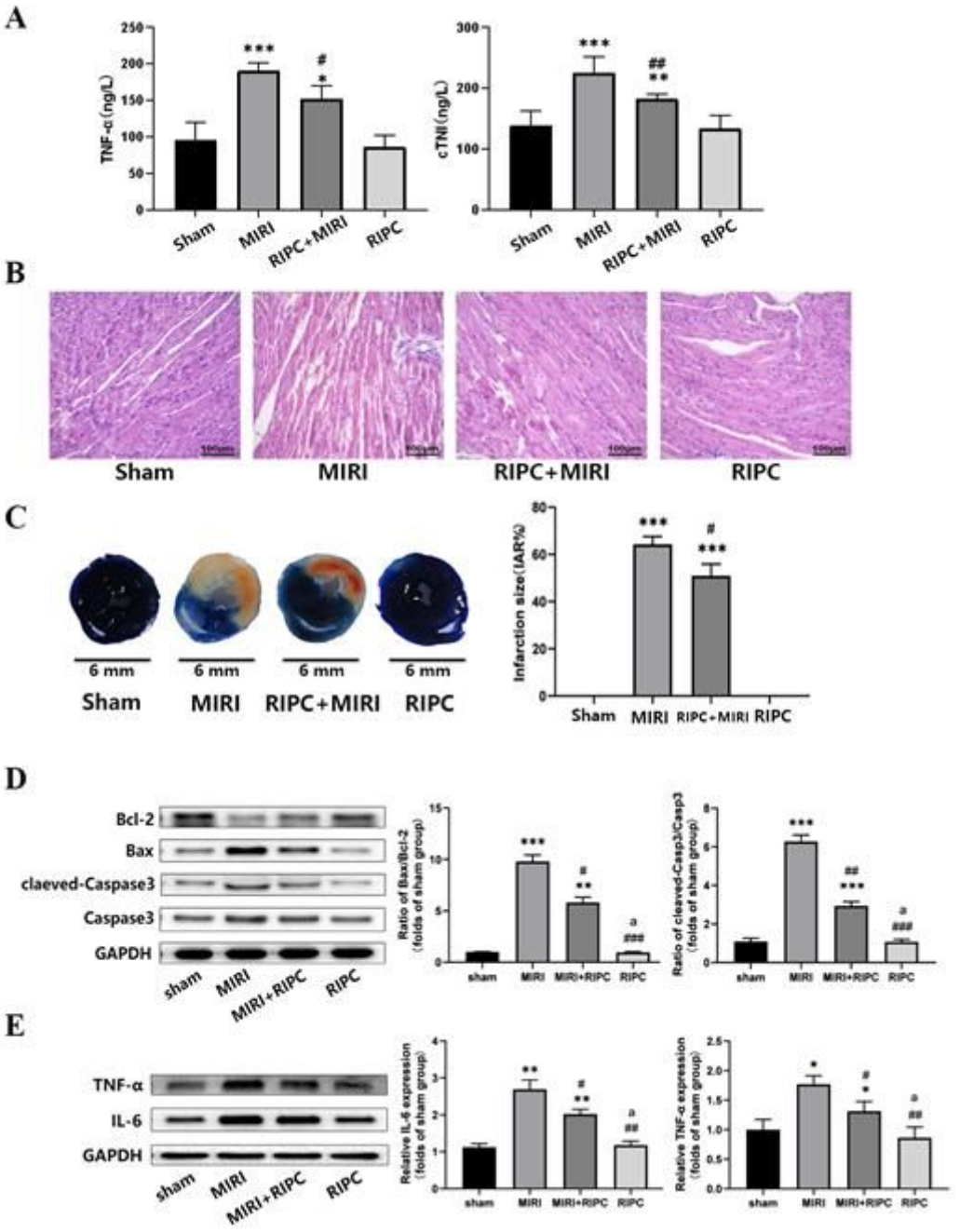

Figure 1

Verification of the protective effect of ischemic preconditioning in vivo. ELISA results of TNF-α and cTNI show RIPC attenuates myocardial injury after ishemia/reperfusion(A),meanwhile Representative Western blot show that RIPC reduces apoptosis and inflammation after MIRI (D, E), RIPC also attenuates harmful myocardial structural changes(B) and myocardial ischemic infarction(C).“*” means compared to Sham group, “#” means compared to MIRI group, “a” means compared to RIPC+MIRI group. *P□0.05,**P□0.01,***P□0.0001.

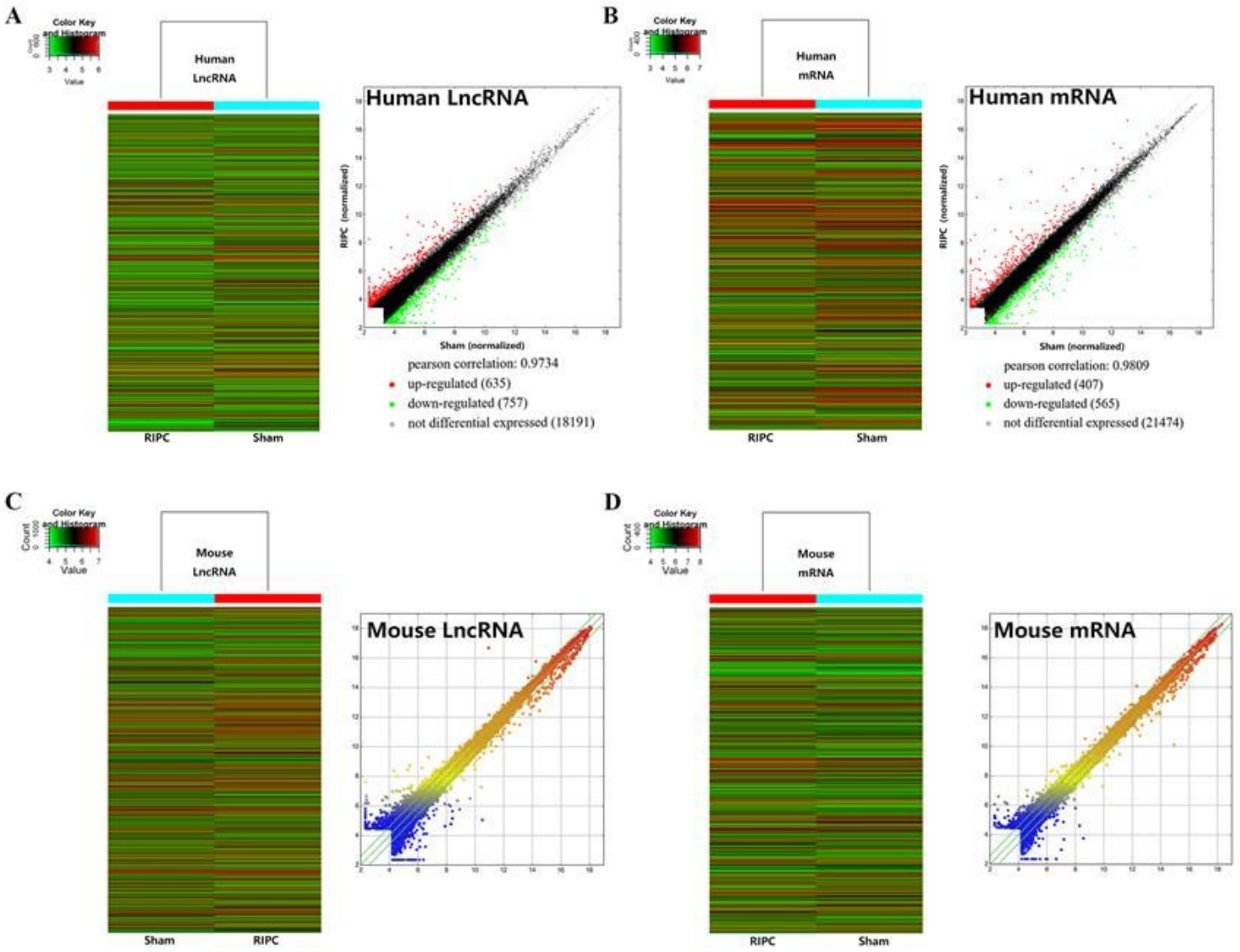

Figure 2

LncRNA and mRNA expression profile comparison between the RIPC group and the Sham group in both human and muose. The hierarchical clustering of all target value human lncRNAs(A) and mRNAs (B). The scatterplot is used for assessing human lncRNA(A) and mRNA(B) expression variation between the RIPC group and the Sham group. The hierarchical clustering of all target value mouse lncRNAs(C) and mRNAs (D). The scatterplot is used for assessing mouse lncRNA(C) and mRNA(D) expression variation between the RIPC group and the Sham group. Green lines inside scatterplot picture are fold change lines (the default FC value is given 2.0). LncRNAs and mRNAs above the top green line are detected up-regulated and below the bottom green line are detected down-regulated, both of them are indicated FC value >2.0 in expression. For example, “red dots” stand for higher relative expression levels(A), and “green dots” stand for low relative expression levels(B).

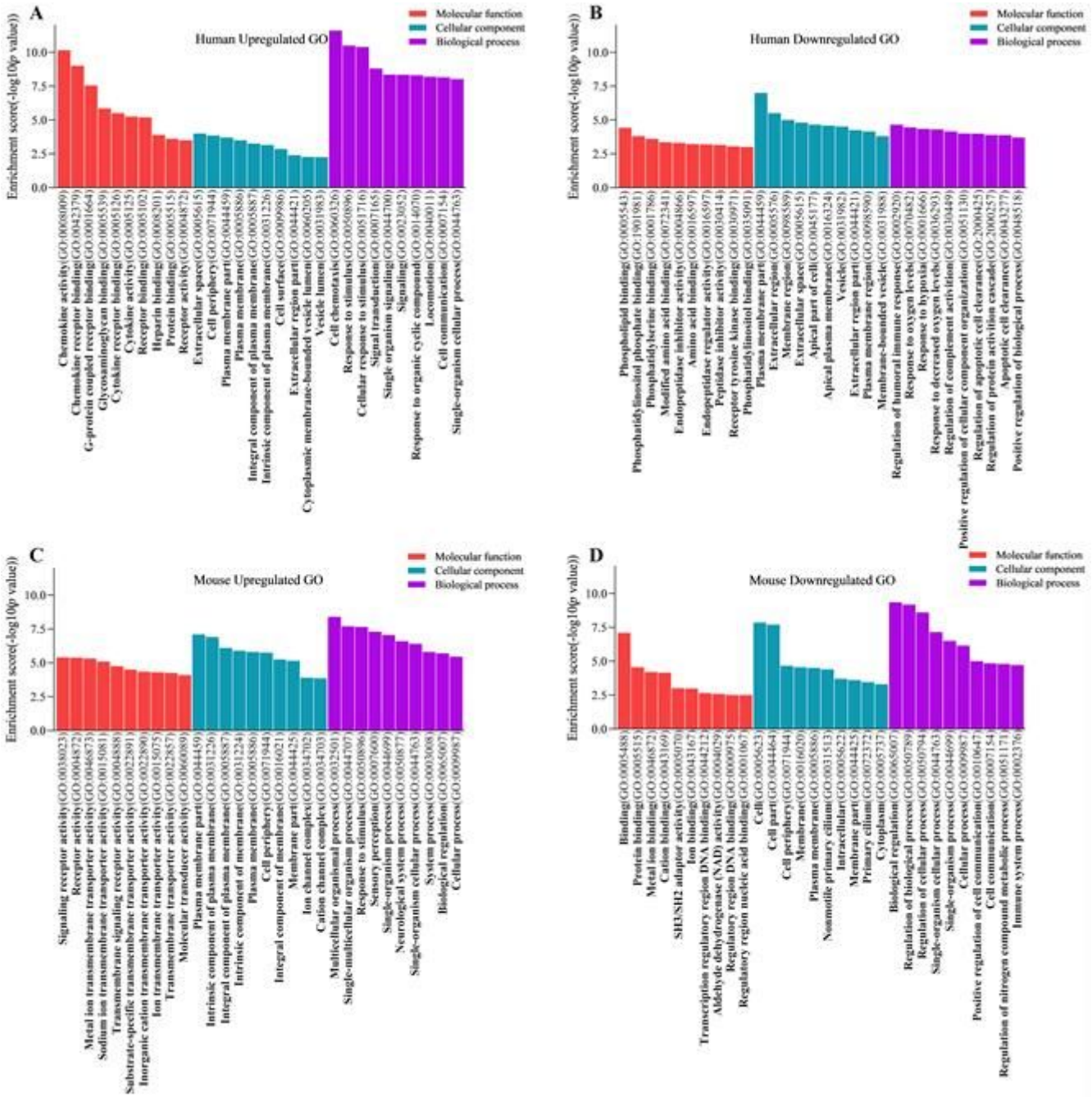

Figure 3

Gene Ontology (GO) analysis of functional classification of the differentially expressed LncRNAs. The GO categories contain 3 domains: biological process (BP), molecular function (MF), and cellular component (CC). The up-regulated GO of human(A), the down-regulated GO of human(B), the up-regulated GO of mouse(C), the down-regulated GO of mouse(D) are arranged in turn. P value denotes the significance of GO term enrichment in the differentially expressed protein-coding RNAs list. Lower the P value is, more significant the GO term is (p value ≤ 0.05 is recommended).

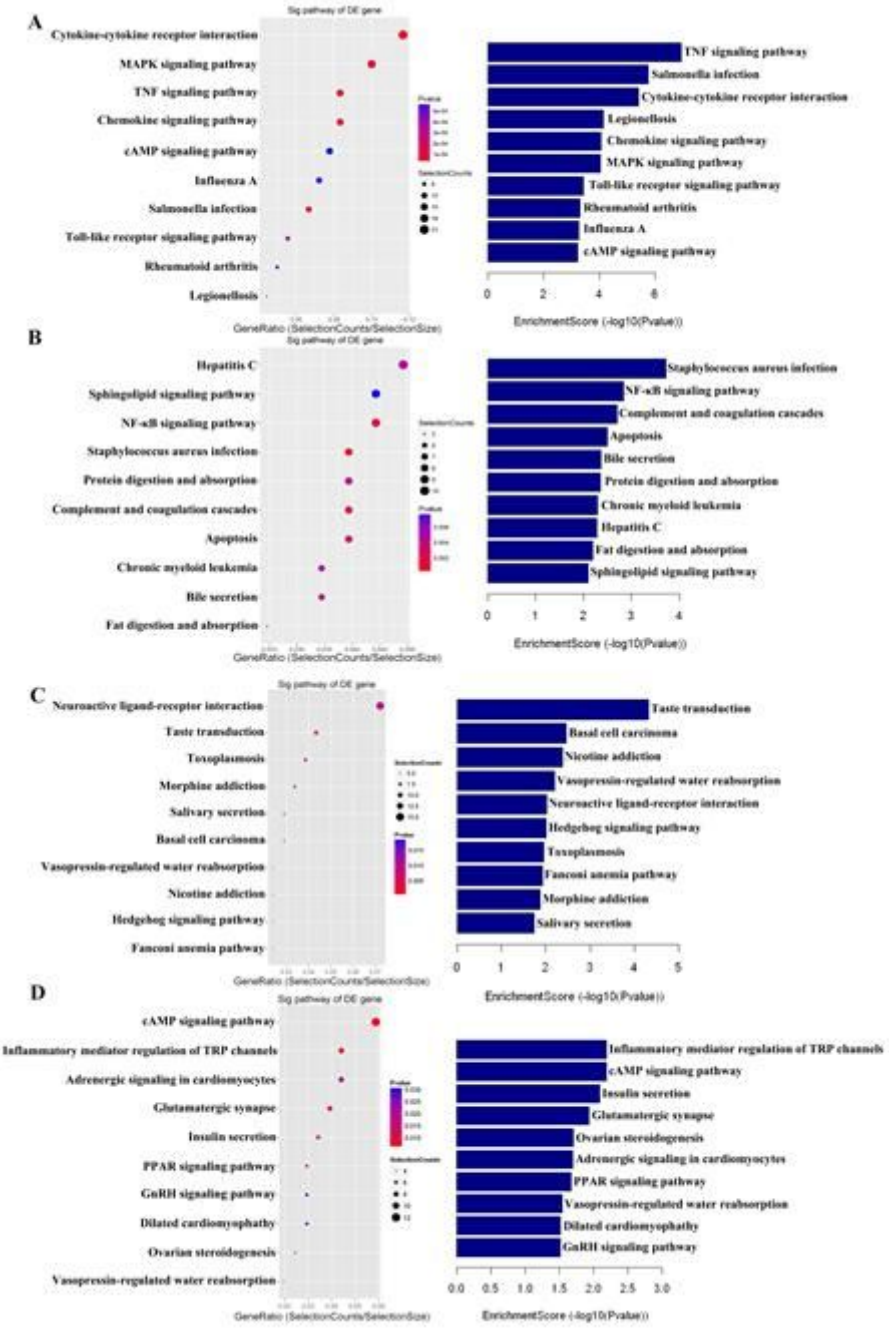

Figure 4

Pathway enrichment analysis. (A, B) The up-regulated gene and down-regulated gene pathway of human. (C, D) The up-regulated gene and down-regulated gene pathway of mouse. This figure shows the top 10 significant pathways of up-regulated and down-regulated genes. The P value (Fisher P value) denotes the significance of the pathway correlated to the conditions. The lower the p value is, the more significant the pathway (the recommended P value cut-off is 0.05). Size of selection counts point means number of corresponding genes. The larger the point is, the more genes involved in this pathway.

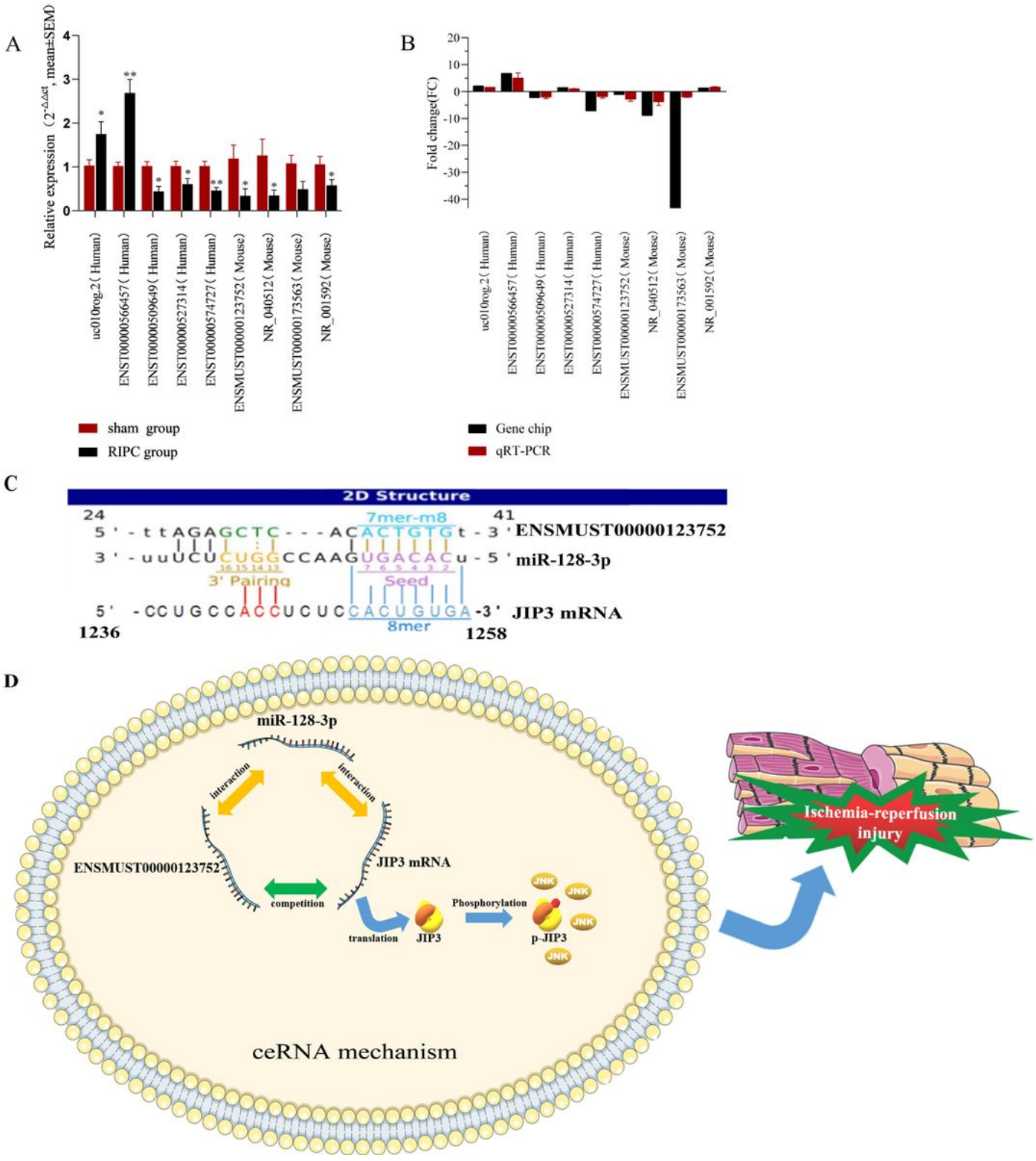

Figure 5

qRT-PCR validation of differential expressed lncRNAs. 8 LncRNAs confirmed by qRT-PCR show to have significant changes between RIPC group and sham group(A). Data are expressed as the mean ± Standard Error of Mean (SEM) of 3 times independent experiments (*P□0.05,**P□0.01). (B) qRT-PCR patterns of 9 lncRNAs are consistent with those of microarray data. Competing binding site is predicted by ceRNA mechanism analysis(C) and interaction is shown in conception map(D).