Altered myocardial gene expression profiling in the ischemic tissues at different time points after cardiac ischemia/reperfusion in rats

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Keywords: *cardiac ischemia/reperfusion injury; differentially expressed genes; lncRNAs; real-time quantitative PCR*

Received: *October 07, 2017* ***Accepted:*** *November 13, 2017* ***Published:*** *January 03, 2018*

ABSTRACT

We used Agilent Gene Expression microarray to analyze differential gene and lncRNA expression patterns in the myocardial ischemia regions during ischemia/reperfusion (I/R)-induced cardiac injury in rats. Male SD rats were assigned into control group, 2 h group (30 min ischemia followed by 2 h reperfusion), 0.5 h (30 min ischemia followed by 0.5h reperfusion) group. We observed that of 18090 lncRNAs, an average of 233 lncRNAs was up-regulated in ischemic tissues of 2 h group, compared with those in control group, while an average of 6115 lncRNAs was down-regulated (with a > 2.0 fold-change and $p < 0.05$). Further, a total of 3135 mRNAs were differentially expressed between control group and 2h group, in which 542 mRNAs were up-regulated and 2593 mRNAs were down-regulated. Some differentially expressed genes were validated by qRT-PCR analyses of select lncRNAs in different time points after cardiac I/R injury. We unveiled that the expressions of lncRNA XR_345533.2, NONRATT025386, NONRATT024318, XR_599241.1, and NONRATT025509 were significantly up-regulated in 2h group compared with control group and 0.5h group, whereas the expression of lncRNA NR_130708.1 was down-regulated after cardiac I/R injury and had no statistically different between 0.5h group and 2h group. Otherwise, the expressions of lncRNA NONRATT028627, NONRATT021959, XR_590005.1 and NONRATT023191 were significantly up-regulated in 2h group compared with 0.5h group. These findings provide evidence for differential expression patterns of mRNAs and lncRNAs in the ischemic tissues after cardiac I/R injury in rats.

INTRODUCTION

Cardiomyocyte death due to ischemia/reperfusion (I/R) injury is well-known to increase morbidity, mortality and medical cost in patients with ischemic heart disease [1–4]. Treatment for this myocardial ischemia-reperfusion disorder and cardiomyocyte death has had limited success. The pathological process leading to cardiomyocyte death is very complicated and our understanding of the mechanisms that underlie the induction of cardiomyocyte death is incomplete [5, 6]. IRI-induced cardiomyocyte death is thought to be triggered by abnormal changes

in gene transcription and translation [7–10]. Data from an increasing number of studies have indicated that IRI dysregulates expression of mRNA and protein for the receptors, enzymes and ion channels in the heart, a phenomenon that may contribute to the induction of cardiomyocyte death [11–14]. However, the molecular mechanisms that underlie this regulation for myocardial ischemia-reperfusion disorder are still unknown.

Recently, long non-coding RNAs (lncRNAs) have become an increased research focus that plays critical roles in many biological processes [15–20]. Recent studies suggest that lncRNAs are often accompanied by important regulatory functions in the heart [21–23]. However, the lncRNAs study for heart is still in its infancy, and the signature and roles of differential gene and lncRNAs expression in the I/R-induced cardiomyocyte death have received relatively little attention. Otherwise, the details regarding differential gene and lncRNAs expression involvement in ischemia myocardial tissues at different time points are unknown. In this study, we tested the differential gene and lncRNAs expression profiles in the myocardial tissues following myocardial ischemia-reperfusion injury in rat and investigated the possible roles of these differential gene and lncRNAs at different stages of ischemia-reperfusion.

RESULTS

Aberrant lncRNA expression in the ischemic tissues 2h after reperfusion

In order to select out possible targets of lncRNAs between model and control group, up to 18090 coding transcripts were detected in the ischemic tissues of the heart at 2h after reperfusion. An average of 233 lncRNAs was up-regulated in ischemic tissues, compared with those in control group, while an average of 6115 lncRNAs was down-regulated (with a > 2.0 fold-change and $p < 0.05$). The distributions of the log2 ratios of lncRNAs between model and control samples were nearly identical. Figure 1 showed the heat maps of the expression ratios (log2 scale) of lncRNAs in the ischemic tissues. The top 20 up-regulated and down-regulated lncRNAs are listed in Tables 1 and 2.

Aberrant mRNA expression in the ischemic tissues after reperfusion

To identify altered genes that may contribute to myocardial I/R injury, we conducted mRNA profiling experiment on the ischemic tissues. The mRNA expressions of the ischemic tissues in the rats were examined using Agilent Rat Gene Expression microarrays that include 27,006 probe sets. The gene expression profiles in I/R group were compared with the corresponding data of control group. We identified that a total of 3135 probe sets were differentially expressed in two groups (with a > 2.0 fold-change and $p < 0.05$), in which 542 probe sets were up-regulated and 2593 probe sets were down-regulated. Figure 2 showed the heat maps of the expression ratios (log2 scale) of mRNAs. The top 20 up-regulated and down-regulated mRNAs from the myocardial ischemic regions are listed in Tables 3 and 4.The maximal and minimal fold change was 25.97 and 2.0, respectively.

Gene ontology annotation for differential expression genes

The differentially expressed genes identified by Agilent Gene Expression microarrays were analyzed by Gene Ontology (GO) annotation. Figure 3 showed the significant enriched GO Terms (Top 30) including three biological functional groups (biological process, cellular component and molecular function). We found that the differentially expressed genes from the myocardial ischemic regions were primarily involved in sensory perception, neurological process, G-protein coupled receptor signaling pathway (Figure 3).

KEGG Pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the differentially expressed genes were performed to identify the molecular changes of the myocardial ischemic regions. Figure 4 showed the significant enriched pathway Terms (Top 30) primarily involved in KEGG pathway including olfactory transduction, neuroactive ligand-receptor interaction, cytokine-cytokine receptor interaction, retinol metabolism, and steriod hormone biosynthesis. Figure 5 showed the differential expression genes were analyzed with GO background significant enrichment. The top five enriched GO biological processes included single-organism process, metabolic process, response to stimulus, biological regulation, and cellular aggregation. The most enriched GO cellular components included extracellular region part, macromolecular complex, membrane-enclosed lumen. The enriched GO molecular functions were catalytic activity, transporter activity, and molecular transducer activity.

Real-time quantitative PCR (RT-qPCR) validation of lncRNAs expression in the myocardial tissues 2h after cardiac I/R injury

To validate the reliability of the microarray results in rats, we analyzed these differentially expressed (DE) lncRNAs, including 5 up-regulated lncRNA and 5 down-regulated lncRNA by RT-qPCR. The ischemic tissues were collected from control group and I/R group (2h after reperfusion). Five up-regulated lncRNAs, including XR_345533.2, NONRATT025386, NONRATT024318,

XR_599241.1, and NONRATT025509 were significantly increased in I/R group compared with control group, whereas one down-regulated lncRNA, NR_130708.1 was significantly decreased (Figure 6). RT-qPCR results of four lncRNA, including NONRATT028627, NONRATT021959, XR_590005.1 and NONRATT023191 were not consistent with data from microarray.

The expression of 10 lncRNA was analyzed in the cardiac tissues at different time points (0.5 h/2 h) after cardiac I/R injury

It is well-known that gene expressions are varied in different time points. We collected myocardial tissue samples 0.5h vs 2h after cardiac I/R injury for RT-qPCR validation. Our results indicated that the expressions of lncRNA XR_345533.2, NONRATT025386, NONRATT024318, XR_599241.1, and NONRATT025509 were significantly up-regulated in 2h group compared with 0.5h group, whereas the expression of lncRNA NR_130708.1 had no statistically different between 0.5h group and 2h group (Figure 6, Figure 7 and Figure 8). Otherwise, the expressions of lncRNA NONRATT028627, NONRATT021959, XR_590005.1 and NONRATT023191 were significantly up-regulated in 2h group compared with 0.5h group.

The expression of 6 lncRNA was analyzed in the cardiac tissues 0.5 h after cardiac I/R injury

Our results indicated that the expression of lncRNA NONRATT025386 was significantly up-regulated in

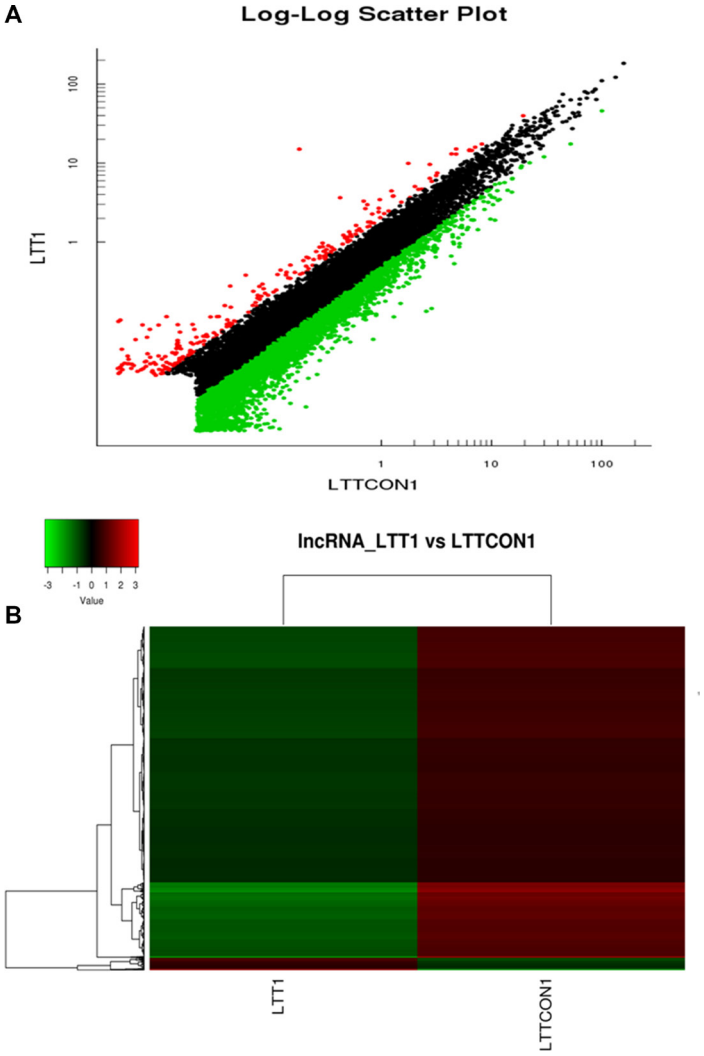

Figure 1: Differential expression lncRNA in the myocardial tissue from the cardiac I/R model. (**A**) Scatter plot for comparing global lncRNA expression profiles in myocardial tissue between the cardiac I/R and control rat. Red color is indicative of up-regulated and blue color of down-regulated genes. Black color of is indicative of not statistical significant difference when it do not pass the cutoff values of 1 and -1 in $\log_2$ scale and $p < 0.05$. (**B**) Heat map showing hierarchical clustering of lncRNA whose expression changes were more than twofold. In clustering analysis, up-and down-regulated genes are colored in red and green, respectively. LTT1 stands for Model group; LTTCON1 stands for control group.

0.5h group compared with control group, whereas the expressions of lncRNA NR_130708.1, NONRATT028627, NONRATT021959, XR_590005.1 and NONRATT023191 were significantly down-regulated in 0.5h group compared with control group (Figure 6 and Figure 7).

DISCUSSION

Cardiomyocyte death due to ischemia/reperfusion injury (IRI) is a great clinical problem. Despite multiple therapeutic strategies, the medical community still faces a challenge to treat cardiac ischemia/reperfusion injury in a complete and definitive way, since the pathogenesis of this ischemia/reperfusion state is very complex. It is essential to discover impactful therapeutic targets by elucidating molecular mechanisms of cardiomyocyte death. Our present work demonstrated many differentially expressed genes, pathways and biological processes in the cardiac ischemic tissues after cardiac I/R injury. By Agilent Rat Gene Expression microarrays, we identified 27,006 mRNAs in the cardiac ischemic tissues from two groups, of which 542 up-regulated and 2593 down-regulated mRNAs (with a > 2.0 fold-change and $p < 0.05$) were identified in response to cardiac ischemia. Similarly,

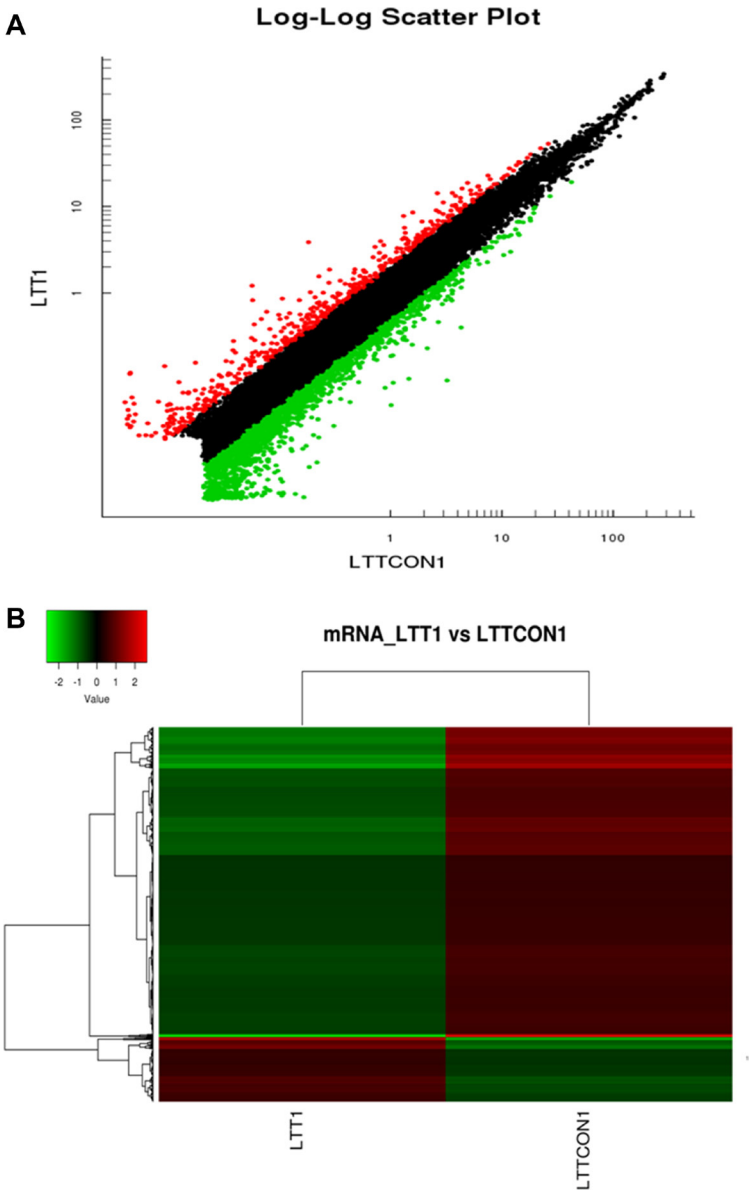

Figure 2: Differential expression genes (mRNA) in the myocardial tissues from the cardiac I/R model. (**A**) Scatter plot for comparing global mRNA genes expression profiles in myocardial tissue between the cardiac I/R and control rat. Red color is indicative of up-regulated and blue color of down-regulated genes. Black color of is indicative of not statistical significant difference when it do not pass the cutoff values of 1 and -1 in $\log_2$ scale and FDR (corrected p-value) < 0.05. (**B**) Heat map showing hierarchical clustering of mRNA whose expression changes were more than twofold. In clustering analysis, up-and down-regulated genes are colored in red and green, respectively. LTT1 stands for Model group; LTTCON1 stands for control group.

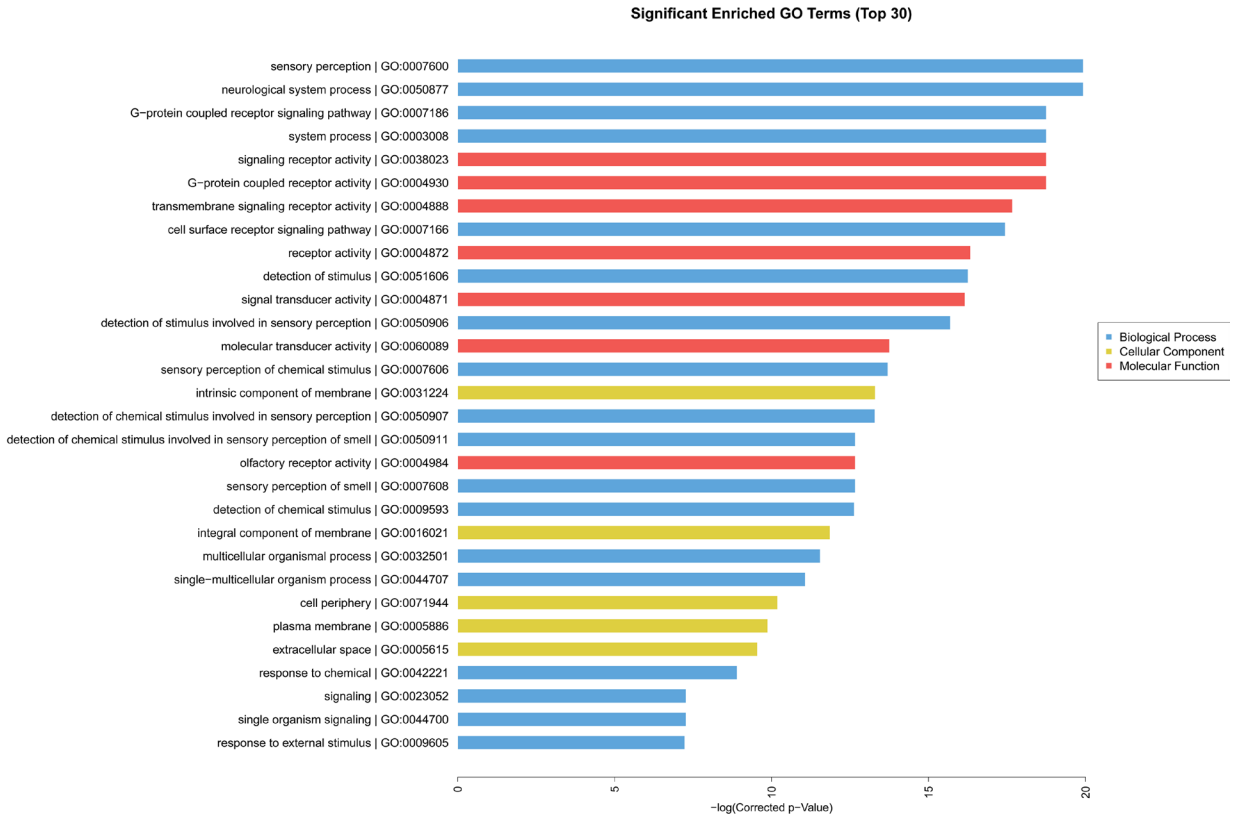

Figure 3: The significant enriched GO Terms (Top 30) were analyzed by Gene Ontology (GO) annotation. Blue bar represents biological process classification; Yellow bar represents cellular component; and Red bar represents molecular function.

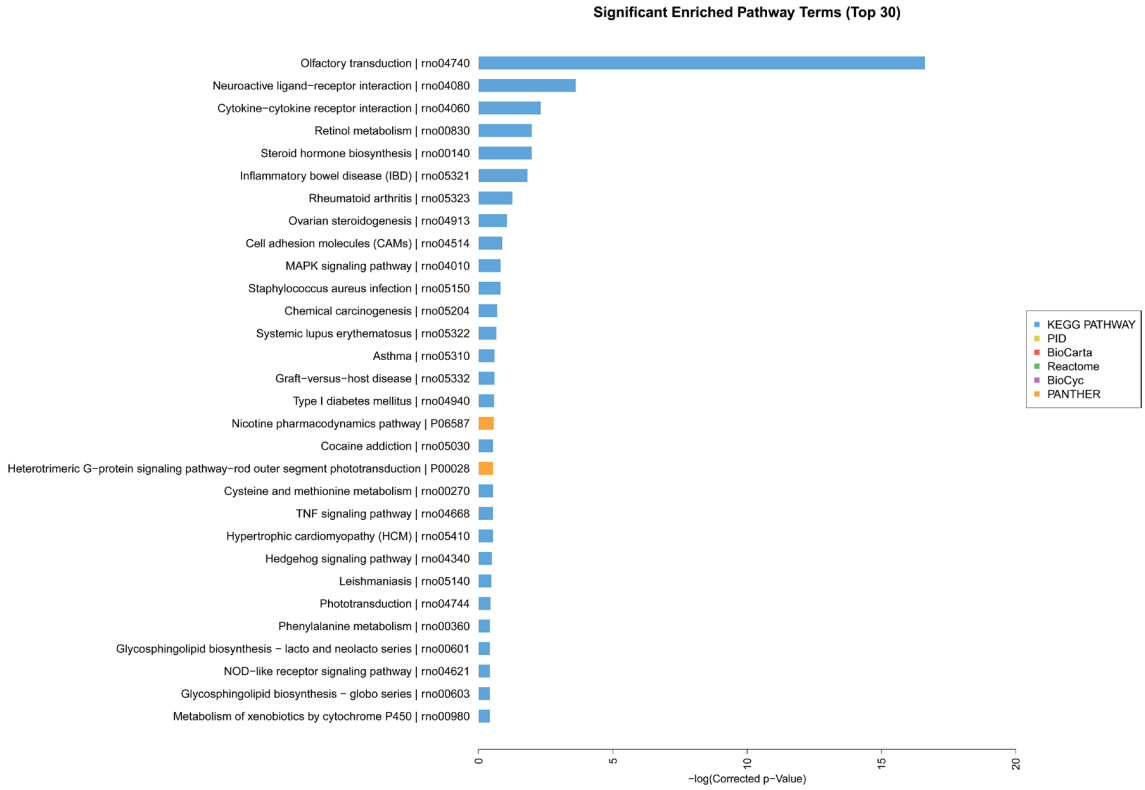

Figure 4: The significant enriched pathway Terms (Top 30) were analyzed by Gene Ontology (GO) annotation. Blue bar represents KEGG pathway and Yellow bar represents Panther.

Table 1: The detail information of the top 20 up-regulated lncRNAs in the ischemic region at 2 h after reperfusion

Lnc RNAs (seqname)	fold change (R/N)	RNA length	Chromosome
NONRATT025386	214.8505	563	6
NONRATT016113	60.64338	900	2
NONRATT018298	44.94348	520	3
NONRATT018300	44.80856	1134	3
NONRATT020994	41.33676	206	4
NONRATT004402	40.78394	546	10
NONRATT028525	31.02353	469	8
NONRATT001626	27.2879	607	1
gi|672028135|ref|XR_594134.1|	20.66773	1408	14
gi|672026098|ref|XR_602081.1|	20.60648	887	11
NONRATT020441	19.0346	485	4
NONRATT011161	18.57801	434	15
NONRATT013793	16.56359	2141	18
gi|672078992|ref|XR_360168.2|	15.72723	1417	15
NONRATT015360	15.30411	301	2
NONRATT013401	15.06407	673	18
NONRATT004403	14.84706	646	10
NONRATT016114	13.83695	436	2
gi|672062648|ref|XR_356473.2|	13.4567	413	8
NONRATT027930	13.28639	585	8

Values are fold changes (FC) in the reperfusion groups (reperfusion 2 h) over the control group (N >2.0-fold; $p < 0.05$ by analysis of variance)

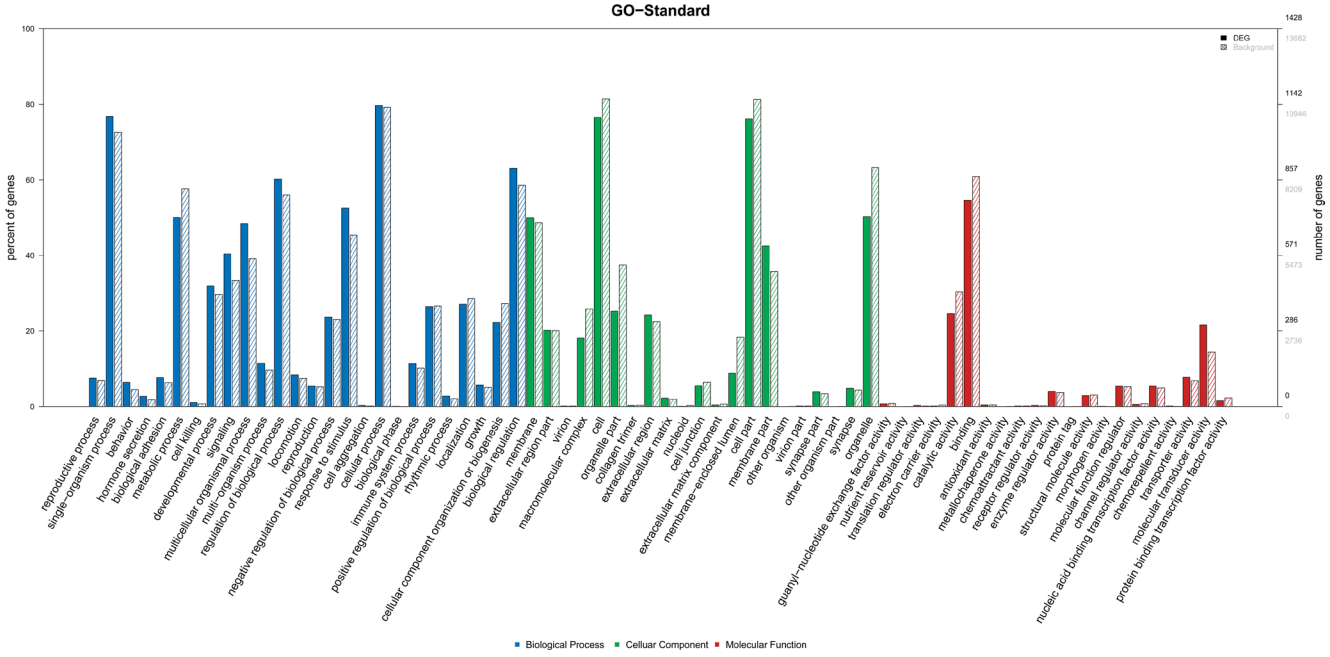

Figure 5: The differential expression genes were analyzed with GO background significant enrichment. Blue bar represents biological process classification; Blue bar represents cellular component; and Red bar represents molecular function. Solid bar represents target genes set, and slash bar represents background genes set.

18090 lncRNAs of which, 233 up-regulated and 6115 down-regulated lncRNAs were identified upon cardiac ischemia. From this list, we confirmed and verified few differentially expressed lncRNAs by RT-qPCR analyses.

Previous studies have suggested that the altered genes in the myocardial ischemic regions are involved in the regulation of cardiac function and have a protective role in I/R-induced cardiocyte apoptosis [24–27]. Yang et al demonstrated that microRNA-21 had an anti-apoptotic role in I/R-induced myocardial damage via the PTEN/Akt-dependent mechanism, suggesting that miR-21 may be a promising agent for the treatment of I/R-induced myocardial injury [27]. Wu et al showed that hypoxia/reoxygenation significantly increased the release of lactate dehydrogenase, levels of malondialdehyde, and cardiomyocyte apoptosis, but these effects were attenuated by an miR-613 mimic, and programmed cell death 10 (PDCD10) was identified as a target gene of miR-613, suggesting that miR-613 inhibits I/R-induced cardiomyocyte apoptosis by targeting PDCD10 [28]. Díaz et al indicated that administration of urocortin-1 and urocortin-2 at the beginning of reperfusion significantly restored cardiac function, and intravenous infusion of urocortin-2 in rat model of I/R mimicked the effect of urocortin-1 on miR-324-3p and miR-139-3p, suggesting that a role of urocortin in myocardial protection may be

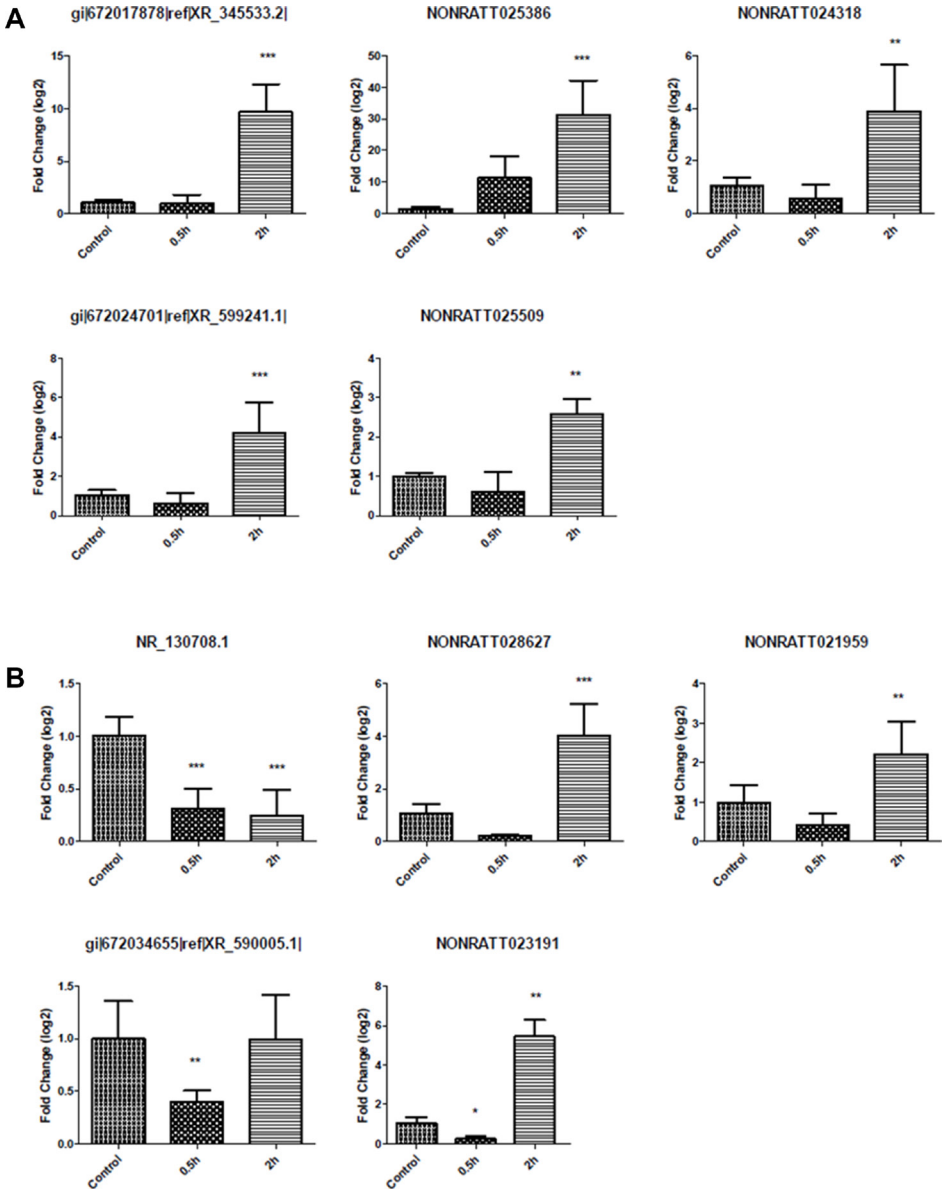

Figure 6: The expression of 10 lncRNA was analyzed in the myocardial tissues at different time points (0.5h/2h) after cardiac ischemia-reperfusion injury. The expressions of lncRNA XR_345533.2, NONRATT025386, NONRATT024318, XR_599241.1, and NONRATT025509 were significantly up-regulated underlying both 0.5h group and 2h group. The expression of lncRNA NR_130708.1 was significantly down-regulated underlying both 0.5h group and 2h group. One-way ANOVA (Dunnelt: Compare all columns vs. naive column). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2: The detail information of the top 20 down-regulated lncRNAs in the ischemic region at 2 h after reperfusion

Lnc RNAs (seqname)	fold change (R/N)	RNA length	Chromosome
gi|672036855|ref|XR_590210.1|	-218.269	395	1
NONRATT002188	-49.5425	455	1
gi|672034598|ref|XR_589980.1|	-45.2882	692	1
gi|672012815|ref|XR_598701.1|	-44.1724	572	1
gi|672036840|ref|XR_590197.1|	-42.3325	1597	1
gi|672033468|ref|XR_589535.1|	-38.0944	910	1
gi|672034607|ref|XR_589989.1|	-34.702	478	1
NONRATT015339	-33.0362	712	2
gi|672016307|ref|XR_599988.1|	-32.6989	886	2
NONRATT017954	-32.2738	380	3
NONRATT001682	-29.1733	423	1
NONRATT010246	-27.7605	1384	15
gi|672034371|ref|XR_589941.1|	-27.4956	322	1
NONRATT000221	-26.2891	564	1
gi|672013705|ref|XR_598835.1|	-26.2395	965	1
gi|672034596|ref|XR_589977.1|	-24.9653	711	1
NONRATT002479	-24.7847	1016	1
NONRATT001101	-23.8465	290	1
gi|672076298|ref|XR_595802.1|	-23.594	1183	14
NONRATT010626	-23.3895	1394	15

Values are fold changes (FC) in the reperfusion groups (reperfusion 2 h) over the control group (N >2.0-fold; $p < 0.05$ by analysis of variance)

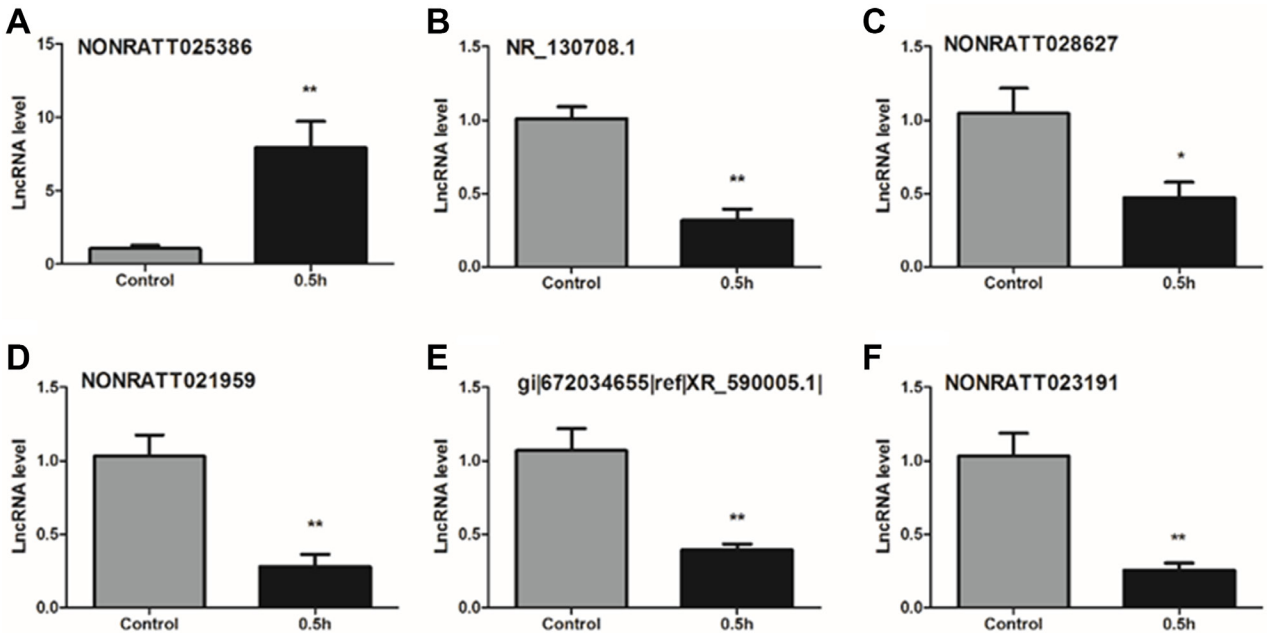

Figure 7: Real-time quantitative PCR (RT-qPCR) validation of 6 deregulated lncRNAs in the myocardium from MIRI rat model. The expressions of lncRNA NONRATT025386 (**A**) was significantly up-regulated underlying 0.5h group. The expression of lncRNA NR_130708.1 (**B**), NONRATT028627 (**C**), NONRATT021959 (**D**), gi|672034655|ref|XR_590005.1| (**E**) and NONRATT023191 (**F**) were significantly down-regulated. Mann-Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$.

involved in posttranscriptional regulation with miRNAs [29]. In this study, we unveiled differential expression of many mRNAs and lncRNAs in the myocardial ischemic regions, further suggesting that these altered genes might be involved in the response to cardiac injury. Although the functions of these mRNA and lncRNAs are not fully known, our findings provide novel insights about the molecular mechanism of I/R-induced myocardial damage.

Among the differentially expressed mRNAs, ASIC3, P2X3R, TRPM8 and ACPP had been reported in the study of heart disease. van Duijnhoven et al reported that the development and characterization of radiolabeled matrix metalloproteinases (MMP)-2/9 sensitive activatable cell-penetrating peptide probes (ACPPs) to assess MMP activity in myocardial remodeling *in vivo*, and found that radiolabeled MMP sensitive ACPP probes enable to assess MMP activity in the course of remodeling post-myocardial infarction [30]. Düzen et al observed marked increases in TRPML1-3, TRPA1, transient receptor potential melastatin subtype (TRPM)1-8, TRPC1-7, TRPV1-6, and PKD2 (TRPP2) gene expressions in non-valvular atrial fibrillation (NVAF) patients, whereas there was no change in PKD1 (TRPP1) gene expression, suggesting that elevated gene expressions of TRP channels may be associated with the pathogenesis of NVAF [31]. Xiong et al pointed that activation of TRPM8 attenuates cold-induced hypertension through ameliorating vascular mitochondrial dysfunction [32]. Zhang et al demonstrated that myocardial ischemic nociceptive signaling mediated by P2X3 receptor in rat stellate ganglion neurons [33]. Pijacka et al showed the purinergic receptors in the carotid body as a new drug target for controlling hypertension

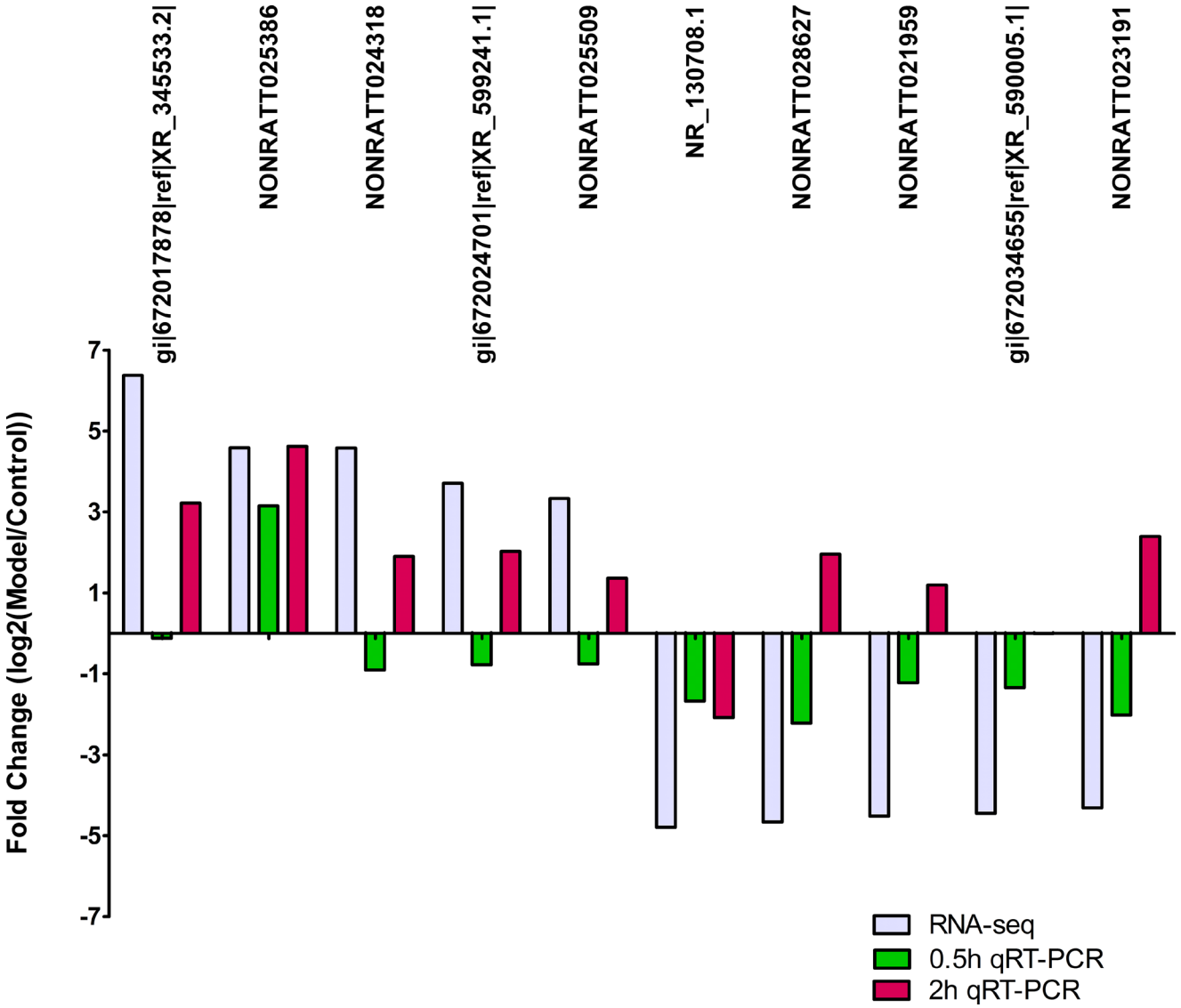

Figure 8: Validation of differential expression lncRNAs of the myocardial tissues at different time points (0.5h/2h) after cardiac ischemia-reperfusion injury. Five up-regulated lncRNAs and five down-regulated lncRNAs were validated by qRT-PCR. The levels of lncRNAs were normalized to GAPDH and expressed as fold of change compared to control group.

Table 3: The detail information of the top 20 up-regulated mRNAs in the ischemic region 2 h after reperfusion

m RNAs (seqname)	fold change (R/N)	GENE_SYMBOL	CHROMOSOMAL_LOCATION
A_64_P140025	474.7933	Tyrp1	chr5:99536404-99536463
A_43_P22979	182.9550	Tyrp1	chr5:99536772-99536831
A_44_P304082	85.08903	Kcnk18	chr1:265764505-265764564
A_42_P798447	75.89409	LOC687797	chr12:41820042-41820101
A_44_P419710	75.37812	Ppef1	chrX:55426098-55426157
A_64_P064833	68.06827	Pirt	chr10:53633404-53633463
A_64_P073143	65.44893	Asic3	chr4:6127828-6127769
A_64_P018681	63.93424	P2rx3	chr3:68228104-68228045
A_44_P556989	59.66698	Ngfr	chr10:84263141-84263082
A_44_P111865	58.96756	Cyp2a3	chr1:81948552-81950011
A_44_P259796	58.00981	Asic3	chr4:6127227-6127168
A_64_P159190	56.45496	Mrgprx1	chr1:97920951-97920892
A_42_P502621	45.42639	Avil	unmapped
A_64_P094230	43.46884	Asb15	chr4_random:386202-386261
A_43_P10303	42.36214	Ppp1r1c	chr3:62620827-62620886
A_64_P041581	41.7290	Trpm8	chr9:87278375-87278434
A_64_P090883	39.48497	Mrgprb4	chr1:97967660-97967601
A_64_P004458	34.19669	Acpp	chr8:109370477-109370418
A_64_P165996	34.07631	Chrna6	chr16:69018847-69018901
A_64_P000192	31.33567	Mrgprx1	chr1:97919998-97919939

Values are fold changes (FC) in the reperfusion groups (reperfusion 2 h) over the control group (N >2.0-fold; $p < 0.05$ by analysis of variance)

[34, 35]. Cheng et al indicated that acid-sensing ion channel 3, but not capsaicin receptor TRPV1, played a protective role in isoproterenol-induced myocardial ischemia in mice [36].

Further, we verified that gene expressions of the myocardial ischemic regions are varied in different time points after cardiac I/R injury by RT-qPCR validation. Our results indicated that the expressions of lncRNA XR_345533.2, NONRATT025386, NONRATT024318, XR_599241.1, and NONRATT025509 were significantly up-regulated in 2h group compared with control group and 0.5h group, whereas the expression of lncRNA NR_130708.1 was significantly down-regulated between 0.5h group and 2h group compared with control group, and had no statistically different between 0.5h group and 2h group. Otherwise, the expressions of lncRNA NONRATT028627, NONRATT021959, and NONRATT023191 were significantly up-regulated in 2h group compared with control and 0.5h groups, whereas the expressions of lncRNA gi|672034655|ref|XR_590005.1| was significantly down-regulated in 0.5h group compared with control group, and had no statistically different between control group and 2h group.

In summary, our data indicate that differential expression patterns of mRNAs and lncRNAs of the ischemic tissues in different time points after cardiac I/R injury in rats, and suggest potential clinical applicability to identify effective biomarkers for the cardiac injury.

MATERIALS AND METHODS

Animals and ethic statement

Male adult Sprague-Dawley rats (SPF class, each weighing 250 ~ 300g) were obtained from the Tongji Laboratory Animal Center (No. 42000600011122), and were housed in cages in groups of three, maintained at 22–25°C with a natural light-dark cycle. The current study was performed in accordance with the directives outlined in the Guidelines for the Care and Use of Laboratory Animals (US National Institutes of Health). Animal care and experimental protocols were approved by the Committee on Animal Care of Tongji Medical University, Wuhan, Hubei, China (IRBID:TJA0804).

Establishment of rat models with myocardial ischemia/reperfusion injury

Myocardial I/R surgery were performed as previously described [6, 37–39]. Briefly, rats were

Table 4: The detail information of the top 20 down-regulated mRNAs in the ischemic region 2 h after reperfusion

m RNAs (seqname)	fold change (R/N)	GENE_SYMBOL	CHROMOSOMAL_LOCATION
A_64_P244020	-53.3920	XM_006227951	chrUn:42644932–42644873
A_64_P166479	-53.2111	XM_006227170	chr1:52447851–52447910
A_64_P055634	-52.9583		chrUn_random:003901566–003901625
A_64_P025366	-52.7065		chr1:051379753–051379812
A_64_P146443	-39.8308		chr16:027206081–027206022
A_64_P221663	-39.7231	XM_003748704	chr1:51977510–51977451
A_64_P043280	-37.7585		chr16:027846591–027846532
A_64_P146268	-34.3418		chr1:051278680–051278621
A_64_P021409	-33.8364	XM_003753181	chr1:48965095–48965154
A_64_P019815	-31.5077		chr1:049995024–049995083
A_64_P122161	-30.8116	XM_006254337	chr17:67813551–67813492
A_44_P974207	-30.6207		chr1:051789811–051789870
A_64_P150351	-30.4985		chrUn:019914712–019914653
A_64_P091294	-30.3642		chr20:038380693–038380752
A_44_P185375	-30.0411	NM_001106764	chr7:13894754–13894695
A_44_P135744	-29.9762	XM_006227354	chrX:78955039–78955098
A_64_P155787	-28.2512		chrUn:003105376–003105317
A_64_P028860	-27.4549		chrUn:002037629–002037688
A_64_P139179	-26.8976		chr1:050493160–050493219
A_64_P016196	-26.7367		chrUn:049752903–049752844

Values are fold changes (FC) in the reperfusion groups (reperfusion 2 h) over the control group (N >2.0-fold; $p < 0.05$ by analysis of variance)

anesthetized; a tracheal intubation was set up. Then an invasive incision was made to expose the heart at the fourth intercostal space. Then the left anterior descending coronary artery was located and ligatured until myocardial ischemia occurred which was indicated by visualizing a marked epicardial cyanosis. After 30 min of myocardial ischemia, the trap of the left anterior artery was opened. Reperfusion was allowed for 0.5 h or 2 h.

Experimental protocols

Experiment A Animals were randomly divided into two groups: control group (Sham, $n = 3$) and I/R group (30 min ischemia followed by 2h reperfusion, $n = 3$). The myocardial ischemic regions were prepared for LncRNA+mRNA Rat Gene Expression Microarray and Real-Time quantitative PCR (RT-qPCR).

Experiment B Rats were randomly assigned to three groups: (1) control group ($n = 6$); (2) 0.5h group (30 min ischemia followed by 0.5h reperfusion, $n = 6$); (3) 2h group (30 min ischemia followed by 2h reperfusion, $n = 6$). The myocardial ischemic regions were prepared for RT-qPCR.

Tissue preparation

After 0.5 h or 2 h reperfusion, rats were immediately decapitated by cervical dislocation. Following decapitation, the myocardial ischemic regions were dissected during a dissection microscope and taken for subsequent analysis. The tissue was flash frozen in liquid nitrogen. Total RNA was isolated using Trizol® reagent (Invitrogen, Carlsbad CA). RNA samples were performed by Ambion mirVana miRNA Isolation Kit for purity and concentration.

Gene expression microarray

High quality samples containing 200 ng of total RNA were used on LncRNA+mRNA Rat Gene Expression microarray (Agilent 8x60K chips) by Capitalbio Technology Corporation. Gene profiling of the myocardial ischemic regions from I/R group and control group were carried out according to the manufacturer's instructions [40–42].

Table 5: Primer sequences for RT-qPCR

Gene	Long	Forward (5′ to 3′)	Reverse (5′ to 3′)
Up			
XR_345533.2	132	GAAAGGTCACGAGGCAAAGG	TCAGCAGATGTCAGGCAAGAA
NONRATT025386	86	GGGTCTGGGGTGGGCTAA	GGAGGTTTCTGAGTGGGATGTG
NONRATT024318	144	AAAGGCTTCGTCGTCCAACA	TCTGCCGATAGTAAAAGACCAATA
XR_599241.1	88	GTGCTCATCAACACGCATTCA	TTCTTAGGGTCTGCCTCCAAC
NONRATT025509	175	CTCCAGCCCCTGTCCAAA	AGCCCCATCCTGAGCACC
Down			
NR_130708.1	225	GAATCCTTCCGCTCTAAATCCC	TCTGCCTTGTTATCTTACCAGTCC
NONRATT028627	208	GAACTGCTTTTCCCACCTGAT	GCCCCTTTTAGTGTCTTGCTT
NONRATT021959	206	AAGAACCCTAAGAGCCCAGGAG	TGACGGAAAACAAGGCAAAGTA
XR_590005.1	238	AGTCGGGTAGTTCTGGCTCTG	AATAGTCCCCTCATCAATCCTTT
NONRATT023191	144	CCCAGCCTGTCGCTTTGA	TGACCCAGCACCCCACTT
GAPDH		CGCTAACATCAAATGGGGTG	TTGCTGACAATCTTGAGGGAG

RT-qPCR: reverse transcriptase quantitative polymerase chain reaction

Real-time quantitative PCR

2 μg of total RNA was extracted from the myocardial ischemic regions responding to I/R using TRIzol reagent (Invitrogen, USA) as described previously [43–47]. The threshold cycle (CT) was used to estimate the amount of target mRNA. The comparative CT method with the formula for relative fold-change = $2^{-\Delta\Delta CT}$was used to quantify the amplified transcripts. The specific forward (F) and reverse (R) primer sequences (Table 5) were designed. Experiments were evaluated in triplicate.

Microarray imaging data, Gene ontology and KEGG pathway analysis

The lncRNA+mRNA array v4.0 data were analyzed by using the Agilent GeneSpring software V13.0. The differentially expressed genes from the myocardial ischemic regions were selected by using the threshold values of ≥ 2 or ≤ −2-fold change, and the tree visualization from the myocardial ischemic regions was performed by using Java Treeview (Stanford University School of Medicine, Stanford, CA, USA) [48, 49]. The lncRNA/mRNA expression profiles from the myocardial ischemic regions between I/R group and control group were screened by volcano plot filtering. The differential lncRNA/mRNA expression from the myocardial ischemic regions was determined with Gene Ontology program. The key regulatory pathways in the myocardial ischemic regions responding to I/R were analyzed using the KEGG pathway analysis.

Statistics analysis

All quantification data are presented as mean ± SEM, and error bars represent SEM. To test for differences between the two groups, Mann-Whitney test were applied for each differentially expressed gene. The statistical analyses were done using *t* test, and $P < 0.05$ was considered statistically significant.

CONFLICTS OF INTEREST

The authors have no conflicts of interest related to this paper.

FUNDING

This work was supported by grants from National Natural Science Foundation of P.R. China (No. 81670240 to H.B. X, 81770283 to M.H. F), National Natural Science Foundation of Hubei Province (No. 2016CFB625 to H.B. X), Research project of Chinese Medicine Bureau in Guangdong Province (No. 20172067 to Q. L), and Medical innovation project in Fujian Province (No. 2017-CX-48 to S.Y. L).

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