Original Paper

Inhibition of LncRNA-HRIM Increases Cell Viability by Regulating Autophagy Levels During Hypoxia/Reoxygenation in Myocytes

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Key Words

Long noncoding RNA • Autophagy • Myocardial Cell • Ischemia/Reperfusion Injury • SiRNA

Abstract

Background/Aims: Ischemia reperfusion (I/R) promotes the severity of cardiomyocyte injury. Long noncoding RNAs (LncRNAs) are key regulators in cardiovascular diseases. However, the association between LncRNAs and myocardial I/R injury has not been thoroughly characterized to date. We attempted to clarify the potential biological role of a LncRNA (E230034O05Rik), which we named **hypoxia/reoxygenation (H/R) injury-related factor in myocytes (HRIM)**, by investigating the differential expression of LncRNAs between groups of myocytes exposed to either a normal level of oxygen or to H/R. **Methods:** Microarray analysis was used to determine analyze the global differential expression of LncRNAs in H9c2 myocytes exposed either to a normal level of oxygen or to H/R. Target LncRNA levels were further verified *in vitro* and *ex vivo* by real-time polymerase chain reaction (qPCR). Cell viability was analyzed using the Cell Counting Kit-8 assay. Autophagy levels were confirmed by Western blotting, transmission electron microscopy, and autophagic double-labeled (mRFP-GFP-LC3) adenovirus analyses. **Results:** Gene expression profiling revealed that 797 LncRNAs and 1898 mRNAs were differentially expressed in the H/R group compared with the normal oxygen group. Among these LncRNAs and mRNAs, 6 upregulated LncRNAs and 2 downregulated LncRNAs in the H/R group were selected and further validated by qPCR *in vitro* and *ex vivo*. Additionally, LncRNA-HRIM was inhibited by specific siRNAs in H9c2 myocytes exposed to H/R. The inhibition of LncRNA-HRIM by siRNA prevented cell death by suppressing excessive autophagic activity in myocytes. This finding suggests a detrimental role of LncRNA-HRIM in the regulation of I/R injury. **Conclusions:** LncRNAs are involved in H/R injury of H9c2 myocytes. Inhibition of LncRNA-HRIM increased cell viability by reducing autophagy in myocytes during H/R.

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Introduction

Acute myocardial infarction (MI) is a common cause of death that results from atherosclerotic coronary heart disease (CHD). Reperfusion therapies, including primary percutaneous coronary intervention or thrombolytic therapy, benefit CHD patients [1]. However, such therapies may cause myocardial ischemia/reperfusion (I/R) injury. Therefore, reduction of reperfusion injury is an important topic in the treatment of MI. However, the pathogenesis of I/R injury has not been thoroughly elucidated to date.

Recent research reported that autophagy is activated during the I/R period and likely contributes to cell death by excessively degrading essential proteins and organelles [2]. During the reperfusion period, inhibition of autophagy with berberine [3] or curcumin [4] significantly mitigates I/R injury, which reduces the scope of MI and improves cardiac function. These findings suggest that excessive autophagy plays an adverse role during I/R period injury. Hence, appropriate regulation of autophagy is beneficial for I/R injury treatment.

A growing body of research revealed that noncoding RNAs are involved in various biological functions in the regulation of pathophysiological conditions, such as cancer [5], autoimmune diseases [6], and inflammation-related illnesses [7]. Moreover, long noncoding RNAs (LncRNAs) and endogenous RNAs that contain over 200 nucleotides can combine with biological molecules (e.g., mRNAs, miRNAs, or proteins) and influence key proteins or signaling pathways in different pathophysiological processes. The pathogenesis of certain diseases may be related to abnormal LncRNA expression [8]. For example, the aberrant expression of LncRNAs, such as Mhrt, is associated with the pathogenesis of ischemic heart failure [8, 9]. However, the role of LncRNAs in cardiac I/R injury remains unclear. In the present study, we investigated the differential expression of LncRNAs in myocytes during I/R using *in vitro* and *ex vivo* models. Furthermore, we determined the effect of LncRNA E230034005Rik on the regulation of autophagy in myocytes. We designated LncRNA E230034005Rik as hypoxia/reoxygenation (H/R) injury-related factor in myocytes (HRIM).

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin (pen/strep, 10, 000 U/ml each) were purchased from GIBCO (Life Technologies, Shanghai China). Anti-GAPDH and LC3B antibodies were procured from Cell Signaling Technology (Danvers, MA USA). Goat anti-rabbit secondary antibodies (Catalog A-21109) used in Western blot were purchased from Invitrogen (Carlsbad, CA USA).

Animals

Male Sprague Dawley rats were obtained from the Shanghai Animal Center Laboratory (Shanghai, China). Animal procedures were performed in accordance with the instructions 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 Wenzhou Medical University (Wenzhou, Zhejiang, China).

Experimental protocol and induction of I/R

Ten Sprague Dawley rats (aged, 8 weeks to 12 weeks, body weight 280 g to 320 g) on a standard diet were randomly divided into two groups: the sham group (sham, $n = 5$) and the I/R group (I/R, $n = 5$). Surgery was performed as described previously [10]. Briefly, rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 μ l/kg). Each rat was completely sedated. The chest was opened via thoracotomy and ribs were removed. The pericardium was ruptured and the aorta was exposed. A steel cannula was later placed into the aorta and secured with a suture. The heart was immediately perfused with Krebs-Henseleit bicarbonate buffer at a constant pressure of 60 mmHg to 70 mmHg at 37°C. Thirty minutes

before the operation, the buffer was bubbled with 95% O₂ and 5% CO₂ to attain a pH of 7.4. The heart was quickly excised and transferred into a Langendorff apparatus while perfusion continued [11]. All hearts were perfused for 20 min before induction of ischemia to attain equilibrated coronary perfusion pressure (CPP). Hearts were subsequently subjected to 30 min of no-flow global ischemia, followed by 120 min of reperfusion [11]. Hearts that showed arrhythmia during the equilibration, had sustained reduction in CPP, or had flow less than 8 ml/min were excluded from the study. ECG, CPP, and perfusion flow parameters were monitored before the induction of ischemia and during reperfusion with a Power lab recorder (AD Instruments, Australia). The successful induction of ischemia was determined by ST segment elevation on an electrocardiogram.

Hypoxia/reoxygenation with H9c2 myocytes

The rat myocardium-derived H9c2 cardiac myoblast cell line was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM containing 4,500 mg/L glucose and supplemented with 10% (v/v) FBS, 10 mM HEPES (Sigma), and 1% pen/strep solution at 37 °C in a humidified 5% CO₂ incubator. To simulate I/R injury, H/R was induced in H9c2 myocytes. The cells were cultured in FBS and glucose-deprived DMEM, and were first exposed to hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂) for 1 h and normoxic conditions for 3 h. Meanwhile, the cells in the control group (without FBS and glucose-deprived DMEM) were maintained under normoxic conditions (21% O₂, 5% CO₂, and 74% N₂) for 4 h.

Microarray and computational analysis

Total RNA was extracted from each sample (3 normal oxygen groups and 3 H/R treatment groups), amplified, and transcribed into fluorescent cRNA with the Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA). Labeled samples were hybridized to the rat LncRNA Array (4 x 44K, ArrayStar, Rockville, MD, USA), which detected the expression profiles of rat genome-wide protein-coding transcripts (KangChen Bio-tech, Shanghai, China). The arrays were washed and then scanned using the Agilent Scanner G2505B. Acquired array images were analyzed with Agilent Feature Extraction Software (version 10.5.1.1). Quantile normalization and subsequent data processing were performed with the GeneSpringGX v11.0 software package (Agilent Technologies). The statistically significant differential expression of LncRNAs was identified using the criteria of a fold change > 1.5 and $p < 0.05$.

Knockdown of LncRNA E230034O05Rik in H9c2 myocytes

To knockdown E230034O05Rik in H9c2 myocytes, H9c2 myocytes were transfected with siRNAs against rat E230034O05Rik (full length rat E230034O05Rik sequence is available at http://www.ncbi.nlm.nih.gov/nuccore/NR_002154). The siRNAs and negative control (NC) siRNA were synthesized by RiboBio (Guangzhou, China). H9c2 myocytes were randomly divided into four groups: the normal oxygen group (control group), the H/R group, the H/R+NC siRNA group and the H/R+siRNA group. The H/R+NC siRNA group was the control for the H/R+siRNA group to eliminate any effects of substance siRNA. H9c2 myocytes were transfected with siRNAs for 48 h in accordance with the manufacturer's instructions and later subjected to H/R treatment as described above.

Gene coexpression network construction

Gene coexpression networks were visually represented to identify interactions among genes. The method was performed as described by Yang [12]. Gene coexpression networks were assembled based on the normalized signal intensity of specific expressed genes. Pearson's correlation coefficients were applied to calculate gene pair correlations on the basis of gene expression. To ensure visual representation, only the strongest correlations (0.99 or greater) were included in the renderings. Each gene corresponded to a node in the gene coexpression network. Two genes connected by an edge indicated a strong correlation (i.e., either positive or negative). Within the network, the node with more edges signified that the gene had more coexpressed genes. Moreover, these genes would be considered in our follow-up studies.

RNA isolation, cDNA synthesis, and TaqMan real-time PCR

H9c2 myocytes were cultured in 12-well plates under similar treatments or in an isolated heart exposed to I/R as mentioned above. mRNA levels were analyzed using SYBR Green reagent kits with gene

specific primers on Applied Biosystems 7300 Real-Time PCR system (USA), following the manufacturer's instructions. Amplification conditions were 94 °C for 7 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. All primer sequences for RT-PCR assays are listed in Table S1. All experiments were performed in triplicate and all samples were normalized with GAPDH.

Assessment of cell viability with a CCK-8 assay

Cell viability was assessed with the Cell Counting Kit-8 (CCK-8, Beyotime Biotech, Jiangsu, China) in accordance with the manufacturer's instructions. The kit contained a water-soluble tetrazolium salt (WST-8) and a substrate of mitochondrial succinate dehydrogenase that converts WST-8 into orange-colored formazan. The activities of these enzymes are proportional to those in living cells. H9c2 myocytes (5×10^3 /well) were seeded in 96-well plates and subjected to both control and H/R culture conditions. At the end of each treatment, the culture medium was replaced with 100 μ L of CCK-8 solution containing 90 μ L of serum-free DMEM and 10 μ L of CCK-8 reagent. Absorbance at 450 nm was measured in each well by visualization of color intensity development.

Transmission electron microscopy

H9c2 myocytes were cultured in P60 dishes ($\sim 1 \times 10^6$) under similar treatments as mentioned above. At the end of each experiment, cells were washed with 0.1 M cacodylate buffer and fixed in 2.5% glutaraldehyde containing 0.1 M cacodylate buffer for 1 h. Cells were scraped off of the plates, collected in Eppendorf tubes and fixed with 1% osmium tetroxide. Cells were dehydrated in a gradient series of ethanol solutions (from 50% to 100%) in Eppendorf tubes and then embedded in epoxy resin. Ultrathin (~ 70 nm) sections were cut and placed on grids. The sections were double-stained with uranyl acetate and lead citrate prior to examination under an electron microscope (JEOL, JEM-1230, Japan).

Protein extraction and Western blot analysis

Cells were lysed with RIPA buffer on ice and then centrifuged at 12,000 rpm for 10 min at 4 °C. Up to 40 μ g of total protein from each sample was loaded for SDS-PAGE separation and later transferred onto PVDF membranes as previously described [13]. The PVDF membranes were blocked in 5% dry milk/TBST and subsequently probed with primary antibodies, including rabbit IgG anti-LC3, at a 1:1000 dilution in 5% dry milk/TBST or anti-GAPDH at a 1:5000 dilution. The secondary antibody was a goat anti-rabbit labeled with Alexa Fluor 680 (1:2000 dilution). The antibody-labeled membranes were scanned for far-red signals by an Odyssey Imaging System (LI-COR, NE, USA). Data were analyzed with Quantity One Software (Bio-Rad, CA, USA). In addition, protein expression levels were quantified by relative densitometry with actin as the loading control.

Statistical analysis

All experiments were repeated at least three times and the data are presented as the mean $\pm$ SD. Data were analyzed by one-way ANOVA. Post-hoc analyses, including the Student-Newman-Keuls method and Dunnett's test, were performed using SPSS 18 software (SPSS Inc., Chicago, IL). The statistical significance was defined as $p < 0.05$.

Results

LncRNA expression profiles of rat H9c2 myocytes after H/R treatment

Microarray analysis was performed on three normal oxygen groups and three H/R groups to investigate the differential expression of LncRNAs. The microarray analysis was conducted by KangChen Bio-tech (Shanghai, China). Compared with the control group, the H/R treatment group had 309 upregulated LncRNAs and 488 downregulated LncRNAs with a >1.5 -fold change and $p < 0.05$ (for all online suppl. material, see www.karger.com/doi/10.1159/000489149, File S1). Additionally, the microarray data have been deposited in the public domain (GEO number: GSE103731). Moreover, we constructed heat maps and performed hierarchical clustering of LncRNA (Fig. 1A) and mRNA (Fig. 1B) expression ratios (log₂ scale), and compared H/R-treated H9c2 myocytes and myocytes that had normal oxygen treatment.

Gene ontological (GO) and pathway analyses

To further reveal the potential roles of LncRNAs in H/R injury, GO analysis was performed to annotate transcripts with terms related to biological processes, cellular component, and molecular function. The 1898 differentially expressed coding genes were analyzed using GO annotations (see online suppl. material, Suppl. Fig. S1). Additionally, module pathway enrichment was analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) to confirm potential module function. A total of 1898 coding genes were identified by ANOVA ($p < 0.05$). KEGG pathway analysis revealed that the majority of LncRNA target genes were associated with sterol biosynthesis and DNA replication pathways (see online suppl. material, Suppl. Fig. S1). Candidate genes for subsequent experiments were selected based on the information yielded by the GO and KEGG pathway analyses.

Gene coexpression network construction

To reveal the potential mechanism for LncRNAs in H/R injury, the 797 LncRNAs and 1898 coding mRNAs were clustered into phenotypically relevant coexpression modules on the basis of LncRNA and gene coexpression networks, where $R > 0.99$. The coexpression networks for eight highly dysregulated LncRNAs are shown in Fig. 1C. The analysis demonstrated that E230034005Rik was coexpressed with coding RNAs (mRNAs) of Zdhhc7, Ptgis, Krt23 and Phactr1, which are linked to the regulation of hypoxia/reoxygenation (H/R) injury in myocytes (Fig. 1C). We confirmed by qPCR that the expression levels of Zdhhc7, Ptgis, Krt23 and Phactr1 were increased in the H/R group compared with the control group. This suggests that Zdhhc7, Ptgis, Krt23 and Phactr1 are coexpressed with E230034005Rik during H/R injury (see online suppl. material, Suppl. Fig. S2).

Validation of misregulated LncRNAs and mRNAs in H/R and I/R treatment

To validate the accuracy of the LncRNA profiles generated by microarray analysis and the success of the H/R and I/R treatment models, we further confirmed the expression levels of 8 LncRNAs and the mRNA expression levels of *nppa*, *bax*, *bcl-2* and *caspase-3* with qPCR analysis. As expected, the expression levels of BC089936, BC168700, *Terc*, E230034005Rik, *Abhd11os*, LOC360231, *nppa*, *bax* and *caspase-3* were significantly increased in the H/R group, whereas the expression levels of *ania-1*, MRAK035284 and *bcl-2* were decreased in the H/R group (Fig. 2A and see online suppl. material, Suppl. Fig. S2). Similar results were observed in Sprague Dawley rats: the expression levels of 6 LncRNAs increased in the I/R group, and the expression levels of 2 LncRNAs decreased in the I/R group (Fig. 2B).

It was difficult to choose a candidate LncRNA for subsequent research based on the large chip data. We selected LncRNA-HRIM for further investigation for the following reasons: (i) LncRNA E230034005Rik was significantly upregulated in the H/R or I/R group compared with the control group, (ii) E230034005Rik is a validated LncRNA and not a pseudogene, and (iii) *Clic1*, the contiguous gene of E230034005Rik, is related to autophagy, which plays an important role in I/R injury. Furthermore, LncRNA-HRIM is co-expressed with *Zdhc7*, *Ptgis*, *Krt23* and *Phactr1* mRNAs, which may be linked to the regulation of hypoxia/reoxygenation (H/R) injury in myocytes. Our results suggest that LncRNA E230034005Rik may be the potential LncRNA that is important for the regulation of hypoxia/reoxygenation (H/R) injury in myocytes.

Genetic characteristics of LncRNA-HRIM

LncRNA-HRIM is a long non-coding RNA that is 1470 bp in size. LncRNA-HRIM is located on 20p12 and has three exons. Its potential regulatory gene is *clic1*, which has been reported to stimulate cell growth both *in vitro* and *in vivo* [14]. We performed RACE and confirmed that LncRNA-HRIM has polyA tail (see online suppl. material, File S2). Furthermore, additional experiments confirmed that the LncRNA-HRIM is expressed in various tissues in rat (see online suppl. material, Suppl. Fig. S2), while, there are low levels of LncRNA-HRIM in human cell samples such as HepG2 and HUVEC. This finding suggests that LncRNA-HRIM has a lack of sequence conservation between species.

Fig. 2. Quantitative real-time PCR validation of 8 differentially expressed lncRNAs and known H/R-regulated protein-coding genes. (A). The 6 upregulated lncRNAs (BC089936, BC168700, *Terc*, E230034005Rik, *Abhd11os*, and LOC360231), 2 downregulated lncRNAs (AF030086, MRAK035284) and I/R-regulated protein-coding gene in the H/R group compared with the normal control *in vitro*, GAPDH was designated an endogenous reference gene. (B) The 6 upregulated lncRNAs (BC089936, BC168700, *Terc*, E230034005Rik, *Abhd11os*, and LOC360231), 2 downregulated lncRNAs (AF030086, MRAK035284) and I/R-regulated protein-coding gene in the I/R group compared with the normal control group *ex vivo*. GAPDH was designated an endogenous reference gene. The lncRNA unit represents the relative expression of the lncRNA compared to GAPDH. Data (mean $\pm$ SD) were obtained from three independent experiments (n=5). *P<0.01 compared with the control group.

Silencing LncRNA-HRIM attenuated H/R injury in H9c2 myocytes

To evaluate the effects of LncRNA-HRIM on H/R injury, three different siRNAs (designated as siRNA-1, siRNA-2, and siRNA-3) were transfected against LncRNA-HRIM in H9c2 myocytes. As depicted in Fig. 3A, all three siRNAs knocked down the expression of LncRNA-HRIM and the inhibitory effect of siRNA-1 was the most significant. The sequences for siRNA-1 is shown (see online suppl. material) in Suppl. Table S1. Compared with cells in the control group, depletion of LncRNA-HRIM by siRNA-1 increased cell viability in H9c2 myocytes during H/R injury. By contrast, the si-LncRNA-control treatment did not affect cell viability (Fig. 3B). These data suggested that inhibition of LncRNA-HRIM can increase cell survival of H9c2 myocytes during the H/R period.

LncRNA-HRIM regulates autophagy in H9c2 myocytes during H/R

Autophagy plays an essential role in myocardial I/R injury [15, 16]. Autophagy is significantly activated during an I/R episode and likely contributes to cell death by excessively degrading essential proteins and organelles [2]. In addition, inhibition of excessive autophagy protects myocardial cells from I/R injury [3]. Therefore, we hypothesized that LncRNA-HRIM similarly regulates autophagic activity. As shown in Fig. 3C and 3D, LncRNA-HRIM expression was decreased using siRNA-1. We observed that the LC3B-II/LC3B-I ratio, which indicates the level of autophagy, was significantly increased in the H/R group compared with the control group. Meanwhile, the LC3B-II/LC3B-I ratio and the total amount of LC3B-II and LC3B-I were markedly decreased in the siRNA-LncRNA-HRIM group compared to the H/R group. Moreover, the number of autophagosomes detected by electron microscopy displayed the same tendency (Fig. 4A). LC3 was tagged and tracked by expression of GFP and mRFP in the tandem GFP-RFP-LC33 adenovirus. The increase or decrease in autophagosome formation is accompanied by the increased or decreased utilization of GFP-RFP-LC33, which displays red and green fluorescence under microscopy. Under reduced pH, GFP degrades from the tandem protein when the autophagosome fuses with a lysosome to form autolysosomes, whereas RFP-LC3 expression is maintained as puncta. Hence, the process from autophagosomes to autolysosomes can be monitored by changes in fluorescence [17]. Both fluorescent proteins were observed after successful infection with GFP-RFP-LC3, and colocalized to resemble yellow puncta (Fig. 4B). In addition to LC3 accumulation, fewer yellow puncta were detected in H9c2 myocytes transfected with si-LncRNA-HRIM than in

Fig. 3. Knockdown of LncRNA-HRIM decreased the ratio of LC3B-I/LC3B-II and increased cell viability of H9c2 myocytes. (A) H9c2 myocytes were transfected with three different siRNAs (designated as siRNA-1, siRNA-2 and siRNA-3) against LncRNA-HRIM. LncRNA-HRIM expression was detected by q-PCR. GAPDH was designed as an endogenous reference gene. (B) Cell viability of H9c2 myocytes in the control, H/R, H/R+NC, and H/R+siRNA-1 groups. (C) Western blot analysis of LC3-I, LC3-II, and GAPDH in H9c2 myocytes. (D) Quantification of panel A by densitometry. GAPDH was used as a loading control. Data (mean $\pm$ SD) were obtained from three independent experiments. * $P < 0.01$ compared with the control group, # $P < 0.01$ compared with the H/R group.

Fig. 4. Downregulation of lncRNA-HRIM decreased autolysosome formation in H9c2 myocytes under H/R compared with normal oxygen treatment. (A) Representative TEM images of intracellular ultrastructures of H9c2 myocytes. Four groups of cells were visualized, including the control, H/R, H/R+NC, and H/R+siRNA-1 groups. Pictures in the left and right columns were taken under 4,200X and 13,500X magnifications, respectively. The arrow designates an autophagosome. (B) Representative images of intracellular autolysosome flux detected by infecting GFP-RFP-LC3 adenovirus in H9c2 myocytes subjected to H/R. The control, H/R, H/R+NC, and H/R+siRNA-1 groups were visualized. Pictures in the right column are a merge from the first two columns. The yellow puncta represent autolysosome formation. Magnification, 200X. (C) Quantification of the average number of autophagosomes in each cell by TEM. (D) Quantification of the average autolysosome flux. Data (mean $\pm$ SD) were obtained from three independent experiments. * $P < 0.01$ compared with the control group, # $P < 0.01$ compared with the H/R group.

the H/R group without si-LncRNA-HRIM. These results further confirmed that depletion of LncRNA-HRIM expression inhibited autolysosome formation, indicating that LncRNA-HRIM mediated autophagy flux in H9c2 myocytes.

Discussion

In this study, we observed that LncRNA expression profiles of H9c2 myocytes changed during the H/R process. We selected 8 LncRNAs that may play critical roles in H/R injury. The differential expression levels of these 8 LncRNAs were validated *in vitro* and *ex vivo* by qPCR, which confirmed the microarray results. We focused on the function of LncRNA-HRIM (E230034005Rik), and demonstrated that inhibition of LncRNA-HRIM expression markedly enhanced myocardial cell activity via reduction of excessive autophagy during H/R. This result suggested that LncRNA-HRIM plays a detrimental role in regulation of H9c2 myocytes survival during the H/R period.

Emerging evidence shows the critical roles of LncRNAs in the initiation and progression of cardiovascular diseases [18, 19]. Mitochondrial LncRNA uc022bqs.1 (LIPCAR) is downregulated during the early stages of MI but is upregulated during the later stages, suggesting that LIPCAR is a novel biomarker of cardiac remodeling and may predict death in patients with heart failure [20]. A recent study [21] showed that compared with healthy volunteers, MI patients have higher levels of hypoxia-inducible factor 1A antisense RNA 2, KQT-like subfamily, member 1 opposite strand/antisense transcript 1 (KCNQ10T1), and metastasis-associated lung adenocarcinoma transcript 1 (MLAT1), as well as lower levels of cyclin-dependent kinase inhibitor 2B antisense RNA 1 (ANRIL). In addition, ANRIL, KCNQ10T1, and MLAT1 levels are lower in patients with ST-segment-elevation MI than in patients with non-ST-segment-elevation MI. Moreover, aberrant LncRNA expression (e.g.,

Mhrt) is associated with the pathogenesis of ischemic heart failure [9]. These data indicate that LncRNAs are involved in the myocardial ischemia process. In the current study, we found that 309 LncRNAs were significantly upregulated and 488 LncRNAs were downregulated in H9c2 myocytes subjected to H/R. This finding suggested that LncRNAs may play a role in heart damage. However, it was difficult to screen all differentially expressed LncRNAs for subsequent research based on the large chip data. We selected 8 LncRNAs to validate for the following reasons: (i) The LncRNAs were significantly upregulated or downregulated in the H/R or I/R group compared with the control group, (ii) Several LncRNAs were validated LncRNAs that had the published sequences, and (iii) the contiguous coding-gene and co-expression of these 8 LncRNAs were related to autophagy or apoptosis, which play important roles in I/R injury.

The E230034005Rik gene, is a long non-coding RNA that is 1470 bp in size. E230034005Rik is located on 20p12 and includes three exons. We selected LncRNA-HRIM (E230034005Rik) for functional studies for the following reasons: (i) LncRNA E230034005Rik was significantly upregulated in the H/R or I/R group compared with the control group, (ii) E230034005Rik is a validated LncRNA and not a pseudogene, (iii) Clic1, the contiguous gene of E230034005Rik, is related to autophagy, which plays an important role in I/R injury and (iv) LncRNA-HRIM was co-expressed with Zdhc7, Ptgs1, Krt23 and Phactr1 mRNAs, which may be linked to the regulation of hypoxia/reoxygenation (H/R) injury in myocytes. Depletion of LncRNA-HRIM with siRNA significantly increased the viability of myocardial cells when myocytes were subjected to H/R. These observations suggested the potential adverse role of LncRNA-HRIM in H9c2 myocytes during H/R.

Most research efforts focus on differential LncRNA expression during the disease process [22, 23]. However, the real mechanism or function of LncRNAs has not been completely clarified. For the current study, we selected LncRNA-HRIM (E230034005Rik) for preliminary functional studies.

Autophagy is a major pathway that responds to various extracellular and intracellular stimuli, such as nutrient deprivation, bacterial infection, and therapeutic treatment [24-27]. Autophagy is a dynamic biological process involved in membrane formation, autophagosome formation, autophagolysosome fusion, and autophagosome degradation, which involve signal transduction, RNA transcription, and protein synthesis. Appropriate autophagy levels are conducive to the survival of myocardial cells subjected to different stress or damage [28]. However, the excessive activation of autophagy degrades some important cell structures, which eventually leads to cell death [29]. Our previous experiments demonstrated that autophagy adversely affects myocardial cells during the H/R period [3, 4] and that inhibition of excessive autophagy increases the viability of myocytes. These results indicated that autophagy regulation is an effective strategy for treating H/R injury.

Several studies support the fact that LncRNAs participate in the regulation of autophagy [30-32]. LncRNA-MEG3 (Maternally Expressed Gene 3) is expressed at a low level in bladder cancer tissues compared with normal controls. Meanwhile, the downregulation of MEG3 increases cell proliferation in bladder cancer by activating autophagy [33]. LncRNA-TGFB2-OT1, as a competing endogenous RNA, regulates autophagy and inflammation in vascular endothelial cells by binding to MIR3960, MIR4488, and MIR4459 [34]. In addition, LncRNA-APF (autophagy promoting factor) regulates miR-188-3p and affects autophagic cell death and myocardial infarction [35]. Therefore, the role of LncRNAs in the regulation of autophagy has attracted the attention of researchers. Previous studies have shown that ZDHHC7 [36, 37], Ptgs1 [38], and Krt23 [39] are involved in regulating autophagy or apoptosis. We observed that these proteins are co-expressed with LncRNA-HRIM by qPCR (see online suppl. material, Suppl. Fig. S2), which suggests that LncRNA-HRIM may be involved in regulation of the autophagic process. However, the target genes of LncRNA-HRIM, and the detailed mechanism need further research to confirm.

Conclusion

The LncRNA profiles of H9c2 myocytes changed extensively during the H/R period. Moreover, inhibition of LncRNA-HRIM increased cell viability partly by inhibiting autophagic activity. This finding suggests that the regulation of LncRNA-HRIM is a promising target for mitigating I/R injury.

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Disclosure Statement

No conflict of interests exists.

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