Silencing of long non-coding RNA HRIM protects against myocardial ischemia/reperfusion injury via inhibition of NF-κB signaling

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Abstract. Myocardial ischemia/reperfusion injury (MI/RI) following cardiac surgery is a leading cause of morbidity and mortality worldwide. The aim of the present study was to investigate the role of long non-coding RNA hypoxia/reoxygenation injury-related factor in myocytes (HRIM) on cardiac function following MI/RI. After establishing an MI/RI model, hemodynamic indices were detected via transthoracic echocardiography. The proliferative and apoptotic capacities of H9C2 cells subjected to oxygen-glucose deprivation/reoxygenation were detected via Cell Counting Kit-8 assay and flow cytometry, respectively. TNF-a, IL-1β, IL-6, lactate dehydrogenase (LDH) and creatine kinase (CK) levels were measured via ELISA. The expression levels of NF-kB-associated proteins were detected via western blotting. The expression levels of HRIM were increased in the myocardial tissue of MI/RI rats and H9C2 cells. The infarct size was significantly increased following induction of MI/RI. Moreover, increased HRIM expression levels suppressed hemodynamics in MI/RI rats. Knockdown of HRIM increased cell proliferation and decreased apoptosis as well as the protein levels of phosphorylated (p)-NF- κ B p65/NF- κ B p65, p-IkB α /IkB α , TNF- α , IL-1 β , IL-6, LDH and CK in H9C2 cells; however, these effects were attenuated via activation of NF-kB signaling. Silencing of HRIM ameliorated MI/RI injury and alleviated inflammation via inactivating the NF-kB signaling pathway.

Introduction

Myocardial ischemia (MI) is characterized by damage of blood vessels (1). According to the World Health Organization, ischemic heart disease has become the leading cause of human mortality worldwide (2), resulting in over 8 million deaths in 2013 (3). Although timely blood reperfusion is the most effective therapy for MI, this can lead to myocardial injury, known as myocardial ischemia/reperfusion injury (MI/RI) (4). MI/RI is associated with myocardial tissue injury, inflammation and heart failure (5-7), and is the leading cause of mortality following cardiac surgery (8). There are few effective drugs for MI/RI (9), primarily due to the poor permeability of the cytomembrane (10). Therefore, identification of novel therapeutic targets to improve the prognosis of patients with MI/RI is necessary.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNA transcripts that are involved in the progression of cardiac disease. For instance, silencing of lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) has been demonstrated to ameliorate acute myocardial infarction via sponging microRNA (miR)-320 (11). In addition, lncRNA 2810403D21Rik/Mirf enhances ischemia/reperfusion (I/R) injury via directly targeting miR-26a (12). Moreover, overexpression of lncRNA taurine-upregulated gene 1 promoted hypoxia-induced injury in cardiomyocytes via the miR-145-5p-Binp3 axis (13). Research has demonstrated that lncRNAs, including lncRNA urothelial carcinoma associated 1 (14), MALAT 1 (15) and hypoxia-inducible factor 1a-antisense RNA 1, are key regulators of MI/RI (16). Hypoxia/reoxygenation (H/R) injury-related factor in myocytes (HRIM; E230034O05Rik gene) is an lncRNA 1,470 bp in length located on chromosome 20p12 (including three exons) (17). Due to the upregulation of E230034005Rik in H/R or I/R, this gene was selected for functional analysis in the present study (18). Hypoxia/reoxygenation injury-related factor in myocytes (HRIM) is co-expressed with autophagyand apoptosis-associated proteins, including zinc finger DHHC-type palmitoyltransferase 7 (19), prostaglandin I2 synthase (20) and keratin, type I cytoskeletal 23 (21). Knockdown of HRIM has been demonstrated to increase cell

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viability via mediating autophagy to protect against myocardial H/R injury (22). Consistently, silencing of HRIM has also been shown to alleviate MI/RI-induced damage (16).

NF-kB is an essential protein transcription factor involved in cell proliferation, differentiation and apoptosis (23). The NF-kB signaling pathway has been demonstrated to be associated with a number of pathological processes in the heart, including inflammation, injury and apoptosis (24). The canonical NF-KB pathway proceeds via activating the IkB kinase complex (IKK). IKK phosphorylates IkB proteins, typically IkBa, leading to dissociation from the NF-κBp65/p50 complex (25,26). The NF-κB p65/p50 complex then translocates to the nucleus, where it activates gene transcription (25,27). p65 is a major subunit of NF- κ B (28). Upregulation of NF-KB p65 can enhance activation of the NF-kB signaling pathway (29), and inhibition of NF-kB p65 activity has been shown to protect against MI/RI in rats (30). Moreover, downregulation of NF-kB attenuated MI/RI in rats (31). However, whether the effects of HRIM are mediated via the NF-kB signaling pathway in MI/RI remains unclear.

The present study aimed to determine the biological function of HRIM in MI/RI rats and H9C2 cells as well as its underlying mechanism of action. These results may provide a novel target for the treatment of MI/RI.

Materials and methods

Animals. Male Sprague-Dawley rats (n=44; weight, 220-260 g; age, 7 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Rats were fed standard chow and water and raised under specific pathogen-free conditions at 22°C and 50% relative humidity with an artificial 12-h light/dark cycle. The present study was approved by the Animal Ethics Committee of Qingdao Municipal Hospital (approval no. 2020-L-054).

MI/RI model in rats. After one week of adjustment, rats were divided into Control (n=10), Sham (n=10) and MI/RI (n=24) groups. Rats without any treatment were defined as the Control group. The Sham group was subjected to all procedures except ligation. The MI/RI model was induced via ligation of the left anterior descending coronary artery (LAD) as previously described, with some modifications (30). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg) via intraperitoneal injection. Subsequently, the LAD was ligated with a 6.0 thread and the ligation line was released after 45 min, followed by reperfusion for 24 h. Thus, an MI/RI model was established. In order to determine the effect of HRIM on MI/RI rats, MI/RI rats were divided into the following groups: MI/RI (MI/RI rats without treatment), MI/RI + small interfering RNA (siRNA) negative control (si-NC; MI/RI rats injected with 200 μ g/kg/d si-NC via the tail vein following surgery) and MI/RI + HRIM siRNA (si-HRIM; MI/RI rats injected with 200 μ g/kg/d si-HRIM via the tail vein following surgery) (n=8/group). Both si-NC (forward, 5'-UUCUCCGAACGU GUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGG AGAATT-3') and si-HRIM (forward, 5'-GCGCCAUUGCUG CAAAUUATT-3' and reverse, 5'-UAAUUUGCAGCAAUG GCGCTT-3') were purchased from Shanghai GenePharma Co., Ltd.

Transthoracic echocardiography (TTE). TTE was performed using a Vevo770 scanner (VisualSonics, Inc.). The left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-diastolic diameter (LVEDD), ventricular end-systolic diameter (LVESD), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum velocity of LV contraction (+dp/dt_{max}) and maximum velocity of LV diastolic function (-dp/dt_{max}) were measured via TTE in rats.

2,3,5-Triphenyltetrazolium chloride (TTC) staining. At one week after establishment of the MI/RI model, rats were anesthetized via intraperitoneal injection of pentobarbital sodium (50 mg/kg) and sacrificed by neck dislocation. Myocardial tissue was isolated, sectioned (thickness, 3 mm), stained with 1% TTC (cat. no. A610558; Sangon Biotech Co., Ltd.) for 30 min at 37°C, and fixed in 10% formalin for 10 min at 37°C. After washing with water, infarct size was quantified using the Motic Med 6.0 Digital Medical Image Analysis system (Motic Instruments). Infarcted myocardium was stained white, whereas surviving myocardium was stained brick red. The infarct size was calculated as follows: Infarct size (%)=(weight of white sections/weight of whole cardiac) x100%.

Hematoxylin-eosin (HE) staining. Myocardial tissue was collected, fixed in 4% paraformaldehyde at 37°C for 24 h, dehydrated with an ethanol gradient (70% ethanol for 2 h, 80% ethanol overnight, 90% ethanol for 2 h, 100% I ethanol for 1 h and 100% II ethanol for 1 h) vitrified with xylene, waxed, embedded in paraffin and cut into sections (thickness, 4 μ m). The sections were then stained with 0.5% hematoxylin for 5 min at 37°C, followed by 0.5% eosin for 1 min at 37°C. The ratio of inflammatory cells/field (5 random fields) in myocardial tissues was calculated by light microscopy (magnification, x400). Stained cells were analyzed using Image-ProPlus (version 6.0; Media Cybernetics, Inc.)

Oxygen-glucose deprivation/reoxygenation (OGD/R)-induced in vitro model. MI/RI was mimicked in vitro by establishing an OGD/R model using myocardial cells (H9C2 cells). H9C2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS at 37°C and 5% CO₂. In order to establish an OGD/R model, serum/glucose-free DMEM was used. H9C2 cells were placed in an anaerobic chamber (95% N₂ and 5% CO₂) at 37°C for 3 h. The medium was then replaced with normal culture medium and incubated for 0, 4 and 8 h to reoxygenate the H9C2 cells. The OGD3h/R0h, OGD3h/R4h and OGD3h/R8h groups received reoxygenation at 3 h post-OGD for 0, 4 and 8 h, respectively. In addition, H9C2 cells without treatment were regarded as the Normal group.

Cell transfection. For transfection experiments, H9C2 cells at >80% confluency were harvested and divided into three groups: OGD/R (H9C2 cells with reoxygenation at 3 h post-OGD for 8 h), OGD/R + si-NC (cells transfected with 50 nmol si-NC for 48 h) and OGD/R + si-HRIM (cells transfected with 50 nmol si-HRIM for 48 h). Asatone (an activator of the NF- κ B pathway; cat. no. HY-N6826;

MedChem Express) (32) was used to further investigate the association between HRIM and the NF- κ B pathway. Thus, the OGD/R + si-HRIM + Asatone group comprised cells transfected with 50 nmol si-HRIM and treated with 20 μ mol/l asatone at 37°C for 48 h. Cell transfection was performed using Lipofectamine[®] 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 25°C.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from myocardial tissue or cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and subsequently reverse-transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, qPCR was performed using the MiScript SYBR Green PCR kit (Qiagen, Inc.) and a ABI 7500HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95°C for 3 min and 40 cycles of 95°C for 15 sec and 60°C for 30 sec, and a final extension step at 72°C for 10 min. Relative expression levels were calculated via the 2^{- $\Delta\Delta$ Cq} method (33). HRIM expression levels were normalized to those of GAPDH. The primer sequences are presented in Table I.

Western blotting. A Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology) was used to extract nuclear and cytoplasmic proteins according to the manufacturer's protocol. Myocardial tissue from Sprague-Dawley rats or H9C2 cells were lysed using lysis buffer on ice to extract the total protein. Protein concentration was determined using a bicinchoninic acid protein concentration assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (20 µg) were separated via 10-12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline, containing 0.01% Tween-20 at 4°C overnight. The membranes were then incubated with the following primary antibodies (all 1:1,000; all Cell Signaling Technology, Inc.) overnight at 4°C: Anti-NF-KB p65 (cat. no. 8242), anti-phosphorylated (p)-NF-κB p65 (cat. no. 3033), anti-IκBα (cat. no. 4812) and anti-p-I κ B α (cat. no. 4812), followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. ab205718; Abcam) for 1 h at 37°C. The protein bands were visualized using enhanced chemiluminescence exposure solution (Pierce; Thermo Fisher Scientific, Inc.) and quantified with Quantity One 1-D analysis software (version 4.62; Bio-Rad Laboratories, Inc.). β-actin (1:1,000; cat. no. sc-517582; Cell Signaling Technology, Inc.) was used as the internal reference.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8) reagent (BD Biosciences) was used to evaluate the proliferation of H9C2 cells according to the manufacturer's instructions. H9C2 cells ($5x10^3$ cells/well) were seeded onto a 96-well plate and subjected to OGD/R. CCK-8 solution (10 μ l/well) was then added and incubated for 2 h at 37°C. The optical density at 450 nm (OD₄₅₀) was measured using a microplate reader (BioTek Instruments Inc.). Wells containing only culture medium and CCK-8 reagent were used as the blank group.

Table I. Primer sequences used for reverse transcriptionquantitative PCR.

Name	Sequence $(5' \rightarrow 3')$
HRIM	F:AGGGGAGGGGGAAAGTAGAA
	R: CACATGGAAGCCAGTGGTCA
GAPDH	F: GACGGCCGCATCTTCTTGT
	R: CACACCGACCTTCACCATTTT

F, forward; R, reverse; HRIM, Hypoxia/reoxygenation injury-related factor in myocytes.

Flow cytometry. After 48 h transfection in 24-well plates $(3x10^5 \text{ cells/well})$, cells were collected in 10 ml centrifuge tubes and centrifuged twice at 1,000 x g for 5 min at 4°C. Subsequently, the supernatant was collected and resuspended in binding buffer (140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES/NaOH, pH 7.4) to adjust the cell density to $1-5x10^6$ cells/ml. Next, 100 μ l cell suspension was cultured with 5 μ l V-FITC and 10 μ l PI for 15 min in the dark at 25°C. An additional 400 μ l 1X binding buffer was added to each tube. The apoptotic rate was then detected using a FACSArray BD Bioanalyzer flow cytometer (BD Biosciences), and data were analyzed via CytoDiff CXP software (version 2.0; Beckman Coulter, Inc.).

ELISA. Following cell transfection, cells from each group were centrifuged at 3,000 x g at 4°C for 10 min and the resulting supernatant was collected. The supernatant was collected and levels of TNF- α , IL-1 β , IL-6, lactate dehydrogenase (LDH) and creatine kinase (CK) were measured via ELISA kits (TNF- α , cat. no. KRC3011; IL-1 β , cat. no. BMS627; IL-6, cat. no. BMS625; all Thermo Fisher Scientific, Inc.; LDH, cat. no. ab183367; CK, cat. no. ab264617; both Abcam). The absorbance of each pore was measured at a wavelength of 450 nm via an enzyme mark sensing instrument (Molecular Devices LLC).

Statistical analysis. Statistical analysis was performed via SPSS software 23.0 (IBM Corp.). Data are presented as the mean \pm SD of three independent repeats. Comparisons between groups were performed via ANOVA followed by post hoc Tukey's multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

MI/RI affects myocardial function and upregulates HRIM expression levels in rats. Once the MI/RI model was established, hemodynamic indices were evaluated via TTE. Compared with the Control and Sham groups, LVEF, LVFS, LVSP, +dp/dt_{max}, and -dp/dt_{max} significantly decreased while LVEDD, LVESD, and LVEDP were significantly increased in the MI/RI group (P<0.01 and P<0.01; Fig. 1A). TTC staining results demonstrated that myocardial infarct size was significantly higher in the MI/RI group compared with the Sham group (P<0.001; Fig. 1B). HE staining revealed that



Figure 1. MI/RI affects myocardial function in rats. (A) Hemodynamics indices were evaluated via transhoracic echocardiography. (B) Myocardial infarct size was identified via 2,3,5-triphenyltetrazolium chloride staining. Scalebar, 2 mm. (C) Ratio of inflammatory cells/field in myocardial tissue was observed via hematoxylin-eosin staining. Scale bar, 100 μ m. (D) Expression levels of HRIM in myocardial tissue were detected via reverse transcription-quantitative PCR. n=3. **P<0.01 and ***P<0.001 vs. Sham. MI/RI, myocardial ischemia/reperfusion injury; HRIM, hypoxia/reoxygenation injury-related factor in myocytes; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVEDD, left ventricular end-diastolic diameter; LVESD, ventricular end-systolic diameter; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; (+dp/dt_{max}, maximum velocity of LV contraction; -dp/dt_{max}, maximum velocity of LV diastolic function.

the ratio of inflammatory cells/field in myocardial tissue was significantly increased in the MI/RI group compared with the Sham group (P<0.001; Fig. 1C). Additionally, HRIM expression levels were significantly upregulated in the MI/RI group compared with the Sham group (P<0.001; Fig. 1D).

OGD/R induces myocardial injury and upregulates HRIM expression levels in myocardial cells. H9C2 cells were subjected to OGD/R to mimic MI/RI conditions in vitro. Following reoxygenation at 3 h post-OGD for 0, 4 and 8 h, the survival rate of H9C2 cells significantly decreased compared with normal cells (P<0.01 and P<0.001; Fig. 2A). RT-qPCR results demonstrated that the expression levels of HRIM in H9C2 cells significantly increased in the OGD3h/R0h, OGD3h/R4h and OGD3h/R8h groups compared with the Normal group (P<0.001; Fig. 2B). The OGD3h/R8h group was selected for subsequent experiments due to the relative high HRIM expression.

In order to examine the role of HRIM, effective HRIM knockdown was performed in H9C2 cells via si-HRIM transfection (P<0.001; Fig. 2C). The CCK-8 assay results demonstrated that the OD₄₅₀ value of the OGD/R group was lower compared with the Normal group at 48 and 72 h post-culturing (P<0.01), and that silencing of HRIM significantly increased the OD₄₅₀ value of H9C2 cells compared with the OGD/R + si-NC group at 48 and 72 h post-culturing (P<0.01; Fig. 2D). Additionally, apoptosis of H9C2 cells significantly increased following OGD/R compared with the Normal group (P<0.001), whereas HRIM

knockdown decreased H9C2 cell apoptosis compared with the OGD/R + si-NC group (Fig. 2E).

Silencing of HRIM protects against inflammation and injury in H9C2 cells. In order to investigate the effect of HRIM knockdown on inflammation in H9C2 cells, levels of IL-1 β , TNF- α and IL-6 were determined via ELISA. Compared with the Normal group, the levels of IL-1 β , TNF- α and IL-6 in H9C2 cells were significantly increased in the OGD/R group (P<0.001); however, HRIM knockdown significantly attenuated these effects (P<0.001; Fig. 3A). Moreover, compared with the Normal group, the levels of LDH and CK in H9C2 cells were significantly elevated in the OGD/R group (P<0.001), and HRIM inhibition significantly decreased these levels in the OGD/R + si-HRIM group (P<0.001; Fig. 3B and C).

Silencing of HRIM inhibits the NF- κ B signaling pathway. In order to evaluate the effect of HRIM inhibition on the NF- κ B signaling pathway in H9C2 cells, the expression levels of NF- κ B-associated proteins were measured via western blotting. The results demonstrated that the levels of p-NF- κ B p65/NF- κ B p65 and p-IkB α /IkB α were significantly increased in the OGD/R group compared with the Normal group (P<0.001). However, compared with the OGD/R + si-NC group, knockdown of HRIM significantly decreased the levels of p-NF- κ B p65 and p-IkB α (P<0.01; Fig. 4A). Furthermore, compared with the Normal group, the levels of cytoplasmic NF- κ B p65 significantly increased (P<0.001), but those of nuclear NF- κ B p65 significantly decreased in the OGD/R



Figure 2. Silencing of HRIM enhances proliferation and attenuates apoptosis of OGD/R cells. Normal group comprised untreated H9C2 cells; OGD3h/R0h, OGD3h/R4h and OGD3h/R4h groups received reoxygenation at 3 h post-OGD for 0, 4 and 8 h, respectively. (A) Cell survival rate was assessed via CCK-8 assay. Expression levels of HRIM in (B) OGD/R and (C) transfected cells were detected via reverse transcription-quantitative PCR. **P<0.01 and ***P<0.001 vs. Normal or si-NC; #*P<0.01 and ##*P<0.001 vs. OGD3h/R0h. (D) OD₄₅₀ value at 0, 24, 48 and 72 h were observed via CCK-8 assay. (E) Apoptosis rate of H9C2 cells detected via flow cytometry. n=3. **P<0.01 and ***P<0.001 vs. Normal; #*P<0.001 vs. OGD/R + si-NC. HRIM, hypoxia/reoxygenation injury-related factor in myocytes; OGD/R, oxygen-glucose deprivation/reoxygenation; CCK-8, Cell Counting Kit-8; OD₄₅₀, optical density at 450 nm; si, small interfering RNA; NC, negative control.



Figure 3. Silencing of HRIM protects against inflammation and injury in OGD/R cells. (A) Levels of IL-1 β , TNF- α , IL-6, (B) LDH and (C) CK in H9C2 cells were measured via ELISA. n=3. ***P<0.001 vs. Normal; ###P<0.001 vs. OGD/R + si-NC. HRIM, hypoxia/reoxygenation injury-related factor in myocytes; OGD/R, oxygen-glucose deprivation/reoxygenation; LDH, lactate dehydrogenase; CK, creatine kinase; si, small interfering RNA; NC, negative control.

group (P<0.001); however, HRIM knockdown attenuated these effects (P<0.01; Fig. 4B).

In order to investigate the association between HRIM and NF- κ B signaling, H9C2 cells were treated with the NF- κ B

signaling pathway activator asatone. CCK-8 assay and flow cytometry results demonstrated that HRIM knockdown significantly increased the OD_{450} value at 48 and 72 h (P<0.001) post-culturing and significantly decreased the apoptosis of



Figure 4. Silencing of HRIM inhibits the NF- κ B signaling pathway in OGD/R cells. Levels of (A) p-NF- κ B p65/NF- κ B p65 and p-IkB α /IkB α and (B) NF- κ B p65 (cyto) and (Nuc) were measured via western blotting. n=3. ***P<0.001 vs. Normal; #P<0.01, ##P<0.001 vs. OGD/R + si-NC. HRIM, hypoxia/reoxygenation injury-related factor in myocytes; OGD/R, oxygen-glucose deprivation/reoxygenation; cyto, cytoplasmic; Nuc, nuclear; si, small interfering RNA; NC, negative control; p, phosphorylated.



Figure 5. Silencing of HRIM regulates the NF- κ B signaling pathway to alleviate injury of myocardial cells in an OGD/R model. (A) Proliferation of H9C2 cells was measured using a Cell Counting Kit-8 assay. (B) Apoptosis of H9C2 cells was detected via flow cytometry. Levels of (C) IL-1 β , TNF- α , IL-6, (D) CK and (E) LDH in H9C2 cells were measured via ELISA. n=3. ###P<0.001 vs. OGD/R + si-NC; & P<0.01 and & & P<0.001 vs. OGD/R + si-HRIM. HRIM, hypoxia/reoxygenation injury-related factor in myocytes; OGD/R, oxygen-glucose deprivation/reoxygenation; CK, creatine kinase; LDH, lactate dehydrogenase; si, small interfering RNA; NC, negative control.

H9C2 cells (P<0.001; Fig. 5A and B). Moreover, HRIM knockdown significantly decreased the levels of TNF- α , IL-1 β , IL-6, CK and LDH in H9C2 cells (P<0.001; Fig. 5C-E). However, the inhibitory effects of HRIM knockdown on H9C2 cells were reversed by treatment with asatone (P<0.01).

Silencing of HRIM inhibits MI/RI injury via inactivating the NF- κ B signaling pathway. The biological function of HRIM in MI/RI rats was investigated. TTE analysis demonstrated that silencing of HRIM significantly increased the LVEF, LVFS, LVSP, +dp/dt_{max} and -dp/dt_{max} (P<0.05), but significantly decreased the LVEDD, LVESD and LVEDP in MI/RI rats compared with the MI/RI + si-NC group (P<0.001; Fig. 6A). Moreover, the ratio of inflammatory cells/field in myocardial tissue was significantly decreased in the MI/RI + si-HRIM group compared with that in the MI/RI + si-NC group (P<0.001; Fig. 6B). HRIM knockdown significantly decreased the expression levels of HRIM in MI/RI rats (P<0.001) compared

with the MI/RI + si-NC group (Fig. 6C). Additionally, knockdown of HRIM significantly decreased the levels of p-NF- κ B p65/NF- κ B p65, p-IkB α /IkB α and cytoplasmic NF- κ B p65 and significantly increased nuclear NF- κ B p65 levels in MI/RI rats compared with the MI/RI + si-NC group (P<0.01; Fig. 6D and E).

Discussion

MI/RI is a major cause of cardiac dysfunction (34). Numerous studies have reported that abnormal expression levels of lncRNAs are associated with elevation of MI/RI (14,35). In the present study, the expression levels of HRIM in myocardial tissue of MI/RI rats were significantly upregulated, which is consistent with previously described lncRNAs (11,16). The expression levels of the lncRNAs necrosis-related factor (36), AK123487 (37) and lncRNA nuclear enriched abundant transcript 1 (38) have previously been shown to be increased



Figure 6. Silencing of HRIM attenuates MI/RI and inhibits the NF- κ B signaling pathway in rats. (A) Hemodynamics indices were evaluated via transthoracic echocardiography. (B) The ratio of inflammatory cells/field in myocardial tissue was observed via hematoxylin-eosin staining. Scale bar, 100 μ m. (C) Expression levels of HRIM in myocardial tissue were detected via reverse transcription-quantitative PCR. Expression levels of (D) p-NF- κ B p65/NF- κ B p65 and p-IkB α /IkB α and (E) NF- κ B p65 (cyto) and (Nuc) were detected via western blotting. n=3. *P<0.05, **P<0.01, ***P<0.001 vs. MI/RI + si-NC. HRIM, hypoxia/reoxygenation injury-related factor in myocytes; MI/RI, myocardial ischemia/reperfusion injury; cyto, cytoplasmic; Nuc, nuclear; si, small interfering RNA; NC, negative control; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVEDD, left ventricular end-diastolic diameter; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; (+dp/dt_{max}, maximum velocity of LV contraction; -dp/dt_{max}, maximum velocity of LV diastolic function; p, phosphorylated.

in MI/RI cardiomyocytes. It was hypothesized that HRIM may serve a key role in the treatment of MI/RI. The present results demonstrated that knockdown of HRIM significantly elevated the LVEF, LVFS, LVSP, +dp/dt_{max} and -dp/dt_{max}, and decreased the LVEDD, LVESD, LVEDP and ratio of inflammatory cells/field in myocardial tissue in MI/RI rats. Hence, these findings indicated that silencing of HRIM may improve cardiac function in MI/RI rats.

An OGD/R-induced model was established to mimic MI/RI in myocardial cells *in vitro*. Following reoxygenation at 3 h post-OGD for 0, 4 and 8 h, the survival rate of H9C2 cells was decreased, whereas HRIM expression levels were increased. A recent study has demonstrated the promoting effect of lncRNAs in the pathogenesis of cardiac diseases, including secretion of proinflammatory cytokines and myocardial cell apoptosis and damage (39). Moreover, knockdown of HRIM has been shown to increase the survival rate of H9C2 cells in H/R via decreasing autophagy in myocytes (40). Downregulation of HRIM has also been demonstrated to promote myocardial cell viability in MI/RI via inducing autophagy (41). Furthermore, inhibition of the lncRNA regulator of reprogramming has been shown to increase cell viability and decrease apoptosis in H9C2 cells (42). The present results indicated that silencing of HRIM ameliorated MI/RI in rats via increasing proliferation and decreasing apoptosis of myocardial cells. Inflammation is a key feature of MI/RI (43). Evidence has demonstrated that inhibition of proinflammatory cytokines can ameliorate MI/RI (44,45). High levels of LDH and CK are key biomarkers of myocardial damage (46). In the present study, knockdown of HRIM significantly decreased the levels of TNF- α , IL-6, IL-1 β , LDH and CK in myocardial cells. Moreover, silencing of HRIM alleviated MI/RI-induced inflammation and damage. These results indicated that HRIM knockdown may protect myocardial cells against MI/RI.

NF-KB participates in inflammatory responses and blockade of the NF-κB signaling pathway has been demonstrated to ameliorate MI/RI (47). In addition, total glucosides of peonies have been shown to alleviate MI/RI in rats via decreasing NF-κB expression levels (48). 17-methoxyl-7-h vdroxy-benzene-furanchalcone protects against MI/RI by decreasing the activity of NF- κ B p65 (46). In the present study, HRIM knockdown decreased the expression levels of NF-kB-associated proteins, including p-NF-kB p65/NF-kBp65 and p-IkB α /IkB α . p65 is a major subunit of NF- κ B (49). Under the action of IKK, IkBa is phosphorylated and then dissociated from the p65/p50 dimer, and p65 is dissociated in the cytoplasm (27). Phosphorylated p65 and p50 are then translocated into the nucleus (50). The phosphorylation levels of IkB α and p65 and translocation of p65 to the nucleus are key to verifying NF- κ B pathway activation (51). A previous study demonstrated that silencing of the lncRNA C2dat1 inhibited focal cerebral I/R injury via suppressing the NF-kB signaling pathway (48). The present study demonstrated that silencing of HRIM suppressed NF-κB activity to alleviate MI/RI in cells, and that treatment with asatone effectively reverses this inhibitory effect. Hence, the present results indicate that silencing of HRIM may protect against MI/RI via suppression of the NF-κB signaling pathway in rats.

There are certain limitations in the present study. Due to the lack of additional data, such as clinical samples with follow-up information, it was difficult to determine the clinical importance of HRIM. Furthermore, although the NF- κ B signaling pathway is regulated via HRIM, further investigation is required to determine whether HRIM is directly involved in NF- κ B signaling.

In summary, the present findings demonstrated that HRIM expression levels were significantly increased in the myocardial tissue of MI/RI rats and OGD/R-induced H9C2 cells. Moreover, knockdown of HRIM decreased inflammation, apoptosis and myocardial injury via inactivating the NF- κ B signaling pathway. Knockdown of HRIM also improved cardiac function in MI/RI rats. Hence, HRIM may act as a potential therapeutic target for MI/RI.

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Availability of data and materials

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

Authors' contributions

LN made substantial contributions to the conception and design of the study. YZ and WP acquired the data. YZ and SL interpreted the data. SL and WP performed the experiments. SL performed the data analysis. LN and WP drafted the manuscript and revised it critically for important intellectual content. YZ revised the manuscript for critically important intellectual content. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was performed with the approval of the Animal Ethics Committee of Qingdao Municipal Hospital (approval no. 2020-L-054).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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