Original Paper

Long Non-Coding RNA H19 Protects H9c2 Cells against Hypoxia-Induced Injury by Targeting MicroRNA-139

Li-Cheng Gong^a Hai-Ming Xu^a Gong-Liang Guo^a Tao Zhang^b Hong Chang^a

^aDepartment of Cardiovascular Internal Medicine, ^bDepartment of Gastrointestinal Surgery, ^cDepartment of Clinical Laboratory, China-Japan Union Hospital of Jilin University, Changchun, China

Key Words

Long non-coding RNA H19 • Acute myocardial infarction • MiR-139 • Sox8 • PI3K/AKT • MTOR • MAPK

Abstract

Background/Aims: Acute myocardial infarction (AMI) occurs when blood supply to the heart is diminished (ischemia) for long time. Ischemia is primarily caused due to hypoxia. The present study evaluated the effects of long non-coding RNA H19 on hypoxic rat H9c2 cells and mouse HL-1 cells. **Methods:** Hypoxia was confirmed by measuring cell viability, migration and invasion, and apoptosis using MTT, Transwell and flow cytometry assays, respectively. H19 expression after hypoxia was estimated by qRT-PCR. We then measured the effects of non-physiologically expressed H19, knockdown of miR-139 with or without H19 silence, and abnormally expressed Sox8 on hypoxia-induced H9c2 cells. Moreover, the interacted miRNA for H19 and downstream target gene were virtually screened and verified. The involved signaling pathways and the effects of abnormally expressed H19 on contractility of HL-1 cells were explored via Western blot analysis. **Results:** Hypoxia induced decreases of cell viability, migration and invasion, increase of cell apoptosis and up-regulation of H19. Knockdown of H19 increased hypoxia-induced injury in H9c2 cells. H19 acted as a sponge for miR-139 and H19 knockdown aggravated hypoxia-induced injury by up-regulating miR-139. Sox8 was identified as a target of miR-139, and its expression was negatively regulated by miR-139. The mechanistic studies revealed that overexpression of Sox8 might decrease hypoxia-induced cell injury by activating the PI3K/AKT/mTOR pathway and MAPK. Besides, Sox8 promoted contractility of HL-1 cells. **Conclusion:** These findings suggest that H19 alleviates hypoxia-induced myocardial cell injury by miR-139-mediated up-regulation of Sox8, along with activation of the PI3K/AKT/mTOR pathway and MAPK.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Acute myocardial infarction (AMI) is one of the most severe manifestations of coronary artery diseases that causes irreversible damage to the heart muscle, and can lead to

Chang Hong

Department of Cardiovascular Internal Medicine, China-Japan Union Hospital of Jilin University, No.126, Xiantai Street, Changchun, (China)
E-Mail changhong0018@126.com

development of heart failure [1, 2]. AMI leads to more than 4 million deaths in Europe and northern Asia [3]. AMI is traditionally divided into ST elevation and non-ST elevation myocardial infarction (MI) [4]. Generally, MI occurs due to prolonged ischemic condition (diminished blood supply to the heart). Myocardial ischemia occurs due to increased myocardial metabolic demand, and/or decreased delivery of oxygen (hypoxia) and nutrients to the myocardium via the coronary circulation [5]. Therefore, the regulation of hypoxia-induced injury to the myocardium and the underlying molecular mechanism are of great importance.

Long non-coding RNAs (lncRNAs) are a diverse class of non-protein-coding transcripts, which play important roles in various biological processes [6, 7]. These biological processes regulated by lncRNAs include invasion, metastasis, apoptosis, DNA damage, and cancer. microRNA (miR/miRNA) silencing, embryonic development, cardiac development, and tumor development [8-11]. Recently, few literatures have focused on the role of lncRNAs in AMI. For instance, circulating level of lncRNA ZFAS1 in AMI subjects was significantly higher than that in non-AMI subjects, whereas the level of lncRNA CDR1AS in AMI subjects was lower than that in non-AMI subjects [12]. Vausort et al. studied expressions of different lncRNAs in patients with MI, and showed that several lncRNAs were abnormally expressed in MI patients [13]. Another study also stated that lncRNA UCA1 promotes cardiomyocyte apoptosis by inhibiting p27 expression [14].

The lncRNA H19, a 2.3 kb lncRNA transcribed from the H19 gene, is highly expressed in several tissues of fetus, but the expression is significantly reduced after birth [15, 16]. Researchers have reported contradictory functions of H19, especially in cancer [15, 17-20]. Initially, H19 is identified as a tumor suppressor [21], however, mounting studies have reported that H19 is overexpressed in multiple types of cancers, including breast cancer [17], bladder cancer [18], and cervical carcinoma [19]. The role of H19 in cell injury has been evaluated in very few studies, for instance, Kim et al. reported that H19 is re-expressed in rat vascular smooth muscle cells after injury [20]. To our knowledge, very little is known about the functional roles of H19 in hypoxia-induced injury to myocardial cells. Here, we studied the expression of H19 in hypoxic H9c2 cells. In addition, we studied the functions and the possible underlying molecular mechanisms of H19 in hypoxic H9c2 cells. Besides, the effects of abnormally expressed H19 on contractility of mouse HL-1 cells were also investigated.

Materials and Methods

Cell culture and treatment

H9c2 cells, derived from rat embryonic ventricular cardiomyocytes, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Carlsbad, CA, USA). Mouse cardiomyocyte HL-1 cells were maintained in StemComb medium containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 0.1 mM noradrenaline (Sigma-Aldrich, St Louis, MO, USA). For normoxic condition, cells were maintained at 37°C in an atmosphere of 95% air and 5% CO₂. To stimulate hypoxia, cells were adapted in a hypoxic incubator containing 94% N₂, 5% CO₂, and 1% O₂.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression level of H19 was measured by using One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China). For the measurement of miR-139 expression, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II were used for reverse transcription and quantitative PCR. For the measurement of mRNA expression level of Sox8, RNA PCR Kit (AMV) Ver. 3.0 and SYBR® Premix Ex Taq™ (both TaKaRa Biotechnology) were used for reverse transcription and quantitative PCR. The relative expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; for H19 or Sox8 mRNA) or U6 (for miR-139) using the 2^{-ΔΔCt} cycle threshold method [22].

Cell transfection

Short-hairpin (sh) RNAs directed against H19 or Sox8, or non-targeting sequence was ligated into the U6/GFP/Neo plasmids (GenePharma, Shanghai, China) and the resultant plasmids were referred to as sh-H19, sh-Sox8 or shNC. Full-length H19 sequences and Sox8 sequences were sub-cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pEX-2 plasmids (GenePharma), respectively, and the resultant plasmids were referred to as pc-H19 and pEX-Sox8. Lipofectamine 3000 reagent (Life Technologies Corporation) was used for cell transfection according to the manufacturer's instructions. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich). Scramble miRs, miR-139 mimic, miR-139 inhibitor and its negative control (NC), synthesized by Life Technologies Corporation, were transfected into cells using Lipofectamine 3000 reagent and the transfected cells were harvested at 48 h post-transfection.

Cell viability, migration and invasion assays

Cell viability was assessed by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In brief, cells (1×10^5 cells/well) were seeded into 96-well plates and cultured for 24 h. After treatments, 20 μ l MTT solution (5 mg/ml; Sigma-Aldrich) was added into each well for 2 h, 24 h, 72 h and 96 h post-hypoxia, and the mixture was incubated at 37°C for additional 4 h. After adding 100 μ l dimethyl sulfoxide, absorbance was read using a Microplate reader (Bio-rad, Richmond, CA, USA) at 490 nm.

Cell migration was determined using a modified two-chamber migration assay with a pore size of 8 μ m. Cells suspended in 200 μ l of serum-free medium were seeded into the upper compartment of 24-well Transwell culture chamber, and 600 μ l of complete medium was added into the lower compartment. After incubation at 37°C, cells were fixed with methanol. Traversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted. The protocol of cell invasion is consistent with that of cell migration except that the membranes were pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA).

Apoptosis assay

Cell apoptosis analysis was performed using propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining. Briefly, cells were washed in phosphate buffered saline (PBS), suspended in binding buffer, and stained with FITC-Annexin V in the presence of 50 μ g/ml RNase A (Sigma-Aldrich) according to the manufacturer's instructions. Flow cytometry analysis was done using a FACScan (Beckman Coulter, Fullerton, CA, USA). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

Luciferase reporter assay

The fragment of Sox8 3' untranslated region (3'UTR) containing the predicted miR-139 binding site was cloned into pGL3 reporter (Promega, Madison, WI, USA) to form the reporter vector Sox8-wild-type (Sox8-Wt). To mutate the miR-139 binding site of miR-139 in Sox8 3'UTR, site-mutations were performed to generate Sox8-mutant type (Sox8-Mt). Then the vectors (Sox8-Wt or Sox8-Mt) and miR-139 mimics or scramble were co-transfected into cells, and Dual Luciferase Reporter Assay System (Promega) was used to test the luciferase activity.

Western blot analysis

Proteins used for western blotting were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). After separation using SDS-PAGE, proteins in the gels were transferred into polyvinylidene difluoride (PVDF) membranes. Then, membranes were blocked with non-fat milk and incubated with primary antibody at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the membranes were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μ l Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured using Image Lab™ software (Bio-Rad).

Statistical analysis

The results of multiple experiments are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed on normalized data using Graphpad 6.0 statistical software. P values were

calculated using one-way analysis of variance. P value of <0.05 was considered to indicate a statistically significant result.

Results

Hypoxia induces cell injury and up-regulation of H19 in H9c2 cells

First, we induced hypoxia in H9c2 cells by incubating the cells in a hypoxic incubator containing 94% N₂, 5% CO₂, and 1% O₂. Then, cell viability, migration, invasion, and apoptosis of hypoxic H9c2 cells and normoxic cells (control) were measured. As compared to the control group, significant decreases in cell viability (P<0.01 and P<0.001 at 72 h and 96 h post-hypoxia, respectively; Fig. 1A), migration (P<0.01; Fig. 1B), and invasion (P<0.01; Fig. 1C) as well as a significant increase in cell apoptosis (P<0.001; Fig. 1D) were observed in the hypoxia groups. Western blot analysis showed that the protein expression of pro-apoptotic proteins (Bax, cleaved caspase-3, and cleaved caspase-9) was increased, whereas expression of anti-apoptotic protein (Bcl-2) was decreased in hypoxic cells as compared to the control group (Fig. 1E). Besides, results of qRT-PCR showed H19 was markedly up-regulated after hypoxia treatments compared to the control group (P<0.001; Fig. 1F). These findings indicate that hypoxia inhibits cell viability, migration, and invasion, and promotes cell apoptosis of H9c2 cells, along with up-regulation of H19.

Hypoxia-induced cell injury is aggravated by H19 knockdown but is attenuated by H19 overexpression

Then we measured the effects of nonphysiologically expressed H19 on hypoxic H9c2 cells. In Fig. 2A, expression level of H19 was significantly decreased by transfection with sh-H19 as compared to the shNC group (P<0.001). Knockdown of H19 using sh-H19 significantly decreased cell viability (P<0.01 and P<0.05 at 72 h and 96 h post-hypoxia, respectively), migration (P<0.05), and invasion (P<0.05), while significantly increased cell

Fig. 1. Hypoxia induces cell injury and up-regulation of H19 in H9c2 cells. H9c2 cells were incubated in a hypoxic incubator containing 94% N₂, 5% CO₂, and 1% O₂ to induce hypoxia. Then, (A) cell viability, (B) cell migration, (C) cell invasion, and (D) cell apoptosis of hypoxic cells and normoxic cells (control group) were measured. (E) Western blot analysis was used to measure the expressions of apoptosis-associated proteins. (F) Expression of H19 was measured by qRT-PCR. Each experiment was repeated three times. **P<0.01, ***P<0.001. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-2: mammalian B cell lymphoma-2; Bax: Bcl-2-associated X protein.

Fig. 2. Hypoxia-induced H9c2 cell injury is aggravated by H19 knockdown but is attenuated by H19 overexpression. Cells in the control group were cultured under normoxia. (A) Effects of H19 knockdown on hypoxia-induced cell injury. (B) Effect of H19 overexpression on hypoxia-induced cell injury. Each experiment was repeated three times. Data are expressed as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-2: B-cell lymphoma-2; Bax: Bcl-2-associated X protein; sh-H19: short-hairpin RNA-suppressed H19; pcDNA3.1: negative control of sh-H19.

apoptosis ($P < 0.05$) in hypoxic cells compared to the hypoxia + shNC groups. Western blot analysis showed that the knockdown of H19 increased the protein expression of Bax, cleaved caspase-3 and cleaved caspase-9, and decreased the expression of Bcl-2 in hypoxic cells. In hypoxic cells, the expression level of H19 was remarkably up-regulated after transfection with pc-H19 compared to the pcDNA3.1 group ($P < 0.001$). Further results showed the effects of H19 overexpression on cell viability, migration, invasion and cell apoptosis were opposite to those of H19 knockdown. Hypoxia-induced cell injury is aggravated by H19 knockdown but is attenuated by H19 overexpression in H9c2 cells.

H19 acts as a sponge of miR-139

Although lncRNAs are functionally and structurally different from miRNAs, few studies have suggested a connection between these two classes of RNAs [16, 23]. By using bioinformatics methods, miR-139 was predicted to bind to H19, and the binding sequences were shown in the Fig. 3A. To verify the possible connection between H19 and miR-139, the relative expression of miR-139 was measured in transfected cells. As shown in Fig. 3B, knockdown of H19 significantly increased the relative expression level of miR-139 as compared with the shNC group ($P < 0.001$), suggesting that H19 negatively regulated the expression of miR-139.

Results indicate that H19 can act as a sponge for miR-139.

The effects of H19 knockdown on hypoxia-induced cell injury are reversed by miR-139 suppression

As miR-139 was identified as a target of H19, we studied the effects of miR-139 on hypoxia-induced cell injury with or without H19 knockdown. After cell transfection, expression level of miR-139 was significantly up-regulated by miR-139 mimic as compared to the scramble group ($P < 0.001$; Fig. 4A) but was markedly down-regulated by miR-139 inhibitor as compared to the NC group ($P < 0.001$; Fig. 4B). Then, the effects of abnormally expressed miR-139 on cell viability, migration, invasion, and apoptosis of hypoxia-treated H9c2 cells were explored. When compared to the hypoxia + NC groups, miR-139 inhibition significantly increased cell viability (both $P < 0.05$ at 72 h and 96 h post-hypoxia; Fig. 4C), migration ($P < 0.05$; Fig. 4D) and invasion ($P < 0.05$; Fig. 4E) but reduced cell apoptosis ($P < 0.01$; Fig. 4F). Likewise, hypoxia-induced alteration of apoptosis-associated proteins was reversed by miR-139 inhibition (Fig. 4G). Subsequently, the effects of miR-139 inhibition on hypoxia-induced injury of H19-silenced H9c2 cells were further explored. As compared to the hypoxia + sh-H19 + NC groups, down-regulation of both miR-139 and H19 expression significantly increased cell viability (both $P < 0.05$ at 72 h and 96 h post-hypoxia; Fig. 4H), migration ($P < 0.01$; Fig. 4I) and invasion ($P < 0.01$; Fig. 4J), but decreased cell apoptosis ($P < 0.05$; Fig. 4K). Likewise, H19 knockdown-induced alterations of apoptosis-associated proteins were all reversed by miR-139 inhibition (Fig. 4L). These findings suggest that H19 knockdown aggravates hypoxia-induced cell injury through up-regulating miR-139 expression.

Sox8 negatively regulated by miR-139

Utilizing the online TargetScan software, possible target genes of miR-139 were virtually screened. Among those targets, we chose Sox8 to deeply investigate. The binding sequences between miR-139 and Sox8 3'UTR were shown in the Fig. 5A. The qRT-PCR and western blot results showed that miR-139 mimic significantly decreased the mRNA and protein expressions of Sox8 ($P < 0.01$; Fig. 5B) while miR-139 inhibitor increased the expression of Sox8 ($P < 0.001$; Fig. 5C), indicating that Sox8 was negatively regulated by miR-139 expression. Dual luciferase reporter assay was performed to verify the targeting between miR-139 and Sox8 3'UTR. As shown in Fig. 5D, luciferase activity was significantly decreased in cells co-transfected with miR-139 mimic and Sox8-Wt, as compared to the co-transfection with Sox8-Wt and scramble miRs ($P < 0.05$). No significant changes were observed in cells co-transfected with Sox8-Mt vector. These findings suggest that Sox8 is a target of miR-139.

Overexpression of Sox8 alleviates hypoxia-induced cell injury of H9c2 cells

After stable transfection, the qRT-PCR and western blot results showed that pEX-Sox8 significantly increased the mRNA and protein expressions of Sox8 ($P < 0.001$; Fig. 6A-6B), while sh-Sox8 significantly decreased the expression of Sox8 ($P < 0.001$; Fig. 6C-D). In further

Fig. 3. H19 acts as a sponge for miR-139. (A) Sequence complementarity between H19 and miR-139-5p. (B) qRT-PCR was used to measure the relative miR-139 expression in H9c2 cells. Each experiment was repeated three times. *** $P < 0.001$. sh-H19: short-hairpin RNA-suppressed H19; shNC: negative control of sh-H19.

Fig. 4. H19 knockdown aggravates hypoxia-induced myocardial injury through up-regulating miR-139. Cells in the control group were cultured under hypoxia. (A–B) Relative miR-139 expression was measured by using qRT-PCR. Effects of miR-139 inhibition on cell viability (C), migration (D), invasion (E), apoptosis (F) and expressions of apoptosis-associated proteins (G) in hypoxia-treated H9c2 cells were measured. Effects of miR-139 inhibition on cell viability (H), migration (I), invasion (J), apoptosis (K) and expressions of apoptosis-associated proteins (L) in cells with H19 knockdown under hypoxia were measured. Each experiment was repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In C, * indicates significance of comparison between the hypoxia + NC group and the hypoxia + miR-139 inhibitor group. In H, * indicates significance of comparison between the hypoxia + sh-H19 + NC group and the hypoxia + shNC + NC group, and # indicates significance of comparison between the hypoxia + sh-H19 + miR-139 inhibitor group and the hypoxia + sh-H19 + NC group. # $P < 0.05$. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-2: mammalian B cell leukemia-2; Bax: Bcl-2-associated X protein; sh-H19: short-hairpin RNA-suppressed H19; shNC: negative control of sh-H19.

In experiments, effects of abnormally expressed Sox8 on cell viability, migration, invasion, and apoptosis of the hypoxic H9c2 cells were explored. The results showed that Sox8 overexpression significantly increased cell viability (both $P < 0.05$ at 72 h and 96 h post-transfection; Fig. 6E), migration ($P < 0.05$; Fig. 6F), and invasion ($P < 0.05$; Fig. 6G); and decreased cell apoptosis ($P < 0.05$; Fig. 6H) as compared to the hypoxia + pEX-2 groups. Western blot results showed the decreased expression of Bcl-2 and the increased expressions of pro-apoptotic proteins, which were induced by hypoxia, were all reversed by Sox8 overexpression (Fig. 6I). However, the effects of Sox8 silence on hypoxia-treated cells were just the opposite. These findings suggest that overexpression of Sox8 alleviates hypoxia-induced cell injury of H9c2 cells.

Fig. 5. Sox8 is a target of miR-139. (A) Sequence complementarity between Sox8 3'-untranslated region (3'UTR) and miR-139-5p. (B-C) mRNA and protein expressions of Sox8 in transfected H9c2 cells. (D) Relative luciferase activity of H9c2 cells co-transfected with Sox8-wild-type (Sox8-Wt) or Sox8-mutated-type (Sox8-Mt) vectors and scramble miRNAs or miR-139 mimic. Each experiment was repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NC: negative control of miR-139 inhibitor.

Fig. 6. Overexpression of Sox8 alleviates hypoxia-induced H9c2 cell injury. Cells in the control group were cultured under normoxia. qRT-PCR was used to measure mRNA expression and western blot was used to measure protein expression of Sox8 in transfected H9c2 cells (A-D). (E) Cell viability, (F) migration, (G) invasion, and (H) apoptosis of H9c2 cells were estimated. (I) Western blot analysis was used to measure the expressions of apoptosis-associated proteins. Each experiment was repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In E, * indicates significance of comparisons compared with the hypoxia + shNC group, and # indicates significance of comparison compared with the hypoxia + pEX-2 group, # $P < 0.05$. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Bcl-2: mammalian B cell lymphoma-2; Bax: Bcl-2-associated X protein; sh-Sox8: short-hairpin RNA-suppressed Sox8; shNC: negative control of sh-Sox8.

Sox8 activates the phosphoinositide 3-kinase (PI3K)/ serine-threonine kinase (AKT)/ mechanistic target of rapamycin (mTOR) pathway and mitogen-activated protein kinase (MAPK)

We then studied the underlying mechanism for the H19-associated modulations using western blot analysis. The results showed that overexpression of Sox8 increased the expressions of phosphorylated PI3K, AKT, mTOR, and MAPK, while the effects of Sox8 inhibition were just the opposite (Fig. 7). These findings indicate that overexpression of Sox8 may alleviate hypoxia-induced cell injury by activating the PI3K/AKT/mTOR pathway and MAPK.

H19 overexpression up-regulates expression of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) in HL-1 cells

Considering the interaction between H19 and miR-139 as well as the interaction between miR-139 and Sox8 are existed in mice, which are shown in the Fig. 3A and Fig. 5A, we finally explored the effects of abnormally expressed H19 on contractility using mouse HL-1 cells. Interestingly, protein expression level of SERCA2a was reduced under hypoxia and further reduced by H19 shNC (Fig. 8A), whereas the down-regulation was reversed by H19 overexpression (Fig. 8B). Results suggest that H19 promotes contractility. The final regulatory circuit of H19 in hypoxia-induced cell injury is shown in Fig. 9.

Discussion

In the present study, we evaluated the effects and the possible underlying mechanism of H19 expression on hypoxia-induced H9c2 cell injury. First of all, hypoxic condition induced injury to H9c2 cells by decreasing cell viability, migration and invasion, and increasing apoptosis as well as up-regulation of H19. Subsequently, hypoxia-induced cell injury was aggravated by H19 knockdown but was attenuated by H19 overexpression. In further experiments, H19 was identified as a sponge for miR-139. Interestingly, the effects of H19 knockdown on the hypoxic cells were reversed by miR-139 suppression. Sox8 was revealed to be

Fig. 7. Sox8 activates the PI3K/AKT/mTOR pathway and MAPK. Cells in the control group were cultured under normoxia. Western blot analysis was used to measure the protein expressions of PI3K, AKT, mTOR, MAPK, and GAPDH as well as phosphorylated proteins. AKT: serine-threonine kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MAPK: mitogen-activated protein kinase; mTOR: mechanistic target of rapamycin; PI3K: phosphoinositide 3-kinase; p-: phosphorylated; sh-Sox8: short-hairpin RNA-suppressed Sox8; shNC: negative control of sh-Sox8; pEX, pEX-2.

Fig. 8. H19 promotes contractility of HL-1 cells. Cells in the control group were cultured under normoxia. (A-B) Western blot analysis was used to measure the protein expressions of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a). GAPDH: glyceraldehyde-3-phosphate dehydrogenase; sh-H19: short-hairpin RNA-suppressed H19; shNC: negative control of sh-H19.

Fig. 9. Regulatory circuit. Solid arrows indicated positive regulation but dotted indicated negative regulation. AKT: serine-threonine kinase; MAPK: mitogen-activated protein kinase; mTOR: mechanistic target of rapamycin; PI3K: phosphoinositide 3-kinase; p: phosphate group.

a target of miR-139 and its expression was negatively regulated by miR-139. Overexpression of Sox8 decreased hypoxia-induced cell injury by activating the PI3K/AKT/mTOR pathway and MAPK. Finally, H19 was identified to promote contractility in HL-1 cells.

Role of lncRNA H19 has been studied in several malignant diseases. For example, Ma et al. reported that H19 promotes proliferation of pancreatic ductal adenocarcinoma cells [24]. Yang et al. showed that overexpression of H19 promotes cell proliferation and decreases apoptosis of gastric cancer cells [25]. In our study, we demonstrated that knockdown of H19 decrease cell viability, migration and invasion, increase apoptosis through mitochondrial- and caspase-dependent pathways in hypoxia-treated H9c2 cells, whereas the effects of H19 overexpression were just the opposite. To our knowledge, this is the first study reporting the effects of H19 on hypoxia-injured H9c2 cells.

We found H19 silence functioned by up-regulating miR-139. In our study, H19 was markedly up-regulated after hypoxia treatments, suggesting the possible involvements in the hypoxia-induced cell injury. miRNAs are small, non-coding RNAs that regulate the expression of protein-encoding genes at the post-transcriptional level. Accumulating evidence has reported the key roles of miRNAs in the process of hypoxia-induced myocardial injury [26-29]. A previous study showed expression of miR-139 was reported to be down-regulated in neurons and rat hearts after hypoxia [30]. Moreover, by using bioinformatics method, human H19 was predicted to bind to miR-139-5p. Thus, we speculated that there might be an interaction between H19 and miR-139. Following results illustrated miR-139 was negatively regulated by H19, and more data proved that the H19 inhibition aggravated hypoxia-induced cell injury by up-regulating miR-139.

We then explored the possible underlying mechanism for the effects of miR-139 on hypoxic cells. Towards this goal, putative target genes of miR-139 were screened using bioinformatics methods. Among those genes, Sox8, belonging to group E of SOX family proteins, is reported to promote cell proliferation of diverse cancer cells [31, 32]. Several researchers have reported that Sox8 is expressed in the developing heart [33, 34]. However, Sox8 has not been studied in hypoxia-induced myocardial injury. In the present study, Sox8 was identified to be a target of miR-139. Meanwhile, overexpression of Sox8 decreased hypoxia-induced cell injury by increasing cell viability, migration, and invasion and decreasing apoptosis. In further experiments, we studied effects of Sox8 on the PI3K/AKT/mTOR signal pathway and MAPK activation. PI3K/AKT signal pathway is an important pathway for protecting myocardial cells against hypoxia-induced injury [35, 36]. Xiao et al. reported that hydrogen sulfide protects myocardial cells against hypoxia-induced injury via mTOR activation [37]. Chen et al. reported that lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation

and Nrf2/ARE complex [38]. Our results showed that overexpression of Sox8 might alleviate hypoxia-induced H9c2 cell injury by activating the PI3K/AKT/mTOR pathway and MAPK, which should be verified by more experiments in the future.

HL-1 cells, established from the AT-1 mouse atrial cardiomyocyte tumor, are a widely used cell line which retains phenotypic characteristics of adult cardiomyocyte, such as contractile phenotype [39]. SERCA2a acting as the ATP-dependent calcium pump is reported to be a prime target for modulating cardiac contractility [40]. In our study, we finally explored the roles of non-physiologically expressed H19 in modulation of contractility by measuring the protein expression of SERCA2a. Results illustrated that hypoxia inhibited contractility of HL-1 cells and the contractility was further inhibited by H19 knockdown. Conversely, overexpression could promote contractility, suggesting the potential protective role of H19 in hypoxia-treated HL-1 cells.

Conclusion

In conclusion, this study demonstrated that knockdown of H19 aggravates hypoxia-induced H9c2 cell injury by decreasing cell viability, migration, invasion and increasing cell apoptosis. Importantly, we found that H19 acted as a sponge for miR-139 and Sox8 was a target of miR-139. Furthermore, we found overexpression of Sox8 might protect H9c2 cells from hypoxia-induced injury by activating the PI3K/AKT/mTOR pathway and MAPK. Finally, H19 was identified to promote contractility in HL-1 cells. Our research is a preliminary study that might provide new insights in the prevention and treatment of AMI. More experiments should be done in other cell types of cardiomyocytes to verify the conclusions.

Disclosure Statement

Authors declare that they have no conflict of interest.

References

- 1 Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, MacCallum C, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB: Executive summary: heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* 2012;125:188-197.
- 2 Teede GW, Jan JE, Cannon CP: Acute myocardial infarction. *Lancet* 2017;389:197-210.
- 3 Nichols M, Townsend N, Scarborough P, Rayner M: Cardiovascular disease in Europe 2014: epidemiological update. *Our Heart J* 2014;35:2950-2959.
- 4 Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD, Katus HA, Lindahl B, Morrow DA, Niemann PM, Johanson P, Hod H, Underwood R, Bax JJ, Bonow RO, Pinto F, Gibbons RJ, Fox KA, Atar D, Newby LK, Galvani M, Hamm CW, Uretsky BF, Steg PG, Wijns W, Bassand JP, Menasche P, Ravkilde J, Ohman EM, Antman EM, Wallentin LC, Armstrong PW, Simoons ML, Januzzi JL, Nieminen MS, Gheorghiade M, Filippatos G, Luepker RV, Fortmann SP, Rosamond WD, Levy D, Wood D, Smith SC, Hu D, Lopez-Sendon JL, Robertson RM, Weaver D, Tendera M, Bove AA, Parkhomenko AN, Vasilieva EJ, Mendis S: Third universal definition of myocardial infarction. *Circulation* 2012;126:2020-2035.
- 5 Nazir S, Tachamo N, Lohani S, Hingorani R, Poudel DR, Donato A: Acute myocardial infarction and antiphospholipid antibody syndrome: a systematic review. *Coron Artery Dis* 2017;28:332-335.
- 6 Ye LC, Zhu X, Qiu JJ, Xu J, Wei Y: Involvement of long non-coding RNA in colorectal cancer: From benchtop to bedside (Review). *Oncol Lett* 2015;9:1039-1045.

- 7 Gu M, Zheng A, Tu W, Zhao J, Li L, Li M, Han S, Hu X, Zhu J, Pan Y, Xu J, Yu Z: Circulating LncRNAs as Novel, Non-Invasive Biomarkers for Prenatal Detection of Fetal Congenital Heart Defects. *Cell Physiol Biochem* 2016;38:1459-1471.
- 8 Yang Z, Li X, Yang Y, He Z, Qu X, Zhang Y: Long noncoding RNAs in the progression, metastasis, and prognosis of osteosarcoma. *Cell Death Dis* 2016;7:e2389.
- 9 Zhou X, Liu S, Cai G, Kong L, Zhang T, Ren Y, Wu Y, Mei M, Zhang L, Wang X: Long Non Coding RNA MALAT1 Promotes Tumor Growth and Metastasis by inducing Epithelial-Mesenchymal Transition in Oral Squamous Cell Carcinoma. *Sci Rep* 2015;5:15972.
- 10 Bouckenheimer J, Assou S, Riquier S, Hou C, Philippe N, Sansac C, Lavabre-Bertrand T, Combes T, Lemaitre JM, Boureux A, De Vos J: Long non-coding RNAs in human early embryonic development and their potential in ART. *Hum Reprod Update* 2016;23:19-40.
- 11 Wang H, Bei Y, Shi J, Xiao J, Kong X: Non-Coding RNAs in Cardiac Aging. *Cell Physiol Biochem* 2017;44:1679-1687.
- 12 Zhang Y, Sun L, Xuan L, Pan Z, Li K, Liu S, Huang Y, Zhao X, Huang L, Wang Z, Hou Y, Li J, Wang L, Han H, Liu Y, Gao F, Zhang Y, Wang S, Du Z, Lu Y, Yang B: Reciprocal Changes of Circulating Long Non-Coding RNAs ZFAS1 and CDR1AS Predict Acute Myocardial Infarction. *Sci Rep* 2016;6:22384.
- 13 Vausort M, Wagner DR, Devaux Y: Long noncoding RNAs in patients with acute myocardial infarction. *Circ Res* 2014;115:668-677.
- 14 Liu Y, Zhou D, Li G, Ming X, Tu Y, Tian J, Lu H, Yu B: Long non coding RNA-UCA1 contributes to cardiomyocyte apoptosis by suppression of p27 expression. *Cell Physiol Biochem* 2015;35:1986-1998.
- 15 Gabory A, Ripoché MA, Yoshimizu T, Dandolo L: The H19 gene: regulation and function of a non-coding RNA. *Cytogenet Genome Res* 2006;113:188-193.
- 16 Li H, Yu B, Li J, Su L, Yan M, Zhu Z, Liu B: Overexpression of long non-coding RNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget* 2014;5:2333-2339.
- 17 Berteaux N, Lottin S, Monte D, Pinte S, Quatannens B, All J, Huettenmarck H, Cury JJ, Dugimont T, Adriaenssens E: H19 mRNA-like noncoding RNA promotes breast cancer cell proliferation through positive control by E2F1. *J Biol Chem* 2005;280:29635-29636.
- 18 Luo M, Li Z, Wang W, Zeng Y, Liu Z, Qiu J: H19 promotes bladder cancer cell proliferation by regulating ID2 expression. *Oncol Rep* 2013;30:1715-1716.
- 19 Kim SJ, Park SE, Lee C, Lee SY, Kim JM, Oh JK: Alterations in promoter usage and expression levels of insulin-like growth factor-II and H19 in human cervical carcinoma exhibiting biallelic expression of IGF-II. *Biochim Biophys Acta* 2006;1758:307-312.
- 20 Kim DK, Zhang L, Dzau VJ, Catt RF: H19, a developmentally regulated gene, is reexpressed in rat vascular smooth muscle cells after injury. *Proc Natl Acad Sci USA* 1994;93:355-360.
- 21 Hao Y, Crenshaw J, Walton J, Newcomb E, Tycko B: Tumour-suppressor activity of H19 RNA. *Nature* 1993;365:767-769.
- 22 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25:402-408.
- 23 Shi M, Chen Y, Luan W, Wang P, Tao T, Zhang J, Qian J, Liu N, You Y: Long non-coding RNA H19 promotes tumor progression by deriving miR-675. *PLoS One* 2014;9:e86295.
- 24 Li L, Tian X, Wang F, Zhang Z, Du C, Xie X, Kornmann M, Yang Y: The long noncoding RNA H19 promotes cell proliferation via E2F-1 in pancreatic ductal adenocarcinoma. *Cancer Biol Ther* 2016;17:1051-1061.
- 25 Yang L, Bi J, Xue X, Zheng L, Zhi K, Hua J, Fang G: Up-regulated long non-coding RNA H19 contributes to proliferation of gastric cancer cells. *Febs j* 2012;279:3159-3165.
- 26 Boon RA, Dimmeler S: MicroRNAs in myocardial infarction. *Nat Rev Cardiol* 2015;12:135-142.
- 27 Yang L, Song S, Lv H: MicroRNA-322 protects hypoxia-induced apoptosis in cardiomyocytes via BDNF gene. *Am J Transl Res* 2016;8:2812-2819.
- 28 Ke ZP, Xu P, Shi Y, Gao AM: MicroRNA-93 inhibits ischemia-reperfusion induced cardiomyocyte apoptosis by targeting PTEN. *Oncotarget* 2016;7:28796-28805.
- 29 Fan ZX, Yang J: The role of microRNAs in regulating myocardial ischemia reperfusion injury. *Saudi Med J* 2015;36:787-793.
- 30 Qu Y, Wu J, Chen D, Zhao F, Liu J, Yang C, Wei D, Ferriero DM, Mu D: MiR-139-5p inhibits HGTD-P and regulates neuronal apoptosis induced by hypoxia-ischemia in neonatal rats. *Neurobiol Dis* 2014;63:184-193.

- 31 Xie C, Han Y, Liu Y, Han L, Liu J: miRNA-124 down-regulates SOX8 expression and suppresses cell proliferation in non-small cell lung cancer. *Int J Clin Exp Pathol* 2014;7:7518-7526.
- 32 Zhang S, Zhu C, Zhu L, Liu H, Liu S, Zhao N, Wu J, Huang X, Zhang Y, Jin J, Ji T, Ding X: Oncogenicity of the transcription factor SOX8 in hepatocellular carcinoma. *Med Oncol* 2014;31:918.
- 33 Bell KM, Western PS, Sinclair AH: SOX8 expression during chick embryogenesis. *Mech Dev* 2000;94:257-260.
- 34 Montero JA, Giron B, Arrechdera H, Cheng YC, Scotting P, Chimal-Monroy J, Garcia-Porrero JA, Hurle JM: Expression of Sox8, Sox9 and Sox10 in the developing valves and autonomic nerves of the embryonic heart. *Mech Dev* 2002;118:199-202.
- 35 Li C, Tian J, Li G, Jiang W, Xing Y, Hou J, Zhu H, Xu H, Zhang G, Liu Z, Ye Z: Asperosaponin VI protects cardiomyocytes from hypoxia-induced apoptosis via activation of the PI3K/Akt and CREB pathways. *Pharmacol* 2010;649:100-107.
- 36 Lin KH, Kuo WW, Jiang AZ, Pai P, Lin JY, Chen WK, Day CH, Shen CY, Padma VV, Huang C: Tetramethylpyrazine Ameliorated Hypoxia-Induced Myocardial Cell Apoptosis via HIF-1 α , p38 and IGFBP3/BNIP3 Inhibition to Upregulate PI3K/Akt Survival Signaling. *Cell Physiol Biochem* 2015;36:334-344.
- 37 Xiao J, Zhu X, Kang B, Xu J, Wu L, Hong J, Zhang Y, Ni X, Wang Z: Hydrogen Sulfide Attenuates Myocardial Hypoxia-Reoxygenation Injury by Inhibiting Autophagy via mTORC1. *Cell Physiol Biochem* 2015;37:2444-2453.
- 38 Chen XQ, Wu SH, Zhou Y, Tang YR: Lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation and Nrf2/ARE complex. *PLoS One* 2013;8:e67120.
- 39 Claycomb WC, Lanson NA, Stallworth BS, Egeland DL, Murphy JB, Bahinski A, Izzo NJ: HL-1 cells. *Proc Natl Acad Sci U S A* 1998;95:2979-2984.
- 40 Park WJ, Oh JG: SERCA2a: a prime target for modulation of cardiac contractility during heart failure. *BMB Rep* 2013;46:237-243.