LncRNA SNHG15 Modulates Ischemia-Reperfusion Injury in Human AC16 Cardiomyocytes Depending on the Regulation of the miR-335-3p/TLR4/NF-κB Pathway

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EXPERIMENTAL STUDY

Summary
Myocardial ischemia-reperfusion (I/R) injury is a serious complication of acute myocardial infarction. Long noncoding RNA (lncRNA) small nucleolar RNA host gene 15 (SNHG15) can regulate I/R-induced cardiomyocyte apoptosis. Here, we investigated the mechanism of SNHG15 activity in I/R-induced cardiomyocyte injury. SNHG15, microRNA (miR)-335-3p, and toll-like receptor 4 (TLR4) were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. Cell viability, proliferation, and apoptosis were gauged by Cell Counting Kit-8 (CCK-8) assay, 5-ethynyl-2´-deoxyuridine (EDU) assay, and flow cytometry, respectively. The direct relationship between miR-335-3p and SNHG15 or TLR4 was validated by dual-luciferase reporter, RNA immunoprecipitation (RIP), and RNA pull-down assays.

SNHG15 was overexpressed in the infarcted area tissues of I/R mice and I/R-stimulated AC16 cells. SNHG15 knockdown alleviated I/R injury in AC16 cells. Mechanistically, SNHG15 directly targeted miR-335-3p, and miR-335-3p was a functional mediator of SNHG15. MiR-335-3p inhibited TLR4 expression by targeting TLR4, and miR-335-3p-mediated inhibition of TLR4 alleviated I/R-induced injury in AC16 cells. Moreover, SNHG15 regulated the TLR4/nuclear factor-κB (NF-κB) signaling pathway through miR-335-3p.

Our findings identify a novel mechanism, the miR-335-3p/TLR4/NF-κB pathway, for the regulation of SNHG15 in myocardial I/R injury.

Key words: Myocardial I/R injury, Acute myocardial infarction, Mechanism

Acute myocardial infarction is a major cause of cardiovascular disease-related mortality worldwide.1 Although the early reperfusion of ischemic myocardial territories is essential to limit infarction size and improve clinical outcomes, it can lead to additional damage known as myocardial ischemia-reperfusion (I/R) injury.2 To develop more effective therapies, it is required to improve our understanding of the molecular basis of myocardial I/R injury.

Long noncoding RNAs (lncRNAs) are a functionally diverse group of RNAs that are implicated in cell homeostasis and dysfunction.3,4 Numerous studies have documented that lncRNAs are frequently dysregulated in human myocardial I/R injury.5,6 For instance, maternally expressed gene 3 (MEG3), RP11-400K9.4, and necrosis-related factor (NRF) contribute to myocardial I/R injury.7,8 Conversely, IncPEAMIR and opioid receptor mu 1 (Oprm 1) have been identified as protective factors against myocardial I/R injury.9,10 Small nuclear RNA host gene 15 (SNHG15), a cancer-related lncRNA,11,12 has been unveiled to affect cerebral I/R injury.13,14 Moreover, Chen, et al. uncovered that SNHG15 aggravated I/R-induced cardiomyocyte apoptosis depending on the regulation of microRNA (miR)-188-5p.15 However, our understanding of the critical role of SNHG15 in myocardial I/R injury has remained incomplete.

MiRNAs are crucial for the regulation of gene expression,16,17 and abnormal miRNA expression is closely associated with myocardial I/R injury.18,19 For example, miR-24 and miR-208a have been unveiled to regulate myocardial I/R injury by targeting their mRNAs.20,21 MiR-335-3p is differently expressed in a mouse model of cerebral I/R injury.22 However, it is still unclear whether miR-335-3p can participate in myocardial I/R injury. Recent evidence has highlighted that lncRNAs involve the post-transcriptional regulation of gene expression by inhibiting microRNA (miRNA) activity.23 Furthermore, it remains undefined whether miR-335-3p can act as a downstream mediator of SNHG15 regulation in myocardial I/R injury.

In this report, our data support the regulatory effect of SNHG15 on I/R-induced cardiomyocyte injury in vitro. Moreover, we demonstrate that SNHG15 regulates the expression of toll-like receptor 4 (TLR4), an overexpressed protein in I/R mice,24 by directly binding to miR-335-3p,

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providing a novel molecular mechanism in myocardial I/R injury.

**Methods**

**Establishment of an *in vivo* model of myocardial I/R injury:** All experimental procedures involving animals were performed in compliance with a protocol approved by the Animal Care and Use Committee of The Affiliated Hospital of Changchun University of Traditional Chinese Medicine. Fourteen male C57BL/6 mice age-matched between 9 and 12 weeks (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were divided into two groups (n = 7 per group): Sham group and I/R group. These mice were used for the establishment of an *in vivo* model of myocardial I/R injury as reported.23) In the I/R group, mice were anesthetized with isoflurane (Aladdin, Shanghai, China), the left thoracic cavity was opened, ischemia was induced by the ligation of the left anterior descending artery with 6-0 silk for 30 minutes, and the knot was untied for reperfusion. In the Sham group, mice were subjected to the operation with nonligation. Following a 24-hour reperfusion, all mice were sacrificed and the infarcted area tissues of the hearts were collected to measure the expression levels of SNHG15, miR-335-3p, and TLR4.

**Cell line:** Human AC16 ventricular cardiomyocytes (Bnbio, Beijing, China) were used in this study. The cells were routinely propagated at 37°C using standard protocols provided by Bnbio in a 5% CO2 incubator (Thermo Fisher Scientific, Hemel Hempstead, UK).

**Establishment of an *in vitro* model of I/R injury:** An *in vitro* model of I/R injury was established by treating AC16 cells with a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). Briefly, AC16 cells grown in 24-well cell culture dishes were maintained in a complete medium at 37°C in a 5% CO2 and 95% air incubator for 24 hours. AC16 cells grown in normoxic conditions were used as controls. Additionally, an *in vitro* model of hypoxia injury was generated by culturing AC16 cells with the glucose-free medium in an anoxic atmosphere (95% N2 and 5% CO2) for 4 hours. Then, the cells were grown in a complete medium at 37°C in a 5% CO2 and 95% air incubator for 24 hours. AC16 cells grown in normoxic conditions were used as controls. Additionally, an *in vitro* model of hypoxia injury was generated by culturing AC16 cells with the glucose-free medium in an anoxic atmosphere (95% N2 and 5% CO2) for 4 hours.

**Plasmids, oligonucleotides, and cell transfection:** Human SNHG15 sequence was synthesized by Genecreate (Wuhan, China) was cloned into the pcDNA3.1 vector (Genomeditech, Shanghai, China) with BamHI I and Xba I sites to produce an SNHG15 overexpression plasmid. The human TLR4 coding sequence (Genecreate) was inserted into the pcDNA3.1 vector opened with Xho I and Apa I sites to generate a TLR4 overexpression plasmid. A scrambled control sequence (Genecreate) was subcloned into the same vector with BamHI I and Xho I sites to create a negative control plasmid. SiRNA-SNHG15 (si-SNHG15), siRNA-TLR4 (si-TLR4), nontarget siRNAs (si-NC or si-con), miR-335-3p mimic and mimic control (miR-NC mimic), and antisense sequence of mature miR-335-3p as miRNA inhibitor (anti-miR-335-3p) and inhibitor control (anti-miR-NC) were purchased from GenePharma (Shanghai, China), and their details were included in the Supplemental Table.

For transient transfection, a mixture of siRNA (80 nM) or/and miRNA mimic (30 nM) or/and miRNA inhibitor (50 nM) or/and plasmid (100 ng), Opti-MEM medium, and Lipofectamine 2000 (all from Thermo Fisher Scientific) was prepared as per the accompanying guidance and then dispensed into each well of 24-well cell culture dishes containing AC16 cells (5 × 103 per well). The transfected cells were directly subjected to OGD/R stimulation in the wells after 24 hours.

**Quantitative real-time polymerase chain reaction (qRT-PCR):** Total RNA was extracted from mouse tissues and cultured AC16 cells using TRIzol reagent based on the recommendations of the manufacturer (Thermo Fisher Scientific). For SNHG15 and TLR4 mRNA analyses, cDNA was randomly primed from 500 ng of RNA using Omniscript RT Kit (Qiagen, Crawley, UK), qRT-PCR was conducted in a 20 μL reaction containing 1 μL of cDNA and specific primers (Supplemental Table) using SYBR Green (Qiagen), and human and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were analyzed for normalization of SNHG15 and TLR4 mRNA. For miR-335-3p and endogenous control U6 analyses, 50 ng of RNA was subjected to stem-loop reverse transcription PCR, and qRT-PCR with designed primers (Supplemental Table) and SYBR Green (Qiagen) was performed as recommended by the manufacturer. Using the comparative Ct method (2-ΔΔCt), the relative expression of RNA species was quantified.

**Subcellular localization assay:** Cytoplasmic and nuclear RNA was extracted from AC16 cells using a Cytoplasmic & Nuclear RNA Purification Kit as per the instructions of the manufacturer (Norgen Biotek, Thorold, ON, Canada).

**Cell viability, proliferation, and apoptosis assays:** For cell viability analysis, cultured AC16 cells were seeded in 96-well cell culture dishes at 5 × 103 per well and maintained in a complete medium. At 24 hours after cell seeding, the Cell Counting Kit-8 (CCK-8) assay was performed as per the manufacturing recommendations (MedChemExpress, Shanghai, China) and the absorbance was read at 450 nM using a standard colorimetric system (Bio-Rad, Munich, Germany). Cell proliferation was evaluated by the 5-ethynyl-2’-deoxyuridine (EDU) assay. Briefly, cultured AC16 cells were incubated with 50 μM EDU (RiboBio, Guangzhou, China) for 2 hours, followed by EDU staining with Apollo 567 (RiboBio). Subsequently, the cells were incubated with 4’,6-diamidino-2-phenylindole (DAPI, RiboBio) for nuclei staining. The proportion of the EDU-positive cells was examined with a Nikon fluorescence microscope (Nikon, Tokyo, Japan). For cell apoptosis analysis, 1 × 104 cultured AC16 cells were stained using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide assay kit based on the recommendations of the manufacturer (Beyotime, Beijing, China). Apoptotic cells were analyzed on a FACScalibur instrument (forward scatter: 0.5°-10°, side scatter: 90°) with CellQuest software (BD Biosciences, Cowley, UK).

**Enzyme-linked immunosorbent assay (ELISA):** The levels of superoxide dismutase (SOD), interleukin 6 (IL-6), malonaldehyde (MDA), and tumor necrosis factor-α (TNF-α) were determined by ELISA with the Human
Figure 1. SNHG15 is overexpressed in I/R mice and I/R-stimulated AC16 cells. A: The expression of SNHG15 was detected by qRT-PCR in the myocardial infarction tissues of I/R mice and Sham mice. *n = 7. B: qRT-PCR showing SNHG15 expression in AC16 cells under a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R), *n = 3 independent biological replicates. C: Subcellular localization assay showed the localization of SNHG15 in AC16 cells. *n = 3 independent biological replicates. "***P < 0.001, ****P < 0.0001.

SOD ELISA Kit (Abcam, Cambridge, UK), Human IL-6 ELISA Kit (Thermo Fisher Scientific), Human MDA ELISA Kit (Abbkine, Wuhan, China), and Human TNF-α ELISA Kit (Abcam), respectively, as per the manufacturing instructions.

Western blot: For western blot, mouse tissues and cultured AC16 cells were homogenized (0°C; 60 minutes) in RIPA buffer (Beyotime). Proteins were resolved by electrophoresis, electroblotted to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and probed with primary antibodies against B cell lymphoma-2 (Bcl-2, #MA5-11757, Invitrogen, Tokyo, Japan), P65 (#33-9900, Invitrogen), IkBα (#4814, Cell Signaling Technology, Danvers, MA, USA), phosphorylated-P65 (p-P65, #MA5-15181, Invitrogen), p-IκBα (#9246, Cell Signaling Technology), Bcl-2 associated X, apoptosis regulator (Bax, ab270742, Abcam), and loading buffer GAPDH (ab8245, Abcam), and then incubated with horseradish peroxidase-coupled antimouse or antirabbit IgG secondary antibody (ab6789 or ab97051, GeNeM, Paris, France) followed by incubation with horseradish peroxidase-coupled antimouse or antirabbit IgG secondary antibody (ab6789 or ab97051, Abcam). Protein bands were visualized by enhanced chemiluminescence (GE Healthcare, Freiburg, Germany) and quantified using the AIDA software (Raytek, Sheffield, UK).

Bioinformatics and dual-luciferase reporter assay: The miRNA-binding sites to SNHG15 were predicted using the web-based program LncBase Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted&miRNAs%5B0%5D=&lncRNAs%5B0%5D=ENSG00000232956&lncRNAs%5B1%5D=ENSG00000232956&threshold=0.7&filters=0&page=5). MiRNA-binding sites to human 3’untranslated region (3’UTR) were predicted using the TargetScan search program (http://www.targetscan.org/vert_71/tdsourcecat=sp_cq_aimsg). The segments of SNHG15 encompassing the target sequence for miR-335-3p or miss-matched target sequence, TLR4 3’UTR, and 3’UTR mutant in the target sequence were synthesized by Genecreate and subcloned into the 3’UTR of Renilla luciferase on the psiCHECK-2 vector (Promega, Mannadale, NSW, Australia). AC16 cells were cotransfected using Opti-MEM medium and Lipofectamine 2000 in quintuplicate with the appropriate luciferase reporter system with a luminometer (Promega).

RNA immunoprecipitation (RIP) and RNA pull-down assays: For RIP experiments, 5 × 10^6 AC16 cells transfected with miR-335-3p mimic or miR-NC mimic were lysed (0°C; 30 minutes) in 100-200 μL of RIPA buffer. In the meantime, a mixture of an antibody against Argonaute2 (Ago2, ab233727, Abcam) or isotype IgG antibody (ab172730, Abcam) and protein A/G magnetic beads (MedChemExpress) was prepared and incubated for 4 hours at 4°C. For RNA pull-down assays, 5 × 10^6 AC16 cells were lysed (0°C; 30 minutes) in 100-200 μL of RIPA buffer. A mixture of biotinylated miR-335-3p mimic (bio-miR-335-3p) or negative control (bio-miR-NC, all from Ribobio) and streptavidin beads (Roche) was prepared and incubated for 4 hours at 4°C. In both assays, cell lysates were incubated with the mixture overnight at 4°C, followed by the collection of the beads. RNA bound to beads was measured by qRT-PCR to quantify SNHG15 and TLR4 enrichment levels.

Statistical analysis: Data were presented as mean ± standard deviation from at least three biological replicates performed in quintuplicate. *P-value < 0.05 was considered significant by a Student’s t-test or analysis for variance (ANOVA) with Tukey’s test. Pearson’s correlation analysis was performed to assess the correlations among SNHG15, miR-335-3p, and TLR4 expression levels in myocardial infarction tissues of I/R mice.

Results

SNHG15 is upregulated in I/R mice and I/R-stimulated AC16 cells: To elucidate the significance of SNHG15 expression in myocardial I/R injury, we firstly established the in vivo and in vitro models of I/R injury. As demonstrated by qRT-PCR, SNHG15 was markedly overexpressed in the myocardial infarction tissues of I/R mice and I/R-stimulated AC16 cells compared with their counterparts (Figure 1A and B). We then adopted subcellular localization assays to evaluate the localization of SNHG15 in AC16 cells. Higher levels of SNHG15 were detected in the cytoplasm than in nuclei (Figure 1C). All of these results suggest that overexpression of SNHG15 might be associated with the pathogenesis of myocardial I/R injury.

Silencing of SNHG15 alleviates I/R-induced injury in AC16 cells: To directly test the functional role of SNHG
15 in myocardial I/R injury, we performed in vitro loss-of-function analyses by silencing its expression with siRNA-SNHG15 (si-SNHG15). Transient transfection of si-SNHG15, but not si-NC controls, remarkably reduced the expression of SNHG15 in I/R-stimulated AC16 cells (Figure 2A). Knockdown of SNHG15 enhanced cell viability in I/R-stimulated AC16 cells as compared with that in the scrambled controls (Figure 2B). SNHG15 knockdown also reduced the production levels of IL-6 and TNF-α in I/R-stimulated AC16 cells, indicating that silencing of SNHG15 suppressed cell inflammation (Figure 2C). Moreover, silencing of SNHG15 repressed cell apoptosis (Figure 2D) and promoted cell proliferation (Figure 2E) in I/R-stimulated AC16 cells. The repression of SNHG15 knockdown on cell apoptosis was also confirmed by the alteration of Bax and Bcl-2 levels (Figure 2F). Furthermore, SNHG15-silenced AC16 cells under I/R injury exhibited higher levels of SOD and lower levels of MDA compared with si-NC controls (Figure 2G and H). Additionally, in agreement with the effects of I/R exposure (Figure 2B-H), increased expression of SNHG15 upon transfection of a SNHG15 plasmid markedly repressed cell viability, repressed proliferation, and enhanced cell inflammation, apoptosis, and oxidative stress (Supplemental Figure 1A-G), implying that I/R exposure might induce cell injury by upregulating SNHG15. Together, these results suggest that silencing of SNHG15 alleviates I/R-induced injury in AC16 cells partially by regulating cell proliferation, apoptosis, inflammation, and oxidative stress.

**SNHG15 directly targets miR-335-3p:** To understand how SNHG15 influenced I/R-induced injury in AC16 cells, we considered the miRNAs that directly bound to SNHG15. Using the computational target prediction program LncBase Predicted v.2, we selected five miRNAs (miR-188-5p, miR-506-5p, miR-324-3p, miR-362-3p, and miR-335-3p) that were associated with the pathogenesis of myocardial I/R injury. Of them, we observed that miR-335-3p expression was the most significantly augmented in SNHG15-silenced AC16 cells (Supplemental Figure 2A). We thus, selected miR-335-3p for further analyses. The predicted data revealed that SNHG15 harbored a binding region for miR-335-3p (Figure 3A). To confirm this, we constructed the SNHG15 wild-type or mutant luciferase reporter and tested them by dual-luciferase reporter assays. Transfection of miR-335-3p markedly reduced the activity of luciferase reporter gene fused on the wild-type target sequence, and this effect was rescued by the mutations in the corresponding binding site (Figure 3B). RIP experiments confirmed that transfection of miR-335-3p mimic did not affect SNHG15 expression (Supplemental Figure 3A) but led to a striking elevation in the enrichment level of SNHG15 in Ago2-containing RNA-induced silencing complexes relative to miR-NC controls (Figure 3C). Furthermore, RNA pull-down assays showed that incubation of cell lysates with biotinylated miR-335-3p mimic (bio-miR-335-3p) caused a significant increase in the enrichment level of SNHG15 in AC16 cells (Figure 3D). Our qRT-PCR data showed that miR-335-3p was strikingly underexpressed in the myocardial infarction tissues of I/R mice and I/R-stimulated AC16 cells (Figure 3E and F). Notably, miR-335-3p expression inversely correlated with SNHG15 levels in I/R mice (Figure 3G). We next elucidated whether SNHG15 influenced miR-335-3p expression in I/R-stimulated AC16 cells. The effectiveness of the SNHG15 overexpression plasmid was validated by qRT-PCR (Figure 3H). As would be expected, in I/R-stimulated AC16 cells, overexpression of SNHG15 induced a significant reduction in the level of endogenous miR-335-3p and knockdown of SNHG15 strongly enhanced miR-335-3p expression (Figure 3I). All of these results establish the notion that SNHG15 targets miR-335-3p by binding to miR-335-3p.

**Silencing of SNHG15 alleviates I/R injury in AC16 cells by upregulating miR-335-3p:** We then asked whether miR-335-3p represented a functional mediator of SNHG15 in regulating I/R injury. To address this, we co-transfected si-SNHG15 and anti-miR-335-3p into AC16 cells before I/R stimulation. Transfection of anti-miR-335-3p significantly reduced miR-335-3p expression in SNHG15-silenced AC16 cells under I/R stimulation (Figure 4A), and SNHG15 expression was not affected by miR-335-3p downregulation (Figure 4B). Reduced expression of miR-335-3p markedly abolished SNHG15 knockdown-driven cell viability enhancement, inflammation inhibition, apoptosis defect, and proliferation promotion (Figure 4C-G) in I/R-stimulated AC16 cells. Furthermore, reduced expression of miR-335-3p counteracted the influence of SNHG15 silencing on SOD and MDA production levels in AC16 cells under I/R stimulation (Figure 4H and I). Taken together, these data indicate that silencing of SNHG15 relieves I/R-induced injury in AC16 cells partially by targeting miR-335-3p.

**MiR-335-3p suppresses the expression of TLR4 by directly targeting TLR4:** To identify the downstream effectors of miR-335-3p, we used the TargetScan search program based on the presence of the binding sites in the 3’UTRs. Among these candidates, we selected seven genes (SIRT1, BACH1, SOCS2, ATG12, HIF1AN, ROCK1, and TLR4) that were linked to the pathogenesis of myocardial I/R injury. Interestingly, of them, we observed that TLR4 mRNA level was the most significantly downregulated in miR-335-3p-overexpressing AC16 cells (Supplemental Figure 2B). Hence, we selected TLR4 for further investigations. The predicted data showed that the 3’UTR of TLR4 mRNA contained eight nucleotides of sequence complementarity to the miR-335-3p seed region (Figure 5A). To determine whether TLR4 was a target of miR-335-3p, we cloned TLR4 3’UTR or the mutations in the binding sequence into the 3’UTR of a luciferase gene and assayed their expression in AC16 cells. The expression from the TLR4 3’UTR reporter, but not that of the reporter carrying a mutant binding region, was significantly repressed by miR-335-3p overexpression (Figure 5B). RIP assays revealed that although miR-335-3p overexpression reduced TLR4 mRNA expression in AC16 cells (Supplemental Figure 3B), overexpression of miR-335-3p resulted in a remarkable increase in the enrichment level of TLR4 mRNA compared with miR-NC controls (Figure 5C). Additionally, RNA pull-down assays showed that incubation of cell lysates with bio-miR-335-3p led to a significant elevation in TLR4 enrichment level in AC16 cells.
Figure 2. Knocking down SNHG15 alleviates I/R injury in AC16 cells. A–H: AC16 cells were transfected with or without si-NC or si-SNHG15 and then exposed to a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). A: SNHG15 expression was gauged by qRT-PCR in treated AC16 cells. B: Viability of treated AC16 cells was assessed by CCK-8 assay. C: The production levels of IL-6 and TNF-α in treated AC16 cells were tested by ELISA. D: Apoptosis of treated AC16 cells was evaluated by flow cytometry and representative images showed the apoptosis rate. E: Proliferation of treated AC16 cells was measured by EDU assay, and representative images showed the proliferation rate. F: Western blot showed the levels of Bax and Bcl-2 in treated AC16 cells. G, H: The production levels of SOD and MDA in treated AC16 cells were tested using the corresponding assay kit. n = 3 independent biological replicates. **P < 0.01, ***P < 0.001, or ****P < 0.0001.
SNHG15 targets miR-335-3p. A: Sequence of miR-335-3p, the binding sequence for miR-335-3p within SNHG15 and the mutation in the target region. B: Relative luciferase activity of SNHG15 wild-type (WT-SNHG15) or mutant (MUT-SNHG15) luciferase reporter was detected in AC16 cells transfected with miR-335-3p mimic or miR-NC mimic. C: AC16 cells were transfected with miR-335-3p mimic or miR-NC mimic and then lysed. RIP experiment was performed to assess SNHG15 enrichment level using an antibody against Ago2 or IgG. n = 3 independent biological replicates. D: RNA pull-down assays showing the enrichment level of SNHG15 using bio-miR-335-3p or bio-miR-NC with cell lysates. n = 3 independent biological replicates. E: Relative miR-335-3p expression was gauged by qRT-PCR in the myocardial infarction tissues of I/R mice and Sham mice. n = 7. F: qRT-PCR of miR-335-3p expression in AC16 cells under a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). n = 3 independent biological replicates. G: Expression correlation between miR-335-3p and SNHG15 in I/R mice was evaluated using the Pearson’s correlation coefficients. H: AC16 cells were transfected with control plasmid pcDNA or SNHG15 overexpression plasmid before I/R stimulation and checked for SNHG15 expression by qRT-PCR. n = 3 independent biological replicates. I: AC16 cells were transfected with si-NC, si-SNHG15, control plasmid pcDNA, or SNHG15 overexpression plasmid before I/R stimulation and checked for miR-335-3p expression by qRT-PCR. n = 3 independent biological replicates. **P < 0.01, ***P < 0.001, or ****P < 0.0001.

relative to bio-miR-NC controls (Figure 5D). The data of qRT-PCR and western blot also showed that TLR4 was overexpressed in I/R mice and I/R-stimulated AC16 cells compared with the corresponding negative controls (Fig-
MiR-335-3p is a downstream mediator of SNHG15 function. A–I: AC16 cells were transfected with or without si-NC, si-SNHG15, si-SNHG15+anti-miR-NC, or si-SNHG15+anti-miR-335-3p, then exposed to a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R), and checked for miR-335-3p and SNHG15 expression by qRT-PCR (A, B), cell viability by CCK-8 assay (C), IL-6 and TNF-α production levels by ELISA (D), cell apoptosis by flow cytometry (E), cell proliferation by EDU assay (F), Bax and Bcl-2 levels by western blot (G), and SOD and MDA production levels by the corresponding assay kit (H, I). n = 3 independent biological replicates. *P < 0.05, **P < 0.01, ***P < 0.001, or ****P < 0.0001.

Figure 4. Having demonstrated that miR-335-3p directly targeted TLR4, we then elucidated if there was an inverse correlation between TLR4 and miR-335-3p levels in the myocardial infarction tissues of I/R mice. As expected, TLR4 levels inversely correlated with miR-335-3p expression (Figure 5H). To validate the direct regulation of miR-335-3p on TLR4 expression, we manipulated miR-335-3p expression by the transfection of miR-335-3p mimic or anti-miR-335-3p, and we confirmed their effectiveness by qRT-PCR (Figure 5I). Remarkably, in I/R-stimulated AC16 cells, increased expression of miR-335-3p resulted in a significant reduction in the level of TLR4 protein, and downregulation of miR-335-3p clearly enhanced TLR4 protein expression (Figure 5J). These results together demonstrate that miR-335-3p directly regulates TLR4 expression through the perfect binding site in the 3’UTR.

MiR-335-3p-mediated inhibition of TLR4 affects I/R-induced injury in AC16 cells: Consistent with the effects of TLR4 knockdown by siRNA targeting TLR4 (si-TLR4)
Figure 5. TLR4 is a direct target of miR-335-3p. A: Sequence of miR-335-3p, the binding sequence for miR-335-3p within TLR4 3’UTR and the mutation in the seed region. B: Dual-luciferase reporter assays were performed in AC16 cells cotransfected with TLR4 3’UTR reporter (WT-TLR4 3’UTR) or mutant reporter (MUT-TLR4 3’UTR) and miR-335-3p mimic or miR-NC mimic. n = 3 independent biological replicates. C: AC16 cells were transfected with miR-335-3p mimic or miR-NC mimic and then lysed. RIP assays were performed by incubating cell lysates with antibody against Ago2 or IgG. n = 3 independent biological replicates. D: RNA pull-down assays showing the enrichment level of TLR4 using bio-miR-335-3p or bio-miR-NC with cell lysates. n = 3 independent biological replicates. E: qRT-PCR of TLR4 mRNA in the myocardial infarction tissues of I/R mice and Sham mice. n = 7. F: Western blot showing TLR4 protein level in the myocardial infarction tissues of I/R mice and Sham mice. n = 3. G: Western blot of TLR4 protein in AC16 cells under a control condition or a condition of oxygen glucose deprivation followed by reperfusion (OGD/R). n = 3 independent biological replicates. H: Expression correlation between miR-335-3p and TLR4 mRNA in I/R mice was evaluated using the Pearson’s correlation coefficients. I, J: qRT-PCR of miR-335-3p expression (I) and western blot showing the level of TLR4 protein (J) in I/R-stimulated AC14 cells transfected with miR-NC mimic, miR-335-3p mimic, anti-miR-NC, or anti-miR-335-3p. n = 3 independent biological replicates. **P < 0.01, ***P < 0.001, or ****P < 0.0001.

(Supplemental Figure 4), overexpression of miR-335-3p by miRNA mimic transfection (Figure 6A) significantly enhanced cell viability (Figure 6B), repressed cell inflammation and apoptosis, and promoted cell proliferation (Figure 6C-F), as well as altered SOD and MDA production (Figure 6G and H) in I/R-stimulated AC14 cells.
Given our data that miR-335-3p suppressed TLR4 expression, we next ascertained whether the reduction of TLR4 might provide an explanation for the regulation of miR-335-3p overexpression on I/R-induced injury in AC14 cells. We overexpressed miR-335-3p in I/R-stimulated AC16 cells together with a construct plasmid expressing TLR4. This plasmid encodes the entire TLR4 coding sequence but lacks the 3′UTR of TLR4, yielding an mRNA that is resistant to miR-335-3p-mediated inhibition of translation. Notably, restored expression of TLR4 (Figure 6A) abrogated miR-335-3p-driven cell viability promotion (Figure 6B), cell inflammation and apoptosis suppression, and cell proliferation enhancement (Figure 6C-F), as well as SOD and MDA production alteration (Figure 6G and H) in I/R-stimulated AC16 cells. All of these data strongly establish that TLR4 is a functional downstream effector of miR-335-3p.

**SNHG15 modulates TLR4 expression through miR-335-3p:** We further investigated whether SNHG15 could regulate TLR4 expression by miR-335-3p. Strikingly, silencing of SNHG15 resulted in reduced levels of TLR4 mRNA and protein in I/R-stimulated AC16 cells, and anti-miR-335-3p transfection abolished the effects (Figure 7A and B). Together, these results point to the role of SNHG15 as a modulator of TLR4 through miR-335-3p.

**SNHG15 regulates the nuclear factor-κB (NF-κB) signaling pathway by the miR-335-3p/TLR4 axis:** The TLR4/NF-κB signaling pathway, an inflammation-related pathway, plays a pivotal role in myocardial I/R injury. Having demonstrated the regulation of the SNHG15/miR-335-3p axis on TLR4 expression, we further elucidated the impact of SNHG15 on NF-κB signaling. In AC16 cells, I/R stimulation significantly activated the pathway, as presented by the increase of p-P65 and p-IκBα levels (Figure 8A). Moreover, silencing of SNHG15 markedly repressed the activation of the pathway in AC16 cells under I/R stimulation, and this effect was strongly abolished by reduced expression of miR-335-3p or overexpression of TLR4 (Figure 8B). All of these results indicate that SNHG15 knockdown blocks NF-κB signaling in I/R-stimulated AC16 cells by the miR-335-3p/TLR4 axis.

**Discussion**

Recently, it has become evident that lncRNA expression is deregulated in myocardial I/R injury, resulting in specific accelerating or repressive events. Knowing the precise actions of these lncRNAs will be essential for developing molecularly targeted interventions. In this report, our data showed that SNHG15 was highly expressed in I/R mice and I/R-stimulated AC16 cells. Previous work showed the important role of SNHG15 in cardiomyocyte apoptosis induced by I/R stimulation. Our results broadened this by demonstrating that SNHG15 modulated I/R-
induced cardiomyocyte injury by affecting cell proliferation, inflammation, apoptosis, and oxidative stress. Our data also showed that SNHG15 expression was markedly upregulated in hypoxia-exposed AC16 cells (Supplemental Figure 5), suggesting that SNHG15 is active not only upon I/R but also upon hypoxia. More importantly, we provided a new molecular explanation, at least in part, for the important regulation of SNHG15 in myocardial I/R injury (Figure 9). Additionally, the main cytoplasmic localization of SNHG15 in AC16 cells could provide the possibility for the interactions between SNHG15 and miRNAs because miRNAs silence gene expression in the RNA-induced silencing complexes in the cytoplasm.16,29)

The critical role of miR-335-3p in human diseases has been widely reported. For instance, dysregulation of miR-335-3p is tightly associated with the development of various cancers, such as acute lymphoblastic leukemia and osteosarcoma.30,31) MiR-335-3p exerts important activity in hypoxia-induced pulmonary arterial hypertension, intervertebral disk degeneration, and age-related neurological diseases.32-34) Our results suggested that SNHG15 regulated myocardial I/R injury in vitro at least partially through miR-335-3p, a regulator in cerebral I/R injury.21) The significant inverse correlation between the levels of miR-335-3p and SNHG15 in I/R mice and I/R-stimulated AC16 cells suggested that SNHG15 might promote the degradation of miR-335-3p by binding to miR-335-3p. Additionally, miR-335-3p did not affect the expression of SNHG15 in I/R-stimulated AC16 cells (Figure 4B), suggesting that SNHG15 upregulation may be the primary event.

TLR4, a member of the TLR family that can mediate the inflammatory response, has been implicated in the development of inflammation-related diseases and cancers.35,36) Overexpression of TLR4 can induce renal and
myocardial I/R injury.\textsuperscript{23,37,38} The TLR4/NF-\kappaB signaling pathway has been well defined, and it exerts a crucial function in myocardial I/R injury.\textsuperscript{26-28} In this report, we first identified TLR4 as a functional effector of miR-335-3p. Moreover, we pointed to the regulation of SNHG15 in TLR4 expression through miR-335-3p, suggesting the SNHG15/miR-335-3p/TLR4 regulatory network. Although it had been reported that miR-335-3p was transactivated via NF-\kappaB,\textsuperscript{32} we discovered that SNHG15 regulated TLR4/NF-\kappaB signaling through miR-335-3p, establishing a negative feedback loop that might be important in human diseases. Chen and colleagues demonstrated that SNHG15 modulated I/R-driven cardiomyocyte apoptosis via miR-188-5p/phosphatase and tensin homolog (PTEN) axis.\textsuperscript{15} In LPS-induced TLR4 signaling, PTEN can activate NF-\kappaB signaling by increasing the association of TLR4.\textsuperscript{39} Moreover, suppression of PTEN downregulates TLR4 expression and thus, regulates inflammation injury in I/R-stressed liver.\textsuperscript{40} These findings prompt that the SNHG15/miR-335-3p/TLR4 and SNHG15/miR-188-5p/PTEN axes may be two interactional regulatory networks in the regulation of SNHG15 in myocardial I/R injury. SNHG15 can bind to many miRNAs, such as miR-188-5p and miR-613, which play key roles in the pathogenesis of myocardial I/R injury.\textsuperscript{31,41} MiR-335-3p can target many genes, such as HIF1AN and ROCK1, which are involved in the pathologic process of myocardial I/R injury.\textsuperscript{33,44}

There may be other miRNA/mRNA networks that remain to be identified in the regulation of SNHG15. Such investigation is limited at present by the lack of \textit{in vivo} analyses using the animal models of myocardial I/R injury to explore whether the new mechanism works \textit{in vivo}. Additionally, TLR4/NF-\kappaB signaling is reported to contribute to myocardial I/R injury via the NLRP3 inflammasome.\textsuperscript{26,45} Zhou, \textit{et al.} uncovered that 22-oxacalcitriol attenuated I/R-induced inflammation in the myocardium by inactivating the TNF-\alpha through the NF-\kappaB pathway.\textsuperscript{46} Further investigations regarding the mechanisms of the regulation of the NF-\kappaB pathway in myocardial I/R injury are warranted.

Our data also showed the influence of the transfection of si-SNHG15, miR-335-3p mimic, anti-miR-335-3p, or pcDNA-TLR4 on the viability of AC16 cells (Supplemental Figure 1H-J), suggesting their regulatory effects in AC16 cells without I/R injury. Overexpression of TLR4 by pcDNA-TLR4 also suppressed AC16 cell proliferation and promoted cell inflammation, apoptosis, and oxidative stress without I/R injury (Supplemental Figure 1K-O), which was similar to the impact of SNHG15 overexpression in AC16 cells. With the finding that SNHG15 overexpression elevated TLR4 expression in AC16 cells (Supplemental Figure 1P), we speculate that SNHG15 overexpression-induced TLR4 upregulation might be similar to simple TLR4 overexpression. According to the ef-
fect of si-SNHG15 in AC16 cells with ($P < 0.01$) or without ($P < 0.05$) I/R injury compared with si-NC controls. We speculate that SNHG15 knockdown may play a more significant role in AC16 cells with I/R injury. Additionally, our results indicated that SNHG15 was upregulated in the myocardial infarction tissues of I/R mice and I/R-stimulated human AC16 cardiomyocytes. Wen and colleagues unveiled the dysregulation of SNHG15 in middle cerebral artery occlusion rats. Furthermore, SNHG15 can participate in the dysfunction of various human cells, such as endothelial cells, neurons, and cancer cells. These findings suggest that SNHG15 might be nonspecific in terms of species and cell types.

In summary, we demonstrate a novel molecular determinant, the miR-335-3p/TLR4/NF-κB pathway, for the modulation of SNHG15 in myocardial I/R injury. The findings provide a rationale for developing SNHG15 as a potential target against myocardial I/R injury.

**Disclosure**

**Conflicts of interest:** The authors declare that they have no conflicts of interest.

**Ethics approval:** The present study was approved by the ethical review committee of The Affiliated Hospital of Changchun University of Traditional Chinese Medicine.

**Availability of data and materials:** The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

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Supplemental Files

Supplemental Table

Supplemental Figures 1-5

Please see supplemental files; https://doi.org/10.1536/ihj.21-511